UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

Facultad de Farmacia

Departamento de Química Orgánica

Cumarinas: Versatilidad estructural y aplicaciones en Química Farmacéutica

Colaboración con el Departamento de Química y Bioquímica de la Facultad de Ciencias de la Universidad de Porto – Portugal

Memoria para optar al título de
Doctora en Farmacia presenta

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CERTIFICAN

Que la Memoria titulada “Cumarinas: Versatilidad estructural y aplicaciones en Química Farmacéutica”, que para optar al grado de Doctora en Farmacia presenta MARIA JOÃO CORREIA PINTO CARVALHO DE MATOS, ha sido realizada bajo nuestra dirección, en el Departamento de Química y Bioquímica de la Facultad de Ciencias de la Universidad de Porto y en el Departamento de Química Orgánica de la Facultad de Farmacia de la Universidad de Santiago de Compostela.

Y considerando que el trabajo constituye tema y labor de Tesis Doctoral, autorizamos su presentación en la Universidad de Santiago de Compostela.

Y para que conste, expedimos el presente certificado en Santiago de Compostela a 27 de mayo de dos mil trece.

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A mis queridos padres

y a mi querido Jose

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"A ciência desenha a onda;

a poesia enche-a de água."

Teixeira de Pascoaes

(1877-1952)

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"I'm a great believer in luck, and I find the harder I work the more I have of it."
Thomas Jefferson

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Resumen

En esta Memoria se describe un trabajo de diseño, síntesis y evaluación farmacológica de derivados cumarinícos de diferente complejidad, mostrando la versatilidad química de este esqueleto y algunas de sus aplicaciones en la Química Farmacéutica. En esta Memoria se hace una selección del trabajo global realizado, mostrando un conjunto de 134 compuestos que han sido los publicados hasta este momento de un total de más de 500. Todos ellos incorporan en una sola estructura el núcleo de la cumarina y del estilbeno, o bien de la cumarina y funciones de tipo éster, amida o carbamato. Modificaciones sencillas de estos esqueletos han permitido además la obtención de compuestos con diferentes sustituyentes (átomos de bromo o cloro, y/o grupos metilo, metoxilo, etoxilo, hidroxilo, nitro, amino, etc) en diversas posiciones de las moléculas. La síntesis de estos compuestos, con pureza y en cantidad suficiente para sus ensayos farmacológicos, se ha realizado utilizando metodologías directas, eficientes y generalizables. Se presentan los resultados obtenidos en la posterior evaluación farmacológica de estos derivados como inhibidores enzimáticos (MAO-A, MAO-B, AChE y tirosinasa), ligandos de adenosina, antioxidantes y antimicrobianos. La selección de los mismos se hizo teniendo en cuenta el conocimiento previo ayudado por herramientas de diseño racional (QSAR y *docking*) y se obtuvieron muy buenos resultados en todos los campos estudiados.

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Summary

This Report describes a work of design, synthesis and pharmacological evaluation of coumarin derivatives of varying complexity, showing the chemical versatility of this skeleton and some of its applications in Pharmaceutical Chemistry. In this Report it was made a selection of the overall work done, showing a set of 134 compounds that have been published so far, of a total of more than 500 compounds. All incorporated in a single structure the core of the coumarin and the stilbene, or the core of the coumarin and ester, amide or carbamate functions. Simple modifications of these skeletons have also allowed the preparation of compounds with different substituents (chlorine or bromine atoms and/or methyl, methoxy, ethoxy, hydroxyl, nitro and amino groups, etc.) in different positions of the molecules. The synthesis of these compounds, in purity and in sufficient quantity to its pharmacological evaluation, was performed using direct, efficient, and generalizable methodologies. In this Report there are presented the results of the subsequent pharmacological evaluation of these derivatives as enzyme inhibitors (MAO-A MAO-B, AChE and tyrosinase), adenosine ligands, antioxidants and antimicrobials. The selection of these derivatives was made taking into account prior knowledge helped by rational design tools (QSAR and *docking*) and there were obtained very good results in all the studied areas.

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I. Índice

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1. Introducción 21
1.1. Estructuras químicas 26
1.1.1. Las cumarinas 26
1.1.1.1. Actividad biológica de las cumarinas 29
1.1.1.2. Síntesis de cumarina 34
1.1.2. Los estilbenos (resveratrol) 37
1.2. Enfermedades neurodegenerativas 41
1.2.1. Enfermedad de Parkinson (EP) 42
1.2.2. Enfermedad de Alzheimer (EA) 46
1.3. Principal diana farmacológica en la Memoria - MAOs 47
1.3.1. Cumarinas y las MAOs 52
2. Antecedentes y objetivos 57
2.1. Antecedentes 59
2.2. Objetivos 61
3. Discusión de resultados 65
3.1. Síntesis 68
3.1.1. Preparación de los esqueletos cumarínicos 68
3.1.1.1. Reacción de Perkin y Perkin-Oglialoro 68
3.1.1.2. Reacción de acoplamiento con paladio 69
3.1.2. Transformaciones funcionales 69
3.1.2.1. Reacción de hidrólisis de grupos éter o éster 69
3.1.2.2. Reacción de halogenación 70
3.1.2.3. Reacción de formación de arilaminas 71
3.1.2.4. Reacción de alquilación – reacción de Williamson 72
3.1.2.5. Preparación de amidas, ésteres y carbamatos 73
3.1.3. Series de compuestos sintetizados 73
3.2. Estudio biológico y relación estructura-actividad (REA) 79
3.3. Aportaciones estructurales y teóricas 81
4. Parte experimental 83
4.1. Artículos publicados 85
4.2. Artículos en fase de publicación 87
6. Conclusiones 325
7. Bibliografía 329

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1. Introducción

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Desde siempre el Hombre ha intentado solucionar los problemas que iban surgiendo en su vida cotidiana. Con la necesidad de curar las enfermedades que le han afectado, el Hombre ha desarrollado preparaciones medicamentosas utilizando a lo largo de los tiempos aquellas estrategias que mejor servían este objetivo. Hasta el siglo XVIII, la conocida como "era de los botánicos",[1] las preparaciones de uso farmacéutico eran, en la gran mayoría de los casos, obtenidas de plantas, en forma de pociones. El descubrimiento de nuevas drogas y tratamientos estaba basado en la utilización de esas mismas plantas y otros productos naturales según la intuición y la observación empírica, siendo el "azar" el principal factor de éxito. Más tarde, siglo XIX, puede entenderse como el comienzo de la era de los medicamentos, se empezaron a aislar, purificar e identificar las sustancias activas como las entidades responsables de la acción de los extractos naturales y posteriormente la "era industrial" ha transformado la producción de nuevos fármacos. El siglo XX, se inicia con la introducción del salvarsán o "bala mágica" de Paul Ehrlich en el tratamiento de la sífilis, y con la utilización de la aspirina en terapéutica. La introducción de nuevas técnicas de elucidación estructural ha permitido el desarrollo de nuevas metodologías de síntesis como estrategia para el descubrimiento de nuevos fármacos y da paso a la denominada "edad de oro" de los medicamentos durante el período 1940-1960 gracias a la introducción de una gran mayoría de clases fármaco-terapéuticas que hoy día constituyen fármacos de gran interés tales como los antibióticos y junto con ellos el concepto de medicina preventiva y la vacunación. Las Ciencias Farmacéuticas se separaron de las Ciencias Médicas y surgieron nuevas áreas de conocimiento que transformaron la Farmacia en un conjunto multidisciplinar de saberes. El crecimiento ha sido exponencial y áreas como la Farmacología, la Biología, la Fisiología, la Biología Molecular, la Genética y la Química pasaron a coordinarse en un intento de dilucidar los mecanismos de acción de los fármacos y establecer las relaciones la estructura y la actividad farmacológica de los mismos. La modificación estructural sobre un compuesto cabeza de serie y/o la búsqueda de nuevos prototipos pasó a ser uno de los aspectos más estudiados[2] con un objetivo claro: mejorar el índice terapéutico de los fármacos, es decir aumentar la actividad y/o disminuir su toxicidad y efectos adversos.

El desarrollo de metodologías sintéticas, de análisis estructural o teóricas como el QSAR y el *docking*, entre otras, han añadido un valor adicional a las Ciencias Farmacéuticas permitiendo un diseño más racional de fármacos y la producción a gran escala.[3] La expansión tecnológica y el recurso a medios de información rápidos y asequibles, han traído progresos increíbles a un área conocida por su dinamismo.

Rápidamente se ha percibido un cambio radical en la industria farmacéutica, que es hoy en día una de las que más presupuesto invierte a nivel mundial. La innovación se ha transformado en el objetivo número uno de las empresas: *ideas* que sean *rentables* y *sostenibles*. Todo esto conlleva sin duda ventajas y desventajas. Existen muchos casos de las conocidas como enfermedades olvidadas que afectan a millones de personas y que, por su bajo aporte económico *versus* elevado gasto en investigación y desarrollo, son también olvidadas por la industria farmacéutica. En su mayoría se trata de enfermedades tropicales infecciosas que afectan fundamentalmente a la población más empobrecida, como por ejemplo la leishmaniosis, la tuberculosis, la enfermedad del sueño, la malaria o la enfermedad de Chagas, que generan un impacto devastador en la humanidad.

El descubrimiento de fármacos se considera una actividad compleja y lenta. Sin embargo, en las últimas décadas, se están desarrollando nuevos enfoques y métodos con la intención de descubrir nuevas entidades químicas de un modo nuevo y más eficiente nuevos paradigmas han tomado fuerza, como el conocimiento claro de la relación molécula-receptor, de mecanismos moleculares/celulares implicados en las enfermedades o de modelos preclínicos que mimetizan de forma más real los procesos bioquímicos y patológicos o de marcadores que monitorizan los efectos terapéuticos. Todas estas herramientas constituyen un buen soporte para la obtención más eficaz de nuevos fármacos más eficaces y seguros.[4] Los químicos farmacéuticos, o químicos medicinales, presentan un papel importante en todo este proceso de cambio. Está en sus manos el conocimiento general de diferentes áreas que permiten el desarrollo de nuevos fármacos. Y su principal ventaja viene de la capacidad de adaptación que los caracteriza.[5] Por otro lado, también la organización de la forma de trabajo de los investigadores viene cambiando en los últimos años. Muchos científicos están trabajando en fundaciones para la

investigación, centros paralelos a la universidad y compañías virtuales, y han surgido nuevas colaboraciones entre grupos de trabajo de universidades e industria. Este aumento de la "libertad de trabajo" y de movilidad hace emerger importantes expectativas para el desarrollo de fármacos. Por otra parte, se están llevando a cabo inversiones significativas en cuanto a espacios modernos, nuevos equipamientos, y personal, en el sentido de aprovechar todo su potencial.[4]

Sin embargo, esta tendencia positiva de cambios en investigación y desarrollo de fármacos no se extiende de forma global a la industria farmacéutica, y la cada vez más baja productividad medida en función del número de nuevos fármacos aprobados por la FDA, limitan de manera muy notable la contratación de nuevos investigadores.[6]

1.1. Estructuras químicas

1.1.1. Las cumarinas

Las cumarinas son una gran clase de lactonas, de origen natural o sintético, constituidas por un anillo de benceno condensado a una anillo α-pirona (Figura 1), y esencialmente poseen un sistema π-π conjugado rico en electrones, que posee buenas propiedades de transporte de carga.[7,8]

El primer representante de esta familia ha sido aislado por Vogel en el año de 1820, del haba de la tonka, cuyo nombre común en Caribe es *cumaru*, que ha dado nombre a esta familia de compuestos. Esta especie de haba es designada en el sistema binominal por *Coumarona odorata* Aube (también conocida por *Dipteryx odorata* Willd).[8] Dicha estructura química es denominada según la IUPAC como 2*H*-cromen-2-ona.

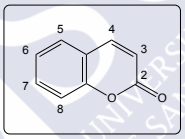


Figura 1. 2*H*-1-benzopiran-2-ona o 2*H*-cromen-2-ona (cumarina).

Las cumarinas se producen de forma natural en plantas y microorganismos, y se han aislado aproximadamente 1000 derivados de más de 800 especies (ejemplos en la Figura 2).

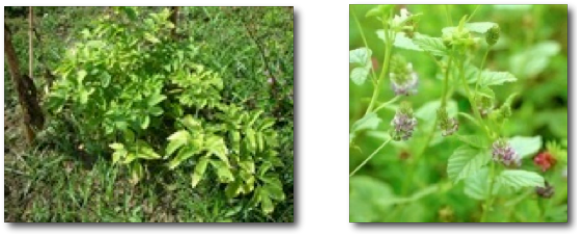


Figura 2. *Angelica archangelica* L. y *Psoralea corylifolia.*

En la naturaleza las cumarinas pueden encontrarse en raíces, hojas, flores o frutos de diferentes familias de las *Angiospermas*, tales como *Apiacea*e, *Rutaceae*, *Asteraceae*, *Umbelliferae*, entre otras (Figura 3).[7]

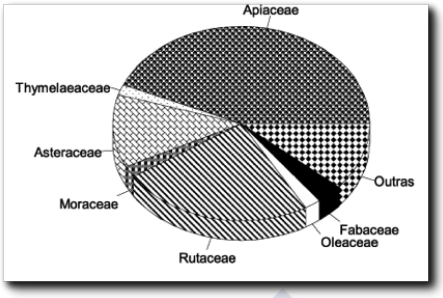


Figura 3. Cumarinas existentes en las principales familias de *Angiospermas*.

El esqueleto cumarínico se originan biosintéticamente por lactonización del ácido cumarínico (2-hidroxi-*Z*-cinámico), según la vía sintética descrita en la Figura 4.[8] La biosíntesis de cumarinas en las plantas superiores empieza con una reacción de 2- o bien 2,4-hidroxilación del ácido cinámico. Los hidroxicinámicos formados son posteriormente glucosilados y como tal se isomerizan posteriormente a los isómeros *E*. La ciclación posterior de los mismos origina finalmente la cumarina o su 7-hidroxi derivado, la umbeliferona. Otros derivados de la cumarina, tales como esculetina (6,7-dihidroxicumarina) o escopoletina (7-hidroxi-6-metoxicumarina) parecen derivar de la oxidación de la umbeliferona y no a partir del correspondiente derivado de ácido hidroxicumárico. (Figura 4).[9]

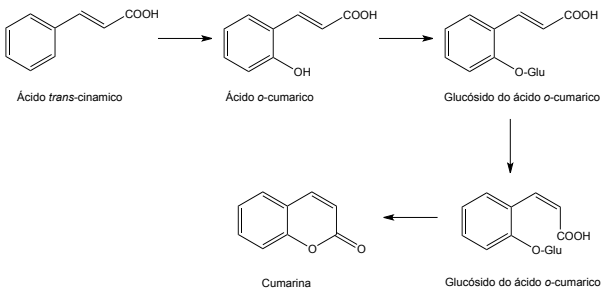


Figura 4. Esquema general de la biosíntesis de la cumarina.

Las cumarinas son, en general, solubles en alcoholes y disolventes orgánicos. Son lactonas y como tales en un medio alcalino sufren hidrólisis con formación de sales y solubilización de la misma. Pueden someterse de nuevo al cierre del anillo de pirona en condiciones ácidas. Presentan un espectro ultravioleta (UV) característico, que es fuertemente influenciado por sus sustituyentes. Presentan fluorescencia a la luz UV de coloraciones entre azul, amarillo o morado y que se intensifican en presencia de vapores de amoníaco.[10]

La diversidad estructural de los compuestos derivados de las cumarinas permite su clasificación en cumarinas simples, o bien en cumarinas más complejas en general condensadas con otros heterociclos: furocumarinas, piranocumarinas, biscumarinas, cumarinolignanos y triscumarinas.[11]

En cualquier caso, sobre la estructura base de la cumarina se pueden introducir sustituyentes de distinta naturaleza, lo que lleva a que existan cumarinas[12] que pueden ser utilizadas, según el tipo de sustituyentes que poseen, con diversas finalidades: aditivos alimentarios, perfumes, cosméticos, productos agroquímicos o bien como fármacos con la finalidad de estudiar, mejorar y ampliar el espectro de su actividad terapéutica.[13,14,15,16]

El anillo cumarínico tiene la capacidad de establecer diverso tipo de interacciones no covalentes que incluyen interacciones hidrófobas, π-π y electrostáticas, así como enlaces de hidrógeno, fuerzas de van der Waals, entre otras, con otras moléculas activas y el sitio de activo en diferentes dianas de organismos vivos. Así, estas moléculas presentan eficacia en una amplia gama de actividades biológicas. Las cumarinas presentan interés farmacológico como anticoagulantes, antioxidantes, antivirales, inhibidores enzimáticos y antibacterianos. Además, la característica única de contener oxígeno en el anillo de lactona también puede hacer de estas moléculas un tipo de ligandos ideales para producir ensamblaje supramolecular y, por lo tanto, naturalmente, ser empleado en el diseño racional y la síntesis de supramoléculas como agentes medicinales, en particular en los campos antibacteriano y antifúngico, en que han demostrado ya un papel importante al que se ha prestado mucha atención en los últimos años. Además, las excelentes capacidades fluorescentes de los compuestos derivados de la cumarina basadas en las propiedades de riqueza en electrones y las

propiedades de un buen transporte de carga favorecidas por un sistema conjugado π-π, las hace interesantes como receptores artificiales de iones, sondas fluorescentes para especies biológicamente importantes y marcadores biológicos para supervisar la actividad de enzimas, complejos eventos biológicos, así como detectores de propiedades farmacológicas y farmacocinéticas precisas.[17]

1.1.1.1. Actividad biológica de las cumarinas

Como ya ha sido referido, desde hace mucho tiempo el estudio de diferentes grupos de trabajo se ha dirigido a la separación y purificación de cumarinas de origen natural, a partir de una gran variedad de plantas, animales y microorganismos, así como hacia la síntesis de nuevos derivados de las cumarinas con el objetivo de explorar un amplio espectro de actividades farmacológicas.[18]

Derivados de las cumarinas se encuentran en casi todas las categorías fármaco-terapéuticas. Algunas cumarinas han sido estudiadas por sus propiedades como cardioprotectores (anticoagulantes y/o vasodilatadores).[19,20] Uno de los casos más conocidos son la warfarina y el acenocumarol, ampliamente utilizados en clínica como anticoagulantes. El carbocromeno, también disponible en la terapéutica actual, es otro ejemplo de una conocida cumarina con actividad cardioprotectora, debido a su acción vasodilatadora e inhibidora de la agregación plaquetaria (Figura 5).[18]

Por otra parte, la modulación del anillo cumarínico, permite obtener interesantes fármacos antiinflamatorios[21,22] y/o antioxidantes[23], sobre todo cuando sobre dicho anillo se introduce un anillo heteroaromático tipo pirazol (Figura 5).[24,25,26]

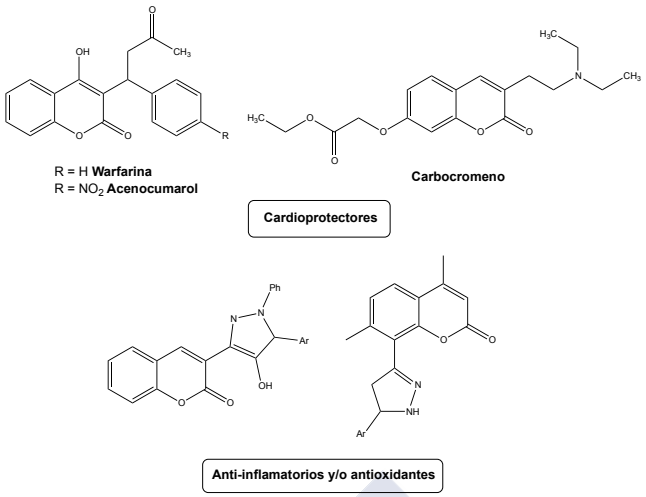


Figura 5. Ejemplos de cumarinas con importancia terapéutica como cardioprotectores, antiinflamatorios y antioxidantes.

Los derivados hidroxilados de cumarinas y flavonoides, tales como la umbeliferona y la esculetina (Figura 6), han sido descritos como inhibidores de la 5α-reductasa y de la tirosinasa.[27,28]

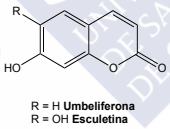


Figura 6. Estructuras de la umbeliferona y de la esculetina.

En otros casos, las cumarinas se han revelado como agentes antivirales. Así, ha sido descrita una interesante actividad antiviral HVC en cumarinas 3- y/o 4-substituidas[29,30,31,32,33,34] y más recientemente en sus ribonucleósidos conjugados.[35] Por su parte, la condensación de un anillo piránico en el enlace *h* ha permitido el diseño de cumarinas análogas de la suksdorfina, compuesto natural con actividad anti-HIV (Figura 7).[36]

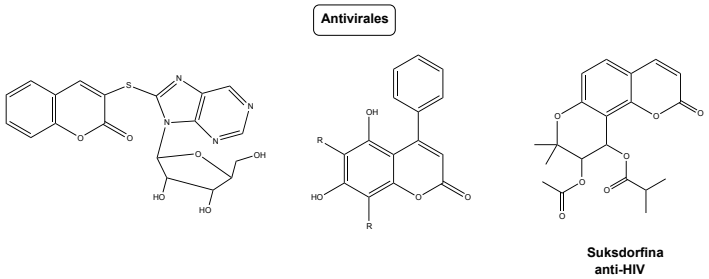


Figura 7. Cumarinas 3/4-sustituidas y análogos tricíclicos, compuestos con actividad antiviral.

Otro campo farmacológico en el que destaca cada vez más el núcleo cumarínico es en el de la terapia anti-cáncer. La geiparvarina (Figura 8) es uno de los ejemplos de compuestos cumarínicos con interés en dicha área. Es un derivado natural aislado de la *Geijera parvioflora* Lindl, conocido por su actividad citostática en ensayos *in vitro*. El núcleo furano de esta molécula ha probado ser esencial para su actividad. Por su parte, cumarinas también en este caso 3 y/o 4-aril/heteroaril substituidas se han descrito como buenos inhibidores de la tubulina.[37,38]

Más recientemente, a partir de la importancia del antimicrobiano novobiocina, se han desarrollado los primeros análogos con actividad anti-cáncer (Figura 8).[39,40]

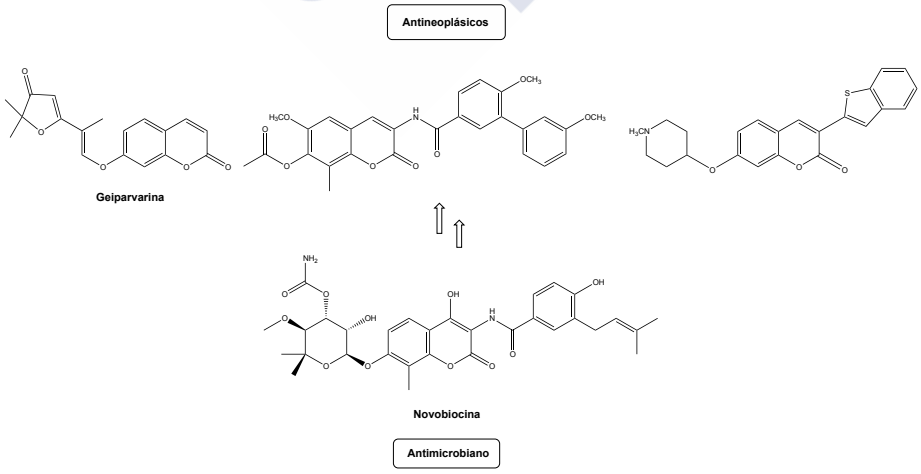


Figura 8. Geiparvarina y otros compuestos anti-cáncer.

Las propiedades antibacterianas de las cumarinas han sido descritas por primera vez en 1945 por Goth y col. cuando se estudió el dicumarol.[41] Es de destacar la actividad antimicrobiana encontrada para diversas cumarinas tales como la ya mencionada novobiocina (Figura 8) y sus análogos, la clorobiocina y la cumermicina A_1. La novobiocina, aislada de *Streptomyces niveus*, es principalmente activa frente a bacterias *Gram*-positivas, si bien no se utiliza en la clínica debido sobre todo a sus efectos secundarios y la difícil solubilidad en agua.[42] Por otro lado, estos tres ejemplos son antibióticos que presentan actividad contra *Staphylococcus aureus* (*S. aureus*) resistente a meticilina (MRSA).[43] El número de bacterias resistentes a múltiples fármacos es cada vez mayor y de especial importancia es el caso de las bacterias *Gram*-positivas MRSA. La capacidad de muchas especies tales como el de *S. aureus* para desarrollar resistencia a prácticamente todos los antibióticos es una preocupación importante.[44] Ello ha provocado el estudio de nuevos antimicrobianos sobre nuevas dianas terapéuticas y en este campo han emergido estructuras cumarínicas que interaccionan con la DNA helicasa,[45,46] y que son activas frente a cepas resistentes a la ciprofloxacina.[47]

Algunos derivados de la cumarina han demostrado además de las actividades antibacterianas, actividades antifúngicas.[48] Un estudio de los derivados cumarínicos sustituidos en el anillo pirona (derivados 3-carboxilados) muestran también importantes actividades.[49] Diferentes cumarinas 4-sustituidas, tal como las 4-clorocumarinas, mostraron también un interesante perfil antimicrobiano.[50]

Por otra parte, es también conocida la actividad antiparasitaria de estructuras derivadas de este anillo, y la creciente demanda de nuevos antiprotozoarios ha provocado la síntesis de nuevos fármacos de estructura flavonoide de los que destacamos la 8-(3-furil)cumarina, compuesto natural con actividad antileishmania,[51] o las 3-cinamoilcumarinas con actividad antimalaria.[52] En otros casos, aunque menos frecuentes, se han desarrollado 4-arilsulfonilmetilcumarinas con marcada actividad tuberculostática (Figura 9).[53,54]

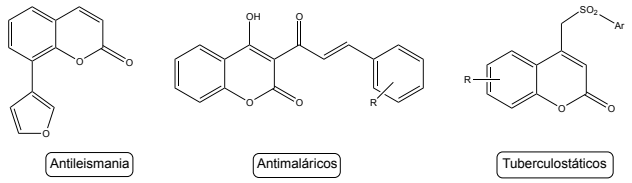


Figura 9. Moléculas con actividad antimicrobiana.

Además de las actividades farmacológicas mencionadas, merece una especial consideración la importancia del anillo cumarínico como prototipo estructural en el diseño de nuevos inhibidores de sistemas enzimáticos muy directamente implicados en el desarrollo las enfermedades neurodegenerativas.[55,56] En 2006, se describió la actividad inhibidora de diversas 6-amidocumarinas (Figura 10) sobre la *β*-secretasa (generalmente referenciada como BACE 1) y su potencial uso en la terapia de la enfermedad de Alzheimer (EA).[57] En el mismo año, se sintetizaron diversas 8-sulfooxicumarinas (Figura 10) que parecen ser útiles en el tratamiento del estrés oxidativo.[58] Los inhibidores de la acetilcolinesterasa (AChE) han sido también ampliamente estudiados por el elevado interés de las cumarinas en enfermedades neurodegenerativas, en especial la EA.[59]

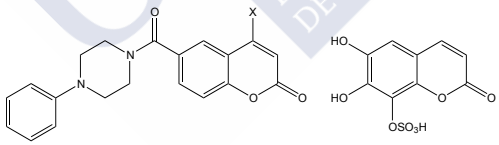


Figura 10. 6-Amidocumarinas y 8-sulfooxicumarinas, compuestos con actividad como inhibidores enzimáticos.

En otros casos se han descrito derivados cumarínicos multidiana con actividad inhibidora de la BACE 1 y de la AChE, ambas dianas de interés en la terapia de la EA (Figura 11).[60]

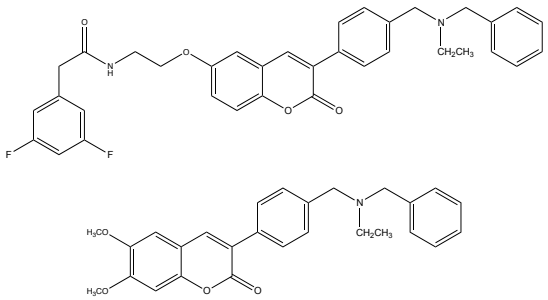


Figura 11. Compuestos multidiana (BACE 1 y AChE).

Como ya ha sido descrito, son muchas más las aplicaciones relacionadas con esta familia de moléculas, sin embargo, la actividad farmacológica de las cumarinas más directamente relacionada con el presente trabajo es su acción inhibidora selectiva de la monoamino oxidasa B (MAO-B)[59,60] y sus posibles aplicaciones terapéuticas,[61,62] encontrándose en los últimos años una interesante actividad inhibidora de la MAO (IMAO) en algunos análogos cumarínicos,[63] análogos a los que nos referiremos más adelante.

Teniendo en mente la etiología compleja de las enfermedades neurodegenerativas, y las diferentes dianas implicadas en ellas, también dedicaremos especial atención en esta Memoria a la actividad inhibitoria dual MAO/AChE y a las propiedades antioxidantes de las cumarinas. También se hará una breve referencia a las cumarinas como ligandos de adenosina.

1.1.1.2. Síntesis de cumarina

La síntesis de cumarinas y de sus derivados es un área de gran y permanente interés por el gran número de compuestos de esta familia y el amplio espectro de actividades biológicas que presentan, tal y como se ha descrito anteriormente. En otros casos son muchas veces utilizadas como productos intermediarios en la síntesis de compuestos con potencial interés farmacológico[64] tales como piranocumarinas,[65] furocumarinas o 2-acil-resorcinoles.[66,67]

Clásicamente han sido estudiados varios procesos de síntesis para la obtención de estos derivados,[68,69] y si bien los primeros métodos presentaban demasiados pasos y en general bajos rendimientos,[70] hoy en día las rutas

sintéticas más utilizadas recurren a reacciones de condensación de **Pechmann**, **Perkin**, **Knoevenagel**, **Reformatsky** o **Wittig** (Figura 12).[71,72,73,74] La primera es, sin duda, la más usada por la simplicidad de los materiales de partida y por los moderados rendimientos obtenidos en la utilización de determinados substratos.[75,76]

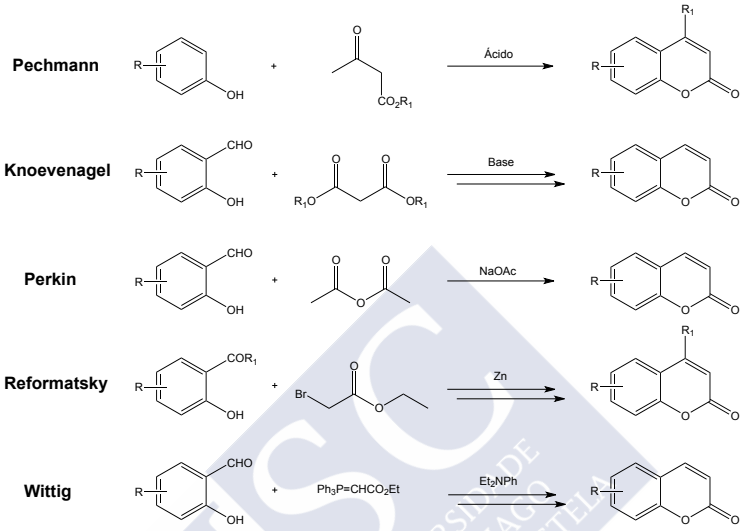


Figura 12. Métodos más comúnmente empleados en la síntesis de las cumarinas.

Hoy en día, sin embargo, es cada vez mayor la utilización de la "*Green Chemistry*" que tiende a disminuir el uso de solventes potencialmente tóxicos y/o caros, recurriendo a la utilización de zeolitas, resinas etc. en las reacciones de Knoevenagel y Wittig,[77] así como a técnicas de irradiación con microondas o la utilización de líquidos iónicos, no detalladas en esta Memoria.[78,79,80]

En general, las cumarinas son obtenidas por condensación de fenoles con *β*-cetoesteres, en presencia de un catalizador ácido. Esta reacción, conocida como reacción de Pechmann, cursa con la formación intermedia del éster fenólico y su posterior ciclación en medio ácido. Esta vía sintética es muy utilizada para la obtención de cumarinas naturales y otras benzopironas de interés biológico o industrial.[81]

La reacción de Perkin consiste en la formación de una cumarina mediante condensación aldólica entre un *orto*-hidroxibenzaldehído y el anhídrido de un ácido o el ácido, en presencia de la sal alcalina del mismo ácido.[82] En un primer

paso, el más rápido de la reacción, la base genera el anión enolato del anhídrido que reacciona con el aldehído (Figura 13).[83]

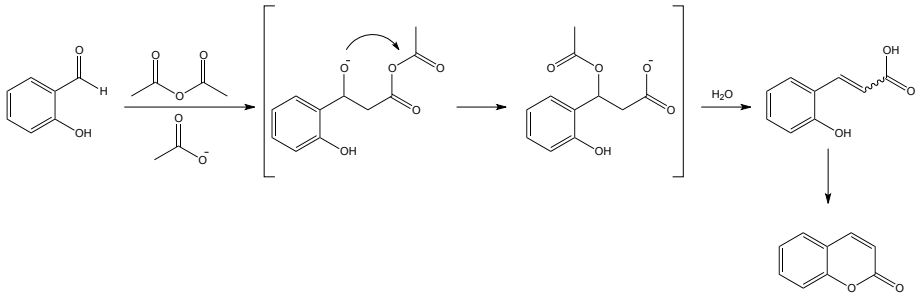


Figura 13. Mecanismo general de la reacción de Perkin.

En estos casos, el objetivo es mejorar las condiciones de reacción, disminuyendo el uso de reactivos, el tiempo de reacción, aumentando a la vez el rendimiento de la misma. Cambios de temperatura han sido muy favorables a esta reacción. Temperaturas muy bajas llevaban a reacciones incompletas, y temperaturas muy altas a mezclas de reacción muy complejas y de difícil purificación. La utilización de la *N,N'*-diciclohexilcarbodiimida (DCC), en dimetilsulfóxido (DMSO), favorece la ciclación posterior para formar el anillo cumarínico. Esta vía permite la reacción *one-pot* de una serie de cumarinas susceptibles de una síntesis en paralelo.

En el caso particular del trabajo realizado en esta Memoria, las reacciones se llevaron a cabo por condensación de salicilaldehídos sustituidos con los ácidos acéticos apropiados, usando DCC como agente deshidratante. Esta es una reacción muy versátil proporcionando diferentes familias de cumarinas sustituidas.

En particular, los derivados acetoxilados de la cumarina se han sintetizado mediante la reacción de Perkin-Oglialoro de los derivados del ácido acético con los respectivos salicilaldehídos, en anhídrido acético (Ac_2O) y acetato potásico (CH_3COOK). La reacción de Perkin-Oglialoro es de hecho la modificación más importante de la reacción tradicional de Perkin, donde se desarrolla la condensación de aldehídos aromáticos con ácidos α-arilacéticos en anhídrido acético y en presencia de una base débil (a través de anhídridos mixtos generados *in situ*) para obtener ácidos α-arilcinámicos.[84]

1.1.2. Los estilbenos (resveratrol)

El resveratrol[85] es un compuesto de origen natural presente en especies de espermatofitas, como las vides, en respuesta a un daño externo o interno, por ejemplo una infección por hongos o exposición a la radiación UV.[86] Su presencia depende mucho del origen geográfico, de la especie o de la forma de cultivo de las vides, entre otros factores.[87] Dado que se encuentra en la piel de la uva y no en la pulpa, existe en mayor concentración en el vino tinto que en el vino blanco (donde el tiempo de contacto con la piel de la uva en la fermentación es mucho más grande – mayor tiempo de maceración).[88] Fue por primera vez detectado en la especie *Vitis vinífera* (Figura 14), en 1976, por Langcake y Pryce[89] y en el vino tinto, en 1992, por Siemann y Creasy.[90]



Figura 14. *Vitis vinifera.*

El interés por este compuesto y sus derivados se incrementó cuando los estudios epidemiológicos establecieron una relación inversa entre el consumo de vino tinto y la incidencia de enfermedades cardiovasculares (paradoja francesa),[91] además de las propiedades hemostáticas y del aumento del HDL circulante descrita para el etanol.[92]

Estructuralmente el resveratrol es el 3,4',5-trihidroxiestilbeno que, debido a la presencia del doble enlace, puede asumir las dos formas isoméricas *trans* y *cis* (Figura 15).

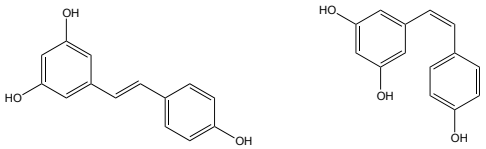


Figura 15. Formas isoméricas del resveratrol (*trans*- y *cis*-resveratrol).

La forma *trans* del resveratrol, parece ser la responsable de las principales propiedades farmacológicas de esta molécula. Es, hoy en día, un producto comercial común, a partir del que se puede obtener por irradiación UV la forma *cis*. El distinto comportamiento de estos dos isómeros depende de su estructura tridimensional.

La extracción del resveratrol y productos similares de las fuentes naturales es costosa en términos de tiempo, dinero y bajos rendimientos, por lo que su extenso estudio y proyección solo han sido posibles después de su síntesis en laboratorio. Hoy en día está bastante bien caracterizado, siendo sus bandas de UV (máxima absorbancia a 307 nm para la forma *trans* y 280 nm para la forma *cis*) y absorción IR (banda de 2800 a 3500 cm^{-1} para el grupo hidroxilo y 965 cm^{-1} para el doble enlace en su forma *trans*) bien definidas.[93]

Por su gran interés farmacológico, el resveratrol ha sido muy ampliamente estudiado en los últimos años.[94,95] Presenta un gran número de actividades destacando sobre todo sus propiedades antineoplásicas,[96] antiinflamatorias,[97] cardioprotectoras (vasodilatador e inhibidor de la agregación plaquetaria)[98] y antioxidantes,[99] mostrando ser muy eficiente convenientemente funcionalizado, como captador de radicales libres.[96,100] Por otra parte, se ha comprobado la actividad inhibidora de la MAO en los isómeros *cis* y *trans* del resveratrol, siendo el *cis*-resveratrol menos efectivo que el *trans*-resveratrol, sobre dicha enzima.[101]

Este interesante perfil farmacológico hace del resveratrol un importante prototipo a partir del cual se ha iniciado la búsqueda de análogos capaces de mejorar y/o incrementar su perfil farmacológico sobre todo en lo que respecta a su potencial aplicación en enfermedades relacionadas con la edad, sobre todo las neurodegenerativas.[102] Por este motivo, en la presente Memoria hemos incorporado dicha estructura a las ya mencionadas cumarinas con las que comparte en gran medida el mismo espectro terapéutico.

A partir de este cabeza de serie, se han desarrollado nuevos polifenoles casi siempre mediante modificaciones muy sencillas del prototipo pero para los que se han encontrado una interesante actividad biológica (Figura 16). De ellas, seguramente la mas estudiada es la actividad antioxidante.[103,104] Recientemente se ha llevado a cabo un estudio que establece la relación entre dicha actividad antioxidante y su relación con la quimioprevención del cáncer,[100,105] mediante variación del número y/o posición de los grupos hidroxilo sobre el esqueleto estilbenico.[106] Un estudio QSAR sobre dicho compuesto corrobora a su vez la influencia de factores estéricos, de tamaño y forma, de los sustituyentes sobre el esqueleto estilbénico.[107]

Por su parte, la introducción de sustituyentes sencillos tales como grupos metoxilo, amino, sulfato o átomos de halógenos, confiere al estilbeno diversas actividades tales como la antioxidante y neuroprotectora (debido a su actividad inhibidora de la agregación amiloide), encontrada también para los metabolitos sulfato conjugados a los que hemos aludido.[104,108]

Recientemente, se ha encontrado una interesante actividad antineoplásica de análogos polimetoxilados, actividad que sin embargo desaparece en los análogos hidroxilados.[109] Por su parte, la introducción de átomos de flúor, así como los grupos amino parecen incrementar dicha actividad.[110]

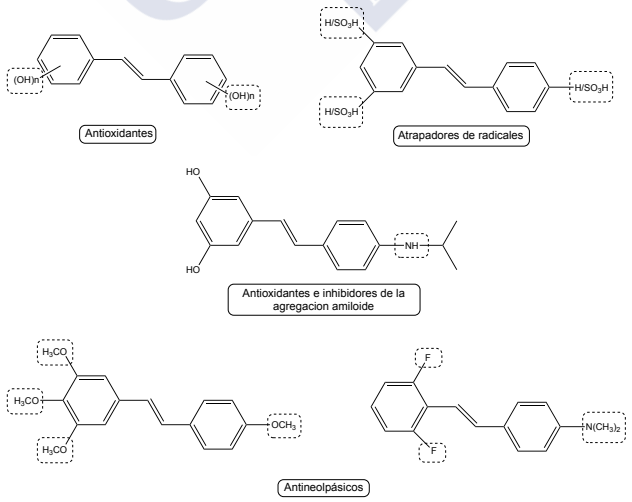


Figura 16. Modificaciones sencillas de la molécula del resveratrol (variación de sustituyentes sobre la estructura *trans*-estilbénica).

Modificaciones más drásticas sobre el resveratrol implican la obtención de imino análogos mediante la substitución isostérica del doble enlace etilénico, que conservan la actividad antioxidante,[111] y para los que se encontró una interesante actividad inhibidora de la agregación β-amiloide (βA) (Figura 17).[112]

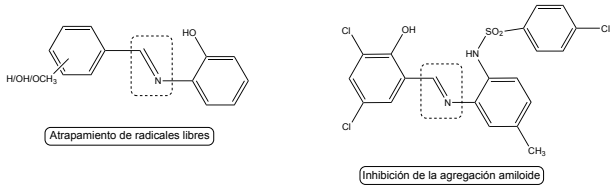


Figura 17. Substitución isostérica del doble enlace etilénico.

Otra interesante modificación sobre el estilbeno, supone la sustitución de uno de los dos anillos bencénicos por un anillo heterocíclico más o menos complejo o la condensación de este con otros ciclos, modificaciones que han permitido la preparación de inhibidores enzimáticos MAO-B como en el caso de los derivados de la estiril-cafeína[113] o de la estiril-isatina (Figura 18).[114]

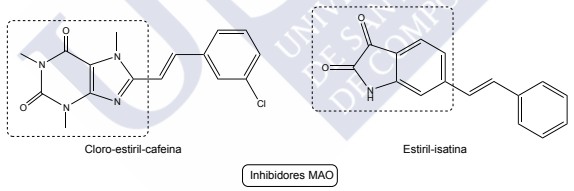


Figura 18. Substitución de uno de los dos anillos bencénicos por biciclos.

A su vez, la introducción de anillos heteroaromáticos del tipo tiofeno o benzotiazol (Figura 19), permite la síntesis de estilbenoides capaces de actuar, por sus propiedades fluorescentes, como pruebas de detección de las placas βA en la diagnosis de la EA.[115] El análogo piridínico Amyvid (Figura 19) es uno de los cuatro agentes utilizados en el diagnóstico de la EA en humanos.[116]

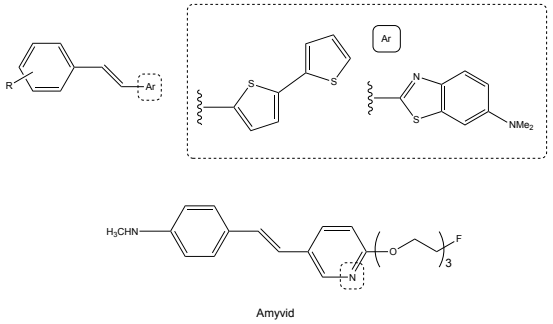


Figura 19. Introducción de anillos heteroaromáticos de tipo tiofeno, benzotiazol o piridina.

1.2. Enfermedades neurodegenerativas

Las enfermedades neurodegenerativas son cada vez más frecuentes con el envejecimiento general de la población mundial. El siglo XX fue testigo de un importante cambio demográfico en la población humana del mundo industrializado que se ha seguido de un cambio similar de la esperanza de vida a edades superiores en Asia, África y América Central y del Sur. La calidad de vida de la población de edad avanzada en la sociedad de hoy es, en gran medida, determinada por el proceso normal de envejecimiento de las neuronas en el sistema nervioso central (SNC) y especialmente por la aparición de enfermedades caracterizadas por la pérdida neuronal acelerada, que tradicionalmente son designadas como enfermedades neurodegenerativas. Estas enfermedades son las principales causas de morbilidad y discapacidad en todo el mundo.

La EA es la más prevalente enfermedad neurodegenerativa seguida de la enfermedad de Parkinson (EP). La EA es la causa más común de demencia senil, que afecta a aproximadamente el 3% de la población entre las edades de 65-74 años y casi el 50% de las personas de 85 años o más.[117,118] La esclerosis lateral amiotrófica (ELA) y la enfermedad de Huntington afectan a un número menor de pacientes, pero tienen consecuencias devastadoras. Un gran número de enfermedades neurodegenerativas raras tienen similares efectos en los pacientes y sus familias que están también afectadas por estas enfermedades. La investigación sobre la patogénesis y el tratamiento de estos trastornos está creciendo de manera exponencial. En gran medida esto ha sido

fomentado por importantes avances en la genética, ya que ahora sabemos que las mutaciones se asocian con un gran número de estas enfermedades. Esto incluye formas familiares de la EA, EP, la demencia fronto-temporal, ELA, enfermedad de Huntington y una variedad de ataxias. La mayor parte de los casos esporádicos de las neurodegeneraciones comunes, tales como EA, EP y ELA parecen ser de origen poligénica y son influenciados por factores ambientales todavía mal caracterizados. El objetivo final del diagnostico y terapéutica es prevenir estas enfermedades, ya sea en individuos en riesgo, antes de la aparición de manifestaciones clínicas o para desarrollar eficaces terapias neuroprotectoras que retardan o detienen la progresión de la enfermedad en las primeras etapas.

Las enfermedades neurodegenerativas son consideradas como uno de los retos más importantes de la medicina de hoy en día debido a su complejidad, la frecuencia de aparición y el desarrollo progresivo. Las enfermedades neurodegenerativas son procesos patológicos que se caracterizan por degeneración y muerte de las neuronas como consecuencia de la acumulación de proteínas anormales en su interior, resultado de la desregulación de diferentes neurotransmisores. Los procesos que generan este acúmulo de proteínas son sumamente complejos y en la actualidad aún no son totalmente conocidos. En función del estado de evolución del paciente este va a presentar síntomas concretos que con el paso del tiempo se van acentuando. Por lo tanto, si las neuronas afectadas pertenecen al hipocampo, zona relacionada con la memoria, el paciente perderá memoria y si el cuadro progresa desarrollará EA. Si la pérdida neuronal se localiza en la sustancia negra, el paciente desarrollará EP por tratarse de un área relacionada con el movimiento; y si lo que se afectan son las neuronas motrices el paciente desarrollará ELA, que se caracteriza por causar una debilidad progresiva.

Diferentes características y procesos bioquímicos tienen en común generar procesos patológicos trágicos y devastadores, siendo necesaria una gran inversión en la investigación para aliviar el sufrimiento humano causado por estas enfermedades.[117,118,119]

1.2.1. Enfermedad de Parkinson (EP)

La principal aplicación terapéutica de los inhibidores de la MAO-B es el tratamiento de la EP, un trastorno neurodegenerativo crónico y progresivo, caracterizado por una sintomatología predominantemente motora acompañada casi siempre de síntomas no motores como depresión y ansiedad, y que es debido a una disminución de los niveles de dopamina (DOPA) en el estriado por disfunción progresiva de neuronas nigroestriadas.[120]

Esta enfermedad ha sido descrita por primera vez en 1817, por James Parkinson, en el "*An Essay on the Shaking Pulse*", como un desorden neurológico severo. Es una de las enfermedades de mayor incidencia en ancianos (prevalencia estimada en 1% de la población por encima de los 60 años de edad) y es caracterizada por cuatro señales esenciales: rigidez, temblor, bradicinesia e inestabilidad postural.[121] Desde un punto de vista patológico, la EP se caracteriza por la pérdida de las neuronas dopaminérgicas de la vía nigroestriada, que se proyectan desde la zona compacta de la sustancia negra (SNC) hasta el estriado. Los síntomas de la EP aparecen cuando se ha destruido en torno al 80% de las neuronas dopaminérgicas de esta vía. Además de esta pérdida neuronal, en la neurofisiopatología de la EP también aparecen cuerpos de Lewy, inclusiones de proteínas citoplasmáticas, que no sólo están presentes en neuronas dopaminérgicas, sino también en sistemas noradrenérgicos (*locus coeruleus*), serotonérgicos (rafe), y colinérgicos (núcleo basal de Meynert). Los cuerpos de Lewy están compuestos por distintas proteínas como la α-sinucleína, la parkina, y la ubiquitina. No son exclusivos de la EP, pudiendo encontrarse también, por ejemplo en la EA, o en la demencia de cuerpos de Lewy. El papel que desempañan los cuerpos de Lewy en la EP no está todavía esclarecido.

La etiología de esta enfermedad está todavía por esclarecer completamente debido a la complejidad y variedad de los factores implicados en su desarrollo. Son posibles causas de la EP la acción de neurotoxinas ambientales, producción de radicales libres, anomalías mitocondriales, predisposición genética, envejecimiento cerebral, o incluso una asociación de más de uno de estos factores. En lo que se refiere a la patogénesis hay abiertas varias hipótesis de investigación, que incluyen distintos mecanismos que parecen estar implicados en el desarrollo de la enfermedad tales como el estrés

oxidativo, uno de los primeros factores de los que se pensó que podía contribuir al desarrollo de esta enfermedad. Basado fundamentalmente en la formación de especies reactivas de oxigeno (ROS) a partir del metabolismo oxidativo de la DOPA, provocando un aumento de los niveles de hierro y una disminución de los niveles del glutatión reducido. Hay evidencias de que la inflamación es otro proceso que podría estar implicado en la patogénesis de la EP. También la excitotoxicidad, un proceso patológico que es capaz de dañar a las neuronas y en el que aumenta la concentración de glutamato, que a través de radicales libres acaba provocando la muerte de las células por distintos mecanismos: apoptosis y autofagia. Por estos motivos, es necesario un gran esfuerzo en el estudio y nuevas alternativas terapéuticas de dicha enfermedad, alternativas que hoy en día, tratan de paliar los síntomas de la EP, a la vez de otras que inhiben la evolución del proceso neurodegenerativo. En otros casos, se recurre a terapias que actúan en más de una diana, produciendo beneficios sintomáticos y neuroprotectores, terapias que suelen ser más efectivas en el tratamiento de esta compleja enfermedad.[122]

En los años 50, Arvid Carlsson demostró que la DOPA era un neurotransmisor y sus niveles en los ganglios basales eran bajos en modelos animales de parkinsonismo. Sus trabajos fueron la base para el desarrollo de distintos ensayos con levodopa (L-DOPA). Gracias a los buenos resultados del primer ensayo controlado de L-DOPA frente a placebo, publicados por Yahr y col. en 1969, este fármaco se convirtió en fundamental para el tratamiento de la EP. La principal estrategia actual para el tratamiento de la EP está enfocada a la restauración de la función dopaminérgica en el tejido estriado del cerebro.[123] El principal objetivo de esta terapia es aumentar/mantener los niveles normales de DOPA a nivel del SNC, donde existe un déficit de la inervación dopaminérgica, como consecuencia de le degeneración de las neuronas dopaminérgicas nigroestriales y consiguiente bajada de las reservas de DOPA.[124]

Desde hace muchos años el aumento de los niveles de DOPA se consigue mediante administración de L-DOPA,[125] el intermediario metabólico precursor de la DOPA, en combinación con inhibidores de la dopa descarboxilasa periférica (DDC). Al mismo tiempo son administrados fármacos agonistas dopaminérgicos que mimetizan los neurotransmisores mediadores de la

DOPA.[91] Esta estrategia es la metodología común para el tratamiento de la enfermedad. Así, el aumento de los niveles de DOPA se puede conseguir con diversas estrategias:

- Facilitando la síntesis de la DOPA;
- Favoreciendo la liberación de la DOPA de los depósitos presinápticos;
- Disminuyendo la recaptación de la DOPA;
- Inhibiendo el catabolismo de la DOPA;
- Estimulando los receptores dopaminérgicos.

Estas terapias dopaminérgicas ofrecen un efectivo alivio de los efectos motores asociados con la enfermedad, especialmente en estados poco avanzados de la misma. Sin embargo, la restitución de los niveles de la DOPA no evita la progresión neurodegenerativa de la EP y con el tiempo progresa la pérdida neuronal y la eficacia de los fármacos si va disminuyendo.[92]

Por este motivo, en los últimos años han surgido nuevas alternativas terapéuticas como la utilización de inhibidores de la catecol-*o*-metiltransferasa (COMT), como el caso de la entacapona, y de inhibidores selectivos de la MAO-B,[126,127] de los cuales están comercializados en España la selegilina[128] y la rasagilina (además de su efecto inhibidor de la MAO-B, es también un importante neuroprotector)[129] y a las que haremos alusión mas adelante.

La selegilina ha sido el primer IMAO-B que se utilizó en clínica. Administrada conjuntamente con L-DOPA permitía reducir la dosis a administrar de ésta. En monoterapia en pacientes con EP en estadios tempranos podía retasar el comienzo del tratamiento con L-DOPA. La selegilina se metaboliza a derivados anfetamínicos, responsables de sus efectos secundarios y de la reducción del efecto neuroprotector. Uno de ellos, la metanfetamina es neurotóxica e interfiere con la acción neuroprotectora de la misma.

La rasagilina es otro IMAO-B, más potente y selectivo, su principal diferencia respecto a la selegilina es que no se metaboliza en anfetamina o metanfetamina. Se metaboliza dando un único compuesto que, a diferencia de lo que ocurre con los metabolitos de la selegilina, el 1-aminoindano ha mostrado actividad neuroprotectora.

1.2.2. Enfermedad de Alzheimer (EA)

La EA, como dicho anteriormente, es una enfermedad neurodegenerativa y progresiva, que supone la causa más común de demencia senil.[130] La demencia es un trastorno cerebral que afecta gravemente la capacidad de una persona de llevar a cabo sus actividades cotidianas. La enfermedad debe su nombre al Dr. Alois Alzheimer que la ha descrito por primera vez en 1906. Sus síntomas incluyen pérdida de la memoria, problemas de lenguaje y comportamiento impredecible. La EA comienza lentamente y progresa al largo del tiempo, primero afectando las partes del cerebro que controlan el pensamiento, la memoria y el lenguaje. La repercusión de esta enfermedad es tal, que se calcula entre 18 y 22 000 000 de personas afectadas a nivel mundial, con una prevalencia media entre el 3 y el 15% y una incidencia anual entre 0,3 y 0,7%.

El desarrollo de la EA, está muy relacionado con los cambios histopatológicos que ocurren en el cerebro de estos pacientes. Estos cambios neuropatológicos, entre los que se citan: la pérdida de neuronas y sinapsis, la angiopatía amiloidea, la placa senil, el cambio neurofibrilar de Alzheimer y el descubrimiento más reciente de las placas no amiliodes "AMY plaque", ocurren o se inician antes del comienzo de la declinación cognitiva propia de la enfermedad (Figura 20).

Existen evidencias, en pacientes con EA, de atrofia del hipocampo, liberación de la proteína Tau por las neuronas lesionadas, uso de sustratos como energía alternativa, depósito excesivo de la proteína βA, presencia del alelo de la apolipoproteína E4 y mutaciones en los genes del precursor de la proteína amiloide (PPA) (cromosoma 21) y la presenilina 1 y 2 (cromosomas 14 y 1 respectivamente).[131]

Aunque esta patología tiene como hemos indicado una etiología múltiple, parece ser fundamentalmente debida a la acumulación de placas βA en el cerebro, lo cual puede promover la degeneración o atrofia de las neuronas colinérgicas, fundamentalmente en la corteza cerebral y en el hipocampo. En consecuencia, clásicamente se ha recurrido al uso de los inhibidores de la AChE para su tratamiento farmacológico.[132,133]

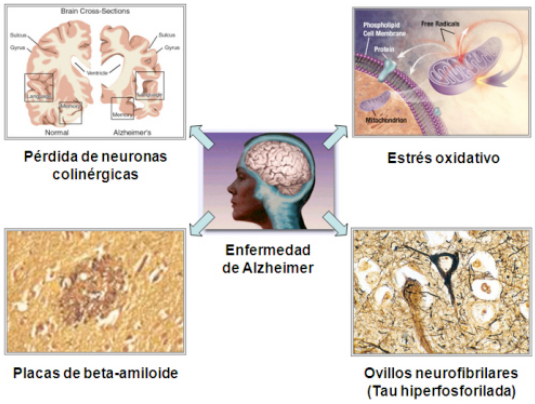


Figura 20. Algunas de las características más comunes de la EA.

En la actualidad, sin embargo, se están generando nuevas expectativas al tener en cuenta otros aspectos relacionados con la etiología de la enfermedad tales como la disminución en los niveles de DOPA, noradrenalina (NA) y serotonina o 5-hidroxitriptamina (5-HT), o bien el aumento de la actividad MAO-B cerebral, lo que origina un incremento de radicales libres, responsables del estrés oxidativo y muerte celular, así como del desarrollo de las placas βA en los enfermos que padecen de EA.[134] Aunque se requieren más estudios para su clarificación, se cree que el efecto beneficioso de los inhibidores selectivos de la MAO-B como la selegilina es debido a un doble efecto: reducción en la formación de radicales libres y de incremento en los niveles de monoaminas en el cerebro de dichos enfermos.[135]

Por la complejidad de la etiología de esta enfermedad, la terapéutica actual se encamina al desarrollo de fármacos mutidiana en concreto a la obtención de moléculas que incorporan fragmentos estructurales responsables de una inhibición de las diferentes enzimas implicadas BACE, MAO-B, y/o de AChE[136,137] y que adicionalmente confieran a las moléculas características antioxidantes.[138,139,140,141] Esta es como indicamos una de las metodologías más prometedoras y en la que nuestro grupo de investigación está trabajando.

1.3. Principal diana farmacológica en la Memoria - MAOs

La MAO (E.C 1.4.3.4) fue descubierta por Mary Hare en 1928 y la denominó tiramina oxidasa. Posteriormente se vio que la oxidación también afectaba a varias etilaminas como la DOPA, NA, adrenalina (A), 5-HT y entonces se reconoció como MAO. Al tener como sustratos a neurotransmisores tan importantes como estos, parece clara la influencia que tendrá esta enzima en distintas funciones y patologías cerebrales.[142]

Las MAO son una familia heterogénea de flavoenzimas que catalizan la desaminación de neurotransmisores y aminas exógenas.[143,144,145] Las MAO tienen unido de forma covalente el cofactor FAD.[146] Las MAO están presentes en las membranas externas de las mitocondrias de células neuronales de la glía, entre otras.[147] La distribución de las MAO no se restringe al SNC sino que también están presentes en la periferia. La MAO-A predomina en hígado y tracto gastrointestinal y la MAO-B en las plaquetas, en el cerebro y en menor cantidad en el hígado. Ambas isoformas están presentes en el cerebro, aunque en distinta proporción (75-80% de MAO-B) y con diferente distribución, la MAO-B es la forma mayoritaria en los ganglios basales y predomina en neuronas serotonérgicas y células de la glía, a diferencia de la MAO-A que abunda en neuronas adrenérgicas, dopaminérgicas y catecolaminérgicas.[148,149]

Como catalizadores de la desaminación oxidativa de las monoaminas, las MAO utilizan el oxígeno para eliminar un grupo amino de una molécula, resultando el correspondiente aldehído y amoniaco, con la generación de peróxido de hidrógeno (Figura 21). Los aldehídos se metabolizan rápidamente a los correspondientes ácidos. Las MAO catalizan la desaminación oxidativa de neurotransmisores y aminas biógenas, actuando sobre aminas primarias, y también en algunas secundarias y terciarias. En esta reacción son formadas especies reactivas de oxigeno (ROS), siendo que compuestos capaces de desarrollar mecanismos antioxidantes de destoxificación pueden ser interesantes adyuvantes en la terapéutica.

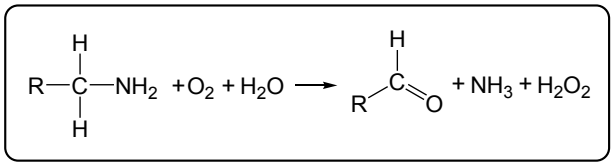


Figura 21. Reacción de desaminación de neurotransmisores por las MAO.

Las dos isoformas denominadas MAO-A y MAO-B (Figura 22)[150,151] han sido identificadas en base a su secuencia de aminoácidos, su estructura tridimensional y la preferencia por determinados sustratos e inhibidores específicos.[152] Aunque compartan 70% de la identidad de sus secuencias de aminoácidos,[153] la MAO-A tiene mayor afinidad por 5-HT, A y NA[154,155] mientras que la MAO-B desamina preferentemente la β-feniletilamina y la bencilamina.[156,157] Estas propiedades determinan la importancia clínica de los inhibidores de la MAO[158] como promisores fármacos para el tratamiento de enfermedades neurodegenerativas, depresión y/o obesidad.[159] Existen evidencias de que con el paso de los años, a medida que progresa el envejecimiento aumenta la actividad cerebral de la MAO-B pero no la de la MAO-A. Los mayores aumentos aparecen en los ganglios basales y en el tálamo, seguidos de la corteza frontal y en menor medida, cerebelo y cortezas parietal y temporal.[160]

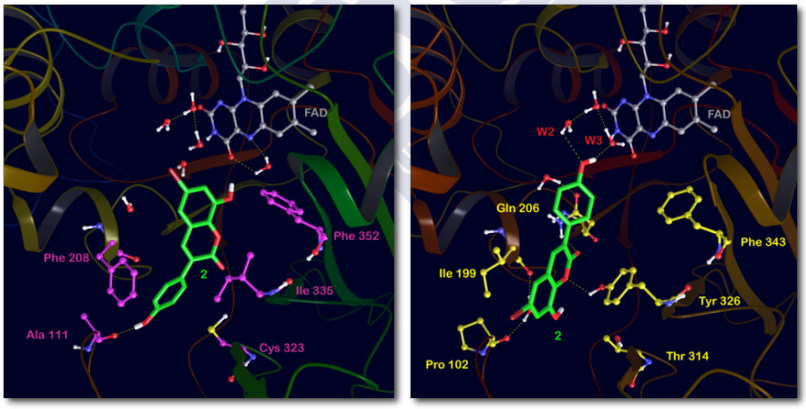


Figura 22. Diagrama virtual de los sitios activos de las isoformas MAO-A y MAO-B con un ligando 3-arilcumarínico (imágenes del trabajo descrito en esta Maemoria).

El centro activo de las MAOs, donde se unirá el sustrato, es una gran cavidad que se extiende desde el sitio de unión del FAD en el centro de la enzima a la superficie de la proteína en el lado opuesto del anillo de adenosina. Esta cavidad es de mayor tamaño en la isoforma MAO-B que en la MAO-A y en realidad, se compone de dos espacios separados: la cavidad del sustrato y la cavidad de entrada, la segunda frente al bucle 99-112. El centro activo de la MAO-B es un ejemplo de flexibilidad ya que puede ser una gran cavidad única o una cavidad dividida en dos según la conformación del aminoácido Ile 199.

Otro aminoácido que también está implicado, aunque no directamente, en la división en dos cavidades es el Tyr 326. De hecho, este par de residuos de aminoácidos junto con los Phe 208-Ile 335 en el caso de la MAO-A determinan diferencias específicas entre sustratos e inhibidores de ambas enzimas.[161]

Los primeros inhibidores de la MAO tales como la isoniazida y iproniazida eran no selectivos pero tras el descubrimiento de las distintas isoformas de la MAO comenzaron a desarrollarse inhibidores relativamente específicos. Los inhibidores MAO-A selectivos (IMAO-A) como la clorgilina o la moclobemida son utilizados en el tratamiento del desórdenes neurológicos tales como la depresión, mientras que los IMAO-B selectivos, tales como la selegilina y la rasagilina, son de utilidad y están autorizados en España para el tratamiento de la EP.[162,163] Estos dos fármacos inhiben de forma selectiva e irreversible a la MAO-B, así que no presentan los efectos no deseados de los inhibidores no selectivos. Al inhibir a la MAO-B, incrementan el tiempo de DOPA en el espacio sináptico, ya que entorno al 80% de la DOPA es metabolizada por esta enzima. Pierden la selectividad por la MAO-B a dosis altas, aumentando las reacciones adversas. Si se administran conjuntamente con L-DOPA pueden aparecer síntomas de hiperactividad dopaminérgica que se solucionan disminuyendo la dosis de L-DOPA administrada. Estos fármacos se pueden utilizar en estadios tempranos de la enfermedad por su posible efecto neuroprotector, ya que durante el metabolismo normal de la DOPA por la MAO-B se pueden generan radicales libres y también para tratar los fenómenos de pérdida de respuesta a L-DOPA.

La selegilina fue el primer IMAO-B que se utilizó en el tratamiento de la EP. Es un derivado de propargilamina que inhibe de forma irreversible a la enzima. La absorción a través del tracto gastrointestinal y la distribución son rápidas. Cruza la barrera hematoencefálica y se acumula en regiones cerebrales ricas en MAO-B. Se metaboliza a N-desmetilselegilina, L-metanfetamina y L-anfetamina.

La rasagilina tiene eficacia mejorada ya que presenta propiedades neuroprotectoras y tiene la ventaja de que se metaboliza en metabolitos no tóxicos. Al igual que la selegilina, es un derivado de propargilamina que inhibe a la enzima de forma irreversible. Se comporta como un inhibidor suicida y la interacción tiene lugar entre el N del grupo propargil y el N-5 del anillo de

isoaloxazina del FAD. Se metaboliza por N-desalquilación a nivel hepático, dando un único compuesto no tóxico el 1-(R)-aminoindano. A diferencia de lo que ocurre con los metabolitos de la selegilina, el 1-aminoindano ha mostrado actividad neuroprotectora.[164] El perfil farmacológico de los IMAO-B no se limita a la inhibición de esta enzima sino que pueden tener un efecto neuroprotector, efecto antioxidante, protección frente a neurotoxinas, aumento de la liberación de DOPA, etc. Algunos de los inhibidores (MAO-A y MAO-B) de referencia están recopilados en la Figura 23.

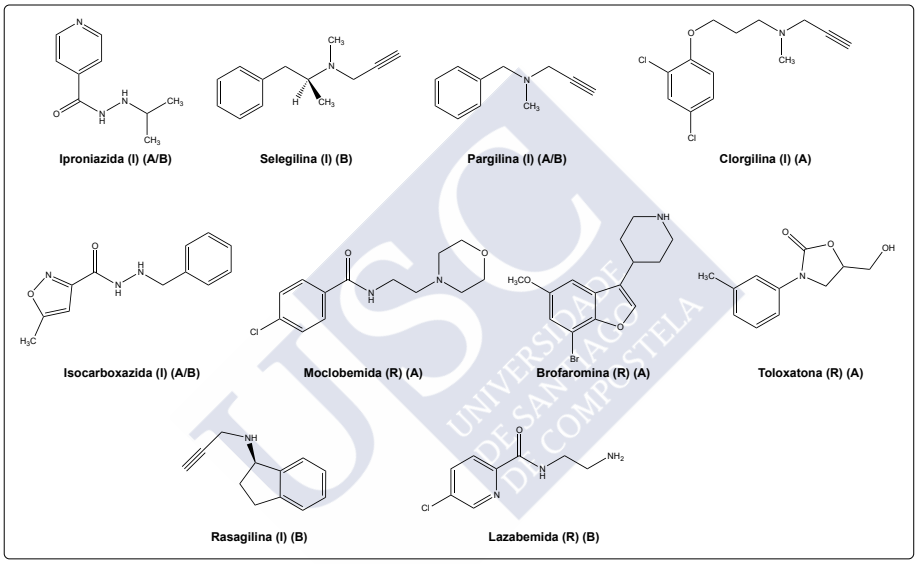


Figura 23. Inhibidores MAO-A y MAO-B reversibles (R) e irreversible (I).

La reacción de desanimación oxidativa catalizada por las MAO-A y MAO-B parece ser el mecanismo mayoritario sufrido por la DOPA a nivel del estriado (Figura 24). Inhibiendo estas enzimas a nivel central, se consigue inhibir la depleción de los niveles de la DOPA y elevar los niveles de DOPA endógena y de DOPA producida por administración de L-DOPA exógena.[165]

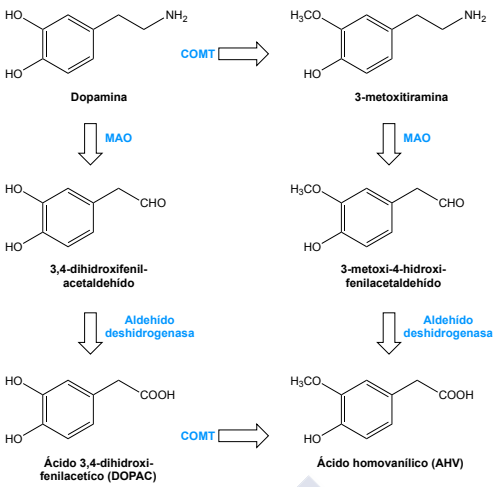


Figura 24. Metabolismo de la DOPA.

1.3.1. Cumarinas y las MAOs

En 1981 se encontró por primera vez una interesante actividad IMAO del núcleo de las cumarinas, cuando un grupo organofosforado fue unido al anillo de la umbeliferona.[166] Pero no fue hasta el 1990 cuando el núcleo de las cumarinas ha sido convertido en un prometedor esqueleto con propiedades inhibidoras de las MAOs.[167,168] En particular, algunas cumarinas aisladas de *Psoralea corylifolia* L., *Peucedanum japonicum* L. y *Monascus anka* K. han sido descritas como IMAOs.[169] Aunque las cumarinas naturales, en general, muestran baja potencia inhibidora de las MAOs, modificaciones sobre estas cumarinas naturales han llevado a la obtención de productos que han sido caracterizados como potentes y selectivos IMAOs. Por ejemplo, el análogo desmetilado de la ya mencionada geiparvarina, una cumarina natural 7-substituida, extraída de las hojas de *Geijera parviflora* L., exhibe una potente y selectiva inhibición de la MAO-B (Figura 25).[170]

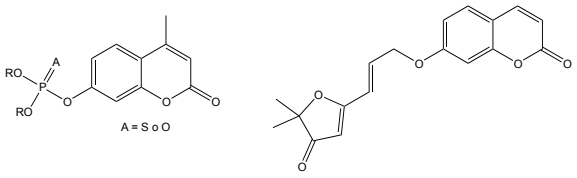


Figura 25. Derivados de la umbeliferona y de la geiparvarina.

Varios grupos de investigación han explorado la importancia del número y posición de los diferentes sustituyentes en el núcleo de cumarina, teniendo en cuenta el volumen estérico, lipofilia y/o propiedades electrónicas, con el fin de mejorar la actividad y la selectividad (Figura 26).

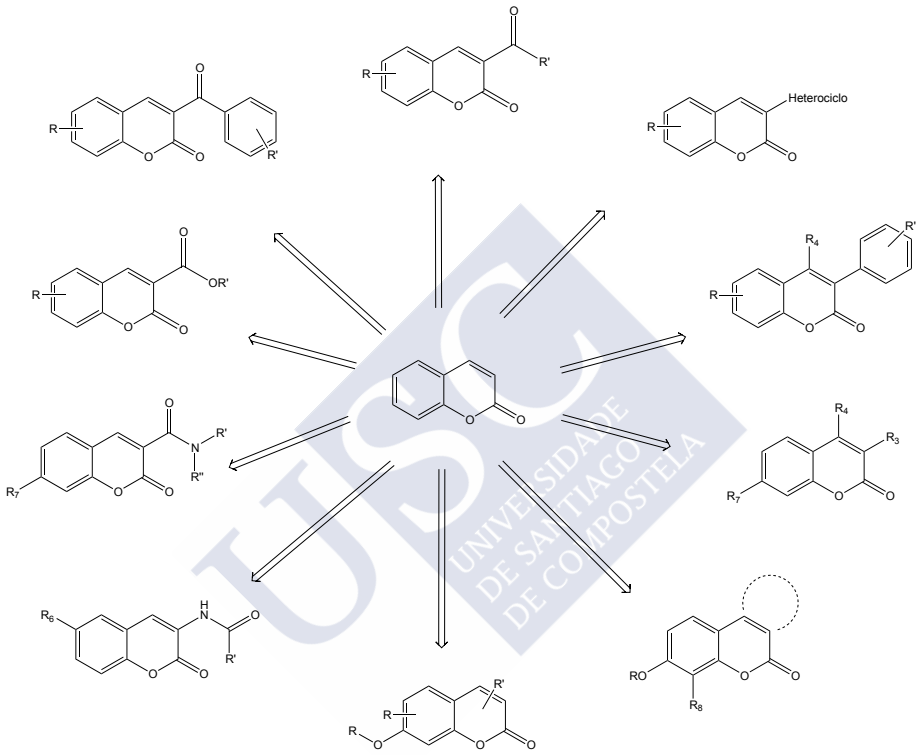


Figura 26. Estructuras químicas de cumarinas estudiadas como IMAOs (parte de las estructuras pertenecen al trabajo descrito en esta Maemoria).

Aunque las posiciones 3 y 7 del anillo de cumarina han sido las más estudiadas, todas las posibilidades de sustitución se han tenido en cuenta y han sido sintetizados y evaluados un gran número de derivados de la cumarina como IMAOs, siendo la mayoría de ellos activos y selectivos frente a la isoenzima MAO-B.[171]

Los primeros estudios de relación estructura-actividad (REA) sobre derivados de las cumarinas han sugerido que la selectividad viene determinada principalmente por la naturaleza de la unión entre la cumarina y el grupo

sustituyente en la posición 7. Una serie de 7-hidroxicumarinas con sustituyentes de tipo éter, éster o carbamato en la posición 7, con tamaño variable y diferente lipofilia, han sido investigados por su actividad inhibidora de las MAO-A y MAO-B. La mayoría de estos compuestos resultó ser preferentemente IMAO-B. Estudios de REA también han demostrado que la lipofilia es una propiedad importante para modular la potencia de inhibición de la MAO-B de las cumarinas anteriormente mencionadas. La presencia de un grupo benciloxi en posición 7 resultó favorecer la unión de las cumarinas a la MAO-B, mientras que un anillo de fenilo π-pobre en electrones en esa misma posición si bien parece mejorar tanto la actividad inhibitoria MAO-A como MAO-B, dando lugar a inhibidores menos selectivos. Por otra parte, se ha demostrado que un grupo alquilsulfoniloxi en la posición 7 proporciona actividad IMAO-A, como es el caso de la esuprona. Este efecto es aún más pronunciado en los grupos fenilsulfonatos, en que la sustitución con grupos aceptores de electrones conduce a más potentes y altamente selectivos IMAO-A. También, B. Rendenbach-Mueller y col. han sintetizado y estudiado el potencial de derivados de éteres y ésteres de ácido sulfónico (Figura 27), comprobando que la introducción de un enlace éster sulfónico, en lugar del puente éter, cambia drásticamente el perfil de actividad de los nuevos compuestos.[51]

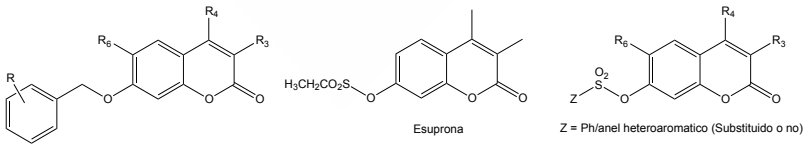


Figura 27. Derivados cumarínicos 7-alkiloxi y sulfoniloxi substituidos.

Sustituciones en las posiciones 3 y/o 4 del núcleo de cumarina también contribuyen a modular la actividad inhibidora y la selectividad MAO-B de los derivados anteriormente descritos. Se puede inferir que el sitio de unión de la MAO-B debe aceptar sustituyentes lipofílicos de tamaño limitado en las posiciones 3 y/o 4 del núcleo de cumarina, mientras que los grupos hidrófobos, hidrófilos y/o más grandes, no son bien tolerados. Sin embargo, los derivados de 7-benziloxicumarinas, teniendo sustituyentes polares debidamente

seleccionados en la posición 4, condujeron a nuevos IMAO-B con un mejor perfil farmacocinético.[172]

B. Rendenbach-Müller y col. también han explorado la importancia de la presencia de un grupo heteroarilalcoxi en la posición 7 del esqueleto de cumarina para modular la actividad contra las isoformas de la MAO. En este estudio, los pequeños grupos tipo alquilo, fenilo o halógeno se introdujeron en las posiciones 3 y 4 del esqueleto. Los mismos autores han evaluado y descrito heteroarilalcoxicumarinas como potentes y selectivos IMAO-B[123,173] en los que se introdujeron pequeños sustituyentes, como grupos metilo, en las posiciones 3 y 4 de este núcleo de cumarina.

Desde el trabajo pionero de H. A. Kadir y col., y el descubrimiento del potencial del esqueleto de la cumarina frente a ambas isoformas de la MAO, han sido sintetizadas nuevas generaciones de moléculas diferentemente sustituidos y evaluadas *in vitro* en ensayos farmacológicos. Algunos de los derivados descritos son compuestos muy activos y selectivos, con CI_{50} en el rango nanoMolar. Sobre la base de estos importantes estudios, hay varios proyectos en curso para el diseño racional de fármacos basado en la estructura de las nuevas moléculas.[174] Estos descubrimientos sin duda facilitarán la obtención de nuevas terapéuticas eficaces y seguras de un problema creciente e importante de los países desarrollados: las enfermedades neurodegenerativas.

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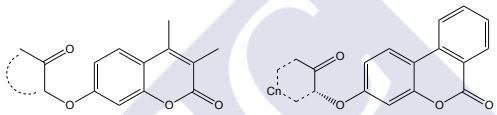
2. Antecedentes y objetivos

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2.1. Antecedentes

En los últimos años se ha hecho un gran esfuerzo por conocer el mecanismo molecular de la interacción de los IMAO con los receptores de las MAO y ello ha originado un elevado número de compuestos con esta actividad.

Nuestro grupo ha elaborado una base de datos con la actividad IMAO experimental de más de 1000 compuestos entre los que había algunas estructuras cumarínicas preparadas en nuestro laboratorio (Figura 28). No solo se encontraron algunos compuestos de interés y con cierta selectividad MAO-A, sino que además se ha podido establecer un modelo QSAR capaz de predecir la actividad de actividad IMAO nuevas moléculas.[178,179]



IMAO-A IMAO-B

Figura 28. Algunos derivados preparados previamente en el laboratorio.

Desde su origen nuestro grupo de investigación viene estudiando el esqueleto cumarínico y su modificación estructural dirigida a diferentes intereses farmacológicos,[18,56,175,176,177,178,179] habiendo realizados varias revisiones recientes sobre este tema.[11,15]

Además de los referidos estudios de actividad IMAO, realizados en colaboración con el *Laboratorio de Farmacología* de nuestra Facultad, estábamos también colaborando en el estudio de otros prototipos cumarínicos, como potenciales cardioprotectoresy/o vasodilatadores.[15] El destacado investigador de este grupo, Francisco Orallo, trabajaba desde hace tiempo en este campo obteniendo excelentes resultados y de manera muy destacada con el resveratrol, demostrando que posee una interesante actividad vasodilatadora e inhibidora de la agregación plaquetaria, propiedades que permiten considerar al mismo como un interesante cardioprotector.[55,87]

A lo largo del trabajo presentado en esta Memoria hemos también tenido en cuenta como referencia para el diseño de algunos de los compuestos estudiados como neuroprotectores, las estructuras de la selegilina o la

rasagilina como IMAO-B, o de la AP2238 o la fenserina como inhibidores de AChE (Figura 29), incorporando sobre el esqueleto cumarínico o 3-arilcumarínico partes estructurales de estos compuestos buscando, en algún caso, fármacos multidiana.

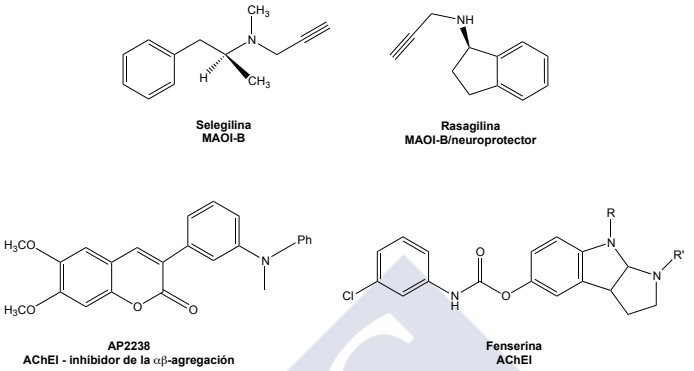


Figura 29. Algunos de los compuestos de referencia usados en el diseño de los nuevos compuestos descritos en esta Memoria.

Utilizamos también como estructuras de referencia la umbeliferona, conocida como inhibidor de la tirosinasa o la novobiocina, conocida como antibacteriano. También las estructuras de la quercetina y de la catequina, importantes antioxidantes cumarínicos, han sido fuente de inspiración para los estudios electroquímicos y de capacidad antioxidante de nuestras moléculas.

2.2. Objetivos

Debido a las propiedades encontradas en el resveratrol y algunas cumarinas,[178] nos ha parecido muy interesante diseñar y sintetizar compuestos que incorporasen en su esqueleto estas dos estructuras y que a su vez pueden considerarse análogos estructurales de las citadas 3,4-benzocumarinas en las que el anillo bencénico es introducido en la posición 3 de la misma (Figura 30).

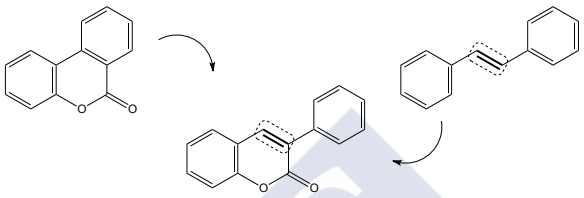


Figura 30. Derivados híbridos cumarina-resveratrol.

Así pues hemos planteado el estudio exhaustivo de estas nuevas estructuras híbridas, 3-arilcumarinas, en las cuales el anillo cumarínico comparte el anillo bencénico y el doble enlace con el resveratrol de forma que este queda bloqueado como isómero *trans.*[179] Además se pensó en ampliar el estudio a híbridos donde la posición 3 estuviese sustituida por diferentes heterociclos (Figura 31).

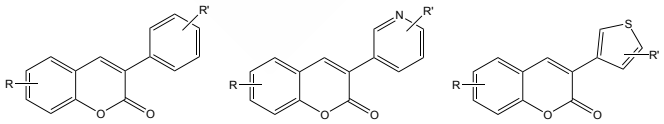


Figura 31. Prototipo estructural del esqueleto de las 3-aril y 3-heteroarilcumarinas (R y R' pueden ser átomos de bromo o cloro, o bien grupos alquilo, alcoxi, hidroxilo, nitro, amino, etc).

Se intentaría la síntesis de estas series utilizando metodologías lo más directas y versátiles posible intentado estrategias de alta eficacia, que nos permitiesen obtener los diferentes compuestos con alta pureza y en cantidad suficiente para los ensayos farmacológicos previstos.

El estudio farmacológico inicial suponía observar la eficacia de series seleccionadas de estos compuestos sobre determinadas dianas con interés en

problemas relacionados con la edad y en particular con trastornos neurodegenerativos. Especialmente MAO y AChE, implicadas de manera muy fundamental en enfermedades tales como la EP o la EA respectivamente.

Además, se pretendía asociar a estas propiedades mas específicas, otras que pudiesen coadyuvar como son las propiedades antioxidantes.

La selección de estas series se realizaría con dos visiones complementarias: a) introducir sustituyentes de diferente naturaleza en distinto número y posición, atendiendo fundamentalmente a variabilidad estérica, electrónica y/o de lipofilia (átomos de cloro o bromo, grupos arilo, alquilo, alcoxilo, hidroxilo, nitro, amino y/o combinaciones de varios de estos átomos y/o grupos, utilizado el diagrama de Craig) y b) utilizando datos precedentes y la lógica bioquímica, además de la ayuda que pudiésemos obtener por el uso de metodologías computacionales tanto de QSAR como de modelado molecular.

En otras series, la posición 3 de la cumarina se substituyó por puentes de tipo éster, amida y carbamato, incorporando rasgos estructurales específicos presentes en moléculas de utilidad clínica. En el sentido de explotar diferencias de reactividad y de actividad, los mismos sustituyentes han sido introducidos en diferentes posiciones del núcleo cumarínico (Figura 32).

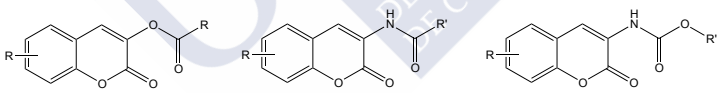


Figura 32. Prototipo estructural de diferentes derivados sintetizados y estudiados (R y R' pueden ser diferentes grupos).

Fue también un marcado objetivo intentar explorar con estas series o con modificaciones específicas de las mismas en algunos casos, otras dianas farmacológicas o intereses terapéuticos. Así, se pensó en explorar actividades quimioterápicas de diferente tipo, otras inhibiciones enzimáticas como la tirosinasa, o interacción con otros receptores como los de adenosina… y esta es todavía una tarea inacabada.

El objetivo global de la Tesis fue realizar un exhaustivo estudio de REA en este grupo de cumarinas y su interés en trastornos neurodegenerativos, siguiendo una metodología cíclica de síntesis, evaluación farmacológica, diseño y de nuevo síntesis, intentando conocer mejor los procesos involucrados

y obtener moléculas de alto interés como nuevos fármacos. Un objetivo colateral pero no olvidado fue también explorar el valor de estas estructuras o análogos muy próximos, con intereses farmacológicos diferentes.

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3. Discusión de resultados

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El trabajo descrito en esta Memoria incluye la síntesis de series seleccionadas de compuestos y su caracterización estructural con la pureza y la calidad necesarias para su estudio farmacológico. Además, incluye estudios teóricos de las mismas. Parte de estos estudios son fruto de interesantes colaboraciones con grupos de la Universidad de Santiago de Compostela (en colaboración con el Prof. Francisco Orallo y la Prof. Dolores Viña han sido realizados estudios de inhibición de la MAO-A, MAO-B, AChE y estudios de neuroprotección, y en colaboración con la Prof. Ysabel Santos y la Prof. Angeles Muñoz han sido realizados estudios antibacterianos), de la Universidad de Cagliari (en colaboración con la Prof. Marcella Corda han sido realizados estudios de inhibición de la tirosinasa), de la Universidad de Coimbra (en colaboración con la Prof. Ana Maria Brett han sido realizados estudios electroquímicos), de la Universidad de Santiago de Chile (en colaboración con el Prof. Claudio Olea han sido realizados estudios de evaluación de la capacidad antioxidante) y de la Universidad de Würzburg (en colaboración con el Prof. Karl-Norbert Klotz han sido realizados estudios en receptores de adenosina). Para tratar de explicar los datos experimentales obtenidos, y extraer información de los ensayos *in vitro* y *in vivo*, han sido realizados estudios de *docking* y estudios teóricos de capacidad de paso de membranas y otros parámetros de ADME (en colaboración con Santiago Vilar y Giulio Ferino). Sin el esfuerzo conjunto no habríamos llevado a cabo todos estos proyectos ni habíamos obtenido los resultados presentes en esta Memoria.

Estos estudios interdisciplinares demuestran que algunos de los compuestos evaluados son más activos y más selectivos que los compuestos de referencia frente a diferentes dianas farmacológicas. Además, nos han permitido establecer interesantes REAs que nos permiten un diseño racional de nuevos fármacos basados en las estructuras de los receptores y en las estructuras de los ligandos de los mismos.

Como ha sido descrito en la Introducción, a lo largo del trabajo experimental presentado en esta Memoria, hemos utilizado la versatilidad de diferentes vías sintéticas para obtener las amplias familias de derivados que han sido posteriormente caracterizados y estudiados. Hemos buscado las rutas sintéticas más rápidas y cuyos reactivos, preferencialmente de bajo coste, se

adaptaban mejor a nuestras necesidades. A continuación son brevemente descritas las reacciones más utilizadas en el trabajo experimental de esta Memoria.

3.1. Síntesis

3.1.1. Preparación de los esqueletos cumarínicos

3.1.1.1. Reacción de Perkin y Perkin-Oglialoro

La etapa clave del planteamiento sintético para la obtención de las 3-aril y 3-heteroarilcumarinas ha sido una reacción de tipo Perkin[180] entre un aldehído salicílico (*orto*-hydroxybenzaldehído) y un ácido aril/heteroarilacético, ambos convenientemente substituidos, en presencia de la sal sódica del ácido.[181] Las condiciones de esta reacción son sencillas, y el rendimiento muy satisfactorio.[182] La versatilidad de esta reacción permitió obtener varias familias de compuestos cuyos rendimientos varían entre 45 y 80%, dependiendo de los sustituyentes de los reactivos de partida. Los productos de reacción obtenidos se purificaron fácilmente por cromatografía de columna.

En el caso de la preparación de las cumarinas planteadas para este trabajo, la síntesis vía Perkin ha sido llevada a cabo en presencia de DCC, usada como agente deshidratante, en DMSO a 110 ºC. Estas condiciones han sido la mejor solución para la preparación de todos los derivados, con excepción a los derivados con sustituyentes hidroxilo y los nitro derivados. Los derivados hidroxilados han sido preparados vía hidrólisis de los respectivos derivados metoxilados, etoxilados o acetoxilados.

Los derivados de tipo “acetoxilo” han sido preparados usando una reacción de Perkin modificada, tipo Perkin-Oglialoro, entre los salicilaldehídos y los ácidos arilacéticos, en anhídrido acético (Ac_2O) y acetato potásico (CH_3COOK).

Los derivados de tipo “nitro” se obtuvieron a través de una reacción de Perkin modificada con nuevas condiciones, utilizando un salicilaldehído y un ácido arilacético, en presencia de hidruro sódico (NaH) en anhídrido acético, por 20 horas.[183]

La presencia de los anillos aromáticos de las cumarinas preparadas es muy clara en los espectros de ^{1}H RMN. Los protones aromáticos aparecen en el espectro alrededor de 6.5-8 ppm, siendo H-4 el protón más desapantallado y el más fácilmente identificable ya que es un singlete debido a la presencia de un grupo arilo en posición 3 de la cumarina.

3.1.1.2. Reacción de acoplamiento con paladio

Al largo del trabajo experimental hemos desarrollado alternativas a las vías sintéticas tradicionales para la obtención de nuestros compuestos. Un protocolo novedoso para la rápida y eficiente síntesis de las 3-arilcumarinas ha sido llevado a cabo, utilizando una reacción de *cross-coupling* catalizada por paladio en la forma de un complejo de paladio. Con esta metodología hemos preparado diferentes derivados a partir de ácidos borónicos y 3-halogenocumarinas. La reacción se ha realizado en presencia de carbonato sódico y una mezcla de DMF/H_2O (1:1), a 110 ^{o}C, por 120-180 minutos. Los rendimientos de reacción son del orden del 60%. Este método directo tiene la desventaja de la limitación de los reactivos de partida. Por esta razón lo hemos utilizado simplemente como vía alternativa a las reacciones que tradicional veníamos utilizando.[184]

3.1.2. Transformaciones funcionales

3.1.2.1. Reacción de hidrólisis de grupos éter o éster

Todos los derivados mono- o poli-metoxilados y/o etoxilados y/o acetoxilados han sido hidrolizados para la obtención de los correspondientes derivados hidroxilados.

Los métodos más comunes para esta transformación recurren a reacciones en la presencia de un ácido de Lewis como el MgI_2,[185] el $AlCl_3$,[186] el BBr_3[187] o el $AlBr_3$. Como fuentes de protones son utilizados ácidos o alcoholes, siendo de uso común el HCl, el MeOH o el EtOH. Estas reacciones se llevan a cabo, en su gran mayoría, utilizando como disolventes diclorometano, acetonitrilo, acetona, tetrahidrofurano o piridina.

Otra forma de hidrolizar los derivados preparados, utilizada por nuestro grupo, es la reacción con el ácido yodhídrico, en presencia de ácido acético y anhídrido acético. Esta reacción se llevó a cabo a reflujo, durante 4-5 horas. Los productos de reacción han sido de difícil purificación, pero con rendimientos muy satisfactorios. La caracterización de los compuestos obtenidos es muy clara por ^{1}H RMN, ya que el/los picos de los grupos –OCH_3 desaparecen claramente de la zona entre 3-4 ppm, apareciendo uno o más picos/bandas muy característicos en la región de los 10 ppm.

Los compuestos acetoxilados originaron los correspondientes derivados hidroxilados mediante la desacetilación en presencia de una solución acuosa de HCl 2N y metanol, en reflujo, por 4 horas. Así, cuando el objetivo del trabajo se centraba en los derivados hidroxilados y no en los metoxilados precursores, seguimos fundamentalmente esta vía porque te trata de una reacción más limpia y más fácil de procesar.

3.1.2.2. Reacción de halogenación

La halogenación de compuestos aromáticos y heteroaromáticos es una importante reacción en síntesis orgánica.[188] Derivados de bromuros de arilo y heteroarilo pueden ser potenciales antioxidantes, antibacterianos o agentes antitumorales.[189,190] Además, la introducción de halógenos en las 3-arilcumarinas hace de estas moléculas precursores interesantes para muchos otros derivados sustituidos. Esto hace de la bromación una reacción muy versátil y, por lo tanto, ampliamente estudiada. Habitualmente son utilizados como agentes de bromación el tribromuro del tetraalquilamonio,[191] el tribromuro de hexametilenotetramina[192] y la *N*-bromosuccinimida (NBS).[193,194] Sin embargo, debido a su disponibilidad y el fácil manejo, este último es el principal agente de bromación. En fase sólida[195] o en reacciones tradicionales, el uso de NBS es un método de monobromación eficaz y versátil de aromáticos y sus derivados, como son las cumarinas.[196,197] Las condiciones de reacción, la simplicidad y la velocidad del proceso son las ventajas de este método, llevado a cabo con rendimientos muy buenos. La bromación sobre el núcleo de la cumarina no substituida ocurre preferentemente en el C-3. Como en las 3-

arilcumarinas esta posición se encuentra bloqueada por un arilo, la posición de bromación sobre estas moléculas dependerá de la naturaleza de los sustituyentes presentes en los anillos aromáticos.[198]

En nuestro caso todos los derivados han sido preparados mediante una reacción de bromación clásica, con NBS y una cantidad catalítica de azobisisobutironitrilo (AIBN), en tetracloruro de carbono (CCl_4). Las reacción se mantuvo a reflujo 24 horas, y los productos obtenidos han sido separados por cromatografía en columna. Los rendimientos de reacción varían entre 40 y el 60%. Siempre que la 3-arilcumarina no presentaba sustituyentes en el grupo 3-arilo, y tenía un metilo en cualquiera de los otros dos anillos, la bromación ocurrió en este grupo metilo, permitiendo la obtención de un derivado bromometilado. Así, en el caso de la 6-metil-3-fenilcumarina, tuvo lugar la bromación bencílica, lo que ha permitido la obtención de la 6-bromometil-3-fenilcumarina.[199]

En el caso de los derivados con sustituyentes activantes (por ejemplo grupos metoxilo) en el 3-arilo la bromación tuvo lugar en una posición *orto* respecto a los mismos. La reacción descrita ha permitido la obtención de un gran número de derivados halogenados en diferentes posiciones del núcleo de la cumarina. Estos pudieron ser, además de nuevos productos, importantes precursores para otros derivados, ya que es posible su substituición.

3.1.2.3. Reacción de formación de arilaminas

- **Por aminación de derivados halogenados**

Las arilaminas primarias pueden tener interés biológico por si mismas y son además importantes intermediarios de reacción en la síntesis de nuevos fármacos. Cuando se obtienen a partir de los correspondientes haluros de arilo se realiza utilizando amoniaco como nucleófilo.[200] Los principales problemas de esta reacción son la necesidad de presión y temperatura elevadas, y que los arinos intermediarios son muy inestables, haciendo esta reacción muy complicada. Por este motivo, la utilización de catalizadores de cobre y otros metales de transición ha sido ampliamente estudiada, pero aún asi sus

condiciones de presión y temperatura son bastante elevadas y la reacción es compatible con muy pocos grupos funcionales.[201] Para evitar estos inconvenientes ha sido desarrollado un método sustituyendo el amoniaco por trifluoracetamida. En este caso el grupo trifluoracetilo se puede eliminar fácilmente en condiciones suaves. El punto clave de esta reacción es el acoplamiento cruzado entre la amida y los haluros de arilo. Distintos ligandos han sido probados para promover el acoplamiento cruzado catalizado por cobre, siendo la *N,N'*-dimetiletilenodiamina (DMEMA) la que mejores rendimientos presenta.[202] En presencia de un yoduro de arilo necesita apenas de 45 ºC para que la reacción ocurra, si bien, en nuestro caso, los bromuros de arilo necesitaron de una temperatura de 75 ºC para optimizar la reacción. El mejor sistema catalítico ha mostrado ser el CuI/DMEDA y la reacción ha sido optimizada mediante tubo sellado a presión. Los bromuros de arilo han pudido ser convertidos en los productos deseados con rendimientos aceptables entre 60-80 %).

El grupo trifluoracetilo formado en la reacción pudo ser eliminado de forma sencilla añadiendo una mezcla de metanol/agua en el tubo de reacción. El éxito de la reacción también fue debido a la presencia de carbonato de potasio (K_2CO_3) en el paso de amidación, que promovió la hidrólisis del grupo trifluoracetilo cuando el agua fue añadida. Teniendo en cuenta todos estos factores, la reacción ha sido llevada a cabo según este nuevo método *one-pot* de síntesis de arilaminas primarias partiendo de haluros de arilo, sin necesidad de aislar intermediarios de reacción.[203]

- **Por reducción de grupos nitro**

Las 3-aminocumarinas se prepararon a partir de las 3-nitrocumarinas previamente sintetizadas o comerciales, en etanol, con Pd/C como catalizador en atmósfera de hidrogeno (H_2). La reacción se ha llevado a cabo a temperatura ambiente por 5 horas. El rendimiento es del orden del 92-98%.

3.1.2.4. Reacción de alquilación – reacción de Williamson

Uno de los objetivos de este trabajo ha sido estudiar la presencia de distintos grupos sustituyentes para estudios de REA para lo que interesó introducir en los compuestos diferentes cadenas alquílicas. Algunos de los derivados hidroxilados han sido transformados en éteres de forma sencilla y eficaz utilizando la reacción de Williamson.[204]

La utilización de una clorocetona como halogenuro de partida ha permitido la preparación numerosos derivados con sustituyentes en las posiciones deseadas. Los datos de ^{1}H RMN permitieron claramente observar la formación de los β-cetoéteres deseados. En todos los casos se ha visto claramente la desaparición del protón hidroxílico. Y la aparición de nuestros grupos a diferentes ppm.

La versatilidad de esta reacción ha permitido introducir en la molécula alcanos, cicloalcanos (en nuestro caso particular de cinco o seis átomos) o bien haloalcanos más o menos grandes.

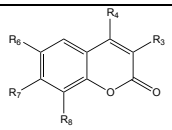
3.1.2.5. Preparación de amidas, ésteres y carbamatos

La segunda parte de los objetivos del trabajo descrito en esta Memoria ha sido la síntesis de cumarinas con funciones amida, éster o carbamato partiendo de las correspondientes aminocumarinas o hidroxicumarinas precursoras. Hemos recurrido a aminocumarinas comerciales sencillas o nitrocumarinas que han sido previamente reducidas. Una reacción de acilación de las amino/hidroxicumarinas con un cloruro de ácido convenientemente sustituido, utilizando piridina y diclorometano, a la temperatura ambiente, durante tres horas, nos permitió obtener las diferentes cumarinas substituidas, con muy buenos rendimientos (80-95%).

3.1.3. Series de compuestos sintetizados

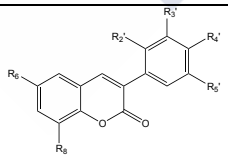
En las siguientes tablas están reunidas todas las estructuras químicas de las moléculas descritas en la publicaciones presentadas en esta Memoria.

Tabla 1. Cumarinas sencillas.



Compuesto	R_3	R_4	R_6	R_7	R_8
1	NO_2	H	H	H	H
2	NO_2	H	H	OCH_3	H
3	NO_2	H	H	H	OCH_3
4	NO_2	H	CH_3	H	H
5	NO_2	H	H	H	CH_3
6	NO_2	H	OCH_3	H	H
7	NO_2	H	H	OCH_3	H
8	NH_2	H	H	H	H
9	NH_2	H	OCH_3	H	H
10	NH_2	H	H	OCH_3	H
11	NH_2	H	H	H	OCH_3
12	NH_2	H	H	OH	H
13	NH_2	H	H	H	OH
14	NH_2	OH	H	H	H

Tabla 2. 3-Arilcumarinas.



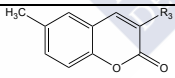
Compuesto	R_6	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
15	H	H	H	H	H	H
16	H	H	H	OCH_3	H	H
17	H	H	H	H	OCH_3	H
18	H	H	H	OH	H	H
19	H	H	H	NO_2	H	H
20	H	H	H	H	NO_2	H
21	H	H	H	NH_2	H	H
22	H	H	H	H	CH_3	H

23	H	H	H	H	Cl	H
24	CH_3	H	H	H	H	H
25	CH_3	H	H	CH_3	H	H
26	CH_3	H	H	H	CH_3	H
27	CH_3	H	OCH_3	H	H	H
28	CH_3	H	H	OCH_3	H	H
29	CH_3	H	H	H	OCH_3	H
30	CH_3	H	H	OCH_3	H	OCH_3
31	CH_3	H	H	OCH_3	OCH_3	H
32	CH_3	H	H	OCH_3	OCH_3	OCH_3
33	CH_3	H	H	Br	OCH_3	H
34	CH_3	H	H	OCH_3	Br	H
35	CH_3	H	Br	OCH_3	H	OCH_3
36	CH_3	H	Br	OCH_3	OCH_3	OCH_3
37	CH_3	H	OH	H	H	H
38	CH_3	H	H	OH	H	H
39	CH_3	H	H	H	OH	H
40	CH_3	H	H	OH	OH	H
41	CH_3	H	Br	H	H	H
42	CH_3	H	H	Br	H	H
43	CH_3	H	H	H	Br	H
44	CH_3	H	H	$C_2H_5O_2$	H	H
45	CH_3	H	H	H	NO_2	H
46	CH_3	H	H	NH_2	H	H
47	CH_3	H	H	H	NH_2	H
48	OCH_3	H	H	H	H	H
49	OCH_3	H	H	CH_3	H	H
50	OCH_3	H	H	H	CH_3	H
51	OCH_3	H	H	H	OCH_3	H
52	OCH_3	H	H	NO_2	H	H
53	OCH_3	H	H	H	NO_2	H
54	OCH_3	H	H	Br	H	H
55	OCH_3	H	H	H	Br	H

56	OH	H	H	H	H	H
57	OH	H	H	H	CH_3	H
58	OH	H	H	Br	H	H
59	OH	H	H	H	Br	H
60	$C_2H_5O_2$	H	H	H	CH_3	H
61	$C_2H_5O_2$	H	H	Br	H	H
62	$C_2H_5O_2$	H	H	H	Br	H
63	C_5H_9O	H	H	H	CH_3	H
64	C_5H_9O	H	H	Br	H	H
65	C_5H_9O	H	H	H	Br	H
66	$C_2H_2O_2Cl$	H	H	Br	H	H
67	$C_2H_2O_2Cl$	H	H	H	Br	H
68	NO_2	H	H	NO_2	H	H
69	NO_2	H	H	H	NO_2	H
70	NO_2	H	H	OCH_3	H	H
71	NO_2	H	H	H	OCH_3	H
72	NO_2	H	H	CH_3	H	H
73	NO_2	H	H	H	CH_3	H
74	NO_2	H	H	Br	H	H
75	NH_2	H	H	H	H	H
76	NH_2	H	H	NH_2	H	H
77	Br	H	H	H	OCH_3	H
78	$BrCH_3$	H	H	H	H	H
79	CH_3	Br	H	H	H	H
80	CH_3	Br	H	H	OCH_3	H
81	CH_3	Br	H	OCH_3	H	OCH_3
82	CH_3	Br	H	OCH_3	OCH_3	OCH_3
83	Br	OCH_3	H	H	H	H
84	Br	OCH_3	H	H	OCH_3	H
85	Br	OCH_3	H	H	CH_3	H
86	Br	OH	H	H	H	H
87	Br	OH	H	H	OH	H
88	Br	OH	H	H	CH_3	H

89	NH_2	OCH_3	H	H	H	H
90	H	OCH_3	H	H	H	H
91	H	OCH_3	H	H	Br	H
92	H	OCH_3	H	H	OCH_3	H
93	H	CH_3	H	H	H	H
94	H	CH_3	H	H	OCH_3	H
95	H	CH_3	H	H	CH_3	H
96	H	CH_3	H	H	Br	H
97	H	CH_3	H	OH	OH	H
98	H	CH_3	H	OH	OH	OH
99	H	CH_3	H	OH	H	OH
100	H	OCH_2CH_3	H	H	H	H
101	H	OCH_2CH_3	H	H	OCH_3	H
102	H	OCH_2CH_3	H	H	CH_3	H
103	H	OH	H	H	H	H
104	H	OH	H	H	OH	H
105	H	OH	H	H	CH_3	H

Tabla 3. 3-Heteroarilcumarinas.



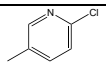
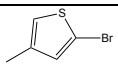
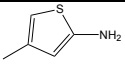
Compuesto	R_3
106	
107	
108	
109	

Tabla 4. (Cumarin-3-il)-4-metilbenzoato.

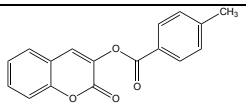
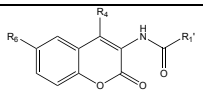
110	

Tabla 5. 3-Amidocumarinas.



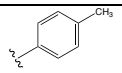
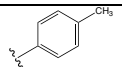
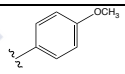
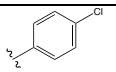
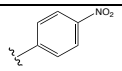
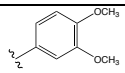
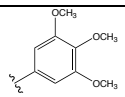
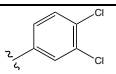
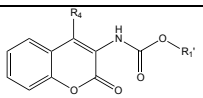
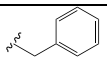
Compuesto	R_4	R_6	$R_{1'}$
111	H	H	CH_3
112	OH	H	CH_3
113	H	H	CH_2Br
114	H	H	CH_2Cl
115	H	CH_3	
116	H	H	
117	H	H	
118	H	H	
119	H	H	
120	H	H	
121	H	H	
122	H	H	
123	H	H	
124	H	H	

Tabla 6. 3-Carbamatocumarinas.



Compuesto	R_4	$R_{1'}$

125	H	CH_3
126	H	CH_2CH_3
127	H	$CH(CH_3)_2$
128	H	$CH_2CH(CH_3)_2$
129	H	CH_2Cl
130	H	CH_2CH_2Br
131	H	
132	H	
133	OH	CH_2CH_3
134	OH	$CH_2CH(CH_3)_2$

3.2. Estudio biológico y relación estructura-actividad (REA)

Después de realizados diferentes ensayos biológicos *in vitro* y en algunos casos *in vivo*, y de un estudio REA más detallado, ha sido posible establecer importantes conclusiones de la relación estructura/actividad de los compuestos preparados. En general los compuestos descritos son potentes inhibidores selectivos de la MAO-B,[205] la diana más estudiada en este trabajo, siendo los que presentan el esqueleto 3-arilcumarina los que demostraron ser los más interesantes.[206,207] De todas las posiciones estudiadas, es muy favorable la introducción de grupos poco voluminosos (por ejemplo metilo, bromo o metoxilo) tanto en posición 6 y/o 8 como en las posiciones 3' y/o 4' del núcleo de la 3-arilcumarina.[208] Grupos de diferente tamaño, como otros anillos o diferentes éteres, han demostrado bajar en gran escala la actividad de dichas moléculas. La 3-arilcumarina por si sola tampoco presenta una actividad notable. Los estudios de *docking* han corroborado la información obtenida experimentalmente. La cavidad de la enzima tiene las dimensiones ideales para nuestras 3-arilcumarinas cuando presentan pequeños sustituyentes en su esqueleto base. A mayores, experimentalmente y a través de dichos estudios teóricos, también hemos sido capaces de concretar las posiciones del anillo en 3 que mejor favorecen la interacción con el receptor. Las posiciones *meta* y *para*, como dicho anteriormente, han demostrado ser las ideales. Los valores

de CI_{50} de estos compuestos son, en muchos casos, del rango de bajo nano y picoMolar. Hemos encontrado que la 3-(3'-bromofenil)-6-metilcumarina (compuesto **42**) es más de 6000 veces más activo (CI_{50} = 134.04 pM) y más de 200 veces más selectivo que la selegilina, usada como compuesto de referencia. Estos datos nos han incentivado a seguir estudiando este tipo de derivados frente a esta diana.

Hemos realizado diferentes estudios electroquímicos[209] y de capacidad antioxidante de algunas de las 3-arilcumarinas hidroxiladas.[210] Los resultados han sido estimulantes, ya que los potenciales de oxidación de estos compuestos son bajos y los ORAC-FL bastante elevados con respecto al trolox, el compuesto de referencia. El compuesto más interesante de la serie estudiada, la 3-(4'-hidroxifenil)-8-hidroxicumarina (compuesto **104**), ha presentado un valor de ORAC-FL de 13,5, conjuntamente al más bajo potencial de oxidación y el 100% de atrapamiento de radicales hidroxilo. La asociación de este tipo de actividad con la ya mencionada actividad IMAO es una importante estrategia en la obtención de fármacos con actividades múltiples.

Avanzando un poco más en el estudio, hemos encontrado algunas 3-amidocumarinas con actividad inhibitoria dual de MAO-B y AChE.[211] El perfil de estos derivados nos ha abierto un camino en el estudio de inhibidores duales y multi-diana. Los derivados con grupos metilo y clorofenil adjuntos al enlace amídico han sido los más interesantes frente a las dos enzimas.

En un estudio posterior algunos de los derivados tipo carbamato han mejorado las actividades IMAO-B obtenidas para las amidas correspondientes, perdiendo algunos de ellos la actividad inhibidora de la AChE. Así que el mejor compuesto de esta serie (bencil(cumarin-3-il)carbamato (compuesto **132**), CI_{50} MAO-B = 45 nM) fue estudiado *in vivo* y los resultados han sido muy esperanzadores.[212] De estas dos series, y buscando ligandos de receptores de adenosina implicados en enfermedades neurodegenerativas, hemos encontrado compuestos con actividades moderadas que nos han incentivado a un estudio más profundo en esta área.[213]

Estudios antibacterianos han sido realizados en diferentes familias de las cumarinas sintetizadas.[214,215] Los resultados frente a bacterias humanas más interesantes han sido obtenidos con cumarinas sencillas y algunas 3-arilcumarinas nitro-sustituidas. De todas las líneas bacterianas estudiadas los

resultados más relevantes han sido encontrados frente a *S. aureus* y *E. coli*, dos de las bacterias más comunes en patologías. Alguna de las moléculas estudiadas ha presentado resultados comparables a los compuestos de referencia, y nos han permitido establecer importantes REAs. En particular, la 3-(3'-metilfenil)-6-nitrocumarina (compuesto **72**) ha presentado un halo de inhibición frente a *S. aureus* de 32 mm y un MIC de 8 μg/mL, valores comparables con la ampicilina (halo = 32 mm y MIC = 2 μg/mL), usada como compuesto de referencia.[215]

Algunas cumarinas sencillas estructuralmente parecidas a la tirosina y algunas 3-arilcumarinas con átomos de bromo en diferentes posiciones han probado tener interés en estudios de inhibición de tirosinasa. Estos compuestos han presentado interesantes perfiles cuando comparados con la umbeliferona, el compuesto de referencia. El compuesto 6-bromo-3-(4´-hidroxifenil)-8-hidroxicumarina (compuesto **87**) ha presentado un valor de inhibición de la tirosinasa inferior a la umbeliferona, pero fue la 3-amino-7-hidroxicumarina (compuesto **12**) el compuesto más activo de todos los estudiados (CI_{50} = 0.05 mM). Este compuesto es 8 veces más activo que el compuesto de referencia.[216,217]

Las rutas sintéticas utilizadas, la caracterización de los compuestos, los ensayos farmacológicos y electroquímicos, y los estudios teóricos y de REA están presentados y discutidos en los respectivos manuscritos, incluidos en el apartado experimental de esta Memoria.

3.3. Aportaciones estructurales y teóricas

A lo largo del trabajo descrito, diferentes estudios teóricos (modelización y semi-empíricos) han aportado información clave de apoyo a los estudios de REA de los compuestos evaluados. Han sido realizados estudios de *docking* recurriendo al uso de los programas MAESTRO y MOE, usando como referencia para la validación de los estudios las estructuras cristalinas de las proteínas encontradas en el PDB.

Simulaciones de *docking* se han realizado utilizando el protocolo Quantum Mechanical Polarized Ligand Docking (QPLD), implementado en

Schrodinger.[218] El algoritmo ha utilizado la metodología *ab initio* para calcular las cargas del ligando dentro del entorno de proteína. En nuestro protocolo QPLD, después de la preparación del ligando y de la proteína, el Glide realizó el primer cálculo de *docking*, con la generación de las cargas iniciales con métodos semiempíricos. En el segundo paso, la teoría funcional de densidad (DFT), con Jaguar, fue aplicado para el tratamiento completo de la mecánica cuántica del ligando (QM). Un re-*docking* de los ligandos mediante el uso de las cargas calculadas por Jaguar y, por último, el algoritmo de QPLD llevaron a la obtención de las poses energéticamente más favorables para cada ligando. Una minimización utilizando el Primer MM-GBSA se llevó a cabo con el único objetivo de minimizar las poses derivadas del QPLD, teniendo en cuenta la flexibilidad de los residuos del *bolsillo* de unión dentro de una distancia de 5 Å a los ligando. La metodología de *docking* mencionada ha demostrado ser muy útil para proponer poses de unión de los derivados estudiados, destacando la importancia de las sustituciones en las posiciones 3, 6 y 8 de la cumarina, bien como de las posiciones *meta* y *para* del anillo aromático en posición 3 de la 3-arilcumarina.[205,208]

Estudios semi-empíricos como el AM1 y el PM3 han sido llevados a cabo para comparar los resultados de los rayos X con los obtenidos por el análisis conformacional seguido por estas metodologías. Los cálculos teóricos dieron resultados que reprodujeron eficazmente la estructura tridimensional (3D) de las moléculas estudiadas por rayos X, lo cual significa que la determinación estructural teórica en la fase gaseosa puede ser extrapolada.[219]

4. Parte experimental

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4.1. Artículos publicados

1. “Regioselective synthesis of different bromo substituted 3-arylcoumarins” (Matos, M.J.*; Delogu, G.; Podda, G.; Santana, L.; Uriarte, E.) – *Synthesis* **2010**, *16*, 2763-2766.

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4. “3-Phenylcoumarin” (Matos, M.J.*; Santana, L.; Uriarte, E.) – *Acta Crystallographica* **2012**, *E68*, o2645.

5. “*N*-(2-Oxo-2*H*-chromen-3-yl)cyclohexanecarboxamide” (Matos, M.J.*; Santana, L.; Uriarte, E.) – *Acta Crystallographica* **2012**, *E68*, o3447-o3448.

6. “Synthesis, NMR characterization, X-ray structural analysis and theoretical calculations of amide and ester derivatives of the coumarin scaffold” (Matos, M.J.*; Uriarte, E.; Santana, L.; Vilar, S.) – *Journal of Molecular Structure* **2013**, *1041*, 144-150.

7. “A new series of 3-phenylcoumarins as potent and selective MAO-B inhibitors” (Matos, M.J.*; Viña, D.; Quezada, E.; Picciau, C.; Delogu, G.; Orallo, F.; Santana, L.; Uriarte, E.) – *Bioorganic & Medicinal Chemistry Letters* 2009, *19*, 3268-3270.

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10. “MAO inhibitory activity modulation: 3-phenylcoumarins versus 3-benzoylcoumarins” (Matos, M.J.; Vázquez-Rodriguez, S.; Uriarte, E.; Santana, L.; Viña, D.*) – *Bioorganic & Medicinal Chemistry Letters* 2011, *21*, 4224-4227.

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12. “8-Substituted-3-arylcoumarins as potent and selective MAO-B inhibitors: synthesis, pharmacological evaluation and docking studies” (Viña, D.; Matos, M.J.*; Ferino, G.*; Cadoni, E.; Laguna, R.; Borges, F.; Uriarte, E.; Santana, L.) – *ChemMedChem* **2012**, *7*, 464-470.

13. “Focusing on new monoamine oxidase inhibitors: differently substituted coumarins as an interesting scaffold” (Matos, M.J.; Viña, D.; Vazquez-Rodriguez, S.; Uriarte, E.; Santana, L.*) – *Current Topics in Medicinal Chemistry* **2012**, *12*, 2210-2239.

14. “Novel (coumarin-3-yl)carbamates as selective MAO-B inhibitors: synthesis, in vitro and in vivo assays, theoretical evaluation of ADME properties and docking study” (Matos, M.J.*; Vilar, S.; Gonzalez-Franco, R.M.; Uriarte, E.; Santana, L.; Friedman, C.; Tatonetti, N.P.; Viña, D.; Fontenla, J.A.) – *European Journal of Medicinal Chemistry* **2013**, *63*, 151-161.

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16. “New halogenated phenylcoumarins as tyrosinase inhibitors” (Matos, M.J.*; Santana, L.; Uriarte, E.; Delogu, G.; Corda, M.; Fadda, M.B.; Era, B.; Fais, A.) – *Bioorganic & Medicinal Chemistry Letters* **2011**, *21*, 3342-3345.

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18. “Targeting adenosine receptors with coumarins: synthesis and binding activities of amide and carbamate derivatives” (Matos, M.J.*; Gaspar, A.;

Kachler, S.; Klotz, K.-N.; Borges, F.; Santana, L.; Uriarte, E.) – *Journal of Pharmacy & Pharmacology* **2013**, *65*, 30-34.

19. "Looking for new targets: simple coumarins as antibacterial agents" (Matos, M.J.*; Vazquez-Rodriguez, S.; Santana, L.; Uriarte, E.; Fuentes-Edfuf, C.; Santos, Y.; Muñoz-Crego, A.) – *Medicinal Chemistry* **2012**, *8*, 1140-1145.

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21. "New hydroxylated 3-arylcoumarins, synthesis and electrochemical study" (Janeiro, P.; Matos, M.J.; Santana, L.; Uriarte, E.; Oliveira-Brett, A.M.*) – *Journal of Electroanalytical Chemistry* **2013**, *689*, 243-251.

"Remarkable antioxidant properties of a series of hydroxy-3-arylcoumarins" (Matos, M.J.*; Pérez-Cruz, F.; Vazquez-Rodriguez, S.; Uriarte, E.; Santana, L.; Borges, F.; Olea-Azar, C.) – *Bioorganic & Medicinal Chemistry* **2013**, *21*, 3900-3906.

4.2. Artículos en fase de publicación

22. New Insights into Functional and Structural Properties of 3-Arylcoumarins as an Interesting Scaffold in MAO-B Inhibition (Matos, M.J.*; Vilar, S.*; García-Morales, V.; Friedman, C.; Uriarte, E.; Santana, L.; Viña, D.), enviado.

23. Synthesis and adenosine receptors binding affinities of a series of 3-arylcoumarins (Matos, M.J.*; Hogger, V.; Gaspar, A.; Kachler, S.; Borges, F.; Uriarte, E.; Santana, L.; Klotz, K.-N.) – *Journal of Pharmacy & Pharmacology* **2013**, aceptado.

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Regioselective Synthesis of Bromo-Substituted 3-Arylcoumarins

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Abstract: Regioselective syntheses of 3-arylcoumarins possessing a bromine substituent either on the 3-aryl ring, the coumarin moiety or on a lateral chain of a coumarin is reported. The regioselectivity is influenced by the substituents present in the substrates. Two different bromination methods are described and compared. Perkin condensation of 5-methylsalicylaldehyde and phenylacetic acid or *para*-methoxyphenylacetic acid affords the desired coumarins. Three different bromine substitution patterns are accessed starting from 5-methylsalicylaldehyde.

Key words: natural products, halogenation, bromine, regioselectivity, coumarins

Phenylcoumarins are synthetic compounds in which an additional phenyl ring is present as a substituent at any position on the coumarin nucleus. They are easily prepared using two general methods: 1) coumarin phenylation,[1] or 2) via construction of the coumarin nucleus with the aryl ring already in place, e.g., by way of Perkin,[2] Pechmann,[3,4] Mukaiyama,[5] Knoevenagel[6] or Perkin–Oglialoro[7] reactions. In the present work we utilize the second method, and specifically the classical Perkin condensation.[8] This represents a direct and general method for preparing 3-phenylcoumarins.

A number of natural products and synthetic analogues featuring the coumarin structural motif display a broad spectrum of biological activity.[9] Structurally, 3-phenylcoumarins can be considered as coumarin–resveratrol hybrids (Figure 1); these are very important molecular frameworks and are synthetically versatile.[10] The C3–C4 double bond of the coumarin nucleus fixes the trans disposition of the *t*-resveratrol-type double bond.[3] The interesting pharmacological properties of *trans*-resveratrol has led to increased interest in synthetic studies toward its analogues.[3,10]

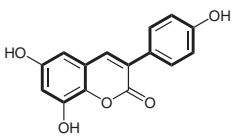


Figure 1 6,8-Dihydroxy-3-(4′-hydroxyphenyl)coumarin (a coumarin–resveratrol hybrid)

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Halogenation of aromatic and heteroaromatic compounds is an important reaction in synthetic organic chemistry.[11] Aryl and heteroaryl bromides are potential antioxidant, antibacterial and antitumor agents.[12] They also represent interesting precursors to many substituted analogues. Hence, bromination reactions are versatile and have been studied extensively. Brominating agents such as tetraalkylammonium tribromide,[13] 1,8-diazabicyclo[5.4.0]undec-7-ene hydrobromide perbromide (DBUHBr$_3$),[14] hexamethylenetetramine tribromide[15] and *N*-bromosuccinimide (NBS)[16,17] are known to facilitate monobromination of aromatic compounds and their derivatives. However, as a result of its ready availability and ease of handling, the latter reagent is the most commonly used brominating agent. The use of *N*-bromosuccinimide in the solid state,[18] or in traditional solution reactions, enables efficient monobromination of aromatics and derivatives such as coumarins.[19,20] *N*-Bromosuccinimide has been used for bromination and/or oxidation reactions.[21–24] It is also used for the conversion of benzaldehydes into the corresponding benzoyl bromides and for bromolactonization of unsaturated acids and their derivatives.[25–27] Mild reaction conditions, good yields, and simple, rapid reactions are some of the advantages of using *N*-bromosuccinimide. It has been reported that bromination of coumarins occurs mainly at C-3, leading to the corresponding monobromo derivatives.[17] In the present compounds this position is blocked by a phenyl ring leading to a change in the reactivity of the coumarin. Side-chain bromination in benzyl or allylic systems can be mediated by free radical initiators, while aromatic bromination requires a catalyst.[28] Electron-rich aromatic compounds are easily brominated using *N*-bromosuccinimide.[18] Several studies on regioselective bromination of coumarins have been performed with the aim of synthesizing bioactive natural products.[29,30]

As part of our research on 3-arylcoumarins, we previously reported the synthesis of 6-methyl-3-arylcoumarins with the aryl ring being mono- (compound **2**), di- or tri-methoxy-substituted.[10] The 3-phenylcoumarins demonstrated important roles in monoamine oxidase (MAO) enzymatic inhibition.[10] We found that the presence of a methoxy group on the 3-phenyl ring was important, especially a *para*-methoxy group, in order to improve the monoamine oxidase inhibitor (MAOI) activity.

In this paper, we report the direct and selective synthesis of various bromo-substituted 6-alkyl-3-arylcoumarins.

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The bromine atom can be installed on the 3-phenyl ring (compound **3**) or on the aromatic ring of the coumarin (compound **5**). In addition, conditions which allow the bromine atom to be substituted on a lateral chain (compound **7**) are described.

3-Arylcoumarins with different substitution patterns were prepared via a direct synthetic route involving the classical Perkin procedure.[8,10,31–33] The reactions were accomplished by condensation of appropriately substituted salicylaldehydes with phenylacetic or *p*-methoxyphenylacetic acids, using *N*,*N*′-dicyclohexylcarbodiimide (DCC) as the dehydrating agent, in dimethyl sulfoxide, at 110 ºC for 24 hours (Scheme 1).[8,31,32] Coumarins **2**[10] and **5** were obtained in yields of 61% and 50%, respectively, by reaction of salicylaldehydes **1** and **4**[8,31,32] with *p*-methoxyphenylacetic acid.

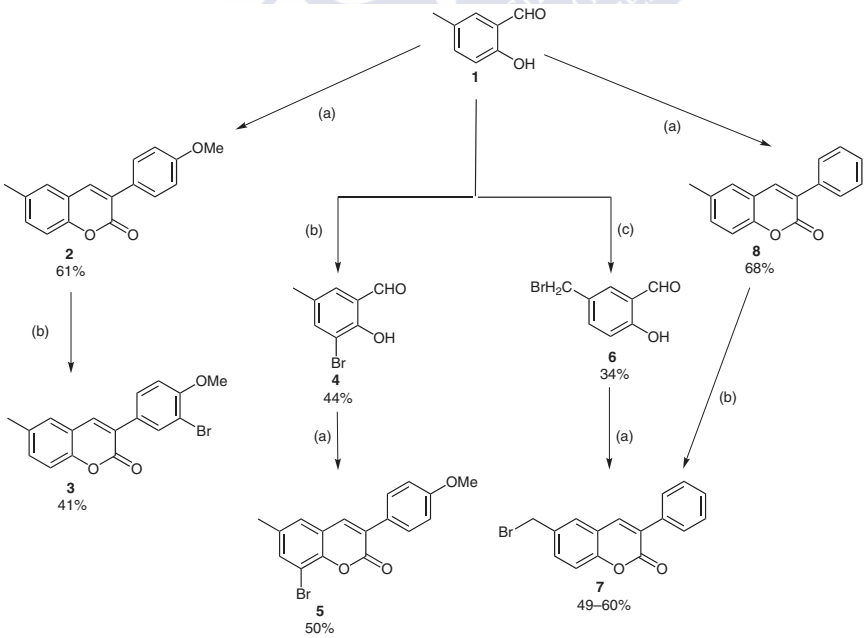
Treatment of 6-methyl-3-(4′-methoxyphenyl)coumarin (**2**) with *N*-bromosuccinimide, at reflux in carbon tetrachloride, using 2,2′-azobis(isobutyronitrile) (AIBN) as the catalyst, afforded brominated phenylcoumarin **3** in a yield of 41%. The regioselective formation of **3** occurred as a result of activation of the *ortho* position in substrate **2** by the electron-donating methoxy group. Under these conditions, the 6-methyl group was not sufficiently activating to direct bromination onto the coumarin moiety.

3-Bromo-2-hydroxy-5-methylbenzaldehyde (**4**),[31–33] the brominated precursor of coumarin **5**, was prepared from **1** in 44% yield, using the same brominating conditions as described above (Scheme 1). The *ortho* position relative to the hydroxy substituent, and *meta* to the aldehyde, was the most active, and as such was the position at which bromination occurred.

5-Bromomethyl-2-hydroxybenzaldehyde (**6**) was obtained in 34% yield via irradiation of precursor **1** and bromine in carbon tetrachloride using a tungsten light source. The ability to brominate salicylaldehyde **1**, regioselectively, enabled the synthesis of 3-arylcoumarins **5** (from **4**) and **7** (from **6**), via the classical Perkin condensation, in 50% and 60% yields, respectively. Thus, bromination of **1** using different reaction conditions allowed the position of the bromine atom in the final coumarin product to be varied.

It is interesting to note that with compounds **1** and **2**, which both possess benzylic protons, treatment with *N*-bromosuccinimide using conditions (b) did not afford the corresponding bromomethyl derivatives. These substrates contain a strong electron-donating group, and hence aromatic substitution occurs instead of benzylic substitution.

On the other hand, unsubsituted 3-phenylcoumarin[34] was prepared and then treated with *N*-bromosuccinimide using the above-mentioned conditions, however, no bromo derivative was obtained. The absence of an activating substituent prevents the electrophilic aromatic substitution occurring. Thus, in a substrate without strong activating groups, the presence of an alkyl substituent could lead to benzylic halogenation instead of aromatic substitution.



Scheme 1 *Reagents and conditions*: (a) phenylacetic acid or *p*-methoxyphenylacetic acid (1.25 equiv), DCC (1.56 equiv), DMSO, 110 ºC, 24 h; (b) NBS (1.2 equiv), AIBN (cat.), CCl_4, reflux, 18 h; (c) Br_2 (1.4 equiv), tungsten light source, CCl_4, 8 h.

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6-Methyl-3-phenylcoumarin (**8**),[10] with no substituent on the 3-phenyl ring, was reacted under the same brominating conditions to afford 6-bromomethyl-3-phenylcoumarin (**7**) in a yield of 49% (Scheme 1). In this case, with no sufficiently strong activating group for aromatic bromination, the bromine atom was instead directed to the lateral chain. This reaction sequence represents another method to obtain bromoalkylcoumarin **7**.

Comparing the formation of arylcoumarins **3** and **7** from 6-methyl-3-arylcoumarins **2** and **8**, respectively, the presence of a methoxy group results in bromination on the 3-aryl ring, whilst the absence of a methoxy group leads to bromine substitution on the lateral chain. Introduction of a bromine atom on the coumarin ring is possible by aromatic bromination of the precursor salicylaldehyde. In this specific case, the hydroxy substituent on the benzene ring directs substitution of the bromine atom at the appropriate position.

In conclusion, convenient procedures have been developed starting from the 5-methylsalicylaldehyde (**1**), that enable bromination on the aryl, methyl or coumarin moieties in differently substituted arylcoumarins. The 3-arylcoumarin products can be used as precursors for other molecules and for pharmacological evaluation.

Melting points were obtained using a Reichert Kofler Thermophan or in capillary tubes with a Büchi 510 apparatus and are uncorrected. ^{13}C (75 MHz) and ^1H NMR (300 MHz) spectra were recorded on a Bruker AMX 300 MHz spectrometer. Chemical shifts (δ, *J* in Hz) are reported in ppm relative to TMS as the internal standard. Mass spectra were obtained using a Hewlett-Packard 5988A spectrometer. Elemental analyses were recorded on a Perkin-Elmer 240B microanalyzer. Silica gel (Merck 60, 230–400 mesh) was used for flash chromatography. Analytical TLC was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm).

3-(3′-Bromo-4′-methoxyphenyl)-6-methylcoumarin (3)

A soln of 6-methyl-3-(4′-methoxyphenyl)coumarin (**2**) (1.0 g, 3.76 mmol), NBS (0.80 g, 4.51 mmol) and AIBN (cat.) in CCl_4 (5.0 mL) was stirred under reflux for 18 h. The resulting soln was filtered to remove succinimide. The solvent was evaporated under vacuum and purified by flash chromatography (hexane–EtOAc, 95:5) to give compound **3**.

White solid; yield: 41%; mp 206–207 °C.

^1H NMR ($CDCl_3$): δ = 2.42 (s, 3 H, CH_3), 3.94 (s, 3 H, OCH_3), 6.97 (d, *J* = 8.7 Hz, 1 H, H-5′), 7.23–7.35 (m, 3 H, H-2′, H-6′, H-5), 7.69–7.74 (m, 2 H, H-7, H-8), 7.89 (s, 1 H, H-4).

^{13}C NMR ($CDCl_3$): δ = 20.8, 56.3, 111.5, 111.6, 116.1, 119.3, 126.3, 127.6, 128.5, 129.0, 132.5, 133.1, 134.2, 139.1, 151.5, 156.2, 160.6.

MS (EI): *m/z* (%) = 347 (18), 346 (98), 345 (19), 344 (100) [M^+], 303 (45), 301 (45), 275 (11), 250 (17), 222 (13), 194 (11), 178 (13), 165 (58), 163 (11), 139 (15), 132 (42), 82 (18), 76 (14), 63 (19), 50 (14).

Anal. Calcd for $C_{17}H_{13}BrO_3$: C, 59.15; H, 3.80. Found: C, 59.10; H, 3.71.

8-Bromo-3-(4′-methoxyphenyl)-6-methylcoumarin (5)

A soln of 3-bromo-2-hydroxy-5-methylbenzaldehyde (**4**) (0.25 g, 1.16 mmol), *p*-methoxyphenylacetic acid (0.24 g, 1.45 mmol) and DCC (0.37 g, 1.81 mmol), in DMSO (2.0 mL), was heated at 100–110 °C in an oil bath for 24 h. Ice (20 g) and AcOH (3.0 mL) were added and the mixture was stirred at r.t. for 2 h, and then extracted with Et_2O (3 × 25 mL). The combined organic layer was washed with 5% aq $NaHCO_3$ soln (50 mL) and H_2O (20 mL), and dried (Na_2SO_4). The solvent was evaporated under vacuum and the residue was purified by flash chromatography (hexane–EtOAc, 9:1) to give coumarin **5**.

White solid; yield: 50%; mp 144–145 °C.

^1H NMR ($CDCl_3$): δ = 2.41 (s, 3 H, CH_3), 3.86 (s, 3 H, OCH_3), 6.98 (d, *J* = 7.1 Hz, 2 H, H-3′, H-5′), 7.26 (s, 1 H, H-7), 7.56 (s, 1 H, H-5), 7.65–7.69 (m, 3 H, H-2′, H-6′, H-4).

^{13}C NMR ($CDCl_3$): δ = 20.5, 55.4, 109.3, 114.0, 120.7, 126.7, 126.9, 128.5, 129.9, 135.2, 137.8, 148.1, 159.9, 160.3.

MS (EI): *m/z* (%) = 346 (99), 345 (15), 344 (100) [M^+], 303 (53), 301 (54), 275 (15), 207 (15), 166 (17), 165 (72), 138 (17), 89 (13), 76 (14), 58 (41).

Anal. Calcd for $C_{17}H_{13}BrO_3$: C, 59.15; H, 3.80. Found: C, 59.27; H, 3.82.

5-Bromomethylsalicylaldehyde (6)

To a soln of 5-methylsalicylaldehyde (**1**) (2.0 g, 14.69 mmol) in CCl_4 (23.0 mL), under reflux and with tungsten light irradiation (300 W, Philips Reflector R125 35°), was added Br_2 (3.29 g, 20.57 mmol) over a period of 3 h. The soln was stirred at r.t. for a further 15 h and the resulting precipitate was filtered and washed with CCl_4 to give aldehyde **6**.

White solid; yield: 34%; mp 148 °C.

^1H NMR ($CDCl_3$): δ = 4.43 (s, 2 H, CH_2), 6.96 (d, *J* = 8.4 Hz, 1 H, H-3), 7.49–7.53 (m, 2 H, H-4, H-6), 9.88 (s, 1 H, CHO), 10.21 (s, 1 H, OH).

^{13}C NMR ($CDCl_3$): δ = 33.2, 115.2, 128.4, 130.9, 131.0, 136.4, 162.0, 191.4.

MS (EI): *m/z* (%) = 216 (14), 215 (61), 214 (100) [M^+], 184 (17), 168 (12), 121 (15), 17 (10).

Anal. Calcd for $C_8H_7BrO_2$: C, 44.68; H, 3.28. Found: C, 44.66; H, 3.23.

6-Bromomethyl-3-phenylcoumarin (7)

Coumarin **7** was obtained in 60% yield starting from 5-bromomethylsalicylaldehyde (**6**), according to the procedure described for the synthesis of compound **5**. The same product was also obtained in 49% yield starting from coumarin **8** using an identical method to that described for the preparation of **3**.

White solid; mp 174–175 °C.

^1H NMR ($CDCl_3$): δ = 4.56 (s, 2 H, CH_2), 7.34–7.72 (m, 8 H, H-2′, H-3′, H-4′, H-5′, H-6′, H-5, H-7, H-8), 7.79 (s, 1 H, H-4).

^{13}C NMR ($CDCl_3$): δ = 32.1, 117.0, 119.7, 128.2, 128.5, 128.9, 129.0, 132.0, 134.3, 134.4, 139.2, 153.1, 160.2.

MS (EI): *m/z* (%) = 315 (23), 314 (100), 179 (21), 176 (40), 152 (12), 118 (19), 89 (15), 76 (16).

Anal. Calcd for $C_{16}H_{11}BrO_2$: C, 60.98; H, 3.52. Found: C, 60.89; H, 3.47.

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Synthesis of 3-arylcoumarins via Suzuki-cross-coupling reactions of 3-chlorocoumarin

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ABSTRACT

A convenient, new, and effective protocol for a rapid synthesis of different substituted 3-arylcoumarins is reported. The developed synthetic route involves Pd-catalyzed cross-coupling reaction, using a catalytic complex Pd-salen. Under these conditions, a series of different substituted boronic acids have been successfully reacted with a coumarin halide to afford the coupling products in good yields.

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Coumarins are an important class of benzopyrones that are found in the vegetable kingdom, either in free or combined state.[1] They occupy an important place in the realm of natural products and synthetic organic chemistry. The diverse biological and pharmaceutical properties of natural and synthetic coumarins as antioxidants,[2] anti-HIV,[3] anticancer,[4] vasorelaxants[5] or enzymatic inhibitors[6] are well-known.[1] In the last few years our research group has been engaged in the synthesis of new biologically active coumarins. In particular, the 3-phenylcoumarin's scaffold has been the focal point of our most recent studies. In fact we have demonstrated that some 3-phenylcoumarins play an important role in the monoamino oxidase (MAO) enzymatic inhibition.[7–9] Accordingly, the interesting application potential of the 3-arylcoumarins in the Health Sciences promotes their preparation as an interesting topic in synthetic organic chemistry.

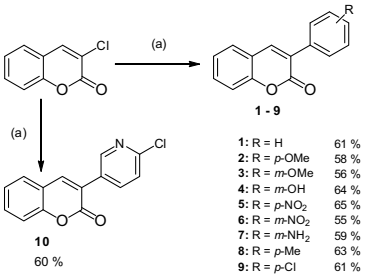
Two different strategies can be used to obtain the phenylcoumarins. One is the construction of the coumarin nucleus by condensation–cyclization-type reactions. Wittig,[10] Pechmann,[5,7,11,12] Perkin[8,9,13–17] or Knoevenagel[18,19] reactions are some of the synthetic routes frequently described to prepare 3-arylcoumarins, starting from phenols or aromatic carbonyl compounds. The classical Perkin condensation is perhaps the most direct and simple method known for the preparation of 3-arylcoumarins.[16] Although this method is often used, it has some drawbacks such as the application of strong acids and high temperatures and sometimes the request of multi-step reactions.

An alternative strategy consists in the direct 3-arylation of the coumarin scaffold by metal cross-coupling reactions. In recent years, palladium-catalyzed coupling reactions have been developing into a versatile and efficient method for the C–C bond formation. Suzuki[20,21] and Stille have described simple synthetic routes to obtain C–C liaisons throughout the coupling arylboronic or organotin compounds with organic halides catalyzed by a palladium-complex. In these reactions, Pd complexes are used as catalysts in which the ligands are P and/or N lone pairs donors or active Pd complexes are generated in situ.[22] In the first case, some of these ligands are expensive, toxic, and sensitive to air and a major challenge lies in the separation of product from the expensive catalyst, much to the dismay of large-scale producers.[22] Ionic liquids,[23] free of organic solvent systems or phase-transfer catalysts[24] have been used to provide a green alternative chemistry to the conventional organic methodologies, but they are not devoid of toxicity. In turn, phosphine ligands[25] had played an important role in C–C bond formation, in homogeneous reaction medium. Several efforts have been made in order to discover air and moisture-stable palladium ligands.[25] However, these ligands are air- and moisture-sensitive and the P–C bond degradation frequently occurs at elevated temperature.[26] The degradation of the catalyst strongly affects conversion and selectivity of the derivatives.[27] So, different methods using phosphine-free ligands have been explored for application in this kind of reactions.[28]

Because of its versatility, palladium catalyzed coupling reactions were widely used to synthesize different arylcoumarins. The arylation in 4-position of the coumarin nucleus is usually achieved by arylation of 4-hydroxycoumarins with arylboronic

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Scheme 1. Synthesis of 3-arylcoumarin derivatives. Reagents and conditions: (a) 3-chlorocoumarin (1.0 equiv), phenylboronic acid/2-chloro-5-pyridineboronic acid (1.25 equiv), sodium carbonate (2.0 equiv), palladium complex (0.5 mol %), DMF/H_2O (1:1), 110 °C, 120–180 min.

acids via C–OH bond activation.[29] Accordingly, the 4-hydroxy group is converted in *pseudo*halides, such as triflates or tosylates which are used instead of halides in a coupling reaction with arylboronic acids (via a Suzuki type reaction). In other cases, the aryl substituents have been successfully incorporated in 4-position of the hydroxycoumarin using organostannanes[30] or organozinc compounds[31] under Stille or Negishi conditions, respectively.

However, the formation of the aryl C–C bond using coumarin as starting material is till now an uncommon subject.[32,33] To our knowledge, few papers have been published so far concerning the 3-arylation of coumarin nucleus by direct coupling under Suzuki conditions: in all these cases, the halide was 3-bromocoumarin. And the catalysts were the classic palladium acetate ($Pd(OAc)_2$), tetrakis(triphenylphosphine)palladium (0) $Pd(PPh3)_4$[34,35], and [1,1′-bis(diphenylphosphino)ferrocene]palladium(II) dichloride.[36,37] Salen ligand has been found to be a highly active catalyst for the Suzuki reaction performed with aryl iodides, bromides, and chlorides, giving excellent yields, in a short reaction time.[38,39] Salen ligand is a bright yellow solid, soluble in polar organic solvents, and effective in C–C bond forming reactions with high selectivity. This symmetrical palladium(II) complex was a product of the reaction of the Salen ligand with palladium acetate (for 20 h at room temperature) in a good yield. This complex promotes a catalytic activity in a low concentration (0.5 mol %).[40] Noteworthy to mention that Salen is a chelating ligand generally used in coordination chemistry and homogeneous catalysis.[41]

Although significant advances have been performed in the metal-catalyzed synthesis, the palladium-catalyzed cross-coupling reaction using 3-chlorocoumarin to afford 3-arylcoumarins has never been described. This constraint encourages us to explore this synthetic strategy. Accordingly, preliminary experiments were done to examine the effect of various ligands for Pd-based coupling.[42,43] The most promising one was *N,N′*-bis(salicylidene)-ethylenediamino-palladium (salen-Pd). In fact, $Pd(OAc)_2$-salen couple in presence of a Na_2CO_3 in DMF/H_2O (1:1) was found to be an efficient catalytic system to obtain a variety of 3-arylcoumarin derivatives (Scheme 1).[40] As coumarins are sensitive substrates to alkaline conditions, and may result in the cleavage of the lactone ring, the solvent, and base for the coupling reaction were carefully studied. To optimize the reaction the influence of the palladium source in the catalytic activity was also performed. $Pd(OAc)_2$ was found to be the best option.[40] After reaction optimization, the influence of the Pd-salen complex in the Suzuki reaction involving cross-coupling of a coumarin halide and different arylboronic acids was investigated. The Suzuki reaction developed herein was found to be effective when using arylboronic acids with a variety of functional groups. Furthermore the reaction works with iodo-, bromo- or chloro-coumarin substrates. Different reaction times are required for each coumarin halide type. Although it is well-known that in Suzuki reaction the chloride derivatives are less reactive than the bromide or the iodide ones, the use of the 3-chlorocoumarin as starting material in the reaction is related with its commercial availability. The synthesis of 3-iodocoumarin and 3-bromocoumarin involves the introduction of other synthetic steps in the overall reaction.

Comparing the synthetic route herein proposed with the classic one, previously reported by us,[7,8] one can verify that both are easy and not too expensive methodologies. The main advantage of this new synthetic approach is that it can afford 3-arylcoumarins with different substitution patterns. By means of the traditional methodology, hydroxyl and nitro derivatives could not be obtained and/or purified. The reaction was not clean and the obtained crude product was a complex mixture with several sub-products and the purification process was worthless. The reaction versatility is the main advantage of this synthetic strategy comparing to the classic Perkin reaction. Compounds **4**–**7**[44–46] and **10**[47] are exclusively prepared by the Suzuki methodology while compounds **1**–**3**, **8**, and **9** can be prepared by both methods (Table 1).[48–50] The proposed synthetic strategy operates from micro- to macro-scale and avoids the formation of undesired sub-products. The palladium complex can be easily and almost totally recuperated at the end of the process. The catalyst is recovered from the reaction mixture when the product is purified by flash chromatography and it is ready to be reused. This subject has great interest because of the toxicity and the cost of palladium complexes.

In conclusion, a novel and versatile route for the synthesis of 3-arylcoumarins is proposed. The palladium cross-coupling reaction can be applied to obtain a series of different functionalized derivatives. In addition, we have shown that *N,N′*-bis(salicylidene)-ethylenediamino-palladium proved to be a good catalyst for the Suzuki reaction of heterocyclic activated chlorides with arylboronic acids, under aerobic conditions. This methodology is an efficient synthetic route for obtaining different 3-arylcoumarins.

Table 1
Synthesis of 3-arylcoumarins (**1**–**10**) by Perkin reaction and/or via Suzuki-cross-coupling reaction

Compounds	Perkin reaction[a]		Suzuki reaction[b]	
	Time (min)	Yield (%)	Time (min)	Yield (%)
1	1440	60	120	61
2	1440	65	120	58
3	1440	68	120	56
4	*	*	180	64
5	*	*	160	65
6	*	*	160	55
7	*	*	160	59
8	1440	70	120	63
9	2160	63	140	61
10	*	*	140	60

* Compounds **4**–**7** and **10** are exclusively prepared by the Suzuki methodology.
[a] Conditions: phenylacetic acids, DCC, DMSO, 110 °C, 24–36 h.
[b] Reactions with 3-chlorocoumarin. Compound **1** was synthesized using 3-bromocoumarin—reaction time: 100 min; yield: 70%.

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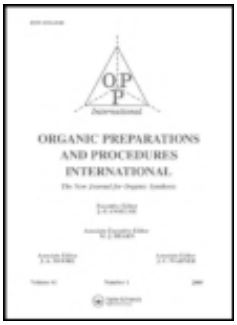
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46. *General method to prepare 3-arylcoumarins*: To a 20 mL two neck round-bottomed flask was added a solution of 3-chlorocoumarin (0.83 mmol), aryl boronic acid (1.04 mmol), Na_2CO_3 (1.66 mmol), and Pd-salen complex (0.5 mol %) in DMF/H_2O (1:1). The reaction mixture was heated at 110 °C for 120–180 min. The reaction was monitored by chromatography. After the completion of the reaction, the mixture was extracted with ethyl acetate (3 × 20 mL). The organic extracts were dried over anhydrous sodium sulphate, filtrated, and the solvent was evaporated under vacuum. The obtained crude product was purified by column chromatography (hexane/ethyl acetate 9:1) to give the coumarins (**1**–**9**). *3-(3′-Nitrophenyl)coumarin* (**6**): yellow solid, yield 55%; mp 252–3 °C. ^{1}H NMR ($CDCl_3$): δ = 7.17–7.22 (m, 2H, H-7, H-8), 7.39–7.48 (m, 3H, H-5, H-6, H-5′), 7.52–7.54 (m, 1H, H-6′), 7.60–7.71 (m, 2H, H-4, H-4′), 8.44 (s, 1H, H-2′). ^{13}C NMR ($CDCl_3$): δ = 110.0, 114.2, 116.7, 117.6, 119.3, 122.8, 125.5, 128.5, 131.1, 132.6, 132.7, 141.4, 149.1, 152.8, 158.6. MS: *m/z* (%) = 268 (26), 267 (M^+, 100), 239 (24), 221 (38), 193 (17) 165 (52), 164 (16), 163 (19), 139 (12), 82 (11). Anal. calcd. for $C_{15}H_9NO_4$ (267.24): C, 67.42; H, 3.39. Found: C, 67.38; H, 3.36. *3-(3′-aminophenyl)coumarin* (**7**): white solid, yield 59%; mp 154–5 °C. ^{1}H NMR ($CDCl_3$): δ = 4.12 (s, 2H, $-NH_2$), 7.27–7.38 (m, 4H, H-2′, H-4′, H-6′, H-8), 7.47 (dd, 2H, H-6, H-7, *J* = 7.74, *J* = 1.31), 7.53–7.58 (m, 2H, H-5, H-5′), 7.88 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$): δ = 116.8, 118.8, 122.4, 125.0, 125.2, 127.2, 127.8, 128.7, 131.9, 133.3, 140.1, 152.6, 155.2, 157.3, 158.8. MS: *m/z* (%) = 239 (10), 238 (19), 237 (M^+, 100), 182 (36), 181 (12), 152 (50), 124 (18), 89 (45), 63 (28), 62 (18). Anal. calcd. for $C_{15}H_{11}NO_2$ (237.25): C, 75.94; H, 4.67. Found: C, 76.00; H, 4.72.
47. *3-(4′-Chloropyridin-3′-yl)coumarin* (**10**): white solid, yield 60%; mp 234–5 °C. ^{1}H NMR ($CDCl_3$): δ = 7.32–7.37 (m, 2H, H-5′, H-8), 7.46 (d, 2H, H-6, H-7, *J* = 7.51), 7.52–7.58 (m, 2H, H-5, H-6′), 7.88 (s, 1H, H-4), 8.58 (s, 1H, H-2′). ^{13}C NMR ($CDCl_3$): δ = 116.8, 118.8, 122.3, 124.7, 125.0, 125.2, 127.2, 131.8, 137.0, 140.0, 147.7, 149.6, 152.7, 157.3. MS: *m/z* (%) = 260 (5), 259 (32), 258 (15), 257 (M^+, 100), 223 (18), 182 (34), 152 (44) 123 (13), 89 (40), 85 (23), 71 (30), 63 (18), 57 (35). Anal. calcd. for $C_{14}H_8ClNO_2$ (257.57): C, 65.26; H, 3.13. Found: C, 65.24; H, 3.10.
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Improved Synthesis of 3-(Aminoaryl)coumarins

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Improved Synthesis of 3-(Aminoaryl)coumarins

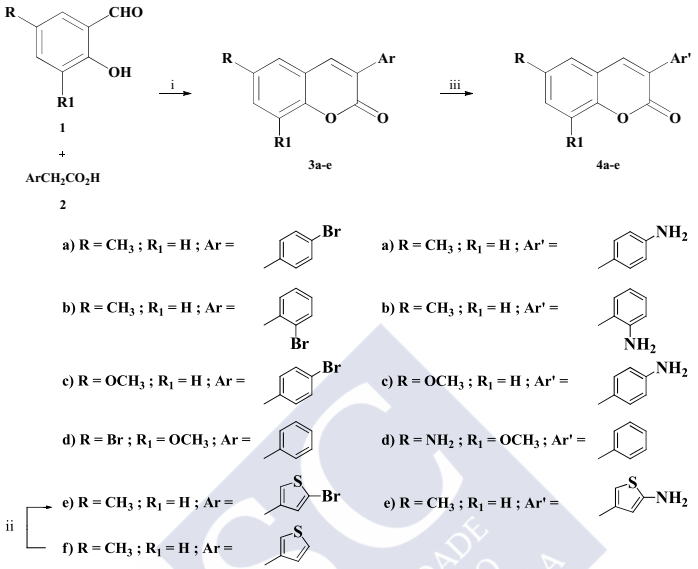
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Coumarins(benzopyrones) are widely distributed in the vegetable kingdom, either in free or combined state,[1] and occupy an important place in the realm of natural products and synthetic organic chemistry.[2] They have been used as antioxidants,[2] anti-HIV,[3] anti-cancer,[4] vasorelaxants,[5] or enzymatic inhibitors.[6] Over the last few years, our research group has paid special attention to the synthesis of new biologically active coumarins as enzymatic inhibitors;[7–12] in particular, 3-arylcoumarins have been the focal point of our recent studies[13,14] as they play an important role in the monoamino oxidase (MAO) inhibition.[7–10] Various strategies have been used to obtain arylcoumarins,[14] *e. g.*, the Wittig,[15] Pechmann,[6,7,16] Perkin,[6–13,17–21] or Knoevenagel[22,23] reactions starting from phenols or aromatic carbonyl compounds. We now describe the synthesis of aminoaryl coumarins **4a**–**e**[24] by the previously unreported copper-catalyzed C-N cross-coupling of bromoaryl coumarins with trifluoroacetamide (TFA).

Perkin condensation of differently substituted 2-hydroxybenzaldehydes (**1**) with arylacetic acids (**2**) afforded the corresponding coumarins (**3a**–**e**)[10,12,25] in 59%–69% yields; brominated compound **3e** had to be prepared by the NBS bromination of the Perkin condensation product **3f**, since the required (4-bromothien-3-yl)acetic acid is not commercially available for the direct Perkin reaction. Since previous studies[26,27] indicated *N*,*N*′-dimethylethylenediamine (DMEDA) to be the best ligand to catalyze the CuI coupling of the coumarinyl aryl bromides[10,12] with TFA, the CuI/DMEDA catalytic system was used to promote the amination of **3a**–**e** with TFA and potassium carbonate in dioxane at 70°C to give aminoaryl coumarins **4a**–**e** in 88%–94% yields. It is likely that this method using TFA could be useful for the amination of other aryl bromides.

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Reagents and conditions: (i) DCC (1.5 equiv.), DMSO, 110ºC, 24 h; (ii) NBS (1.2 equiv), AIBN, CCl_4, reflux, 18 h; (iii) CuI (5 mol%), K_2CO_3 (2.0 equiv.), molecular sieves (0.5 g), trifluoroacetamide (1.5 equiv.), DMEDA (10 mol%), dioxane, 75ºC, 24 h; methanol/water, r.t., 5 h.

Scheme 1

In conclusion, a novel and versatile route for the synthesis of 3-(aminoaryl)coumarins has been developed. The advantage of Cu-catalyzed cross-coupling method compared with classical cross-coupling reactions[28–31] is its versatility, allowing the preparation of differently functionalized derivatives in addition to the use of trifluoroacetamide as a good ammonia substitute and of CuI/DMEDA as an effective catalytic system for the amination reaction of activated bromides, under mild conditions.

Experimental Section

Melting points were obtained using a Reichert Kofler-Thermophan or in capillary tubes with a Büchi 510 apparatus and are uncorrected.^{13}C (75.4 MHz) and ^{1}H NMR (300 MHz) spectra were recorded on a Bruker AMX 300 MHz spectrometer. Chemical shifts (δ, J in Hz) are reported in ppm relative to TMS as the internal standard. Mass spectra were obtained using a Hewlett-Packard 5988A spectrometer. Elemental analyses were carried out on a Perkin-Elmer 240B microanalyzer. Silica gel (Merck 60, 230–400 mesh) was used

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for flash chromatography. Analytical TLC was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm). The preparation and characterization of **3c**, **3d**, and **3f** have been described in references 10, 12, and 25.

General Procedure for the Preparation of 3-Arylcoumarins 3a, 3b, and 3e. A solution of the substituted 2-hydroxybenzaldehyde (**1**, 7.34 mmol), the corresponding arylacetic acid (**2**, 9.18 mmol) and *N*,*N*-dicyclohexylcarbodiimide (DCC, 2.36 g, 11.46 mmol), in DMSO (5 ml), was heated at 110°C in an oil bath for 24 h. Ice (20 g) and AcOH (10 ml) were added and the mixture was stirred at r.t. for 2 h, and then extracted with Et_2O (3 × 25 ml). The combined organic layer was washed with 5% aq. $NaHCO_3$ solution (50 ml) and H_2O (20 ml), and dried (Na_2SO_4). The solvent was evaporated under vacuum and the residue was purified by flash chromatography (hexane/EtOAc, 9:1) to give the desired coumarins.

3-(4-Bromophenyl)-6-methylcoumarin (3a). Colorless solid, 62% yield, mp. 197°C–198°C. ^{1}H NMR ($CDCl_3$): δ 2.44 (s, 3H, CH_3), 7.24 (dd, J = 7.9, 1.6 Hz, 1H, H-7), 7.36 (dd, J = 7.9, 1.7 Hz, 2H, H-5, H-8), 7.56–7.62 (m, 4H, H-2′, H-3′, H-5′, H-6′), 7.78 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$): δ 20.8, 116.2, 119.2, 123.0, 127.7, 130.1, 131.6, 132.8, 133.7, 134.3, 139.9, 151.7, 160.5. MS (EI): *m/z* (%) = 317 (18), 316 (99), 315 (19), 314 (100) [M$^+$], 288 (34), 287 (22), 286 (34), 285 (17), 179 (17), 178 (36), 152 (9), 118 (14), 89 (11), 76 (12).

Anal. Calcd for $C_{16}H_{11}BrO_2$: C, 60.98; H, 3.52. Found: C, 61.00; H, 3.56.

3-(2-Bromophenyl)-6-methylcoumarin (3b). Colorless solid, 59% yield, mp. 141°C–142°C. ^{1}H NMR ($CDCl_3$): δ 2.45 (s, 3H, CH_3), 7.26–7.29 (m, 1H, H-4′), 7.31–7.39 (m, 2H, H-7, H-8); 7.32–7.49 (m, 3H, H-5, H-5′, H-6′); 7.69 (d, J = 7.8, 1H, H-3′); 7.71 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$): δ 20.8, 116.4, 118.7, 123.6, 127.4, 127.9, 128.6, 130.1, 131.3, 132.9, 133.0, 134.3, 135.9, 142.6, 152.0, 160.0. MS (EI): *m/z* (%) = 316 (5), 315 (38), 314 (100) [M$^+$], 236 (32), 235 (80), 178 (22), 117 (7), 89 (7), 76 (9).

Anal. Calcd for $C_{16}H_{11}BrO_2$: C, 60.98; H, 3.52. Found: C, 60.93; H, 3.48.

Preparation of the 3-(4-Bromothien-3-yl)-6-methylcoumarin (3e). A solution of 6-methyl-3-(thien-3-yl)coumarin **3f** (1.0 g, 3.76 mmol), NBS (0.8 g, 4.51 mmol), and AIBN (cat.) in CCl_4 (5 ml) was stirred under reflux for 18 h. The resulting suspension was filtered to remove succinimide. The solvent was evaporated under vacuum and purified by flash chromatography (hexane/EtOAc, 95:5) to give the desired bromo derivative **3e** (1.0 g, 75%) as a colorless solid, mp. 175°C–176°C. ^{1}H NMR ($CDCl_3$): δ 2.43 (s, 3H, CH_3), 7.25–7.37 (m, 5H, H-5, H-7, H-8, H-2′, H-5′), 7.9 (s, 1H, H-4). ^{13}C RMN ($CDCl_3$): δ 20.8, 112.0, 116.4, 118.8, 122.2, 125.6, 127.8, 129.3, 132.9, 134.3, 134.5, 142.6, 151.8, 160.2. MS (EI): *m/z* (%) = 320 (100) [M$^+$], 242 (18), 241 (10), 184 (18) 152 (8), 138 (11), 115 (10), 92 (12), 63 (11), 58 (14).

Anal. Calcd for $C_{14}H_9BrO_2S$: C, 52.35; H, 2.82. Found: C, 52.32; H, 2.79.

General Procedure for the Preparation of 3-(Aminoaryl)coumarins 4a–e. To a dry 20 ml two-neck round-bottom flask, a catalytic amount of CuI (8 mg, 5 mol%), K_2CO_3 (0.276 g, 2.0 mmol), and molecular sieves (4 AMS, 0.500 g) were added. The two-neck round-bottom flask was evacuated and back filled with argon. Then, the bromo coumarin (0.263 g, 1.0 mmol), 2,2,2-trifluoroacetamide (0.170 g, 1.5 mmol), DMEDA (0.012 ml, 10 mol%), and dioxane (2 ml) were added and the mixture was stirred for 24 h, at 75°C. The mixture was then cooled to room temperature and a mixture of methanol/H_2O (3:3 ml) was added. The suspension was stirred for 5 h and then extracted with EtOAc (3 × 20 ml). The

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residue obtained after evaporation of the solvent was purified by column chromatography (hexane/EtOAc, 9:1) to give the desired aminocoumarins.

3-(4-Aminophenyl)-6-methylcoumarin (4a). Colorless solid; yield 92%; mp. 191°C–192°C. ^{1}H NMR ($CDCl_3$): δ 2.43 (s, 3H, CH_3), 3.70 (s, 2H, NH_2), 7.24–7.36 (m, 3H, H-3′, H-5′, H-6), 7.54–7.65 (m, 4H, H-2′, H-6′, H-5, H-7), 7.76 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$): δ 21.8, 114.4, 115.2, 117.0, 122.3, 124.6, 124.9, 127.4, 129.6, 130.2, 132.0, 135.3, 139.9, 142.0, 157.8, 160.0. MS (EI): *m/z* 252 (21), 251 (100) [M^+], 236 (20), 152 (21), 134 (45), 129 (23), 112 (31).

Anal. Calcd for $C_{16}H_{13}NO_2$: C, 76.48; H, 5.21. Found: C, 76.41; H, 5.19.

3-(2-Aminophenyl)-6-methylcoumarin (4b). Colorless solid, 90% yield, mp. 139°C–140°C. ^{1}H NMR ($CDCl_3$): δ 2.43 (s, 3H, CH_3), 3.99 (s, 2H, NH_2), 7.09–7.12 (m, 1H, H-3′), 7.28–7.33 (m, 2H, H-4′, H-5′), 7.37–7.43 (m, 2H, H-7, H-8), 7.57–7.60 (m, 1H, H-6′), 7.67 (d, $J = 1.9$, 1H, H-5), 7.80 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$): δ 25.1, 113.4, 116.4, 119.4, 124.5, 127.4, 127.9, 130.1, 131.4, 131.6, 132.9, 133.1, 134.1, 140.0, 142.6, 155.9. MS (EI): *m/z* (%) = 252 (20), 251 (89) [M^+], 236 (29), 235 (12), 178 (23), 152 (33), 76 (13).

Anal. Calcd for $C_{16}H_{13}NO_2$: C, 76.48; H, 5.21. Found: C, 76.44; 5.19.

6-Amino-8-methoxy-3-phenylcoumarin (4d). Pale yellow solid, 88% yield, mp. 172°C–173°C. ^{1}H NMR ($CDCl_3$): δ 3.85 (s, 3H, -CH_3), 3.97 (s, 2H, -NH_2), 6.97 (s, 2H, H-7), 7.13 (s, 1H, H-5), 7.24–7.26 (m, 3H, H-2′, H-4′, H-6′), 7.63–7.69 (m, 2H, H-3′, H-5′) 7.81 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$): δ 56.4, 113.87, 115.9, 116.5, 121.2, 121.4, 126.4, 129.1, 129.8, 136.9, 147.5, 159.4, 160.3. MS (EI): *m/z* (%) = 268 (23), 267 (100) [M^+], 248 (9), 182 (13), 167 (16), 139 (17).

Ana. Calcd for $C_{16}H_{13}O_3$: C, 71.90; H, 4.90. Found: C, 71.91; H, 4.92.

3-(5-Aminothien-3-yl)-6-methylcoumarin (4e). Colorless solid, 94% yield, mp. 170°C–171°C. ^{1}H NMR ($CDCl_3$): δ 2.46 (s, 3H, CH_3), 7.27 (s, 1H, H-5′), 7.29 (s, 2H, NH_2), 7.36 (s, 1H, H-2′), 7.40 (dd, $J = 7.2$, 2.0 Hz, 2H, H-7, H-8,), 7.56 (d, $J = 1.9$ Hz, 1H, H-5), 7.93 (s, 1H, H-4). ^{13}C RMN ($CDCl_3$): δ 22.0, 116.7, 119.8, 122.0, 124.8, 125.6, 126.6, 127.2, 132.3, 135.2, 138.9, 147.2, 150.61, 160.3. MS (EI): *m/z* (%) = 258 (15), 257 (100) [M^+], 242 (18), 152 (10).

Anal. Calcd for $C_{14}H_{11}NO_2S$: C, 65.35; H, 4.31. Found: C, 65.32; H, 4.29.

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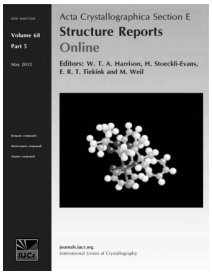
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3-Phenylcoumarin

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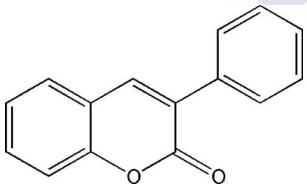
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Key indicators: single-crystal X-ray study; T = 100 K; mean σ(C–C) = 0.002 Å; R factor = 0.040; wR factor = 0.104; data-to-parameter ratio = 12.5.

In the title compound, $C_{15}H_{10}O_2$, a 3-phenyl derivative of the coumarin (also known as 2*H*-chromen-2-one or 2*H*-1-benzopyran-2-one) scaffold, the $C_p-C_p-C_c-C_c$ torsion angle between the coumarin (c) ring system and the phenyl (p) ring is −47.6 (2)°.

Related literature

For the synthesis of the title compound, see: Matos, Santana *et al.*. *et al.* (2011); Matos, Terán *et al.*. *et al.* (2011). For examples of biological activity of coumarin derivatives, see: Borges *et al.* (2009); Matos *et al.* (2009, 2010); Matos, Santana *et al.*. *et al.* (2011); Matos, Terán *et al.*. *et al.* (2011); Viña, Matos, Ferino *et al.* (2012); Viña, Matos Yáñez *et al.* (2012).



Experimental

Crystal data

$C_{15}H_{10}O_2$
M_r = 222.23
Monoclinic, $C2/c$
a = 18.469 (4) Å
b = 5.9596 (12) Å
c = 19.274 (4) Å
β = 99.079 (3)°
V = 2094.9 (7) Å^3
Z = 8
Mo $K\alpha$ radiation
μ = 0.09 mm^{-1}
T = 100 K
0.27 × 0.22 × 0.09 mm

Data collection

Bruker SMART CCD 1000 diffractometer
Absorption correction: multi-scan (*SADABS*; Bruker, 2002)
T_{min} = 0.876, T_{max} = 1
9021 measured reflections
1924 independent reflections
1492 reflections with $I > 2\sigma(I)$
R_{int} = 0.042

Refinement

$R[F^2 > 2\sigma(F^2)]$ = 0.040
$wR(F^2)$ = 0.104
S = 1.03
1924 reflections
154 parameters
H-atom parameters constrained
$\Delta\rho_{max}$ = 0.21 e Å^{-3}
$\Delta\rho_{min}$ = −0.26 e Å^{-3}

Data collection: *SMART* (Bruker, 2002); cell refinement: *SAINT* (Bruker, 2002); data reduction: *SAINT*; program(s) used to solve structure: *SIR97* (Altomare *et al.*, 1999); program(s) used to refine structure: *SHELXL97* (Sheldrick, 2008); molecular graphics: *ORTEP-3 for Windows* (Farrugia, 1997); software used to prepare material for publication: *WinGX* (Farrugia, 1999).

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Supplementary data and figures for this paper are available from the IUCr electronic archives (Reference: ZJ2091).

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supplementary materials

3-Phenylcoumarin

Maria J. Matos, Lourdes Santana and Eugenio Uriarte

Comment

Coumarin derivatives are very interesting molecules due to the biological properties that they may display (Borges *et al.* 2009 and Matos *et al.* 2009, 2010, 2011, 2012). The title structure is a 3-phenyl coumarin derivative that posses one aromatic ring linked at that position. Therefore, the X-ray analysis of this compound (figure 1) aims to contribute to the elucidation of structural requirements needed to understand the partial planarity of the compound (coumarin nucleus) and the torsion of the 3-phenyl ring. From the single-crystal diffraction measurements one can conclude that the experimental bond lengths are within normal values with the average the molecule bond lengths. The planarity of the coumarin moiety is also evident by the torsion angles values between their carbons. Also, the angle C5—C6—C7—C8 is from -47.6°, typical of the torsion permitted by the rotation of the 3-phenyl ring. Packing diagram of the structure allows the interpretation of the spatial orientation of the molecules (figure 2).

Experimental

3-Phenylcoumarin was prepared according to the protocol described by Matos *et al.* (2011). Perkin reaction gave the desired coumarin derivative. A solution of 2-hydroxybenzaldehyde (0.9 g, 7.37 mmol) and the phenylacetic acid (1.25 g, 9.21 mmol) in dimethyl sulfoxide (15 ml) was prepared. Dicyclohexylcarbodiimide (DCC, 2.37 g, 11.50 mmol) was added, and the mixture was heated at 110 °C for 24 h. Ice (100 ml) and acetic acid (10 ml) were added to the reaction mixture. After keeping it at room temperature for 2 h, the mixture was extracted with ether (3 *x* 25 ml). The organic layer was extracted with sodium bicarbonate solution (50 ml, 5%) and then water (20 ml). The solvent was evaporated under vacuum, and the dry residue was purified by flash chromatography (hexane/ethyl acetate 9:1). A white solid was obtained in a yield of 67% (1.1 g). Suitable crystals for X-ray studies were grown from slow evaporation from acetone/ethanol: Mp. 131–132 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.34–7.54 (5*H*, *m*, 6-H, 8-H, 9-H, 11-H, 13-H), 7.56–7.66 (2*H*, *m*, 10-H, 12-H), 7.72–7.80 (2*H*, *m*, 5-H, 7-H), 7.90 (1*H*, *s*, 4-H); ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 116.5, 119.7, 124.5, 127.9, 128.4, 128.5, 128.5, 128.9, 131.4, 134.7, 139.9, 153.5, 160.6; DEPT: 116.5, 124.5, 127.9, 128.4, 128.5, 128.5, 131.4, 139.9; MS *m/z* 223 ([M + 1]$^+$, 16), 222 (M^+, 100). Anal. Calcd for $C_{15}H_{10}O_2$: C, 81.07; H, 4.54. Found: C, 81.02; H, 4.52.

Refinement

Refinement of F^2 against ALL reflections. The weighted *R*-factor *wR* and goodness of fit *S* are based on F^2, conventional *R*-factors *R* are based on *F*, with *F* set to zero for negative *F*2. The threshold expression of $F^2 > 2\sigma(F^2)$ is used only for calculating *R*- factors(gt) *etc*. and is not relevant to the choice of reflections for refinement. *R*-factors based on *F*2 are statistically about twice as large as those based on *F*, and *R*- factors based on ALL data will be even larger.

Computing details

Data collection: BRUKER Smart; cell refinement: BRUKER Smart; data reduction: BRUKER Saint; program(s) used to solve structure: *SIR97* (Giacovazzo *et al.*, 1997); program(s) used to refine structure: *SHELXL97* (Sheldrick, 2008); molecular graphics: *ORTEP-3 for Windows* (Farrugia, 1997); software used to prepare material for publication: *WinGX*

publication routines (Farrugia, 1999).

fig1.tif

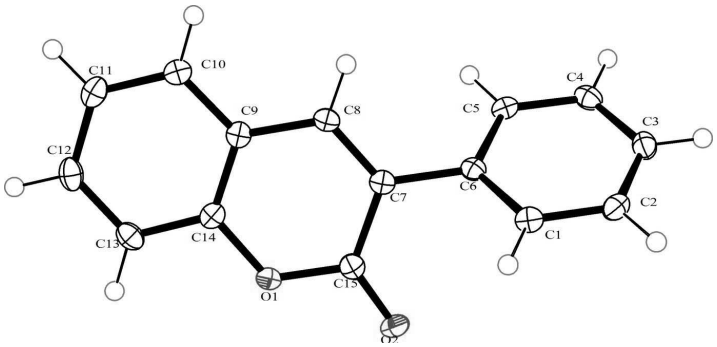


Figure 1

The molecular structure of the title compound with the atom-numbering scheme. Displacement ellipsoids are drawn at the 50% probability level.

fig2.tif

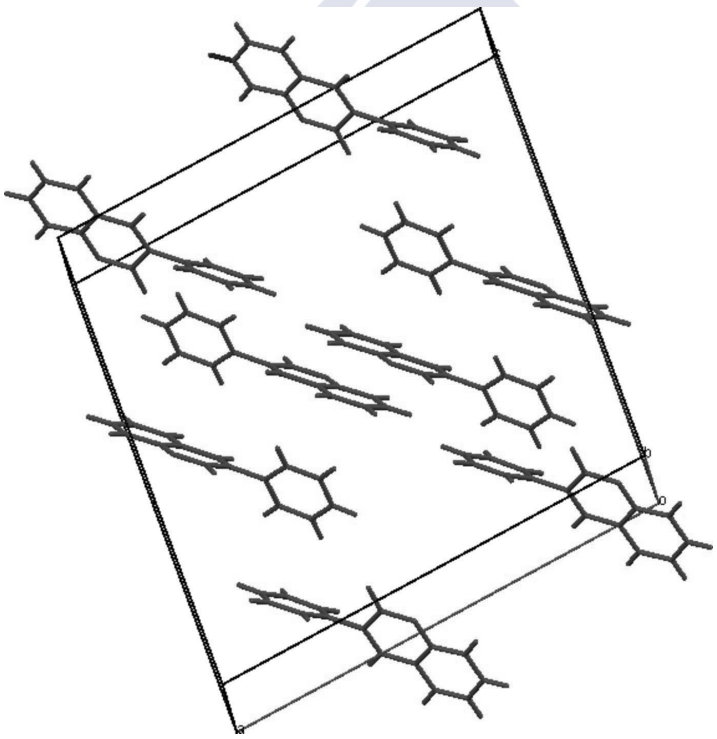


Figure 2

A packing diagram of the title structure viewed along the *b* axis.

(I)

Crystal data

$C_{15}H_{10}O_2$	$F(000)$ = 928
M_r = 222.23	F(000) = 928
Monoclinic, *C*2/*c*	D_x = 1.409 Mg m^{-3}
Hall symbol: -C 2yc	Mo $K\alpha$ radiation, λ = 0.7107 Å
a = 18.469 (4) Å	Cell parameters from 1813 reflections
b = 5.9596 (12) Å	θ = 2.8–26.2°
c = 19.274 (4) Å	μ = 0.09 mm^{-1}
β = 99.079 (3)°	*T* = 100 K
V = 2094.9 (7) Å^3	Prism, colourless
Z = 8	0.27 × 0.22 × 0.09 mm

Data collection

Smart-CCD-1000 BRUKER diffractometer	9021 measured reflections
	1924 independent reflections
Radiation source: fine-focus sealed tube	1492 reflections with $I > 2\sigma(I)$
Graphite monochromator	R_{int} = 0.042
ω scans	θ_{max} = 25.4°, θ_{min} = 2.1°
Absorption correction: multi-scan BRUKER *SADABS*	*h* = −22→21
	k = 0→7
T_{min} = 0.876, T_{max} = 1	*l* = 0→23

Refinement

Refinement on F^2	Secondary atom site location: difference Fourier map
Least-squares matrix: full	
$R[F^2 > 2\sigma(F^2)]$ = 0.040	Hydrogen site location: inferred from neighbouring sites
$wR(F^2)$ = 0.104	
S = 1.03	H-atom parameters constrained
1924 reflections	$w = 1/[\sigma^2(F_o^2) + (0.0528P)^2 + 1.3579P]$ where $P = (F_o^2 + 2F_c^2)/3$
154 parameters	
0 restraints	$(\Delta/\sigma)_{max}$ = 0.001
Primary atom site location: structure-invariant direct methods	$\Delta\rho_{max}$ = 0.21 e Å^{-3}
	$\Delta\rho_{min}$ = −0.26 e Å^{-3}

Special details

Geometry. All s.u.'s (except the s.u. in the dihedral angle between two l.s. planes) are estimated using the full covariance matrix. The cell s.u.'s are taken into account individually in the estimation of s.u.'s in distances, angles and torsion angles; correlations between s.u.'s in cell parameters are only used when they are defined by crystal symmetry. An approximate (isotropic) treatment of cell s.u.'s is used for estimating s.u.'s involving l.s. planes.

Refinement. Refinement of F^2 against ALL reflections. The weighted *R*-factor *wR* and goodness of fit *S* are based on F^2, conventional *R*-factors *R* are based on *F*, with *F* set to zero for negative F^2. The threshold expression of $F^2 > 2\sigma(F^2)$ is used only for calculating *R*-factors(gt) *etc*. and is not relevant to the choice of reflections for refinement. *R*-factors based on F^2 are statistically about twice as large as those based on *F*, and *R*- factors based on ALL data will be even larger.

Fractional atomic coordinates and isotropic or equivalent isotropic displacement parameters (Å^2)

	x	*y*	*z*	U_{iso}*/U_{eq}
C1	0.21370 (8)	0.2410 (3)	0.36971 (8)	0.0168 (4)
H1	0.219	0.3712	0.3985	0.02*
C2	0.15672 (9)	0.2280 (3)	0.31395 (8)	0.0184 (4)
H2	0.1223	0.3472	0.3054	0.022*

C3	0.14979 (9)	0.0409 (3)	0.27041 (8)	0.0192 (4)	
H3	0.1115	0.0337	0.2313	0.023*	
C4	0.19876 (9)	−0.1348 (3)	0.28427 (8)	0.0188 (4)	
H4	0.1939	−0.263	0.2546	0.023*	
C5	0.25492 (9)	−0.1254 (3)	0.34124 (8)	0.0166 (4)	
H5	0.2876	−0.2485	0.3511	0.02*	
C6	0.26348 (8)	0.0646 (3)	0.38409 (8)	0.0145 (4)	
C7	0.32522 (8)	0.0774 (3)	0.44333 (8)	0.0146 (3)	
C8	0.34288 (8)	−0.0903 (3)	0.49004 (8)	0.0145 (3)	
H8	0.3139	−0.2228	0.4858	0.017*	
C9	0.40398 (8)	−0.0744 (3)	0.54588 (8)	0.0145 (4)	
C10	0.42315 (8)	−0.2408 (3)	0.59681 (8)	0.0168 (4)	
H10	0.3949	−0.3744	0.5955	0.02*	
C11	0.48286 (9)	−0.2116 (3)	0.64877 (8)	0.0201 (4)	
H11	0.4954	−0.3246	0.6834	0.024*	
C12	0.52497 (9)	−0.0168 (3)	0.65072 (8)	0.0206 (4)	
H12	0.5664	0.001	0.6863	0.025*	
C13	0.50708 (9)	0.1506 (3)	0.60137 (8)	0.0190 (4)	
H13	0.5357	0.2835	0.6026	0.023*	
C14	0.44646 (8)	0.1196 (3)	0.55016 (8)	0.0152 (4)	
C15	0.37106 (8)	0.2799 (3)	0.44882 (8)	0.0161 (4)	
O1	0.42953 (6)	0.29055 (18)	0.50224 (5)	0.0177 (3)	
O2	0.36238 (6)	0.43926 (18)	0.40948 (6)	0.0220 (3)	

Atomic displacement parameters (Å²)

	U^{11}	U^{22}	U^{33}	U^{12}	U^{13}	U^{23}
C1	0.0191 (8)	0.0165 (8)	0.0160 (8)	0.0006 (7)	0.0062 (7)	−0.0010 (7)
C2	0.0167 (8)	0.0186 (9)	0.0206 (8)	0.0025 (7)	0.0050 (7)	0.0052 (7)
C3	0.0166 (8)	0.0250 (9)	0.0156 (8)	−0.0040 (7)	0.0013 (6)	0.0034 (7)
C4	0.0215 (9)	0.0188 (9)	0.0167 (8)	−0.0050 (7)	0.0051 (7)	−0.0029 (7)
C5	0.0188 (8)	0.0134 (8)	0.0182 (8)	0.0012 (7)	0.0050 (7)	0.0020 (6)
C6	0.0144 (8)	0.0156 (8)	0.0144 (8)	−0.0023 (6)	0.0054 (6)	0.0004 (6)
C7	0.0149 (8)	0.0147 (8)	0.0152 (8)	0.0012 (6)	0.0055 (6)	−0.0020 (6)
C8	0.0154 (8)	0.0132 (8)	0.0160 (8)	0.0001 (6)	0.0058 (6)	−0.0028 (6)
C9	0.0138 (8)	0.0167 (8)	0.0140 (8)	0.0030 (6)	0.0056 (6)	−0.0019 (6)
C10	0.0174 (8)	0.0166 (8)	0.0177 (8)	0.0012 (7)	0.0066 (7)	0.0003 (7)
C11	0.0216 (9)	0.0225 (9)	0.0163 (8)	0.0063 (7)	0.0037 (7)	0.0017 (7)
C12	0.0158 (8)	0.0283 (10)	0.0171 (8)	0.0035 (7)	0.0010 (6)	−0.0041 (7)
C13	0.0174 (8)	0.0201 (9)	0.0199 (9)	−0.0013 (7)	0.0046 (7)	−0.0057 (7)
C14	0.0168 (8)	0.0162 (8)	0.0136 (8)	0.0038 (7)	0.0057 (6)	−0.0005 (6)
C15	0.0154 (8)	0.0161 (8)	0.0171 (8)	0.0020 (7)	0.0038 (6)	−0.0025 (7)
O1	0.0202 (6)	0.0138 (6)	0.0185 (6)	−0.0026 (5)	0.0011 (5)	0.0000 (5)
O2	0.0239 (6)	0.0148 (6)	0.0265 (6)	−0.0003 (5)	0.0017 (5)	0.0053 (5)

Geometric parameters (Å, º)

C1—C2	1.382 (2)	C8—H8	0.95
C1—C6	1.395 (2)	C9—C14	1.392 (2)
C1—H1	0.95	C9—C10	1.401 (2)

126	C2—C3	1.390 (2)	C10—C11	1.379 (2)
127	C2—H2	0.95	C10—H10	0.95
128	C3—C4	1.382 (2)	C11—C12	1.395 (2)
129	C3—H3	0.95	C11—H11	0.95
130	C4—C5	1.387 (2)	C12—C13	1.382 (2)
131	C4—H4	0.95	C12—H12	0.95
132	C5—C6	1.396 (2)	C13—C14	1.383 (2)
133	C5—H5	0.95	C13—H13	0.95
134	C6—C7	1.483 (2)	C14—O1	1.3776 (18)
135	C7—C8	1.350 (2)	C15—O2	1.2102 (19)
136	C7—C15	1.468 (2)	C15—O1	1.3709 (19)
137	C8—C9	1.434 (2)		
138				
139	C2—C1—C6	120.61 (15)	C9—C8—H8	119
140	C2—C1—H1	119.7	C14—C9—C10	117.94 (14)
141	C6—C1—H1	119.7	C14—C9—C8	117.93 (14)
142	C1—C2—C3	120.03 (15)	C10—C9—C8	124.13 (15)
143	C1—C2—H2	120	C11—C10—C9	120.29 (15)
144	C3—C2—H2	120	C11—C10—H10	119.9
145	C4—C3—C2	119.76 (15)	C9—C10—H10	119.9
146	C4—C3—H3	120.1	C10—C11—C12	120.22 (15)
147	C2—C3—H3	120.1	C10—C11—H11	119.9
148	C3—C4—C5	120.48 (15)	C12—C11—H11	119.9
149	C3—C4—H4	119.8	C13—C12—C11	120.71 (15)
150	C5—C4—H4	119.8	C13—C12—H12	119.6
151	C4—C5—C6	120.11 (15)	C11—C12—H12	119.6
152	C4—C5—H5	119.9	C12—C13—C14	118.25 (15)
153	C6—C5—H5	119.9	C12—C13—H13	120.9
154	C1—C6—C5	118.96 (14)	C14—C13—H13	120.9
155	C1—C6—C7	121.09 (14)	O1—C14—C13	116.88 (14)
156	C5—C6—C7	119.94 (14)	O1—C14—C9	120.55 (14)
157	C8—C7—C15	118.97 (14)	C13—C14—C9	122.57 (14)
158	C8—C7—C6	123.41 (14)	O2—C15—O1	116.41 (14)
159	C15—C7—C6	117.56 (14)	O2—C15—C7	125.59 (15)
160	C7—C8—C9	122.02 (15)	O1—C15—C7	117.99 (14)
161	C7—C8—H8	119	C15—O1—C14	122.53 (12)
162				
163	C6—C1—C2—C3	−1.8 (2)	C9—C10—C11—C12	−0.5 (2)
164	C1—C2—C3—C4	1.9 (2)	C10—C11—C12—C13	0.8 (2)
165	C2—C3—C4—C5	−0.2 (2)	C11—C12—C13—C14	0.0 (2)
166	C3—C4—C5—C6	−1.6 (2)	C12—C13—C14—O1	179.25 (13)
167	C2—C1—C6—C5	0.0 (2)	C12—C13—C14—C9	−1.2 (2)
168	C2—C1—C6—C7	179.41 (14)	C10—C9—C14—O1	−178.93 (13)
169	C4—C5—C6—C1	1.6 (2)	C8—C9—C14—O1	0.4 (2)
170	C4—C5—C6—C7	−177.73 (14)	C10—C9—C14—C13	1.5 (2)
171	C1—C6—C7—C8	133.05 (16)	C8—C9—C14—C13	−179.15 (13)
172	C5—C6—C7—C8	−47.6 (2)	C8—C7—C15—O2	178.09 (15)
173	C1—C6—C7—C15	−49.86 (19)	C6—C7—C15—O2	0.9 (2)
174	C5—C6—C7—C15	129.50 (15)	C8—C7—C15—O1	−0.9 (2)

C15—C7—C8—C9	1.5 (2)	C6—C7—C15—O1	−178.17 (12)
C6—C7—C8—C9	178.59 (14)	O2—C15—O1—C14	−179.01 (13)
C7—C8—C9—C14	−1.3 (2)	C7—C15—O1—C14	0.1 (2)
C7—C8—C9—C10	178.02 (14)	C13—C14—O1—C15	179.72 (13)
C14—C9—C10—C11	−0.6 (2)	C9—C14—O1—C15	0.1 (2)
C8—C9—C10—C11	−179.96 (14)		

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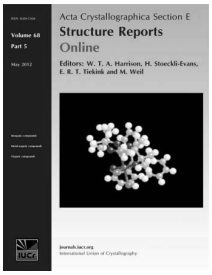
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N-(2-Oxo-2*H*-chromen-3-yl)cyclohexanecarboxamide

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N-(2-Oxo-2*H*-chromen-3-yl)cyclohexanecarboxamide

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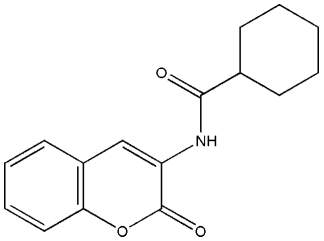
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Key indicators: single-crystal X-ray study; T = 100 K; mean σ(C–C) = 0.002 Å; R factor = 0.045; wR factor = 0.113; data-to-parameter ratio = 13.4.

In the title compound, $C_{16}H_{17}NO_3$, the coumarin moiety is essentially planar [maximum deviation from the mean plane formed by the C and O atoms of the coumarin = 0.0183 (12) Å] and that the cyclohexane ring adopts the usual chair conformation. The dihedral angle between the mean plane of the coumarin residue and the plane of the amide residue (defined as the N, C and O atoms) is 18.9 (2)°. There are two intramolecular hydrogen bonds involving the amide group. In one, the N atom acts as donor to the ketonic O atom and in the other, the amide O atom acts as acceptor of a C—H group of the coumarin. In the crystal, molecules are linked into inversion dimers by pairs of N—H···O contacts and these dimers are linked into pairs by weak C—H···O hydrogen bonds. The combination of these interactions creates a chain of rings which runs parallel to [2$\bar{1}$0]. C—H···π and π–π [centroid–centroid distance = 3.8654 (10) Å] interactions are also observed.

Related literature

For the synthesis of the title compound, see: Viña, Matos, Ferino *et al.* (2012); Viña, Matos, Yáñez *et al.* (2012). For the biological activity of coumarin derivatives, see: Borges *et al.* (2009); Matos *et al.* (2009, 2010); Matos, Santana *et al.* (2011); Matos, Terán *et al.* (2011). For graph-set analysis of hydrogen bonds, see: Bernstein *et al.*, (1995)



Experimental

Crystal data

$C_{16}H_{17}NO_3$
M_r = 271.31
Triclinic, $P\bar{1}$
a = 6.4486 (6) Å
b = 9.6324 (11) Å
c = 11.0837 (11) Å
α = 83.061 (6)°
β = 89.134 (5)°
γ = 73.987 (5)°
V = 656.79 (12) Å^3
Z = 2
Mo $K\alpha$ radiation
μ = 0.10 mm^{-1}
T = 100 K
0.48 × 0.45 × 0.09 mm

Data collection

Bruker X8 APEXII KappaCCD diffractometer
Absorption correction: multi-scan (*SADABS*; Bruker, 2012)
T_{min} = 0.910, T_{max} = 1.000
9698 measured reflections
2487 independent reflections
1834 reflections with $I > 2\sigma(I)$
R_{int} = 0.044

Refinement

$R[F^2 > 2\sigma(F^2)]$ = 0.045
$wR(F^2)$ = 0.113
S = 1.06
2487 reflections
185 parameters
H atoms treated by a mixture of independent and constrained refinement
$\Delta\rho_{max}$ = 0.20 e Å^{-3}
$\Delta\rho_{min}$ = −0.25 e Å^{-3}

Table 1
Hydrogen-bond geometry (Å, °).

*Cg*1 and *Cg*2 are the centroids of the O1/C2–C5/C10 and C5–C10 rings, respectively.

D—H···A	D—H	H···A	D···A	D—H···A
N12—H12···O11	0.87 (2)	2.346 (19)	2.6990 (18)	104.7 (14)
N12—H12···O11^{i}	0.87 (2)	2.098 (18)	2.9303 (16)	160.8 (17)
C4—H4···O14	0.95	2.37	2.9094 (19)	115
C7—H7···O14^{ii}	0.95	2.57	3.473 (2)	158
C16—H16*B*···*Cg*1^{iii}	0.99	2.81	3.5732 (17)	134
C17—H17*B*···*Cg*2^{iii}	0.99	2.70	3.5876 (19)	149

Symmetry codes: (i) $-x+1, -y, -z$; (ii) $-x-1, -y+1, -z$; (iii) $-x, -y, -z$.

Data collection: *APEX2* (Bruker, 2012); cell refinement: *SAINT* (Bruker, 2012); data reduction: *SAINT*; program(s) used to solve structure: *SIR97* (Altomare *et al.*, 1999); program(s) used to refine structure: *SHELXL97* (Sheldrick, 2008); molecular graphics: *PLATON* (Spek, 2009); software used to prepare material for publication: *WinGX* (Farrugia, 2012).

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Supplementary data and figures for this paper are available from the IUCr electronic archives (Reference: GO2076).

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supplementary materials

Acta Cryst. (2012). E**68**, o3447–o3448 [doi:10.1107/S1600536812047903]

N-(2-Oxo-2*H*-chromen-3-yl)cyclohexanecarboxamide

Maria J. Matos, Lourdes Santana and Eugenio Uriarte

Comment

Coumarin derivatives are very interesting molecules due to the biological properties that they may display (Borges *et al.* 2009; Matos *et al.* 2009, 2010; Matos, Santana *et al.*, 2011); Matos, Terán *et al.*, 2011). The title structure is a 3-substituted coumarin derivative that posses one cyclohexane ring linked by an amidic bridge at that position. Therefore, the X-ray analysis of this compound (figure 1) aims to contribute to the elucidation of structural requirements needed to understand the partial planarity of the compound (coumarin nucleus) and the torsion of the 3-substituent. Also, the X-ray analysis allows understanding the chair conformation of the cyclohexane. From the single-crystal diffraction measurements it can be concluded that the experimental bond lengths are within normal values with the average the molecule bond lengths. The planarity of the coumarin moiety is also evident by the torsion angles values between their carbons and oxygen atoms. The torsion angles C4—C3—N12—C13 (-21.3°), C3—N12—C13—C15 (-173.68°) and N12—C13—C15—C16 (-162.13°) are typical of the torsion permitted by the rotation of the amidic group at position 3. Also, the torsion angle C15—C16—C17—C18 (56.43°) is typical of a chair conformation of a cyclohexane ring.

There are intramolecular short contacts N12-H12···011 and C4–H4···O14.

The molecules are linked to form centrosymmetric $R^2_2(10)$ dimers, (Bernstein *et al.*, 1995), by the N12-H12···O11(-x+1,-y,-z) hydrogen bond. The molecules are also link into R^2_2 pairs, (Bernstein *et al.*, 1995), by the weak C7···H7···O14 (-x-1,-y+1,-z) hydrogen bond.

Combination of these pair of interactions creates a chain of rings which runs parallel to $[2\bar{1}0]$

There are C–H···π interactions between C16 and C17 and the centroids of the rings containing O11 and C9 respectively at (-x,-y,-z).

In addition there is π–π stacking between the rings containg O1 at (x,y,z) and (-x,-y+1,-z) in which the centroid to centroid distance is 3.8654 (10)Å, the perpendicular distance between the rings is 3.4428 (6)Å and the offset is 1.757Å.

Experimental

N-(coumarin-3-yl)cyclohexanecarboxamide was prepared according to the protocol described by (Viña, Matos, Ferino *et al.* 2012; Viña, Matos, Yáñez *et al.* 2012). To a solution of 3-aminocoumarin (1 mmol) and pyridine (1.1 mmol) in dichlorometane (9 ml), the corresponding acid chloride (1.1 mmol) was added dropwise and the reaction was stirred, at room temperature, for 3 h. The solvent was evaporated under vacuum and the dry residue was purified by FC (hexane/ethyl acetate 9:1). A pale yellow solid was obtained in a yield of 72%. Suitable crystals for X-ray studies were grown from slow evaporation from acetone/ethanol: Mp 180–181 °C.

Refinement

Refinement of F^2 against ALL reflections. The weighted *R*-factor *wR* and goodness of fit *S* are based on F^2, conventional *R*-factors *R* are based on *F*, with *F* set to zero for negative *F*2. The threshold expression of $F^2 > 2\sigma(F^2)$ is used only for

calculating *R*- factors(gt) *etc*. and is not relevant to the choice of reflections for refinement. *R*-factors based on *F*2 are statistically about twice as large as those based on *F*, and *R*- factors based on ALL data will be even larger.

Computing details

Data collection: *APEX2* (Bruker, 2012); cell refinement: *SAINT* (Bruker, 2012); data reduction: *SAINT* (Bruker, 2012); program(s) used to solve structure: *SIR97* (Altomare *et al.*, 1999); program(s) used to refine structure: *SHELXL97* (Sheldrick, 2008); molecular graphics: *PLATON* (Spek, 2009); software used to prepare material for publication: *WinGX* (Farrugia, 2012).

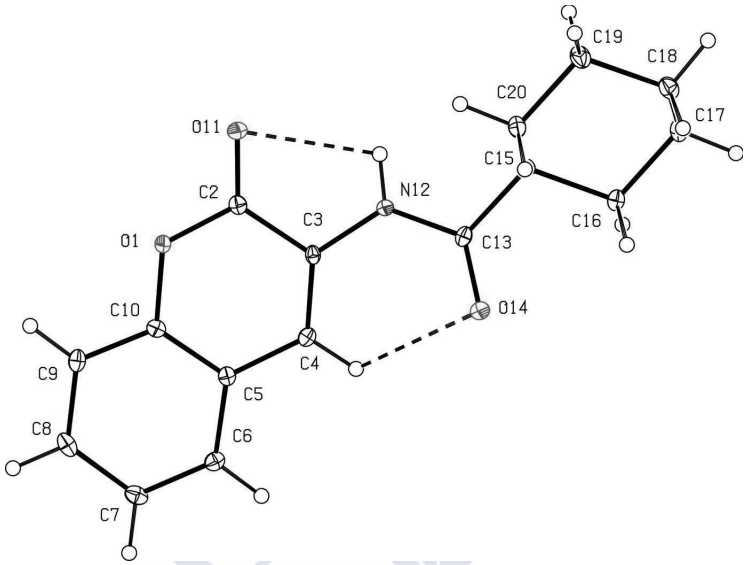


Figure 1

The molecular structure of the title compound with the atom-numbering scheme. Displacement ellipsoids are drawn at the 30%probability level. The dashed lines indicate intramolecular close contacts.

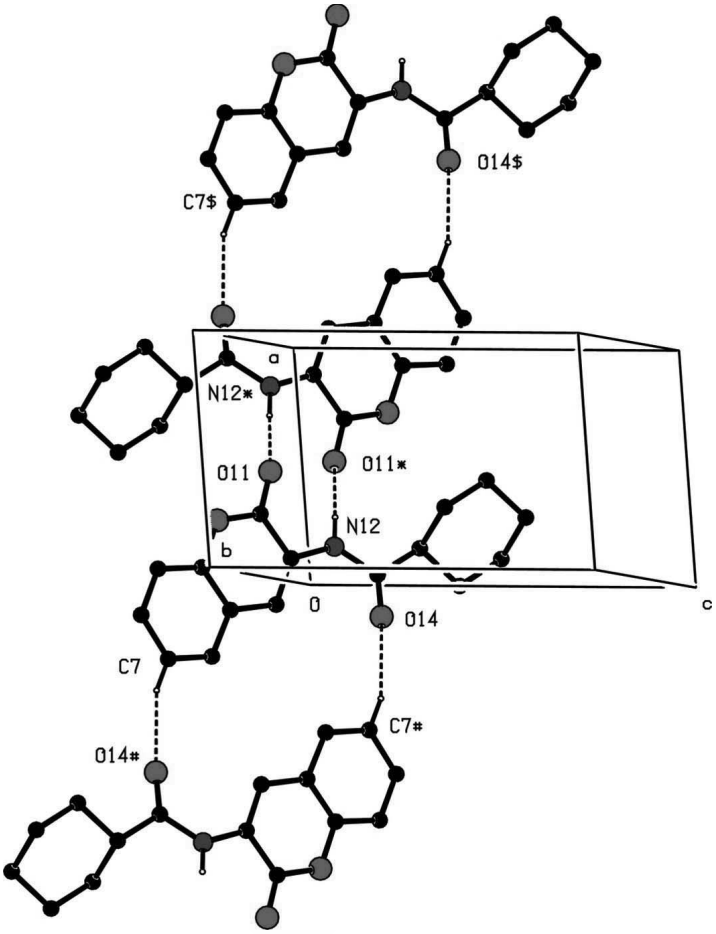


Figure 2

Part of the crystal structure of (2) showing the chain formed by molecules linked by $R^2_2(10)$ and $R^2_2(18)$ rings which runs parallel to the the {2$\bar{1}$0]. Atoms labelled with an asterisk (*), hash (#) or dollar ($), are at(-x+1,-y,-z), (-x-1,-y+1,-z) and x+2,y-1,z respectively. Hydrogen atoms not involved in the hydrogen bonding have been omitted.

***N*-(2-Oxo-2*H*-chromen-3-yl)cyclohexanecarboxamide**

Crystal data

$C_{16}H_{17}NO_3$	α = 83.061 (6)°
M_r = 271.31	β = 89.134 (5)°
Triclinic, $P\bar{1}$	γ = 73.987 (5)°
Hall symbol: -P 1	V = 656.79 (12) Å^3
a = 6.4486 (6) Å	Z = 2
b = 9.6324 (11) Å	F(000) = 288
c = 11.0837 (11) Å	F(000) = 288

D_x = 1.372 Mg m^{-3}
Melting point: 100 K
Mo $K\alpha$ radiation, λ = 0.71073 Å
Cell parameters from 1680 reflections
θ = 2.7–26.3°

μ = 0.10 mm^{-1}
T = 100 K
Plate, colourless
0.48 × 0.45 × 0.09 mm

Data collection

Bruker X8 APEXII KappaCCD diffractometer
Radiation source: fine-focus sealed tube
Graphite monochromator
ω and phi scans
Absorption correction: multi-scan (*SADABS*; Bruker, 2012)
T_{min} = 0.910, T_{max} = 1.000

9698 measured reflections
2487 independent reflections
1834 reflections with $I > 2\sigma(I)$
R_{int} = 0.044
θ_{max} = 25.7°, θ_{min} = 1.9°
h = −7→7
k = −11→11
l = 0→13

Refinement

Refinement on F^2
Least-squares matrix: full
$R[F^2 > 2\sigma(F^2)]$ = 0.045
$wR(F^2)$ = 0.113
S = 1.06
2487 reflections
185 parameters
0 restraints
Primary atom site location: structure-invariant direct methods

Secondary atom site location: difference Fourier map
Hydrogen site location: inferred from neighbouring sites
H atoms treated by a mixture of independent and constrained refinement
$w = 1/[\sigma^2(F_o^2) + (0.0577P)^2]$ where $P = (F_o^2 + 2F_c^2)/3$
$(\Delta/\sigma)_{max} < 0.001$
$\Delta\rho_{max}$ = 0.20 e Å^{-3}
$\Delta\rho_{min}$ = −0.25 e Å^{-3}

Special details

Experimental. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.27–1.95 (10H, m, 2H-2, 2H-3, 2H-4, 2H-5, 2H-6), 2.33–2.40 (1H, m H-1), 7.25–7.29 (2H, m, H-6, H-8), 7.33 (1H, dd, H-7, J=8.5, J=1.5), 7.46 (1H, dd, H-5, J=8.5, J=1.6), 8.12 (1H, s, H-4), 8.70 (1H, s, –NH); ^{13}C NMR (75.47?MHz, CDCl3): δ 25.6, 25.7, 29.7, 43.0, 116.6, 120.2, 123.4, 124.3, 125.4, 128.0, 129.8, 150.1, 159.2, 175.9; DEPT: 25.6, 25.7, 29.7, 43.0, 116.6, 120.2, 124.3, 125.4, 128.0; MS m/z 272 ([M + 1]+, 16), 271 (M+, 100). Anal. Calcd for C16H17NO3: C, 70.83; H, 6.32. Found: C, 70.85; H, 6.35.

Geometry. All s.u.'s (except the s.u. in the dihedral angle between two l.s. planes) are estimated using the full covariance matrix. The cell s.u.'s are taken into account individually in the estimation of s.u.'s in distances, angles and torsion angles; correlations between s.u.'s in cell parameters are only used when they are defined by crystal symmetry. An approximate (isotropic) treatment of cell s.u.'s is used for estimating s.u.'s involving l.s. planes.

Refinement. Refinement of F^2 against ALL reflections. The weighted R-factor wR and goodness of fit S are based on F^2, conventional R-factors R are based on F, with F set to zero for negative F^2. The threshold expression of $F^2 > 2\sigma(F^2)$ is used only for calculating R-factors(gt) *etc*. and is not relevant to the choice of reflections for refinement. R-factors based on F^2 are statistically about twice as large as those based on F, and R- factors based on ALL data will be even larger.

Fractional atomic coordinates and isotropic or equivalent isotropic displacement parameters (Å^2)

	x	*y*	*z*	U_{iso}*/U_{eq}
O1	0.24398 (15)	0.35744 (12)	−0.13872 (9)	0.0151 (3)
C2	0.2812 (2)	0.23542 (18)	−0.05704 (14)	0.0135 (4)
C3	0.0969 (2)	0.20233 (18)	0.00628 (14)	0.0124 (4)
C4	−0.1049 (2)	0.28990 (18)	−0.01851 (13)	0.0136 (4)
H4	−0.2245	0.2668	0.0216	0.016*
C5	−0.1385 (2)	0.41752 (18)	−0.10531 (13)	0.0128 (4)
C6	−0.3423 (2)	0.51517 (19)	−0.13683 (14)	0.0157 (4)

H6	−0.4679	0.4963	−0.1007	0.019*
C7	−0.3625 (3)	0.63721 (19)	−0.21886 (14)	0.0177 (4)
H7	−0.5009	0.7028	−0.2381	0.021*
C8	−0.1796 (3)	0.66474 (19)	−0.27396 (14)	0.0185 (4)
H8	−0.1938	0.7492	−0.3307	0.022*
C9	0.0224 (3)	0.56983 (18)	−0.24641 (14)	0.0165 (4)
H9	0.1473	0.5875	−0.2843	0.02*
C10	0.0385 (2)	0.44942 (18)	−0.16300 (14)	0.0135 (4)
O11	0.46651 (16)	0.16189 (12)	−0.04220 (10)	0.0183 (3)
N12	0.1571 (2)	0.07624 (16)	0.08823 (12)	0.0147 (3)
H12	0.285 (3)	0.019 (2)	0.0806 (15)	0.028 (5)*
C13	0.0426 (2)	0.03698 (18)	0.18526 (14)	0.0139 (4)
O14	−0.13975 (16)	0.10822 (13)	0.20623 (10)	0.0219 (3)
C15	0.1649 (2)	−0.09965 (18)	0.26441 (14)	0.0131 (4)
H15	0.24	−0.1722	0.2095	0.016*
C16	0.0122 (2)	−0.16661 (18)	0.34364 (14)	0.0160 (4)
H16A	−0.0669	−0.0959	0.3977	0.019*
H16B	−0.0948	−0.1882	0.2911	0.019*
C17	0.1367 (2)	−0.30629 (19)	0.42045 (15)	0.0188 (4)
H17A	0.0353	−0.3448	0.4735	0.023*
H17B	0.2042	−0.3802	0.3664	0.023*
C18	0.3111 (3)	−0.27969 (19)	0.49876 (15)	0.0201 (4)
H18A	0.2425	−0.2148	0.5595	0.024*
H18B	0.3954	−0.3732	0.5431	0.024*
C19	0.4615 (3)	−0.21078 (19)	0.42127 (15)	0.0212 (4)
H19A	0.5427	−0.2807	0.3672	0.025*
H19B	0.567	−0.1889	0.4748	0.025*
C20	0.3377 (2)	−0.07084 (19)	0.34427 (14)	0.0174 (4)
H20A	0.4394	−0.0317	0.292	0.021*
H20B	0.2681	0.0028	0.3981	0.021*

Atomic displacement parameters (Å²)

	U^{11}	U^{22}	U^{33}	U^{12}	U^{13}	U^{23}
O1	0.0132 (6)	0.0145 (7)	0.0169 (6)	−0.0041 (5)	0.0012 (5)	0.0019 (5)
C2	0.0175 (8)	0.0115 (10)	0.0127 (9)	−0.0048 (7)	0.0005 (7)	−0.0039 (7)
C3	0.0168 (8)	0.0117 (10)	0.0096 (8)	−0.0053 (7)	0.0014 (6)	−0.0015 (7)
C4	0.0145 (8)	0.0160 (10)	0.0117 (9)	−0.0058 (7)	0.0013 (7)	−0.0037 (8)
C5	0.0170 (8)	0.0126 (10)	0.0098 (9)	−0.0048 (7)	0.0007 (7)	−0.0039 (7)
C6	0.0153 (8)	0.0196 (11)	0.0125 (9)	−0.0044 (7)	0.0004 (7)	−0.0043 (8)
C7	0.0199 (9)	0.0175 (10)	0.0133 (9)	0.0000 (7)	−0.0041 (7)	−0.0037 (8)
C8	0.0290 (9)	0.0128 (10)	0.0137 (9)	−0.0060 (8)	−0.0040 (7)	−0.0007 (8)
C9	0.0207 (9)	0.0178 (10)	0.0138 (9)	−0.0099 (7)	0.0015 (7)	−0.0022 (8)
C10	0.0150 (8)	0.0136 (10)	0.0117 (9)	−0.0029 (7)	−0.0016 (6)	−0.0031 (7)
O11	0.0129 (6)	0.0174 (7)	0.0229 (7)	−0.0024 (5)	0.0014 (5)	−0.0005 (5)
N12	0.0126 (7)	0.0145 (8)	0.0149 (8)	−0.0011 (6)	0.0024 (6)	0.0005 (6)
C13	0.0157 (8)	0.0161 (10)	0.0123 (9)	−0.0079 (7)	0.0021 (7)	−0.0036 (7)
O14	0.0162 (6)	0.0218 (8)	0.0230 (7)	−0.0005 (5)	0.0046 (5)	0.0035 (6)
C15	0.0161 (8)	0.0118 (10)	0.0112 (8)	−0.0036 (7)	0.0008 (6)	−0.0014 (7)
C16	0.0182 (8)	0.0169 (10)	0.0148 (9)	−0.0080 (7)	0.0019 (7)	−0.0019 (8)

C17	0.0241 (9)	0.0178 (11)	0.0156 (9)	−0.0083 (7)	0.0029 (7)	−0.0011 (8)
C18	0.0248 (9)	0.0174 (11)	0.0165 (9)	−0.0041 (8)	−0.0006 (7)	0.0003 (8)
C19	0.0213 (9)	0.0221 (11)	0.0194 (10)	−0.0067 (8)	−0.0049 (7)	0.0029 (8)
C20	0.0192 (8)	0.0172 (10)	0.0165 (9)	−0.0075 (7)	−0.0011 (7)	0.0013 (8)

Geometric parameters (Å, º)

O1—C2	1.3610 (19)	C13—O14	1.2220 (17)
O1—C10	1.3852 (17)	C13—C15	1.514 (2)
C2—O11	1.2115 (17)	C15—C16	1.531 (2)
C2—C3	1.462 (2)	C15—C20	1.537 (2)
C3—C4	1.3523 (19)	C15—H15	1
C3—N12	1.391 (2)	C16—C17	1.526 (2)
C4—C5	1.434 (2)	C16—H16A	0.99
C4—H4	0.95	C16—H16B	0.99
C5—C10	1.389 (2)	C17—C18	1.525 (2)
C5—C6	1.410 (2)	C17—H17A	0.99
C6—C7	1.373 (2)	C17—H17B	0.99
C6—H6	0.95	C18—C19	1.521 (2)
C7—C8	1.395 (2)	C18—H18A	0.99
C7—H7	0.95	C18—H18B	0.99
C8—C9	1.383 (2)	C19—C20	1.527 (2)
C8—H8	0.95	C19—H19A	0.99
C9—C10	1.374 (2)	C19—H19B	0.99
C9—H9	0.95	C20—H20A	0.99
N12—C13	1.370 (2)	C20—H20B	0.99
N12—H12	0.867 (17)		
C2—O1—C10	121.98 (12)	C13—C15—C20	111.68 (14)
O11—C2—O1	117.05 (14)	C16—C15—C20	110.15 (13)
O11—C2—C3	124.73 (15)	C13—C15—H15	107.8
O1—C2—C3	118.23 (13)	C16—C15—H15	107.8
C4—C3—N12	127.29 (15)	C20—C15—H15	107.8
C4—C3—C2	120.24 (15)	C17—C16—C15	111.00 (12)
N12—C3—C2	112.46 (13)	C17—C16—H16A	109.4
C3—C4—C5	120.11 (15)	C15—C16—H16A	109.4
C3—C4—H4	119.9	C17—C16—H16B	109.4
C5—C4—H4	119.9	C15—C16—H16B	109.4
C10—C5—C6	116.77 (15)	H16A—C16—H16B	108
C10—C5—C4	119.08 (14)	C18—C17—C16	111.32 (14)
C6—C5—C4	124.15 (15)	C18—C17—H17A	109.4
C7—C6—C5	121.11 (15)	C16—C17—H17A	109.4
C7—C6—H6	119.4	C18—C17—H17B	109.4
C5—C6—H6	119.4	C16—C17—H17B	109.4
C6—C7—C8	119.91 (15)	H17A—C17—H17B	108
C6—C7—H7	120	C19—C18—C17	111.01 (14)
C8—C7—H7	120	C19—C18—H18A	109.4
C9—C8—C7	120.39 (16)	C17—C18—H18A	109.4
C9—C8—H8	119.8	C19—C18—H18B	109.4
C7—C8—H8	119.8	C17—C18—H18B	109.4

C10—C9—C8	118.56 (15)	H18A—C18—H18B	108
C10—C9—H9	120.7	C18—C19—C20	111.67 (14)
C8—C9—H9	120.7	C18—C19—H19A	109.3
C9—C10—O1	116.42 (14)	C20—C19—H19A	109.3
C9—C10—C5	123.25 (14)	C18—C19—H19B	109.3
O1—C10—C5	120.33 (15)	C20—C19—H19B	109.3
C13—N12—C3	127.25 (13)	H19A—C19—H19B	107.9
C13—N12—H12	115.9 (12)	C19—C20—C15	110.64 (14)
C3—N12—H12	116.6 (12)	C19—C20—H20A	109.5
O14—C13—N12	122.46 (15)	C15—C20—H20A	109.5
O14—C13—C15	123.73 (14)	C19—C20—H20B	109.5
N12—C13—C15	113.80 (13)	C15—C20—H20B	109.5
C13—C15—C16	111.49 (12)	H20A—C20—H20B	108.1
C10—O1—C2—O11	179.93 (13)	C4—C5—C10—C9	−179.32 (15)
C10—O1—C2—C3	−0.1 (2)	C6—C5—C10—O1	−178.81 (13)
O11—C2—C3—C4	−178.64 (15)	C4—C5—C10—O1	1.3 (2)
O1—C2—C3—C4	1.4 (2)	C4—C3—N12—C13	−21.3 (3)
O11—C2—C3—N12	0.8 (2)	C2—C3—N12—C13	159.30 (15)
O1—C2—C3—N12	−179.14 (13)	C3—N12—C13—O14	5.0 (3)
N12—C3—C4—C5	179.33 (14)	C3—N12—C13—C15	−173.68 (14)
C2—C3—C4—C5	−1.3 (2)	O14—C13—C15—C16	20.2 (2)
C3—C4—C5—C10	0.0 (2)	N12—C13—C15—C16	−161.13 (14)
C3—C4—C5—C6	−179.90 (14)	O14—C13—C15—C20	−103.50 (18)
C10—C5—C6—C7	−1.3 (2)	N12—C13—C15—C20	75.17 (17)
C4—C5—C6—C7	178.56 (15)	C13—C15—C16—C17	178.50 (13)
C5—C6—C7—C8	1.1 (2)	C20—C15—C16—C17	−56.93 (18)
C6—C7—C8—C9	0.0 (2)	C15—C16—C17—C18	56.43 (18)
C7—C8—C9—C10	−0.7 (2)	C16—C17—C18—C19	−55.19 (18)
C8—C9—C10—O1	179.81 (13)	C17—C18—C19—C20	55.31 (19)
C8—C9—C10—C5	0.4 (2)	C18—C19—C20—C15	−56.28 (18)
C2—O1—C10—C9	179.36 (13)	C13—C15—C20—C19	−178.89 (13)
C2—O1—C10—C5	−1.2 (2)	C16—C15—C20—C19	56.65 (17)
C6—C5—C10—C9	0.6 (2)		

Hydrogen-bond geometry (Å, º)

Cg1 and Cg2 are the centroids of the O1/C2–C5/C10 and C5–C10 rings, respectively.

D—H···*A*	*D*—H	H···*A*	*D*···*A*	*D*—H···*A*
N12—H12···O11	0.87 (2)	2.346 (19)	2.6990 (18)	104.7 (14)
N12—H12···O11^{i}	0.87 (2)	2.098 (18)	2.9303 (16)	160.8 (17)
C4—H4···O14	0.95	2.37	2.9094 (19)	115
C7—H7···O14^{ii}	0.95	2.57	3.473 (2)	158
C16—H16*B*···*Cg*1^{iii}	0.99	2.81	3.5732 (17)	134
C17—H17*B*···*Cg*2^{iii}	0.99	2.70	3.5876 (19)	149

Symmetry codes: (i) −*x*+1, −*y*, −*z*; (ii) −*x*−1, −*y*+1, −*z*; (iii) −*x*, −*y*, −*z*.

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Synthesis, NMR characterization, X-ray structural analysis and theoretical calculations of amide and ester derivatives of the coumarin scaffold

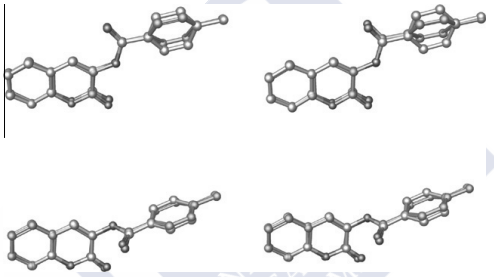
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HIGHLIGHTS

- Compound 1 (4-Methyl-N-(coumarin-3-yl)benzamide) was synthesized.
- Compound 2 (coumarin-3-yl)-4-methylbenzoate was synthesized.
- ^{1}H and ^{13}C NMR and X-ray diffractometry determined the molecular structures.
- Semiempirical calculations presented an alternative to determine the 3D structures.
- AM1 and PM3 yielded results reproducing the whole 3D structure of both molecules.

GRAPHICAL ABSTRACT



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ABSTRACT

Compounds 1 (4-methyl-N-(coumarin-3-yl)benzamide) and 2 ((coumarin-3-yl)-4-methylbenzoate) were synthesized by linking the coumarin system (3-aminocoumarin or 3-hydroxycoumarin, respectively) to a p-toluoylchloride. ^{1}H and ^{13}C NMR and X-ray diffractometry determined the molecular structures of both derivatives. The X-ray results were compared to those obtained by conformational analysis followed by semiempirical methodologies (AM1 and PM3). The theoretical calculations yielded results reproducing the whole three-dimensional (3D) structure of both molecules in a good agreement with X-ray structural analysis. The global structures of the two compounds are very similar in the two studied environments, meaning that the structural determination in the gas phase can be extrapolated. A comparative study between compounds 1 and 2, based on the structural results, was carried out.

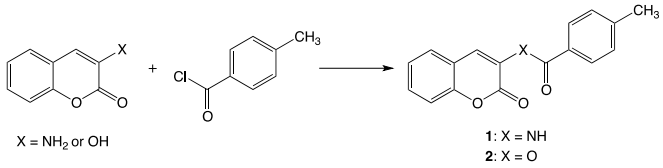
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1. Introduction

The complex etiology and the actual prevalence of neurodegenerative diseases have led to an intensive search for compounds that interact with some specific receptors. In particular, in our research group we have paid special attention to compounds that can inhibit monoamine oxidase B (MAO-B isoform) and acetylcholinesterase (AChE) enzymes. In the last years we are synthesizing and studying an important family of compounds, the coumarin derivatives, focusing on those targets. Coumarin (2H-1-benzopyran-2-one) is a natural compound that can be found in several plants like tonka bean, vanilla grass and sweet woodruff [1–3]. Derivatives of this benzopyrone are of pharmaceutical interest because they have been showing different important biological activities [1–3]. Coumarins have been described as anticancer, anti-inflammatory, antimicrobial, cardioprotective, vasorelaxant and antioxidant agents [4–13]. Furthermore, several coumarins showing a significant MAO inhibitory activity, in some cases accompanied by AChE inhibitory activity, have been reported by our research group, and some of them are suggested as potential drugs with application on neurodegenerative diseases [14–20]. Due to the biological importance of these derivatives, the synthesis and characterization

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Scheme 1. Synthesis of compounds 1 and 2. Reagents and conditions: Pyridine, dicloromethane, 0 °C to r.t., 4 h.

Table 1
Crystal data and structure refinement parameters for compounds 1 and 2.

	Compound 1	Compound 2
Empirical formula	C17H13NO3	C17H12O4
Formula weight	279.28	280.27
Temperature	100(2) K	100(2) K
Wavelength	0.71073 Å	0.71073 Å
Crystal system	Triclinic	Triclinic
Space group	P-1	P-1
Unit cell dimensions	a = 7.9932(4) Å, α = 113.893(3)°	a = 6.7771(7) Å, α = 91.563(8)°
	b = 9.2074(6) Å, β = 97.003(3)°	b = 7.0925(9) Å, β = 91.436(7)°
	c = 10.3711(7) Å, γ = 101.145(3)°	c = 13.9318(18) Å, γ = 101.984(7)°
Volume	667.43(7) Å3	654.49(14) Å3
Z	2	2
Density (calculated)	1.390 Mg/m3	1.422 Mg/m3
Absorption coefficient	0.096 mm−1	0.102 mm−1
F(0 0 0)	292	292
Crystal size	0.38 × 0.30 × 0.25 mm3	0.47 × 0.37 × 0.14 mm3
Theta range for data collection	2.20–26.37°	2.93–26.02°
Index ranges	$-9 \leqslant h \leqslant 9, -11 \leqslant k \leqslant 10, 0 \leqslant l \leqslant 12$	$-8 \leqslant h \leqslant 8, -8 \leqslant k \leqslant 8, 0 \leqslant l \leqslant 17$
Reflections collected	9965	14,492
Independent reflections	2715 [R(int) = 0.0326]	2573 [R(int) = 0.0479]
Completeness to theta = 26.37°	99.6%	99.8%
Absorption correction	Semi-empirical from equivalents	Semi-empirical from equivalents
Max. and min. transmission	0.9773 and 0.8884	1.0000 and 0.9573
Refinement method	Full-matrix least-squares on F2	Full-matrix least-squares on F2
Data/restraints/parameters	2715/0/196	2573/0/191
Goodness-of-fit on F2	1.078	1.084
Final R indices [I > 2sigma(I)]	R1 = 0.0445, wR2 = 0.1049	R1 = 0.0438, wR2 = 0.1045
R indices (all data)	R1 = 0.0622, wR2 = 0.1132	R1 = 0.0610, wR2 = 0.1127
Largest diff. peak and hole	0.218 and −0.271 e Å−3	0.237 and −0.259 e Å−3

of coumarins is a topic of interest. The interaction between a specific molecule, a drug candidate, and a receptor occurs through recognition between the different compounds involved. An important step in the study of molecular interaction processes is the determination of the structure of the potential drugs. This means carrying out an analysis of the spatial arrangement of the different atomic groups and their chemical properties. In this way, the first step must be to obtain information about the intramolecular features responsible for the 3D structure of the molecule under study. The X-ray structure is an important tool to better understand the interaction of the compound with the active site of the enzyme.

According to the latest findings related to the conformational preferences of these molecules, it is interesting to investigate the 3-D structure of novel 3-substituted coumarins by using both experimental and theoretical approaches. Computational approaches can offer an alternative solution to determine the three-dimensional molecular structure of the compounds under study. Depending on the molecular complexity and the precision required for the study, different types of calculations can be carried out. Molecular mechanics methods [21] could be appropriate for the calculation of the 3-D structure of large molecules while more accurate quantum mechanical methods, such as ab initio calculations [22], would require long periods of time making the complexity of the calculations a limiting factor to be applicable in large systems. An intermediate level of accuracy is provided by the so-called semiempirical quantum chemical calculations that use some parameters from empirical data [23]. Among these methods, the rapid, inexpensive and user-friendly AM1 and PM3 are probably two of the most popular methodologies [24,25]. The analysis of semiempirical methods results is an interesting tool for reproducing the experimental geometry of specific molecules for further studies directed to the molecular modeling and design of active molecules.

In the current work described here, experimental and theoretical structural analysis of two synthesized 3-substituted coumarin derivatives, presenting at that position an amide (compound 1) or an ester (compound 2), were undertaken. Their structures were characterized by experimental methods such as NMR spectrometry and were finally determined by X-ray diffractometry and theoretical calculations combining conformational analysis with AM1 and PM3 methods. The comparison between the theoretical and the crystal structure for both compounds showed a high level of similarity. This fact manifested the capability of the theoretical methods to reproduce the experimental molecular geometry being an alternative methodology to obtain three-dimensional information when the crystal structure is not available.

2. Experimental section

2.1. Material and measurements

Melting points were determined using a Reichert Kofler thermopan or in capillary tubes on a Büchi 510 apparatus and are

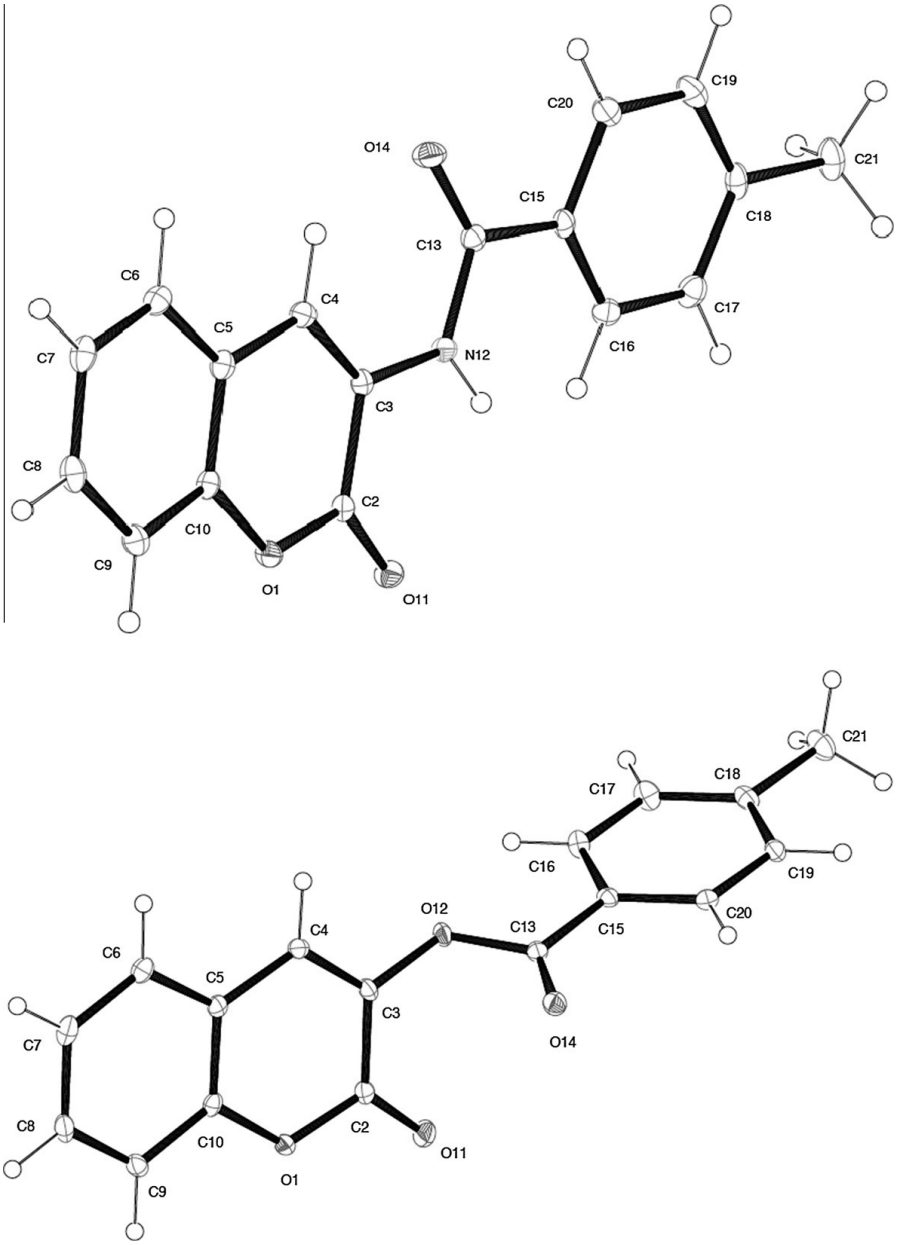


Fig. 1. Molecular structures of compounds 1 and 2 with the atom-numbering scheme. Displacement ellipsoids are drawn at the 30% probability level.

uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX spectrometer at 300 and 75.47 MHz, respectively, using TMS as internal standard (chemical shifts in δ values, J in Hz) and DMSO-d6 as solvent. Mass spectra were obtained using a Hewlett Packard 5972-MSD spectrometer. Elemental analyses were performed using a Perkin–Elmer 240B microanalyser and were within ±0.4% of calculated values in all cases. Silica gel (Merck 60, 230–00 mesh) was used for flash chromatography (FC). Analytical thin layer chromatography (TLC) was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm). The purity of compounds was assessed by HPLC and was found to be higher than 95%.

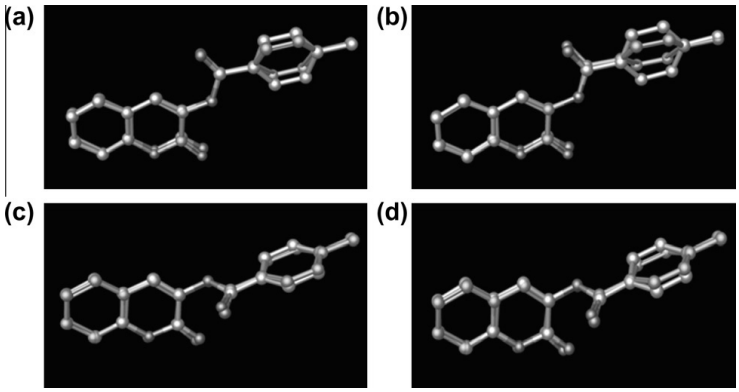


Fig. 2. Superposition of the crystal structure (grey carbons) and the theoretical conformations (green carbons) obtained through AM1 (panels a and c) and PM3 (panels b and d) for compounds 1 and 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
RMSD between the conformations obtained through theoretical methods and the X-ray structures for compounds 1 and 2. RMSD values are shown for distances [Å], bond angles [°], dihedral angles [°] and the heavy atoms coordinates (hydrogens were not taken into account).

	Compound 1		Compound 2	
RMSD	AM1	PM3	AM1	PM3
Distance	0.014	0.015	0.015	0.011
Angles	1.79	2.56	2.010	2.521
Dihedral angles	9.02	15.41	3.584	4.507
Heavy atoms coordinates	0.254	0.369	0.178	0.246

Table 5
Hydrogen bonds for compound 1 [Å and °].

D—H···A	d(D—H)	d(H···A)	d(D···A)	<(DHA)
N(12)–H(12)···O(11)#1	0.835(17)	2.498(18)	3.2711(17)	154.4(15)

Symmetry transformations used to generate equivalent atoms: #1 −x + 1, −y, −z + 1.

2.2. Synthesis of compounds 1 and 2

Compounds 1 and 2 (Scheme 1) were prepared according to the protocol described by us [16]. Detailed protocol is described in Supplementary data.

4-Methyl-N-(coumarin-3-yl)benzamide (1). Pale yellow crystals. Mp 187–188 °C. ^{1}H NMR: 2.49 (s, 3H, CH3); 7.33 (s, 1H, H-4); 7.38 (d, 2H, J = 7.6 Hz, H-6, H-8); 7.50 (d, 2H, J = 8.5 Hz, H-3′, H-5′); 7.76 (d, 1H, J = 7.6 Hz, H-7); 7.83–7.88 (m, 3H, H-2′, H-5, H-6′); 8.62 (s, 1H, NH). ^{13}C NMR: 21.32 (CH3); 116.35 (C-8); 119.75 (C-4a); 124.57 (C-8a); 125.49 (C-4); 126.62 (C-5); 126.81 (C-6); 127.94 (C-2′,C-6′); 128.45 (C-7); 129.64 (C-3′, C-5′); 130.59 (C-1′); 130.98 (C-3); 130.92 (C-4′); 143.04 (C-2); 1450.53 (NH—C=O). DEPT: 21.32 (CH3); 116.35 (C-8); 125.49 (C-4); 126.62 (C-5); 126.81 (C-6); 127.94 (C-2′, C-6′); 128.45 (C-7); 129.64 (C-3′, C-5′). EI MS m/z (%): 280 (9), 279 (M+, 50), 239 (20), 134 (23), 112 (20), 98 (42) Elem. Anal. Calc. for C17H13NO3: C, 73.11; H, 4.69. Found: C, 73.09; H, 4.65.

(Coumarin-3-yl)-4-methylbenzoate (2). Pale yellow crystals. Mp 168–169 °C. ^{1}H NMR: 2.43 (s, 3H, CH3); 7.44 (d, 2H, J = 7.5 Hz, H-6, H-8); 7.51 (d, 2H, J = 8.1 Hz, H-3′, H-5′); 7.64 (d, 1H, J = 7.4 Hz, H-7); 7.75 (d, 1H, J = 7.6 Hz, H-5); 8.0 (d, 2H, J = 8.2 Hz, H-2′, H-6′); 8.20 (s, 1H, H-4). ^{13}C NMR: 21.30 (CH3); 124.98 (C-8); 125.39 (C-4a); 128.71 (C-4); 129.45 (C-1′); 129.93 (C-6); 130.23 (C-5); 131.47 (C-7); 132.30 (C-3′, C-5′); 134.27 (C-2′, C-6′); 135.71 (C-3); 137.65 (C-4′); 145.51 (C-8a); 159.21 (C-2); 163.77 (O—C=O). EI MS m/z (%): 280 (M+, 8), 119 (100), 91 (29), 77 (8), 65 (12). Elem. Anal. Calc. for C17H12O4: C, 70.58; H, 4.13. Found: C, 70.60; H, 4.13.

2.3. X-ray data collection and reduction

Pale yellow prismatic crystals of compounds 1 (C17H13NO3) and 2 (C17H12O4) were mounted on a glass fiber for data collection. Cell constants and orientation matrix were obtained by least-squares refinement of the diffraction data for 9965 and 14,492 reflections in the range of 2.20–26.37° and 2.93–26.02°, respectively, measured on a Bruker APEX2 [26]. Data were collected by the ω scan technique at 100 K using radiation (λ = 0.71073 Å), and were corrected for Lorentz and polarization effects. The crystal data and structure refinement were detailed in Table 1 (compounds 1 and 2, respectively). A semiempirical absorption correction was also performed.

2.4. Structure solution and refinement

The structures were solved by direct methods [27], which revealed the positions of all non-hydrogen atoms, and were refined on F2 by a full-matrix least-squares procedure using anisotropic displacement parameters. The program used to refine the structures was SHELXL97 [28]. All hydrogen atoms were located from difference Fourier maps and were refined isotropically. Atomic scattering factors were taken from International Tables for X-ray Crystallography [29]. Molecular graphics were generated with Platon [30]. Finally, the software used to prepare material for publication was WinGX publication routines [31]. A summary of the crystals data, experimental details, and refinements results is given in Table 1.

2.5. Theoretical calculations

A stochastic conformational search was carried out with the help of MOE software [32]. Partial charges were calculated using MMFF94 force field. The RMS gradient was 0.005 with an interaction limit of 500. The conformations with the lowest energy were representative of the calculation and were optimized with MOPA-

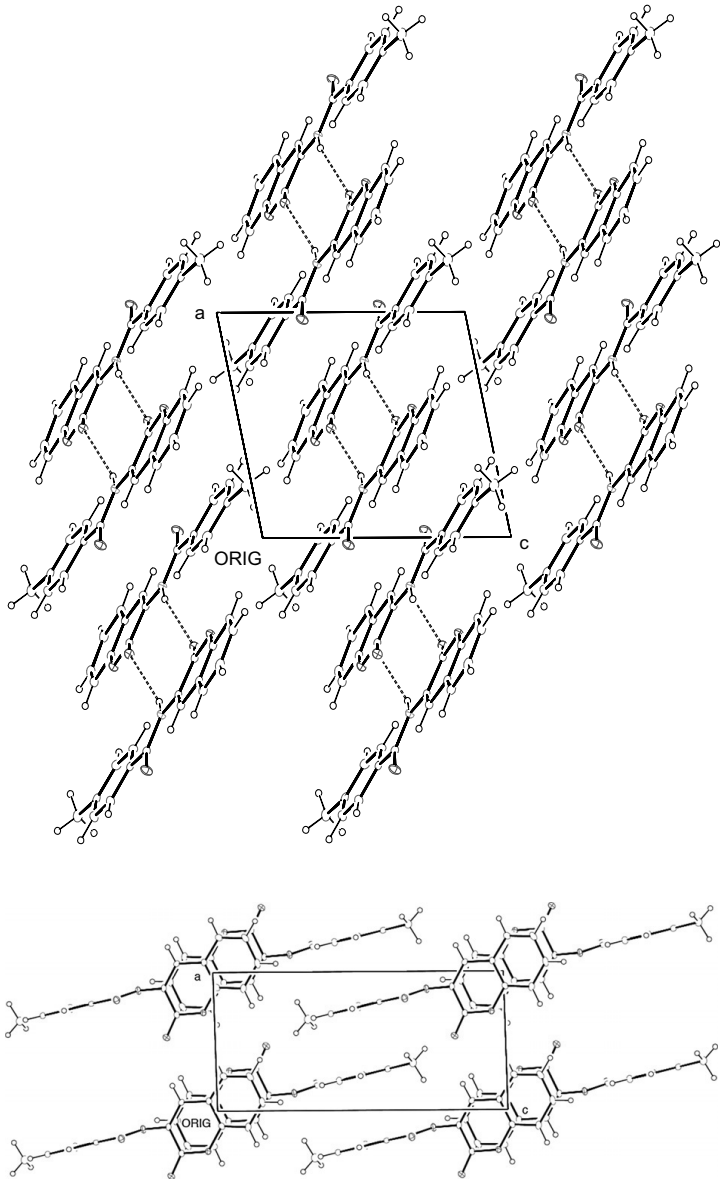


Fig. 3. Packing diagram of both structures viewed along the b axis.

C in the Schrödinger package [33,34] using AM1 and PM3 semiempirical methods. The calculations were carried out in gas phase.

3. Results and discussion

Compound 1 was prepared in 71% yield, by amidation reaction of the 3-aminocoumarin with the corresponding benzoyl chloride (Scheme 1) [16]. Compound 2 was prepared in 69% yield, by an esterification reaction of the 3-hydroxycoumarin with the corresponding benzoyl chloride (Scheme 1). Examination of the information obtained from the NMR spectra enabled us to assign the chemical shifts of the different protons and carbons for both compounds. Also, the molecular structures of both compounds were resolved by X-ray diffractometry and are shown in Fig. 1, together with the atomic numbering scheme used.

As it can be observed in Fig. 1, both molecules adopted some similar aspects in the conformation of the crystal. Both coumarin nucleuses and aromatic rings attached to the amidic or the ester

bridges in both molecules are planar, as expected. The main difference between the molecular structures of compounds 1 and 2 is that in the first case the substitution at position 3 of the coumarin scaffold is almost aligned with the substituent, and in the second case it is almost perpendicular to it. The torsion angles between the mean planes of compound 1 C4–C3–N12–C13 (−5.7°), C3–N12–C13–C15 (−179.68°) and N12–C13–C15–C20 (−171.17°) are typical of the torsion permitted by the rotation of the amidic group at position 3. The torsion angles between the mean planes of compound 2 C4–C3–O12–C13 (−72.52°), C3–O12–C13–C15 (171.82°) and O12–C13–C15–C16 (−4.95°) are typical of the torsion permitted by the rotation of the ester group at position 3. The greatest conformational freedom of the molecules resides, therefore, in the amidic bridge of compound 1 and in the ester bridge of compound 2, composed by C3–N12–C13–C15 and C3–O12–C13–C15, respectively.

In addition, many interesting intramolecular contacts are present in both compounds. In particular, looking to Fig. 1 compound 1 shows interesting C4H···O14, C20H···O14 contacts while compound 2 shows a C16H···O12 contact. Moreover in compound 1 a short contact between two hydrogens (that bonded to C16, more negatively charged and that bonded to N12 more positively charged) is present. Some references about these contacts, referred as non-conventional H-bond (H···H and CH···O/N contacts), have been already suggested and described [35,36].

Compound 1 is structurally similar to the compound described by Jotani et al. [37]. The phenyl ring of that molecule (N-(2-oxo-2H-chromen-3-yl)benzamide) forms a dihedral angle of 7.69° with the fused ring system. In compound 1, as in that molecule, a dihedral angel of 5.84° is formed between the phenyl ring and the fused ring system. The observed conformation is also stabilized by the described intramolecular N—H···O and C—H···O interactions. Dong et al. also described amidic compounds structurally related with these derivatives [38]. As described in that manuscript, the complexity of the molecules (voluminous substituents – a benzyl group at position 3 and a phenylamine at position 4) changed the related position of the coumarin nucleus and the benzylamide at position 3.

Tables 2 and 3 (presented in Supplementary data) list the bond lengths, bond angles and dihedral angles obtained for both compounds 1 and 2, respectively, according to the X-ray analysis. The results for the theoretical calculations (conformational analysis with the refinement of the minimum energy structures through AM1 and PM3 methods), for both compounds, are also included in the tables.

The final structures obtained through semiempirical methods are highly coincident with the data extracted through X-ray. For the compound 1, two possible conformational states are obtained differing in the orientation of the 4-methylphenyl system regarding the plane of the coumarin ring. The dihedral angle value for O(14)–C(13)–C(15)–C(20) calculated through AM1 method can be 36.2 or −37.1 Å for the conformers. A conformational equilibrium resulting in two different conformations was also obtained for the compound 2 through the theoretical calculations. Both possible conformational states differ in the orientation of the carbonyl group regarding to the plane of the coumarin ring. The dihedral angle value in C(3)–O(12)–C(13)–O(14) could be −7.7 or 7.2 Å for the conformers (AM1 method). Fig. 2 shows the superposition of the crystal structure and the conformations obtained through AM1 and PM3 for both compounds (conformers with a O(14)–C(13)–C(15)–C(20) dihedral angle value of 36.2 for the compound 1 and C(3)–O(12)–C(13)–O(14) dihedral value of −7.7 for the compound 2, using AM1 method). The comparison between the theoretical and the crystal structure showed a high level of similarity. The RMSD (root mean square deviation) values between the heavy atoms coordinates are shown in Table 4. AM1 and PM3 yielded similar reproduction of the bond lengths whereas AM1 calculation afforded more coincident results with the crystal for angles and dihedral angles (see Table 4). However, the results described in this article do not justify the selection of one of the methods. Similar results comparing the X-ray and theoretical conformations in piperazine and coumarin derivatives were previously reported by our research group [24,25].

Another important structural feature, which distinguishes the studied molecules, is the capacity of formation of N(12)–H(12)···O(11) intermolecular hydrogen bonds of compound 1, detailed in Table 5. The presence of these hydrogen bonds and the conformation of the substituent at position 3 of the coumarin nucleus, distinguish the packing diagram of both molecules. Packing diagram of the structures allows the interpretation of the spatial orientation of the molecules and are shown in Fig. 3.

4. Conclusion

In summary, we have determined and analyzed the entire structural parameters in the crystalline state of two coumarin derivatives (with amide or ester functions at position 3), and proved that the results are well reproduced by conformational analysis and semiempirical AM1 and PM3 methods. Theoretical approaches can be an alternative methodology to determine the 3D conformation for this type of derivatives when the crystal structure is not available. We can also conclude that it is possible to modulate the relative position of the coumarin scaffold and the aromatic ring at position 3 by modifying the chemical substituent between both moieties.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molstruc.2013.03.014.

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A new series of 3-phenylcoumarins as potent and selective MAO-B inhibitors

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ABSTRACT

6-Methyl-3-phenylcoumarins **3–6** were designed, synthesized and evaluated as monoamine oxidase A and B (MAO-A and MAO-B) inhibitors. The synthesis of these new compounds (resveratrol–coumarin hybrids) was carried out with good yield by a Perkin reaction, from the 5-methylsalicylaldehyde and the corresponding phenylacetic acid. They show high selectivity to the MAO-B isoenzyme, with IC_{50} values in the nanomolar range. Compound **5** is the most active compound and is several times more potent and selective than the reference compound, *R*-(−)-deprenyl.

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Coumarins (or benzopyrones) are a large family of compounds, of natural and synthetic origin, that show numerous biological activities.[1] Recent studies pay special attention to their antioxidative, anticarcinogenic and enzymatic inhibition properties.[2–6] In regard to the monoamine oxidase (MAO) inhibition, the recent findings revealed that MAO-A and MAO-B affinity and selectivity can be efficiently modulated by appropriate substitutions in the coumarin ring, in particular in the 3:4 and 6:7 positions.[7–11]

The resveratrol (3,4′,5-trihydroxystilbene) is a phytoalexin of natural origin present in spermatophytes species such as vines, in response to damage. Resveratrol was already studied as antioxidant, anti-inflammatory, cardioprotective (vasodilatory and platelet antiaggregatory activities), anticancer, enzymatic inhibitor, proving to be very efficient in a large group of in vitro, ex vivo and/or in vivo experiments.[12–15] The resveratrol's cis and trans isomers are inhibitors of the MAO activity. *cis*-Resveratrol is less effective than *trans*-resveratrol as inhibitor of MAO-A and MAO-B activities.[16]

Due to these coincident properties, it seems to be interesting to design and synthesize hybrids that incorporate the skeleton of those two kinds of molecules.[17,18] In the present compounds the resveratrol nucleus is blocked by the coumarin ring and can only assume the trans isomeric form. A series of these molecules, with different number and position of methoxy groups in the 3-phenyl ring (compounds **3–6**), was synthesized and evaluated as MAO inhibitors (iMAO). These modifications were studied to find out how these changes can contribute to the biological activity of these molecules, helping to understand a structure–activity relationship (SAR).

MAO is a FAD-containing enzyme with two known isoforms (MAO-A and MAO-B) and is present in the mitochondrial outer membrane of glial, neuronal and other cells.[19] MAO enzymes intervene in the monoamines degradation and carry out an important physiologic function in the adrenaline, noradrenaline and serotonin deamination (preferentially MAO-A) and in the β-phenylethylamine and benzylamine deamination (preferentially MAO-B).[20] This enzymatic function increases the synaptic concentration of these neurotransmitters and conditions to a great extent the neurone's excitement of those possessing receptors for these mediators.[21]

The iMAO are a class of compounds that act by blocking the MAO enzymatic action, being used by several years in the treatment of the depression and anxiety diseases (iMAO-A) or in Parkinson's disease (iMAO-B).[22] Nowadays is being studied also in the Alzheimer's disease.[23]

The active sites of these two isoenzymes (MAO-A and MAO-B) are not completely known and for this reason there is a lack of information in respect of how the inhibitors act selectively in one of the two of them.[10] Recent X-ray crystal structures of MAO-B[24,25] and MAO-A,[21,26] completed with irreversible and reversible inhibitors, can be used in the future as a tool to the structural basis to help understanding the selective enzyme–ligand recognition and drug development of iMAOs.[21]

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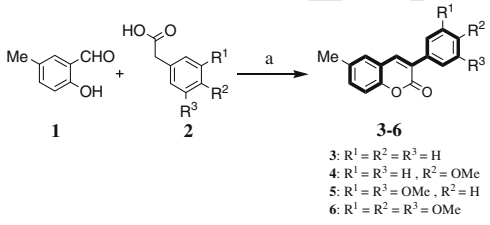
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With the aim of finding out new structural features for the MAO inhibitory activity and selectivity, we decided in this work to explore the importance of the number and position of different methoxy groups under the benzenic ring in 3-position (compounds **4**[27,28]**–6**), to establish a relation between them and with the non substituted analogue (compound **3**[27–29]).

The preparation of these 6-methyl-3-phenylcoumarins was performed via the classical Perkin reaction.[29] This reaction was carried out by condensation of the 5-methylsalicylaldehyde **1** and the conveniently substituted phenylacetic acids **2**, with *N,N′*-dicyclohexylcarbodiimide (DCC) as dehydrating agent, under DMSO reflux, during 24 h (Scheme 1). The reaction to obtain **3–6** is very clean and the yields are between 60% and 70%.[30–33] The obtained products are easy to purify by flash chromatography, using a mixture of hexane/ethyl acetate in a proportion 9:1 as eluent.

The inhibitory MAO activity of compounds **3–6** was evaluated in vitro by the measurement of the enzymatic activity of human recombinant MAO isoforms in BTI insect cells infected with baculovirus.[8,34] Then, the IC_{50} values and MAO-B selectivity ratios [IC_{50} (MAO-A)]/[IC_{50} (MAO-B)] for inhibitory effects of both, new compounds and reference inhibitors, were calculated (Table 1).[35]

The prepared series of compounds proved to be selective as inhibitor of the MAO-B isoenzyme. Compound **3**, none substituted in the phenyl ring, is by itself very active and selective against MAO-B isoenzyme. Compound **4** (with a *p*-methoxy group) has a MAO-B IC_{50} similar to the *R*-(−)-deprenyl (reference MAO-B inhibitor) and is more selective than this one. The most potent molecule of this family is compound **5**, bearing two methoxy groups in 3′- and 5′-positions (IC_{50} = 8.98 ± 1.42 nM). This one is two times more active and several times more iMAO-B selective than the *R*-(−)-deprenyl. Compound **6**, with 3 methoxy groups, is more active than **3** (none substituted) but it loses activity in respect to the mono and dimethoxy derivatives (compounds **4** and **5**, respectively). None of the described compounds showed a MAO-A inhibitory activity for the highest concentration tested (100 μM). This iMAO-B selectivity is an important factor to discriminate the potential therapeutic application of this kind of molecules.



Scheme 1. Reagents and conditions: (a) DCC, DMSO, 110 °C, 24 h.

Table 1
MAO-A and MAO-B inhibitory activity results for compounds **3–6** and reference compounds

Compd	MAO-A IC_{50}	MAO-B IC_{50}	Ratio
3	*	283.75 ± 0.98 nM	>352[b]
4	*	13.05 ± 0.90 nM	>7663[b]
5	*	8.98 ± 1.42 nM	>11,136[b]
6	*	160.64 ± 1.01 nM	>623[b]
R-(−)-Deprenyl	67.25 ± 1.02 μM[a]	19.60 ± 0.86 nM	3431
Iproniazide	6.56 ± 0.76 μM	7.54 ± 0.36 μM	0.87

* Inactive at 100 μM (highest concentration tested). At higher concentrations compounds precipitate.

[a] $P < 0.01$ versus the corresponding IC_{50} values obtained against MAO-B, as determined by ANOVA/Dunnett's.

[b] Values obtained under the assumption that the corresponding IC_{50} against MAO-A is the highest concentration tested (100 μM).

Comparing the iMAO-B activities of **3** and **4**, the introduction of a *p*-methoxy group increases the inhibitory activity. Substitution with two methoxy groups in the 3′- and 5′-positions on the phenyl ring, compound **5**, improves the iMAO-B activity. Increasing the number of methoxy substituent to three, compound **6**, decreases the enzymatic inhibitory activity. However, compound **6** is even better than none substituted coumarin **3**. The presence of methoxy substituent in the 3-phenyl ring seems to be important to modulate and improve the inhibitory enzymatic activity of the 6-methyl-3-phenylcoumarins.

These hybrid compounds with resveratrol–coumarin skeleton show high selectivity against MAO-B isoenzyme, being active in the nanomolar range. Introduction of methoxy groups in the phenyl ring improves the activity, giving more active and selective compounds than the reference ones. These modifications, which we studying more deeply, can improve the pharmacologic profile of the synthesized coumarins in the Parkinson's disease.

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30. 6-Methyl-3-phenylcumarin **(3)**. It was obtained with a yield of 68%. Mp 148–149 °C (biblio. 145–147 °C, 149–150 °C). ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 2.42 (s, 3H, $-CH_3$), 7.27 (m, 1H, H-7), 7.34 (m, 2H, H-4′ and H-8), 7.43 (m, 3H, H-2′,H-4′ and H-5), 7.70 (dd, 2H, H-1′ and H-5′, *J* = 7.7 and 1.8), 7.77 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 21.29, 116.67, 119.57, 128.17, 128.70, 128.95, 129.02, 129.26, 132.95, 134.65, 135.35, 140.39, 152.16, 161.31. DEPT ($CDCl_3$) δ (ppm): 21.29, 100.60, 116.67, 128.17, 128.95, 129.02, 129.26, 132.95, 140.39. MS *m*/*z* (%): 236 (M^+, 100), 208 (50), 178 (8), 165 (8) 76 (6), 51 (69). Anal. Calcd for $C_{16}H_{12}O_2$: C, 81.34; H, 5.12; O, 13.54. Found: C, 81.44; H, 4.84.
31. 3-(4′-Methoxy)phenyl-6-methylcumarin **(4)**. It was obtained with a yield of 61%. Mp 144–145°C (biblio. 143 °C). ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 2.40 (s, 3H, $-CH_3$), 3.84 (s, 3H, $-OCH_3$), 6.96 (dd, 2H, H-3′ and H-5′, *J* = 6.8 and 2.1), 7.26 (m, 3H, H-4, H-7 and H-8), 7.66 (m, 3H, H-3, H-2′ and H-6′). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.75, 55.32, 113.85, 116.04, 119.54, 127.19, 127.46, 127.66, 129.77, 132.02, 134.03, 138.47, 151.41, 160.05, 160.96. DEPT ($CDCl_3$) δ (ppm): 20.76, 55.32, 113.85, 116.04, 127.46, 129.78, 132.01, 138.48. MS *m*/*z* (%): 266 (M^+, 100), 223 (60), 195 (24), 165 (16), 152 (13), 115 (5), 89 (5), 63 (7), 50 (6). Anal. Calcd for $C_{17}H_{14}O_3$: C, 76.68; H, 5.30; O, 18.02. Found: C, 76.88; H, 5.32.
32. 3-(3′,5′-Dimethoxy)phenyl-6-methylcumarin **(5)**. It was obtained with a yield of 60%. Mp 110–111°C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 2.40 (s, 3H, $-CH_3$), 3.82 (s, 6H, $-(OCH_3)_2$), 6.50 (t, 1H, H-4′, *J* = 2.1), 6.83 (d, 2H, H-2′ and H-6′, *J* = 2.1), 7.22–7,33 (m, 3H, H-5, H-7 and H-8), 7.74 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.72, 55.41, 100.89, 106.72, 116.06, 119.22, 127.69, 127.93, 132.49, 134.10, 136.68, 140.02, 151.60, 160.48, 160.61. DEPT ($CDCl_3$) δ (ppm): 20.72, 55.40, 100.89, 106,71, 116.06, 127.69, 132.49, 140.02. MS *m*/*z* (%): 296 (M^+, 100), 295 (17), 267 (16), 210 (8), 181 (22), 152 (13), 139 (6), 105 (8), 76 (9). Anal. Calcd for $C_{18}H_{16}O_4$: C, 72.96; H, 5.44; O, 21.60. Found: C, 73.23; H, 4.90.
33. 6-Methyl-3-(3′,4′,5′-trimethoxy)phenylcumarin **(6)**. It was obtained with a yield of 70%. Mp 165–166 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 2.43 (s, 3H, $-CH_3$), 3.90 (s, 3H, $-OCH_3$), 3,92 (s, 6H, $-(OCH_3)_2$), 6.93 (d, 2H, H-2′and H-6′, *J* = 2.1), 7.24–7.35 (m, 3H, H-5, H-7 and H-8), 7.76 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.70, 56.19, 60.72, 105.94, 116.03, 119.24, 127.48, 127.58, 130.04, 130.44, 132.38, 134.12, 139.48, 151.45, 153.02, 160.66. DEPT ($CDCl_3$) δ (ppm): 20.76, 56.23, 60.77, 105.97, 116.08, 127.63, 132.43, 139.54. MS *m*/*z* (%): 326 (M^+, 100), 311 (89), 283 (43), 253 (12), 225 (30), 197 (10), 169 (10), 148 (5), 115 (8). Anal. Calcd for $C_{19}H_{18}O_5$: C, 69.93; H, 5.56; O, 24.51. Found: C, 70.14; H, 5.58.
34. *Determination of human monoamine oxidase (hMAO) isoform activity*: The effects of the test compounds on hMAO isoform enzymatic activity were evaluated by a fluorimetric method following the experimental protocol previously described by us. Briefly, 0.1 mL of sodium phosphate buffer (0.05 M, pH 7.4) containing the test drugs in various concentrations and adequate amounts of recombinant hMAO-A or hMAO-B required and adjusted to obtain in our experimental conditions the same reaction velocity [165 pmol of *p*-tyramine/min (hMAO-A: 1.1 μg protein; specific activity: 150 nmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min/mg protein; hMAO-B: 7.5 μg protein; specific activity: 22 nmol of *p*-tyramine transformed/min/mg protein)] were placed in the dark fluorimeter chamber and incubated for 15 min at 37 °C. The reaction was started by adding (final concentrations) 200 μM Amplex® Red reagent, 1 U/mL horseradish peroxidase and 1 mM *p*-tyramine. The production of H_2O_2 and, consequently, of resorufin was quantified at 37 °C in a multidetection microplate fluorescence reader (FLX800™, Bio-Tek® Instruments, Inc., Winooski, VT, USA) based on the fluorescence generated (excitation, 545 nm, emission, 590 nm) over a 15 min period, in which the fluorescence increased linearly.Control experiments were carried out simultaneously by replacing the test drugs with appropriate dilutions of the vehicles. In addition, the possible capacity of the above test drugs to modify the fluorescence generated in the reaction mixture due to non-enzymatic inhibition (e.g., for directly reacting with Amplex® Red reagent) was determined by adding these drugs to solutions containing only the Amplex® Red reagent in a sodium phosphate buffer.The specific fluorescence emission (used to obtain the final results) was calculated after subtraction of the background activity, which was determined from vials containing all components except the hMAO isoforms, which were replaced by a sodium phosphate buffer solution.
On the other hand, in our experiments and under our experimental conditions, the control activity of hMAO-A and hMAO-B (using *p*-tyramine as a common substrate for both isoforms) was 165 ± 2 pmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min (*n* = 20).
35. All IC_{50} values shown in the table are expressed as means ± SEM from five experiments.

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Synthesis and evaluation of 6-methyl-3-phenylcoumarins as potent and selective MAO-B inhibitors

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ABSTRACT

A series of 6-methyl-3-phenylcoumarins **3-6** were synthesized and evaluated as monoamine oxidase A and B (MAO-A and MAO-B) inhibitors. A comparative study between the three possible mono methoxy 3-phenyl derivatives and the *p*-hydroxy analogue is reported. The synthesis of these new resveratrol-coumarin hybrids was carried out by a Perkin reaction between the 5-methylsalicylaldehyde and the corresponding phenylacetic acids. The *p*-methoxy substituted compound **3** was hydrolyzed to **6** by a traditional reaction with hydriodic acid. The prepared compounds show high selectivity to the MAO-B isoenzyme, some of them with IC_{50} values in the low nanomolar range. Compound **4**, with the methoxy group in meta position, is the most active of this series, with an IC_{50} against MAO-B of 0.80 nM, and is several times more potent and MAO-B selective than the *R*-(−)-deprenyl (reference compound).

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Coumarins are present in remarkable amounts in the nature. They have attracted considerable interest due to their numerous biological activities depending on their substitution pattern.[1] These compounds have been shown to possess antioxidative and anticarcinogenic properties and to inhibit several enzymes.[2–6] Some coumarin derivatives of natural and synthetic origin have been characterized as monoamine oxidase inhibitors (MAOIs).[7–12]

Monoamine oxidase (MAO) is an FAD-containing enzyme bound to the mitochondrial outer membrane of neuronal, glial, and other cells.[10,13] This enzyme regulates levels of biogenic amines (including neurotransmitters) in the brain and the peripheral tissues by catalyzing their deamination.[11] MAO exists as two distinct enzymatic isoforms, MAO-A and MAO-B, based on their substrate and inhibitor specificities.[14,15]

MAO-A preferentially deaminates serotonin, adrenaline and noradrenaline. That isoenzyme is irreversibly inhibited by low concentrations of clorgyline. MAO-B preferentially deaminates β-phenylethylamine and benzylamine and is irreversibly inhibited by *R*-(−)-deprenyl.[16] The MAOIs have been used for several years in the treatment of depression and anxiety diseases (MAO-A inhibitors) and in Parkinson's disease (MAO-B inhibitors).[17]

Resveratrol, structurally 3,4′,5-trihydroxystilbene, is a natural phenolic component of *Vitis vinifera* L. and other spermatophyte species, produced in response to an exterior or interior damage.[18] Resveratrol shows a large number of pharmacological activities, including antiinflammatory, antioxidant, anticancer, and cardioprotective properties and enzyme inhibition.[19–23] *cis* and *trans*-resveratrol proved to be MAO activity inhibitors, the trans isomer being more effective than the cis.[24]

Because of their similar characteristics, it was interesting to design and synthesize hybrids that incorporate the nucleus of the coumarins and resveratrol molecules.[19,25] In previous work, our research group had reported a comparative study of the importance to the MAOI activity of the different number of methoxy groups on the phenyl ring in the 3 position of coumarin. This study contributed to establish a relationship between them and with the non-substituted analogue.[8] Based on this, and with the aim of helping to better understand a structure/activity relationship for the MAO inhibitory activity and selectivity, in this paper we report the synthesis and evaluation of a new series. Maintaining the 6-methyl-3-phenylcoumarin structure, the three possible different positions of one methoxy group in 3-phenyl ring were explored. We also explored the importance of the hydrolysis of this methoxy group.

The synthesis of the 6-methyl-3-phenylcoumarins was carried out via the classical Perkin reaction.[26] This reaction is performed by condensation of the 5-methylsalicylaldehyde **1** and the appropriately substituted phenylacetic acids **2**, with *N,N′*-dicyclohexylcarbodiimide (DCC) as dehydrating agent, in DMSO, at 110 °C, for

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24 h (Scheme 1). Compounds **3**,[8] **4**[27] and **5**[28] were obtained in yields of 61%, 53%, and 59%, respectively. The reaction mixture was purified by flash chromatography, using hexane/ethyl acetate, in a proportion of 9:1, as eluent.

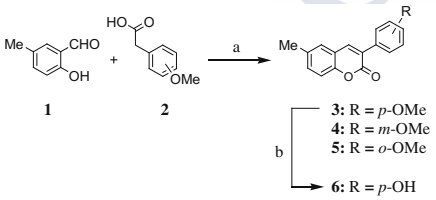
The *p*-methoxy derivative **3** was hydrolyzed with hydriodic acid, in the presence of acetic acid and acetic anhydride, at 110 °C, for 5 h (Scheme 1). The residue was purified by crystallization of acetonitrile, and the phenol derivative **6**[29] was obtained with a yield of 63%.

MAO inhibiting activity of compounds **3-6** was evaluated in vitro by the measurement of the enzymatic activity of human recombinant MAO isoforms in BTI insect cells infected with baculovirus.[8] Then, the IC_{50} values and MAO-B selectivity ratio [IC_{50} (MAO-A)]/[IC_{50} (MAO-B)] for inhibitory effects of both new compounds and reference inhibitors were calculated (Table 1).[30]

The resveratrol–coumarin hybrid compounds **3**, **4,** and **6** showed high selectivity for the MAO-B isoenzyme and inhibitory activity in the nano to picomolar range. Compound **4** was the most active compound of this series, making the *meta* methoxy position the most interesting position at which to improve the MAO-B-inhibiting activity. Compound **5** has no MAOI activity up to the highest tested concentration, proving that the methoxy group in the *ortho* position is not favorable to the measured enzymatic inhibition. Changes on the methoxy substituent position on the phenyl ring in coumarin's 3 position can modulate the pharmacologic potential of the synthesized coumarins.

Comparing compound **3** with its hydroxyl derivative **6**, it was shown that the hydrolysis of methoxy groups is not, in this case, a strategy to improve the MAOI activity. The IC_{50} of compound **6** for inhibition of MAO-B activity is approximately 10 times bigger than compound **3**. However, this value is also in the nanomolar range, and the molecule is also a potent MAOI, selective for the MAO-B isoenzyme.

In conclusion, the synthesized resveratrol–coumarin hybrid compounds show high selectivity for the MAO-B isoenzyme. Most of them present activity in the low nanomolar range. The introduction of one *meta* methoxy group in the 3-phenyl ring improves several times the MAO-B inhibitory activity in respect to *ortho* and *para* positions. Compound **4** is about 24 times more active that *R*-(−)-deprenyl, and several times more selective than this drug. The hydrolysis of methoxy groups is not a strategy to get better MAOI activity. These studied modifications can interestingly improve the pharmacologic potential of the 3-phenylcoumarins in the treatment of Parkinson's disease.



Scheme 1. Reagents and conditions: (a) DCC, DMSO, 110 °C, 24 h; (b) HI, AcOH, Ac_2O, 110 °C, 5 h.

Table 1
MAO-A and MAO-B inhibition by the prepared compounds **3–6** and for the reference compounds

Compounds	MAO-A IC_{50}	MAO-B IC_{50}	Ratio
3	*	13.05 ± 0.90 nM	>7663[b]
4	*	802.60 ± 53.75 pM	>124,595[b]
5	*	*	
6	*	155.59 ± 17.09 nM	>643[b]
R-(−)-Deprenyl	67.25 ± 1.02 μM[a]	19.60 ± 0.86 nM	3431
Iproniazid	6.56 ± 0.76 μM	7.54 ± 0.36 μM	0.87

* Inactive at 100 μM (highest concentration tested). At higher concentrations the compounds precipitate.

[a] P <0.01 versus the corresponding IC_{50} values obtained against MAO-B, as determined by ANOVA/Dunnett's.

[b] Values obtained under the assumption that the corresponding IC_{50} against MAO-A is the highest concentration tested (100 μM).

Acknowledgments

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27. *3-(3′-Methoxy)phenyl-6-methylcumarin* (**4**): It was obtained with a yield of 53%. Mp 84–85 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 2.44 (s, 3H, $-CH_3$), 3.88 (s, 1H, $-OCH_3$), 6.97 (m, 1H, H-4′), 7.26–7.42 (m, 6H, H-5, H-7, H-8, H-2′, H-5′ and H-6′) 7.78 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.77, 55.37, 114.15, 114.38, 116.10, 119.28, 120.86, 127.70, 129.43, 132.48, 134.12, 136.12, 139.91, 140.10, 151.60, 159.45 160.66. MS *m/z* (%): 267 (48), 266 (M$^+$, 100), 239 (16), 238 (70), 237 (20), 195 (48), 194 (16), 166 (10), 165 (29), 152 (23). Anal. Calcd for $C_{17}H_{14}O_3$: C, 76.68; H, 5.30. Found: C, 76.76; H, 5.21.
28. *3-(2′-Methoxy)phenyl-6-methylcumarin* (**5**): It was obtained with a yield of 59%. Mp 177–178 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 2.41 (s, 3H, $-CH_3$), 3.82 (s, 1H, $-OCH_3$), 7.02 (m, 2H, H-3′, H-4′), 7.24–7.41 (m, 5H, H-5, H-7, H-8, H-5′ and H-6′) 7.69 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.80, 55.81, 111.31, 116.21,

119.24, 120.57, 124.20, 126.36, 127.58, 130.14, 130.80, 132.21, 133.89, 141.84, 151.82, 157.22 160.55. MS *m/z* (%): 267 (22), 266 (M^+, 100), 265 (10), 249 (29), 237 (22), 235 (14), 223 (22), 220 (12), 195 (29), 173 (26), 165 (25), 152 (17), 145 (19), 118 (19). Anal. Calcd for $C_{17}H_{14}O_3$: C, 76.68; H, 5.30. Found: C, 76.76; H, 5.22.

29. *3-(4′-Hydroxy)phenyl-6-methylcumarin* (**6**): It was obtained with a yield of 63%. Mp 217–218 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 2.37 (s, 3H, $-CH_3$), 6.84 (d, 2H, H-3′ and H-5′, *J* = 8.8), 7.31 (d, 1H, H-8, *J* = 8.4), 7.40 (dd, 1H, H-7, *J* = 1.9 and 8.4), 7.56 (m, 3H, H-2′, H-6′ and H-5), 8.06 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.31, 115.04, 115.52, 119.45, 125.29, 126.66, 127.92, 129.83, 132.00, 133.67, 138.46, 150.79, 157.95, 160.04. MS *m/z* (%): 253 (13), 252 (M^+, 75), 224 (58), 223 (26), 165 (12) 152 (15), 143 (23), 99 (37), 98 (25), 83 (12), 70 (20), 56 (100), 55 (27). Anal. Calcd for $C_{16}H_{12}O_3$: C, 76.18; H, 4.79. Found: C, 75.99; H, 4.69.

30. All IC_{50} values shown in the table are expressed as means ± SEM from five experiments.

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New halogenated 3-phenylcoumarins as potent and selective MAO-B inhibitors

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ABSTRACT

With the aim to find out the structural features for the MAO inhibitory activity and selectivity, in the present communication we report the synthesis and pharmacological evaluation of a new series of bromo-6-methyl-3-phenylcoumarin derivatives (with bromo atom in both different benzene rings of the skeleton) with and without different number of methoxy substituent at the 3-phenyl ring. The methoxy substituents were introduced, in this new scaffold, in the *meta* and/or *para* positions of the 3-phenyl ring. The synthesized compounds **3–7** were evaluated as MAO-A and B inhibitors using *R*-(−)-deprenyl (selegiline) and iproniazide as reference inhibitors, showing, most of them, MAO-B inhibitory activities in the low nanomolar range. Compounds **4** (IC_{50} = 11.05 nM), **5** (IC_{50} = 3.23 nM) and **6** (IC_{50} = 7.12 nM) show higher activity than selegiline (IC_{50} = 19.60 nM) and higher MAO-B selectivity, with more than 9050-fold, 30,960-fold and 14,045-fold inhibition levels, with respect to the MAO-A isoform.
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Coumarins are a large family of compounds, of natural and synthetic origin, that presents different pharmacological activities.[1] Due to their structural variability, they occupy an important place in the realm of natural products and synthetic organic chemistry. Representatives of these groups of compounds are found to occur in the vegetable kingdom, either in the free or in the combined state.[2] Recent studies pay special attention to their antioxidative, anticancer, and enzymatic inhibition properties.[3–6] Some coumarins proved to be monoamine oxidase (MAO) inhibitors (MAOI).[7] Recent findings revealed that MAO-A and MAO-B affinity and selectivity can be efficiently modulated by appropriate substitutions in the coumarin moiety. The 3/4 and 6/7 positions are particularly suitable to these modifications.[8–14]

Resveratrol, 3,4′,5-trihydroxystilbene, is a natural polyphenolic compound present in grapes and red wine.[15] In in vitro, ex vivo, and in vivo experiments resveratrol has shown important biological activities including antiinflammatory, antioxidant, anticancer, and cardioprotective properties, besides inhibitory activity towards several enzymes.[15–20]

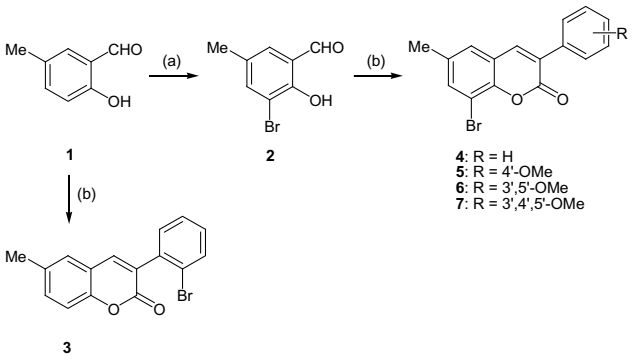
Therefore this compound has been attracting a huge interest since the last decade. Recently, it has been demonstrated that resveratrol also has MAO inhibitory activity.[16,21] To date, most pharmacological studies have considered the *trans* isomer to be more effective than the *cis*.[22]

MAOs are flavoenzymes (FAD-containing enzyme) bound to the outer mitochondrial membrane of neuronal, glial, and other cells.[23,24] These enzymes are responsible for the oxidative deamination of neurotransmitters and dietary amines.[25,26] Two isoforms, namely MAO-A and MAO-B, have been identified basis on their amino acid sequences, three-dimensional structure, substrate preference and inhibitor selectivity.[20,27] MAO-A has a higher affinity for serotonin and noradrenaline, while MAO-B preferentially deaminates phenylethylamine and benzylamine.[28] These properties determine the clinical interest of MAOIs. Selective MAO-A inhibitors, such as clorgyline (irreversible) and moclobemide (reversible), are used for the treatment of neurological disorders, like depression and anxiety,[29,30] whilst selective and irreversible MAO-B inhibitors, such as selegiline and rasagiline, are useful for the treatment of Parkinson's[31,32] and Alzheimer's diseases.[33,34] As the ideal drug candidate has not been attained, an intensive search for new and innovative MAOIs is still needed. This effort has considerably increased in recent years.[11]

In this context, and in an attempt to develop novel MAO-B selective inhibitors, we had previously synthesized 3-arylcoumarin derivatives in which both the coumarin and the resveratrol templates were present. These compounds proved to be potent and selective MAO-B inhibitors.[8,9] In this Letter, a subsequent project was developed based on a 6-methyl-3-phenylcoumarin scaffold, with a bromo pattern in both benzenic rings of the skeleton, and with several methoxyl substituents located in the 3-phenyl ring (Scheme 1).

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Scheme 1. Reagents and conditions: (a) NBS, AIBN, CCl_4, reflux, 18 h; (b) phenylacetic acids, DCC, DMSO, 110 °C, 24 h.

Table 1
MAO-A and MAO-B inhibitory activity results for compounds **3–7** and reference compounds

Compounds	MAO-A IC_{50} (nM)	MAO-B IC_{50} (nM)	Ratio
3	*	$4.3 \times 10^3 \pm 0.29 \times 10^3$	>23[b]
4	*	11.05 ± 0.81	>9050[b]
5	*	3.23 ± 0.49	>30,960[b]
6	*	7.12 ± 0.01	>14,045[b]
7	$31.20 \times 10^3 \pm 2.09 \times 10^3$	$4.89 \times 10^3 \pm 0.22 \times 10^3$	6.4
R-(−)-Deprenyl	$67.25 \times 10^3 \pm 1.02 \times 10^3$[a]	19.60 ± 0.86	3431
Iproniazide	$6.56 \times 10^3 \pm 0.76 \times 10^3$	$7.54 \times 10^3 \pm 0.36 \times 10^3$	0.87

* Inactive at 100 μM (highest concentration tested). At higher concentrations the compounds precipitate.
[a] $P < 0.01$ versus the corresponding IC_{50} values obtained against MAO-B, as determined by ANOVA/Dunnett's.
[b] Values obtained under the assumption that the corresponding IC_{50} against MAO-A is the highest concentration tested (100 μM).

The coumarin derivatives **3–7** were efficiently synthesized according to the synthetic protocol outlined in Scheme 1. The experimental details are given in Ref. 35. The treatment of the precursor **1** with *N*-bromosuccinimide (NBS) in carbon tetrachloride (CCl_4) under reflux, using 2,2′-azo-bis-iso-butyronitrile (AIBN) as catalyst, afforded the bromo derivative **2**[36] in a yield of 44%. The preparation of the 8-bromo-6-methyl-3-phenylcoumarins (**4–7**) was performed via the classical Perkin reaction.[8,9,37–39] This reaction occurs by condensation of the 3-bromo-5-methylsalicylaldehyde (**2**) and the conveniently substituted phenylacetic acids, with *N*,*N*′-dicyclohexylcarbodiimide (DCC) as dehydrating agent, in dimethyl sulfoxide (DMSO), at 110 °C and during 24 h (Scheme 1).[8,9] The reaction is efficient and the compounds **4–7** were obtained with yields between 45% and 50%. Compound **3** was prepared in the same conditions described for compounds **4–7**, but starting from the 5-methylsalicylaldehyde **1** and the *o*-bromophenylacetic acid.

The inhibitory MAO activity of compounds **3–7** was evaluated in vitro by the measurement of the enzymatic activity of human recombinant MAO isoforms in BTI insect cells infected with baculovirus.[8,9,40] Then, the IC_{50} values and MAO-B selectivity ratios [IC_{50} (MAO-A)]/[IC_{50} (MAO-B)] for inhibitory effects of both new compounds and reference inhibitors were calculated (Table 1).[40–42]

In the present communication, the effect of the introduction of a halogen substituent into the 3-phenylcoumarin was studied. In fact a superior MAOI activity, regarding the non-halogenated compounds, was observed.[7,8] It was shown that the introduction of a bromo substituent enhance the MAO-B inhibitory properties (potency and selectivity) of the recently described 6-methyl-3-phenylcoumarin (IC_{50} = 284 nM).[7]

As it is shown in the Table 1, compound **3**, with the halogen atom in the 3-phenyl ring, has an IC_{50} in the micromolar range (IC_{50} = 4.3 μM). When compared with the 6-methyl-3-phenylcoumarin, compound **3** lost at least 15 times the MAO-B inhibitory activity. The structural change obtained with compound **4**, with the halogen atom in the coumarin nucleus, leads to a significant increase of the MAO-B activity (IC_{50} = 11.0 nM). In fact it was greater than the IC_{50} found for 6-methyl-3-phenylcoumarin and compound **3**. So, as consequence, the MAO-B selectivity had increased too (see Table 1). A change of the bromo atom position in the 3-phenylcoumarin nucleus, from 2′ to 8, was the other strategy performed to improve the activity (compounds **5–7**). Compound **5**, with a *p*-methoxy substituent in the 3-phenyl ring, was the most potent and selective molecule, against MAO-B isoenzyme, of this series, with an IC_{50} in the low nanomolar range (IC_{50} = 3.2 nM). This compound is six times more active and to a great extent more selective than the *R*-(−)-deprenyl (IC_{50} = 19.6 nM, reference MAO-B inhibitor). Compound **6**, with two methoxyl groups in the 3′ and 5′ positions, reveal also to be a potent MAOI-B. Its activity (IC_{50} = 7.1 nM) is still better than the non-phenylsubstituted compound **4**. Compound **7**, with three methoxyl groups, present a loss of activity (activity in the micromolar range) and selectivity in regard to the mono and dimethoxyl derivatives (compounds **5** and **6**, respectively). Compounds **3–6** do not exhibit MAO-A inhibitory activity for the highest tested concentration (100 μM). The MAO selectivity is an important factor to discriminate the potential therapeutic application of this kind of molecules. In summary one can say that the presence of a bromo atom in 8-position and a restricted number of methoxyl substituents (one or two) in the 3-phenyl ring seems to be important chemical features to modulate and improve the inhibitory enzymatic activity of the 6-methyl-3-phenylcoumarins.

In conclusion, in the present study it was shown that the synthesized resveratrol–coumarin hybrid compounds have high

selectivity for the MAO-B isoenzyme. Most of them present MAO-B inhibitory activity is in the low nanomolar range. The presence of a bromo atom in position 8 of the coumarin improves the activity respect to the presence of a bromo atom linked to the 3-phenyl ring. The introduction of one *para*-methoxy group in the 3-phenyl ring of the 8-bromo-6-methyl-3-phenylcoumarin improve to a great extent the MAO-B inhibitory activity respect to the other prepared derivatives. In fact, the introduction of a bromo atom improves the pharmacologic potential of the 6-methyl-3-phenylcoumarins confirming that this lead could be effectively optimized in a candidate for the treatment of neurodegenerative diseases. These finds have encouraged us to continue the efforts towards the optimization of the pharmacologic profile of 6-methyl-3-phenylcoumarin.

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35. *3-(2-Bromophenyl)-6-methylcoumarin* (**3**): A solution of 2-hydroxy-6-methylbenzaldehyde (**1**, 1.0 g, 7.34 mmol) and 2-bromophenylacetic acid (1.98 g, 9.18 mmol) in DMSO and DCC (2.36 g, 11.46 mmol) was heated in an oil-bath at 110 °C for 24 h. Triturate ice (100 mL) and acetic acid (10.0 mL) were added to the reaction mixture. After keeping it at room temperature for 2 h, the mixture was extracted with ether (3 × 25 mL). The organic layer was extracted with sodium bicarbonate solution (50 mL, 5%) and then water (20 mL). The solvent was evaporated under vacuum and the dry residue was purified by FC (hexane/ethyl acetate 9:1) to give **3** (1.36 g, 59%) as a white solid. Mp 141–142 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.45 (s, 3H, $-CH_3$), 7.28 (m, 1H, H-4′); 7.35 (m, 2H, H-7, H-8); 7.40 (m, 3H, H-5, H-5′, H-6′); 7.69 (d, 1H, H-3′); 7.71 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.8, 116.4, 118.7, 123.6, 127.4, 127.9, 128.6, 130.1, 131.3, 132.9, 133.0, 134.3, 135.9, 142.6, 152.0, 160.0. MS *m/z* (%): 316 (5), 315 (38), 314 (M^+, 100), 236 (32), 235 (80), 178 (22), 117 (7), 89 (7), 76 (9). Anal. Calcd for $C_{16}H_{11}BrO_2$: C, 60.98; H, 3.52. Found: C, 60.92; H, 3.47.
General procedure for the preparation of 8-bromo-6-methyl-3-phenylcoumarins (**4**–**7**): A solution of 3-bromo-2-hydroxy-6-methylbenzaldehyde (**2**, 0.56 mmol) and the correspondent phenylacetic acid (0.70 mmol) in DMSO and DCC (0.87 mmol) was heated in an oil-bath at 110 °C for 24 h. Triturate ice (20 mL) and acetic acid (3.0 mL) were added to the reaction mixture. After keeping it at room temperature for 2 h, the mixture was extracted with ether (3 × 25 mL). The organic layer was extracted with sodium bicarbonate solution (50 mL, 5%) and then water (20 mL). The solvent was evaporated under vacuum and the dry residue was purified by FC (hexane/ethyl acetate 9:1) to give a white solid.
8-Bromo-6-methyl-3-phenylcoumarin (**4**): It was obtained with a yield of 45%. Mp 158–159 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 2.41 (s, 3H, $-CH_3$), 7.28 (d, 1H, H-7, *J* = 2.3), 7.45 (m, 3H, H-3′, H-4′, H-5′), 7.58 (m, 1H, H-5), 7.70 (m, 3H, H-4, H-2′, H-6′). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.5, 109.4, 120.5, 127.1, 128.5, 129.0, 129.1, 134.3, 135.2, 135.6, 139.3, 148.3, 159.7. MS *m/z* (%): 316 (17), 315 (42), 314 (M^+, 100), 313 (99), 288 (36), 286 (15), 285 (36), 207 (30), 178 (26), 89 (10), 76 (10). Anal. Calcd for $C_{16}H_{11}BrO_2$: C, 60.98; H, 3.52. Found: C, 61.01; H, 3.56.
8-Bromo-6-methyl-3-(4′-methoxyphenyl)coumarin (**5**): It was obtained with a yield of 47%. Mp 144–145 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 2.40 (s, 3H, $-CH_3$), 3.87 (s, 3H, $-OCH_3$), 6.98 (d, 2H, H-3′, H-5′, *J* = 7.1), 7.26 (s, 1H, H-7), 7.56 (s, 1H, H-5), 7.67 (dd, 3H, H-4, H-2′, H-6′, *J* = 6.9 and *J* = 1.1). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.5, 55.4, 109.3, 114.0, 120.7, 126.7, 126.9, 128.5, 129.9, 135.2, 137.8, 148.1, 159.9, 160.3. MS *m/z* (%): 346 (99), 345 (15), 344 (M^+, 100), 303 (53), 301 (54), 275 (15), 207 (15), 166 (17), 165 (72), 138 (17), 89 (13), 76 (14), 58 (41). Anal. Calcd for $C_{17}H_{13}BrO_3$: C, 59.15; H, 3.80. Found: C, 59.17; H, 3.86.
8-Bromo-6-methyl-3-(3′,5′-dimethoxyphenyl) coumarin (**6**): It was obtained with a yield of 46%. Mp 165–166 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 2.40 (s, 3H, $-CH_3$), 3.83 (s, 6H, $(-OCH_3)_2$), 6.51 (t, 1H, H-4′ *J* = 2.3), 6.83 (d, 2H, H-2′, H-6′, *J* = 2.3), 7.26 (s, 1H, H-7), 7.57 (s, 1H, H-5), 7.70 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 21.0, 56.0, 101.6, 107.2, 109.9, 120.8, 127.6, 129.2, 135.7, 136.1, 136.6, 140.0, 148.7, 160.0, 161.2. MS *m/z* (%): 376 (99), 375 (26), 374 (M^+, 100), 238 (37), 209 (14), 181 (36), 165 (30), 153 (18), 152 (41), 151 (17), 126 (15), 76 (22). Anal. Calcd for $C_{18}H_{15}BrO_4$: C, 57.62; H, 4.03. Found: C, 57.63; H, 3.98.
8-Bromo-6-methyl-3-(3′,4′,5′-trimethoxyphenyl) coumarin (**7**): It was obtained with a yield of 50%. Mp 167–168 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 2.41 (s, 3H, $-CH_3$), 3.91 (d, 9H, $(-OCH_3)_3$, *J* = 5.8), 6.92 (s, 2H, H-2′, H-6′), 7.28 (d, 1H, H-7, *J* = 6.5), 7.59 (d, 1H, H-5, *J* = 1.3), 7.70 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.5, 56.1, 56.3, 60.9, 106.0, 106.2, 109.4, 120.4, 127.0, 128.7, 129.7, 135.3, 135.6, 138.9, 148.1, 153.1, 159.7. MS *m/z* (%): 407 (21), 406 (99), 405 (21), 404 (M^+, 100), 391 (56), 389 (56), 363 (19), 361 (19), 303 (21), 247 (13), 187 (15), 186 (15), 139 (19). Anal. Calcd for $C_{19}H_{17}BrO_5$: C, 56.31; H, 4.23. Found: C, 56.28; H, 4.19.
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40. *Determination of human monoamine oxidase (hMAO) isoform activity*: The effects of the test compounds on hMAO isoform enzymatic activity were evaluated by a fluorimetric method. Briefly, 0.1 mL of sodium phosphate buffer (0.05 M, pH 7.4) containing the test drugs in various concentrations and adequate amounts of recombinant hMAO-A or hMAO-B required and adjusted to obtain in our experimental conditions the same reaction velocity [165 pmol of *p*-tyramine/min (hMAO-A: 1.1 μg protein; specific activity: 150 nmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min/mg protein; hMAO-B: 7.5 μg protein; specific activity: 22 nmol of *p*-tyramine transformed/min/mg protein)] were placed in the dark fluorimeter chamber and incubated for 15 min at 37 °C. The reaction was started by adding (final concentrations) 200 μM Amplex® Red reagent, 1 U/mL horseradish peroxidase and 1 mM *p*-tyramine. The production of H_2O_2 and, consequently, of resorufin was quantified at 37 °C in a multidetection microplate fluorescence reader (FLX800™, Bio-Tek® Instruments, Inc., Winooski, VT, USA) based on the fluorescence generated (excitation, 545 nm, emission, 590 nm) over a 15 min period, in which the fluorescence increased linearly. Control experiments were

carried out simultaneously by replacing the test drugs with appropriate dilutions of the vehicles. In addition, the possible capacity of the above test drugs to modify the fluorescence generated in the reaction mixture due to non-enzymatic inhibition (e.g., for directly reacting with Amplex® Red reagent) was determined by adding these drugs to solutions containing only the Amplex® Red reagent in a sodium phosphate buffer. The specific fluorescence emission (used to obtain the final results) was calculated after subtraction of the background activity, which was determined from vials containing all components except the hMAO isoforms, which were replaced by a sodium phosphate buffer solution. On the other hand, in our experiments and under our experimental conditions, the control activity of hMAO-A and hMAO-B (using *p*-tyramine as a common substrate for both isoforms) was 165 ± 2 pmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min ($n = 20$).

41. All IC_{50} values shown in the table are expressed as means ± SEM from five experiments.
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MAO inhibitory activity modulation: 3-Phenylcoumarins versus 3-benzoylcoumarins

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ABSTRACT

With the aim of finding the structural features for the human MAO inhibitory activity and selectivity, in the present communication we report the synthesis, pharmacological evaluation and a comparative study of a new series of 3-phenylcoumarins (compounds **1**–**4**) and 3-benzoylcoumarins (compounds **5**–**8**). A bromo atom and a methoxy/hydroxy substituent were introduced in these scaffolds, at six and eight positions of the coumarin moiety, respectively. The synthesized compounds **1–8** were evaluated as MAO-A and B inhibitors using R-(−)-deprenyl and iproniazide as reference compounds. The presence or absence of a carbonyl group between the coumarin and the phenyl substituent in 3 position remarks, respectively, the MAO-A or MAO-B inhibitory activity. Some of the new compounds showed MAO-B inhibitory activities in the low nanomolar range. Compound **2** (IC_{50} = 1.35 nM) showed higher inhibitory activity than the R-(−)-deprenyl (IC_{50} = 19.60 nM) and higher MAO-B selectivity, with more than 74,074-fold inhibition level, respecting to the MAO-A isoform.

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Coumarins, chalcone and their natural and/or synthetic derivatives are biologically interesting compounds because of their structural diversity. Due to this variability, these heterocyclic compounds occupy an important role not only in the Organic Chemistry but also in the Medicinal Chemistry realm.[1–6] They are described as anticancer, anti-inflammatory, antimicrobial, and antioxidant agents.[7–16] A number of studies pay special attention to coumarin derivatives as monoamine oxidase (MAO)[17–23] inhibitors. Recently, chalcone structure has also been identified as a valid scaffold for monoamine oxidase inhibitors (MAOI).[24] Therefore, recent findings reveal that MAO-A and MAO-B affinity and selectivity can be efficiently modulated by appropriate substitutions in different positions of the coumarin and chalcone moiety (Fig. 1).[19,25–27]

MAO is a FAD-containing enzyme (flavoenzyme) bound to the outer mitochondrial membrane in neuronal, glial and many other cells.[28,29] Two isoforms namely as MAO-A and MAO-B have been identified based on their amino acid sequences, three-dimensional structure, substrate preference and inhibitor selectivity.[30,31] These isoenzymes are responsible for the oxidative deamination of neurotransmitters and dietary amines. Therefore, they are responsible for the regulation of intracellular levels of biogenic amines in the brain and the peripheral tissues.[32,33] MAO-B preferentially deaminates phenylethylamine and benzylamine, while MAO-A has a higher affinity for noradrenaline and serotonin.[34,35] Despite of these differences, dopamine and tyramine are common substrates for both isoforms. These properties determine the pharmacological interest of MAOIs. Selective and irreversible MAO-B inhibitors, such as selegiline (R-(−)-deprenyl) and rasagiline are useful compounds for the treatment of Parkinson[36,37] and Alzheimer's diseases.[38,39] Selective MAO-A inhibitors, such as clorgyline (irreversible) and moclobemide (reversible), are useful for the treatment of neurological disorders, such as depression and anxiety.[40,41] All these findings have led us to an intensive search for novel, selective and efficient MAO inhibitors.

With the aim of finding novel and selective MAO-B inhibitors, we have previously synthesized 3-arylcoumarin derivatives in which both the coumarin nucleus and a 3-phenyl ring were differently substituted. The experimental data show that those compounds are potent and selective MAO-B inhibitors.[20–23] In particular, the 6,8-disubstituted coumarins proved to be very interesting derivatives.[22] Based on the previous 3-phenylcoumarins experimental results, in this paper we describe a new project with a comparative study between 3-phenylcoumarins (compounds **1**–**4**) and 3-benzoylcoumarins (compounds **5**–**8**), which are

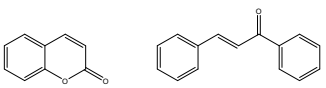
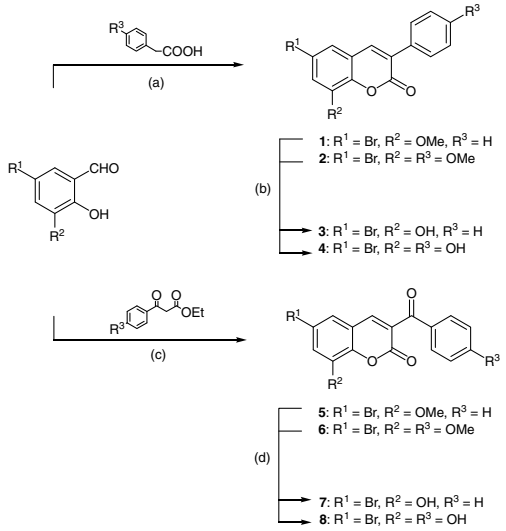


Figure 1. Chemical structures of coumarin and *trans*-chalcone.

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Scheme 1. Reagents and conditions: (a) DCC, DMSO, 110 °C, 24 h; (b) HI, AcOH, Ac_2O, 0 °C to reflux, 4 h; (c) ethanol, piperidine, reflux, 2–5 h; (d) BBr_3, DCM, 80 °C, 48 h.

interesting semi-rigid chalcones with the α,β-unsaturated double bond included in the coumarin skeleton (Scheme 1).

The 6-bromo-8-methoxycoumarins[42] were efficiently synthesized by Perkin[43–45] (**1** and **2**) and Knoevenagel[46,47] (**5** and **6**) reactions. The hydroxy derivatives (**3**, **4**, **7** and **8**)[42] were obtained by two different hydrolysis reactions,[48–50] according to the synthetic protocol outlined in Scheme 1. Treatment of the corresponding salicylaldehyde and the conveniently substituted phenylacetic acid with *N,N'*-dicyclohexylcarbodiimide (DCC) as dehydrating agent, in dimethyl sulfoxide (DMSO) at 110 °C during 24 h, afforded the 3-phenylcoumarins **1** and **2**. The consequent hydrolysis of **1** and **2** in acetic acid and acetic anhydride, with hydriodic acid 57%, for 4 h, yielded the hydroxy derivatives **3** and **4**.[42] The synthesis of the 3-benzoylcoumarins **5** and **6** was performed via condensation of the conveniently substituted salicylaldehyde with the corresponding β-ketoester, in ethanol at reflux temperature for 5 or 2 h respectively, using piperidine as basic catalyst. The resulting methoxy derivatives were treated with boron tribromide at 80 °C for 48 h, to give the corresponding hydroxy derivatives **7** and **8**. Starting from the same salicylaldehyde, we can afford two series, differing just in the presence (compounds **5**–**8**) or absence (compounds **1**–**4**) of a carbonyl group between the phenyl substituent at the 3 position and the coumarin scaffold.

The inhibitory MAO activity of compounds **1–8** was evaluated *in vitro* by the measurement of the enzymatic activity of human recombinant MAO isoforms expressed in BTI insect cells infected with baculovirus.[51] Subsequently, the IC_{50} values and MAO-B selectivity indexes [IC_{50} (MAO-A)]/[IC_{50} (MAO-B)] for inhibitory effects of both new types of compounds and reference inhibitors were calculated (Table 1).[51–53]

In the present communication, the effect of the presence or the absence of a carbonyl group between the coumarin and the 3-phenyl ring is studied. Substituents and their positions in the 3-phenylcoumarin nucleus have been selected based on previous results which showed very high MAOI activity for some derivatives. It was shown that introduction of both bromo and methoxy substituents at 6 and 8 positions respectively (compounds **1** and **2**) enhances the MAO-B inhibitory properties (potency and selectivity) of the described 3-phenylcoumarins, comparing to some of the other previously described compounds.[20–22] In addition, the introduction of a substituent in the *para* position of the 3-phenyl ring might help to improve the activity. Consequently, when this substituent is a methoxy group (compound **2**), the MAO-B inhibitory activity and selectivity improve in a great extent respecting to the other derivatives. However, replacement of the methoxy groups for hydroxyl groups at the indicated positions decreases the activity (compounds **3** and **4**) validating the previous information we already had[21] and it can even decrease selectivity. On the other hand, when we analyze the second series where a 3-benzoyl group has replaced the 3-phenyl substituent, the introduced carbonyl group decreases the MAOI activity against B isoform. The 6-bromo-8-hydroxy derivatives **7** and **8** are more active than the corresponding 6-bromo-8-methoxy ones (compounds **5** and **6**), being the structure activity relationship just the opposite of the 3-phenylcoumarins. Compounds **5** and **6** do not present any MAO inhibitory activity at the higher tested concentration (100 μM). However, compounds **7** and **8** increase the affinity for the MAO-A receptor, losing the MAO-B selectivity of the first series. A small change in the structure causes a big change in the affinity of the molecules for the receptor. These preliminary results allow us to understand slightly better interactions between molecule and receptor and the molecular fragments that are essential to maintain and improve the MAO activity and selectivity.

As conclusion, it was verified an important lost of the MAO-B inhibitory activity and selectivity when the 3-phenyl skeleton is substituted for the 3-benzoyl one. However, in some of the 3-benzoyl derivatives it was shown not only inactivity against MAO-B

Table 1
MAO-A and MAO-B inhibitory activity results for the synthesized compounds **1–8** and reference inhibitors

Compounds	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Selectivity Index
1	*	$83.48x10^{-3} \pm 5.60x10^{-3}$	>1,197[b]
2	*	$1.35x10^{-3} \pm 0.09x10^{-3}$	>74,074[b]
3	*	30.91 ± 2.09	>3.2[b]
4	20.74 ± 1.40	16.87 ± 1.14	1.2
5	*	*	–
6	*	*	–
7	46.81 ± 3.18[a]	73.92 ± 4.99	0.63
8	19.17 ± 1.29	**	0.19[c]
R-(−)-Deprenyl	67.25 ± 1.02[a]	$19.60x10^{-3} \pm 0.86x10^{-3}$	3,431
Iproniazide	6.56 ± 0.76	7.54 ± 0.36	0.87

* Inactive at 100 μM (highest concentration tested). At higher concentrations compound precipitate.
** 100 μM inhibits enzymatic activity around (by approximately) 45–50%. At higher concentrations compound precipitate.
[a] $P < 0.05$ versus the corresponding IC_{50} values obtained against MAO-B, as determined by ANOVA/Dunnett's.
[b] Values obtained under the assumption that the corresponding IC_{50} against MAO-A is the highest concentration tested (100 μM).
[c] Value obtained under the assumption that the corresponding IC_{50} against MAO-B is the highest tested concentration (100 μM).

isoenzyme, but showed MAO-A inhibitory activity and selectivity. Therefore, selectivity seems to depend on the nature of the coumarins' substituent. In the present study it was shown that 6-bromo-8-methoxy-3-phenylcoumarins are an interesting scaffold for MAO-B inhibitory studies, whereas the 6-bromo-8-hydroxy-3-benzoylcoumarins are an interesting moiety for MAO-A inhibitory ones. Compound **2**, with a *p*-methoxy substituent in the 3-phenyl ring was the most potent and selective molecule of the first series against MAO-B isoenzyme, with an IC_{50} in the low nanomolar range (IC_{50} = 1.35 nM). This compound is fifteen times more active and several times more selective than the R-(−)-deprenyl (IC_{50} = 19.6 nM, reference MAO-B inhibitor). The MAO selectivity is an important factor to discriminate the different potential therapeutic applications of these molecules. These findings encourage us to continue the efforts towards the optimization of the pharmacological profile of these structural types as important scaffolds in the neurodegenerative diseases realm.

Acknowledgments

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42. *General procedure for the preparation of 3-phenylcoumarins (**1** and **2**)*: a solution of the conveniently substituted salicylaldehyde (0.56 mmol) and the correspondent phenylacetic acid (0.70 mmol) in DMSO and DCC (0.87 mmol) was heated in an oil-bath at 110 °C for 24 h. Triturate ice (20 mL) and acetic acid (3.0 mL) were added to the reaction mixture. After keeping it at room temperature for 2 h, the mixture was extracted with ether (3 x 25 mL). The organic layer was extracted with sodium bicarbonate solution (50 mL, 5%) and then with water (20 mL). The solvent was evaporated under vacuum and the dry residue was purified by FC (hexane/ethyl acetate 9:1) to give the desired compound.
*6-Bromo-8-methoxy-3-phenylcoumarin (**1**)*: it was obtained with a yield of 50%. Mp 153–154 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), J (Hz): 3.97 (s, 3H, –OCH_3), 7.16 (d, 1H, H-7, *J* = 2.0), 7.26 (d, 1H, H-5, *J* = 2.0), 7.43–7.48 (m, 3H, H-3', H-4', H-5'), 7.68–7.73 (m, 3H, H-2', H-6', H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 57.2, 117.0, 117.3, 121.9, 122.1, 129.2, 129.8, 130.3, 134.8, 139.1, 142.8, 148.3, 160.0. MS *m*/*z* (%): 332 (99), 331 (30), 330 (M^+, 100), 304 (40), 302 (40), 261 (25), 259 (26), 194 (16), 165 (12), 153 (14), 152 (88), 151 (23), 102 (19), 76 (34). Anal. Calcd for $C_{16}H_{11}BrO_3$: C, 58.03; H, 3.35. Found: C, 58.01; H, 3.30.
*6-Bromo-8-methoxy-3-(4'-methoxyphenyl)coumarin (**2**)*: it was obtained with a yield of 53%. Mp 184–185 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), J (Hz): 3.85 (s, 3H, –OCH_3), 3.96 (s, 3H, –OCH_3), 6.93–6.96 (m, 2H, H-3', H-5'), 7.12 (d, 1H, H-7, *J* = 1.8), 7.23 (d, 1H, H-5, *J* = 2.0), 7.63–7.67 (m, 2H, H-2', H-6'), 7.69 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 55.7, 56.8, 114.2, 116.2, 116.8, 121.5, 126.8, 129.4, 130.2, 130.8, 137.3, 142.2, 147.8, 159.8, 160.6. MS *m*/*z* (%): 363 (19), 362 (M^+, 100), 361 (19), 360 (59), 334 (24), 332 (23), 319 (33), 317 (34), 291 (11), 289 (11), 182 (18), 167 (17), 139 (21), 91 (11). Anal. Calcd for $C_{17}H_{13}BrO_4$: C, 56.53; H 3.63. Found: C, 56.55; H, 3.68.
*General procedure for the preparation of hydroxylated 3-phenylcoumarins (**3** and **4**)*. a solution of the corresponding methoxy-3-phenylcoumarin (0.50 mmol) in acetic acid (5.0 mL) and acetic anhydride (5.0 mL), at 0 °C, was prepared. Hydriodic acid 57% (10.0 mL) was added dropwise. The mixture was stirred under reflux temperature for 3 h. The solvent was evaporated under vacuum and the dry residue was purified by recrystallization with CH_3CN.
*6-Bromo-8-hydroxy-3-phenylcoumarin (**3**)*: it was obtained with a yield of 61%. Mp 160–161 °C. ^{1}H NMR (DMSO-d_6) δ (ppm), J (Hz): 7.19 (d, 1H, H-7, *J* = 2.0), 7.43–7.49 (m, 4H, H-5, H-3', H-4', H-5'), 7.70 (dd, 2H, H-2', H-6', *J* = 7.6, *J* = 1.6), 8.13 (s, 1H, H-4), 10.79 (s, 1H, -OH). ^{13}C NMR (DMSO-d_6) δ (ppm): 115.5, 119.9, 120.4, 121.8, 127.9, 128.2, 128.5, 128.8, 134.4, 139.7, 141.1, 145.6, 159.1. MS *m*/*z* (%): 319 (16), 318 (99), 317 (17), 316 (M^+, 100), 291 (12), 290 (78), 289 (13), 288 (81), 153 (22), 152 (51), 151 (12), 76 (25). Anal. Calcd for $C_{15}H_9BrO_3$: C, 56.81; H, 2.86. Found: C, 56.78; H, 2.82.
*6-Bromo-8-hydroxy-3-(4'-hydroxyphenyl)coumarin (**4**)*: It was obtained with a yield of 53%. Mp 249–250 °C. ^{1}H NMR (DMSO-d_6) δ (ppm), J (Hz): 6.83 (d, 2H, H-3', H-5', *J* = 8.8), 7.14 (t, 1H, H-7, *J* = 1.5), 7.36 (d, 1H, H-5, *J* = 2.0), 7.56 (d, 2H, H-2', H-6', *J* = 8.5), 8.00 (s, 1H, H-4). ^{13}C NMR (DMSO-d_6) δ (ppm): 115.6, 116.0, 119.9, 120.6, 122.5, 125.4, 128.2, 130.4, 138.0, 141.2, 146.0, 158.6, 159.8. MS *m*/*z* (%): 335 (35), 334 (99), 333 (45), 332 (M^+, 100), 307 (31), 306 (99), 305 (35), 304 (99), 225 (26), 197 (29), 169 (35), 168 (46), 153 (24), 141 (21), 140 (16), 139 (51), 118 (17), 115 (28), 98 (13), 89 (14), 84 (18), 84 (46), 75 (11), 63 (13). Anal. Calcd for $C_{15}H_9BrO_4$: C, 54.08; H, 2.72. Found: C, 54.00; H 2.69.
*General procedure for the preparation of 3-benzoylcoumarins (**5** and **6**)*. To a solution of the appropriate β-ketoester and the corresponding salicylaldehyde in ethanol was added piperidine in catalytic amount. The reaction mixture was refluxed for 2–5 h and after completion, the reaction was cooled and the precipitated was filtered and washed with cold ethanol and ether to afford the desired compound. Compounds were further recrystallized in methanol/CH_2Cl_2.
*6-Bromo-8-methoxy-3-benzoylcoumarin (**5**)*: it was obtained with a yield of 49%. Mp 207–208 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), J (Hz): 3.82 (s, 3H, –OCH_3), 7.09 (s, 1H, H-7), 7.14 (s, 1H, H-5), 7.24–7.51 (m, 3H, H-3', H-4', H-5'), 7.69 (d, 2H, H-2', H-6', *J* = 7.3), 7.78 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 57.2, 116.6, 118.6, 120.5, 123.0, 128.0, 129.2, 130.0, 134.5, 136.3, 143.3, 144.6, 147.8, 157.8, 191.9. MS *m*/*z* (%): 360 (18), 359 (M^+, 58), 358 (18), 357 (58), 105 (100), 77 (70). Anal. Calcd for $C_{17}H_{11}BrO_4$: C, 56.85; H, 3.09; Found: C, 56.79; H, 3.06.

6-Bromo-8-methoxy-3-(4′-methoxybenzoyl)coumarin (**6**): It was obtained with a yield of 90%. Mp 224–225 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), J (Hz): 3.71 (s, 3H, $-OCH_3$), 3.82 (s, 3H, $-OCH_3$), 6.77 (d, 2H, H-3', H-5', J = 8.9), 7.14–7.16 (m, 2H, H-5, H-7), 7.69 (d, 2H, H-2', H-6', J = 8.9), 7.72 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 55.5, 56.1, 92.8, 95.2, 103.8, 113.7, 121.2, 129.7, 132.1, 141.5, 157.9, 158.3, 159.2, 163.8, 165.8, 190.6. MS m/z (%): 389 (87), 388 (17), 387 (M^+, 90), 135 (100), 77 (56). Anal. Calcd for $C_{18}H_{13}BrO_5$: C, 55.55; H, 3.37. Found: C, 55.53; H, 3.34.

*General procedure for the preparation of hydroxylated 3-benzoylcoumarins (**7** and **8**).* To the corresponding methoxy-3-benzoylcoumarin in DCM (1 mmol), BBr_3 in DCM (20 mmol) was added in a Schlenk tube. Tube was sealed, and the reaction mixture was heated at 80 °C for 48 h. The resulting crude was treated with MeOH and rotated to dryness. The obtained precipitated was recrystallized in MeOH to afford the desired hydroxy derivative.

*6-Bromo-8-hydroxy-3-benzoylcoumarin (**7**)*: it was obtained with a yield of 98%. Mp 240–242 °C. ^{1}H NMR (DMSO-d_6) δ (ppm), J (Hz): 7.29 (d, J = 2.2 Hz, 1H, H-7), 7.61–7.39 (m, 3H, H-3', H-4', H-5'), 7.76–7.67 (m, 1H, H-5), 7.93 (d, 2H, H-2', H6', J = 8.0), 8.30 (s, 1H, H-4), 10.92 (s, 1H, -OH). ^{13}C NMR (DMSO-d_6) δ (ppm): 39.3, 39.5, 39.7, 39.9, 40.1, 40.4, 40.6, 116.2, 120.9, 121.8, 122.0, 127.8, 129.2, 130.0, 134.5, 136.3, 142.7, 144.8, 146.3, 157.9, 191.9. MS m/z (%): 345 (46), 343 (M^+, 45), 105 (100), 77 (58). Anal. Calcd for $C_{16}H_9BrO_4$: C, 55.68; H, 2.63. Found: C, 55.63; H, 2.59.

*6-Bromo-8-hydroxy-3-(4′-hydroxybenzoyl)coumarin (**8**)*: it was obtained with a yield of 40%. Mp 293–295 °C. ^{1}H NMR (DMSO-d_6) δ (ppm), J (Hz): 6.86 (d, 2H, H-3', H-5', J = 8.7), 7.27 (d, 1H, H-7, J = 2.2), 7.46 (d, 1H, H-5 J = 2.2), 7.82 (d, 2H, H-2', H-6' J = 8.7), 8.18 (s, 1H, H-4), 10.62 (s, 1H, -OH), 10.89 (s, 1H, -OH). ^{13}C NMR (DMSO-d_6) δ (ppm): 115.9, 116.2, 121.0, 121.5, 121.6, 127.6, 128.5, 133.0, 142.7, 143.5, 146.3, 157.9, 163.6, 190.0. MS m/z (%): 362 (29), 360 (M^+, 30), 121 (100), 93 (16), 65 (15). Anal. Calcd for $C_{16}H_9BrO_5$: C, 53.21; H, 2.51. Found: C, 52.97; H, 4.41.

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51. Determination of human monoamine oxidase (hMAO) isoform activity. The effects of the tested compounds on hMAO isoform enzymatic activity were evaluated by a fluorimetric method. Briefly, 0.1 mL of sodium phosphate buffer (0.05 M, pH 7.4) containing the tested drugs in several concentrations and adequate amounts of recombinant hMAO-A or hMAO-B required and adjusted to obtain in our experimental conditions the same reaction velocity [165 pmol of *p*-tyramine/min (hMAO-A: 1.1 mg protein; specific activity: 150 nmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min/mg protein; hMAO-B: 7.5 mg protein; specific activity: 22 nmol of *p*-tyramine transformed/min/mg protein)] were placed in the dark fluorimeter chamber and incubated for 15 min at 37 °C. The reaction was started by adding (final concentrations) 200 μM AmplexÒ Red reagent, 1 U/mL horseradish peroxidase and 1 mM *p*-tyramine. The production of H_2O_2 and, consequently, of resorufin was quantified at 37 °C in a multidetection microplate fluorescence reader (FLX800TM, Bio-TekÒ Instruments, Inc., Winooski, VT, USA) based on the fluorescence generated (excitation, 545 nm, emission, 590 nm) over a 15 min period, in which the fluorescence increased linearly. Control experiments were carried out simultaneously by replacing the tested drugs with appropriate dilutions of the vehicles. In addition, the possible capacity of the above tested drugs for modifying the fluorescence generated in the reaction mixture due to non-enzymatic inhibition (e.g., for directly reacting with AmplexÒ Red reagent) was determined by adding these drugs to solutions containing only the AmplexÒ Red reagent in a sodium phosphate buffer. The specific fluorescence emission (used to obtain the final results) was calculated after subtraction of the background activity, which was determined from vials containing all components except the hMAO isoforms, which were replaced by a sodium phosphate buffer solution.
52. All IC_{50} values shown in the table are expressed as means ± SEM from five experiments.
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Synthesis and Study of a Series of 3-Arylcoumarins as Potent and Selective Monoamine Oxidase B Inhibitors

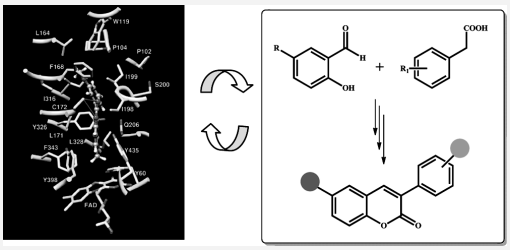
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ABSTRACT: New series of 6-substituted-3-arylcoumarins displaying several alkyl, hydroxyl, halogen, and alkoxy groups in the two benzene rings have been designed, synthesized, and evaluated in vitro as human monoamine oxidase A and B (hMAO-A and hMAO-B) inhibitors. Most of the studied compounds showed a high affinity and selectivity to the hMAO-B isoenzyme, with IC_{50} values on nanomolar and picomolar range. Ten of the 22 described compounds displayed higher MAO-B inhibitory activity and selectivity than selegiline. Coumarin 7 is the most active compound of this series, being 64 times more active than selegiline and also showing the highest hMAO-B specificity. In addition, docking experiments were carried out on hMAO-A and h-MAO-B structures. This study provided new information about the enzyme−inhibitor interaction and the potential therapeutic application of this 3-arylcoumarin scaffold.



■ INTRODUCTION

Coumarins are a wide family of compounds present in remarkable amounts in the nature.[1,2] Representative compounds occur for instance in the vegetable kingdom, either in free or combined state.[1,2] Because of their structural variability, these heterocyclic compounds occupy an important role not only in organic chemistry but also in medicinal chemistry.[3,4] Therefore, coumarins have been attracting considerable interest because of their numerous biological activities. In the literature, coumarin derivatives have been described as anticancer,[5] antioxidant, or anti-inflammatory,[6] vasorelaxant,[7] antimicrobial,[8,9] antiviral,[10,11] and enzymatic inhibitors.[12−14] In particular, some coumarins had been previously described as monoamine oxidase inhibitors.[15−17]

Monoamine oxidases (MAOs) are FAD-depending enzymes found in the outer mitochondrial membrane of neuronal, glial, and other mammalian cells.[18] These enzymes are responsible for catalyzing the oxidative deamination of neurotransmitters and dietary amines, regulating intracellular levels of biogenic amines in the brain and the peripheral tissues.[19,20] Two enzymatic isoforms, named MAO-A and MAO-B, have been identified on the basis of their amino acid sequences,[21] three-dimensional structure,[22] tissue distribution,[23] inhibitor selectivity,[24] and substrate preferences.[25] The hMAO-A isoform has a higher affinity for serotonin and noradrenaline, whereas hMAO-B isoform preferentially deaminates β-phenylethylamines and benzylamine.[26,27] On the other hand, dopamine and tyramine are common substrates for both isoforms. These physiologic properties determine the clinical interest of MAO inhibitors. Selective MAO-A inhibitors, such as clorgyline (irreversible) and moclobemide (reversible), are used for the treatment of neurological disorders like depression and anxiety,[28] while selective and irreversible MAO-B inhibitors, such as selegiline and rasagiline, are used in the therapy of Parkinson's disease.[29,30] All of these aspects have led to an intensive search for novel MAO inhibitors. Additionally, the description of the crystal structure of the two MAO isoforms by Binda et al. has provided relevant information about the selective interactions and the pharmacophoric requirements needed for the design of potent and selective inhibitors.[31−34] In recent years, interest in selective hMAO-B inhibitors has significantly intensified due to the discovery that expression levels of this isoenzyme in neuronal tissue increase 4-fold with age, resulting in an increment of dopamine metabolism, as well as of production of hydrogen peroxide, which cause oxidative stress and may play a relevant role in the etiology of neurodegenerative diseases.[35] As a result, MAO-B inhibitors would be useful as adjuvant for the treatment of Parkinson's and Alzheimer's diseases. For instance, it has been reported that selegiline protects neuronal cells from the consequences of the oxidative stress in these patients.[36]

In the past few years, a broad consensus has been reached concerning the necessity for the research of new, more potent, and less toxic MAO-B selective inhibitors. This research has resulted in a significant number of compounds with different

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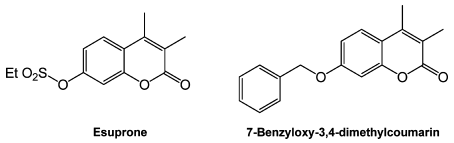


Figure 1. Coumarin-based MAO-A and MAO-B inhibitors.

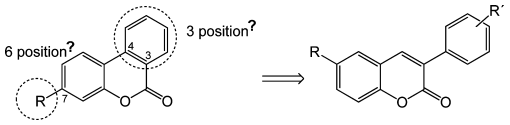


Figure 2. Planed modifications and newly synthesized coumarins.

structural scaffolds.[37−42] Therefore, the coumarin nucleus has emerged in the 1990s as a promising scaffold for MAO inhibitors.[43] Structure−selectivity relationship studies about coumarin derivatives suggested that selectivity was mainly determined by the nature of the linkage between the coumarin and the lipophilic aryl groups in the position 7.[44] Therefore, the alkylsulfonyloxy group provided MAO-A inhibitors like esuprone, while a benzyloxy substituent led to potent and selective MAO-B inhibitors, such as 7-benzyloxy-3,4-dimethylcoumarin (Figure 1).[45]

In relation to this research, we have previously reported interesting MAO inhibitory properties for several 7-substituted coumarins containing a benzene ring fused at 3,4 positions (Figure 2).[46] This condensation, in general, increased the MAO-A and MAO-B inhibitory activities, and when an additional acetonyloxy group was present in the 7 position, this resulted in compounds with very high MAO-B selectivity. These results led us to analyze the importance of the aryl substituent under the pyrone ring and the above-mentioned C7 substituent, synthesizing a series of 3-arylcoumarin derivatives (Figure 2). The substitution at C7 was removed and a smaller substituent was introduced at C6 and the 3,4-condensed benzene ring has moved at C3, including different substituent groups on it.[47−49] The high MAO-B selectivity found for this new 3-arylcoumarin scaffold encouraged us to synthesize and study the MAO inhibitory activity of new analogues, in which a variety of groups with different size, electronic, and lipophilic properties were introduced in both aromatic rings, in order to clarify the influence of the substitution pattern in the MAO inhibitory activity and selectivity of the 3-arylcoumarin skeleton.

Finally, on the basis of the significant structural information currently available on MAOs,[50−52] and to analyze the structural requirements for MAO activity and selectivity, docking experiments were carried out on hMAO-A and hMAO-B crystallographic structures.

■ CHEMISTRY

The coumarin derivatives **1**−**22** were efficiently synthesized according to the protocol outlined in Scheme 1. The chemical structure of the compounds is organized in Table 1. The general reaction conditions and the compounds characterization are described in the Experimental Section.

Perkin condensation of different *ortho*-hydroxybenzaldehydes with the adequate arylacetic acid, using *N*,*N*′-dicyclohexylcarbodiimide (DCC) as dehydrating agent,[47,48] afforded the 3-arylcoumarins **1**, **2**, and **6**−**14**. The treatment of the 3-(methoxyphenyl)-6-methylcoumarins **9**,[47] **10**,[48] **12**, and **14** with *N*-bromosuccinimide (NBS), in carbon tetrachloride (CCl_4), under reflux, using 2,2′-azo-bis-iso-butyronitrile (AIBN) as catalyst,[49] afforded the bromomethoxy derivatives **15**−**18**. In addition, compounds **3**, **19**−**21** were obtained by acidic hydrolysis of the respective methoxy derivatives **2**, **9**, **10**, and **11**, using hydriodic acid 57% in the presence of acetic acid and acetic anhydride.[48] Finally, the Williamson reaction[45] of the hydroxycoumarin **3** with chloroacetone or cyclopentyl bromide, gave the corresponding ethers **4** and **5**, respectively. The Williamson reaction of the hydroxycoumarin **20** with a chloroacetone gave the corresponding derivative **22**.

■ BIOCHEMISTRY

The biological evaluation of the test drugs on hMAO activity was investigated by measuring their effects on the production of hydrogen peroxide (H_2O_2) from *p*-tyramine (a common substrate for h-MAO-A and hMAO-B), using the Amplex Red MAO assay kit (Molecular Probes, Inc., Eugene, Oregon, USA) and microsomal MAO isoforms prepared from insect cells (BTI-TN-5B1−4) infected with recombinant baculovirus containing cDNA inserts for hMAO-A or hMAO-B (Sigma-Aldrich Química SA, Alcobendas, Spain) were used as source for the two microsomal MAO isoforms.[53] The production of H_2O_2 catalyzed by the two MAO isoforms can be detected using 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red reagent), a nonfluorescent and highly sensitive probe that reacts with H_2O_2 in the presence of horseradish peroxidase to produce a fluorescent product, resorufin. New compounds and reference inhibitors were unable to react directly with the Amplex Red reagent, which indicates that these drugs do not interfere with the measurements. On the other hand, in our experiments and under our experimental conditions, hMAO-A displayed a Michaelis constant (K_m) equal to 457.17 ± 38.62 μM and a maximum reaction velocity (V_{max}) in the control group of 185.67 ± 12.06 (nmol *p*-tyramine/min)/mg protein, whereas hMAO-B showed a K_m of 220.33 ± 32.80 μM and V_{max} of 24.32 ± 1.97 (nmol *p*-tyramine/min)/mg protein (n = 5). Most tested compounds, concentration-dependently inhibited this enzymatic control activity (Table 1).

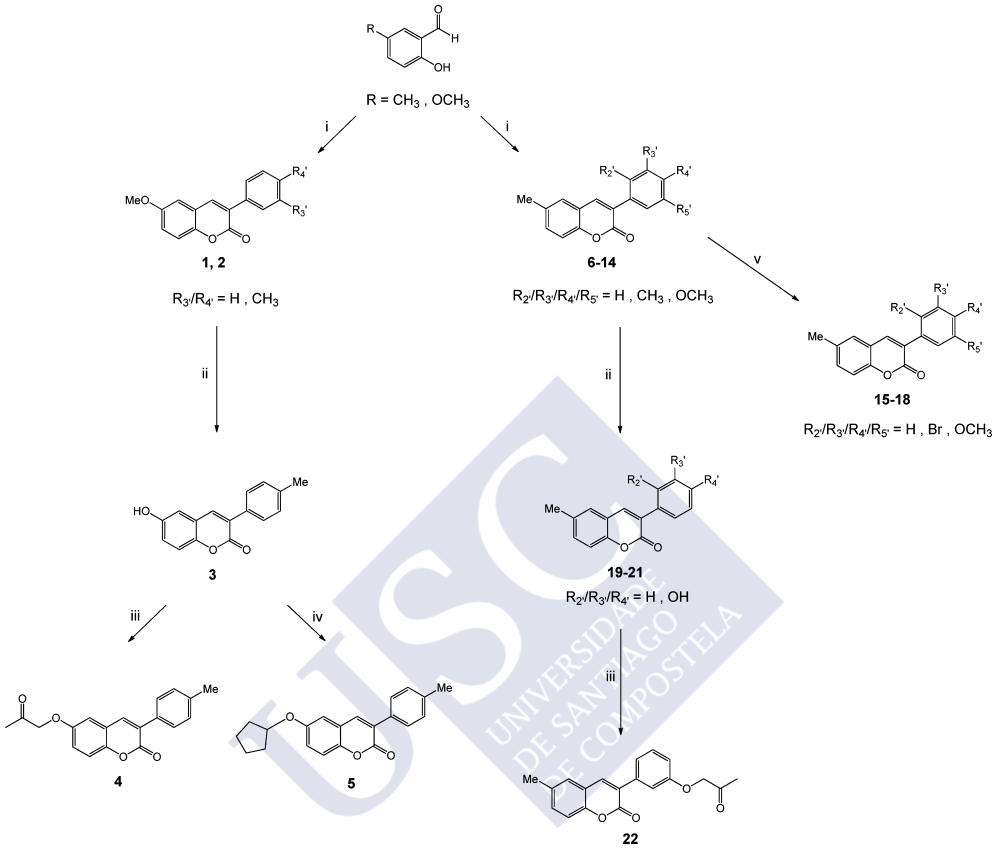
■ DOCKING STUDIES

To obtain information about enzyme−inhibitor interactions that help us to explain the structural requirements for MAO activity and selectivity of 3-arylcoumarin scaffold, a docking study was performed. The crystallographic structure of MAO-B in complex with a coumarin inhibitor (pdb code 2V61)[52] and of MAO-A in complex with harmine (pdb code 2ZX5)[51] were used to dock the derivates under study. The docking simulations were carried out using the DOCK v. 6.3 package.[54]

To validate our docking protocol, we used the 7-(3-chlorobenzyloxy)-4-[(methylamino)methyl]coumarin cocrystallized with the MAO-B enzyme,[52] as well as two other MAO inhibitors (3-[(4-fluorophenyl)carbamoyl]coumarin and 3-[(4-methanesulfonylphenyl)carbamoyl]coumarin), all of them previously subjected to molecular modeling studies and having bulky substituents at the 3-position as is the case for the new coumarin derivates reported in our manuscript (Figure 3).[15]

Although the proposed docking methodology is able to reproduce the crystallographic orientation of the MAO-B

Scheme 1[a]



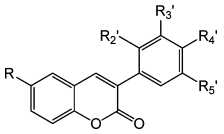
[a] Reagents and conditions: (i) substituted phenylacetic acid, DCC, DMSO, 110 °C, 24 h; (ii) HI, AcOH, Ac_2O, reflux, 3 h; (iii) chloroacetone, K_2CO_3, acetone, reflux, 16 h; (iv) cyclopentyl bromide, K_2CO_3, acetone, reflux, 24 h; (v) NBS, AIBN, CCl_4, reflux, 18 h.

coumarinic inhibitor (pdb code 2V61), the successful redocking of the crystallographic inhibitor does not warrant a realistic binding prediction of the new inhibitors proposed. To study whether our methodology is able or not to reproduce the previously described binding mode of noncrystallographic inhibitors, we redocked the last two above-mentioned compounds. This yielded that the obtained binding modes are in agreement with the results previously reported by Chimenti et al.[15]

During the validation of the docking protocol, we also included 3, 5, and 6 structural water molecules in the receptor structure, yielding similar scoring and orientations for the compounds as in the water depleted receptor structure. This is the main reason we did not use any structural water molecule in the modeling of the compounds included in the paper. Furthermore, the use of the water depleted receptor would allow the ligands to explore the lower region of the binding pocket. In this way, the docking algorithm is able to explore orientations of the inhibitors with the 3-substituent oriented to the upper subpocket and to the FAD cofactor. Despite the fact that water molecules were not considered during the ligands orientation step, the predicted poses were rescored using the DOCK Amber-based scoring function, which takes into account the solvent effect using a continuous solvent model and account for small structural rearrangements on the predicted complexes structures.[55,56] These results support the use of our protocol for the prediction and analysis of the binding mode of the coumarin derivatives described in this study.

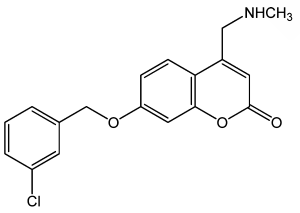
The molecular docking studies were done for different compounds of the studied series. Although we mainly focused the present results on two representative compounds, the coumarin derivatives **9** and **10**, with substituents in *para* and *meta* positions (Figure 4). They interact with the isoenzyme in the well-known binding pocket,[32,33,50,52] with the substituent at C6 pointing to the FAD cofactor, Phe343, and Tyr60. The coumarin ring is oriented toward the bottom of the substrate cavity, interacting with the FAD cofactor as well as with Tyr398, Tyr435, and Gln206 through van der Waals and hydrophobic interactions, including $\pi-\pi$ interactions with these tyrosine residues. In addition, the predicted orientation of the coumarin moiety allows the interaction of its oxygen atoms of the coumarin moiety

Table 1. Structure and hMAO Inhibitory Activity in Vitro of the 3-Arylcoumarin Derivatives 1−22 and Reference Compounds[a]

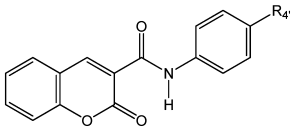


compd	R	R2′	R3′	R4′	R5′	IC_{50} hMAO-A (μM)	IC_{50} hMAO-B	SI[d]
1	OCH_3	H	CH_3	H	H	*b*	17.05 ± 0.88 nM	>5882[e]
2	OCH_3	H	H	CH_3	H	*b*	1.52 ± 0.08 nM	>6789[e]
3	OH	H	H	CH_3	H	24.90 ± 1.33	67.10 ± 2.99 nM	372
4	$C_3H_5O_2$	H	H	CH_3	H	*b*	5.52 ± 0.29 μM	>18[e]
5	C_5H_9O	H	H	CH_3	H	*b*	14.47 ± 0.78 μM	>6.9[e]
6	CH_3	H	H	H	H	*b*	283.75 ± 19.90 nM	>352[e]
7	CH_3	H	H	CH_3	H	*b*	0.31 ± 0.02 nM	>333333[e]
8	CH_3	H	CH_3	H	H	*c*	15.01 ± 0.83 nM	>6667[e]
9	CH_3	H	H	OCH_3	H	*b*	13.05 ± 0.90 nM	>7663
10	CH_3	H	OCH_3	H	H	*b*	0.80 ± 0.05 nM	>125000
11	CH_3	OCH_3	H	H	H	*b*	*b*	
12	CH_3	H	OCH_3	H	OCH_3	*b*	8.98 ± 1.42 nM	>11136[e]
13	CH_3	H	OCH_3	OCH_3	H	25.14 ± 1.68	2.73 ± 0.12 nM	9209
14	CH_3	H	OCH_3	OCH_3	OCH_3	*b*	160.64 ± 1.01 nM	>623[e]
15	CH_3	H	Br	OCH_3	H	*b*	0.74 ± 0.02 nM	>135870[e]
16	CH_3	H	OCH_3	Br	H	*b*	3.25 ± 0.17 nM	>31250[e]
17	CH_3	Br	OCH_3	H	OCH_3	*b*	54.03 ± 3.91 μM	>1.9[e]
18	CH_3	Br	OCH_3	OCH_3	OCH_3	*b*	*b*	
19	CH_3	H	H	OH	H	*b*	155.59 ± 17.09 nM	>643[e]
20	CH_3	H	OH	H	H	35.04 ± 1.88	650.03 ± 34.82 nM	54
21	CH_3	OH	H	H	H	21.58 ± 1.16	120.02 ± 6.43 nM	180
22	CH_3	H	$C_3H_5O_2$	H	H	*c*	180.04 ± 9.65 nM	>555[e]
Selegiline						67.25 ± 1.02	19.60 ± 0.86 nM	3431
Iproniazide						6.56 ± 0.76	7.54 ± 0.36 μM	0.87

[a] Each IC_{50} value is the mean ± SEM from five experiments (n = 5). [b] Inactive at 100 μM (highest concentration tested), at higher concentrations the compounds precipitate. [c] 100 μM inhibits the corresponding hMAO activity by approximately 40−45%, at higher concentrations the compounds precipitate. [d] Selectivity index: MAO-B selectivity ratios [IC_{50} (MAO-A)]/[IC_{50} (MAO-B)] for inhibitory effects of both new compounds and reference inhibitors. [e] Values obtained under the assumption that the corresponding IC_{50} against MAO-A is the highest concentration tested (100 μM).



7-(3-chlorobenzyloxy)-4-[(methylamino)methyl]coumarin



$R_{4'}$ = F ; 3-[(4-Fluorophenyl)carbamoyl]coumarin
$R_{4'}$ = SO_2CH_3 ; 3-[(4-Methanesulfonylphenyl)carbamoyl]coumarin

Figure 3. Compounds used to validate the docking protocol.

with Cys172, showing a favorable geometry for the formation of hydrogen bonds between the ligand and the receptor. Moreover, the coumarin core also interacts with Ile198, Ile199, and Leu171. Finally, the substituted 3-phenyl ring is oriented to an entrance cavity, an hydrophobic subpocket existing only in the MAO-B isoform, which is defined by Leu171, Ile199, Tyr326, Phe168, Ile316, Trp119, Pro102, and Pro104. This is probably one explanation to the MAO-B selectivity of the described compounds.

■ RESULTS AND DISCUSSION

All described coumarins in this report (compounds **1**−**22**) were efficiently synthesized and evaluated for their ability to

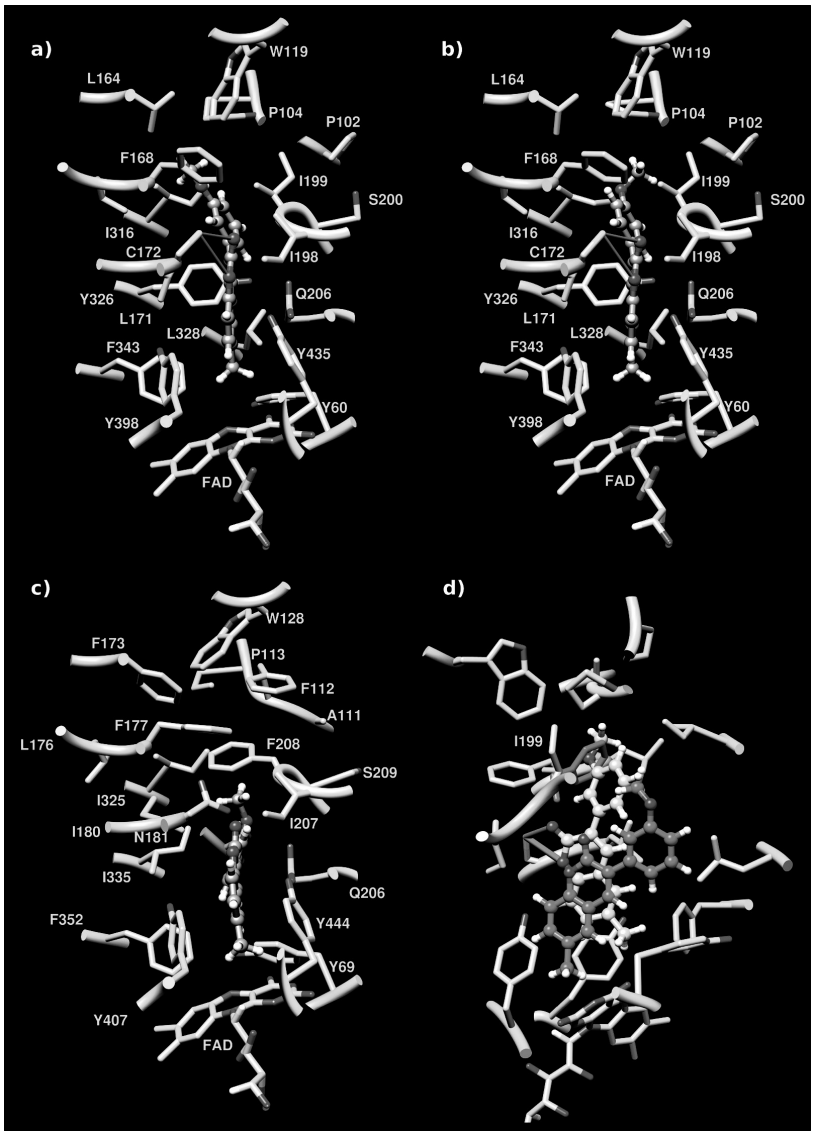


Figure 4. Molecular docking studies of coumarin derivatives **9** and **10**. Most stable binding poses of compound **10** (a) and **9** (b) into MAO-B binding site, and compound **10** (c) into MAO-A binding site. Compounds are colored by atom type, and predicted H-bonds are represented as green pseudobonds. Superposition of binding poses of compound **10** (d) to both isoenzymes, MAO-A (ligand and Phe208 in magenta), and MAO-B.

inhibit the A and B isoforms of hMAO. The corresponding IC_{50} values and MAO-B selectivity ratios $[IC_{50}$ (MAO-A)$]/[IC_{50}$ (MAO-B)] are shown in Table 1. The chemical structures of the newly designed compounds, as well as the biological and docking results, can help us with an interesting SAR study. From the experimental results, it can be observed that most of the tested compounds are selective inhibitors toward MAO-B, with IC_{50} values in the low micro, nano, and picomolar range. Ten of the 22 tested compounds have similar or better IC_{50} than the selegiline (IC_{50} = 19.60 nM, reference MAO-B inhibitor). Two of the most active compounds are the coumarin derivatives **7** and **10**, with IC_{50} against hMAO-B of 0.31 and 0.80 nM, respectively. Both compounds **7** and **10** are *para* or *meta* substituted in the 3-phenyl ring with a methyl or a methoxy group, respectively. With the aim

of introducing a group with different physicochemical properties under the 3-phenyl ring of the coumarin moiety, we have brominated the different 3-methoxyphenyl derivatives. Therefore, compound **15** has the *para* and *meta* positions substituted with a methoxy and a bromo groups, respectively. This compound proved to be one of the three most active compounds, with IC_{50} against hMAO-B of 0.74 nM. This new substitution makes this compound a better MAO-B inhibitor than the corresponding derivative without the bromo atom, compound **9**, with an IC_{50} of 13.05 nM. On the other hand, compound **16**, with the *para* and *meta* positions substituted with a bromo and a methoxy groups, respectively, seems to lose MAO-B inhibitory activity. So, we can infer that the presence of an electron donor at the *para* position is an important modification to improve the activity. From the biological data, the coumarin **7**, with a *p*-methyl substituent in the 3-phenyl ring and a 6-methyl substituent in the coumarin aromatic ring, was the most potent and selective one against MAO-B isoenzyme. This compound was 63 times more active than the selegiline, resulting also much more selective than it (selectivity MAO-B index >333333).

The activity data of new reported compounds point out that almost all the substitutions at *ortho* position of the 3-phenyl ring seem to be unfavorable for the activity. Compounds **11** and **18**, with a methoxy or a bromo substituent, respectively, at the *ortho* position are inactive at 100 μM (highest concentration tested). The corresponding compound **14**, compared with compound **18** but without the *ortho*-bromo atom, has an activity in the nanomolar range. Compound **17**, also with a bromo atom at *ortho* position, has an MAO-B inhibitory activity in the high micromolar range, while their correspondent nonbrominated compound **12**, with a "free" *ortho* position, has an activity against MAO-B in the low nanomolar range. It seems clear that the presence of different substituents in the *ortho* position annulated or strongly decreased the inhibitory MAO-B activity. An exception occurs when the *ortho* position is substituted with a hydroxyl group (compound **21**). In this case, the methoxy derivative (compound **11**) has no activity against MAO-B and the hydroxyl one has an IC_{50} in the nanomolar range. This is going to be explained later in the discussion of the Docking Studies. Moreover, inhibitory MAO-B activity increases when the small lipophilic groups (methoxy or methyl) are included at *meta* or *para* positions. Nevertheless, the dimethoxy-substituted compound **13** is less active than the *meta*-monosubstituted one (compound **10**). In addition, a hydroxyl group incorporated in *para*- or *meta*-positions of the phenyl ring of the moiety seems to be less favorable for the MAO-B inhibition than a methoxy or methyl group at the same position, as it can be seen by comparing the IC_{50} values of compounds **7**−**10** with those of compounds **19** and **20**, respectively. Finally, a methyl group in the 6 position of the coumarin moiety (compound **7**) provides better MAO-B inhibitory activity than a methoxy group (compound **2**) or a hydroxyl group (compound **3**). Also, bulkier alkoxy substituents at C6, such as an 2-oxopropoxy group (compound **4**) or a cyclopentyloxy group (compound **5**), significantly reduced the activity, being better tolerated in the 3-phenyl ring, in particular in the *meta* position (compound **22**). Almost all the studied compounds with substituents in the 3-phenyl ring proved to be most active against the MAO-B than the nonsubstituted 6-methyl-3-phenylcoumarin **6** (IC_{50} = 283 nM). Compounds substituted in the *ortho* position (excepting compound **21**, already mentioned), compound **20** and compounds bulky substituted (compounds **4** and **5**) in position 6 were the only exception.

On the basis of the docking results, the proposed binding mode for this series of compounds in the pocket of hMAO-B is consistent with the observation that only small substituents are tolerated at position 6, as can be seen analyzing the results of the compounds **4** and **5**. The increase in the size of substituents at C6 results in a disruption of the proposed binding mode because part of the space occupied by the coumarin ring will be used by this bulky substituent, resulting in the loss of the key interactions to stabilize the ligand−enzyme complex.

Additionally, the poses predicted by the docking simulations show a small angle between the planes defined by the coumarin and the 3-phenyl ring. These conformations, which are sterically prohibited when a methoxy group is included at 2′ position of the 3-phenyl ring, are however permitted for a hydroxyl group at the same position. These conformational changes can explain why the *ortho* position substitutions of the 3-phenyl ring considerably decreased or abolished the activity and why the compound **21** has activity against MAO-B, whereas the corresponding methoxy derivative **11** is inactive at the highest tested concentration against the same enzyme (Table 1). Moreover, the docking results indicate that a substituent at *para*- or *meta*-position of the 3-phenyl ring makes the compound occupy the above-mentioned hydrophobic subpocket (Figure 4a and 4b).

Finally, the comparison between the predicted binding mode of compound **10** to MAO-A (Figure 4c) with its binding mode to MAO-B (Figure 4d) corroborates that entrance cavity only exits in MAO-B because in MAO-A its formation is prevented by the presence of the Phe208 residue instead of the Ile199 present in the same position of MAO-B.

On the basis of the docking studies information, the high MAO-B selectivity of these series of compounds could be explained by taking into account two factors: the ability of the compounds to recognize and exploit the entrance cavity in MAO-B and the fact that, according to predicted binding mode, in MAO-A these compounds should occupy a more distant position into the binding pocket to fit in the cavity and interact with the binding points (ligand in magenta ball and sticks, Figure 4d). This movement to a deeper region in the cavity results in the displacement of some water molecules conserved in the X-ray structures of the MAOs, which is not necessary for the binding to MAO-B, according to the proposed models. This displacement of the water molecules is an energetically expensive process that negatively influences the formation of stable complexes between the inhibitors and MAO-A.

■ CONCLUSION

In this paper, we have used the Perkin reaction as a key step of a good methodology for the efficient and general synthesis of a selected series of 3-arylcoumarins. Most of the studied compounds show a high affinity and selectivity for the MAO-B isoenzyme. Several compounds of these series proved to have much higher MAO-B inhibitory activity and selectivity than the selegiline. The docking studies performed on these compounds show that their interactions with the MAO-B binding pocket are more intense than those with the MAO-A one. The docking results are in accord with the biological data and allowed us to support an interesting SAR study. Additionally, all the results show that a small substitution at C6 (methyl or methoxy groups) of the coumarin core seems to be important when a phenyl group is located at C3. A bulkier group at C6 (compounds **4** and **5**) drastically decreases the MAO-B inhibitory activity. With a small

substituent at C6, both kind and position of substituents included in the 3-aryl group play a crucial role in activity and selectivity. *Meta* and *para* substituents were the most favorable positions for the desired activity. Currently, further research is going to be conducted on the 3-arylcoumarin scaffold as potential agents for the treatment of Parkinson's disease.

■ EXPERIMENTAL SECTION

Chemistry. Melting points were determined using a Reichert Kofler thermopan or in capillary tubes on a Büchi 510 apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 1640FT spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX spectrometer at 300 and 75.47 MHz, respectively, using TMS as internal standard (chemical shifts in δ values, J in Hz). Mass spectra were obtained using a Hewlett-Packard 5988A spectrometer. Elemental analyses were performed using a Perkin-Elmer 240B microanalyser and were within ±0.4% of calculated values in all cases. Silica gel (Merck 60, 230−00 mesh) was used for flash chromatography (FC). Analytical thin layer chromatography (TLC) was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm). The purity of compounds **1**−**22** was assessed by HPLC and was found to be higher than 95%.

General Procedure for the Preparation of 3-Phenylcoumarins (1, 2, 6−14). A solution of 2-hydroxy-6-methylbenzaldehyde/2-hydroxy-6-methoxybenzaldehyde (7.34 mmol) and the corresponding phenylacetic acid (9.18 mmol) in dimethyl sulfoxide (15 mL) was prepared. *N*,*N*′-Dicyclohexylcarbodiimide (11.46 mmol) was added, and the mixture was heated in an oil bath at 110 °C for 24 h. Ice (100 mL) and acetic acid (10 mL) were added to the reaction mixture. After keeping it at room temperature for 2 h, the mixture was extracted with ether (3 × 25 mL). The organic layer was extracted with sodium bicarbonate solution (50 mL, 5%) and then water (20 mL). The solvent was evaporated under vacuum, and the dry residue was purified by FC (hexane/ethyl acetate 9:1).

*6-Methoxy-3-(3-methylphenyl)coumarin (**1**).* Yield 79%; mp 98−99 °C. ^{1}H NMR ($CDCl_3$) 2.46 (s, 3H, $-CH_3$), 3.90 (s, 3H, $-OCH_3$), 7.01 (d, 1H, H-5, J = 2.8), 7.14 (dd, 1H, H-7, J = 9.0, J = 2.9), 7.25−7.41 (m, 3H, H-5′, H-4′, H-8), 7.50−7.54 (m, 2H, H-2′, H-6′), 7.79 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 21.5, 55.8, 109.9, 117.4, 119.0, 120.0, 125.7, 128.3, 128.8, 129.2, 129.6, 134.7, 138.0, 139.6, 147.9, 156.1, 160.7. MS m/z 267 ([M + 1]$^+$, 30), 266 (M$^+$, 100), 238 (38), 195 (25), 167 (14), 165 (17), 152 (24). Anal. Calcd for $C_{17}H_{14}O_3$: C, 76.68; H, 5.30. Found: C, 76.72; H, 5.38.

*6-Methoxy-3-(4-methylphenyl)coumarin (**2**).* Yield 76%; mp 130−131 °C. ^{1}H NMR ($CDCl_3$) 2.40 (s, 3H, $-CH_3$), 3.86 (s, 3H, $-OCH_3$), 7.01 (d, 1H, H-7, J = 3.9), 7.13−7.17 (m, 2H, H-5, H-8), 7.30−7.35 (m, 2H, H-3′, H-5′), 7.65 (d, 2H, H-2′, H-6′, J = 8.1), 7.78 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 21.3, 55.8, 109.8, 117.4, 118.9, 120.1, 128.4, 128.6, 129.2, 131.9, 138.9, 139.0, 147.9, 156.1, 160.8. MS m/z 267 ([M + 1]$^+$, 20), 266 (M$^+$, 100), 238 (25), 195 (18), 165 (9), 152 (16). Anal. Calcd for $C_{17}H_{14}O_3$: C, 76.68; H, 5.30. Found: C, 76.67; H, 5.23.

*6-Methyl-3-(4-methylphenyl)coumarin (**7**).* Yield 72%; mp: 139−140 °C. ^{1}H NMR ($CDCl_3$) 2.40 (s, 6H, $(-CH_3)_2$), 7.21−7.30 (m, 5H, H-5, H-7, H-8, H-3′, H-5′), 7.60 (d, 2H, H-2′, H-6′, J = 8.2), 7.72 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 21.1, 21.6, 116.4, 119.7, 127.9, 128.4, 128.7, 129.4, 132.2, 132.5, 134.3, 139.0, 139.4, 139.5, 151.8, 161.2. MS m/z 251 ([M + 1]$^+$, 32), 250 (M$^+$, 100), 222 (56), 221 (24), 178 (27), 150 (12), 136 (12), 124 (19). Anal. Calcd for $C_{17}H_{14}O_2$: C, 81.58; H, 5.64. Found: C, 81.52; H, 5.60.

*6-Methyl-3-(3-methylphenyl)coumarin (**8**).* Yield 75%; mp 84−85 °C. ^{1}H NMR ($CDCl_3$) 2.39 (s, 6H, $-(CH_3)_2$), 6.98−7.02 (m, 3H, H-4′, H-7, H-8), 7.13−7.24 (m, 4H, H-2′, H-5, H-5′, H-6′), 7.72 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 20.8, 21.5, 116.1, 119.4, 125.6, 127.6, 128.3, 129.2, 129.5, 132.3, 134.1, 134.8, 138.0, 139.5, 139.8, 151.6, 160.8. MS m/z 251 ([M + 1]$^+$, 27), 250 (M$^+$, 100), 222 (72), 221 (39), 178 (32), 150 (10), 136 (10), 124 (10). Anal. Calcd for $C_{17}H_{14}O_2$: C, 81.58; H, 5.64. Found: C, 81.56; H, 5.62.

General Procedure for the Preparation of 3-(Bromomethoxyphenyl)coumarins (15−18). A solution of 3-(methoxyphenyl)-coumarins (3.76 mmol), NBS (4.51 mmol), and AIBN (cat.) in CCl_4 (5 mL) was stirred under reflux for 18 h. The resulting solution was filtered to remove the succinimide. The solvent was evaporated under vacuum and purified by flash chromatography (hexane/ethyl acetate 95:5).

*3-(3-Bromo-4-methoxyphenyl)-6-methylcoumarin (**15**).* Yield: 41%; mp 206−207 °C. ^{1}H NMR ($CDCl_3$) 2.42 (s, 3H, $-CH_3$), 3.94 (s, 3H, $-OCH_3$), 6.97 (d, 1H, H-5′ J = 8.7), 7.23−7.35 (m, 3H, H-2′, H-6′, H-5), 7.69−7.74 (m, 2H, H-7, H-8), 7.89 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 20.8, 56.3, 111.5, 111.6, 116.1, 119.3, 126.3, 127.6, 128.5, 129.0, 132.5, 133.1, 134.2, 139.1, 151.5, 156.2, 160.6. MS m/z (%) 347 (18), 346 (98), 345 ([M + 1]$^+$, 19), 344 (M$^+$, 100), 303 (45), 301 (45), 275 (11), 250 (17), 222 (13), 194 (11), 178 (13), 165 (58), 163 (11), 139 (15), 132 (42), 82 (18), 76 (14), 63 (19), 50 (14). Anal. Calcd for $C_{17}H_{13}BrO_3$: C, 59.15; H, 3.80. Found: C, 59.10; H, 3.72.

*3-(4-Bromo-3-methoxyphenyl)-6-methylcoumarin (**16**).* Yield: 51%; mp 139−140 °C. ^{1}H NMR ($CDCl_3$) 2.49 (s, 3H, $-CH_3$), 3.89 (s, 3H, $-OCH_3$), 6.49 (dd, 1H, H-7, J = 7.4, J = 1.3), 7.08−7.12 (m, 3H, H-2′, H-6′, H-8), 7.32−7.42 (m, 2H, H-5, H-5′), 7.74 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 33.1, 55.4, 114.2, 114.7, 117.1, 119.7, 120.9, 126.8, 128.2, 128.7, 129.6, 132.2, 134.3, 135.7, 139.4, 153.2, 159.5. MS m/z (%) 346 (45), 345 ([M + 1]$^+$, 10), 344 (M$^+$, 100), 266 (49), 265 (45), 238 (24), 237 (67), 194 (42), 165 (29), 133 (28). Anal. Calcd for $C_{17}H_{14}O_2$: C, 59.15; H, 3.80. Found: C, 59.17; H, 3.83.

*3-(2-Bromo-3,5-dimethoxyphenyl)-6-methylcoumarin (**17**).* Yield: 46%; mp 178−179 °C. ^{1}H NMR ($CDCl_3$) 2.40 (s, 3H, $-CH_3$), 3.79 (s, 3H, $-OCH_3$), 3.87 (s, 3H, $-OCH_3$), 6.51 (s, 2H, H-4′, H-6′), 7.26−7.33 (m, 3H, H-5, H-7, H-8), 7.60 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 20.8, 55.6, 56.4, 100.1, 104.3, 107.4, 116.4, 118.7, 127.9, 129.0, 132.8, 134.2, 137.6, 142.3, 152.1, 157.0, 159.7. MS m/z (%) 377 (42), 376 ([M + 1]$^+$, 18), 375 (M$^+$, 100), 282 (59), 279 (14), 239 (11) 208 (9), 152 (10), 118 (9), 58 (12). Anal. Calcd for $C_{18}H_{15}BrO_4$: C, 57.62; H, 4.03. Found: C, 57.61; H, 4.08.

*3-(2-Bromo-3,4,5-trimethoxyphenyl)-6-methylcoumarin (**18**).* Yield: 50%; mp 167−168 °C. ^{1}H NMR ($CDCl_3$) 2.40 (s, 3H, $-CH_3$), 3.83 (s, 3H, $-OCH_3$), 3.90 (s, 6H, $-(OCH_3)_2$), 6.72 (s, 1H, H-6′), 7.25 (d, 1H, H-8, J = 8.4), 7.29 (d, 1H, H-5, J = 1.9), 7.34 (dd, 1H, H-7, J = 8.4, J = 2.0), 7.63 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 20.8, 55.6, 61.1, 61.1, 110.1, 110.4, 116.4, 118.7, 127.8, 128.4, 131.2, 132.9, 134.3, 142.7, 143.4, 151.2, 152.0, 152.7, 160.1. MS m/z (%) 408 (32), 407 ([M + 1]$^+$, 12), 406 (M$^+$, 68), 404 (5) 326 (22), 325 (59), 282 (15), 264 (15) 169 (9), 139 (10). Anal. Calcd for $C_{19}H_{17}BrO_5$: C, 56.31; H, 4.23. Found: C, 56.38; H, 4.28.

General Procedure for the Preparation of Hydroxy-3-phenylcoumarins (3, 20−21). A solution of substituted 6-methoxy-3-phenylcoumarin (0.50 mmol) in acetic acid (5 mL) and acetic anhydride (5 mL), at 0 °C, was prepared. Hydriodic acid 57% (10 mL) was added dropwise. The mixture was stirred, under reflux temperature, for 3 h. The solvent was evaporated under vacuum, and the dry residue was purified by CH_3CN crystallization.

*6-Hydroxy-3-(4-methylphenyl)coumarin (**3**).* Yield 61%; mp 214−215 °C. ^{1}H NMR (DMSO-d_6) 2.32 (s, 3H, $-CH_3$), 7.01 (d, 1H, H-7, J = 8.8, J = 2.9), 7.07 (d, 1H, H-5, J = 2.8), 7.21−7.26 (m, 3H, H-3′, H-5′, H-8), 7.59 (d, 2H, H-2′, H-6′, J = 8.1), 8.08 (s, 1H, H-4), 9.77 (s, 1H, −OH). ^{13}C NMR (DMSO-d_6) 21.3, 113.0, 117.1, 120.0, 120.5, 127.2, 128.8, 129.2, 132.3, 138.5, 140.4, 146.7, 154.2, 160.5. MS m/z (%) 253 ([M + 1]$^+$, 51), 252 (M$^+$, 100), 225 (35), 224 (96), 223 (62) 195 (13), 181 (17), 165 (19), 152 (31), 139 (10), 125 (15), 115 (15). Anal. Calcd for $C_{16}H_{12}O_3$: C, 76.18; H, 4.79. Found: C, 76.11; H, 4.77.

*3-(3-Hydroxyphenyl)-6-methylcoumarin (**20**).* Yield 53%; mp 160−161 °C. ^{1}H NMR (DMSO-d_6) 2.32 (s, 3H, −CH_3), 6.77 (dd, 1H, H-4′, J = 7.1, J = 2.4), 7.05−7.07 (m, 2H, H-5, H-8), 7.21−7.25 (m, 2H, H-5′, H-6′), 7.38 (dd, 1H, H-7, J = 8.5, J = 1.8), 7.50 (s, 1H, H-2′), 8.08 (s, 1H, H-4), 9.52 (s, 1H, −OH). ^{13}C NMR (DMSO-d_6) 20.8, 115.9, 116.1, 119.6, 127.3, 128.7, 129.7, 133.0, 134.2, 136.4, 140.8, 151.5, 157.5, 160.2. MS m/z 254 (12), 253 ([M + 1]$^+$, 63), 252 (M$^+$, 73), 251 (15), 225 (46), 223 (53), 195 (33), 194 (18), 181 (29), 178 (17), 166 (12), 165 (42), 152 (43), 139 (13), 126 (24), 111 (19), 63 (16), 51 (16). Anal. Calcd for $C_{16}H_{12}O_3$: C, 76.18; H, 4.79. Found: C, 76.20; H, 4.83.

*3-(2-Hydroxyphenyl)-6-methylcoumarin (**21**).* Yield 56%; mp 167−168 °C. ^{1}H NMR (DMSO-d_6) 2.38 (s, 3H, −CH_3), 6.30−6.35 (m, 2H, H-3′, H-5′), 7.22−7.24 (d, 1H, H-5, J = 1.5), 7.29−7.31 (m, 2H, H-4′, H-6′), 7.34 (d, 1H, H-8, J = 8.4), 7.44 (dd, 1H, H-7, J = 8.4, J = 1.5), 7.97 (s, 1H, H-4), 9.60 (s, 1H, −OH). ^{13}C NMR (DMSO-d_6) 20.3, 115.6, 118.7, 119.0, 122.3, 125.9, 128.0, 129.6, 130.8, 132.3, 133.6, 141.8, 151.2, 155.0, 159.5. MS m/z 253 ([M + 1]$^+$, 18), 252 (M$^+$, 100), 251 (10), 234 (12), 223 (58), 195 (35), 194 (5), 181 (18), 165 (17), 152 (18), 126 (5), 63 (6), 51 (6). Anal. Calcd for $C_{16}H_{12}O_3$: C, 76.18; H, 4.79. Found: C, 76.20; H, 4.81.

General Procedure for the Preparation of 2-Oxopropoxy-6-phenylcoumarin (4, 22). Under a suspension of anhydrous K_2CO_3 (0.25 mmol) and the corresponding hydroxycoumarin (0.13 mmol), in anhydrous acetone (3 mL), the chloroketone (0.25 mmol) was added. The suspension was stirred, at reflux temperature, for 16 h. The mixture was cooled, and the precipitate was recovered by filtration and washed with anhydrous acetone (3 × 40 mL). The solvent was evaporated under vacuum, and the dry residue was purified by FC (hexane/ethyl acetate 85:15).

*3-(4-Methylphenyl)-6-(2-oxopropoxy)coumarin (**4**).* Yield 74%; mp 128−129 °C. ^{1}H NMR ($CDCl_3$) 2.33 (s, 3H, −CH_3), 2.42 (s, 3H, −CH_3), 4.64 (s, 2H, −CH_2), 6.96 (d, 1H, H-5, J = 2.9), 7.14 (dd, 1H, H-7, J = 9.0, J = 2.9), 7.28−7.33 (m, 3H, H-3′, H-5′, H-8), 7.60−7.65 (m, 2H, H-2′, H-6′), 7.74 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 21.3, 26.6, 73.5, 111.0, 117.7, 119.2, 120.2, 128.4, 129.0, 129.2, 131.7, 138.6, 139.1, 148.4, 154.2, 160.6, 204.7. MS m/z 309 ([M + 1]$^+$, 31), 308 (M$^+$, 100), 266 (12), 265 (50), 236 (15), 235 (26), 207 (31) 195 (12), 179 (16), 178 (16), 165 (11), 153 (12), 129 (20). Anal. Calcd for $C_{19}H_{16}O_4$: C, 74.01; H, 5.23. Found: C, 73.90; H, 5.18.

*6-Methyl-3-[3-(2-oxopropoxy)phenyl]coumarin (**22**).* Yield 71%; mp 107−108 °C. ^{1}H NMR ($CDCl_3$) 2.31 (s, 3H, −CH_3), 2.43 (s, 3H, −CH_3), 4.61 (s, 2H, −CH_2), 6.94 (d, 1H, H-5, J = 1.2), 7.24−7.42 (m, 6H, H-7, H-8, H-2′, H-4′, H-5′, H-6′), 7.77 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 20.8, 26.6, 73.1, 114.8, 115.0, 116.1, 119.2, 121.9, 127.7, 129.7, 132.6, 134.2, 136.4, 140.2, 151.6, 157.6, 160.6, 205.3. MS m/z 309 ([M + 1]$^+$, 14), 308 (M$^+$, 70), 266 (55), 265 (50), 237 (29), 236 (21), 235 (20), 178 (24), 165 (10). Anal. Calcd for $C_{19}H_{16}O_4$: C, 74.01; H, 5.23. Found: C, 74.08; H, 5.30.

Preparation of 6-(2-Cyclopentyloxy)-3-(4-methylphenyl)-coumarin (5). Under a suspension of anhydrous K_2CO_3 (0.25 mmol) and the 6-hydroxycoumarin **3** (0.13 mmol), in anhydrous acetone (3.0 mL), the cyclopentyl bromide (0.25 mmol) was added. The suspension was stirred, at reflux temperature, for 24 h. The mixture was cooled, and the precipitate was recovered by filtration and washed with anhydrous acetone (3 × 40.0 mL). The solvent was evaporated under vacuum, and the dry residue was purified by FC (hexane/ethyl acetate 9:1). Yield 58%; mp 147−148 °C. ^{1}H NMR ($CDCl_3$) 1.37−1.99 (m, 8H, (−CH_2)$_4$), 2.52 (s, 3H, −CH_3), 4.89 (s, 1H, H-1″), 7.05 (d, 1H, H-5, J = 2.7), 7.17 (dd, 1H, H-7, J = 9.0, J = 2.7), 7.36−7.40 (m, 3H, H-3′, H-5′, H-8), 7.72 (d, 2H, H-2′, H-6′, J = 8.1), 7.84 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 21.3, 24.0, 32.8, 80.0, 111.8, 117.3, 120.1, 120.3, 128.4, 128.4, 129.1, 132.0, 138.8, 139.2, 147.6, 154.6, 160.9. MS m/z 321 ([M + 1]$^+$, 7), 320 (M$^+$, 28), 253 (19), 252 (100), 224 (38), 223 (13), 152 (11). Anal. Calcd for $C_{21}H_{20}O_3$: C, 78.75; H, 6.29. Found: C, 78.69; H, 6.24.

Determination of hMAO Isoform Activity. Briefly, 0.1 mL of sodium phosphate buffer (0.05 M, pH 7.4) containing different concentrations of the test drugs (new compounds or reference inhibitors) in various concentrations and adequate amounts of recombinant hMAO-A or hMAO-B required and adjusted to obtain in our experimental conditions the same reaction velocity, i.e., to oxidize (in the control group) the same concentration of substrate: 165 pmol of *p*-tyramine/min (hMAO-A, 1.1 μg protein; specific activity, 150 nmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min/mg protein; hMAO-B, 7.5 μg protein; specific activity, 22 nmol of *p*-tyramine transformed/min/mg protein) were incubated for 15 min at 37 °C in a flat-black-bottom 96-well microtest plate and placed in the dark fluorimeter chamber. After this incubation period, the reaction was started by adding (final concentrations) 200 μM Amplex Red reagent, 1 U/mL horseradish peroxidase, and 1 mM *p*-tyramine. The production of H_2O_2 and, consequently, of resorufin was quantified at 37 °C in a multidetection microplate fluorescence reader (FLX800, Bio-Tek Instruments, Inc., Winooski, VT, USA) based on the fluorescence generated (excitation, 545 nm, emission, 590 nm) over a 15 min period, in which the fluorescence increased linearly.

Control experiments were carried out simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions of the vehicles. In addition, the possible capacity of the above test drugs to modify the fluorescence generated in the reaction mixture due to nonenzymatic inhibition (e.g., for directly reacting with Amplex Red reagent) was determined by adding these drugs to solutions containing only the Amplex Red reagent in a sodium phosphate buffer. To determine the kinetic parameters of hMAO-A and hMAO-B (K_m and V_{max}), the corresponding enzymatic activity of both isoforms was evaluated (under the experimental conditions described above) in the presence of a number (a wide range) of *p*-tyramine concentrations.

The specific fluorescence emission (used to obtain the final results) was calculated after subtraction of the background activity, which was determined from vials containing all components except the hMAO isoforms, which were replaced by a sodium phosphate buffer solution. In our experimental conditions, this background activity was practically negligible.

Molecular Docking Simulations. The crystallographic structure of MAO-B in complex with a coumarin inhibitor (pdb code 2V61)[52] and the MAO-A in complex with harmine (pdb code 2ZX5)[51] were used to dock the coumarins under study. For both isoenzymes, hydrogen atoms and charges were added to the receptor structure using the UCSF Chimera,[57] and the atomic charges of the FAD cofactor were calculated using GAMESS.[58]

Three-dimensional conformers for the compounds were generated using the OMEGA software.[59] A maximum of 50000 conformations per molecule were generated using an energy window of 10 kcal. All rotatable bonds were considered during the torsion search, using the Merck Molecular Force Field (MMFF), and duplicate conformers were discarded based on a root-mean-square (rms) value of 0.5 Å. A maximum number of 300 conformers were saved for each compound. Afterward, AM1-BCC charges were added to each conformer using the MOLCHARGE program, which is part of the QUACPAC package.[60]

Rigid docking was carried out for each ligand conformer using the DOCK 6.3 package.[54] A maximum of 5000 orientations per ligand was assayed. The energy grid-based scoring function was selected for poses quality evaluation. The five lowest scored poses for each ligand conformer were saved, allowing for a maximum of 1500 saved poses for each compound. Afterward, the five lowest scored conformers of each ligand were rescored, using the DOCK Amber-based scored function, considering only the flexibility of the ligand. Finally, the pose with the lowest Amber-based scoring value for each compound was rescored, with the same scoring function, considering both the ligand and the binding. We chose the Amber-based scoring function to take

into account small structural rearrangements on both ligand and receptor as well as the effect of the solvent through a GB/SA continuum model for the solvation free energy.[55,56] Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR001081).

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■ DEDICATION

This article is dedicated to the memory of Prof. Francisco Orallo

■ ABBREVIATIONS USED

hMAO, human monoamine oxidase; FAD, flavin adenine dinucleotide; IC_{50}, half maximal inhibitory concentration; cDNA, cDNA; SAR, structure−activity relationship; PDB, Protein Data Bank; HPLC, high-performance liquid chromatography

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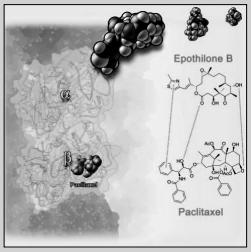
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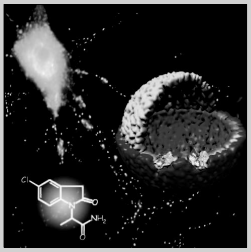
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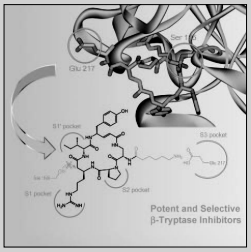
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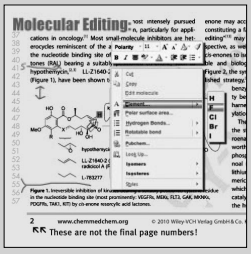
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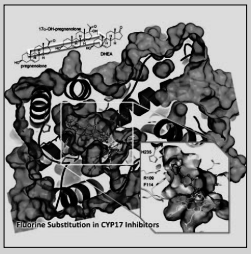


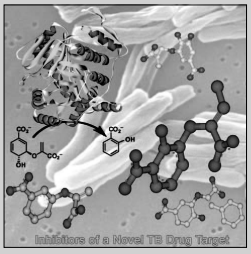


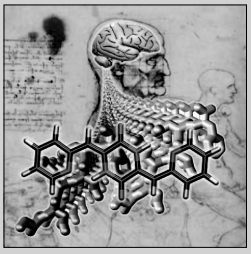


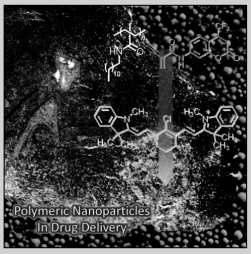


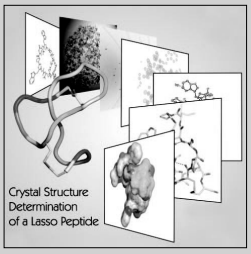


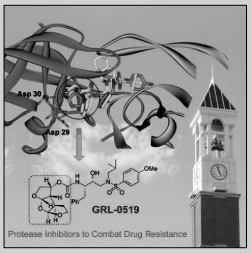


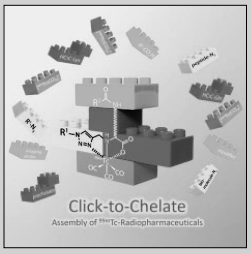












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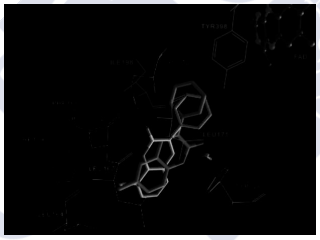
CHEMISTRY ENABLING DRUG DISCOVERY

Table of Contents

D. Viña, M. J. Matos, G. Ferino,* E. Cadoni, R. Laguna, F. Borges, E. Uriarte, L. Santana*

464 – 470

8-Substituted 3-Arylcoumarins as Potent and Selective MAO-B Inhibitors: Synthesis, Pharmacological Evaluation, and Docking Studies



B-selective! With the aim of finding new selective MAO-B inhibitors, herein we report the synthesis, in vitro evaluation, and a docking simulation of a new series of 3-arylcoumarins variously substituted at the 8-position as potential inhibitors of hMAO-A and B. Most of the studied compounds have high affinity and selectivity for hMAO-B, with IC_{50} values in the low micro- to nanomolar range.

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8-Substituted 3-Arylcoumarins as Potent and Selective MAO-B Inhibitors: Synthesis, Pharmacological Evaluation, and Docking Studies

Dolores Viña,[d] Maria J. Matos,*[a, c] Giulio Ferino,*[b] Enzo Cadoni,[b] Reyes Laguna,[d] Fernanda Borges,[a] Eugenio Uriarte,[c] and Lourdes Santana[c]

Neurodegenerative disorders are becoming more prevalent given the increase in the aging population. This has inspired active research in the development of new drugs that could mark an important advance in the treatment of complex diseases such as Alzheimer's and Parkinson's. With the aim of finding new MAO-B-selective inhibitors, we report the synthesis, in vitro evaluation, and docking simulation of a new series of 3-arylcoumarins variously substituted at the 8-position. Most of the studied compounds show high affinity and selectivity for the hMAO-B isoform, with IC_{50} values in the low micro- to nanomolar range. Some of them have greater hMAO-B inhibitory activity and selectivity than the reference compound, selegiline. Compounds **7** and **8** are the most active of this series, with compound **8** being fivefold more potent against MAO-B and severalfold more selective than selegiline. Docking experiments were carried out with hMAO-B crystal structures, providing new information about the enzyme–inhibitor interaction and the potential therapeutic application of the new 8-substituted 3-arylcoumarins.

Introduction

With the increase in the aging population in modern society, neurodegenerative diseases (NDs) are becoming increasingly prevalent pathologies. Maintaining the quality of life for the world's aging population is among the most important challenges in medicine today. Alzheimer's disease (AD) is the most prevalent of the NDs, followed by Parkinson's disease (PD). The development of effective neuroprotective therapies that either slow or halt disease progression in the earliest stages, or palliate the appearance of symptoms, is one of the main goals for researchers in this area.

Increased activity in monoamine oxidase A and B (MAO-A and MAO-B) is associated with decreased quantities of endogenous and exogenous monoamines. The two isoforms of MAO are responsible for catalyzing the oxidative deamination of neurotransmitters and dietary amines, thus regulating the intracellular levels of biogenic amines in the brain and peripheral tissues.[1,2] MAOs are FAD-dependent enzymes found in the outer mitochondrial membrane of various cell types such as neuronal, glial, and other mammalian cells.[3] MAO-A and B have been identified and differentiated through their primary sequences,[4] three-dimensional structures,[5] tissue distribution,[6] inhibitor selectivity,[7] and substrate preferences.[8] Due to their affinity for different substrates, these enzymes are involved in different pathologies such as anxiety and depression (MAO-A), and PD and AD (MAO-B). The MAO-A isoform has higher affinity for serotonin and noradrenalin, whereas the MAO-B isoform preferentially deaminates β-phenylethylamines and benzylamine.[9,10] Dopamine and tyramine are common substrates for both isoforms. These physiological properties therefore determine the clinical interest of MAO inhibitors. Selective MAO-A inhibitors such as clorgyline (irreversible) and moclobemide (reversible) are used for the treatment of neurological disorders,[11] whereas selective and irreversible MAO-B inhibitors such as selegiline and rasagiline are used for the treatment of NDs.[12–14]

Interest in selective MAO inhibitors, especially those for MAO-B, has increased significantly in recent years. This is related to the recent discovery that the increase in MAO-B activity is associated with gliosis, which can result in high levels of hydrogen peroxide and oxidative free radicals. It was reported that the expression levels of this isoform in neuronal tissue increase fourfold with age. The increment of dopamine metabolism, as well as the described oxidative stress, may play a role in the etiology of ND.[15,16] Rasagiline is a good MAO-B inhibitor due to its capacity to protect neuronal cells from the consequences of oxidative stress in patients.[16,17]

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Osthole (7-methoxy-8-isopentenoxycoumarin), an active constituent of *Cnidium monnieri* (L.) *Cusson* was reported in 1996 to have various pharmacological activities.[18] It has shown protective effects in animal models of central nervous system (CNS) diseases owing to its anti-oxidative and anti-apoptotic activity.[19]

Several natural and synthetic coumarins[20–24] have been cited since the 1990s as having a promising scaffold for MAO inhibition.[25–28] Structure–selectivity relationship studies suggested that selectivity is modulated by the nature and position of the linkage between the coumarin moiety and the lipophilic aryl groups.[29] Substitution patterns at the 3- and 7-positions have been thoroughly studied. Some 3-arylcoumarins with various substituents in either the 3-aryl or coumarin rings were also reported as potent and selective MAO-B inhibitors.[30–34] In those studies, small substituents (especially methyl groups) at position 6 of the coumarin moiety and the introduction of an aryl ring at position 3 of the scaffold proved to be important for potent MAO-B inhibitory activity and selectivity.[35,36]

Alternatively, it was reported that alkylsulfonyloxy groups at position 7 of the coumarin moiety produce MAO-A inhibitors such as esuprone, while a benzyloxy substituent at the same position leads to potent and selective MAO-B inhibitors.[25–27,29,37,38] The presence of an acetonyloxy group at position 7 with a benzene ring fused at positions 3 or 4 led to compounds with very high MAO-B selectivity.[39] Therefore, it is clear that substitutions on the coumarin moiety can modulate MAO-A and B activity and selectivity. Furthermore, work related to the chromone ring, an isomer of the coumarin scaffold, has also been carried out. Those with substituents at position 3 of the γ-pyrone nucleus act preferentially as MAO-B inhibitors, with IC_{50} values in the nanomolar to micromolar range.[40]

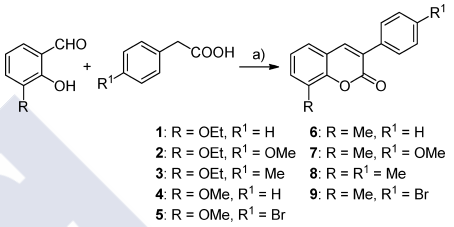
Taking into account previous studies, and particularly the very high MAO-B selectivity observed for 6-substituted 3-arylcoumarins,[30–32,35] we continued our exploration of substitutions on the coumarin ring system. We were motivated to synthesize and study the MAO inhibitory activity of new analogues in which a range of groups of various size and lipophilicity were introduced at position 8 of the coumarin aromatic ring and at position 4 of the 3-aryl ring. Both aromatic rings were substituted in order to clarify the influence of the position and substitution pattern on MAO inhibitory activity and selectivity by the 3-arylcoumarins. Given that there are no other published studies regarding 8-substituted coumarins, this is an interesting scaffold to explore.

Crystal structures of the two MAO isoforms have provided relevant information about the selective interactions and pharmacophore requirements for the design of potent and selective inhibitors.[41–46] Based on the significant structural information that is currently available,[47,48] and to analyze the structural requirements for MAO activity and selectivity, docking experiments were carried out on crystallographic structures of the human MAO-B isoform, hMAO-B. These studies are very helpful for corroborating and clarifying the experimental data obtained in order to develop a rational drug design project.

Results

Chemistry

Coumarin derivatives **1**–**9** were efficiently synthesized according to the protocol outlined in Scheme 1.[31–33,49] The structures of the described compounds were confirmed by ^{1}H NMR, ^{13}C NMR, mass spectrometry, elemental analyses, and melting points. The general reaction conditions and compound characterization data are described below in the Experimental Section.



Scheme 1. *Reagents and conditions:* a) DCC, DMSO, 110 °C, 24 h.

Perkin condensation of various *ortho*-hydroxybenzaldehydes with the appropriate arylacetic acid, using *N*,*N*′-dicyclohexylcarbodiimide (DCC) as dehydrating agent, was the key step to afford the desired 3-arylcoumarins. This is a very easy and versatile reaction that affords different families of substituted coumarins with a wide range of substituent patterns.

Pharmacology: inhibition of MAO

Evaluation of the test compounds on hMAO activity was performed by measuring their effects on the production of hydrogen peroxide from *para*-tyramine (a common substrate for hMAO-A and hMAO-B) using the Amplex Red MAO assay kit (Molecular Probes Inc., Eugene, OR, USA) and microsomal MAO isoforms prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for hMAO-A or hMAO-B (Sigma–Aldrich Química S.A., Alcobendas, Spain).[50]

The production of hydrogen peroxide catalyzed by the two MAO isoforms can be detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a non-fluorescent and highly sensitive probe that reacts with H_2O_2 in the presence of horseradish peroxidase to produce a fluorescent product, resorufin. New compounds and reference inhibitors were unable to react directly with the Amplex Red reagent, indicating that these drugs do not interfere with the assay measurements. In our experiments and under specific conditions, hMAO-A displayed a Michaelis constant (K_M) of 457.17 ± 38.62 μM and a maximum reaction velocity (V_{max}) in the control group of 185.67 ± 12.06 (nmol *p*-tyramine) min^{-1} (mg protein)$^{-1}$, whereas hMAO-B showed a K_M of 220.33 ± 32.80 μM and a V_{max} of 24.32 ± 1.97 (nmol *p*-tyramine) min^{-1} (mg protein)$^{-1}$ ($n = 5$).

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Most tested compounds inhibited this enzymatic control activity in a concentration-dependent manner (Table 1).

Table 1. Structure and in vitro hMAO inhibitory activity of 3-arylcoumarin derivatives **1–9** and reference compounds.

Compd	IC_{50} [nM][a]		SI[b]
	hMAO-A	hMAO-B	
1	*	$1.06\times10^3\pm0.71\times10^3$	>94[c]
2	*	208.82 ± 13.98	>479[c]
3	*	148.53 ± 9.96	>673[c]
4	**	150.02 ± 10.04	>667[c]
5	*	$3.31\times10^3\pm0.22\times10^3$	>30[c]
6	$6.26\times10^3\pm0.23\times10^3$	78.28 ± 5.25	79
7	$21.60\times10^3\pm0.97\times10^3$	3.43 ± 0.23	6.297
8	**	4.51 ± 0.24	>22.173[c]
9	*	19.90 ± 1.33	>5.025[c]
selegiline	$67.25\times10^3\pm1.02\times10^3$	19.60 ± 0.86	3.431
iproniazide	$6.56\times10^3\pm0.76\times10^3$	$7.54\times10^3\pm0.36\times10^3$	0.87
rasagiline	$16.44\times10^3\pm0.85\times10^3$	68.96 ± 3.63	238

[a] Values represent the mean ±SEM of five experiments ($n=5$). *: Inactive at 100 μM (highest concentration tested); at higher concentrations the compounds precipitate. **: 100 μM inhibits the corresponding hMAO activity by ~40–45%; at higher concentrations the compounds precipitate. [b] Selectivity index: MAO-B selectivity ratios $[IC_{50(MAO\text{-}A)}]/[IC_{50(MAO\text{-}B)}]$ for inhibitory effects of both new compounds and reference inhibitors. [c] Values obtained under the assumption that the corresponding IC_{50} value against MAO-A is the highest concentration tested (100 μM).

Table 2 lists the results of the reversibility and irreversibility tests for compound **8** and reference inhibitors. hMAO-B inhibition is irreversible in the presence of **8** as shown by the lack of restored enzyme activity after repeated washing. Similar results were obtained for selegiline. In contrast, significant recovery of hMAO-B activity was observed after repeated washing of isatin, indicating that this drug is a reversible inhibitor of hMAO-B.

Table 2. Reversibility and irreversibility of hMAO-B inhibition by derivative **8** and reference inhibitors.

Compd	hMAO-B inhibition [%][a]	
	Before washing	After repeated washing
8 (10 nM)	53.05 ± 3.58	57.15 ± 3.86
selegiline (20 nM)	56.34 ± 5.37	58.67 ± 6.60
isatin (33 μM)	63.45 ± 4.28	23.10 ± 0.52[b]

[a] Values represent the mean ±SEM of five experiments ($n=5$). [b] Level of statistical significance: $p<0.01$ versus the corresponding percent hMAO-B inhibition before washing, as determined by ANOVA/Dunnett's test.

Molecular docking studies

Docking studies were performed to better understand the manner in which 8-substituted 3-arylcoumarins interact with the hMAO-B binding pocket. The X-ray crystal structure of hMAO-B in complex with 4-carboxaldehyde-7-(3-chlorobenzyloxy)coumarin (c17) was used as the model for docking calculations (PDB code: 2V60).[48] Water molecules close to the binding site were considered for the experiments because they form an extensive hydrogen bond network amongst each other and with the enzyme; moreover, some of these water molecules proximal to the FAD cofactor appear to be highly conserved in hMAO-B isoforms.[51]

QM-polarized ligand docking (QPLD)[52] followed by post-docking processing operated with Prime MM-GBSA[53] was used as protocol for this study. Briefly, in the QPLD step, the ligand was first docked into the enzyme using Glide;[54] quantum mechanical calculations were then made to take into account the polarization of the charges in the ligand by the enzyme. Finally, ligands with improved charges were re-docked into the enzyme (see the Experimental Section below for details). The best pose of each compound derived from QPLD was subjected to an energy calculation and minimization with Prime MM-GBSA. Prime was used in this study with the sole aim of minimizing the input poses derived from QPLD, taking into account a flexible enzyme region defined by a distance of 5 Å from the ligand. We validated our docking protocol by re-docking the co-crystallized coumarin c17 inside the binding cleft. The best docking pose shows an RMSD of 0.47 Å for heavy atoms.

The first simulation was made with the aim of visualizing the binding mode of the most active compounds (Figure 1a). The best docking poses were retrieved for compounds **7**, **8**, and **9**, which manifest the same pattern. The coumarin ring occupies the substrate cleft facing the FAD cofactor, leaving the 3-arylcoumarin moiety directed toward the entry cavity. All three poses are stabilized by a hydrogen bond between the carbonyl oxygen atom of the coumarin and Cys172. Tyr326 is involved in a π–π stacking interaction with 3-arylcoumarin rings. Conversely, good van der Waals and electrostatic interactions with Phe168, Leu171, Ile198, Ile199, Ile316, Phe343, Tyr398, and Thy435 were observed.

In the second docking calculation we investigated the best-pose solutions adopted from 8-ethoxy-3-arylcoumarins (Figure 1b). The patterns adopted from compounds **1**, **2**, and **3** could be considered generally the same, while small differences can be observed between the most active compounds **2** and **3** with respect to compound **1**. The hydrogen bond with Cys172 was maintained for all the three coumarins. The methoxy oxygen atom of compound **2** acts as a hydrogen bond acceptor with a water molecule, forming a hydrogen bond at a distance of 1.91 Å. For compound **3**, Tyr326 contributes to ligand stabilization by forming a π–π stacking interaction with the 3-aryl ring of the coumarin. Furthermore, the ethoxy group at position 8 is positioned in a different manner for compound **1** than its positioning for coumarins **2** and **3**. Coumarin **1** therefore shows good hydrophobic pattern recognition only with Trp119 and Phe103, whereas compounds **2** and **3** interact with Trp119, Leu164, Leu167, Phe168, and Ile316 (Figure 1b). Regarding the presence of a bulkier (relative to methoxy or methyl) hydrophobic group at position 8, the *para*-3-aryl substituent seems to modulate the inhibitory activity of coumarin derivatives.

Finally, with the aim of analyzing the differences between substitutions at positions 8 and 6 of the coumarin nucleus, we

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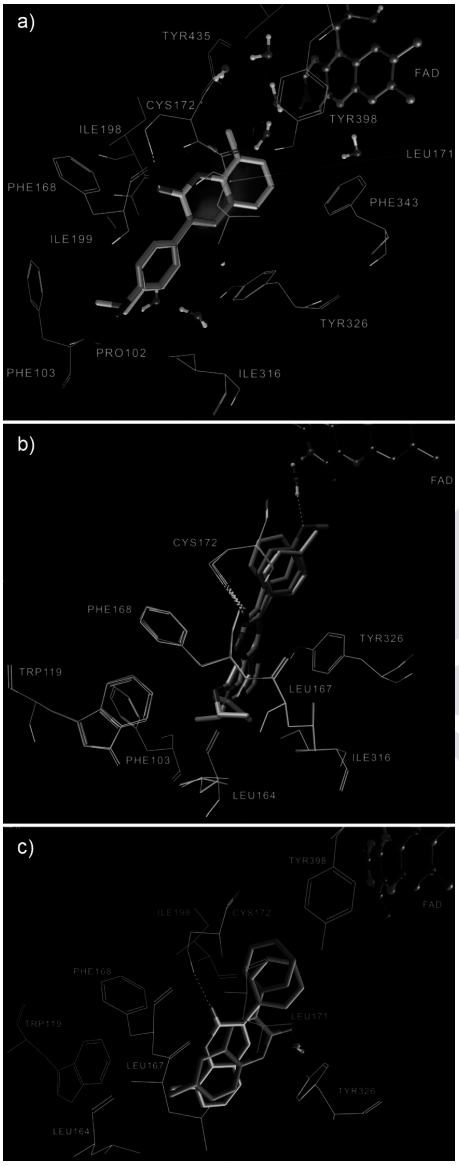


Figure 1. a) Comparison of the most stable binding modes of compounds **7**, **8**, and **9** into hMAO-B (PDB code: 2V60). The binding pocket surface is colored according to residue property: green for hydrophobic residues and turquoise for polar uncharged residues. Ligands are represented in tube format; carbon atoms are yellow for **7**, green for **8**, and purple for **9**. The FAD cofactor and water molecules are depicted in ball-and-stick format; carbon atoms are grey. Interacting residues are represented in wire, with carbon atoms in grey. The hydrogen bond is displayed as a yellow dotted line, and nonpolar hydrogen atoms are omitted. b) Superimpositions of the best-pose solutions for compounds **1**, **2**, and **3** (shown in tube format) docked into hMAO-B (PDB code: 2V60). Coumarins and the interacting residues with their ethoxy groups are colored by the same scheme: green for **1**, purple for **2**, and turquoise for **3**. Tyr326 and Cys172 are also shown. All residues are displayed in wire format. The water molecule involved in hydrogen bonding (yellow dotted line) and FAD are represented in ball-and-stick format with carbon atoms in grey. c) Best poses of compound **6** and 6-methyl-3-phenylcoumarin into hMAO-B (PDB code: 2V60). The ligands are displayed in tube format and are colored in accordance with the residues with which they interact (green for **6** and purple for 6-methyl-3-phenylcoumarin). Residues colored in yellow interact with both ligands (see text for discussion). Cys172 (carbon atom in green) and a water molecule (ball and stick) are also shown. The hydrogen bond is displayed as a yellow dotted line. FAD is shown in ball-and-stick format with carbon atoms in grey. Only hydrogen atoms involved in hydrogen bonding are displayed.

analyzed the most stable pose retrieved for compound **6** and compared it with the results obtained for the analogue 6-methyl-3-phenylcoumarin, previously reported by us[28] (Figure 1c). Interestingly, both structures share the phenyl ring facing the FAD cofactor, directed toward the aromatic cage formed by Tyr398 and Tyr435, and leave the methyl group occupying the same position in the binding pocket. In both structures the methyl group is involved in hydrophobic interactions with the residues Trp119, Leu164, Leu167, and Phe168, which form part of highly hydrophobic region of the entrance cavity in the enzyme. For compound **6** the carbonyl fragment is situated toward Cys172, establishing a hydrogen bond with a distance of 1.99 Å. Strong hydrophobic interactions were also observed with Tyr326. Regarding 6-methyl-3-phenylcoumarin, the first relevant contribution to complex energy stabilization is related to the hydrogen bond formed with a water molecule situated 2.08 Å from the oxygen atom of the carbonyl group. Indeed, the carbonyl group is placed in a different manner relative to **6**. The 3-phenyl moiety of 6-methyl-3-phenylcoumarin is also involved in favorable hydrophobic interactions with Leu171, Ile198, and Tyr398. This could partially explain why compound **6** shows better MAO-B inhibitory activity than the previously described 6-methyl-substituted analogue.[30]

Discussion

All the described coumarins (compounds **1**–**9**) were efficiently synthesized by an easy and versatile methodology (Scheme 1). They were also evaluated for their ability to inhibit the A and B isoforms of hMAO. The corresponding IC_{50} values and MAO-B selectivity ratios $[IC_{50(MAO\text{-}A)}]/[IC_{50(MAO\text{-}B)}]$ are listed in Table 1.

Position 8 of the 3-arylcoumarin moiety has never been described as a potential location for modulating the inhibitory activities of MAO-A and MAO-B. Analysis of the experimental data revealed that seven of the nine tested compounds are selective inhibitors of MAO-B, with IC_{50} values in the low micro- and nanomolar ranges. Three compounds of this series have similar or better IC_{50} values than selegiline (IC_{50} = 19.60 nM), the most potent MAO-B inhibitor used as reference. The most active compounds of this series are the coumarin derivatives **7** (IC_{50} = 3.43 nM) and **8** (IC_{50} = 4.51 nM), which are about sixfold more active than the reference compound. Compound **8** is

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also severalfold more selective than selegiline. The steric bulk of the substituent at position 8 seems to be important for modulating MAO-B inhibitory activity. All the described methyl derivatives showed better inhibition results than the corresponding methoxy or ethoxy derivatives. As shown in Figure 1a, those compounds exhibit a unique binding pattern, manifesting good interactions with key residues in the binding pocket. These results are in accordance with experimental data.

In comparing the 8-methyl derivatives **6**–**9**, it can be observed that the introduction of various substituents at the *para* position of the 3-phenyl ring is a good strategy for improving the desired MAO-B inhibitory activity. Moreover, with a methoxy, methyl, or bromo substituent at that position, the compounds are very selective for the MAO-B isoform (compounds **7**, **8**, and **9**, respectively). Compounds **6** and **7** are better MAO-B inhibitors than the previously reported analogues 6-methyl-3-phenylcoumarin ($IC_{50}=283$ nM) and 3-(4′-methoxyphenyl)-6-methylcoumarin ($IC_{50}=13$ nM), respectively.[28] These data indicate that substitution of the 3-phenylcoumarin at position 8 could be a good strategy for searching out better MAO-B inhibitors than those previously described.

The chemical structures of the newly designed compounds, along with the results of biological and docking studies, can help with a SAR study. The activity data for these compounds indicate that the varied nature of the substitutions at position 8 of the aromatic ring of the coumarin moiety would be more favorable for modulating the activity. Moreover, MAO-B inhibitory activity also increases if small lipophilic and electron-donating groups (methoxy or methyl) are included at position 4 of the 3-phenyl ring.

Conclusions

We used the Perkin reaction as a suitable method for the efficient and general synthesis of a series of 8-substituted 3-arylcoumarins. Most of the compounds studied show high affinity and selectivity for the MAO-B isoform. Docking studies performed for these compounds allowed us to establish and corroborate the nature of the interactions between the compounds and the MAO-B enzyme. Both the type and position of the substituents on the 3-aryl group and at position 8 of the coumarin moiety play a crucial role in both activity and selectivity. Further research into this interesting scaffold is currently ongoing.

Experimental Section

General methods

Starting materials and reagents were obtained from commercial suppliers and were used without purification (Sigma–Aldrich). Melting points (mp) are uncorrected and were determined with a Reichert Kofler thermopan or in capillary tubes in a Büchi 510 apparatus. ^{1}H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) spectra were recorded with a Bruker AMX spectrometer using [D_6]DMSO or $CDCl_3$ as solvent. Chemical shifts (δ) are expressed in ppm using TMS as an internal standard. Coupling constants (J) are expressed in Hz. Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets), and m (multiplet). Mass spectrometry was carried out with a Kratos MS-50 or a Varian MAT-711 spectrometer. Elemental analyses were performed with a PerkinElmer 240B microanalyzer and are within ± 0.4% of calculated values in all cases. The analytical results show ≥ 95% purity for all compounds. Flash chromatography (FC) was performed on silica gel (Merck 60, 230–400 mesh); analytical TLC was performed on pre-coated silica gel plates (Merck 60 F_{254}). Organic solutions were dried over anhydrous Na_2SO_4. Concentration and evaporation of the solvent after reaction or extraction was carried out on a rotary evaporator (Büchi Rotavapor) operating at reduced pressure.

Synthesis

General synthetic methodology for 3-phenylcoumarins 1–9: A solution of 3-ethoxy-2-hydroxybenzaldehyde/2-hydroxy-3-methoxybenzaldehyde/2-hydroxy-3-methylbenzaldehyde (7.4 mmol) and the corresponding phenylacetic acid (9.2 mmol) in DMSO (15 mL) was prepared. *N*,*N*′-Dicyclohexylcarbodiimide (11.5 mmol) was added, and the mixture was heated in an oil bath at 110 °C for 24 h. Triturate ice (100 mL) and acetic acid (10 mL) were added to the reaction mixture. After keeping it at room temperature for 2 h, the mixture was extracted with Et_2O (3×25 mL). The precipitated dicyclohexylurea was filtered off. The organic layer was washed with a solution of sodium bicarbonate (50.0 mL, 5%). The organic phase was stirred for 1 h with 5% aqueous sodium metabisulfite to remove the unreacted hydroxybenzaldehyde, then washed with H_2O (20 mL) and dried (Na_2SO_4). The solvent was evaporated under vacuum, and the dry residue was purified by FC (hexane/EtOAc 9:1).

8-Ethoxy-3-phenylcoumarin (1): Pale-yellow solid (700 mg, 55%): $R_f=0.33$ (hexane/EtOAc, 8:2); mp: 117–118 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=1.50$ (t, $J=7.0$ Hz, 3H, CH_3), 4.21 (dd, $J=14.0$, 7.0 Hz, 2H, CH_2), 7.09 (t, $J=6.9$ Hz, 2H, H-6, H-7), 7.21 (t, $J=7.8$ Hz, 1H, H-5), 7.42–7.48 (m, 3H, H-3′, H-4′, H-5′), 7.72 (dd, $J=7.7$, 1.4 Hz, 2H, H-2′, H-6′), 7.79 ppm (s, 1H, H-4); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=$ 14.8, 65.0, 114.5, 119.3, 120.4, 124.3, 128.3, 128.4, 128.5, 128.8, 134.8, 140.1, 143.4, 146.3, 160.2 ppm; IR (KBr): $\tilde{\nu}=2850$, 1718, 1699, 1468 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 267 (22) $[M+H]^+$, 266 (100) $[M^+]$; HRMS-FAB: *m/z* $[M+H]^+$ calcd for $C_{17}H_{14}O_3$: 267.1021, found: 267.1017; Anal. calcd for $C_{17}H_{14}O_3$: C 76.68, H 5.30, found: C 76.66, H 5.28.

8-Ethoxy-3-(4′-methoxyphenyl)coumarin (2): White solid (870 mg, 61%): $R_f=0.21$ (hexane/EtOAc, 8:2); mp: 99–100 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=1.50$ (t, $J=7.0$ Hz, 3H, CH_3), 3.84 (s, 3H, OCH_3), 4.19 (dd, $J=14.0$, 7.0 Hz, 2H, CH_2), 6.84–7.26 (m, 5H, H-3′, H-5′, H-5, H-6, H-7), 7.69 (t, $J=7.7$ Hz, 2H, H-2′, H-6′), 7.73 ppm (s, 1H, H-4); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=$14.8, 55.4, 64.8, 113.8, 114.0, 119.1, 120.49, 124.2, 127.1, 127.8, 129.7, 129.8, 138.6, 138.7, 146.2, 160.0 ppm; IR (KBr): $\tilde{\nu}=2927$, 2841, 1716, 1460 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 297 (18) $[M+H]^+$, 296 (100) $[M^+]$; HRMS-FAB: *m/z* $[M+H]^+$ calcd for $C_{18}H_{16}O_4$: 297.1127, found: 297.1117; Anal. calcd for $C_{18}H_{16}O_4$: C 72.96, H 5.44, found: C 72.91, H 5.39.

8-Ethoxy-3-(4′-methylphenyl)coumarin (3): White solid (650 mg, 48%): $R_f=0.30$ (hexane/EtOAc, 8:2); mp: 109–110 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=1.51$ (td, $J=7.0$, 1.8 Hz, 3H, CH_3), 2.39 (s, 3H, CH_3), 4.20 (dd, $J=7.0$, 1.8 Hz, 2H, CH_2), 706–7.10 (m, 2H, H-5, H-6), 7.18 (dd, $J=8.0$, 1.9 Hz, 1H, H-7), 7.26 (d, $J=1.6$ Hz, 2H, H-3′, H-5′), 7.61 (dd, $J=8.1$, 1.8 Hz, 2H, H-2′, H-6′), 7.74 ppm (s, 1H, H-4); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=14.8$, 21.3, 65.0, 114.4, 119.2, 120.5, 124.3, 128.3, 128.4, 129.2, 131.9, 138.8, 139.4, 143.3, 146.3,

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160.3 ppm; IR (KBr): $\tilde{\nu}$ = 2857, 1707, 1663, 1470 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 281 (27) $[M+H]^+$, 280 (100) $[M^+]$; HRMS-FAB: *m/z* $[M+H]^+$ calcd for $C_{18}H_{16}O_3$: 281.1178, found: 281.1167; Anal. calcd for $C_{18}H_{16}O_3$: C 77.12, H 5.75, found: C 77.07, H 5.80.

3-(4′-Bromophenyl)-8-methoxycoumarin (5): Pale-yellow solid (880 mg, 62%): R_f = 0.24 (hexane/EtOAc, 8:2); mp: 169–170 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.92 (s, 3H, -OCH_3), 7.07–7.12 (m, 3H, H-5, H-6, H-7), 7.15 (dd, *J* = 7.8, 1.5 Hz, 2H, H-2′, H-6′), 7.58 (dd, *J* = 7.8, 1.5 Hz, 2H, H-3′, H-5′), 7.80 ppm (s, 1H, H-4); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 56.0, 113.4, 119.3, 120.1, 123.2, 124.5, 127.4, 130.1, 131.6, 133.5, 140.0, 141.8, 147.3, 161.7 ppm; IR (KBr): $\tilde{\nu}$ = 2850, 1701, 1660, 1068 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 331 (22) $[M+H]^+$, 330 (100) $[M^+]$; HRMS-FAB: *m/z* $[M+H]^+$ calcd for $C_{16}H_{11}BrO_3$: 330.9970, found: 330.9969; Anal. calcd for $C_{16}H_{11}BrO_3$: C 55.04, H 3.75, found: C 55.05, H 3.77.

3-(4′-Methoxyphenyl)-8-methylcoumarin (7): Pale-yellow solid (610 mg, 66%): R_f = 0.33 (hexane/EtOAc, 8:2); mp: 100–101 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.50 (s, 3H, CH_3), 3.86 (s, 3H, OCH_3), 6.98 (dd, *J* = 8.9, 1.2 Hz, 2H, H-3′, H-5′), 7.18–7.20 (m, 1H, H-7), 7.37 (d, *J* = 7.7 Hz, 2H, H-5, H-6), 7.66–7.76 ppm (m, 3H, H-2′, H-6′, H-4); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 15.5, 55.4, 113.9, 119.5, 124.0, 125.4, 125.8, 127.2, 127.4, 129.8, 132.3, 139.0, 151.6, 160.0, 160.9 ppm; IR (KBr): $\tilde{\nu}$ = 2837, 1708, 1462 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 267 (31) $[M+H]^+$, 266 (100) $[M^+]$; HRMS-FAB: *m/z* $[M+H]^+$ calcd for $C_{17}H_{14}O_3$: 267.1021, found: 267.1013; Anal. calcd for $C_{17}H_{14}O_3$: C 76.68, H 5.30, found: C 76.58, H 5.40.

3-(4′-Methylphenyl)-8-methylcoumarin (8): White solid (580 mg, 67%): R_f = 0.42 (hexane/EtOAc, 8:2); mp: 111–112 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.40 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 7.16 (d, *J* = 7.5 Hz, 1H, H-7), 7.23–7.26 (m, 2H, H-5, H-6), 7.37 (d, *J* = 7.5 Hz, 2H, H-3′, H-5′), 7.62 (d, *J* = 8.1 Hz, 2H, H-2′, H-6′), 7.77 ppm (s, 1H, H-4); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 15.4, 21.2, 119.4, 124.0, 125.5, 125.8, 128.4, 129.1, 131.9, 132.5, 138.8, 139.5, 139.7, 151.8, 160.8 ppm; IR (KBr): $\tilde{\nu}$ = 1697, 1460 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 251 (27) $[M+H]^+$, 250 (98) $[M^+]$; HRMS-FAB: *m/z* $[M+H]^+$ calcd for $C_{17}H_{14}O_2$: 251.1072, found: 251.1062; Anal. calcd for $C_{17}H_{14}O_2$: C 81.58, H 5.64, found: C 81.61, H 5.72.

3-(4′-Bromophenyl)-8-methylcoumarin (9): White solid (1.2 g, 65%): R_f = 0.42 (hexane/EtOAc, 8:2); mp: 193–194 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.51 (s, 3H, CH_3), 7.22 (t, *J* = 7.7 Hz, 1H, H-7), 7.38–7.42 (m, 2H, H-5, H-6), 7.57–7.60 (m, 4H, H-2′, H-3′, H-5′, H-6′), 7.82 ppm (s, 1H, H-4); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 15.4, 119.2, 123.0, 124.2, 125.7, 126.0, 126.7, 130.1, 131.6, 133.0, 133.7, 140.4, 151.9, 160.4 ppm; IR (KBr): $\tilde{\nu}$ = 1706, 1462, 1065 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 315 (25) $[M+H]^+$, 314 (95) $[M^+]$. HRMS-FAB: *m/z* $[M+H]^+$ calcd for $C_{16}H_{11}BrO_2$: 315.0021, found: 315.0024; Anal. calcd for $C_{16}H_{11}BrO_2$: C 60.98, H 3.52, found: C 60.90, H 3.43.

Determination of MAO isoform activity

The effects of the new synthesized compounds on hMAO isoform activity were evaluated with a fluorimetric method previously described by us.[38] Briefly, sodium phosphate buffer (0.1 mL, 0.05 M, pH 7.4) containing the test drugs (new compounds or reference inhibitors) at various concentrations and adequate amounts of recombinant hMAO-A or hMAO-B required and adjusted to obtain the same reaction velocity under our experimental conditions [hMAO-A: 1.1 mg protein, specific activity: 150 (nmol *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde) min^{-1} (mg protein)$^{-1}$; hMAO-B: 7.5 mg protein, specific activity: 22 (nmol *p*-tyramine transformed) min^{-1} mg protein)$^{-1}$] were incubated for 15 min at 37 °C in a flat- and black-bottomed 96-well microplate, placed in the dark fluorimeter chamber. After this incubation period, the reaction was started by adding (final concentrations) 200 μM Amplex Red reagent, 1 U mL^{-1} horseradish peroxidase and 1 mM *p*-tyramine. The production of H_2O_2 and, consequently, of resorufin was quantified at 37 °C in a multidetection microplate fluorescence reader (FLX800, Bio-Tek Instruments Inc., Winooski, VT, USA) based on the fluorescence generated ($\lambda_{excitation}$: 545 nm, $\lambda_{emission}$: 590 nm) over a 15 min period, in which the fluorescence increased linearly. Control experiments were carried out simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions of the vehicles. In addition, the potential capacity of the above test drugs to modify the fluorescence generated in the reaction mixture through non-enzymatic inhibition (e.g., reacting directly with the Amplex Red reagent) was determined by adding these drugs to solutions containing only the Amplex Red reagent in a sodium phosphate buffer. The specific fluorescence emission (used to obtain the final results) was calculated after subtraction of the background activity, which was determined from vials containing all components except the hMAO isoforms, which were replaced by a sodium phosphate buffer solution.

Reversibility and irreversibility experiments

To evaluate whether compound **8** and reference inhibitors are reversible or irreversible hMAO-B inhibitors, an effective centrifugation–ultrafiltration method (so-called repeated washing) was used.[55]

Molecular docking

The Schrödinger 2011 package was employed for all computer calculations in this work.

Protein and ligand preparation

The Protein Preparation Wizard[56] workflow was used to prepare the hMAO-B crystal structure (PDB code: 2V60) as the target in docking simulations. Water molecules beyond 5 Å from the ligand were deleted, and hydrogen atoms were added and subsequently minimized using the OPLS_2005 force field. The refinement stage of the protein reorients the hydroxy groups, amide groups of asparagine and glutamine residues, and optimizes the protonation state of histidines. Furthermore the orientation of water molecules was varied in order to optimize hydrogen bonding.
Coumarin derivatives were prepared according to the LigPrep tool of Maestro 9.1.[57] Different ionization states at pH 7.0 ± 2.0 and tautomers were generated. The MMFFs force field was used for the minimization of structures.

QM-polarized ligand docking procedure

The grid box was centered in the co-crystallized ligand c17 with a length of 20 Å and a receptor van der Waals scaling of 1.0. The initial docking was performed with Glide at Standard Precision (SP) level, generating the initial charges on the ligands with semiempirical method; eight poses per ligand were retained. Ab initio density functional theory (DFT) was used for the full quantum mechanical (QM) treatment of the ligand. Ligand charges were calculated by Jaguar[58] using the 6-31G*/LACVP* basis set, B3LYP density functional, and Ultrafine SCF accuracy level. Finally, Glide with extra

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precision (XP) mode was used to re-dock the ligands with improved QM charges, returning the best four poses for each ligand.

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Keywords: coumarins · heterocycles · molecular modeling · monoamine oxidase · perkin reaction

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Focusing on New Monoamine Oxidase Inhibitors: Differently Substituted Coumarins As An Interesting Scaffold

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Abstract: It is commonly accepted that monoamine oxidase (MAO) may play a critical role in the regulation of the central nervous system activity and contribute to the pathogenesis of human neurodegenerative and depressive disorders. This has encouraged an active research in the development of new drugs, MAO inhibitors, since they may represent an important advance in the treatment of complex diseases such as Alzheimer and Parkinson, which are becoming prevalent pathologies due to the increase of aging population of developed countries societies. Different research groups are intensively working in this area with the aim of finding new MAO selective inhibitors. Differently substituted coumarins have been synthesized and evaluated as MAO-A and MAO-B inhibitors. The purpose of this review is to summarize the findings reported in this area, particularly focuses on the coumarin scaffold.

Keywords: Coumarins, Alzheimer disease, parkinson disease, monoamine oxidase, MAO inhibitors.

1. INTRODUCTION

Monoamine oxidase (MAO) is a FAD-depending enzyme found in the outer mitochondrial membrane of neuronal, glial and other mammalian cells [1]. This enzyme is responsible for catalyzing the oxidative deamination of neurotransmitters and dietary amines, regulating intracellular levels of biogenic amines in the brain and the peripheral tissues [2, 3]. It is well-known that MAO plays a critical role in the regulation of central nervous system activity and contributes to the pathogenesis of human neurodegenerative and depressive disorders [4]. Fifty years ago the first generation of MAO inhibitors (MAOIs) was developed and applied in therapy as anti-depressive compounds. However, for many years MAOIs were considered useless in therapy due to the serious side effects induced by these drugs [5]. Later, two enzymatic isoforms, named MAO-A and MAO-B, have been identified on the basis of their amino acid sequences [6], three-dimensional structure [7], tissue distribution [8], inhibitor selectivity [9] and substrate preferences [10]. The MAO-A isoform has higher affinity for serotonin and noradrenaline, whereas the MAO-B isoform, preferentially deaminates β-phenylethylamines and benzylamine [11, 12]. On the other hand, dopamine and tyramine are common substrates for both isoforms. As a result of these physiological properties, MAOIs became again the center of scientific and pharmacological interest, providing new drugs for the therapy of Parkinson´s diseases (PD), Alzheimer´s disease (AD) and various types of depression [13]. Additionally, the description of the crystal structure of the two MAO isoforms by C. Binda *et al.* has provided relevant information about the selective interactions and the pharmacophoric requirements needed for the design of potent and selective inhibitors [14-18].

MAO-A plays an important role in neuropsychiatric and behavioural disorders. The importance of this isoform is suggested by the aggressive phenotype seen in male mice deficient in MAO-A and in males in a Dutch kindred bearing a spontaneous mutation (resulting in a premature stop) in the *mao*-A gene [19]. Similarly, maltreated children, whose genotype confers low levels of MAO-A expression, more often develop conduct disorder, antisocial personality and adult violent crime than children with a high-activity MAO-A genotype do [20]. Relatively modest changes in MAO-A activity/function can have important neuropsychiatric consequences as demonstrated by the fact that [^{11}C]-harmine-labeled is elevated by only 34% throughout the brain of untreated depressed patients compared to controls, yet it appears to be the major contributor to the monoamine metabolism in these same patients [21]. Depression not only may promote cognitive impairment, but also may be a risk factor for AD [22]. Not surprisingly, MAO-A which is often a target for the treatment of depression, is also a potential risk factor for late-onset AD [23-26]. In contrast to irreversible MAOI-A as clorgyline, reversible inhibitors are better tolerated and have been particularly efficacious in treating depression [27] and cognitive disorders [28] in the elderly. In addition, inhibition of MAO-A activity protect against striatal damage produced by the mitochondrial poison malonate and appears to rely on attenuation of dopamine-derived reactive oxygen species (ROS) [29], while apoptosis following serum starvation is reduced in MAO-A deficient cortical brain cells [30].

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This manuscript is dedicated to Prof Francisco Orallo in memoriam, who had introduced and incentivated us in the study of the MAOIs.

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On the other hand, it has been demonstrated that selective and irreversible MAOIs-B, such as selegiline and rasagiline, are important molecules in the therapy of PD as well as in patients with mild AD-type dementia [31-33]. Both MAO-B positive astrocytes [34] and ROS [35] have been found in the vicinity of β-amyloid (Aβ) plaques. Selegiline and rasagiline can also exert neuroprotection at lower concentrations than those required for inhibition of the enzyme [36]. It has been associated with activation of Bcl-2 family members, interaction with the mitochondrial pore complex and modulation of amyloid precursor protein cleavage [37].

The importance of the discovery of drugs associated with neurodegenerative diseases (ND) has emerged with the significant increase of life expectancy.

Coumarins are a wide family of compounds present in remarkable amounts in the nature [38, 39]. Representative compounds occur for instance in the vegetable kingdom, either in free or combined state [40, 41]. The versatile and efficient synthetic accessibility and the substitution capability make them privileged scaffolds not only in organic chemistry but also in the medicinal chemistry field [42]. In the literature, coumarin derivatives have been described as anticancer [43, 44], antioxidant or anti-inflammatory [45, 46], vasorelaxant [47], antimicrobial [48, 49], antiviral [50, 51], and enzymatic inhibitors [52-54].

In 1981, it was found for the first time an interesting MAOI activity of the coumarin moiety, when an organophosphate group was attached to the umbelliferone ring [55]. But it was only in the 1990´s when the coumarin nucleus emerged as a promising scaffold for MAOIs [56]. In particular, some coumarins isolated from *Psoralea corylifolia* L., *Peucedanum japonicum* L. or *Monascus anka* K. have been described as MAOIs [57-59]. Although natural coumarins generally show low MAO inhibitory potency, properly modified natural coumarins have been characterized as potent and selective MAOIs. For example, the desmethyl congener of geiparvarin, a natural 7-substitued coumarin from the leaves of *Geijera parviflora* L., exhibites potent and selective MAO-B inhibition Fig. (**1**) [60].

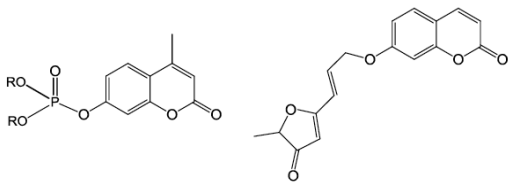


Fig. (1). Umbelliferone and geiparvarin derivatives.

Several research groups have been exploring the importance of the number and position of different substituents on the coumarin nucleus, taking into account volume, lipophilicity, steric impediment, electron withdrawing and electron donating characteristics. Different substituents were attached to the coumarin skeleton with the aim of improving both activity and selectivity Fig. (**2**). Although positions 3 and 7 of the coumarin ring were the most studied, all the substitution possibilities were taken into account and a big number of coumarin derivatives have been synthesized and evaluated as MAOIs, being most of them active and selective against MAO-B isoenzyme [61-81].

It is important to mention that the IC_{50} values given in the following tables resulted from different experimental settings.

2. COUMARIN DERIVATIVES AND MAO ACTIVITY

The first structure-activity relationship studies (SAR) about coumarin derivatives suggested that selectivity was mainly determined by the nature of the linkage between the coumarin and the substituent group at position 7. A series of 7-hydroxycoumarin derivatives **I** bearing at position 7 ether, ester or carbamate functions of varying size and lipophilicity have been investigated for their *in vitro* MAO-A and -B inhibitory activities. Most of these compounds resulted to be preferentially MAOIs-B. SAR studies also showed that lipophilicity is an important property to modulate the MAO-B inhibition potency of the above mentioned coumarins [61-68]. A 7-benzyloxy group proved to be the favouring substituent for the binding of coumarins to MAO-B, whereas a π-electron-poor phenyl ring at position 7 seems to improve both MAO-A and MAO-B inhibitiory activities, leading to more active but slightly less selective inhibitors [62]. A. Carotti *et al.* described a large family of compounds related to these structures. On the other hand, it has been proved that an alkylsulfonyloxy group at position 7 provided MAOIs-A like esuprone. This effect is even more pronounced in the phenyl sulfonates in which the substitution with electron withdrawing groups lead to more potent and highly selective MAOIs-A. Also, B. Rendenbach-Mueller *et al.* synthesized and studied the potential of ether derivatives and sulfonic acid esters Fig. (**3**), checking that introduction of a sulfonic ester linkage, instead of the ether bridge, dramatically changed the activity profile of the new compounds [63].

Substitutions at position 3 and/or 4 of the coumarin nucleus also contribute to modulate MAO-B inhibitory activity and A/B selectivity of the previously described derivatives. It can be inferred that the MAO-B binding site should accept lipophilic substituents of limited size at positions 3 and/or 4 of the coumarin nucleus, whereas hydrophilic or larger hydrophobic groups are not well tolerated [62]. However, derivatives of 7-benzyloxycoumarins bearing properly selected polar substituents at position 4 led to new MAOIs-B with an improved pharmacokinetic profile and a higher druggability [64].

B. Rendenbach-Müller *et al.* also explored the importance of the presence of a heteroarylalkoxy group at position 7 of the coumarin scaffold to modulate the activity against MAO isoforms [56]. In this study, small alkyl, phenyl or halogen groups were introduced at positions 3 and 4 of the skeleton. The same authors evaluated and described heteroarylalkoxycoumarins as potent and selective MAOIs-B [63, 65, 68]. Only small substituents, as methyl groups, were introduced at positions 3 and 4 of this coumarin nucleus.

Based on previously described SAR studies, introduction of cycloaliphatic or benzene rings fused at 3,4-positions was performed on 7-hydroxycoumarin derivatives. A. Carotti *et al.* and B. Rendenbach-Mueller *et al.* described series of these derivatives with interesting MAO-B properties [62, 65,

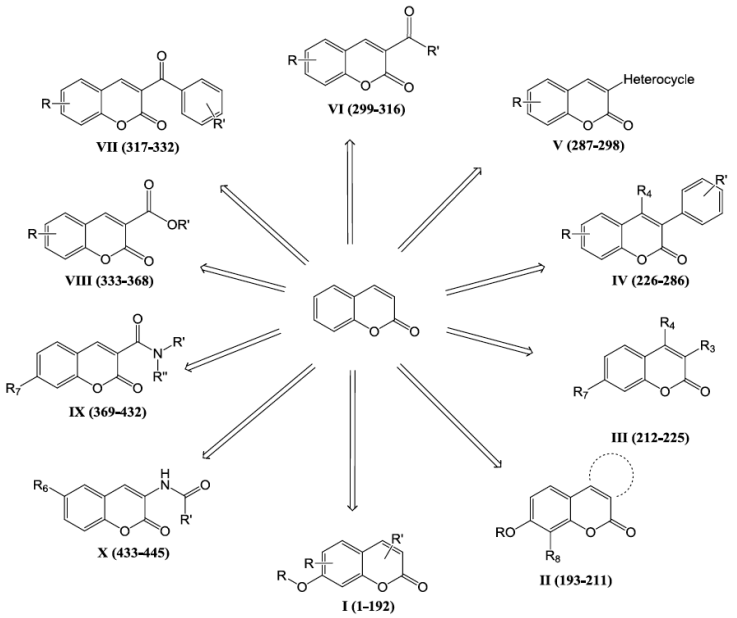


Fig. (2). Chemical structures of known coumarins as MAOIs.

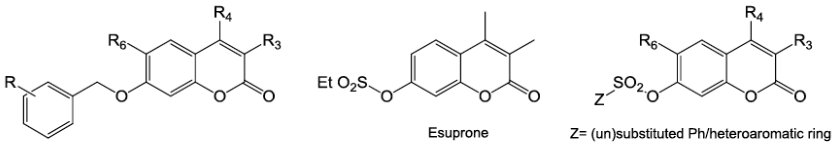


Fig. (3). 7-Alkyloxy and sulfonyloxy coumarin derivatives.

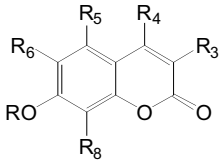
66]. L. Santana *et al.* have reported that annelation with 5- and 6-membered rings on the coumarin nucleus, in addition to 7-acetonyloxy-substitutents, showed higher activity against both MAO isoforms than the 7-hydroxy precursors [66]. These derivatives presented different MAO-A/MAO-B selectivity profiles, as shown in the (Table **2**).

In order to complete the study of 7-substituted coumarin derivatives, A. Carotti *et al.* synthesized and evaluated the *in vitro* biological MAO activity of novel series of coumarins bearing different substituents at position 7. Such substituents consisted of a phenyl ring linked to a coumarin core by bridges of different size, length and lipophilic and electronic nature. In general, isosteric variations of the oxymethylene linker lead to inhibitors with lower MAO-B affinity and B/A selectivity [62, 67].

The reported results for 7-substituted coumarins containing a benzene ring fused at 3,4 positions encouraged M. J. Matos *et al.* to analyze the importance of the aryl substituent on the pyrone ring, synthesizing a series of 3-arylcoumarin derivatives **IV**. The substitution at C7 was removed and small substituents were introduced either at C6 [69-74] or/and C8 [75, 76] positions. The high MAO-B selectivity found for this new 3-arylcoumarin scaffold encouraged the authors to synthesize and study the MAO inhibitory activity of new analogues. A variety of groups with different size, electronic and lipophilic properties were introduced in both aromatic rings, in order to clarify the influence of the substitution pattern in the MAO inhibitory activity and selectivity of the 3-arylcoumarin skeleton [69-76]. Most active compounds present *para* or *meta* substitutions in the 3-phenyl ring. The best compound of the described series is the 6-methyl-3-(4'-methylphenyl)coumarin (compound **245**), with an IC_{50} of 0.31 nM. This compound proved to be not only 64 times more active *in vitro* than selegiline (the reference compound) but also several times more selective than it (compound **245** is > 97 fold more selective than selegiline) [72].

Additionally, a hydroxyl group at position 4 of the coumarin skeleton was introduced. This modification allowed to consider the similarity of the core structure of the 4-hydroxycoumarin comparing with the 2-hydroxyisoflavone. Taking into account the obtained results, it could be estab-

Table 1. Chemical Structures and MAO Inhibition Data of 7-hydroxycoumarin Derivatives I (Compounds 1-192)



Cpds	R	R_3	R_4	R_5	R_6	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
1	H	H	CH_3	H	H	OCH_3	80.30	> 100	66
2	H	H	CH_3	H	H	CH_3	> 100	71.32	66
3	H	H	CH_3	H	H	H	> 100	80.41	66
4	H	NH_2	H	H	H	OCH_3	> 100	> 100	66
5		CH_3	CH_3	H	H	H	11.0	0.004	61
6		CH_3	CH_3	H	H	H	9.20	0.94	61
7		CH_3	CH_3	H	H	H	5.90	0.11	61
8		CH_3	CH_3	H	H	H	7.70	0.014	61
9		CH_3	CH_3	H	H	H	4.10	0.004	61
10		CH_3	CH_3	H	H	H	20 (15%)	0.028	61
11		CH_3	CH_3	H	H	H	10 (0%)	10 (27%)	61
12		CH_3	CH_3	H	H	H	34.0	0.89	61
13		CH_3	CH_3	H	H	H	0.70	0.84	61
14		CH_3	CH_3	H	H	H	1.13	0.14	61
15		H	H	H	H	H	> 100	0.044	66
16		H	CH_3	H	H	H	0.041	0.006	66
17		H	CH_3	H	H	OCH_3	2.66	0.86	66
18		H	CH_3	H	H	CH_3	0.007	0.011	66
19		H	CH_3	H	H	H	43.0	> 100	66
20		H	CH_3	H	H	OCH_3	15.45	> 100	66
21		H	CH_3	H	H	CH_3	0.40	0.004	66
22	CH_3	CH_3	CH_3	H	H	H	0.071	0.0005	63

(Table 1) contd….

Cpds	R	R_3	R_4	R_5	R_6	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
23		CH_3	CH_3	H	H	H	31.60	3.24	67
24		Cl	CH_3	H	H	H	> 10	0.004	56
25		H	H	H	H	H	6.76	0.055	62
26		CH_3	H	H	H	H	5.50	0.007	67
27		H	CH_3	H	H	H	1.95	0.018	62
28		CH_3	CH_3	H	H	H	0.69	0.004	62
29		H	OH	H	H	H	15 (26%)	1.58	62
30		H	CF_3	H	H	H	> 5	1.38	62
31			CH_3	H	H	H	3 (4%)	3 (8%)	62
32		H		H	H	H	> 4	> 4	62
33		CH_3	CH_3	H	H	CH_3	0.56	3.31	62
34		CH_3	CH_3	H		H	> 0.4	> 0.4	62
35		CH_3	CH_3	H	OH	H	112.20	3.09	62
36		CH_3	CH_3	H	H	H	3.55	0.32	62
37		CH_3	CH_3	H	H	H	> 0.54	0.001	56
38		CH_3	CH_3	H	H	H	0.39	0.001	56
39		CH_3	CH_3	H	H	H	0.56	0.001	56
40		CH_3	CH_3	H	H	H	> 10	0.004	56
41		H	H	H	H	H	> 10	0.002	56
42		H	CH_3	H	H	H	> 10	0.005	56
43		CH_3	H	H	H	H	> 10	0.003	56
44		CH_3	CH_3	H	H	H	> 10	0.007	56
45		CH_3	CH_3	H	Cl	H	> 10	0.031	56
46		CH_3	CH_3	H	H	CH_3	> 100	> 100	63
47		CH_3	CH_3	H	H	H	> 10	10.0	63
48		CH_3	CH_3	H	H	H	> 10	0.032	56
49		CH_3	CH_3	H	H	H	0.30	0.001	56
50		CH_3	CH_3	H	H	H	> 10	0.002	56

(Table 1) contd….

Cpds	R	R_3	R_4	R_5	R_6	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
51		CH_3	CH_3	H	H	H	1.51	0.004	62
52		CH_3	CH_3	H	H	H	2.10	0.0006	56
53		CH_3	CH_3	H	H	H	5.89	0.011	62
54		CH_3	CH_3	H	H	H	0.58	0.003	62
55		CH_3	CH_3	H	H	H	0.12	0.003	62
56		CH_3	CH_3	H	H	H	0.12	0.001	62
57		CH_3	CH_3	H	H	H	0.68	0.003	62
58		CH_3	CH_3	H	H	H	0.11	0.007	62
59		CH_3	CH_3	H	H	H	0.69	0.006	62
60		CH_3	H	H	H	H	12.59	0.005	67
61		CH_3	CH_3	H	H	H	1.12	0.003	62
62		CH_3	CH_3	H	H	H	0.12	0.003	62
63		H	CH_3	H	H	H	0.13	0.013	62
64		CH_3	CH_3	H	H	H	0.076	0.003	62
65		CH_3	CH_3	H	H	H	0.83	0.009	62
66		CH_3	CH_3	H	H	H	0.18	0.006	62
67		CH_3	CH_3	H	H	H	1.0	0.035	62
68		CH_3	CH_3	H	H	H	7.08	0.25	62
69		CH_3	CH_3	H	H	H	0.42	0.010	62
70		CH_3	CH_3	H	H	H	0.42	0.023	62
71		CH_3	CH_3	H	H	H	0.22	0.011	62
72		CH_3	CH_3	H	H	H	0.10	0.004	62
73		CH_3	CH_3	H	H	H	1.0	0.006	62
74		CH_3	CH_3	H	CH_2CH_3	H	> 10	0.010	56
75		CH_3	CH_3	H	H	H	10.0	0.071	67

(Table 1) contd….

Cpds	R	R_3	R_4	R_5	R_6	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
76		CH_3	CH_3	H	H	H	9.77	0.018	67
77		H	H	H	H	H	1.50	0.004	56
78		CH_3	CH_3	H	H	H	1.20	0.004	56
79		CH_3	CH_3	H	H	H	10.23	0.027	67
80		CH_3	CH_3	H	H	H	31.62	0.11	67
81		H	H	H	H	H	100.0	0.058	67
82		CH_3	CH_3	H	H	H	1.80	0.009	63
83		CH_3	CH_3	H	Cl	H	> 10	0.0007	65
84		CH_3	CH_3	H	H	H	> 10	0.011	65
85		CH_3	CH_3	H	H	H	10.0	0.008	65
86		CH_3	CH_3	H	H	H	> 10	0.002	65
87		CH_3	CH_3	H	H	H	110.0	> 100	63
88		CH_3	CH_3	H	H	H	0.008	5.0	63
89		CH_3	CH_3	H	H	H	0.016	2.20	63
90		CH_3	CH_3	H	H	H	0.034	> 10	63
91		H	H	H	H	H	0.45	54.95	67
92		CH_3	CH_3	H	H	H	0.024	1.10	63
93		CH_3	CH_3	H	H	H	0.047	15 (17%)	62
94		CH_3	CH_3	H	H	H	0.071	16.98	62
95		CH_3	CH_3	H	H	H	0.005	0.004	63
96		CH_3	CH_3	H	H	H	0.008	> 100	63
97		CH_3	CH_3	H	H	H	0.006	> 100	63
98		CH_3	CH_3	H	H	H	0.013	2 (20%)	62
99		CH_3	CH_3	H	H	H	2.0	100.0	67
100		CH_3	CH_3	H	H	H	2.82	0.87	67
101		CH_3	CH_3	H	H	H	> 10	0.017	65
102		CH_3	CH_3	H	H	H	0.35	0.0005	65
103		CH_3	CH_3	H	H	H	0.23	0.0005	65
104		CH_3	CH_3	H	H	H	> 10	0.001	65

(Table 1) contd….

Cpds	R	R_3	R_4	R_5	R_6	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
105		CH_3	CH_3	H	H	H	10.0	0.003	65
106		CH_3	CH_3	H	H	H	3.20	0.006	65
107		CH_3	CH_3	H	H	H	10.0	0.001	68
108		CH_3	CH_3	H	H	H	> 10	0.003	65
109		Cl	CH_3	H	Cl	H	> 10	0.002	65
110		CH_3	CH_3	H	H	H	10.0	0.001	65
111		Cl	CH_3	H	Cl	H	> 10	0.002	65
112		CH_3	CH_3	H	H	H	1.50	0.002	65
113		CH_3	CH_3	H	H	H	10.0	0.002	65
114		CH_3	CH_3	H	H	H	10.0	0.005	65
115		CH_3	CH_3	H	H	H	6.0	0.0007	65
116		CH_3	CH_3	H	H	H	2.90	0.001	65
117		CH_3	CH_3	H	H	H	10.0	0.002	65
118		CH_3	CH_3	H	H	H	> 10	0.001	65
119		CH_3	CH_3	H	H	H	> 10	0.001	65
120		CH_3	CH_3	H	H	H	7.0	0.001	65
121		CH_3	CH_3	H	H	H	> 10	0.001	65
122		CH_3	CH_3	H	H	H	> 10	0.001	65
123		CH_3	CH_3	H	H	H	> 10	0.014	65
124		CH_3	CH_3	H	H	H	20.0	0.0006	65
125		CH_3	CH_3	H	H	H	5.0	0.004	65
126		CH_3	CH_3	H	H	H	10.0	0.005	65
127		CH_3	CH_3	H	H	H	> 10	0.002	65

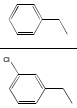
(Table 1) contd….

Cpds	R	R_3	R_4	R_5	R_6	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
128		Cl	CH_3	H	H	H	> 10	0.002	65
129		CH_3	CH_3	H	CH_2CH_3	H	> 10	0.009	65
130		CH_3	CH_3	H	H	H	> 10	0.010	65
131		CH_3	CH_3	H	H	H	5.60	0.015	65
132		CH_3	CH_3	H	H	H	10.0	0.001	65
133		CH_3	CH_3	H	H	H	> 10	0.008	65
134		CH_3	CH_3	H	H	H	4.0	0.004	65
135		CH_3	CH_3	H	H	H	> 10	0.001	65
136		CH_3	CH_3	H	H	H	7.0	0.001	65
137		CH_3	CH_3	H	H	H	> 10	0.013	65
138		CH_3	CH_3	H	H	H	> 10	0.001	68
139		CH_3	CH_3	H	Cl	H	> 10	0.003	68
140		CH_3	CH_3	H	H	H	3.60	0.003	65
141		CH_3	CH_3	H	CH_2CH_3	H	> 10	0.007	65
142		CH_3	H	H	H	H	> 10	0.015	65
143		Cl	CH_3	H	H	H	> 10	0.006	65
144		CH_3	CH_3	H	Br	H	> 10	0.001	65
145		CH_3	CH_3	H	H	H	> 10	0.002	68
146		H		H	H	H	1.44	0.040	64
147		H		H	H	H	2.50	0.090	64
148		H		H	H	H	> 100	> 10	64

(Table 1) contd….

Cpds	R	R_3	R_4	R_5	R_6	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
149		H		H	H	H	8.0	0.20	64
150		H		H	H	H	26.0	0.030	64
151		H		H	H	H	70.0	0.050	64
152		H		H	H	H	4.27	1.0	64
153		H		H	H	H	2.80	0.015	64
154		H		H	H	H	0.50	0.024	64
155		H		H	H	H	> 100	> 10	64
156		H		H	H	H	> 100	5.0	64
157		H		H	H	H	23.10	0.40	64
158		H		H	H	H	> 100	0.040	64
159		H		H	H	H	> 100	> 10	64
160		H		H	H	H	21.0	1.30	64
161		H		H	H	H	26.50	2.0	64
162		H		H	H	H	6.26	0.10	64
163		H		H	H	H	2.0	0.10	64
164		H		H	H	H	15.40	0.028	64
165		H		H	H	H	5.94	0.013	64
166		H		H	H	H	13.50	0.018	64

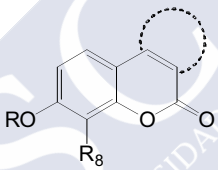
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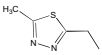
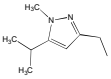
Cpds	R	R_3	R_4	R_5	R_6	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
167		H		H	H	H	5.40	0.020	64
168		H		H	H	H	22.60	0.10	64
169		H		H	H	H	91.0	3.80	64
170		H		H	H	H	> 100	3.60	64
171		H		H	H	H	100.0	1.80	64
172		H		H	H	H	50.80	1.12	64
173		H		H	H	H	> 100	0.51	64
174		H		H	H	H	> 100	8.0	64
175		H		H	H	H	4.40	0.021	64
176		H		H	H	H	2.0	0.015	64
177		H		H	H	H	0.20	0.23	64
178		H		H	H	H	0.47	0.016	64
179		H		H	H	H	100.0	0.68	64
180		H		H	H	H	10 (35%)	0.21	64
181		H		H	H	H	99.80	16.10	64
182		H		H	H	H	10 (5%)	9.60	64
183		H		H	H	H	> 100	2.40	64
184		H		H	H	H	22.70	1.80	64
185		H		H	H	H	35.0	0.38	64
186		H		H	H	H	> 100	0.85	64

(Table 1) contd….

Cpds	R	R_3	R_4	R_5	R_6	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
187		H		H	H	H	31.80	0.25	64
188		H		H	H	H	74.0	0.30	64
189		H		H	H	H	25.90	0.46	64
190	H	H	H	H		H	23.44	2.04	62
191	H	CH_3	CH_3	H		H	112.20	3.09	62
192	H	CH_3	CH_3		H	H	30 (35%)	30 (18%)	62

Table 2. Chemical Structures and MAO Inhibition Data of Substituted Coumarins with 3,4-fused Cycloaliphatic or Benzene Rings II (Compounds 193-211)

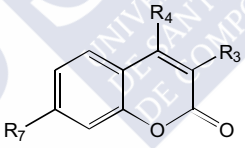


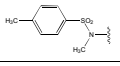
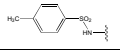
Cpds	R	R_3	R_4	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
193				H	1.58	0.003	62
194				H	1.58	0.003	62
195				H	1 (7%)	0.050	62
196				H	> 10	0.007	65
197				H	> 10	0.10	65
198	H			H	48.20	> 100	66
199	H			OCH_3	63.53	> 100	66
200	H			CH_3	> 100	> 100	66
201	H			OCH_3	> 100	50.20	66
202	H			CH_3	60.11	> 100	66
203				OCH_3	0.12	12.98	66

(Table 2) contd….

Cpds	R	R_3	R_4	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
204				CH_3	0.24	0.17	66
205				OCH_3	0.37	12.12	66
206				CH_3	0.004	17.30	66
207				OCH_3	11.93	9.36	66
208				CH_3	2.56	28.13	66
209				CH_3	0.13	1.18	66
210				CH_3	0.70	0.24	66
211				CH_3	2.38	0.001	66

Table 3. Chemical Structures and MAO Inhibition Data of Coumarins Bearing a Carbo, Thio or Aza Spacer at Position 7 III (212-225)

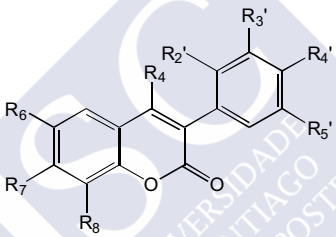


Cpds	R_7	R_3	R_4	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
212		CH_3	CH_3	0.41	0.028	62
213		CH_3	CH_3	10.0	0.040	67
214		CH_3	CH_3	18.62	2.75	67
215		CH_3	CH_3	4.57	31.62	67
216		CH_3	CH_3	67.61	40 (29%)	62
217		CH_3	CH_3	1.38	0.19	62
218		CH_3	CH_3	1.58	0.16	62
219		CH_3	CH_3	7.08	0.46	67

(Table 3) contd….

Cpds	R_7	R_3	R_4	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref.
220		H	H	0.39	0.085	62
221		H	H	41.69	2.14	62
222		H	H	3.80	1.29	67
223		H	H	6.17	0.58	67
224		H	H	21.88	25.70	67
225		H	H	9.55	2.40	67

Table 4. Chemical Structures and MAO Inhibition Data of Differently Substituted 3-arylcoumarins IV (Compounds 226-286)



Cpds	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	R_4	R_6	R_7	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
226	H	H	H	H	H	H	H	H	> 100	11.81	74
227	OH	H	H	H	H	Cl	H	H	> 100	0.44	69
228	H	OH	H	H	H	Cl	H	H	> 100	0.21	69
229	H	H	OH	H	H	Cl	H	H	> 100	0.13	69
230	OCH_3	H	H	H	H	Cl	H	H	> 100	> 100	69
231	H	OCH_3	H	H	H	Cl	H	H	> 100	0.001	69
232	H	H	OCH_3	H	H	H	H	H	> 100	0.004	69
233	OH	H	H	H	H	Br	H	H	> 100	0.48	69
234	H	OH	H	H	H	Br	H	H	> 100	0.29	69
235	H	H	OH	H	H	Br	H	H	> 100	0.64	69
236	OCH_3	H	H	H	H	Br	H	H	> 100	> 100	69
237	H	OCH_3	H	H	H	Br	H	H	> 100	0.002	69
238	H	H	OCH_3	H	H	Br	H	H	> 100	0.006	69
239	H	H	H	H	H	Br	H	OCH_3	> 100	0.083	73
240	H	H	H	H	H	Br	H	OH	> 100	30.91	73
241	H	H	OCH_3	H	H	Br	H	OCH_3	> 100	1.35	73

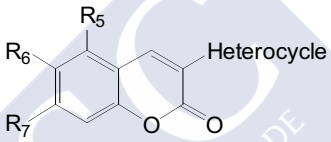
(Table 4) contd….

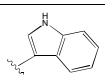
Cpds	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	R_4	R_6	R_7	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
242	H	H	OH	H	H	Br	H	OH	20.74	16.87	73
243	H	H	H	H	H	CH_2Br	H	H	> 100	0.043	69
244	H	H	H	H	H	CH_3	H	H	> 100	0.28	72
245	H	H	CH_3	H	H	CH_3	H	H	> 100	$0.31x\ 10^{-3}$	72
246	H	CH_3	H	H	H	CH_3	H	H	> 100	0.015	72
247	H	OCH_3	H	H	H	CH_3	H	H	> 100	$0.80x\ 10^{-3}$	72
248	H	OH	H	H	H	CH_3	H	H	35.04	0.65	72
249	H	H	OCH_3	H	H	CH_3	H	H	> 100	0.013	72
250	H	H	OH	H	H	CH_3	H	H	> 100	0.16	72
251	H	OCH_3	OCH_3	H	H	CH_3	H	H	25.14	0.003	72
252	H	OCH_3	H	OCH_3	H	CH_3	H	H	> 100	0.009	72
253	H	OCH_3	OCH_3	OCH_3	H	CH_3	H	H	> 100	0.16	72
254	H	Br	OCH_3	H	H	CH_3	H	H	> 100	$0.74x\ 10^{-3}$	72
255	H	OCH_3	Br	H	H	CH_3	H	H	> 100	0.003	72
256	H		H	H	H	CH_3	H	H	> 100	0.18	72
257	OCH_3	H	H	H	H	CH_3	H	H	> 100	> 100	72
258	OH	H	H	H	H	CH_3	H	H	21.58	0.12	72
259	Br	H	H	H	H	CH_3	H	H	> 100	4.34	75
260	Br	OCH_3	H	OCH_3	H	CH_3	H	H	> 100	54.03	72
261	Br	OCH_3	OCH_3	OCH_3	H	CH_3	H	H	> 100	> 100	72
262	H	H	H	H	H	CH_3	H	Br	> 100	0.011	75
263	H	H	OCH_3	H	H	CH_3	H	Br	> 100	0.003	75
264	H	OCH_3	H	OCH_3	H	CH_3	H	Br	> 100	0.007	75
265	H	OCH_3	OCH_3	OCH_3	H	CH_3	H	Br	31.20	4.89	75
266	H	CH_3	H	H	H	OCH_3	H	H	> 100	0.018	72
267	H	H	CH_3	H	H	OCH_3	H	H	> 100	0.002	72
268	H	H	CH_3	H	H	OH	H	H	24.90	0.067	72
269	H	H	CH_3	H	H		H	H	> 100	5.52	72
270	H	H	CH_3	H	H		H	H	> 100	14.47	72
271	H	H	H	H	H	H	H	CH_3	6.26	0.078	76
272	H	H	OCH_3	H	H	H	H	CH_3	21.60	0.003	76
273	H	H	CH_3	H	H	H	H	CH_3	> 100	0.005	76
274	H	H	Br	H	H	H	H	CH_3	> 100	0.020	76
275	H	H	H	H	H	H	H	OCH_3	> 100	0.15	76
276	H	H	Br	H	H	H	H	OCH_3	> 100	0.003	76
277	H	H	H	H	H	H	H	OCH_2CH_3	> 100	1.06	76

(Table 4) contd….

Cpds	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	R_4	R_6	R_7	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
278	H	H	OCH_3	H	H	H	H	OCH_2CH_3	> 100	0.21	76
279	H	H	CH_3	H	H	H	H	OCH_2CH_3	> 100	0.15	76
280	H	H	H	H	OH	H	H	H	> 100	> 100	77
281	H	H	OCH_3	H	OH	H	H	H	> 100	69.59	77
282	H	H	OCH_3	H	OH	CH_3	H	H	> 100	32.04	77
283	H	H	OCH_3	H	OH	Cl	H	H	> 100	> 100	77
284	H	Cl	OCH_3	H	OH	H	H	H	> 100	9.26	77
285	H	Cl	OCH_3	H	OH	CH_3	H	H	> 100	42.68	77
286	H	Cl	OCH_3	H	OH	Cl	H	H	> 100	2.79	77

Table 5. Chemical Structures and MAO Inhibition Data of Differently Substituted 3-heteroarylcoumarins V (Compounds 287-298)

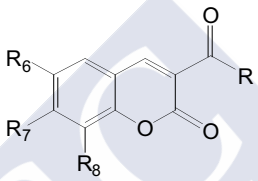


Cpds	R_3	R_5	R_6	R_7	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
287		H	H	H	> 100	6.37	79
288		H	OCH_3	H	> 100	0.63	79
289		H	H	OCH_3	> 100	0.26	79
290		OCH_3	H	OCH_3	> 100	> 100	79
291		H	H	H	> 100	13.30	79
292		H	OCH_3	H	> 100	0.23	79
293		H	H	OCH_3	4.16	0.056	79
294		OCH_3	H	OCH_3	> 100	> 100	79
295		H	H	H	> 100	1.92	79

(Table 5) contd….

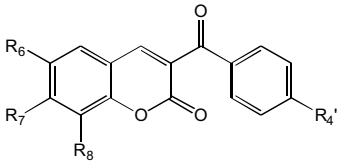
Cpds	R_3	R_5	R_6	R_7	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
296		H	OCH_3	H	> 100	> 100	79
297		H	H	OCH_3	9.67	0.046	79
298		OCH_3	H	OCH_3	> 100	> 100	79

Table 6. Chemical Structures and MAO Inhibition Data of 3-carbonylcoumarin Derivatives VI (Compounds 299-316)



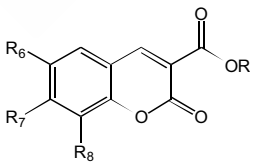
Cpds	R	R_6	R_7	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
299	CH_3	H	H	H	> 100	14.45	78
300	CH_3	H	H	OH	39.36	42.13	78
301	CH_3	H	OH	H	> 100	> 100	78
302	CH_3	OH	H	H	100 (45%)	64.17	78
303	CH_3	H	OCH_3	H	100 (45%)	15.40	78
304	CH_3	H	$N(CH_2CH_3)_2$	H	> 100	18.07	78
305	CH_3	CH_3	H	H	> 100	100 (45%)	78
306	CH_3	Cl	H	H	> 100	17.72	78
307	CH_3	Br	H	H	51.63	100 (45%)	78
308	CH_3			H	100 (45%)	2.01	78
309	CH_3	Cl	H	Br	55.16	0.14	78
310	CH_3	Br	H	Br	> 100	0.20	78
311	CH_3	I	H	I	> 100	>100	78
312	Cl	H	H	H	0.060	0.12	80
313	Cl	CH_3	H	H	0.029	0.10	80
314	Cl	Br	H	H	0.020	0.11	80
315	Cl	Cl	H	H	0.030	0.010	80
316	Cl	NO_2	H	H	0.029	0.10	80

Table 7. Chemical Structures and MAO Inhibition Data of 3-benzoylcoumarin Derivatives VII (Compounds 317-332)



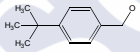
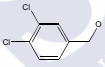
Cpds	$R_{4'}$	R_6	R_7	R_8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
317	H	H	H	H	> 100	> 100	78
318	H	Br	H	H	> 100	> 100	78
319	H	Br	H	OCH_3	> 100	> 100	73
320	H	Br	H	OH	46.81	73.92	73
321	OH	Br	H	OH	19.17	> 100	73
322	OCH_3	Br	H	OCH_3	> 100	> 100	73
323	H	H	H	OH	40.47	27.11	78
324	H	OH	H	H	51.66	17.97	78
325	H	H	OH	H	> 100	> 100	78
326	H	H	OCH_3	H	> 100	100 (45%)	78
327	H	H	$N(CH_2CH_3)_2$	H	> 100	23.84	78
328	H	CH_3	H	H	> 100	100 (45%)	78
329	H	Cl	H	H	> 100	> 100	78
330	H	Cl	H	Br	> 100	100 (45%)	78
331	H	Br	H	Br	> 100	100 (45%)	78
332	H	I	H	I	> 100	> 100	78

Table 8. Chemical Structures and MAO Inhibition Data of 3-carboxycoumarin Derivatives VIII (Compounds 333-368)



Cpds	R	R^6	R^7	R^8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
333	CH_2CH_3	H	H	H	0.23	5.01	80
334	CH_2CH_3	H	H	OH	40.55	14.81	78
335	CH_2CH_3	H	OH	H	> 100	> 100	78
336	CH_2CH_3	OH	H	H	49.07	10.06	78
337	CH_2CH_3	H	OCH_3	H	100 (45%)	23.46	78

(Table 8) contd….

Cpds	R	R^6	R^7	R^8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
338	CH_2CH_3	OCH_3	H	H	> 100	21.63	78
339	CH_2CH_3	H	$N(CH_2CH_3)_2$	H	> 100	45.52	78
340	CH_2CH_3	Cl	H	Br	> 100	17.51	78
341	CH_2CH_3	Br	H	Br	> 100	100 (45%)	78
342	CH_2CH_3	I	H	I	> 100	> 100	78
343	CH_2CH_3	H		H	46.35	0.021	78
344	CH_2CH_3	CH_3	H	H	0.19	1.0	80
345	CH_2CH_3	Br	H	H	0.21	1.0	80
346	CH_2CH_3	Cl	H	H	0.50	1.0	80
347	CH_2CH_3	NO_2	H	H	0.26	1.0	80
348	CH_2CH_3	H		H	> 100	0.007	78
349	CH_2CH_3	H		H	> 100	0.061	78
350	CH_2CH_3	H		H	> 100	3.75	78
351	CH_2CH_3	H		H	> 100	0.009	78
352	H	H	H	H	15.14	0.017	80
353	H	CH_3	H	H	12.02	0.019	80
354	H	Br	H	H	1.0	0.033	80
355	H	Cl	H	H	0.50	0.023	80
356	H	NO_2	H	H	0.068	0.019	80
357	H	H	H	OH	59.61	100 (45%)	78
358	H	OH	H	H	40.20	100 (45%)	78
359	H	H	OCH_3	H	> 100	100 (45%)	78
360	H	OCH_3	H	H	> 100	> 100	78
361	H	H	$N(CH_2CH_3)_2$	H	> 100	> 100	78
362	H	Cl	H	Br	> 100	> 100	78
363	H	Br	H	Br	> 100	> 100	78
364	H	I	H	I	> 100	17.78	78
365	H	H		H	> 100	8.62	78
366	H	H		H	> 100	1.80	78

(Table 8) contd….

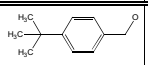
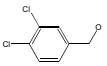
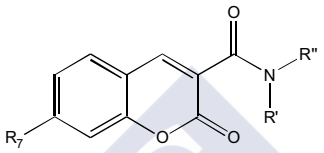
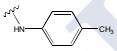
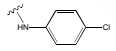
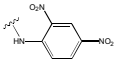
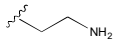
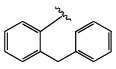
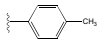
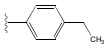
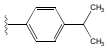
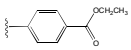
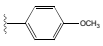
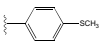
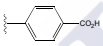
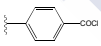
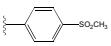
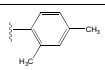
Cpds	R	R^6	R^7	R^8	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
367	H	H		H	> 100	> 100	78
368	H	H		H	> 100	0.41	78

Table 9. Chemical Structures and MAO Inhibition Data of 3-carboxyhydrazide and 3-carboxamide Coumarin Derivatives IX (Compounds 369-432)

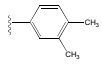
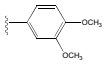
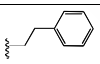
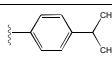


Cpds	R'	R''	R7	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
369	NH_2	H	H	> 100	0.003	78
370		H	H	41.40	4.52	78
371		H	H	39.56	> 100	78
372		H	H	58.43	> 100	78
373		H	H	48.02	1.19	78
374		H	H	60.49	56.12	78
375		H	H	> 100	0.50	81
376		H	H	> 100	25.36	81
377		H	H	> 100	3.27	81
378		H	H	> 100	> 100	81
379		H	H	> 100	7.68	81
380		H	H	13.12	0.76	81
381		H	H	> 100	0.91	81

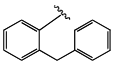
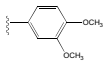
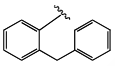
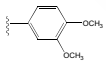
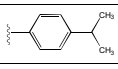
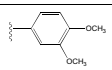
(Table 9) contd….

Cpds	R'	R''	R7	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
382		H	H	14.50	0.60	81
383		H	H	> 100	0.64	81
384		H	H	> 100	0.25	81
385		H	H	> 100	0.25	81
386		H	H	19.45	0.71	81
387		H	H	15.67	0.89	81
388		H	H	15.14	0.65	81
389		H	H	15.98	> 100	81
390		H	H	> 100	0.82	81
391		H	H	14.76	2.84	81
392		H	H	13.06	0.067	81
393		H	H	9.97	1.14	81
394		H	H	9.67	> 100	81
395		H	H	9.33	0.001	81
396		H	H	> 100	0.80	81
397		H	H	> 100	19.07	81
398		H	H	15.95	22.45	81
399		H	H	12.32	13.74	81
400		H	H	20.63	18.27	81
401		H	H	9.79	1.29	81

(Table 9) contd….

Cpds	R'	R''	R7	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
402		H	H	12.76	9.23	81
403		H	H	18.77	0.38	81
404		H	H	9.45	0.86	81
405		H	H	17.56	0.70	81
406		H	H	61.46	4.18	81
407		H	H	> 100	0.46	81
408		H	H	16.04	0.86	81
409		H	H	11.43	0.58	81
410		H	H	> 100	1.96	81
411		H	H	> 100	> 100	81
412		CH_3	H	> 100	8.89	81
413		CH_3	H	> 100	> 100	81
414			H	> 100	> 100	81
415		H		> 100	64.45	81
416		H		63.52	> 100	81
417		H		58.64	9.59	81
418		H		60.93	3.69	81
419		H		61.38	16.54	81

(Table 9) contd….

Cpds	R'	R''	R7	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
420		H		51.89	63.52	81
421		H		49.75	61.49	81
422		H		> 100	4.39	81
423		H		52.89	3.36	81
424		H		> 100	0.99	81
425		H		41.40	4.52	81
426		H		58.49	3.45	81
427		H		55.83	> 100	81
428		H		59.56	41.64	81
429		H		49.20	2.87	81
430		H		52.62	21.56	81
431		H		> 100	> 100	81
432		CH_3		> 100	2.37	81

lished a potential therapeutic application of these flavonoids/coumarins analogues as a new interesting scaffold with interest against age-related neurodegenerative diseases such as PD or AD [77].

G. Delogu *et al.* synthesized new coumarin derivatives **V** in which the 3-aryl ring is changed for a heteroaryl group and explored the importance of the number and position of methoxy groups on (1H)-benzopyran structure [79]. In general, these derivatives were found to be selective for the MAO-B isoform, with 7-methoxy-3-heteroarylcoumarins exhibiting inhibition potencies in the low nanoMolar range [79].

Substitution of the coumarin nucleus at position 3 has been widely carried out with phenyl and methyl groups, but less data have been reported with polar groups like ketone alkylic (Table **6**), ketone arylic (Table **7**) and carboxylic acid or ethyl esther (Table **8**). D. Secci *et al.* have dedicated particular attention to the study of the influence of these groups on MAO inhibitory activity and on A/B selectivity. For these derivatives, the steric hindrance of the substituent at position 6 is particulary important, in which only small substituents are tolerated [78, 80]. 3-Carbonyl chloride coumarins showed a high potency against both isoforms [80].

By analyzing the data reported in the previous (Table **6**), it can be seen that 3-methylketone coumarin derivatives showed better inhibitory activity and selectivity against MAO-B than their corresponding arylketones **VII** (Table **7**). Likewise, an important lost of the MAO-B inhibitory activity was verified when the 3-phenyl skeleton was substituted for the 3-benzoyl one [73]. Therefore, the simple introduction of a carbonyl bridge between the coumarin and the 3-phenyl ring changes dramatically the activity profiles.

Compounds bearing a CO_2Et moiety at C3 position, improved the inhibitory activity and selectivity towards MAO-B. However, the hydrolysis of ethyl esters to the corresponding carboxylic acids decreased the selectivity and the inhibitory activity towards MAO-B [78, 80]. Results of MAO activity are showed in (Table **8**).

D. Secci *et al.* also described a series of 3-carboxy hydrazide coumarin derivatives [78] as well as a series of 3-carboxamide coumarin derivatives (Table **9**) [81]. It can be seen that the majority of the compounds show a selective inhibitory activity toward the MAO-B isoenzyme. Substitution at position 7 on the coumarin ring by a benzyloxy group leads to a decrease in the activity against both MAO isoforms. Inhibitory activity increased when the nitrogen is substituted by a phenyl group.

Taking into account the activity of the above mentionated amido coumaris, M. J. Matos *et al.* synthetized analogs **X** and studied the possibility of linking the amidic group to the coumarin by the nitrogen atom [74]. These compounds could be considered inverse amidic derivatives with respect to the last described molecules **IX**. In spite of these considerations, the new compounds resulted to be poor MAOIs. However, some of them showed AChE inhibitory activity, being considered therefore as dual MAO/AChE inhibitors [74]. Compounds with such dual inhibitory activities are expected to have potential for the treatment of AD because they might decrease β-amyloid deposition [82].

3. SYNTHETIC METHODOLOGIES

Due to the interest of coumarins in industry as additives in food, agrochemicals, cosmetics and pharmaceuticals, synthesis of the coumarin template is a topic of current interest in organic chemistry. Different methodologies can be used to obtain the described compounds, depending on the starting materials and on the desired final substituents. Classically, the most important synthetic methodologies to obtain simple coumarins are the Pechmann, Perkin, Knoevenagel and Wittig reactions.

The Pechmann reaction allows to obtain the coumarin scaffold by condensation of phenols with β-ketoesters, in the presence of acid catalysts (usually sulphuric acid) (Scheme **1**). Although this is the most classical synthetic route to obtain the coumarin nucleus with alkyl, aryl, halogen, halogenomethyl or hydroxy substituents, some slight modifications to the traditional Pechmann reaction were performed, depending on the stability and reactivity of the used phenols and β-dicarbonyls [62, 64, 66, 83-85]. Several efforts are still being devoted to improve its performance by the use of specific catalysts and/or microwave radiation in ionic liquids media or solvent-free conditions [86-89]. The use of perchloric acid as the condensing agent allows, for example, the synthesis of differently substituted 7-hydroxycoumarin derivatives [90].

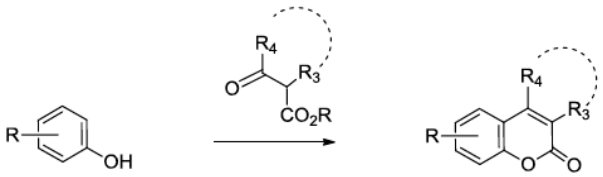
The Perkin reaction is an aldolic condensation of an *ortho*-hydroxybenzaldehyde and the conveniently substituted acid anhydrides, in the presence of an alkali salt of the acid (Scheme **2**). This methodology is perhaps the most direct and simple method known for the preparation of 3-arylcou marins. However, the overall yields of the final products in these reactions are invariably low and the variety of substrates is, in most cases, limited [47]. Thus, a lightly modified version of this methodology is commonly used for the synthesis of the 3-aryl or 3-heteroaryl substituted coumarins [69-73, 75, 76, 91]. In this reaction, the coumarin nucleus is obtained starting from the *ortho*-hydroxybenzaldehyde and the conveniently substituted arylacetic acid, in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC), the dehydrating agent, under DMSO reflux.

The Knoevenagel reaction is a condensation reaction that affords the coumarin derivatives starting from *ortho*-hydroxybenzaldehydes and active methylene compounds, in presence of ammonia or amines (Scheme **2**). The reaction is usually catalysed by weak bases or by suitable combinations of amines (usually piperidine) and carboxylic or Lewis acids under homogeneous conditions [78, 81]. The use of Meldrum´s acid in presence ionic liquids, results a simple and alternative method to obtain 3-carboxycoumarins [92]. A large list of coumarins has been synthesised using the Knoevenagel condensation. Benzoyl coumarins [73], as well as amino [93], amides [94], and 3-alkylamino substituted coumarins are some examples of the potential of this methodology [56, 68].

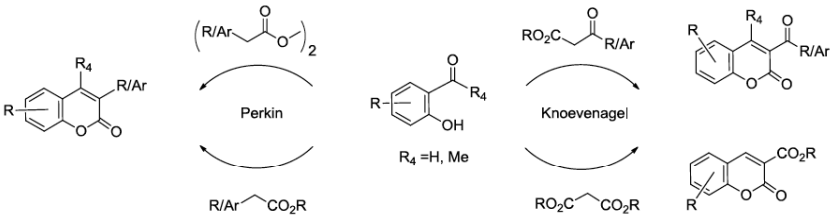
In order to afford multigram scale synthesis and easy purification, F. Chimenti *et al.* described the preparation of the 3-carboxycoumarin scaffold by the heterocyclization reaction of 2-hydroxybenzaldoximes and carbon suboxide (Scheme **3**) [80]. The basic hydrolysis of the carboxamide intermediate and the subsequent functionalization of the carboxylic acid intermediate, affords carboxycoumarin derivatives with high MAO inhibitory activity.

Wittig reaction is also an efficient and easy method for the synthesis of coumarins (Scheme **4**). This method consists in the reaction of *ortho*-hydroxycarbonyl aromatic compounds with phosphonium ylides and the subsequent intramolecular cyclization [95]. This type of reaction is a useful method to obtain prenylated coumarins [96, 97]. Also, aryl-, alkoxy-, or carboxycoumarin derivatives can be obtained via Wittig reaction, under classical conditions or improved protocols [70, 98, 99]. A variety of nitro/amine coumarins was prepared using microwave irradiation in solvent free conditions [96] and large combinatorial libraries of 3-halogenocoumarins were obtained by *one-pot* three component bromination/Wittig/cyclization process [100].

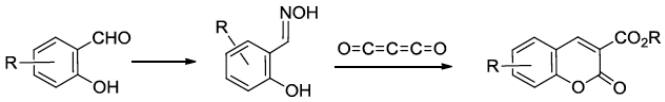
The palladium catalysed chemistry is an alternative methodology for the preparation of 4-substituted coumarin derivatives. 4-Aryl/alkylcoumarins can be obtained from the corresponding phenols by palladium-catalysis reaction with the appropriated propiolates or acrylates [101-103].



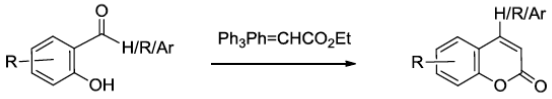
Scheme 1. Pechmann reaction.



Scheme 2. Perkin and Knoevenagel reactions.



Scheme 3. Heterocyclization reaction of 2-hydroxybenzaldoximes and carbon suboxide.



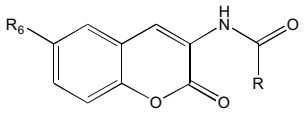
Scheme 4. Wittig reaction.

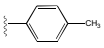
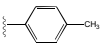
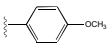
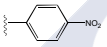
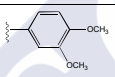
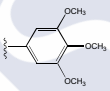
Special mention must be made regarding the arylcoumarin derivatives. Additionally to the above mentioned cyclo-condensation reactions, 3- and or 4-arylcoumarins can be obtained, starting from halogen or hydroxycoumarins by direct cross-coupling palladium arylation. The most common halogencoumarin used as starting material is the 3-bromo derivative. Therefore, libraries of 3-arylcoumarins were prepared starting from the 3-bromocoumarin by Pd-catalyzed coupling with boronic esters, acetylenes, or vinyl compounds [104]. However, very recently our research group has used for the first time the 3-chlorocoumarin as starting material and has prepared different 3-arylcoumarins by a Pd-catalyzed cross-coupling reaction using a catalytic complex Pd-salen [105].

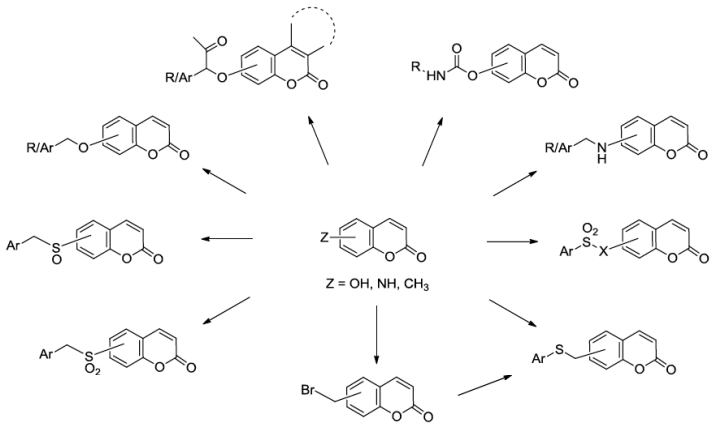
Other starting materials for the arylation of the coumarin ring are the 4-hydroxycoumarin derivatives. Due to the special reactivity of the 4-hydroxycoumarin ring, the coupling arylation can take place in either 3 or 4 positions, depending on the reaction conditions. The general palladium protocol or the Suzuki-Miyaura couplings are an efficient method for coupling 4-hydroxycoumarins. Through the intermediated triflate and tosylate derivatives, 4-arylcoumarins can be prepared in high yields [98, 106, 107]. However, the coupling arylation can take place also at position 3. Reaction of 4-hydroxycoumarins with iodobenzene diacetate gives the 3-phenyliodonium zwitterion intermediates which couple with substituted arylboronic acids in the palladium-catalyzed Suzuki-type reactions [77]. The excellent yields make this method a valuable tool for generating diversified 4-hydroxy-3-arylcoumarin derivatives. According to these methodologies, when the starting compounds are 4-hydroxy-3-bromocoumarins, it is possible to design an efficient protocol for the synthesis of 3- and/or 4-arylcoumarin derivatives [108].

The SAR studies performed with coumarin derivatives suggested that activity and selectivity were mainly determined by the characteristics of the substituents in the coumarin moiety [61, 64]. For this reason, during the last years new synthetic strategies have been described using as starting materials the previously synthesized coumarin ring. Most of the coumarin derivatives were prepared by nucleophilic substitution of simple hydroxy, amine or halogencoumarins under reflux of acetone, xylene, ethanol, or other solvents in the presence of potassium carbonate (Scheme **5**). Therefore, coumarins with benzyloxy [64, 84] and some alkylsulfonyloxy [67] groups were extensively synthesized as active and

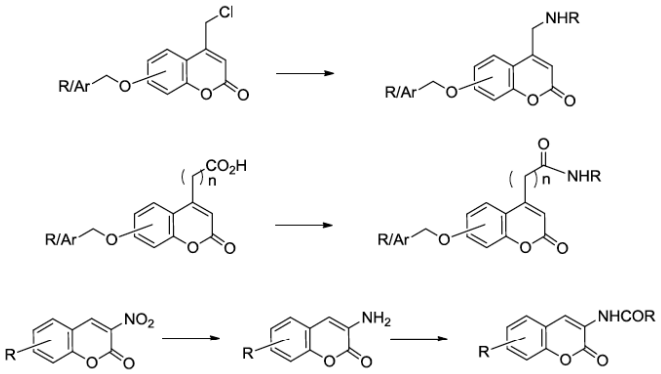
Table 10. Chemical Structures and MAO Inhibition Data of 3-carboxamidocoumarin Derivatives X (Compounds 433-445)



Cpds	R_6	R	MAO-A IC_{50} (μM)	MAO-B IC_{50} (μM)	Ref
433	H		> 100	0.76	74
434	CH_3		45.97	0.17	74
435	H		> 100	7.96	74
436	H		> 100	30.50	74
437	H		> 100	1.95	74
438	H		> 100	> 100	74
439	H		> 100	58.38	74
440	H		> 100	72.58	74
441	H		> 100	5.95	74
442	H		> 100	49.96	74
443	H	CH_3	38.93	> 100	74
444	H	CH_2Br	> 100	> 100	74
445	H	CH_2Cl	> 100	> 100	74



Scheme 5. Synthetic strategies using as starting materials the previously synthesized coumarin ring.



Scheme 6. Functionalization of coumarins to afford the corresponding amino, carboxamido or amidocoumarin derivatives.

selective MAO inhibitors. The etherification of hydroxycoumarins and aminocoumarins were commonly performed by Williamson-type reactions [74]. So, different substituted bromides were furnished target coumarins in the presence of potassium carbonate and ethanol, under reflux [64, 66, 67, 84]. The same etherification reaction occurs adding suitable bromides and potassium carbonate in dry acetone, using *N,N'*-dicyclohexyl-18-crown-6-ether as chelating agent [81]. This type of reaction was also possible starting from the formation of the bromomethylcoumarin and the subsequent nucleophilic displacement of bromide by hydroxy or amine substituted molecules (for example phenol or aniline) [62]. In general, these etherifications were the most common substitutions under the coumarin nucleus to afford the desired MAO activity. The synthesis of alkylsulfonyloxy derivatives was carried out starting from the corresponding hydroxycoumarins with a conveniently substituted sulfonyl chloride in pyridine [67]. The thioether derivatives were prepared by refluxing bromides and thiols in MeCN in the presence of potassium carbonate. Sulfoxide and sulfone derivatives were obtained by oxidation of thioethers with sodium periodate or potassium permanganate, respectively [61]. Some functionalizations can also be performed by microwave irradiation [84].

Halogeno, carboxy or nitrocoumarins can also be used as starting synthons for the subsequent functionalization affording an array of the corresponding amino, carboxamido or amidocoumarins, compounds with inhibitory MAO activity (Scheme **6**).

Finally it must be mentioned that 3-aryl and 3-benzoylcoumarins can be further substituted in different positions of both aromatic rings using the same aforementioned methodologies [69-73, 75, 76]. Bromination of the derivatives was performed with *N*-bromosuccinimide (NBS), in carbon tetrachloride (CCl_4) under reflux, using 2,2'-azo-bis-iso-butyronitrile (AIBN) as catalyst [69, 70]. The methoxy/ethoxy derivatives can be hydrolyzed either with hydriodic acid, in the presence of acetic acid and acetic anhydride or BBr_3 in DCM [69, 72, 73, 75]. The Williamson reaction of the hydroxyl substituted arylcoumarins, as described for simple coumarins, was performed with differently substituted chlorides or bromide to give the corresponding ethers [72].

3. CONCLUSION

Since the pioneering work of H. A. Kadir *et al.* [55], and the discovery of the potential of the coumarin scaffold against both MAO isoforms, new generations of differently substituted molecules have been synthesized and evaluated *in vitro* in pharmacological assays. Some of the described derivatives are very active and selective compounds, with IC_{50} in the low nanomolar range. These findings pave the way for new promise for efficient and safe therapeutic solutions of an increasing and important problem of the developed countries: the neurodegenerative diseases. Furthermore, based on these important studies, there are several ongoing projects for the rational structure-based drug design of new molecules.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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Original article

Novel (coumarin-3-yl)carbamates as selective MAO-B inhibitors: Synthesis, *in vitro* and *in vivo* assays, theoretical evaluation of ADME properties and docking study

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ABSTRACT

A series of (coumarin-3-yl)carbamates was synthesized and evaluated *in vitro* as monoamine oxidase (MAO-A and MAO-B) inhibitors. Most of the new compounds selectively inhibited MAO-B isoenzyme with IC_{50} values in the micro or nanoMolar ranges. Since these compounds must achieve the brain cells, theoretical evaluation of ADME properties were also carried out. Compound **8** (benzyl(coumarin-3-yl)carbamate), which presented the most interesting *in vitro* MAO-B inhibitory profile (IC_{50} against MAO-B = 45 nM), was subjected to further studies. This *in vitro* MAO-B inhibitory activity is comparable with that of the selegiline, the reference compound (IC_{50} against MAO-B = 20 nM). Taking into account the *in vitro* results of compound **8**, *in vivo* assays and docking calculations were also carried out for this derivative.

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1. Introduction

Quality of life of the aging population in the today's society is, to a great extent, determined by the normal aging process of neurons in the central nervous system (CNS) and especially in diseases characterized by accelerated neuronal loss, which are traditionally designated as neurodegenerative diseases (ND). These diseases are major contributors to disability and morbidity around the world. Alzheimer's disease (AD) is the most prevalent ND followed by Parkinson's disease (PD). AD is the most common cause of senile dementia, affecting approximately 3% of the population between the ages of 65–74 and nearly 50% of those 85 years and older [1–4]. PD, the second most important ND, is characterized by loss of dopaminergic neurons in the nigrostriatal pathway. These neurons extend from the zona compacta of the substantia nigra (SNC) to the striatum. The symptoms of PD, resting tremor, rigidity and bradykinesia, appear when about 80% of dopaminergic neurons have been destroyed [5]. The etiology is not completely known, but several risk factors have been identified, including environmental factors, genetics, and age. Several research hypotheses exist regarding the pathogenesis, including different mechanisms that may be involved in the development of disease, such as oxidative stress, inflammation, excitotoxicity, mitochondrial dysfunction and proteolytic stress [6–8]. In the 50's, dopamine (DA) was described as a neurotransmitter, which showed low levels in the basal ganglia in animal models of parkinsonism. After the success of a placebo-controlled trial, levo-dopa (LD) became an essential drug for the treatment of PD [9]. Even today the standard treatment of PD is the combination of LD with inhibitors of peripheral dopa decarboxylase (DDC), to avoid the formation of DA in the periphery. LD is also associated to inhibitors of the enzyme catechol-O-methyltransferase (COMT) and prevents the formation of 3-O-methyldopa (3-OMD) [10]. Among other drugs that can be used

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to treat PD, selective inhibitors of monoamine oxidase B (MAO-B) play an important role [11–13].

Interest in selective monoamine oxidase inhibitors (MAOIs), especially those for MAO-B, has increased significantly in recent years. This is related to the recent discovery an increase in MAO-B activity is associated with gliosis, which can result in high levels of hydrogen peroxide and oxidative free radicals. Evidence exists that as aging progresses activity of MAO-B in the brain increases, but not so for MAO-A. The largest increases are in the basal ganglia and thalamus, followed by the frontal cortex and, to a lesser extent, cerebellum and parietal and temporal cortices [14].

Increased activity of MAO-A and MAO-B is associated with loss of endogenous and exogenous monoamine amounts. The two isoforms of the MAO enzyme are responsible for catalyzing the oxidative deamination of neurotransmitters and dietary amines, regulating intracellular levels of biogenic amines in the brain and the peripheral tissues [15,16]. MAOs are FAD-depending enzymes found in the outer mitochondrial membrane of different cells, such as neuronal, glial and other mammalian cells [17]. MAO-A and MAO-B have been characterized and compared according to their amino acid sequences [18], three-dimensional structure [19], tissue distribution [20], inhibitor selectivity [21] and substrate preferences [22]. The similarities of the genes encoding the two isoforms of MAO suggest that both derived from a common ancestral gene. Overall MAO-A and MAO-B share 70% amino acid sequence, however similarity is higher in some regions, including the pentapeptide (Ser-Gly-Gly-Cys-Tyr) that allows the covalent attachment of FAD through cysteine. DA and tyramine are common substrates for both the MAO-A and MAO-B isoforms. However, the MAO-A isoform has a higher affinity for serotonin and noradrenaline, while the MAO-B isoform preferentially deaminates β-phenylethylamines and benzylamine [23].Due to their general affinity for different substrates, these enzymes are involved in different pathologies such as anxiety and depression (MAO-A), and PD and AD (MAO-B). Selective MAO-A inhibitors, such as clorgyline (irreversible) and moclobemide (reversible), are used for the treatment of neurological disorders [24], whereas selective and irreversible MAO-B inhibitors, such as selegiline and rasagiline, are used in the treatment of ND [25]. Several studies point to the neuroprotective role of MAO-B inhibitors (MAOI-B) in PD and their modifying effect by delaying the progression of the disease. Rasagiline, a selective MAOI-B, has been reported to retard further deterioration of cognitive functions, displaying an important neuroprotective activity against the consequences of oxidative stress in patients [26,27].

Coumarins are a large family of compounds from both natural and synthetic origin and display a variety of pharmacological properties. Due to their structural variability and versatile synthetic methodologies they occupy an important place in the realm of natural products and synthetic organic chemistry. Recent studies pay special attention to their antioxidative and enzymatic inhibition properties, regarding their potential against ND [28]. Numerous functionalized coumarins have been displaying potent MAO and/or AChE inhibitory activities and some of them have been proposed for treating AD [29–31]. Substitution in position 3 of the coumarin nucleus modulates MAO-B as well as AChE inhibitory activity. Our research group has reported, in several studies, the importance to the MAO inhibitory activity of different substituents on the phenyl ring (directly linked to the coumarin or spaced with a carbonyl or amide group) at position 3 of the coumarin moiety (Fig 1) [32–37]. Also, we have verified an important loss of the MAO-B inhibitory activity and selectivity when the 3-phenyl group was substituted for the 3-benzoyl one. Therefore, the presence or absence of a carbonyl group between the coumarin and the phenyl substituent at position 3 modulated, respectively, the MAO-A or MAO-B inhibitory activity (Fig. 1) [38]. Introduction of an amidic group between the 3-substituted phenyl ring and the coumarin afforded compounds that showed a selective inhibitory activity toward *h*MAO-B [30,39], some of them also described as dual inhibitors of MAO and AChE in the microMolar range [30].

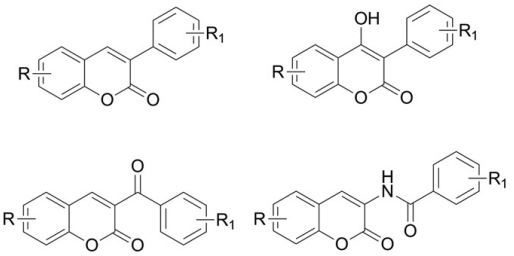


Fig. 1. 3-Substituted coumarins with MAO activity which have been previously reported by our research group.

Taking into account previous studies [32–38], our current work is focused on modifying the molecular structure of our very potent MAO-B inhibitors by introducing new functional groups at position 3 to improve their activities and potential for treating PD. In addition, preliminary studies established that carbamates (showing alkyl or aryl groups) were moderate MAO-B substrates [40] and series of coumarins, incorporating this type of substituents at position 7, differing for size and hydrophobicity have been also reported [41].

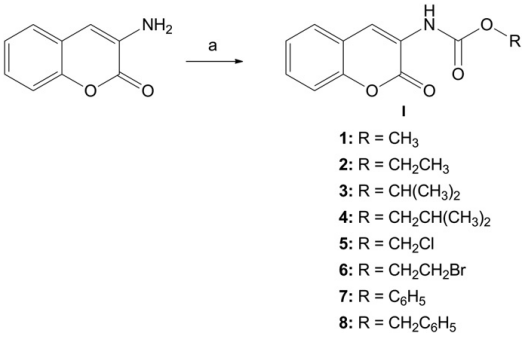
Guided by the wealth of information on structure-affinity and structure selectivity relationships (SAFIRs and SSRs, respectively) we have been encouraged to synthesized and study the *in vitro* MAO-A and MAO-B inhibitory activity of new (coumarin-3-yl) carbamate derivatives, in which a variety of groups with different size, electronic properties and lipophilicity were introduced. This limited, albeit crucial, structural modification, if tolerated, would have enabled us to identify new MAO-B inhibitors with an improved pharmacokinetic profile and a higher druggability.

The best MAO-B inhibitor analogue *in vitro* (compound 8) was studied *in silico* and *in vivo*, in order to gain a better understanding of its activity and to assess its potential for PD. In addition, compound **8** exhibited appropriate pharmacologic features that are presented and discussed in this paper, along with new SAFIRs, SSRs, and docking studies that provided a consistent picture of the main binding interactions modulating the MAO inhibitory potency and MAO-B isoform selectivity of this new class of coumarins. These studies help to corroborate and clarify the previously obtained *in vitro* experimental data and promote rational drug design.

2. Results and discussion

2.1. Chemistry

All the described (coumarin-3-yl)carbamates (compounds **1**–**8**) were efficiently synthesized by a versatile methodology and evaluated for their ability to inhibit the A and B isoforms of *h*MAO. The chemical structure and general synthetic procedure of coumarin derivatives **1**–**8**, with general structure **I**, were outlined in Scheme 1. A direct acylation reaction of the commercially available 3-aminocoumarin with the conveniently substituted acid chloride, using pyridine in dichloromethane, at room temperature, for 3 h, afforded the differently substituted (coumarin-3-yl)carbamates. Reaction conditions and compound characterization are described in the Experimental section.



Scheme 1. Reagents and conditions: a) substituted acid chloride, pyridine, DCM, r. t., 3 h.

2.2. Pharmacology

2.2.1. In vitro inhibition of MAO

All the described (coumarin-3-yl)carbamates (compounds **1**–**8**) were evaluated for their ability to inhibit the A and B isoforms of *h*MAO. The corresponding IC_{50} values and *h*MAO-B selectivity ratios [IC_{50} (*h*MAO-A)]/[IC_{50} (*h*MAO-B)] are shown in Table 1.

The biological evaluation of the test drugs on *h*MAO activity was investigated by measuring their effects on the production of hydrogen peroxide (H_2O_2) from *p*-tyramine (a common substrate for *h*MAO-A and *h*MAO-B), using the Amplex® Red MAO assay kit (Molecular Probes, Inc., Eugene, Oregon, USA) and microsomal MAO isoforms prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for *h*MAO-A or *h*MAO-B (Sigma–Aldrich Química S.A., Alcobendas, Spain) [42]. The production of H_2O_2 catalyzed by the two MAO isoforms can be detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red reagent), a non-fluorescent and highly sensitive probe that reacts with H_2O_2 in the presence of horseradish peroxidase to produce a fluorescent product, resorufin. New compounds and reference inhibitors were unable to react directly with the Amplex® Red reagent, which indicates that these drugs do not interfere with the measurements. On the other hand, in our experiments and under specific conditions, *h*MAO-A displayed a Michaelis constant (K_m) equal to 457.17 ± 38.62 μM and a maximum reaction velocity (V_{max}) in the control group of 185.67 ± 12.06 (nmol*p*-tyramine/min)/mg protein, whereas *h*MAO-B showed a K_m of 220.33 ± 32.80 μM and V_{max} of 24.32 ± 1.97 (nmol*p*-tyramine/min)/mg protein ($n = 5$). Most of the tested compounds concentration-dependently inhibited this enzymatic control activity (Table 1).

Table 1
*In vitro h*MAO-A and *h*MAO-B inhibitory Activities of (Coumarin-3-yl)carbamates **1**–**8** and reference compounds.[a]

Compounds	IC_{50}*h*MAO-A (μM)	IC_{50}*h*MAO-B (μM)	S. I.[b]
1	*	**	–
2	*	33.38 ± 2.25	>3.0[c]
3	*	6.63 ± 0.45	>15[c]
4	*	0.185 ± 0.012	>541[c]
5	*	*	–
6	*	5.08 ± 0.34	>19[c]
7	*	24.30 ± 1.64	>4.1[c]
8	*	0.045 ± 0.003	>2,222[c]
Selegiline	67.25 ± 1.02	0.020 ± 0.009	3431
Iproniazide	6.56 ± 0.76	7.54 ± 0.36	0.87
Rasagiline	16.44 ± 0.85	0.069 ± 0.004	238

*Inactive at 100 μM (highest concentration tested); at higher concentrations the compounds precipitate.
**100 μM inhibits the corresponding *h*MAO activity by approximately 40–45%, at higher concentrations the compounds precipitate.
[a] Each IC_{50}value is the mean ± S.E.M. from five experiments ($n = 5$).
[b] Selectivity index: *h*MAO-B selectivity ratios [IC_{50} (*h*MAO-A)]/[IC_{50} (*h*MAO-B)] for inhibitory effects of both new compounds and reference inhibitors.
[c] Values obtained under the assumption that the corresponding IC_{50} against *h*MAO-A is the highest concentration tested (100 μM).

As reported in Table 1, none of the studied (coumarin-3-yl)carbamates showed activity against MAO-A isoform at the highest tested concentration (100 μM). Activity against MAO-B isoform depended on the substituents linked to the carbamate group. Therefore, the steric bulkiness of the carbamate's alkyl substituent determinates the activity of these derivatives. Ethyl (coumarin-3-yl) carbamate (compound **2**) resulted more active against MAO-B than the methyl (coumarin-3-yl)carbamate (compound **1**), which is inactive. Isopropyl (coumarin-3-yl)carbamate (compound **3**) proved to be more active than the ethyl derivative (compound **2**). In the same way, isobutyl (coumarin-3-yl)carbamate (compound **4**) proved to be approximately 36 times more potent than the isopropyl (coumarin-3-yl)carbamate (compound **3**). One possible explanation for the activity's modulation is the importance of the volume that these additional atoms occupy in the MAO-B binding pocket. The results of the docking calculations corroborated this statement.

The introduction of a chlorine atom on the methyl group of compound **1** (compound **5**) does not seem to improve the activity. Compound **5** is inactive at the maximum tested concentration (100 μM). However, the introduction of a bromine atom on the ethyl of compound **2** (compound **6**) improved activity over six times.

Compounds **3** and **6**, with an isopropyl or a bromoethyl attached to the carbamate, presented a similar profile against MAO-B, with IC_{50} values of 6.63 and 5.08 μM, respectively.

The last strategy for the MAO-B activity modulation was the introduction of an aromatic ring to the carbamate linker. Compounds **7** and **8**, presenting an aromatic ring directly connected to the carbamate or linked by a methylene bridge, respectively, were prepared. Compound **8** proved to be 540 times more active than compound **7**. Compounds **2** and **7**, presenting an ethyl group or a benzene ring attached, respectively, to the carbamate, showed IC_{50} values against MAO-B in the same range (33.38 and 24.30 μM, respectively). Compound **8** (IC_{50}*h*MAO-B = 45 nM) presented an activity in the same range of selegiline (IC_{50}*h*MAO-B = 20 nM) and rasagiline (IC_{50}*h*MAO-B = 69 nM), presenting a high selectivity comparing to the reference compounds. These data encourage us to deeply study compound **8** as an interesting candidate for in vivo assays.

In general, the presented derivatives are not better MAO-B inhibitors than some of the previously described 3-arylcoumarins [36]. However, some of these new compounds proved to be better MAO-B inhibitors than the previously described amide derivatives [35]. These data are an important observation to follow up a rational design of more potent and selective 3-substituted coumarin derivatives.

2.2.2. In vivo assays

2.2.2.1. - Unpretreated mice. In vivo activity of derivative **8**, which resulted the most active compound of the series in the *in vitro* assays, was evaluated on unpretreated mice. This test was performed to observe the results of the administration of selegiline and compound **8** in normal mice. Doses of 10 mg/kg of compound **8** did not present significant effect on the motor activity, velocity, time moving and straightening of the animals respecting to the control group. However, selegiline caused a significant decrease of these parameters when the same dose (10 mg/kg) was used (Fig. 2).

Selegiline, which was used as MAOI-B drug reference, is metabolized to amphetamine derivatives. Therefore, it could be

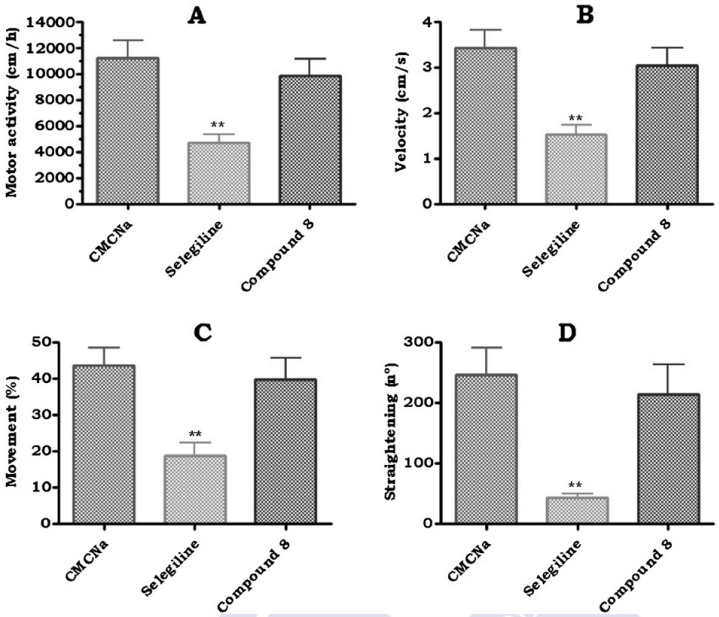


Fig. 2. Results obtained in unpretreated mice. A) Motor activity; B) Velocity; C) Movement; D) Straightening. $^{**}p < 0.01$.

expected an increase in motor activity. However, in our experiments, acutely administered and at the used dosage, significantly reduced motor activity. In the literature mixed results were found regarding its effects on motor activity [11–13].

2.2.2.2. - Potentiation effect of L-dopa/carbidopa (LD/CD) in mice pretreated with reserpine. This model aims to highlight the possible potentiation of the effect of LD in mice treated with reserpine. If the new molecule acted as a MAO-B inhibitor in vivo at the tested dose, by inhibiting the metabolism of DA, an increase in the residence time of the DA in nigrostriatal synaptic cleft would be observed. Reserpine blocks the VMAT (vesicular monoamine transporter), preventing proper storage of synthesized DA, causing depletion of dopaminergic nerve terminals and including the nigrostriatal pathway, leading to hypokinesia in experimental animals. It is important to notice that reserpine is not very selective and also affects the vesicular storage of NA and 5-HT.

Following this model, selegiline (10 mg/kg) significantly increased motor activity regarding the control group as well as the others analyzed parameters. Increasing the average speed of animals, the percentage of time moving and the number of straightening, were although quite variable. Compound **8** (10 mg/kg) showed a slight tendency to increase the motor activity as well as the percentage of time moving (Fig. 3).

In order to check the tendency of the compound **8**, the assays were repeated at a higher dose (100 mg/kg) (Fig. 4).

Increasing the dose it can be appreciated an increase in motor activity of animals treated with the compound **8**, although no statistically significant differences were obtained. The rest of the analyzed parameters: velocity, percentage of time and straightening movement behavior of the mice treated with compound **8**, followed the same pattern as in the case of motor activity.

By analyzing how motor activity, velocity and movement have evolved over time, it was possible to notice that the start of activity in mice treated with selegiline was much faster than in mice treated with compound **8**. Nevertheless, in the last minute, all the parameters evaluated for animals treated with compound **8** were comparable with those registered for mice treated with selegiline. Also, statistically significant differences were obtained compared to vehicle treated animals (Fig. 5).

During the performance of all assays, mortality in experimental animals was monitored for 24 h and 48 h, after the execution thereof. In mice treated with reserpine, the mortality was drastically reduced by administration of compound **8** at the dose of 100 mg/kg. Therefore, increasing the dosage not only improved locomotor activity, but also reduced mortality.

The observation that the compound **8** showed in vivo activity, even at high concentrations, confirmed the interest of its *in vitro* activity. Analyzing these data, it was possible to prove that compound **8** not only achieved the proposed target, but also it was capable to mimic the effects of the reference drug.

2.3. Molecular docking studies

Molecular docking simulations were performed for the most active coumarin **8**, in order to understand the enzyme-inhibitor key interactions that contribute to the most stable complex conformation. A similar docking protocol was recently used by our group [35] where the crystal structure of the *h*MAO-B in complex with 7-(3-chlorobenzyloxy)-4-carboxaldehyde-coumarin **c17** (PDB: 2V60 – Fig. 6) [43,44] was taken into account. The water molecule that establishes a hydrogen bond with the co-crystallized coumarin **c17** in the *h*MAO-B was retained and QM-polarized ligand docking combined with Prime MM-GBSA was performed with the help of Maestro software [45–47].

The validation of the docking protocol was carried out through the calculation of the RMSD (root mean square deviation) of the heavy atoms coordinates between the theoretical and experimental

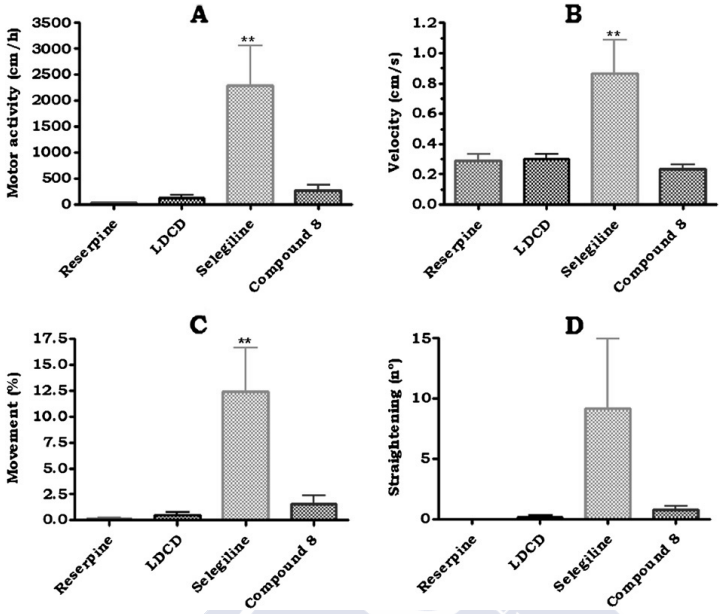


Fig. 3. Results obtained in mice pretreated with reserpine, LD/CD and selegiline or compound **8** (10 mg/kg). A) Motor activity; B) Velocity; C) Movement; D) Straightening. $^{**}p < 0.01$.

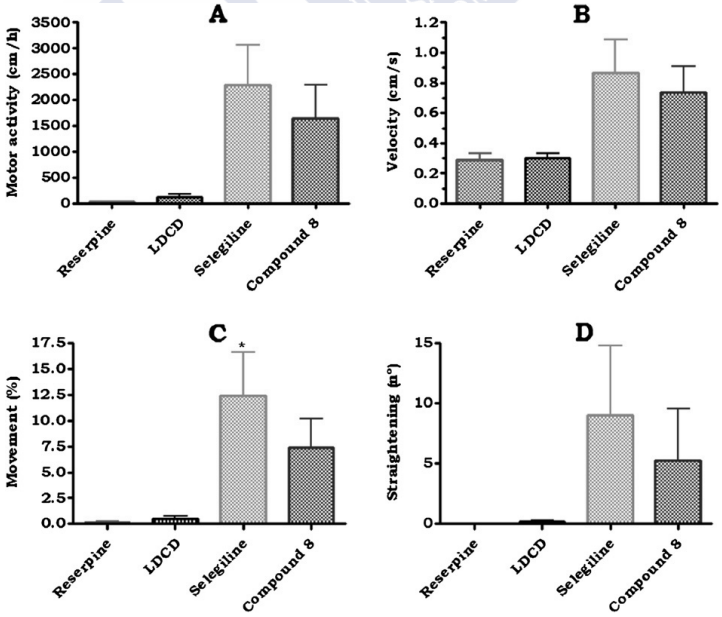


Fig. 4. Results obtained in mice pretreated with reserpine, LD/CD and selegiline (10 mg/kg) or compound **8** (100 mg/kg). A) Motor activity; B) Velocity; C) Movement; D) Straightening. $^{*}p < 0.05$.

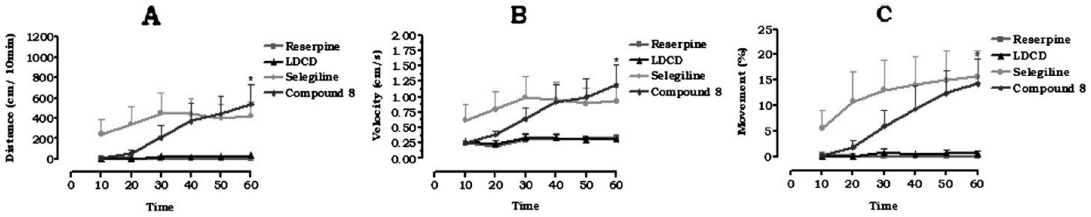


Fig. 5. Evolution of motor activity, velocity and movement of mice treated with reserpine, LD/CD and further treated with compound **8** (100 mg/kg) or selegiline (10 mg/kg). $^{*}p < 0.05$.

conformations of the ligands from different crystal structures (PDB: 2V60, 2XFN, 1OJ9, 1OJD, 2V61, 2V5Z, 2BK3, 1OJA, 4A79, 3PO7) [47].The docking was able to successfully reproduce the crystallographic orientation of the different *h*MAO-B inhibitors showing a RMSD value of 0.44 for the coumarin derivative **c17** in the 2V60 structure (RMSD values in Table 2).

The validated docking protocol was applied to the coumarin **8** and the most favourable docking conformation was retrieved from the calculation. The coumarin ring is slightly placed towards the bottom comparing to the crystallized ligand **c17** (Fig. 7). The phenyl ring in the benzopyrone system is oriented towards the FAD cofactor whereas the benzyl group is directed towards the hydrophobic pocket in the entrance cavity establishing hydrophobic interactions with residues Phe103, Pro104, Trp119, Leu164, Leu167, Phe168, Ile199 and Ile316. Different residues interact with the ligand through *van der Waals* interactions (Fig. 7b), mainly defined by Ile199, Leu171, Gln206, Tyr326, Ile198, Tyr398 and Phe168. The analysis of the binding mode also showed arene-H interactions between the residue Tyr326 and the benzopyrone ring as well as between Ile316 and the phenyl ring placed in the hydrophobic area. The results extracted from docking simulations for the compound **8** are in agreement with the binding modes described for coumarins in previous publications [35,36].

Docking studies for the *h*MAO-A have been recently published [36,39]. In this article, to study the selectivity of compound **8** towards the *h*MAO-B we also docked the compound to the *h*MAO-A. According to docking calculations, the *h*MAO-A cavity with water molecules did not have enough space to appropriately accommodate compound **8**. However, we also carried out molecular docking with no water molecules. The RMSD between the calculated and the crystal coordinates for the ligand harmine was 0.54 Å. Molecular docking results without water molecules in the *h*MAO-A showed a conformation for compound **8** placed deeply in the cavity (see Fig. 7c). This theoretical conformation would have to displace three water molecules preserved in the *h*MAO-A crystal structure that can play an important energetic role in the complex stabilization. This necessary water displacement for the compound **8** to bind the *h*MAO-A could be an energetically unfavourable process that limits ligand binding potential, explaining the lack of activity shown by compound **8** in the experimental assays. The results are in agreement with a previous publication in which the coumarin ring was shifted towards a deeper region in the case of *h*MAO-A [36].

On the other hand, both isoenzymes present some different residues in the pocket, such as the Phe208 residue in the *h*MAO-A instead of the Ile199 in the *h*MAO-B that affect the shape and nature of the cavity. This fact plays an important role and influences the stabilization of a particular ligand inside the protein pocket.

Through molecular docking simulations we analyzed the possible binding mode for the compound **8** and proposed, as said before, that the coumarin ring is directed towards the FAD cofactor in the *h*MAO-B and the benzyl group is positioned inside the hydrophobic entrance cavity in a similar way to the co-crystallized coumarin **c17** in the 2V60 structure. However, the length of the compound **8** causes the coumarin ring's position to shift towards the FAD compared to the coumarin **c17** in 2V60. On the other hand, hydrophobic substituents in the carbamate group are well accommodated in the hydrophobic entrance cavity. The usefulness of the described docking protocol is supported by the fact that docking simulations previously published showed similar binding modes for this type of compounds [35,36].On the other hand, the selectivity of some coumarins towards *h*MAO-B has been recently studied [36]. Interactions with a deeper region were found with the MAO-A, which can exert different influence in the stabilization of the ligand-protein complexes.

2.4. Theoretical evaluation of ADME properties

In order to better understand the overall properties of the described compounds, the lipophilicity (expressed as the octanol/water partition coefficient and herein called log *P*), was calculated using the Molinspiration property calculation program [48]. The

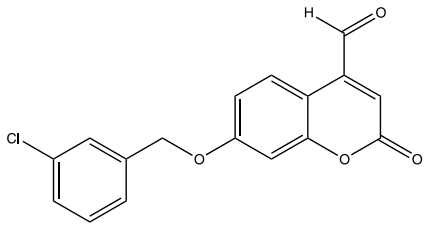


Fig. 6. Chemical structure of **c17** (7-(3-chlorobenzyloxy)-4-carboxaldehyde-coumarin).

Table 2
RMSD values between different co-crystallized ligands with *h*MAO-B and the calculated conformations through molecular docking (Å).

PDB code	RMSD (root mean square deviation)
2V60	0.44
2XFN	0.56
1OJ9	0.59
1OJD	1.17
2V61	1.30
2V5Z	1.46
2BK3	1.61
1OJA	1.87
4A79	2.20
3PO7	2.62

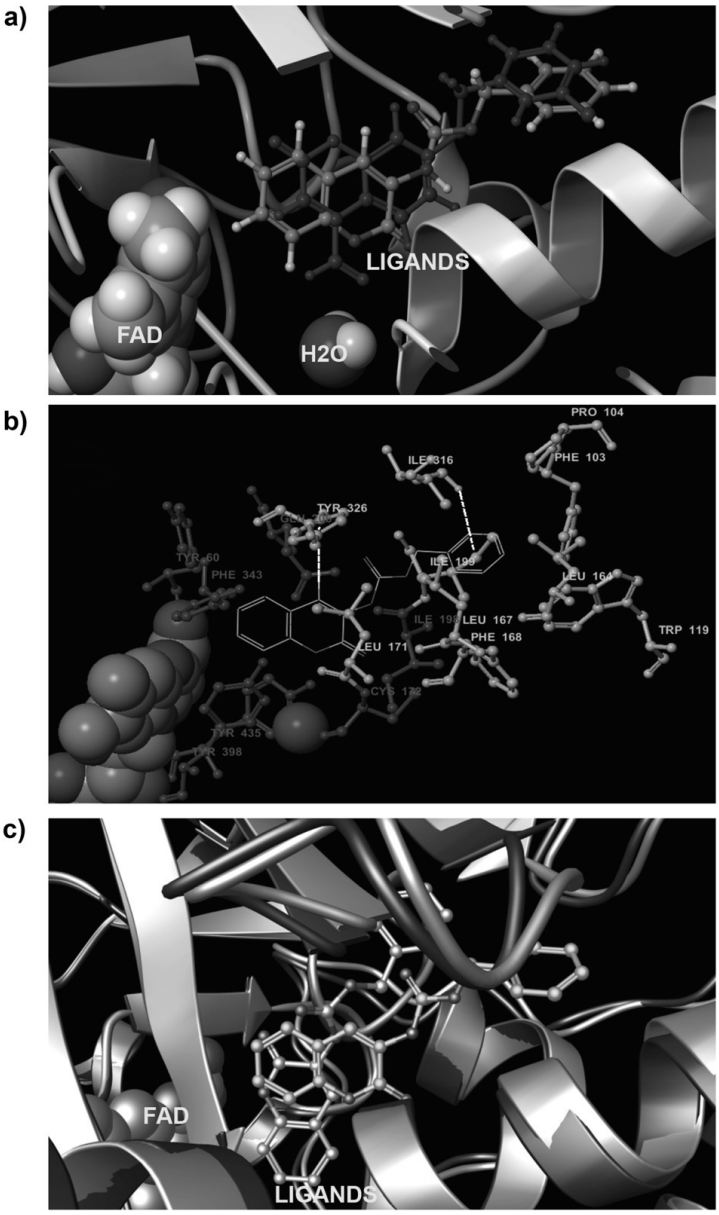


Fig. 7. a) Comparison of the co-crystallized ligand **c17** (purple color) and the calculated pose for the compound **8** (colored by element, green carbons) by the docking simulation in the *h*MAO-B . b) Analysis of the binding mode proposed for the compound **8** in the *h*MAO-B. FAD cofactor and residues in purple establish *van der Waals* interactions with the ligand. Residues in yellow establish both *van der Waals* and hydrophobic forces. Arene-H interactions with Tyr326 and Ile316 are represented in white. The FAD cofactor and the water molecule are represented in CPK. c) Comparison of the conformation of compound **8** in the *h*MAO-B (green carbons; grey ribbons) and *h*MAO-A (pink carbons; blue ribbons). The compound is placed in a deeper region in the *h*MAO-A. However, for this buried conformation to happen the compound would have to displace three water molecules preserved in the *h*MAO-A crystal structure that establish an H-bond network with the protein. The water displacement could negatively influence the ligand–protein complex formation explaining the lack of activity shown in *in vitro* assays. According to docking results, this process is not necessary for the binding to *h*MAO-B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Structural properties of the (coumarin-3-yl)carbamates derivatives **1**–**8**.[a]

Comp.	log *P*	Molecular weight	TPSA	H-bond acceptors	H-bond donors	Volume
1	1.65	219.20	68.54	5	1	185.52
2	2.25	233.22	68.54	5	1	202.32
3	2.39	247.25	68.54	5	1	218.91
4	3.00	261.28	68.54	5	1	235.71
5	2.24	253.64	68.54	5	1	199.30
6	2.61	312.12	68.54	5	1	220.45
7	3.02	281.27	68.54	5	1	240.37
8	3.24	295.29	68.54	5	1	257.17

[a] log *P* – octanol/water partition coefficient; TPSA – topological polar surface area. The data was determined with Molinspiration calculation software [48].

theoretical prediction of ADME properties (molecular weight, log *P*, number of hydrogen donors and acceptors) of all the studied compounds (**1**–**8**) was carried out and is presented in Table 3 [49,50].

From the data presented in Table 3, it was remarkable that all the described coumarin derivatives possess log *P* values compatible with those required to cross membranes. TPSA, described to be a predictive indicator of membrane penetration, was also found to be positive. In addition, it can be observed that no violations of Lipinski's rule (molecular weight, log *P*, number of hydrogen donors and acceptors) were found. As MAOIs have to pass different membranes and reach the CNS, this supports the potential of these derivatives as drug candidates.

3. Conclusion

In this study, a general and efficient synthesis of a new series of (coumarin-3-yl)carbamates was developed. Most of the synthesized derivatives were selective inhibitors of MAO-B isoform in the micro or nanoMolar range. In addition, it can be observed that no violations of Lipinski's rule were observed for all the studied derivatives. Therefore, the described compounds may present desirable ADME properties. Benzyl(coumarin-3-yl)carbamate (compound **8**), the most active compound *in vitro* against MAO-B, was evaluated *in vivo*. In mice pretreated with reserpine, the administration of compound 8 (100 mg/kg) resulted in an increase of the motor activity, velocity and movement comparable with selegiline, in the last minute of the experiment. Molecular docking simulations were also performed to study the binding mode of the compound **8** inside the *h*MAO-B, further analyzing isoenzyme selectivity and rationalizing the drug-design of this type of derivatives.

4. Experimental protocols

4.1. General

Starting materials and reagents were obtained from commercial suppliers and were used without further purification (Sigma–Aldrich). Melting points (mp) are uncorrected and were determined with a Reichert Koflerthermopan or in capillary tubes in a Büchi 510 apparatus. ^{1}H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) spectra were recorded with a Bruker AMX spectrometer using DMSO-d_6 or $CDCl_3$as solvent. Chemical shifts (δ) are expressed in ppm using TMS as an internal standard. Coupling constants (J) are expressed in Hz. Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets), and m (multiplet). Mass spectrometry was carried out with a Hewlett–Packard-5972-MSD spectrometer. Elemental analyses were performed with a PerkinElmer 240B microanalyzer and are within 0.4% of calculated values in all cases. Flash chromatography (FC) was performed on silica gel (Merck 60, 230–400 mesh); analytical TLC was performed on pre-coated silica gel plates (Merck 60 F254). Organic solutions were dried over anhydrous Na_2SO_4. Concentration and evaporation of the solvent after reaction or extraction was carried out on a rotary evaporator (Büchi Rotavapor) operating at reduced pressure. The analytical results showed >95% purity for all compounds.

4.2. General methodology to prepare (coumarin-3-yl)carbamates **1**–**8**

3-Aminocoumarin (1 mmol) was dissolved in dichloromethane (9 mL) and pyridine (1.1 mmol) was added. The mixture was cooled to 0 °C. The corresponding acid chloride (1.1 mmol) was added dropwise at this temperature, and the mixture was stirred, at room temperature, for 3 h. The solvent was evaporated under vacuum and the dry residue was purified by FC (hexane/ethyl acetate 9:1) to give the desired (coumarin-3-yl)carbamates **1**–**8** [51,52].

4.2.1. Methyl (coumarin-3-yl)carbamate (**1**)

Orange needles. Yield 64%. mp 130–131 °C. ^{1}H NMR (300 MHz; DMSO-d_6) δ (ppm): 3.68 (s, 3H, OCH_3), 7.42 (m, 1H, H-6), 8.05 (t, 1H, J = 7.5 Hz, H-8), 8.23 (s, 1H, H-4), 8.55 (td, 1H, J = 7.7, J = 1.4 Hz, H-7), 8.90 (dd, 1H, J = 7.5, J = 1.3 Hz, H-5), 9.18 (s, 1H, NH). ^{13}C NMR (75 MHz; DMSO-d_6) δ: 62.1, 90.7, 115.6, 116.0, 122.7, 127.2, 127.3, 142.4, 145.9, 146.0, 159.2. EI-MS *m/z* 220 (15), 219 (M^+, 100), 188 (22), 187 (96), 147 (22), 132 (82), 104 (25), 77 (28), 59 (11). Anal. Calcd. for $C_{11}H_9NO_4$: C, 63.16; H, 4.24. Found: C, 63.14; H, 4.21.

4.2.2. Ethyl (coumarin-3-yl)carbamate (**2**)

Pale yellow needles. Yield 74%. mp 113–114 °C. ^{1}H NMR (300 MHz; $CDCl_3$) δ (ppm): 1.31 (t, 3H, J = 7.2 Hz, CH_3), 4.24 (dd, 2H, J = 14.2, J = 7.1 Hz, CH_2), 7.24–7.30 (m, 2H, H-6, H-8), 7.39 (td, 1H, J = 7.3, J = 1.0 Hz, H-7), 7.46 (dd, 1H, J = 7.7, J = 1.5 Hz, H-5), 7.49 (s, 1H, NH), 8.27 (s, 1H, H-4). ^{13}C NMR (75 MHz; $CDCl_3$) δ: 14.4, 61.9, 116.3, 119.3, 120.8, 124.3, 125.0, 127.4, 129.2, 149.6, 153.3, 158.5. EI-MS *m/z* 234 (38), 233 (M^+, 100), 161 (12), 134 (27), 98 (50), 84 (30), 69 (41), 56 (66). Anal. Calcd. for $C_{12}H_{11}NO_4$: C, 61.80; H, 4.75. Found: C, 61.83; H, 4.78.

4.2.3. Isopropyl (coumarin-3-yl)carbamate (**3**)

Orange needles. Yield 65%. mp 94–95 °C. ^{1}H NMR (300 MHz; DMSO-d_6) δ (ppm): 1.24 (dd, 6H, J = 6.2, J = 1.5 Hz, OCH_3), 4.90 (td, 1H, J = 6.2, J = 1.5 Hz, CH), 7.29–7.37 (m, 1H, H-6), 7.40 (d, 1H, J = 6.0 Hz, H-8), 7.45-7.52 (m, 1H, H-7), 7.68 (d, 1H, J = 6.0 Hz, H-5), 8.22 (s, 1H, H-4), 8.90 (s, 1H, NH). ^{13}C NMR (75 MHz; DMSO-d_6) δ: 21.3, 62.1, 100.7, 115.6, 116.0, 122.7, 127.2, 127.3, 142.4, 145.9, 146.0, 159.2. EI-MS *m/z*248 (9), 247 (M^+, 20), 220 (15), 219 (100), 188 (22), 187 (96), 147 (22), 132 (82), 104 (25), 77 (28), 59 (11). Anal. Calcd. for $C_{13}H_{13}NO_4$: C, 63.15; H, 5.30. Found: C, 63.14; H, 5.28.

4.2.4. Chloromethyl (coumarin-3-yl)carbamate (**5**)

Yellow needles. Yield 75%. mp 129–130 °C. ^{1}H NMR (300 MHz; DMSO-d_6) δ (ppm): 5.97 (s, 2H, CH_2Cl), 7.31–7.41 (m, 2H, H-6, H-8), 7.52 (dd, 1H, J = 7.7, J = 1.2 Hz, H-7), 7.73 (dd, 1H, J = 7.7, J = 1.2 Hz, H-5), 8.27 (s. 1H, H-4), 9.87 (s, 1H, NH). ^{13}C NMR (75 MHz; DMSO-d_6) δ: 71.2, 99.8, 116.0, 119.3, 124.0, 125.1, 128.2, 130.3, 150.3, 151.7, 157.3. EI-MS *m/z*255 (33), 254 (12), 253 (M^+, 71), 188 (52), 187 (100), 174 (58), 160 (22), 132 (88), 104 (35), 77 (35), 51 (30). Anal. Calcd. for $C_{11}H_8ClNO_4$: C, 52.09; H, 3.18. Found: C, 53.00; H, 3.21.

4.2.5. 2-Bromoethyl (coumarin-3-yl)carbamate (**6**)

Pale yellow needles. Yield 71%. mp 129–130 °C. ^{1}H NMR (300 MHz; DMSO-d_6) δ (ppm): 3.73 (t, 2H, J = 5.6 Hz, CH_2Br), 4.45 (t, 2H, J = 5.6 Hz, CH_2), 7.30-7.41 (m, 2H, H-6, H-8), 7.50 (dd, 1H, J = 7.7,

J = 1.5 Hz, H-7), 7.70 (dd, 1H, J = 7.7, J = 1.4 Hz, H-5), 8.22 (s, 1H, H-4), 9.25 (s, 1H, NH). ^{13}C NMR (75 MHz; DMSO-d_6) δ: 22.4, 64.9, 116.0, 118.7, 124.5, 125.1, 128.0, 129.9, 134.3, 149.9, 153.3, 157.5. EI-MS m/z314 (8), 313 (M^+, 29), 312 (4), 311 (28), 187 (24), 174 (27), 161 (23), 132 (100), 109 (38), 107 (40), 103 (20), 77 (60), 51 (48). Anal. Calcd. for $C_{12}H_{10}BrNO_4$: C, 46.18; H, 3.23. Found: C, 46.20; 3.25.

4.2.6. Benzyl (coumarin-3-yl)carbamate (**8**)

Pale yellow needles. Yield 80%. mp 107–108 °C. ^{1}H NMR (300 MHz; $CDCl_3$) δ(ppm): 5.21 (s, 3H, CH_2), 7.27–7.36 (m, 3H, H-6, H-7, H-8), 7.38-7.41 (m, 5H, H-2′, H-3′, H-4′, H-5′, H6′), 7.47 (dd, 1H, J = 7.7, J = 1.5 Hz, H-5), 7.60 (s, 1H, H-4) 8.30 (s, 1H, NH). ^{13}C NMR (75 MHz; $CDCl_3$) δ: 67.5, 116.3, 119.8, 121.0, 124.1, 125.1, 127.4, 128.2, 128.5, 128.7, 129.2, 135.5, 149.6, 153.0, 158.3. EI-MS m/z296 (25), 295 (M^+, 100), 251 (12), 91 (58). Anal. Calcd. for $C_{17}H_{13}NO_4$: C, 68.42; H, 4.42. Found: C, 68.45; H, 4.46.

4.3. Determination of MAO isoforms activity

4.3.1. In vitro assays

The effects of the new synthesized compounds on *h*MAO isoform enzymatic activity were evaluated by a fluorimetric method following the experimental protocol previously described by us [42]. Briefly, 0.1 mL of sodium phosphate buffer (0.05 M, pH 7.4) containing the test drugs (new compounds or reference inhibitors) in various concentrations and adequate amounts of recombinant *h*MAO-A or *h*MAO-B required and adjusted to obtain in our experimental conditions the same reaction velocity, (*h*MAO-A: 1.1 mg protein; specific activity: 150 nmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min/mg protein; *h*MAO-B: 7.5 mg protein; specific activity: 22 nmol of *p*-tyramine transformed/min/mg protein) were incubated for 15 min at 37 °C in a flat-blackbottom 96-well microtest™ plate, placed in the dark fluorimeter chamber. After this incubation period, the reaction was started by adding (final concentrations) 200 μM Amplex® Red reagent, 1 U/mL horseradish peroxidase and 1 mM *p*-tyramine. The production of H_2O_2 and, consequently, of resorufin was quantified at 37 °C in a multidetection microplate fluorescence reader (FLX800™, Bio-Tek® Instruments, Inc., Winooski, VT, USA) based on the fluorescence generated (excitation, 545 nm, emission, 590 nm) over a 15 min period, in which the fluorescence increased linearly. Control experiments were carried out simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions of the vehicles. In addition, the possible capacity of the above test drugs to modify the fluorescence generated in the reaction mixture due to non-enzymatic inhibition (e.g., for directly reacting with Amplex® Red reagent) was determined by adding these drugs to solutions containing only the Amplex® Red reagent in a sodium phosphate buffer. The specific fluorescence emission (used to obtain the final results) was calculated after subtraction of the background activity, which was determined from vials containing all components except the *h*MAO isoforms, which were replaced by a sodium phosphate buffer solution.

4.3.2. In vivo assays

4.3.2.1. Animals. Charles River "CD1" mice weighing 30 ± 5 g were housed in groups of 4 or 8 animals under regulated conditions (light/dark cycle between 8.00 and 20.00 h at 21 ± 2 °C) in standard Makrolon cages (215 × 465 × 145 mm^3). The animals received standard laboratory chow and tap water ad libitum until the beginning of the experiments.

4.3.2.2. Drugs and chemicals. Reserpine, LD (200 mg/kg) and selegiline (10 mg/kg) were supplied by Sigma–Aldrich (St. Louis, MO, USA) and CD (50 mg/kg) was kindly provided by DupontPharma. Sodium carboxymethyl cellulose (CMCNa) was supplied by Merck (Madrid, Spain). All the used chemicals were of analytical grade. In behavioral experiments all compounds were administered in0.01 mL/g intraperitoneal (ip) injections. A suspension of 1% CMCNa was used as control whileselegiline was used as MAO-B drug baseline. Compound **8** was tested at doses of 10 and 100 mg/kg, in a suspension of 1% CMCNa.

4.3.2.3. Locomotor activity. The locomotor activity of animals in square arenas (50 × 50 × 30 cm^3) under a video camera suspended from the ceiling was recorded over a 1 h period between 10.00 and 14.00 h and analyzed using a computerized animal observation system (EthovisionV. 3.1.16, Noldus Information Technology, Wageningen, The Netherlands), located in a separated room. The variables considered were distance traveled (in cm), velocity (cm/s), % time moving during the test run and straightening.

4.3.2.4. Protocol. Animal models for study were twofold:

- Unpretreated mice: Compound **8** was tested by ip administration at doses of 10 mg/kg or 100 mg/kg in untreated mice, comparing with the vehicle (1% CMCNa) and selegiline (10 mg/kg). The pharmacological effect was evaluated during 1 h immediately after administration.
- Potentiation effect of LD/CD in mice pretreated with reserpine: This model involves the administration of reserpine (2.5 mg/kg, ip) to the animals 22 h before the completion of each trial. After this time, the vehicle (1% CMCNa) or new molecules under test were administered. LD/CD (200:50 mg/kg) were administered 30 min later. Locomotor activity was evaluated 1 h after the last administration during a period of 1 h.

4.3.2.5. Expression of results, plotting and statistical analysis. Results are expressed as mean ± standard error of the mean (x ± SEM). The minimum number of animals used in each assay was 8 (n = 8). The graphical representation and statistical analysis were performed using GraphPad Prism (V 4.3) (San Diego, USA). Statistically significant differences were determined by one-way ANOVA (treatment) followed by the Dunnet test or by two-way ANOVA (treatment-time) followed by Boferroni test.

4.4. Molecular docking

Maestro software available in Schrödinger 2011 package [45] was used to carry out all the molecular docking simulations.

4.4.1. Protein and ligand preparation

Protein Preparation Workflow in Maestro software [45] was used to pre-process the crystal structure of the *h*MAO-B in complex with the coumarin **c17** (PDB code: 2V60) and the crystal structure of the *h*MAO-A with the ligand harmine (PDB code: 2Z5X). Only the water molecule that establishes a hydrogen bond with the co-crystallized coumarin was retained in the calculations for the *h*MAO-B. Two different protocols were followed in the *h*MAO-A: 1) no water molecules were retained in the cavity protein and 2) only waters within 5 Å from the ligand were retained. Hydrogen were added and energy minimized with the OPLS_2005 force field. Optimizations of the hydrogen bonding network and the protonation states of some residues were also carried out through this procedure.

The dataset of different ligands belonging to *h*MAO-B and *h*MAO-A as well as the coumarin derivative **8** were prepared with the LigPrep module [53]. Protonation states at pH 7.0 ± 2.0 and tautomers were generated and optimized using OPLS_2005 force field.

Table 4
Experimental and calculated affinities (p*Ki*) for different known ligands.

Compounds[a]	*h*MAO-B		Compounds	*h*MAO-A	
	Experimental p*Ki*[b]	Calculated p*Ki*		Experimental p*Ki*	Calculated p*Ki*[c]
2V60	6.40	7.65	2Z5X	8.30	7.60
2V61	7.00	7.89	CHEMBL1813518	5.81	7.26
2V5Z	6.35	7.12	CHEMBL466872	7.21	7.28
2BK3	6.10	6.92	CHEMBL1645551	6.30	7.55
1OJA	5.52	5.89	CHEMBL602466	7.29	6.53
3PO7	5.51	6.38	CHEMBL1645547	8.44	7.81
CHEMBL1813523	8.52	7.41	CHEMBL1645545	8.41	7.73
CHEMBL1642678	9.55	7.09			
CHEMBL466872	8.40	7.00			

	*h*MAO-B		*h*MAO-A	
	Experimental IC_{50}	Calculated p*Ki*[d]	Experimental IC_{50}	Calculated p*Ki*
Compound **8**	0.045 μM	7.01	Inactive at 100 μM	6.69

[a] PDB and CHEMBL codes.
[b] p*Ki* values extracted from Binding DB.
[c] Calculated values using the *h*MAO-A docking with no water in the pocket (for the crystallized harmine in 2Z5X, both *h*MAO-A docking protocols were taken into account).
[d] Calculated p*Ki* for compound **8** is provided through the equations with $r = 0.42$ and $r = 0.41$ for the *h*MAO-B and *h*MAO-A respectively.

4.4.2. Ligand docking procedure

A receptor grid was calculated and centred in the co-crystallized ligand **c17** in the *h*MAO-B and in the harmine in the *h*MAO-A with an outer box length of 20 Å. The receptor *van der Waals* scaling factor was 1.0 with a partial charge cut-off of 0.25. The docking protocol included QM-polarized ligand docking [46]. The ligands were docked to the protein structure using Standard Precision (SP) level and three poses per ligand were retained. New partial charges of the ligands inside the protein pocket were calculated with the help of Jaguar module [54]. The ligands with the improved charges were redocked using Extra Precision (XP) mode and three poses per ligand were retained. Finally, the best pose according to the energy score E_{model} was optimized using MM-GBSA in Prime module [53] taking into account a flexible protein region defined by 5 Å from the ligand.

Average MMGBSA free energy of binding calculated from the three complexes extracted from QM-polarized docking was calibrated with the experimental affinity (p*Ki*) for different known ligands (data shown in Table 4).

Acknowledgments

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CONCISE ARTICLE

3-Substituted coumarins as dual inhibitors of AChE and MAO for the treatment of Alzheimer's disease

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The complex etiology of Alzheimer's disease (AD) has encouraged active research in the development of multi-target drugs with two or more complementary biological activities, since they may represent an important advance in the treatment of this disease. A series of 3-substituted coumarins were synthesized and evaluated as monoamino oxidases (MAO) and acetylcholinesterase (AChE) inhibitors. Most of the 3-benzamide coumarin derivatives inhibited both MAO-B and AChE with values in the micromolar range. It might be a promising direction for developing novel drugs as potential agents for the treatment of AD patients.

1. Introduction

The frequency, morbidity and complexity of neurodegenerative diseases make them the greatest therapeutic challenge to medicine today. Among them, Alzheimer's disease (AD) is the most common cause of senile dementia, affecting approximately 3% of the population between the ages of 65–74 and nearly 50% of those 85 years and older.[1] This disease is characterized by a decreased number of cholinergic neurons in the hippocampus and amygdala, which is clinically reflected in the appearance of specific symptoms: progressive impairment in memory and intellectual ability to perform basic activities of daily living.[2] Although the etiology of AD is not completely known, deposits of aberrant proteins, namely β-amyloid (Aβ) and τ-protein, oxidative stress, dyshomeostasis of biometals and low levels of acetylcholine (ACh) seem to play significant roles.[3] Monoamine oxidase B (MAO-B) activity is also increased in association with gliosis, which can result in higher levels of H_2O_2 and oxidative free radicals which are a possible source of oxidative stress for vulnerable neurons affected by AD.[4] Anti-acetylcholinesterase (anti-AChE) agents such as tacrine, galantamine, and donepezil have shown a modest improvement in memory and cognitive function but do not appear to prevent or slow the progressive neurodegeneration.[5] On the other hand, rasagiline, a selective MAO-B inhibitor, has been reported to retard the further deterioration of cognitive functions, displaying neuroprotective activity.[6] Because of the complexity of this disease, drugs hitting a single target may be inadequate for the treatment of AD. It has encouraged active research in the development of multifunctional drugs with two or more complementary biological activities, since they may represent an important advance in the treatment of the disease.[7,8] Recently, a new bifunctional drug, ladostigil, was developed by combining the neuroprotective effects of rasagiline with the cholinesterase inhibitory activity in a single molecule. Ladostigil inhibits both cholinesterase (acetylcholinesterase and butyrylcholinesterase) and brain monoamine oxidases (MAO-A and MAO-B).[9]

Coumarins are a large family of compounds, of natural and synthetic origin, that display a variety of pharmacological properties. Due to their structural variability they occupy an important place in the realm of natural products and synthetic organic chemistry. Recent studies pay special attention to their antioxidative and enzymatic inhibition properties.[10] Numerous functionalized coumarins have been presented as potent MAO and/or AChE inhibitors and some of them have been proposed for treating AD.[11,12] Substitution in position 3 of the coumarin nucleus modulated AChE as well as MAO-B inhibitory activity.[12] 3-(Piperazine-1-carbonyl)coumarins showed significant anti-AChE activities.[13] Phenyl, methyl, carboxylic acids, ethyl ester or acyl chloride groups in that position resulted in potent MAO inhibitors.[14] Our research group has reported, in several studies, the importance of the MAO inhibitory activity of different substituents on the phenyl ring in position 3 of the coumarin (Fig. 1—**I**).[15–18] Our work is now focused on modifying the molecular structure of these potent MAO-B inhibitors, introducing either an arylamide group (amidic group between the coumarin and the 3-phenyl ring) (Fig. 1—**II**) or an alkylamide group in that position (Fig. 1—**III**) in order to provide them with additional biological properties, such as AChE inhibition, becoming useful for treating AD.

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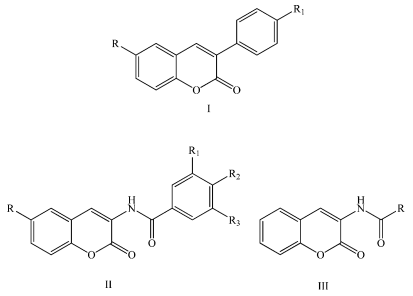
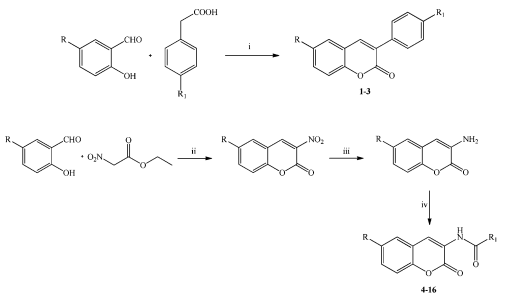


Fig. 1 General structures of the coumarin derivatives.

2. Chemistry

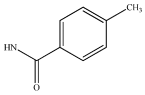
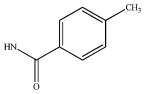
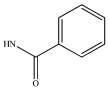
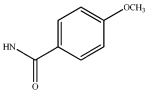
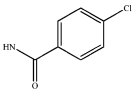
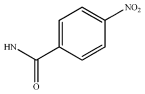
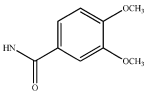
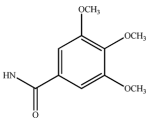
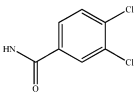
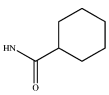
Coumarin derivatives **1–3**, with general structures **I**, were synthesized according to the protocol outlined in Scheme 1, under Perkin condensation conditions, starting from the corresponding *ortho*-hydroxybenzaldehyde and the substituted arylacetic acid.[15–20] Compounds **4–16**, with general structures **II** and **III**, were also efficiently synthesized according to the protocol outlined in Scheme 1.[21–28] Coumarins **4–16** were prepared starting from a condensation of a commercially available salicylaldehyde and the ethyl nitroacetate. The reaction was performed in a dry Schlenk tube, with acetic anhydride as solvent, in the presence of sodium hydride, at room temperature, for three hours. The 3-aminocoumarins were prepared from previously synthesized 3-nitrocoumarins, in ethanol, with Pd/C as catalyst under H_2 atmosphere. An acylation reaction of the 3-aminocoumarin with the conveniently substituted acid chloride, using pyridine and dichloromethane, at room temperature, for three hours, afforded the differently substituted 3-amidocoumarins **4–16**.

The chemical structure of the synthesized compounds **1–16** is detailed in Table 1. Reaction conditions and compound characterization are described in the Experimental section.



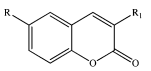
Scheme 1 *Reagents and conditions*: (i) DCC, DMSO, 110 °C, 24 h; (ii) NaH, Ac_2O, r. t., 3 h; (iii) H_2, EtOH, Pd/C, r. t., 3 h; (iv) substituted acid chloride, pyridine, DCM, r. t., 3 h.

Table 1 Structures of the studied compounds **1–16**

Compounds	*R*	R_1
1	H	
2	CH_3	
3	CH_3	
4	CH_3	
5	H	
6	H	
7	H	
8	H	
9	H	
10	H	
11	H	
12	H	
13	H	

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Table 1 (*Contd.*)

Compounds	*R*	R_1
		
14	H	
15	H	
16	H	

3. Pharmacology

3.1. Inhibition of MAO

The potential effects of the newly synthesized compounds on hMAO activity were investigated by measuring their effects on the production of hydrogen peroxide (H_2O_2) from *p*-tyramine, using the Amplex Red MAO assay kit (Molecular Probes, Inc., Eugene, Oregon, USA) and MAO isoforms in microsomes prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for hMAO-A or hMAO-B (Sigma-Aldrich Química S.A., Alcobendas, Spain). The inhibition of hMAO activity was evaluated using the above method following the general procedure described previously by us.[29] The test compounds did not show any interference with the reagents used for a biochemical assay. The control activity of hMAO-A and hMAO-B using *p*-tyramine as the common substrate was 165 ± 2 pmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde per min ($n = 20$).

The results of the hMAO-A and hMAO-B inhibition studies with our compounds are reported in Table 2 together with the MAO-B selectivity index. Enzymatic assays revealed that most of the test compounds were moderate to potent hMAO inhibitors at either low micromolar to nanomolar concentrations, showing selectivity toward hMAO-B.

3.2. Inhibition of AChE

The method of Ellman[30] was used to determine the *in vitro* cholinesterase activity. The activity was measured by the increase in absorbance at 412 nm due to the yellow color of 5-mercapto-2-nitrobenzoic acid produced by the reaction of thiocholine with dithiobisnitrobenzoate. Thiocholine was generated from acetylthiocholine using human recombinant AChE expressed in HEK 293 cells (Sigma-Aldrich Química S.A., Alcobendas, Spain). The control activity from hAChE using acetylthiocholine as substrate was 0.66 ± 0.13 mM of acetylthiocholine to oxidized thiocholine per min ($n = 20$).

Assays were performed with a blank containing all components except AChE, in order to account for nonenzymatic reaction. The reaction rates were compared and the percent inhibition due to the presence of test compounds was calculated and the IC_{50} values are reported in Table 3. These data show that some of the compounds exhibited moderate inhibitory activity against the AChE.

Table 2 IC_{50} values and hMAO-B selectivity index for the new compounds and reference inhibitors on the enzymatic activity of human recombinant MAO isoforms expressed in baculovirus-infected BTI insect cells[a]

Compounds	MAO-A (IC_{50})	MAO-B (IC_{50})	SI MAO-B[e]
1	[c]	11.81 ± 0.80 μM	>8.5[f]
2	[c]	283.75 ± 25.50 nM	>352[f]
3	[c]	308.52 ± 20.69 pM	>324,128[f]
4	45.97 ± 3.11 μM[a]	0.17 ± 0.01 μM	270
5	[c]	7.96 ± 0.53 μM	>12.6[f]
6	[c]	0.76 ± 0.05 μM	>131[f]
7	[c]	30.50 ± 2.06 μM	>3.3[f]
8	[c]	1.95 ± 0.13 μM	>52[f]
9	[c]	[c]	
10	[c]	58.38 ± 3.91 μM	>1.7[f]
11	[c]	72.58 ± 4.90 μM	>1.4[f]
12	[c]	5.95 ± 0.40 μM	>16.8[f]
13	[c]	49.96 ± 3.35 μM	>2.0[f]
14	38.93 ± 2.63 μM	[d]	<0.40[f]
15	[c]	[c]	
16	[c]	[c]	
R-(−)-Deprenyl	67.25 ± 1.02 μM[b]	14.80 ± 0.99 nM	4,544
Iproniazide	6.56 ± 0.76 μM	7.54 ± 0.36 μM	0.87

[a] Each IC_{50} value is the mean ± SEM from five experiments ($n = 5$). [b] Level of statistical significance: $P < 0.01$ *versus* the corresponding IC_{50} values obtained against MAO-B, as determined by ANOVA/Dunnett's. [c] Inactive at 100 μM (highest concentration tested). At higher concentrations the compounds precipitate. [d] 100 μM inhibits enzymatic activity by approximately 40–45%. [e] SI MAO-B = [IC_{50}(MAO-A)]/[IC_{50}(MAO-B)]. [f] Values obtained under the assumption that the corresponding IC_{50} against either MAO-A or MAO-B is the highest concentration tested (100 μM).

4. Results and discussion

Compounds **1–16** were prepared and tested for their *in vitro* inhibitory activity against MAO-A and -B isoforms, in

Table 3 IC_{50} values index for the new compounds and reference inhibitors on the enzymatic activity of human recombinant AChE expressed in HEK 293 cells[a]

Compounds	IC_{50} AChE/μM
1	[b]
2	[b]
3	[b]
4	41.37 ± 2.80
5	18.38 ± 1.23
6	[b]
7	69.47 ± 4.65
8	18.71 ± 0.84
9	12.89 ± 0.70
10	[b]
11	[b]
12	15.01 ± 0.68
13	[b]
14	[b]
15	[b]
16	[b]
Tacrine	0.15 ± 0.01
Eserine	0.16 ± 0.01

[a] Each IC_{50} value is the mean ± SEM from five experiments ($n = 5$). [b] Inactive at 100 μM (highest concentration tested). At higher concentrations the compounds precipitate.

microsomes prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for MAO-A or -B as well as against AChE recombinant, expressed in HEK 293 cells.

Aryl substitutions in position 3 of the coumarin moiety proved to be important for the MAO-B activity and selectivity.[15–18] Also, the presence of an amidic group in the same position was an important structural change to improve the AChE inhibitory activity.[13] Therefore, the introduction of both structural fragments in that position was thought as a possible strategy to develop dual MAO-B/AChE inhibitors.

According to our previous results, 3-arylcoumarins **1–3** showed activity against MAO-B isoform in the micro, nano and picomolar range. The introduction of a methyl group in the six position of the coumarin moiety improved the MAO-B activity and selectivity approximately 50-fold (comparing compounds **2** and **1**). Additionally, the introduction of another methyl group in position *para* of the 3-aryl ring of the same scaffold increased the MAO-B inhibitory activity and the selectivity almost 1000-fold (comparing compounds **3** and **2**). None of the above-mentioned compounds showed inhibitory activity against AChE.

Taking into account that compound **3** was the most potent MAO-B inhibitor, an amidic group was introduced between the coumarin moiety and the 3-aryl ring. This new derivative (compound **4**) presented inhibitory activity against AChE and both MAO isoforms, in the micromolar range. Despite these results, the presence of both methyl groups in the six position and the amidic group between the coumarin and 3-aryl ring leads to an unwanted loss of MAO selectivity.

Considering the 3-benzamidecoumarins as a new scaffold with the desirable dual MAO-/AChE inhibitory activities, modifications under this skeleton were done in order to improve the MAO-B selectivity and the AChE inhibitory activity. The elimination of the methyl group in the six position (compound **5**) leads to a selective MAO-B inhibitor, with an increase in AChE inhibitory activity. Based on these experimental data, the importance of the substitutions under the aryl ring of the 3-benzamidecoumarins was studied. Unsubstituted 3-benzamide coumarin (compound **6**) improved the MAO-B activity approximately 10-fold compared with compound **5**. However, this compound proved to be inactive against AChE, showing that the substituents under the aryl ring are important to modulate the AChE inhibitory activity. Therefore, different substituents were introduced in several positions of this ring. A methoxy group incorporated in the *para* position of the ring (compound **7**) seems to be less favorable for the AChE and MAO-B inhibition than a methyl group (compound **5**). A chloro group in the same position allows a new compound (**8**) with similar AChE inhibitory activity to compound **5** and a slight increase of the inhibitory MAO-B activity. Introduction of a nitro group at the same position (compound **9**) leads to a slightly more active compound against AChE, but inactive against MAO-B.

Another strategy to modulate the dual AChE/MAO-B activities was the introduction of more than one substituent under the aryl ring. Introduction of additional methoxy groups in this ring leads to inactive AChE inhibitory compounds (**10**, **11**), also with a decrease in the MAO-B inhibitory activity. However, introduction of a second chloro atom in the *meta* position of the aryl ring resulted in a compound (**12**) with similar AChE and MAO-B inhibitory activities to the mono-substituted derivative (compound **8**). From the experimental results it can be observed that the nature, number and position of the substituents in the aryl ring are important to modulate the AChE and MAO-B inhibitory activities.

Finally, in order to determine the importance of the aryl ring, it was replaced with an alkyl or haloalkyl group (compounds **13–16**). None of these compounds presented inhibitory activity against AChE. Compound **13**, with a cycloalkyl linked to the amidic group, instead of an aromatic ring, also presented inhibitory activity against MAO-B. However, this activity is approximately 66-fold less than that presented by compound **6**. Substitution by a less bulky group leads to a compound (**14**) which presents inhibitory activity against MAO-A, in the micromolar range. Introduction of a haloalkyl group in the same position leads to inactive compounds against both MAO isoforms and AChE (compounds **15** and **16**).

5. Experimental

5.1. General methods

Starting materials and reagents were obtained from commercial suppliers and were used without purification. Melting points (mp) are uncorrected and were determined with a Reichert Kofler thermopan or in capillary tubes in a Buchi 510 apparatus. ^{1}H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) spectra were recorded with a Bruker AMX spectrometer using DMSO-d_6 or $CDCl_3$ as solvent. Chemical shifts (δ) are expressed in parts per million (ppm) using TMS as an internal standard. Coupling constants J are expressed in hertz (Hz). Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets) and m (multiplet). Mass spectrometry was carried out with a Kratos MS-50 or a Varian MAT-711 spectrometer. Elemental analyses were performed by a Perkin-Elmer 240B microanalyzer and were within $\pm$0.4% of calculated values in all cases. The analytical results were $\geq$95% purity for all compounds. Flash Chromatography (FC) was performed on silica gel (Merck 60, 230–400 mesh); analytical TLC was performed on precoated silica gel plates (Merck 60 F254). Organic solutions were dried over anhydrous sodium sulfate. Concentration and evaporation of the solvent after reaction or extraction were carried out on a rotary evaporator (Büchi Rotavapor) operating at reduced pressure.

5.2. Synthesis

5.2.1. General synthetic methodology for the 3-amidocoumarin moiety (4–16). General methodology to prepare 3-nitrocoumarins: In a 20-mL dry Schlenk tube, to a solution of the conveniently substituted salicylaldehyde (2.5 mmol) and ethyl nitroacetate (2.5 mmol), in acetic anhydride, NaH (2.5 mmol) was added in small portions, and the reaction mixture was stirred for 3 hours, at room temperature. The obtained crude was filtered and washed with diethyl ether. The solid was then purified by flash chromatography (hexane/ethyl acetate 9 : 1) to give the desired coumarins.

General methodology to prepare 3-aminocoumarins: the previously prepared 3-nitrocoumarin (2.5 mmol) was dissolved in ethanol and a catalytic amount of Pd/C was added to the

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mixture. The solution was stirred, at room temperature, under H_2 atmosphere, for 5 hours. After the completion of the reaction, the mixture was filtered to eliminate the catalyst. The obtained crude was then purified by flash chromatography (hexane/ethyl acetate 9 : 1) to give the desired coumarins.

General methodology to prepare 3-amidocoumarins: To a solution of 3-aminocoumarin (1 mmol) and pyridine (1.1 mmol) in dichloromethane (9 mL), the corresponding acid chloride (1.1 mmol) was added dropwise and the reaction was stirred, at room temperature, for 3 hours. The solvent was evaporated under vacuum and the dry residue was purified by FC (hexane/ethyl acetate 9 : 1).

*3-(4′-Methoxybenzamide)-6-methylcoumarin (**4**).* (0.61 g, 72%); cream solid; mp 125–126 °C; ^{1}H NMR (300 MHz, DMSO-d_6) 2.27 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 6.86 (s, 1H, *H*-4), 7.06–7.32 (m, 5H, *H*-5, *H*-7, *H*-8, *H*-3′, *H*-5′), 7.93 (d, J = 8.4 Hz, 1H, *H*-2′), 8.04 (d, J = 8.2 Hz, 1H, *H*-6′), 8.08 (s, 1H, N*H*); ^{13}C NMR (75.4 MHz, DMSO-d_6) 21.4, 21.8, 117.1, 119.9, 121.8, 122.3, 126.4, 127.5, 128.9, 132.3, 135.2, 137.4, 141.3, 150.6, 160.1, 165.4; EI-MS (*m*/*z*): 294 [M + 1]$^+$, 293 [M$^+$]; Anal. calcd for $C_{18}H_{15}NO_3$: C 73.71, H 5.15. Found: C 73.70, H 5.13%.

*3-(3′,4′-Dimethoxybenzamide)coumarin (**10**).* (0.72 g, 81%); white solid; mp 187–188 °C; ^{1}H NMR (300 MHz, DMSO-d_6) 3.83 (s, 6H, (OCH_3)$_2$), 7.09 (d, J = 8.5 Hz, 1H, *H*-5′), 7.33–7.44 (m, 2H, *H*-6, *H*-6′), 7.51–7.61 (m, 3H, *H*-2′, *H*-7, *H*-8) 7.74 (d, J = 7.7 Hz, 1H, *H*-5), 8.55 (s, 1H, *H*-4), 9.51 (s, 1H, N*H*); ^{13}C NMR (75.4 MHz, DMSO-d_6) 55.8, 55.9, 98.2, 101.4, 110.9, 111.2, 111.3, 116.1, 121.3, 124.4, 125.2, 127.1, 128.2, 130.3, 148.6, 150.2, 153.0, 165.8; EI-MS (*m*/*z*): 326 [M + 1]$^+$, 325 [M$^+$]; Anal. calcd for $C_{18}H_{15}NO_5$: C 66.46, H 4.65. Found: C 66.49, H 4.68%.

*3-(3′,4′,5′-Trimethoxybenzamide)coumarin (**11**).* (0,55, 65%); white solid; mp 145–146 °C; ^{1}H NMR (300 MHz, DMSO-d_6) 3.73 (s, 3H, OCH_3), 3.86 (s, 6H, (OCH_3)$_2$), 7.37–7.45 (m, 4H, *H*-2′, *H*-6′, *H*-6, *H*-8), 7.54 (d, J = 7.1 Hz, 1H, *H*-5), 7.75 (dd, J = 7.2; 1.4 Hz, 1H, *H*-7), 8.52 (s, 1H, *H*-4), 9.7 (s, 1H, N*H*); ^{13}C NMR (75.4 MHz, DMSO-d_6) 57.8, 60.8, 106.0, 116.7, 119.9, 124.8, 125.7, 128.8, 128.9, 129.4, 131.1, 141.5, 151.1, 153.4, 158.5, 166.1; EI-MS (*m*/*z*): 356 [M + 1]$^+$, 355 [M$^+$]; Anal. calcd for $C_{19}H_{17}NO_6$: C 64.22, H 4.82. Found: C 64.25, H 4.85%.

*3-(3′,4′-Dichlorobenzamide)coumarin (**12**).* (0.76 g, 89%); light yellow solid; mp 262–263 °C; ^{1}H NMR (300 MHz, DMSO-d_6) 7.37–7.45 (m, 2H, *H*-6, *H*-8), 7.55 (d, J = 8.5 Hz, 1H, *H*-5′), 7.76–7.83 (m, 1H, *H*-5), 7.90 (d, J = 8.4 Hz, 1H, *H*-6′), 7.75 (dd, J = 7.7; 1.4 Hz, 1H, *H*-7), 8.19 (s, 1H, *H*-4), 8.58 (s, 1, *H*-2′), 10.0 (s, 1H, N*H*); ^{13}C NMR (75.4 MHz, DMSO-d_6) 116.5, 116.7, 120.0, 121.8, 125.7, 126.0, 127.9, 128.3, 128.8, 129.9, 133.2, 133.8, 136.3, 136.9, 158.4, 166.1; EI-MS (*m*/*z*): 334 [M + 1]$^+$, 333 [M$^+$]: Anal. calcd for $C_{16}H_9Cl_2NO_3$: C 57.51, H 2.71. Found: C 57.53, H 2.73%.

*2-Bromo-*N*-(coumarin-3-yl)acetamide (**15**).* (0.70 g, 76%); white solid; mp 199–200 °C; ^{1}H NMR (300 MHz, DMSO-d_6) 4.28 (s, 2H, CH_2), 7.30–7.40 (m, 2H, *H*-6, *H*-8), 7.52 (dd, J = 7.3; 1.6 Hz, 1H, *H*-7), 7.72 (dd, J = 7.8; 1.4 Hz, 1H, *H*-5), 8.63 (s, 1H, *H*-4), 10.22 (s, 1H, N*H*); ^{13}C NMR (75.4 MHz, DMSO-d_6) 62.2, 116.7, 120.2, 122.9, 124.2, 125.8, 128.7, 130.5, 150.3, 158.5, 172.4; EI-MS (*m*/*z*): 282 [M + 1]$^+$, 281 [M$^+$]; Anal. calcd for $C_{11}H_8BrNO_3$: C 46.84, H 2.86. Found: C 46.87, H 2.90%.

5.3. Determination of MAO isoforms activity

The effects of the new synthesized compounds on hMAO isoform enzymatic activity were evaluated by a fluorimetric method following the experimental protocol previously described by us.[19]

Briefly, 0.1 mL of sodium phosphate buffer (0.05 M, pH 7.4) containing the test drugs (new compounds or reference inhibitors) in various concentrations and adequate amounts of recombinant hMAO-A or hMAO-B, required and adjusted to obtain in our experimental conditions the same reaction velocity (hMAO-A: 1.1 mg protein; specific activity: 150 nmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min/mg protein; hMAO-B: 7.5 mg protein; specific activity: 22 nmol of *p*-tyramine transformed/min/mg protein), were incubated for 15 min at 37 °C in a flat-black bottom 96-well microtest plate, placed in the dark fluorimeter chamber. After this incubation period, the reaction was started by adding (final concentrations) 200 mM Amplex Red reagent, 1 U mL^{-1} horseradish peroxidase and 1 mM *p*-tyramine. The production of H_2O_2 and, consequently, of resorufin was quantified at 37 °C in a multidetection microplate fluorescence reader (FLX800, BioTek Instruments, Inc., Winooski, VT, USA) based on the fluorescence generated (excitation, 545 nm, emission, 590 nm) over a 15 min period, in which the fluorescence increased linearly.

Control experiments were carried out simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions of the vehicles. In addition, the possible capacity of the above test drugs to modify the fluorescence generated in the reaction mixture due to non-enzymatic inhibition (*e.g.*, for directly reacting with Amplex Red reagent) was determined by adding these drugs to solutions containing only the Amplex Red reagent in a sodium phosphate buffer.

The specific fluorescence emission (used to obtain the final results) was calculated after subtraction of the background activity, which was determined from vials containing all components except the hMAO isoforms, which were replaced by a sodium phosphate buffer solution.

5.4. Determination of AChE activity

The AChE activity was evaluated by studying the hydrolysis of acetylthiocholine following the method of Ellman.[30] The assay solution consisted of a 50 mM phosphate buffer pH = 7.4, with the addition of 0.25 mM 5,5′-dithio-bis(2-nitrobenzoic acid), 0.165 unit per mL recombinant acetylcholinesterase expressed in HEK 293 (Sigma-Aldrich Química S.A., Alcobendas, Spain), and 5 mM of substrate (acetylthiocholine iodide). Test compounds were added to the buffer and preincubated at 37 °C with the enzyme for 5 min followed by the addition of cromogene and substrate. The activity was determined by measuring the increase in absorbance at 412 nm at 1 min intervals for 10 min at 37 °C.

Control experiments were carried out simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions of the vehicles. The specific

absorbance (used to obtain the final results) was calculated after subtraction of the background activity, which was determined from vials containing all components except the AChE, which was replaced by a sodium phosphate buffer solution.

Conclusions

In the current study, a series of 3-substituted coumarin derivatives were synthesized and evaluated as inhibitors of MAO and AChE. In general, and in accordance with previous results, an aryl ring in that position, additionally to the substitution in the six position of the coumarin, led to interesting MAO inhibitors which lack the activity on AChE. Introduction of an amidic group between the 3-substituted aryl rings and the coumarin afforded compounds which can behave as dual inhibitors of MAO (mainly MAO-B) and AChE in the micromolar range. Replacement of the aryl ring for an alkylamidic group afforded inactive compounds against MAO and AChE. According to our results, 3-benzamidecoumarins proved to be interesting dual inhibitors, suggesting a therapeutic opportunity worth exploring.

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New halogenated phenylcoumarins as tyrosinase inhibitors

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ABSTRACT

With the aim to find out structural features for the tyrosinase inhibitory activity, in the present communication we report the synthesis and pharmacological evaluation of a new series of phenylcoumarin derivatives with different number of hydroxyl or ether groups and bromo substituent in the scaffold. The synthesized compounds **5–12** were evaluated as mushroom tyrosinase inhibitors showing, two of them, lower IC_{50} than the umbelliferone. Compound **12** (IC_{50} = 215 μM) is the best tyrosinase inhibitor of this series.

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Tyrosinase (EC 1.14.18.1) is a multifunctional dinuclear copper centre enzyme widely distributed in nature and mainly involved in the formation of pigments such as melanins and other polyphenolic compounds.[1] Tyrosinase oxidizes phenols and diphenols using a catalytic mechanism that depends on the presence of copper at its active site.[1] In fungi and vertebrates, tyrosinase catalyzes the two initial steps for the formation of the melanin pigments (melanogenesis), starting from the tyrosine.[2] This enzyme catalyses the conversion of tyrosine to DOPA and the oxidation of the resultant DOPA.[2] So, this enzyme is responsible for the pigmentation of the skin, eyes and hair.[3] In fact, tyrosinase inhibitors have been used as depigmenting agents for the treatment or prevention of hyperpigmentation disorders.[4] Also, tyrosinase is involved in the process to maintain the appearance, flavour, texture and nutritional value of many fresh-cut products.[5] The enzyme extracted from the mushroom *A. bisporus* has high homology with the mammalian one. So, it is suited as a model for melanogenesis and tyrosinase bio-pathways studies.

Coumarins are a large family of compounds, of natural and synthetic origin, which presents different pharmacological activities.[6] Structurally they are lactones of the cinnamic acid. Due to their structural variability, they occupy an important place in the realm of natural products and synthetic organic chemistry.[7] Recent studies pay special attention to their antioxidative, antiinflamatory, anticancer and enzymatic inhibition properties.[8–14]

Resveratrol –3,4′,5-trihydroxystilbene—is a natural polyphenolic compound present in grapes and red wine.[15] It is a phytoalexin produced by some species in response to an external or internal damage (such as a fungal infection).[15,16] In in vitro, ex vivo and in vivo experiments, resveratrol has shown important pharmacological activities including antiinflammatory, antioxidant, anticancer and cardioprotective properties, besides inhibitory activity towards several enzymes.[15–19] Therefore, this compound has been attracting a huge pharmacological interest since the last decade.

In recent studies, some coumarins proved to be mushroom tyrosinase inhibitors.[20,21] In these studies, esculetin and umbelliferone exhibited some of the strongest inhibitory activities of the tested series. Masamoto et al. investigated the structure–activity relationship of 18 coumarins for their inhibitory activity against mushroom tyrosinase. They found that esculetin exhibited the strongest inhibitory activity of those series.[20] Recently, and in contrast with the Masamoto's findings, Sollai et al. have shown that esculetin is considered to be a tyrosinase substrate rather than an inhibitor, whereas umbelliferone seems to be an inhibitor of the mentioned oxidase.[22] These recent findings revealed that tyrosinase affinity can be efficiently modulated by appropriate substitutions in the coumarin moiety. The introduction of hydroxyl groups in different positions is particularly suitable to these modifications.[20,21] Recently, it has been demonstrated that also resveratrol and other stilbenes[23] have inhibitory effects against mushroom tyrosinase activity.[24] Resveratrol showed stronger DOPA oxidase inhibitory activity than kojic acid (reference inhibitor).[25] In fact, until the last years, polyphenols are the largest scaffold in tyrosinase inhibition.[23,26] Their activity depends on

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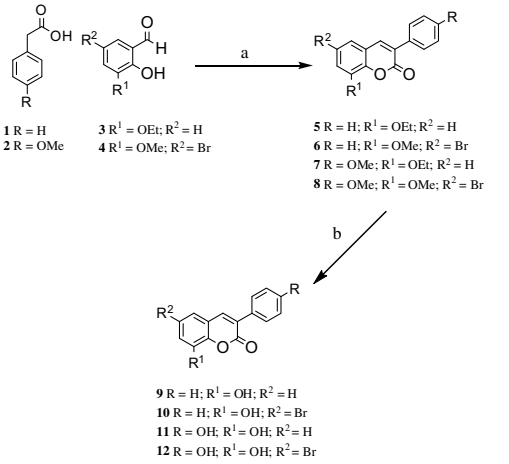
the presence and position of additional substituents whether a polyphenol may act as an inhibitor.[26] So, it was proved that both stilbene and coumarin derivatives show interesting inhibitory effects against tyrosinase.

Tyrosinase inhibitors could have broad applications. As the ideal drug candidate has not been attained, an intensive search for new and innovative tyrosinase inhibitors is still needed. This effort has considerably increased in recent years. In this context, and in an attempt to develop novel tyrosinase inhibitors, we had previously synthesized and described 3-arylcoumarin derivatives in which both the coumarin and the resveratrol templates were present.[21]

Resveratrol (Fig. 1, A) and umbelliferone (B) are very good tyrosinase inhibitors.[2,20] Umbelliferone-resveratrol hybrid (C) and 3-phenylumbelliferone (D) are less active than the mentioned compounds (IC_{50} 3.68 mM and >10, respectively). Although, the previously derivative described by us 6,8,3′,4′,5′-pentahydroxy proved to be the most active compound of the evaluated 3-arylcoumarin series, with an IC_{50} of 0.27 mM.[21] This molecule have two hydroxyl groups under the coumarin ring, being like the coumarin-resveratrol hybrid (E).

Based on these results,[21] in the present work we proposed to continue the 3-phenylcoumarin scaffold study, with different substitutions like methoxyl, ethoxyl, hydroxyl and/or bromo under the 6, 8 and 4′ positions (Scheme 1). We decided to explore the importance of the number and position of ether and hydroxyl groups located in the 3-phenyl ring or in the aromatic ring of the coumarin and, at the same time, the bromination of the coumarin moiety.

The coumarin derivatives **5–12**[27] were efficiently synthesized according to the synthetic protocol outlined in Scheme 1. The 3-phenylcoumarins **5–8** were prepared starting from the conveniently substituted phenylacetic acid **1** or **2** and the appropriate salicylaldehyde **3** or **4**, using dicyclohexylcarbodiimide (DCC) as dehydrating agent, by Perkin reaction,[28–31] in dimethylsulfoxide (DMSO). These reactions gave 55%, 59%, 61% and 60% yield, respectively. Hydrolysis of the methoxyl/ethoxyl groups, by treatment with hydriodic acid 57% in acetic acid/acetic anhydride (1:1),[32] gave the hydroxyl derivatives **9**, **10**, **11** and **12** in 64%, 60%, 61% and 53% yield, respectively. The obtained products were easy to purify by flash chromatography, using a mixture of hexane/ethyl acetate, in a proportion 9:1, as eluent.



Scheme 1. Reagents and conditions: (a) DCC, DMSO, 110 °C, 12 h; (b) HI/AcOH/Ac_2O, 0 °C–rt, 3 h.

The tyrosinase inhibitory activity of compounds **5–12** was evaluated in vitro by the measurement of the enzymatic activity of mushroom tyrosinase enzyme extracted from the mushroom specie *A. bisporus*.[33] Then, the IC_{50} values for inhibitory effects of the new compounds were calculated (Table 1).

In the present communication, the effect of the introduction of a halogen substituent into different hydroxy-3-phenylcoumarins was proposed. In fact, a higher tyrosinase inhibitory activity, regarding the non-halogenated compounds, was observed. The structural change obtained from compound **9** to compound **10** and from **11** to **12**, with the bromo atom in the coumarin nucleus, leads to a significant increase of the tyrosinase activity (from milimolar to micromolar range).

As it is shown in the Table 1, compound **12** is the most active compound of this series. This compound, with a bromo atom and two hydroxyl groups in the 3-phenylcoumarin moiety, has an IC_{50} in the micromolar range (IC_{50} = 215 μM). As expected, the more hydroxyl groups in the coumarin moiety, the better activity the compounds have. When compared with compound **10**, the introduction of one hydroxyl group more in compound **12** increases at least 1.5 times the tyrosinase inhibitory activity. Compound **12** shows, in fact, a lightly higher inhibitory activity than the 6,8-dihydroxy-3-(3′,4′,5′-trihydroxyphenyl)coumarin (IC_{50} = 270 μM), previously described by us.[21] The presence in 6 and 4′

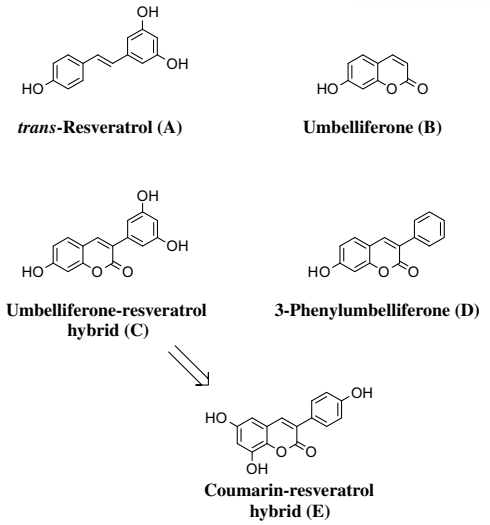


Figure 1. Tyrosinase inhibitors with coumarin/resveratrol derivative structures.

Table 1
Inhibitory effect of compounds **5–12** and umbelliferone on mushroom tyrosinase activity

Compounds	IC_{50} (mM) (L-DOPA 0.5 mM)
5	2.07 ± 0.07
6	>5.0
7	>5.0
8	1.36 ± 0.12
9	>5.0
10	0.302 ± 0.002
11	1.30 ± 0.08
12	0.215 ± 0.085
Umbelliferone	0.42[a]

[a] Obtained from data in Ref.[21]

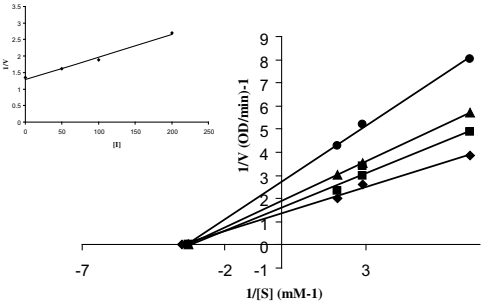


Figure 2. Lineweaver–Burk plots for inhibition of compound **12** on mushroom tyrosinase for catalysis of L-DOPA. Inhibitor concentrations were 0 (♦), 0.050 mM (■), 0.100 mM (▲), 0.200 mM (●). The inset represents the secondary plot of $1/V_{max}$ versus concentration of compound **12**, to determine the inhibition constant (K_i).

positions, at the same time, of a bromo and a hydroxyl substituent, respectively, contributed to an increase of the inhibitory activity. The experimental results show that the methoxyl and ethoxyl derivatives (compounds **5–8**) do not present any significant activity against the tested enzyme. These results suggest that the bromination of the hydroxycoumarins, in spite of the bromination of methoxycoumarins, could be an important step in the synthesis of novel tyrosinase inhibitors.

The inhibitory mechanism of the compound **12** was determined using a Lineweaver–Burk double reciprocal plot (Figure ure2). The data, displayed as a plot of 1/V versus 1/[S], gave three straight lines with different slopes and a horizontal line that intersected at the same point. With an increase in compound concentration, the V_{max} value decreased, whereas the K_m value was unchanged, suggesting that this compound is a non-competitive tyrosinase inhibitor. The inhibition constant of this compound (K_I = 0.189 mM) was determined by plotting the intercept values versus the concentration of the corresponding compound, as shown in Figure 2.

In conclusion, in the present study it was shown that the synthesized coumarin-resveratrol hybrid compounds have inhibitory activity against mushroom tyrosinase. Some of them present tyrosinase inhibitory activity in the micromolar range. The presence of a bromo atom in position 6 of the hydroxycoumarins improves the inhibitory activity respect to the other synthesized derivatives. So, the introduction of a bromo atom improves the pharmacological potential of these 3-phenylcoumarins, confirming that this lead could be effectively optimized in a candidate for the treatment of some hyperpigmentation skin diseases. These finds have encouraged us to continue the efforts towards the optimization of the pharmacological profile of these 3-phenylcoumarins.

Acknowledgement

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27. *8-Ethoxy-3-phenylcoumarin* (**5**). It was obtained with a yield of 55% mp 117–118 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 1.50 (t, 3H, –CH_3, *J* = 7.0), 4.21 (dd, 2H, –CH_2, *J* = 14.0 and *J* = 7.0), 7.09 (t, 2H, H-6, H-7, *J* = 6.9), 7.21 (t, 1H, H-5, *J* = 7.8), 7.42–7.48 (m, 3H, H-3′, H-4′, H-5′), 7.72 (dd, 2H, H-2′, H-6′, *J* = 7.7 and *J* = 1.4), 7.79 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.8, 65.0, 114.5, 119.3, 120.4, 124.3, 128.3, 128.4, 128.5, 128.8, 134.8, 140.1, 143.4, 146.3, 160.2. MS *m*/*z* (%): 267 (16), 266 (M^+, 83), 239 (16), 238 (20), 212 (14) 211 (20), 181 (15), 153 (25). Anal. Calcd for $C_{17}H_{14}O_3$: C, 76.68; H 5.30. Found: C, 76.66; H, 5.35.
6-Bromo-8-methoxy-3-phenylcoumarin (**6**). It was obtained with a yield of 59% mp 153–154 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 3.97 (s, 3H, –OCH_3), 7.16 (d, 1H, H-7, *J* = 2.0), 7.26 (d, 1H, H-5, *J* = 2.0), 7.42–7.47 (m, 3H, H-3′, H-4′, H-5′), 7.68–7.78 (m, 3H, H-2′, H-6′, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 57.2, 117.0, 117.3, 121.9, 122.1, 129.2, 129.8, 130.3, 134.9, 139.1, 142.9, 148.3, 160.0. MS *m*/*z* (%): 332 (99), 331 (30), 330 (M^+, 100), 304 (40), 302 (40), 261 (25), 259 (26), 194 (16), 165 (12), 153 (14), 151 (23), 102 (19), 76 (34). Anal. Calcd for $C_{16}H_{11}BrO_3$: C, 58.03; H, 3.35. Found: C, 58.01; H, 3.30.
8-Ethoxy-3-(4′-methoxyphenyl)coumarin (**7**). It was obtained with a yield of 61% mp 99–100 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 1.50 (t, 3H, –CH_3, *J* = 7.0), 3.84 (s, 3H, –OCH_3), 4.19 (dd, 2H, –CH_2, *J* = 14.0, *J* = 7.0), 6.84–7.26 (m, 5H, H-3′, H-5′, H-5, H-6, H-7), 7.69 (t, 2H, H-2′, H-6′, *J* = 7.7), 7.73 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.8, 55.4, 64.8, 113.8, 114.0, 119.1, 120.49, 124.2, 127.1, 127.8, 129.7, 129.8, 138.6, 138.7, 146.2, 160.0. MS *m*/*z* (%): 297 (35), 296 (M^+, 100), 268 (54), 240 (47) 225 (45), 197 (13), 152 (11), 139 (15). Anal. Calcd for $C_{18}H_{16}O_4$: C, 72.96; H, 5.44. Found: C, 72.91; H, 5.39.
6-Bromo-8-methoxy-3-(4′-methoxyphenyl)coumarin (**8**). It was obtained with a yield of 60% mp 183–184 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 3.85 (s, 3H, –OCH_3), 3.96 (s, 3H, –OCH_3), 6.94–6.98 (m, 2H, H-3′, H-5′), 7.12 (d, 1H, H-7, *J* = 1.8), 7.23 (dd, 1H, H-5, *J* = 2.1 and *J* = 0.9), 7.63–7.69 (m, 3H, H-4, H-2′, H-6′). ^{13}C NMR ($CDCl_3$) δ (ppm): 55.7, 56.8, 114.2, 116.2, 116.8, 121.5, 126.8, 129.4, 130.2, 130.8, 137.3, 142.2, 147.8, 159.8, 160.6. MS *m*/*z* (%): 363 (19), 362 (M^+, 100), 361 (19), 334 (24), 332 (23), 319 (33), 317 (34), 291 (11), 289 (11), 182 (18), 167 (17), 139 (21), 91 (11). Anal. Calcd for $C_{17}H_{13}BrO_4$: C, 56.53; H 3.63. Found: C, 56.55; H, 3.68.
8-Hydroxy-3-phenylcoumarin (**9**). It was obtained with a yield of 64% mp 199–200 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 7.14–7.19 (m, 3H, H-5, H-6, H-7), 7.40–7.49 (m, 3H, H-3′, H-4′, H-5′), 7.70–7.78 (m, 2H, H-2′, H-6′), 8.19 (s, 1H, H-4), 10.25 (s, 1H, –OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 118.0, 118.6, 120.4, 124.6, 126.7, 128.2, 128.5, 134.7, 141.0, 141.7, 144.3, 159.6. MS *m*/*z* (%): 239 (16), 238 (M^+, 100), 210 (80), 181 (13), 153 (22) 152 (20), 105 (9), 76 (15), 51 (6). Anal. Calcd for $C_{15}H_{10}O_3$: C, 75.62; H, 4.23. Found: C, 75.57; H, 4.28.
6-Bromo-8-hydroxy-3-phenylcoumarin (**10**). It was obtained with a yield of 60% mp 163–164 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 7.19 (d, 1H, H-7, *J* = 2.0), 7.42–7.51 (m, 4H, H-5, H-3′, H-4′, H-5′), 7.70 (dd, 2H, H-2′, H-6′, *J* = 7.6 and *J* = 1.6), 8.13 (s, 1H, H-4), 10.79 (s, 1H, –OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 115.5, 119.9, 120.4, 121.8, 127.9, 128.3, 128.5, 128.8, 134.4, 139.7, 141.1, 145.6, 159.1. MS *m*/*z* (%):

318 (99), 317 (17), 316 (M^+, 100), 291 (12), 289 (13), 153 (22), 152 (51), 151 (12), 76 (25). Anal. Calcd for $C_{15}H_9BrO_3$: C, 56.81; H, 2.86. Found: C, 56.77; H, 2.81.

8-hydroxy-3-(4′-hydroxy)phenylcoumarin (**11**). It was obtained with a yield of 61% mp 237–238 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 6.81-6.85 (m, 2H, H-3′, H-5′), 7.03-7.12 (m, 3H, H-5, H-6, H-7), 7.55–7.59 (m, 2H, H-2′, H-6′), 8.05 (s, 1H, H-4), 9.80 (s, 1H, –OH), 10.15 (s, 1H, –OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 115.3, 117.8, 118.6, 120.9, 124.7, 125.6, 126.8, 130.1, 139.2, 141.6, 144.5, 158.2, 160.1. MS *m*/*z* (%): 255 (18), 254 (M^+, 100), 227 (16), 226 (98), 197 (15), 169 (12), 115 (12). Anal. Calcd for $C_{15}H_{10}O_4$: C, 70.83; H, 3.87. Found: C, 70.80; H, 3.83.

6-Bromo-8-hydroxy-3-(4′-hydroxyphenyl)coumarin (**12**). It was obtained with a yield of 53% mp 249–250 °C. ^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz): 6.83 (d, 2H, H-3′, H-5′, *J* = 8.8), 7.14 (t, 1H, H-7, *J* = 1.5), 7.36 (d, 1H, H-5, *J* = 2.0), 7.56 (d, 2H, H-2′, H-6′, *J* = 8.5), 8.00 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ (ppm): 115.6, 116.0, 119.9, 120.6, 122.5, 125.4, 128.2, 130.4, 138.0, 141.2, 146.0, 158.6, 159.8. MS *m*/*z* (%): 334 (99), 333 (45), 332 (M^+, 100), 307 (31), 305 (35), 225 (26), 197 (29), 169 (35), 168 (46), 153 (24), 141 (21), 140 (16), 118 (17), 115 (28), 98 (13), 89 (14), 84 (18), 75 (11), 63 (13). Anal. Calcd for $C_{15}H_9BrO_4$: C, 54.08; H 2.72. Found: C 54.01, H 2.69.

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33. Pre-incubation with the enzyme: 1/15 M phosphoric acid buffer solution (pH 6.8, 1.8 mL), an aqueous solution of mushroom tyrosinase (1000 U/mL, Sigma Chemical Co., 0.1 mL) and DMSO (0.1 mL) with or without the sample. The mixture was incubated at 25 °C for 10 min. Then, a 1.05 mM of L-3,4-dihydroxyphenylalanine (DOPA) solution (1 mL) was added and the reaction was monitored at 475 nm, for 5 min. The percent of tyrosinase activity inhibition was calculated as: inhibition (%) = (A−B)/A × 100, where A represents the difference in the absorbance of control sample between 0.5 and 1.0 min, and B represents the difference in absorbance of the test sample between 0.5 and 1.0 min. The IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determinate by interpolation of dose–response curves. The mushroom tyrosinase activity was determinate by spectrophotometric assays (Varian Cary 50). Umbelliferone was used as a reference tyrosinase inhibitor.

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Tyrosine-like condensed derivatives as tyrosinase inhibitors

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Keywords
depigmenting agents; mixed-type inhibitor; tyrosinase inhibition; tyrosine-like compounds

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Abstract

Objectives We report the pharmacological evaluation of a new series of 3-aminocoumarins differently substituted with hydroxyl groups, which have been synthesized because they include in their structures the tyrosine fragment (tyrosine-like compounds), with the aim of discovering structural features necessary for tyrosinase inhibitory activity.

Methods The synthesized compounds **4** and **7–9** were evaluated *in vitro* as mushroom tyrosinase inhibitors.

Key findings Two of the described compounds showed lower IC50 (concentration giving 50% inhibition of tyrosinase activity) than umbelliferone, used as a reference compound.

Conclusions Compound **7** (IC50 = 53 μM) was the best tyrosinase inhibitor of this small series, having an IC50 value 10-fold lower than umbelliferone. Compound **7** (3-amino-7-hydroxycoumarin) had amino and hydroxyl groups precisely mimicking the same positions that both groups occupy on the tyrosine molecule.

Introduction

Tyrosinase (EC 1.14.18.1) is a multifunctional dinuclear copper centre metalloenzyme widely distributed in nature.[1] This enzyme catalyses two distinct reactions of conversion of the tyrosine: 3′-hydroxylation of L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA) and oxidation of the resultant L-DOPA into DOPA quinone, which further polymerizes spontaneously into melanin.[2] Most melanin-biosynthesis inhibitors are phenol or catechol analogues, which are structurally similar to tyrosinase substrates: tyrosine or L-DOPA.[3] Therefore, tyrosine-like molecules could be an interesting scaffold to the tyrosinase inhibition process. Tyrosinase is mainly involved in the formation of pigments such as melanins and other polyphenolic compounds.[1] Tyrosinase oxidizes phenols and diphenols using a catalytic mechanism dependent on the presence of copper at its active site.[1,2] This enzyme is responsible for unwanted browning of fruits and vegetables.[4] Therefore, it is involved in the process of maintaining the appearance, flavour, texture and nutritional value of many fresh-cut products.[4] Tyrosinase is also responsible for the colouring of skin, hair and eyes in animals, including humans.[5] In fact, tyrosinase inhibitors have been used as depigmenting agents for the treatment or prevention of hyperpigmentation disorders.[6] In recent years, many tyrosinase inhibitors have been reported, including vitamin C (ascorbic acid), kojic acid, umbelliferone, resveratrol, hydroquinone and oxyresveratrol.[7–12] Because of the structural similarity, umbelliferone was used as a reference inhibitor (Figure 1).

Coumarins are a large family of compounds, of natural and synthetic origin, which present different pharmacological actions.[13] Chemically they are lactones of cinnamic acid. Their structural variety is responsible for the important place that they occupy in the natural product and synthetic organic chemistry realm.[14] Some important studies pay special attention to their antioxidative, anti-cancer anti-inflammatory, cardioprotective and enzymatic inhibitory properties.[15–21] In recent studies, some coumarins proved to be mushroom tyrosinase inhibitors.[22,23] In those studies, esculetin (6,7-dihydroxycoumarin) and umbelliferone (7-hydroxycoumarin) exhibited some of the strongest inhibitory activity of the tested series, with esculetin proving to be the strongest inhibitor of the series.[22] Recently, and in contrast to the initial findings, Sollai *et al.* showed that esculetin is a tyrosinase substrate rather than an inhibitor, whereas umbelliferone seems to be an inhibitor of tyrosinase.[24] These studies revealed that tyrosinase affinity

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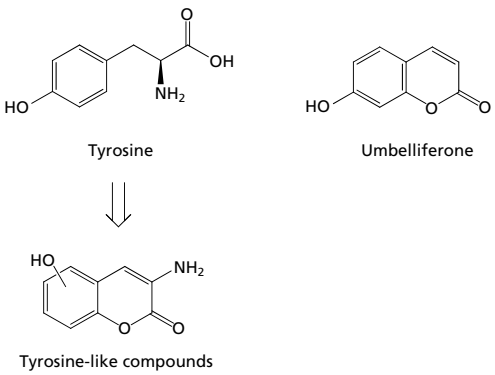


Figure 1 Chemical structures of tyrosine (tyrosinase substrate), tyrosine-like condensed molecules and umbelliferone (tyrosinase inhibitor).

can be efficiently modulated by appropriate substitutions in the coumarin moiety. The introduction of different numbers of hydroxyl groups in different positions of the coumarin is one of the most important modifications.[22,23] Also, until recently, polyphenols were the largest scaffold in tyrosinase inhibition.[25,26]

Tyrosinase inhibitors could have broad applications. As the ideal drug candidate has not been attained, an intensive search for new tyrosinase inhibitors is still needed. This effort has considerably increased in the recent years. In this context, and in an attempt to develop novel tyrosinase inhibitors, we had previously synthesized and described 3-arylcoumarin derivatives in which both the coumarin and the resveratrol templates were present.[23,27] Based on this work, and with the aim of finding new structure–activity features, we propose the study of a series of tyrosine-like condensed molecules (Figure 1). The similarity of these compounds to the tyrosinase substrate is the novelty of this study. The proposed compounds are structurally related to the amino acid tyrosine, the natural substrate of this enzyme. Tyrosinase inhibitors based on the aminocoumarin scaffold have not been previously studied.

Materials and Methods

We synthesized (Figure 2) and evaluated a small series of 3-aminocoumarins. We decided to explore the importance of the position of a hydroxyl group under the 3-aminocoumarin moiety, based on the idea of mimicking the molecular structure of the tyrosinase substrate.

The coumarin derivatives **4** and **7**–**9**[28–30] were efficiently synthesized according to the protocol outlined in Figure 2. Starting from different substituted commercially available salicylaldehydes and ethyl nitroacetate, we afforded 3-nitrocoumarins **1**–**3** in good yields (75–95%). They were synthesized in a dry Schlenk tube, with acetic anhydride as solvent, in the presence of sodium hydride, at room temperature, for 3 h. The reaction mixture was purified by flash chromatography, using hexane/AcOEt (9 : 1) as eluent. The 3-aminocoumarins **4**–**6** were prepared from previously synthesized 3-nitrocoumarins, in EtOH, with Pd/C as catalyst, under H_2 atmosphere. The obtained products were purified by crystallization in AcOEt to give the desired 3-aminocoumarins, in a yield of 95%. The hydroxy derivatives **7** and **8** were obtained from the methoxy derivatives (compounds **5** and **6**, respectively) by a hydrolysis reaction with hydriodic acid, in the presence of acetic acid and acetic anhydride, at 110°C, for 5 h. The residue was purified by crystallization of acetonitrile, and the hydroxy derivatives were obtained in a yield of 60%. Compound **9**[31] was obtained via reduction reaction, under the same conditions as described above, of the commercially available 4-hydroxy-3-nitrocoumarin, in a yield of 93%.

The biological assays were carried out using the following protocol. Pre-incubation with the enzyme was carried out: 1/15 M phosphoric acid buffer solution (pH 6.8, 1.8 ml), an aqueous solution of mushroom tyrosinase (1000 U/ml; Sigma Chemical Co., Milan, Italy, 0.1 ml) and dimethyl sulfoxide (DMSO) (0.1 ml) with or without the sample. The mixture was incubated at 25°C for 10 min. Then, 1.5 mM L-DOPA solution (1 ml) was added and the reaction was monitored at 475 nm for 5 min. The percent of tyrosinase activity inhibition was calculated as: inhibition (%) = $(A - B)/A \times 100$, where A represents the difference in the absorbance of control sample between 0.5 and 1.0 min, and B represents the difference in absorbance of the test sample between 0.5 and 1.0 min. The mushroom tyrosinase activity was determined by spectrophotometric assays (Varian Cary 50). Umbelliferone was used as a reference tyrosinase inhibitor.

Statistical methods

All experiments were carried out three times. Continuous variables were expressed as mean ± SD. The IC50 value (concentration giving 50% inhibition of tyrosinase activity) was determined by interpolation of dose–response curves and all data were statistically evaluated using Student's *t*-test or Mann–Whitney test (Statistica 6; Statsoft Tulsa, Tulsa, OK, USA). To assess the slopes of curves in Figure 3 the Kruskal–Wallis test followed by Dunn's post-hoc test (Statistica 6; Statsoft Tulsa) was used. The criterion for statistical significance was generally taken as $P < 0.05$.

Results

The tyrosinase inhibitory activity of compounds **4** and **7**–**9** was evaluated *in vitro* by measuring the enzymatic activity of

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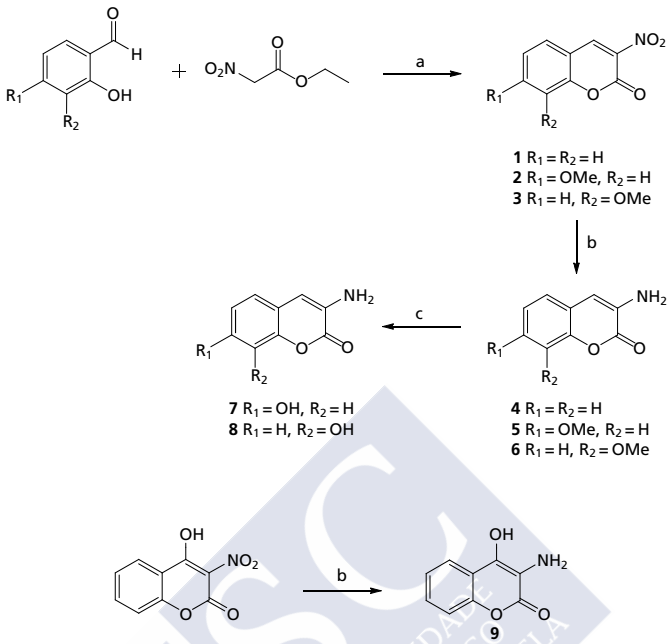


Figure 2 Protocols for synthesis of coumarin derivatives. *Reagents and conditions:* (a) acetic anhydride, NaH, r.t., 3 h; (b) H_2, EtOH, Pd/C, r.t., 5 h; (c) HI, AcOH, Ac_2O, 110°C, 5 h.

mushroom tyrosinase enzyme extracted from the mushroom *Agaricus bisporus*. Then, the IC50 values for the inhibitory effects of the new compounds were calculated (Table 1).

In the presence of compound **7**, kinetic studies on mushroom tyrosinase, using a Lineweaver–Burk double reciprocal plot (Figure 3), showed that compound **7** was a mixed-type inhibitor. Increasing the concentration of **7** resulted in a family of lines with different slope and intercept, which intersected in the second quadrant. This behaviour showed that compound **7** can bind not only with free enzyme but also with the enzyme–substrate complex, with different equilibrium constants. The inhibition constants for the inhibitor binding with free enzyme, K_I (2.14 mM), and with enzyme–substrate complex, K_{IS} (0.78 mM), were obtained from the linear secondary plots of $1/V_{max}$ and K_m/V_{max} versus the concentration of compound **7**, respectively.

Discussion

In this communication, the possible tyrosinase inhibitory effect of coumarins that incorporate a portion of tyrosine on their skeletons was described. The introduction of a hydroxyl substituent into different positions of the 3-aminocoumarin moiety was studied. In this way a small series of compounds that are both tyrosine (tyrosinase substrate) and umbelliferone (tyrosinase inhibitor) analogues was obtained.

It is known that umbelliferone (7-hydroxycumarin) has a tyrosinase inhibitor effect (IC50 = 0.42 mM), despite having no amino group in its position 3. The 3-aminocoumarin (compound **4**) was synthesized and evaluated and did not show tyrosinase inhibitory activity. Then, other coumarins were prepared, maintaining the amino group in the 3-position, also incorporating a hydroxyl group in different positions of the coumarin skeleton.

Different synthetic methodologies were carried out to obtain compounds **7** and **8**, which have the hydroxyl group in positions 7 and 8, respectively, of the coumarin nucleus (benzene ring) and compound **9**, with the hydroxyl group in position 4 of the coumarin (pyrone ring). As shown in Table 1, compound **7** was the most active compound of this series, with an IC50 value in the micromolar range (53 μM). This compound was more than 10 times more active than umbelliferone, the reference compound. Compound **7** had the amino and hydroxyl groups that precisely mimicked the same positions that both groups occupy on the tyrosine molecule. Compound **8** was inactive against tyrosinase whereas

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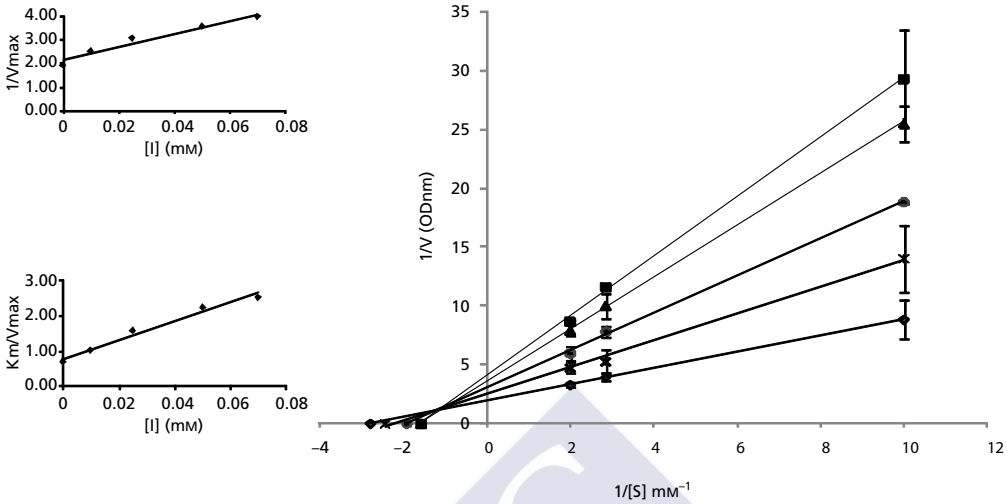


Figure 3 Lineweaver–Burk plots for inhibition of compound **7** on mushroom tyrosinase for catalysis of L-DOPA. Inhibitor concentrations were 0 (◆), 0.010 mM (●), 0.025 mM (x), 0.050 mM (▲), 0.070 mM (■). The insets are the secondary plots of $1/V_{max}$ and K_m/V_{max} versus concentration of compound **7**, respectively.

Table 1 Inhibitory effect of compounds **4** and **7**–**9** and umbelliferone on mushroom tyrosinase activity

Compounds	IC50 (mM) (L-DOPA 0.5 mM)
4	>5.0
7	0.05 ± 0.01
8	>5.0
9	0.25 ± 0.003
Umbelliferone	0.42[a]

[a]Obtained from Fais *et al*.[23]. These results are average results of three experiments.

compound **9** was active against this enzyme (IC50 = 0.26 mM). Compound **9** presented only slightly higher tyrosinase inhibitory activity than umbelliferone.

Therefore, it can be inferred that the tyrosinase inhibitory activity depends on the position of the hydroxyl group in the coumarin moiety. Also, the presence of the hydroxyl group at the same position that of tyrosine and umbelliferone is important to the inhibitory activity.

Conclusions

This study showed that some of the synthesized tyrosine-like condensed derivatives have inhibitory activity against mushroom tyrosinase. The two active compounds present tyrosinase inhibitory activity in the micromolar range. The presence of a hydroxyl group in the seven position of the 3-aminocoumarin, the same position as in tyrosine and umbelliferone, improves the inhibitory activity with respect to the other synthesized derivatives and the reference compound. So, the introduction of hydroxyl groups improves the pharmacological potential of these 3-aminocoumarins, confirming that this lead could be effectively optimized in a candidate for the treatment of some hyperpigmentation skin diseases. These findings have encouraged us to continue the effort towards the optimization of the pharmacological profile of these coumarins.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Research Paper

Targeting adenosine receptors with coumarins: synthesis and binding activities of amide and carbamate derivatives

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Keywords
adenosine receptor ligands; amide derivatives; binding affinity; carbamate derivatives; coumarins

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Abstract

Objectives With the aim of finding the structural features governing binding activity and selectivity against adenosine receptors (ARs), several 3-subtituted coumarins with amide (compounds **3**–**6**) and carbamate (**7**–**9**) functions were synthesized. To study its possible influence on the binding activity and selectivity, a hydroxyl substituent was also introduced at position 4 of the coumarin moiety.

Methods A new series of coumarins (**3**–**9**) were synthesized and evaluated by radioligand binding studies towards ARs.

Key findings None of the 4-hydroxy derivatives (**4**, **8** and **9**) showed binding affinity for any of the ARs. None of the compounds interacted with the hA$_{2B}$ AR (K_i > 100 000 nM). Compounds **3**, **5**, **6** and **7** had different activity profiles with dissimilar binding affinity and selectivity towards human A_1, A_{2A} and A_3 ARs.

Conclusions The most remarkable derivative is compound **7**, which presents the best affinity and selectivity for the A_3 adenosine receptor (K_i = 5500 nM).

Introduction

Coumarins and their natural and synthetic derivatives are pharmacologically interesting compounds due to their structural diversity.[1,2] Due to the synthetic accessibility and substitution variability these heterocyclic compounds play an important role not only in organic chemistry but also in the field of medicinal chemistry.[3–10] In fact, coumarins have been previously described as anti-cancer, antiviral, anti-inflammatory, antimicrobial, enzyme-inhibitory and anti-oxidant agents.[3–18]

Adenosine displays a wide number of physiological actions through activation of four different receptors: A_1, A_{2A}, A_{2B} and A_3,[19] These four subtypes are cell membrane-bound G-protein-coupled receptors that regulate diverse physiological functions.[20,21] Adenosine receptors (ARs) have been recognized as important pharmacological targets since they are involved in diverse pathological processes such as sepsis, inflammation, myocardial infarction, asthma, diabetes, obesity and neurodegenerative diseases.[22,23]

In recent years, the search for innovative ligands showing selectivity and potency towards individual subtypes of AR has been intensified as the role of these receptors in many areas is continuously expanding.[24,25] In particular, A_3AR has been the subject of intensive research during the last decade as it is recognized as being a potential therapeutic target due to its contribution to important pathological processes.[26] Evidence has been acquired supporting the significant role of this AR subtype in inflammation, cell growth and immunosuppression.[27] Therefore, A_3AR agonists represent a new therapeutic window for cancer, cerebral ischaemia and myocardial ischaemia,[28] while A_3AR antagonists can be effective as anti-inflammatory, anti-asthma and anti-glaucoma treatment.[29]

Since AR ligands have broad applications, and an ideal drug candidate has not yet been attained, intensive research in this area is still needed. This effort has considerably increased in recent years. In this context, and with the aim of finding new structure–activity features, this work based on coumarin derivatives was carried out. To our knowledge, no information has been published so far concerning the coumarin scaffold as a putative ligand of the ARs. Therefore, this is an innovative study, performed for the first time, on this interesting and versatile moiety. Our groups have recently proposed chromone derivatives, which are isomers of coumarins, to be a valid scaffold for the design of novel AR ligands.[30,31] These recent findings, and the lack of data on the coumarin scaffold,

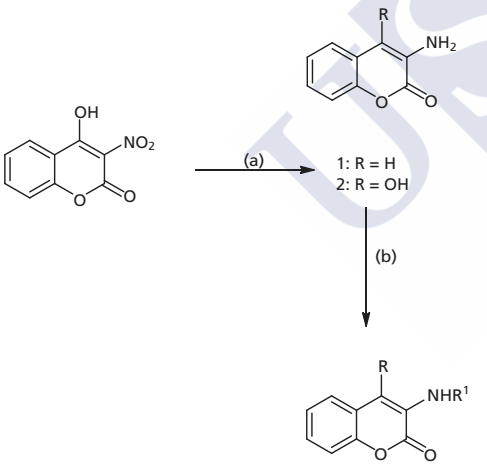
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encouraged us to explore the ARs' binding activity of a selected series of coumarin derivatives.

Materials and Methods

Chemistry

The coumarin derivatives were efficiently synthesized according to the protocol outlined in Figure 1. Coumarins **3–9** were prepared starting from the commercially available 3-aminocoumarin (compound **1**) or from 3-amino-4-hydroxycoumarin (compound **2**), which was previously synthesized.[32–35] The 3-amino-4-hydroxycoumarin **2** was prepared under a reduction reaction of the commercially available 3-nitro-4-hydroxycoumarin, in ethanol, with Pd/C as catalyst, under H_2 atmosphere, with a yield of 90%. An acylation reaction of the 3-aminocoumarins **1** or **2** with the conveniently substituted acid chloride or chloroformate, using pyridine in dichloromethane from 0°C to room temperature, stirring overnight, afforded the differently substituted 3-amidocoumarins (**3–6**) or 3-carbamatecoumarins (**7–9**), in yields between 77 and 85%. The reaction conditions and chemical characterization of the new compounds are detailed in the experimental section.[32–35]



3: R = H ; R^1 = $COCH_3$
4: R = OH ; R^1 = $COCH_3$
5: R = H ; R^1 = $COCH_2Br$
6: R = H ; R^1 = $COCH_2Cl$
7: R = H ; R^1 = $COOCH_2CH(CH_3)_2$
8: R = OH ; R^1 = $COOCH_2CH(CH_3)_2$
9: R = OH ; R^1 = $COOCH_2CH_3$

Figure 1 Protocol for synthesis of coumarin derivatives. Reagents and conditions: (a) H_2, EtOH, Pd/C, r.t., 5 h; (b) R^1Cl, pyridine, dichloromethane, 0°C to r.t, overnight.

Synthetic methodologies

Melting points were determined using a Reichert Kofler thermopan or in capillary tubes on a Büchi 510 apparatus (Flawil, Switzerland) and are uncorrected. IR spectra were recorded on a Perkin-Elmer 1640FT spectrophotometer (Waltham, MA, USA). ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX spectrometer at 300 and 75.47 MHz, respectively, using tetramethylsilane (TMS) as internal standard (chemical shifts in δ-values, J in Hz). Mass spectra were obtained using a Hewlett-Packard 5988A spectrometer. Elemental analyses were performed using a Perkin-Elmer 240B microanalyser and were within $\pm$ 0.4% of calculated values in all cases. Silica gel (Merck 60, 230–00 mesh) was used for flash chromatography (FC). Analytical thin-layer chromatography (TLC) was performed on plates pre-coated with silica gel (Merck 60 F254, 0.25 mm). The purity of compounds was assessed by high-performance liquid chromatography (HPLC) coupled with a diode array detector (DAD) on a Thermo Quest Spectrasystem (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a P4000 pump, an UV6000 UV-Vis diode array detector and an SN4000 interface operated via a personal computer. Instrument software ChromQuest 5.0 (Thermo Fisher Scientific, Waltham, MA, USA) was used for data acquisition. Different analytical columns and mobile phases (all solvents were HPLC grade) were tested. The mobile phase was H_2O : CH_3CN (70 : 30) and an Eclipse xdb C18 column (5 µm particle size, 0.46 mm i.d., 25 cm length; Agilent Technologies, Santa Clara, CA, USA) was used. The purity of the compounds was found to be higher than 95%.

Preparation of 3-amino-4-hydroxycoumarin (2)

The commercially available 4-hydroxy-3-nitrocoumarin (Sigma-Aldrich, St. Louis, MO, USA) (2.5 mmol) was dissolved in ethanol and a catalytic amount of Pd/C was added to the mixture (Sigma-Aldrich). The solution was stirred, at room temperature, under H_2 atmosphere, for 5 h. After the completion of the reaction, the mixture was filtered to eliminate the catalyst. The obtained crude product was then purified by FC (hexane–ethyl acetate, 9 : 1) to give the desired coumarin **2**, in a yield of 90%.[32]

General procedure for the preparation of 3-substituted coumarins (3–9)

The 3-aminocoumarin (Sigma-Aldrich) (**1**) or 3-amino-4-hydroxycoumarin (**2**) (1 mmol) was dissolved in dichloromethane (9 ml), then pyridine (Sigma-Aldrich) (1.1 mmol) was added and the mixture was cooled to 0°C. Differently substituted acid chloride or chloroformate (Sigma-Aldrich) (1.1 mmol) was added drop-wise at this temperature, and the mixture was stirred overnight at room temperature. The batch was evaporated and purified by column chromatography (hexane–ethyl acetate, 9 : 1) to give the desired compound.

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Table 1 Affinity (K_i), for binding to human A_1, A_{2A} and A_3 adenosine receptors expressed in CHO cells, of coumarins **3–9**, in radioligand binding assays

	K_i (nM)			Selectivity	
Compound	*h*A_1	*h*A_{2A}	*h*A_3	*h*A_1/*h*A_3	*h*A_{2A}/*h*A_3
3	53 900 (35 900–81 100)	>30 000	7 160 (5 700–9 000)	7.5	>4.2
4	>100 000	>100 000	>100 000	–	–
5	25 000 (21 600–29 000)	>30 000	27 800 (22 600–34 300)	>0.9	>1.1
6	7 760 (4 070–14 800)	36 600 (20 500–65 500)	13 700 (12 000–15 600)	0.57	2.7
7	>100 000	>100 000	5 500 (3 490–8 670)	>18.2	>18.2
8	>100 000	>100 000	>100 000	–	–
9	>100 000	>30 000	>100 000	–	–

These results are the average of three experiments.

Biological assays

The affinity of compounds **3–9** for the human AR subtypes *h*A_1, *h*A_{2A}, *h*A_3, was determined with radioligand competition experiments in Chinese hamster ovary (CHO) cells that were stably transfected with the individual receptor subtypes.[36,37] The radioligands used were 1 nM (2R, 3R,4S,5R)-2-(2-Chloro-6-cyclopentylamino-purin-9-yl)-5-hydroxymethyl-tetrahydro-3,4-diol ([^{3}H]CCPA) for *h*A_1, 10 nM (1-(6-amino-9H-purin-9-yl)-1-deoxy-N-ethyl-β-D-ribofuronamide) ([^{3}H]NECA) for *h*A_{2A}, and 1 nM 2-(1-hexynyl)-N^6-methyladenosine [3H] ([^{3}H]HEMADO) for *h*A_3 receptors. The results were expressed as K_is (dissociation constants), which were calculated with the program SCTFIT.[38] Due to the lack of a suitable radioligand for *h*A_{2B} receptors, the potency of antagonists at the *h*A_{2B} receptor (expressed on CHO cells) was determined by inhibition of NECA-stimulated adenylyl cyclase activity.[36]

Statistical analysis

K_i values (Table 1) are reported as geometric means of three independent experiments with each tested concentration of compound measured in duplicate. As an interval estimate for the dissociation constants, 95% confidence intervals are given in parentheses.

Results

Structural identification

3-(Isobutylcarbamate)coumarin (**7**): Yield 78%. Mp 92–93°C. ^{1}H NMR δppm ($CDCl_3$-300 MHz): 1.39 (6H, d, J = 6.8 Hz, 2xCH_3), 2.38 (1H, s, CH), 4.39 (2H, d, J = 6.7 Hz, CH_2), 7.69 (1H, s, H-4), 7.70–7.74 (2H, m, H-6, H-8), 7.80 (1H, dd, J = 7.3, 1.8 Hz, H-7), 7.88 (1H, dd, J = 7.6, 1.8 Hz, H-5), 8.7 (1H, s, NH). ^{13}C NMR δppm ($CDCl_3$-75 MHz): 19.6, 28.5, 72.6, 116.9, 120.5, 121.4, 125.0, 125.7, 128.1, 129.8, 150.2, 154.1, 159.1. MS (ESI): 262 (7), 261 (M^+, 42), 188 (23), 161 (70), 133 (28), 57 (41). Anal. Calcd for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79. Found: C, 64.39; H, 5.83.

4-Hydroxy-3-(isobutylcarbamate)coumarin (**8**): Yield 77%. Mp 103–104°C. ^{1}H NMR δppm ($CDCl_3$-300 MHz): 0.96 (6H, d, J = 6.6 Hz, 2xCH_3), 2.00 (1H, s, CH), 4.00 (2H, d, J = 7.0 Hz, CH_2), 7.28–7.37 (2H, m, H-6, H-8), 7.52–7.62 (1H, m, H-7), 7.93 (1H, dd, J = 7.9, 2.0 Hz, H-5), 7.48 (1H, s, NH), 12.38 (1H, s, OH). ^{13}C NMR δppm ($CDCl_3$-75 MHz): 18.9, 27.9, 73.6, 104.0, 116.2, 117.1, 123.8, 124.6, 131.2, 150.1, 150.3, 157.1, 160.6. MS (ESI): 278 (7), 277 (M^+, 40), 203 (27), 177 (100), 148 (15), 121 (65), 88 (18), 57 (50). Anal. Calcd for $C_{14}H_{15}NO_5$: C, 60.64; H, 5.45. Found: C, 60.60; H, 5.41.

3-(Ethylcarbamate)-4-hydroxycoumarin (**9**): Yield 85%. Mp 152–153°C. ^{1}H NMR δppm ($CDCl_3$-300 MHz): 1.35 (3H, s, CH_3), 4.28 (2H, s, CH_2), 7.24–7.35 (4H, m, H-5, H-6, H-7, H-8), 7.93 (1H, s, NH), 12.35 (1H, s, OH). ^{13}C NMR δppm ($CDCl_3$-75 MHz): 14.4, 63.7, 104.1, 116.2, 117.1, 123.8, 124.6, 131.2, 150.1, 150.3, 157.0, 160.6. MS (ESI): 250 (10), 249 (M^+, 43), 203 (59), 177 (30), 148 (41), 121 (61), 65 (13). Anal. Calcd for $C_{12}H_{11}NO_5$: C, 57.83; H, 4.45. Found: C, 57.85; H, 4.47.

Biological results

The data from radioligand binding experiments for compounds **3–9** are summarized in Table 1. Details for pharmacological experiments are described in Materials and Methods and in previous work.[36–38]

Discussion

A novel coumarin series possessing a common planar N-C = O framework, represented by an amidic or a carbamate group at position 3, were studied for their ability to bind to ARs. In addition, the presence or the absence of a hydroxyl group at position 4 was also explored in these preliminary studies.

The experimental results (Table 1) reveal that none of the 4-hydroxy derivatives (compounds **4**, **8** and **9**) have binding affinity for any AR subtype. The presence of a hydroxyl function at position 4 of the coumarin scaffold is not tolerated,

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notwithstanding the presence of acetamide (compound **4**), isobutylcarbamate (compound **8**) or ethylcarbamate (compound **9**) at position 3. On the other hand, for compounds **3**, **5**, **6** and **7** different activity profiles with diverse binding affinity and selectivity towards human hA_1, hA_{2A} and hA_3 ARs were observed. Compound **3**, with an acetamide group at position 3 of the coumarin moiety, has a higher affinity to hA_3 AR (K_i = 7160 nM) than to hA_1 (K_i = 53 900 nM) and no affinity for hA_{2A} and hA_{2B}. Replacing the acetamide substituent with bromoacetamide (compound **5**) decreased the binding affinity and selectivity for hA_3 AR (hA_1 K_i = 25 000 nM and hA_3 K_i = 27 800 nM). Nevertheless it is interesting to note that the affinity for hA_1 AR is enhanced by this substitution. The substitution of a bromine for a chlorine atom leads to compound **6**, which is almost twice as selective for hA_1 than hA_3 AR and about five times more selective for hA_1 than hA_{2A} AR. The substitution of the acetamide group (compound **3**) for an isobutylcarbamate function (compound **7**) allows one to obtain a very active and selective compound for hA_3 ARs (K_i = 5500 nM; Selectivity index (SI) hA_1/hA_3 and hA_{2A}/hA_3 > 18.2). The change of the amide to an amide-like functional group (carbamate) in the coumarin nucleus brings about a noticeable affinity and selectivity for hA_3 ARs. The additional oxygen of the carbamate functionality, which exerts a unique steric and electronic environmental, seems to have an important role for the ligand–receptor interaction. The interesting result found for compound **7** provides a stimulus for the design and synthesis of new coumarin-based hA_3 AR selective ligands.

Conclusions

Evidence was acquired demonstrating that coumarin is a potential scaffold for the design of novel AR ligands. A detailed analysis of the experimental results obtained so far allow us to conclude that the affinity and/or selectivity of the coumarins towards ARs may be greatly influenced by the nature of their substituents. Compound **7** exhibits the best performance and can be considered as the starting point for the design and synthesis of new coumarins as hA_3 AR selective ligands. The easy synthetic routes and the decoration capability of coumarins make them a privileged structure for drug discovery concerning ARs.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Looking for New Targets: Simple Coumarins as Antibacterial Agents

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Abstract: The dramatic worldwide increase of dangerous infections by resistant and multi-resistant microbes makes the search of new molecules and new chemical entities an important topic in Medicinal Chemistry. As the ideal drug candidate has not been attained, an intensive search for new and innovative antimicrobials is still needed. A small series of 3-amino/nitrocoumarins without substitutions or substituted by methyl or methoxy groups at different positions were synthesized and evaluated for their antibacterial and antifungal activities against clinical isolates of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* strains. Some of these structurally simple molecules exhibited antibacterial activity. The preliminary SAR study showed that the antibacterial activity against *E. coli* and *S. aureus* was dependent on the kind and position of the substitution pattern at the coumarin moiety.

Keywords: Aminocoumarins, nitrocoumarins, coumarin synthesis, antibacterial assays, inhibition zones, minimum inhibitory concentrations, *S. aureus*, *E. coli*.

INTRODUCTION

In recent years great advances have been made in our ability to design and test new products derived from chemical synthesis. Some of these products are based on a diversity of molecules found and extracted from natural sources. Nitrogen-containing heterocyclic compounds are very interesting structural units for both the organic chemists and the biochemists [1]. Among other different heterocyclic compounds, coumarins are a large family of compounds associated with remarkable pharmacological activities. Their structural variety is responsible for the important place that they occupy in the natural products and synthetic organic chemistry realm [2,3]. Recent studies pay special attention to their anti-inflammatory, antioxidative, cardioprotective, anticancer, antimicrobial and enzymatic inhibition properties [4-15]. Novobiocin, chlorobiocin and coumermycin A_1 (Fig. **1**) are important natural occurring antibiotics, presenting the coumarin nucleus in their skeleton. This family of compounds has a 4-hydroxy-8-methyl/chlorocoumarin core etherified and aminated in the 7 and 3 positions, respectively. These compounds antagonize the B subunit of the essential *E. coli* DNA gyrase supertwisting activity *in vitro* and the bacterial multiplication [16]. They are not used in clinic because of their relatively weak activity against the Gram-negative bacteria, side effects and poor water solubility [17]. On the other hand, these three examples are antibiotics with activity against methicillin-resistant *S. aureus* (MRSA) [17]. MRSA are often an important problem for severe infections in patients, especially immunesuppressed people, during their stays in hospitals [18].

It has been proved that some other coumarin derivatives have other antibacterial and antifungal activities [19-21]. Different 4-substituted coumarins, such as 4-chlorocoumarins, exhibit an interesting antimicrobial profile [22,23]. Also, coumarin derivatives (benzopyrone moiety) could be considered structurally similar to quinolone drugs (benzopyridone moiety). These drugs have been widely used in clinic, with broad antimicrobial spectrum, including many encountered pathogens (Fig. **2**) [24]. This fact makes the study of quinolone-like compounds an interesting topic on this research area.

3-Nitrocouamrins and related compounds have been chosen as the subject for a QSAR study [25]. For those studies literature data containing results of microbiological activity screening of 33 coumarin derivatives against *C. albicans* and *S. aureus* have been selected. There are no more compounds in the literature related to this scaffold and these activities. Therefore, it seems to be a good area to explore.

The dramatic worldwide increase of dangerous infections by resistant and multi-resistant microbes makes, therefore, the search of new molecules and new chemical entities an important topic in Medicinal Chemistry [26,27]. As the ideal drug candidate has not been attained, an intensive search for new and innovative antimicrobials is still needed. Unfortunately, due to economic reasons, pharmaceutical companies have dropped considerably this effort in recent years [28].

RESULTS AND DISCUSSION

Based on previous findings, in the chemical structure of the novobiocin and their derivatives (Fig. **1**) and in our experience with 3-substituted coumarins [22,23,29,30], in

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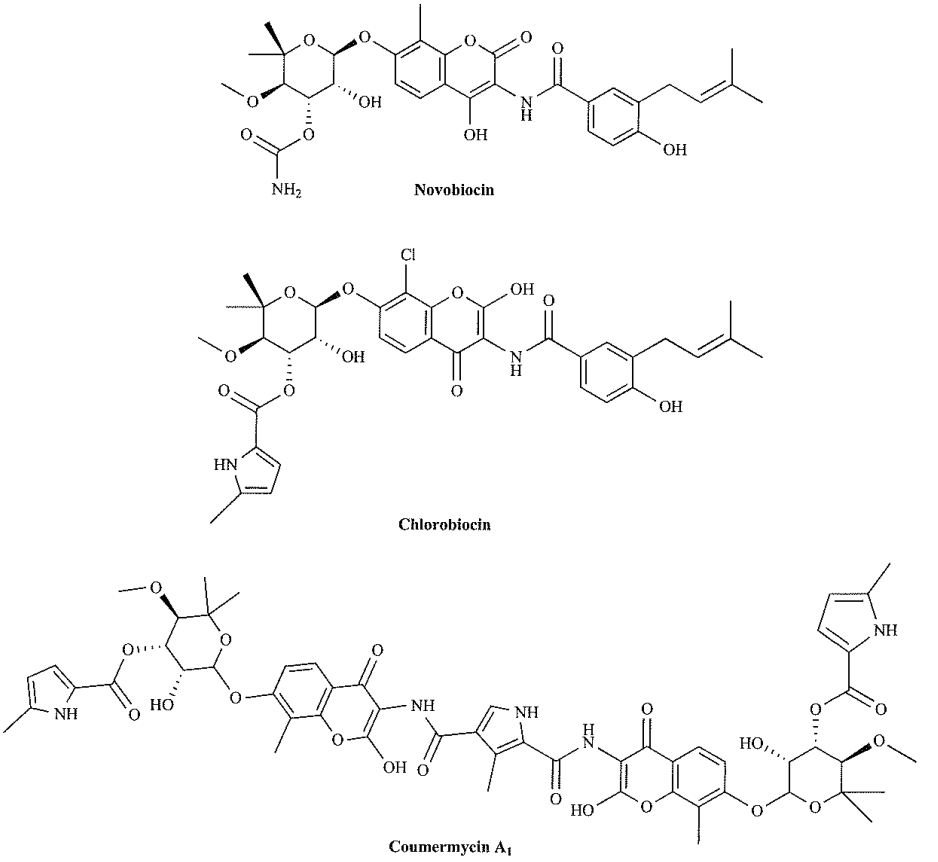


Fig. (1). Chemical structure of some coumarin derivatives with potent antimicrobial activity.

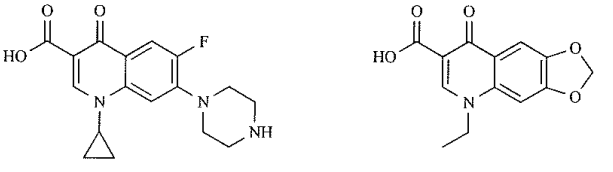
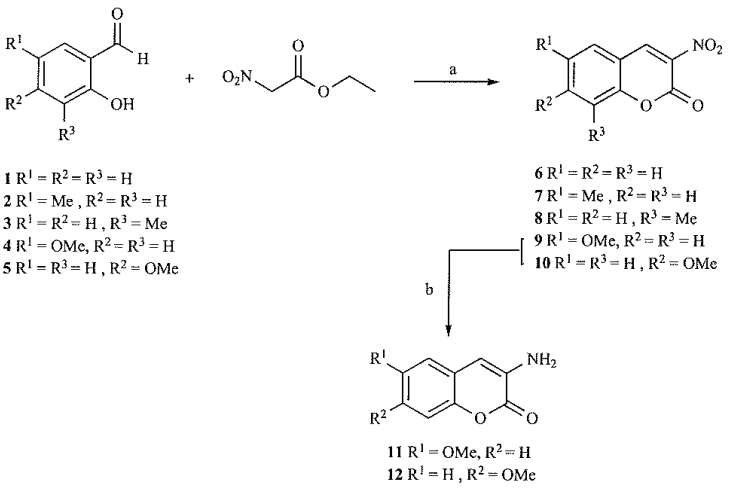


Fig. (2). Chemical structure of two quinolone derivatives.

rience with 3-substituted coumarins [22,23,29,30], in the present work we have proposed the synthesis and antimicrobial evaluation of different substituted 3-aminocoumarins (Scheme **1**). With the aim of mimetizing the novobiocin structure we have proposed the inclusion of a simple ether (methoxy group) in the 3-aminocoumarin moiety. Derivatives **11** and **12** were synthesized and evaluated. These two derivatives proved to have no activity against the tested bacteria. Therefore, we have centralized our work in the 3-nitrocoumarin scaffold. It was described that some nitrogen-containing moieties could increase significantly the antimicrobial efficiency and broaden their antimicrobial spectrum [20]. With the aim of finding out structural features for the antimicrobial activity and selectivity, we decided to explore also the importance of the nature and position of small groups (methoxy and methyl substituents) at 6, 7 or 8 position of the phenyl ring of the 3-nitrocoumarin moiety.

The coumarin derivatives **6-12** [31,32] were efficiently synthesized according to the synthetic protocol outlined in Scheme **1**. Starting from different commercially available salicylaldehydes and the ethyl nitroacetate, we obtained 3-



1 $R^1 = R^2 = R^3 = H$
2 $R^1 = Me$, $R^2 = R^3 = H$
3 $R^1 = R^2 = H$, $R^3 = Me$
4 $R^1 = OMe$, $R^2 = R^3 = H$
5 $R^1 = R^3 = H$, $R^2 = OMe$

6 $R^1 = R^2 = R^3 = H$
7 $R^1 = Me$, $R^2 = R^3 = H$
8 $R^1 = R^2 = H$, $R^3 = Me$
9 $R^1 = OMe$, $R^2 = R^3 = H$
10 $R^1 = R^3 = H$, $R^2 = OMe$

11 $R^1 = OMe$, $R^2 = H$
12 $R^1 = H$, $R^2 = OMe$

Scheme 1. *Reagents and conditions:* (a) acetic anhydride, NaH, r.t., 3 h, yields: 75-90 %; (b) H_2, ethanol, r.t., 5 h, yields: 95 %.

Table 1. ***In vitro*** **Antibacterial Activity[a] of 3-amino/nitro-coumarins 6-12 (A) Expressed as Inhibition Zones (mm).[b] (B) Expressed as MIC (μg/mL)**

Compounds	Method A (mm)		Method B (μg/mL)	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
6	16	12	32	64
7	15	12	16	64
8	15	11	32	64
9	13	NA	32	128
10	11	NA	32	256
11	NA	NA	> 256	> 256
12	NA	NA	> 256	> 256
Oxolinic acid	26	31	2	< 1
Ampicillin	32	23	2	8

[a] These results are average results of three experiments.
[b] The compounds were used at the concentration of 100 μg/disk and the inhibition zones are stated in mm.
NA = not active; diameter of inhibition zone ≤ 5 mm.

nitrocoumarins **6-10** in good yields (75-90 %). They were synthesized in a dry Schlenk tube, with acetic anhydride as solvent, in the presence of sodium hydride at room temperature for three hours. The reaction mixture was purified by flash chromatography, using hexane/ethyl acetate as eluent, in a 9:1 proportion. The 3-aminocoumarins **11** and **12** were prepared from the previously synthesized 3-nitrocoumarin (compounds **9** and **10**, respectively), in ethanol and using Pd/C as catalyst under H_2 atmosphere. The obtained products were purified by column chromatography (hexane/ethyl acetate 9:1) to give the desired aminocoumarins in a 95% yield.

Two different methodologies were employed in the antimicrobial studies: disk diffusion test and microdilution method. Clinical antimicrobial drugs –oxolinic acid (quinolone antibiotic, Fig. **2**) and ampicillin (*β*-lactamic antibiotic)– were used as positive controls. In the first case, the antibacterial and antifungal activities of the compounds **6-12** were evaluated *in vitro* by the measurement (in mm) of the inhibition zone of a Gram-positive bacterium (*S. aureus*), a Gram-negative bacterium (*E. coli*) and a yeast (*C. albicans*), using the disk diffusion test. The antibacterial and antifungal activities were tested at concentrations of 100μg/disk, in DMSO. The bacterial/fungal cultures were inoculated and incubated according to the described protocol. The inhibition zones values for growth inhibitory effects of the new compounds and controls were measured in millimetres (Table **1** – method A).

The obtained results revealed that the studied 3-aminocoumarins (compounds **11** and **12**) are inactive against all the tested strains in the *in vitro* assays, while the 3-nitrocoumarins (compounds **6-10**) can, in some cases, efficiently inhibit their growth. Compounds **7** and **8**, with a methyl group at 6 and 8 positions respectively, and compound **6**, without any substituents in the phenyl ring, are all active against both tested bacteria. Compounds **9** and **10**, with a methoxy group at 6 and 7 positions respectively, did not show any antimicrobial activity against *E. coli* by the first methodology. Also, they resulted to be less active against the *S. aureus* than the other nitro derivative compounds. In spite of the interesting results against the two tested bacteria, the entire series is inactive against the yeast *C. albicans*. The experimental data revealed that some of the tested compounds showed interesting activity against the two tested bacteria when compared with the standards ampicillin and oxolinic acid and that the different nature and position of the substituent in the coumarin scaffold changes the selectivity for both bacteria.

In order to deeply study the antibacterial activities and quantify the previously mentioned information, minimum inhibitory concentrations (MICs) of **6-12** against *S. aureus* and *E. coli* were also determined. This study was done against the same bacteria strains, by the microdilution method with serial dilutions (from 512 to 1 μg/mL) of the synthesized and the reference compounds. The MICs are summarized in table 1 – method B. MICs against *C. albicans* of compounds **6-12** were not evaluated taking into account the lack of inhibition zones in the previous disk diffusion test. Almost all the results are congruent with the ones found for the first methodology. The small differences between the two methodologies could be due to the experimental conditions. Some compounds have problems in their diffusion in agar. So, the first one is only a good preliminary orientation for the studies. Analyzing the MICs results, all the five nitro substituted studied compounds presented activity against the two tested bacteria. From the data we can infer that the studied compounds have higher antibacterial activity against *S. aureus* (16-32 μg/mL) than against *E. coli* (64-256 μg/mL), as we previously verified. Compounds **9** and **10**, with a methoxy substituent in the phenyl ring of the coumarin, were not selective against *S. aureus*, as it seems in the first study. However they resulted to be less active against *E. coli* (128 and 256 μg/mL, respectively) than compounds **6-8** (64 μg/mL), with a methyl substituent or without any substitution. The nature of the substituent seems to be important for the activity against *E. coli*. With this methodology, compound **7**, with a methyl group at position 6, proved to have half of the minimum inhibitory concentration against *S. aureus* than the other methyl substituted compound **8** and the unsubstituted compound **6**. The position of the methyl substituent seems to be relevant against *S. aureus*.

CONCLUSION

In conclusion, in the present study it was shown that all the synthesized 3-nitrocoumarins have inhibitory activity against *S. aureus* and *E. coli*. MICs determination proved that the tested compounds, against both bacteria species, presented different profiles due to the substituents. The effect against *E. coli* seems to depend on the type of substituent in the phenyl ring of the coumarin moiety (methyl > methoxy). The effect against *S. aureus* seems to depend on the position of the methyl substitution (position 6 > position 8). The pharmacological potential of these 3-nitrocoumarins confirms that this lead could be effectively optimized into a drug candidate for the treatment of some bacterial diseases. These findings have encouraged us to continue the efforts towards the optimization of the antimicrobial profile of these coumarins.

EXPERIMENTAL

General Methods

Melting points (mp) are uncorrected and were determined with a Reichert Kofler thermopan or in capillary tubes in a Buchi 510 apparatus. ^{1}H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) spectra were recorder with a Bruker AMX spectrometer. Chemical shifts (d) are expressed in parts per million (ppm) using TMS as an internal standard. Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets) and m (multiplet). Mass spectrometry was carried out with a Kratos MS-50 or a Varian MAT-711 spectrometer. Elemental analyses were performed by a Perkin-Elmer 240B microanalyzer and were within ±0.4% of calculated values in all cases. Flash Chromatography (FC) was performed on silica gel (Merck 60, 230-400 mesh); analytical TLC was performed on precoated silica gel plates (Merck 60 F254).

General Methodology to Prepare 3-nitrocoumarins

In a 20 mL dry Schlenk tube, to a solution of the conveniently substituted salicylaldehyde (2.46 mmol) and ethyl nitroacetate (2.46 mmol), in acetic anhydride, NaH (2.46 mmol) was added, in small portions, and the reaction mixture was stirred for 3 hours, at room temperature. The obtained crude was filtered and washed with diethyl ether. The solid was then purified by flash chromatography (hexane/ethyl acetate 9:1) to give the desired coumarins (**6–10**).

8-Methyl-3-nitrocoumarin (**8**). It was obtained a white solid with a yield of 78 %. Mp: 147-148 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.49 (s, 3H, CH_3), 7.35 (t, 1H, H-6, J=7.6 Hz), 7.58 (dd, 1H, H-7, J=7.6, 1.6 Hz), 7.62 (dd, 1H, H-5, J=7.6, 1.5 Hz), 8.73 (s, 1H, H-4). ^{13}C NMR (75.4 MHz, $CDCl_3$) δ (ppm): 15.32, 115.90, 125.49, 126.89, 128.33, 135.25, 137.44, 142.74, 153.21, 156.23. ESI-MS m/z: 206 (12), 205 (M^+, 100), 147 (13), 103 (54), 102 (12), 77 (30), 51 (14). Anal. Calcd. for $C_{10}H_7NO_4$: C, 58.54; H, 3.44. Found: C, 58.52; H, 5.39.

General Methodology to Prepare 3-aminocoumarins

The previously prepared 3-nitrocoumarin (2.46 mmol) was dissolved in ethanol and a calatytic amount of Pd/C was added to the mixture. The solution was stirred, at room temperature, under H_2 atmosphere, for 5 hours. After the completion of the reaction, the mixture was filtered to eliminate the catalyst. The obtained crude was then purified by flash chromatography (hexane/ethyl acetate 9:1) to give the desired coumarins (**11** and **12**).

Biological Assays

Disk Diffusion Test: The antimicrobial activity of the compounds was assayed by the disk diffusion method, following the procedures of the Clinical and Laboratory Standards Institute (CLSI, 2006). The inoculum was prepared by making a saline suspension of colonies from a 24 h Mueller Hinton Agar (MHA) (Cultimed, Spain) plate culture of the microorganisms, containing approximately $1x10^8$ colony forming units (CFU)/mL (OD_{620} of 0,09). Colony counts on inoculum suspension were verified by the plate dilution method using MHA plates and counting the bacterial colonies produced. The entire surface of the MHA plate was inoculated by streaking with the swab containing the inoculum. Antimicrobial solution (concentration of 10000 μg/mL) was prepared by using DMSO as solvent. Sterile disks of 6 mm diameter (Liofilchem, Italy) embedded in the drug at a final concentration of 100 μg/disk were kept on agar surface. Sterile disks embedded with DMSO were used as a negative control. The plates were incubated at 37 °C for 24 h. Zones of inhibition were measured in millimeter.

Disk Minimum Inhibitory Concentrations (MICs): The MICs were evaluated using the broth microdilution method, following the procedures of the Clinical and Laboratory Standards Institute (CLSI, 2006). Broth microdilution tests were performed with 96 sterile flat-bottom microtiter plates (Becton Dickinson Labware Europe). Antimicrobials were serially diluted (512 to 1 μg/mL) in Mueller-Hinton Broth (MHB) (Cultimed, Spain) and then one hundred microliters of each dilution were deposited into each well. The inoculum was prepared by making a MHB suspension of colonies from a 24 h MHA (Cultimed, Spain) plate culture of the microorganisms, containing approximately $1x10^8$ colony forming units (CFU)/mL (OD_{620} of 0,09). The inoculum was diluted 1:100 in MHB to yield $1x10^6$ CFU/mL. Ten microliters of this suspension was inoculated into the each well. Colony counts on inoculum suspension were verified by the plate dilution method using MHA plates and counting the bacterial colonies produced. MHB with and without inoculum was used as growth-control and negative control, respectively. The plates were incubated at 37 °C for 24 h. The MIC was established comparing the amount of growth in the wells containing the antimicrobial agents with the amount of growth in the growth-control wells both with the unaided eye and by using a photometric device (OD_{620}).

CONFLICT OF INTEREST

The authors(s) confirm that this article content has no conflict of interest.

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Article

Synthesis and Structure-Activity Relationships of Novel Amino/Nitro Substituted 3-Arylcoumarins as Antibacterial Agents

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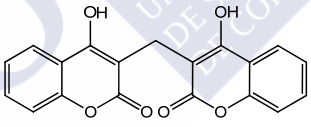
Abstract: A new series of amino/nitro-substituted 3-arylcoumarins were synthesized and their antibacterial activity against clinical isolates of *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) was evaluated. Some of these molecules exhibited antibacterial activity against *S. aureus* comparable to the standards used (oxolinic acid and ampicillin). The preliminary structure-activity relationship (SAR) study showed that the antibacterial activity against *S. aureus* depends on the position of the 3-arylcoumarin substitution pattern. With the aim of finding the structural features for the antibacterial activity and selectivity, in the present manuscript different positions of nitro, methyl, methoxy, amino and bromo substituents on the 3-arylcoumarin scaffold were reported.

Keywords: amino/nitro substituted 3-arylcoumarins; Perkin reaction; antibacterial assays; *S. aureus*; *E. coli*

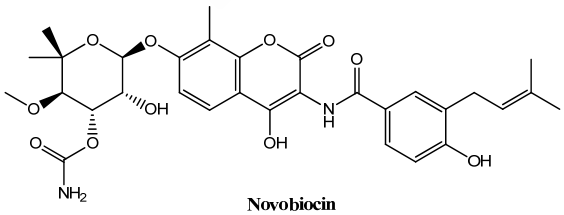
1. Introduction

Drug discovery is considered a complex and slow activity. However, with the intention of discovering new chemical entities in a new and more efficient way, medicinal chemistry researchers have developed new approaches and methods. Some of the new products are based on diversity molecules found and extracted from natural sources. Coumarins are a large family of compounds of natural or synthetic origin, associated with remarkable pharmacological activities [1,2]. They occur naturally in plants and microorganisms and approximately 1,000 coumarin derivatives have been isolated from over 800 species [2]. The fused heterocyclic framework of coumarins has been used as a prototype scaffold for the synthesis of a wide variety of analogues in order to study and improve their biological properties. Its structural variety is responsible for the important place they occupy in the realm of natural products and synthetic organic chemistry [2,3]. Some coumarins have been studied for their antimicrobial [4–7], cardioprotective [8], anticancer [9,10], antioxidative [11] and enzymatic inhibition properties [12–17]. Goth described the antibacterial properties of coumarins for the first time in 1945 when he studied the dicoumarol (Figure 1) [18]. Some other coumarin derivatives have proven to display antibacterial and antifungal activities [19–22]. A study of coumarin derivatives substituted on the pyrone ring indicated that 3-carboxyl derivatives show significant activities [23]. Different 4-substituted coumarins, such as 4-chlorocoumarins, exhibit also an interesting antimicrobial profile [24,25]. Novobiocin (Figure 1), chlorobiocin and coumermycin A_1 are important natural occurring antibiotics in which the coumarin nucleus is present in their skeleton [26].

Figure 1. Chemical structures of dicoumarol and novobiocin.



Dicoumarol



Novobiocin

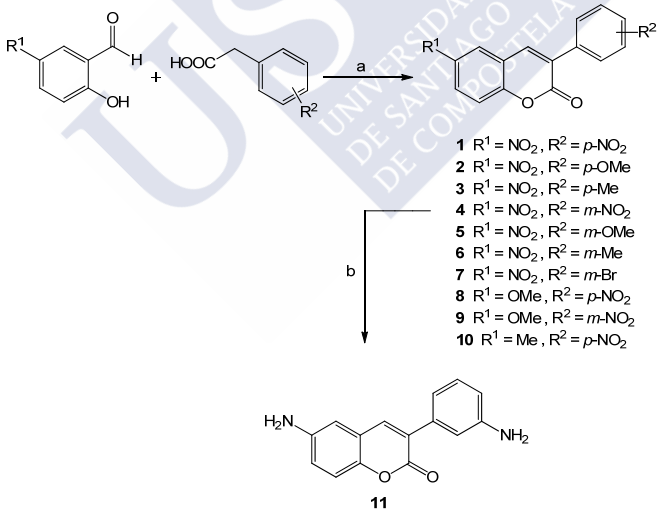
The most active of them is novobiocin, isolated from *Streptomyces niveus* and which is mainly active against Gram-positive bacteria. Although they antagonize the B subunit of the essential *E. coli* DNA gyrase supertwisting activity *in vitro* and the bacterial multiplication, they are not used in the clinic due to their relatively weak activity against Gram-negative bacteria, side effects and poor water solubility [4,26]. On the other hand, these three examples are antibiotics, which present activity against methicillin-resistant *S. aureus* (MRSA) [26]. The number of multidrug-resistant (MDR) bacteria is increasing and the Gram-positive MRSA are of particular importance. The ability of

S. aureus species to develop resistance to virtually all antibiotics is a major concern and the discovery of new antimicrobial agents is essential to combat this problem [27]. MRSA are often an important problem for severe infections in patients, especially if they are immunosuppressed, during long stays in hospitals [27]. The dramatic worldwide increase of dangerous infections by resistant and multi-resistant microbes makes, therefore, the search of new molecules and new chemical entities an important topic in medicinal chemistry [28,29]. As the ideal drug candidate has not been attained, an intensive search for new and innovative antimicrobials is still needed. Unfortunately, due to economic reasons, pharmaceutical companies have considerably decreased this effort in recent years [30].

2. Results and Discussion

Based on previous findings in the area [24,25], and in our experience with substituted 3-arylcoumarins [15,31,32], in the present work we proposed the synthesis and antibacterial evaluation of a series of different substituted amino/nitro 3-arylcoumarins (Scheme 1). With the aim of finding the structural features for the antibacterial activity and selectivity, we decided to explore the importance of the nature and position of small groups (methoxy, bromo, nitro, amino and methyl substituents) into both coumarin nucleus and 3-aryl ring (Scheme 1).

Scheme 1. Synthesis of 3-arylcoumarins (**1**–**11**).



Reagents and conditions: (a) NaH, acetic anhydride, r.t., 3 h; (b) H_2, Pd/C, ethanol, r.t., 3 h.

New derivatives **2**, **4**–**7** and **9**–**11**, as well as the already described compounds **1**, **3** and **8** [33–36], were efficiently synthesized according to the synthetic protocol outlined in Scheme 1. 3-Arylcoumarins **1**–**10** were synthesized in a dry Schlenk tube, in presence of sodium hydride and with acetic anhydride as solvent, at room temperature for three hours. The reaction mixture was purified by flash chromatography, using hexane/ethyl acetate as eluent in a proportion of 9:1. Starting from different commercially available substituted salicylaldehydes and the respectively substituted phenylacetic acids, we obtained ten derivates in good yields (60–75%). The derivative **11** was obtained

from **4**, in ethanol, with palladium/carbon as catalyst and under hydrogen atmosphere for three hours. The reaction mixture was purified by flash chromatography, using hexane/ethyl acetate as eluent, in a proportion of 9:1. We obtained the desired derivate in good yield (80%). Then, two different methodologies were employed for the antibacterial evaluation of these compounds: disk diffusion test and microdilution method. Clinical antimicrobial drugs–oxolinic acid and ampicillin–were used as positive controls. The inhibition zones for growth inhibitory effects of the new compounds and controls were measured in millimetres (Table 1, method A). The minimum inhibitory concentrations (MICs) of the new compounds and controls were measured in microgram/millilitre (Table 1, method B).

Table 1. *In vitro* antibacterial activity [a] of amino/nitro substituted 3-arylcoumarins **1**–**11** (**A**) expressed as inhibition zones (mm) [b] and (**B**) expressed as MICs (μg/mL).

Compounds	Method A (mm)		Method B (μg/mL)	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
1	19	NA	128	-
2	25	NA	64	-
3	22	NA	32	-
4	22	NA	32	-
5	25	NA	64	-
6	32	NA	8	-
7	14	NA	256	-
8	NA	NA	>512	-
9	NA	NA	>512	-
10	NA	NA	>512	-
11	18	NA	128	-
Oxolinic acid	26	31	2	<1
Ampicillin	32	23	2	8

[a] These results are average results of three experiments; [b] The compounds were used at the concentration of 100 μg/disk and the inhibition zones are stated in mm; NA = not active; diameter of inhibition zone ≤ 5 mm.

The obtained inhibition zones revealed that all the 6-nitro derivatives (compounds **1**–**7**) presented antibacterial activity against *S. aureus*, showing inhibition zones ranging between 14 and 32 mm. The amino derivative **11** presented activity against the same bacterial strain. The 3-arylcoumarins nitro substituted only in the 3-aryl ring (compounds **8**–**10**) were inactive against *S. aureus* in the *in vitro* disk diffusion test assays, independently of the relative position of this nitro group (*para* or *meta* positions). From the data, it is shown that compound **6** and ampicillin presented the same inhibition zone against *S. aureus* (32 mm) and a higher inhibition zone than the oxolinic acid (26 mm). Hence, the preliminary experimental data revealed that some of the tested compounds showed a very interesting activity profile against *S. aureus* when compared with the standards ampicillin and oxolinic acid. In addition, the different nature and position of the substituent in the coumarin scaffold seems to influence the antibacterial activity.

In order to deeply study the antibacterial activities and quantify the previously mentioned information, MICs of compounds **1**–**11** against *S. aureus* were also determined. This second methodology (method B) is used to clarify and refine the previously results obtained by method A, which could be influenced by differences in the diffusion of the compounds. This study was performed

against the same bacteria strain, by a microdilution assay with serial dilutions (from 512 to 1 μg/mL) of the synthesized and the reference compounds. MICs of compounds **1**–**11** against *E. coli* were not evaluated, taking into account the lack of inhibition zones in the previous disk diffusion test (inhibition zones ≤ 5 mm). Analysing the MICs results for *S. aureus*, it is clear that the presence of a nitro substituent at the six position of coumarin moiety (compounds **1**–**7**) is more beneficial than its presence only at *meta* or *para* positions of the 3-aryl ring (compounds **8**–**10**).

Compounds with nitro substituents either in the coumarin moiety and in the aryl group (compounds **1** and **4**) are not better than compounds where the nitro in the aryl ring is substituted for a methyl or a methoxy group (compounds **2** and **3** compared to compound **1**, and compounds **5** and **6** compared to compound **4**). However, compound **4** (MIC = 32 μg/mL), with the nitro substituent in *meta* position of the 3-aryl ring, is better than compound **1** (MIC = 128 μg/mL), with the same substituent in *para* position. The presence of a methyl group in the *meta* position is the best substitution when one nitro substituent is present at six position of the coumarin moiety (compound **6**, MIC = 8 μg/mL). In general, *meta* substitutions are more favourable than *para*, being the *meta*-methyl substituent the most active, followed by *meta*-nitro and finally *meta*-methoxy. There is one exception to this conclusion. The introduction of a bromo atom at the same position was the worst substitution of the studied 3-aryl-6-nitrocoumarins (compound **7**, MIC = 256 μg/mL). A *para*-methyl substituent in the 3-aryl ring (compound **3**, MIC = 32 μg/mL) is the best substitution in this *para* position. Therefore, the *para*-methyl substitution is better than the *para*-nitro and *para*-methoxy substitutions. Comparing the two positions in the 3-aryl ring, the substitutions by methyl groups are more favourable for the desired activity. The substitution of nitro by amino groups significantly decreases the activity of the described compounds (comparing compounds **4** and **11**).

In order to evaluate if there was a correlation between the activity and the lipophilicity, and to better understand the overall properties of the described compounds, logP (octanol/water partition coefficient) values were calculated using the Molinspiration property calculation program [37]. The results are presented in Table 2.

Table 2. LogP (octanol/water partition coefficient) values calculated for the amino/nitro substituted 3-arylcoumarins **1**–**11** [a].

Compounds	LogP
1	3,631
2	3,729
3	4,120
4	3,607
5	3,705
6	4,096
7	4,457
8	3,729
9	3,705
10	4,120
11	1,841

[a] The data was determined with Molinspiration calculation software [37].

Analysing the obtained data, it can be concluded that there are no linear correlations between the lipophilicity and the activity of the compounds. Compounds **5** and **6** are two of the best compounds against *S. aureus*, presenting intermediate, but different, logP values (3.705 and 4.096 respectively). The compounds with the lowest (compound **11**, 1.841) and the highest (compound **7**, 4.457) logP presented activity. Compound **9**, with the same logP as compound **5**, resulted to be inactive. Also, compound **10**, with the same lipophilicity profile as compound **3** (logP = 4.12), resulted to be inactive. Therefore, there are no clear correlation between activity and logP values. It is important to highlight that the calculated logP values for all the described compounds are lower than 5.

3. Experimental

3.1. Chemistry

Merck supplied the entire chemicals used in the synthesis. Melting points (mp) are uncorrected were determined using a Reichert Kofler thermopan or in capillary tubes on a Büchi 510 apparatus and are uncorrected. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75.4 MHz) spectra were recorded with a Bruker AMX spectrometer. Chemical shifts (δ) are expressed in parts per million (ppm) using TMS as an internal standard. Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets) and m (multiplet). Mass spectrometry was carried out with a Kratos MS-50 or a Varian MAT-711 spectrometer. Elemental analyses were performed by a Perkin-Elmer 240B microanalyzer and were within ±0.4% of calculated values in all cases. Flash Chromatography (FC) was performed on silica gel (Merck 60, 230–400 mesh); analytical TLC was performed on precoated silica gel plates (Merck 60 F254).

General Procedure for the preparation of 3-arylcoumarins **1**–**10**. In a 20 mL dry Schlenk tube, to a solution of the conveniently substituted salicylaldehyde (2.46 mmol) and the arylacetic acid (2.46 mmol), in acetic anhydride (6 mL), NaH (2.46 mmol) was added in small portions, and the reaction mixture was stirred for 3 hours, at room temperature. The obtained crude was filtered and washed with diethyl ether. The solid was then purified by flash chromatography (hexane/ethyl acetate 9:1) to give the desired coumarins **1**–**10**.

3-(4′-Methoxyphenyl)-6-nitrocoumarin (**2**). Pale yellow solid, 75% yield. Mp: 62–63 °C. ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.80 (s, 3H, OCH_3), 7.05 (d, 2H, H-3′, H-5′, J = 9.45 Hz), 7.62–7.69 (m, 3H, H-2′, H-6′, H-8), 8.36 (s, 1H, H-4), 8.73 (d, 2H, H-5, H-7, J = 2.93 Hz). ^{13}C-NMR ($CDCl_3$) δ (ppm): 55.9, 116.4, 119.7, 127.9, 128.4, 128.6, 129.2, 132.2, 132.5, 134.3, 139.1, 139.4, 151.5, 155.8, 161.2. ESI-MS *m/z*: 297 (M^+, 100). Anal. Calcd. for $C_{16}H_{11}NO_5$: C, 64.65; H, 3.73; Found: C, 64.67; H, 3.76.

3-(3′-Nitrophenyl)-6-nitrocoumarin (**4**). Pale yellow solid, 69% yield. Mp: 87–88 °C. ^{1}H-NMR ($CDCl_3$) δ (ppm): 7.57–7.63 (m, 2H, H-8, H-6′), 7.71–7.77 (m, 1H, H-5′), 8.15 (s, 1H, H-4), 8.42-8.63 (m, 4H, H-4′, H-2, H-5, H-7). ^{13}C-NMR ($CDCl_3$) δ (ppm): 118.1, 120.2, 123.1, 123.3, 124.3. 124.5, 124.7, 129.4, 133.6, 135.1, 141.2, 144.6, 147.9, 159.2, 160.5. ESI-MS *m/z*: 312 (M^+, 100). Anal. Calcd. for $C_{15}H_8N_2O_6$: C, 50.70; H, 2.58. Found: C, 50.72; H, 2.60.

3-(3′-Methoxyphenyl)-6-nitrocoumarin (**5**). Pale yellow solid, 71% yield. Mp: 53–54 °C. ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.71 (s, 3H, OCH_3), 6.80 (s, 1H, H-2′), 7.17–7.22 (m, 3H, H-4′, H-5′, H-6′), 7.57 (d, 1H, H-8, *J* = 6.5 Hz), 7.77 (s, 1H, H-5), 8.19 (d, 1H, H-7, *J* = 6.5 Hz), 8.39 (s, 1H, H-4). ^{13}C-NMR ($CDCl_3$) δ (ppm): 55.4, 84.5, 112.4, 115.5, 122.0, 123.5, 125.8, 126.8, 129.5, 129.7, 136.9, 145.7, 153.2, 159.6, 168.7, 173.0. ESI-MS *m/z*: 297 (M^+, 100). Anal. Calcd. for $C_{16}H_{11}NO_5$: C, 64.65; H, 3.73. Found: C, 64.63; H, 3.70.

3-(3′-Methylphenyl)-6-nitrocoumarin (**6**). Pale yellow solid, 69% yield. Mp: 55–56 °C. ^{1}H-NMR ($CDCl_3$) δ (ppm): 2.25 (s, 3H, CH_3), 6.90–7.30 (m, 5H, H-2′, H-4′, H-5′, H-6′, H-8), 7.75 (s, 1H, H-4), 8.44 (d, 1H, H-7, *J* = 2.8 Hz), 8.62 (d, 1H, H-5, *J* = 2.8 Hz). ^{13}C-NMR ($CDCl_3$) δ (ppm): 21.5, 118.4, 123.3, 124.5, 124.6, 124.8, 125.9, 126.6, 128.3, 128.5, 132.7, 138.4, 141.1, 144.6, 159.3, 160.9. ESI-MS *m/z*: 281 (M^+, 100). Anal. Calcd. for $C_{16}H_{11}NO_4$: C, 68.32; H, 3.94. Found: C, 68.31; H, 3.92.

3-(3′-Bromophenyl)-6-nitrocoumarin (**7**). Pale yellow solid, 60% yield. Mp: 240–241 °C. ^{1}H-NMR ($CDCl_3$) δ (ppm): 7.12–7.26 (m, 2H, H-4′, H-5′), 7.46–7.71 (m, 5H, H-2′, H-6′, H-5, H-7, H-8), 7.78 (s, 1H, H-4). ^{13}C-NMR ($CDCl_3$) δ (ppm): 117.9, 122.7, 123.1, 124.5, 124.6, 124.8, 127.9, 129.5, 129.7, 130.8, 134.6, 140.7, 144.6, 159.1, 161.1. ESI-MS *m/z*: 345 (M^+, 100). Anal. Calcd. for $C_{15}H_8BrNO_4$: C, 52.05; H, 2.33. Found: C, 52.01; H, 2.30.

6-Methoxy-3-(3′-nitrophenyl)coumarin (**9**). Pale yellow solid, 73% yield. Mp: 84–85 °C. ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.77 (s, 3H, OCH_3), 7.01–7.10 (m, 2H, H-5, H-7), 7.54–7.59 (m, 2H, H-5′, H-6′), 7.71 (d, 1H, H-8, *J* = 7.6 Hz), 8.06–8.09 (m, 2H, H-2′, H-4′), 8.15 (s, 1H, H-4). ^{13}C-NMR ($CDCl_3$) δ (ppm): 56.7, 115.0, 116.8, 122.3, 124.9, 128.7, 130.2, 134.2, 135.5, 137.2, 138.0, 143.3, 148.3, 156.8, 167.7, 172.7. ESI-MS *m/z*: 297 (M^+, 100). Anal. Calcd. for $C_{16}H_{11}NO_5$: C, 64.65; H, 3.73. Found: C, 64.63; H, 3.70.

6-Methyl-3-(4′-nitrophenyl)coumarin (**10**). Pale yellow solid, 75% yield. Mp: 100–101 °C. ^{1}H-NMR ($CDCl_3$) δ (ppm): 2.36 (s, 3H, CH_3), 7.21 (d, 2H, H-7, H-8, *J* = 8.2 Hz), 7.38 (s, 1H, H-5), 7.63–7.70 (m, 2H, H-2′, H-6′), 7.99–8.20 (m, 2H, H-3′, H-5′), 8.38 (s, 1H, H-4). ^{13}C-NMR ($CDCl_3$) δ (ppm): 20.6, 122.6, 123.9, 125.1, 127.9, 130.2, 131.4, 136.4, 136.6, 136.8, 137.2, 148.1, 149.3, 170.2, 190.6. ESI-MS *m/z*: 281 (M^+, 98). Anal. Calcd. for $C_{16}H_{11}NO_4$: C, 68.32; H, 3.94. Found: C, 68.38; H, 3.99.

Procedure for the Preparation of 3-(3′-Aminophenyl)-6-Aminocoumarin (**11**)

The previously prepared 3-(3′-nitrophenyl)-6-nitrocoumarin (**4**, 2.46 mmol) was dissolved in ethanol (5 mL) and a catalytic amount of Pd/C was added to the mixture. The solution was stirred, at room temperature, under a H_2 atmosphere, for 3 h. After completion of the reaction, the mixture was filtered to eliminate the catalyst. The obtained crude was then purified by flash chromatography (hexane/ethyl acetate 9:1) to give the desired coumarin **11** as a white solid in 80% yield. Mp: 105–106 °C. ^{1}H-NMR ($CDCl_3$) δ (ppm): 4.02 (s, 2H, NH_2), 6.50–6.55 (m, 1H, H-4′) 6.56 (s, 1H, H-2′), 6.69–6.73 (m, 1H, H-6′) 6.80 (dd, 2H, H-5, H-7, *J* = 7.8, *J* = 2.4 Hz), 7.30–7.38 (m, 2H, H-5′, H-8), 8.4 (s, 1H, H-4). ^{13}C-NMR ($CDCl_3$) δ (ppm): 114.9, 115.7, 116.7, 120.9, 123.6, 125.4, 127.2, 128.6,

133.7, 135.8, 136.2, 143.3, 145.5, 149.7, 161.9. ESI-MS *m/z*: 252 (M^+, 100). Anal. Calcd. for $C_{15}H_{12}N_2O_2$: C, 71.42; H, 4.79. Found: C, 71.45; H, 4.82.

3.2. Biological Assays

Disk Diffusion Test: The antimicrobial activity of the compounds was assayed by the disk diffusion method, following the procedures of the Clinical and Laboratory Standards Institute (CLSI, 2006). The inoculum was prepared as a saline suspension of colonies from a 24 h Mueller Hinton Agar (MHA) (Cultimed, Barcelona, Spain) plate culture of the microorganisms, containing approximately 1×10^8 colony forming units (CFU)/mL (OD_{620} of 0,09). Colony counts on inoculum suspension were verified by the plate dilution method using MHA plates and counting the bacterial colonies produced. The entire surface of the MHA plate was inoculated by streaking with the swab containing the inoculum. Antimicrobial solution (concentration of 10000 μg/mL) was prepared using DMSO as solvent. Sterile disks of 6 mm diameter (Liofilchem, Roseto degli Abruzzi, Italy) embedded in the drug at a final concentration of 100 μg/disk were kept on agar surface. Sterile disks embedded with DMSO were used as a negative control. The plates were incubated at 37 °C for 24 h. Zones of inhibition were measured in millimeter.

Disk Minimum Inhibitory Concentrations (*MIC*s): The MICs were evaluated using the broth microdilution method, following the procedures of the Clinical and Laboratory Standards Institute (CLSI, 2006). Broth microdilution tests were performed with 96 sterile flat-bottom microtiter plates (Becton Dickinson Labware Europe, Madrid, Spain). Antimicrobials were serially diluted (512 to 1 μg/mL) in Mueller-Hinton Broth (MHB) (Cultimed) and then one hundred microliters of each dilution were deposited into each well. The inoculum was prepared by making a MHB suspension of colonies from a 24 h MHA (Cultimed) plate culture of the microorganisms, containing approximately 1×10^8 colony forming units (CFU)/mL (OD_{620} of 0,09). The inoculum was diluted 1:100 in MHB to yield 1×10^6 CFU/mL. Ten microliters of this suspension was inoculated into the each well. Colony counts on inoculum suspension were verified by the plate dilution method using MHA plates and counting the bacterial colonies produced. MHB with and without inoculum was used as growth-control and negative control, respectively. The plates were incubated at 37 °C for 24 h. The MIC was established comparing the amount of growth in the wells containing the antimicrobial agents with the amount of growth in the growth-control wells both with the unaided eye and by using a photometric device (OD_{620}).

4. Conclusions

In conclusion, in the present study it was shown that eight out of the eleven synthesized amino/nitro-substituted 3-arylcoumarins have inhibitory activity against *S. aureus*. MIC determinations proved that the tested compounds presented different profiles against *S. aureus* due to their substituents, being 3-(3′-methylphenyl)-6-nitrocoumarin (**6**) the best one. A nitro substituent at the 6-position of the coumarin moiety seems to be essential for the antibacterial activity of this kind of compounds. The introduction of an amino substituent seems to decrease the described activity. The pharmacological potential of these amino/nitro-substituted 3-arylcoumarins confirms that this scaffold can be effectively optimized into a candidate for the treatment of some bacterial infectious diseases.

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Sample Availability: Samples of the compounds are available from the authors.

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New hydroxylated 3-arylcoumarins, synthesis and electrochemical study

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ABSTRACT

A selected series of new different substituted 3-arylcoumarins have been designed, synthesized, and evaluated their electrochemical redox mechanisms. The relevant structural information about coumarins was considered in order to better understand the structure/electrochemical relationship. The influence of bromine, methyl or other hydroxyl groups in different positions of the coumarin scaffold, by cyclic, differential pulse and square wave voltammetry at a glassy carbon electrode at different pHs was investigated. A comparative study of differently substituted 8-hydroxy-3-arylcoumarins was performed.

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1. Introduction

In recent years, phytochemical compounds have achieved great interest due to their presence in bioactive dairy products. Fruits, vegetables, oil and wine have been studied as health protectors because of the antioxidant potential of their phenolic compounds.

Recent studies have shown that many dietary phenolic compound constituents derived from plants are more effective antioxidants *in vitro* than vitamins E or C, and thus might contribute significantly to the protective effects *in vivo*. These compounds are known to be the most common secondary metabolites in the vegetable kingdom [1–3]. Studies demonstrated the antioxidant properties of phenolic compound constituents of plants, and their help in the identification of the active constituents in beverages, vegetables and fruit that may help sustain antioxidant status and protect against free radical damage [1].

Phenolic compounds are a very wide group of compounds important in hormones, vitamins, and food antioxidants. Their mechanism of action as antioxidants seems to involve the ability of phenols to scavenge radicals by an electron transfer process in which phenol is converted into a phenoxyl radical. A simple method was developed for estimating flavonoid antioxidant activity involving the quantitative correlation between lipid peroxidation inhibition and the half-wave potential ($E_{1/2}$) [4].

Most phenolic compounds can be electrochemically oxidized due to the hydroxyl groups attached to the aromatic rings. It is known that pH is one of the most significant factor determining the antioxidant activity of phenolic compounds. The dependence of the phenol derivatives oxidation potential, $E_{1/2}$, on solution pH has been studied thoroughly for different classes of polyphenols using a glassy carbon electrode [5–11].

These parameters gave information not only for evaluating the antioxidant potentialities of polyphenols but also for understanding their reaction mechanisms. Cyclic voltammetry has also been used for the evaluation of the antioxidant capacity of several polyphenols and their mixtures [12].

The activity of phenols towards free radical protection is important due to their scavenging role, related to their ability to react with radicals much more rapidly than with other organic substrates. Interest in the potential health benefits associated with dietary consumption of phenolic compounds has increased significantly in the last decades.

A well know and extensively studied polyphenolic natural phytoalexin is resveratrol [13]. This 3,4′,5-trihydroxystilbene is produced by some spermatophytes species, such as vines, in response to damage. Resveratrol has already been studied for its antioxidant, anti-inflammatory, cardio-protective (vasodilator and platelet anti-aggregator), anticancer and enzymatic inhibitory properties, proving to be very efficient in a large group of *in vitro*, *ex vivo* and/or *in vivo* experiments [14,15].

The fusion of a pyrone with a benzene ring gives rise to a class of heterocyclic compounds known as benzopyrones or coumarins [16]. Coumarins are a wide group of compounds present in remarkable amounts in the nature [17]. Representatives of this group occur in the vegetable kingdom, either in free or combined state [18]. Due to their structural variability, they are an elite class of compounds which occupy an important role in synthetic

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organic chemistry [19]. Coumarins have been attracting considerable interest due to their numerous biological activities, usually associated to low toxicity, depending on their substitution pattern [20]. There are many possible permutations offered by substitution and conjugation, and this readily explains why so many synthetic analogues featuring coumarin structural motif are investigated due to their wide range of biological properties [21]. In the literature, coumarins are described as anticancer [22,23], antioxidant [24,25], antimicrobial [26], antiviral [27], vasorelaxant [28], anti-inflammatory [29] and enzymatic inhibitors [30–33]. Indeed, some coumarins are now commercially available as medicines.

Natural products have a special ability to interact with more than one target [29]. In medicinal chemistry, this represents a significant source of inspiration for the design and synthesis of properly functionalized analogues with the aim of improving the pharmacological profile. Limited data are available to demonstrate the antioxidant activities of coumarins [34,35].

In this study an efficient methodology of synthesis followed by the investigation of the electrochemical mechanism of oxidation of several coumarin-resveratrol hybrids was undertaken. The electrochemical mechanisms were studied for a wide range of solution conditions, using cyclic, differential and square wave voltammetry. The information on these mechanisms was obtained at different pH and is shown to play a crucial role in understanding its antioxidant activity.

2. Experimental

2.1. Materials and methods

The coumarin-resveratrol hybrid derivatives **1**–**10** [33,36–38] were efficiently synthesized according to the protocol outlined in Scheme 1. The general conditions and the compounds characterization are described below.

Perkin condensation of differently substituted *ortho*-hydroxybenzaldehydes with the corresponding arylacetic acids, using *N,N′*-dicyclohexylcarbodiimide (DCC) as dehydrating agent, in DMSO, afforded the 3-phenylcoumarins **1**–**5**. Compounds **6**–**10** were synthesized starting from the respective methoxy/ethoxy derivatives **1**–**5** by hydrolysis reaction, using hydriodic acid 57%.

Melting points were determined using a Reichert Kofler thermopan or in capillary tubes on a Büchi 510 apparatus. IR spectra were recorded on a Perkin-Elmer 1640FT spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX spectrometer at 300 and 75.47 MHz, respectively, using TMS as internal standard (chemical shifts in δ values, J in Hz).

Mass spectra were obtained using a Hewlett Packard 5988A spectrometer. Elemental analyses were performed using a Perkin-Elmer 240B microanalyser and were within ±0.4% of calculated values in all cases. Silica gel (Merck 60, 230–00 mesh) was used for flash chromatography (FC).

Analytical thin layer chromatography (TLC) was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm).

All supporting electrolyte solutions were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity $\leqslant 0.1$ μS cm^{-1}), Table 1. Experiments were carried out at room temperature (25 ± 1 °C) and in the presence of dissolved oxygen.

The pH measurements were carried out with a Crison micropH 2001 pH-meter with an Ingold combined glass electrode. All experiments were done at room temperature (25 ± 1 °C) and microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pippettes (Rainin Instrument Co. Inc., Woburn, USA).

2.2. General procedure for the preparation of 3-phenylcoumarins (1–5)

To a solution of the (7.34 mmol) hydroxybenzaldehyde and (9.18 mmol) phenylacetic acid in (15 mL) dimethyl sulfoxide, (11.46 mmol) *N,N′*-dicyclohexylcarbodiimide was added and the mixture was heated in an oil-bath at 110 °C for 24 h. Triturate ice (100 mL) and acetic acid (10 mL) were added to the reaction mixture. After keeping it at room temperature for 2 h, the mixture was extracted with ether (3 × 25 mL). The organic layer was extracted with sodium bicarbonate solution (50 mL, 5%) and then water (20 mL). The solvent was dried with sodium sulfate and evaporated under vacuum. The residue was purified by FC (hexane/ethyl acetate 9:1).

2.2.1. 8-Ethoxy-3-phenylcoumarin (**1**)

Yield 55%; mp 117–118 °C. ^{1}H NMR ($CDCl_3$): 1.50 (t, 3H, –CH_3, J = 7.0), 4.21 (dd, 2H, –CH_2, J = 14.0, 7.0), 7.09 (t, 2H, H-6, H-7, J = 6.9), 7.21 (t, 1H, H-5, J = 7.8), 7.42–7.48 (m, 3H, H-3′, H-4′, H-5′), 7.72 (dd, 2H, H-2′, H-6′, J = 7.7, 1.4), 7.79 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$): 14.8, 65.0, 114.5, 119.3, 120.4, 124.3, 128.3, 128.4, 128.5, 128.8, 134.8, 140.1, 143.4, 146.3, 160.2. MS m/z (%): 267 ([M+1]$^+$, 16), 266 (M$^+$, 83), 239 (16), 238 (20), 212 (14) 211 (20), 181 (15), 153 (25). Anal. Calcd for $C_{17}H_{14}O_3$: C, 76.68; H, 5.30. Found: C, 76.66; H, 5.28.

2.2.2. 8-Ethoxy-3-(4′-methoxyphenyl)coumarin (**2**)

Yield 61%; mp 99–100 °C. ^{1}H NMR ($CDCl_3$): 1.50 (t, 3H, –CH_3, J = 7.0), 3.84 (s, 3H, –OCH_3), 4.19 (dd, 2H, –CH_2, J = 14.0, 7.0), 6.84–7.26 (m, 5H, H-3′, H-5′, H-5, H-6, H-7), 7.69 (t, 2H, H-2′, H-6′, J = 7.7), 7.73 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$): 14.8, 55.4, 64.8, 113.8, 114.0, 119.0, 120.5, 124.2, 127.0, 127.8, 129.7, 129.8, 138.6, 138.7, 146.2, 160.0. MS m/z (%): 297 ([M+1]$^+$, 35), 296 (M$^+$, 100), 268 (54), 240 (47) 225 (45), 197 (13), 152 (11), 139 (15). Anal. Calcd for $C_{18}H_{16}O_4$: C, 72.96; H, 5.44. Found: C, 72.91; H, 5.39.

2.2.3. 8-Ethoxy-3-(4′-methylphenyl)coumarin (**3**)

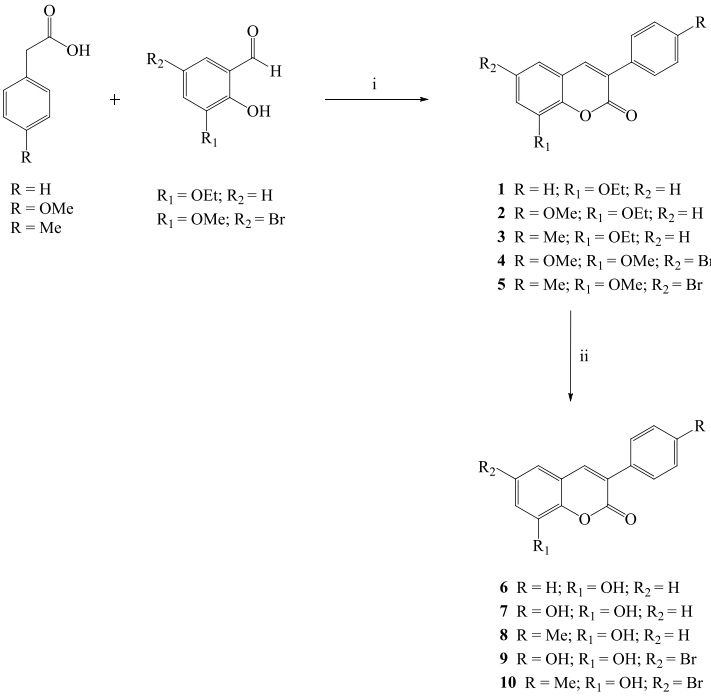
Yield 48%; mp: 109–110 °C. ^{1}H NMR ($CDCl_3$): 1.51 (td, 3H, –CH_3, J = 7.0, J = 1.8), 2.39 (s, 3H, –CH_3), 4.20 (dd, 2H, –CH_2, J = 7.0, J = 1.8), 7.06–7.10 (m, 2H, H-5, H-6), 7.18 (dd, 1H, H-7, J = 8.0, 1.9), 7.26 (d, 2H, H-3′, H-5′, J = 1.6), 7.61 (dd, 2H, H-2′, H-6′, J = 8.1, 1.8), 7.74 (d, 1H, H-4, J = 1.9). ^{13}C NMR ($CDCl_3$): 14.81, 21.30, 64.96, 114.37, 119.24, 120.51, 124.26, 128.34, 128.39, 129.15, 131.88, 138.83, 139.39, 143.31, 146.32, 160.26. MS m/z (%): 281 ([M+1]$^+$, 16), 280 (M$^+$, 80), 252 (78), 225 (16), 224 (56), 223 (20), 165 (30), 142 (45), 115 (17). Anal. Calcd for $C_{18}H_{16}O_3$: C, 77.12; H, 5.75. Found: C, 77.07; H, 5.80.

2.2.4. 6-Bromo-8-methoxy-3-(4′-methoxyphenyl)coumarin (**4**)

Yield 53%; mp 144–145 °C. ^{1}H NMR ($CDCl_3$): 3.85 (s, 3H, –OCH_3), 3.96 (s, 3H, –OCH_3), 6.93–6.96 (m, 2H, H-3′, H-5′), 7.12 (d, 1H, H-7, J = 1.8), 7.23 (d, 1H, H-5, J = 2.0), 7.63–7.67 (m, 2H, H-2′, H-6′), 7.69 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$): 55.7, 56.8, 114.2, 116.2, 116.8, 121.5, 126.8, 129.4, 130.2, 130.8, 137.3, 142.2, 147.8, 159.8, 160.6. MS m/z (%): 363 ([M+1]$^+$, 19), 362 (M$^+$, 100), 361 (19), 360 (59), 334 (24), 332 (23), 319 (33), 317 (34), 291 (11), 289 (11), 182 (18), 167 (17), 139 (21). Anal. Calcd for $C_{17}H_{13}BrO_4$: C, 56.53; H, 3.63. Found: C, 56.55; H, 3.68.

2.2.5. 6-Bromo-8-methoxy-3-(4′-methylphenyl)coumarin (**5**)

Yield 56%; mp 164–165 °C. ^{1}H NMR ($CDCl_3$): 2.40 (s, 3H, –CH_3), 3.98 (s, 3H, –OCH_3), 7.15 (s, 1H, H-7), 7.22–7.28 (m, 3H, H-3′, H-5′, H-5), 7.61 (d, 2H, H-2′, H-6′, J = 8.2), 7.67 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$): 21.3, 56.5, 116.0, 116.1, 116.6, 121.3, 128.4, 129.2, 129.6, 131.3, 137.6, 137.8, 139.3, 147.6, 159.4. MS m/z (%): 347 (25), 346 (98), 345 ([M+1]$^+$, 26), 344 (M$^+$, 100), 318 (47), 316 (46), 275



Scheme 1. Synthesis experimental conditions: (i) DCC, DMSO, 110 °C, 24 h; (ii) HI, AcOH, Ac_2O, reflux, 3 h.

Table 1
Supporting electrolytes.

pH	Composition
4.5	HAcO + NaAcO
7.0	NaH_2PO_4 + Na_2HPO_4
8.5	NaH_2PO_4 + Na_2HPO_4

(18), 273 (18), 208 (11), 166 (52), 165 (43), 115 (15), 83 (14). Anal. Calcd for $C_{17}H_{13}BrO_3$: C, 59.15; H, 3.80. Found: C, 59.11; H, 3.87.

2.3. General procedure for the preparation of hydroxy-3-phenylcoumarins (6–10)

To a solution of (0.50 mmol) substituted methoxy/ethoxy-3-phenylcoumarin in (5 mL) acetic acid and (5 mL) acetic anhydride at 0 °C, (10 mL) hydriodic acid 57% was added dropwise. The mixture was stirred, under reflux, for 3 h. The solvent was evaporated under vacuum and the dry residue was purified by CH_3CN crystallization.

2.3.1. 8-Hydroxy-3-phenylcoumarin (**6**)

Yield 64%; mp 199–200 °C [33].

2.3.2. 8-Hydroxy-3-(4′-hydroxyphenyl)coumarin (**7**)

Yield 41%; mp 237–238 °C [33].

2.3.3. 8-Hydroxy-3-(4′-methylphenyl)coumarin (**8**)

Yield 56%; mp 199–200 °C. ^{1}H NMR ($CDCl_3$): 2.33 (s, 3H, –CH_3), 7.06 (dd, 2H, H-5, H-6, J = 6.8, 2.9), 7.10–7.21 (m, 3H, H-3′, H-5′, H-7), 7.25 (d, 2H, H-2′, H-6′, J = 8.0), 8.15 (s, 1H, H-4), 10.22 (s, 1H, –OH). ^{13}C NMR ($CDCl_3$): 20.8, 117.8, 118.5, 120.4, 124.5, 126.6, 128.3, 128.8, 131.8, 138.0, 140.3, 141.5, 144.2, 159.7. MS m/z (%): 253 ([M+1]$^+$, 38), 252 (M$^+$, 100), 225 (26), 223 (23), 165 (15), 153 (12), 152 (25), 115 (11). Anal. Calcd for $C_{16}H_{12}O_3$: C, 76.08; H, 4.79. Found: C, 76.02; H, 4.83.

2.3.4. 6-Bromo-8-hydroxy-3-(4′-hydroxyphenyl)coumarin (**9**)

Yield 56%; mp 249–259 °C. ^{1}H NMR ($CDCl_3$): 6.83 (d, 2H, H-3′, H-5′, J = 8.8), 7.14 (d, 1H, H-7, J = 1.9), 7.36 (d, 1H, H-5, J = 2.0), 7.56 (d, 2H, H-2′, H-6′, J = 8.5), 8.00 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$): 115.6, 116.0, 119.9, 120.6, 122.5, 125.4, 128.2, 130.4, 138.0, 141.2, 146.0, 158.6, 159.8; MS m/z (%): 335 (35), 334 (99), 333 ([M+1]$^+$, 45), 332 (M$^+$, 100), 307 (31), 305 (35), 225 (26), 197 (29), 169 (35), 168 (46), 153 (24), 141 (21), 140 (16), 139 (51), 118 (17), 115 (28), 84 (18), 84 (46). Anal. Calcd for $C_{15}H_9BrO_4$: C, 54.08; H, 2.72. Found: C, 54.05; H, 2.69.

2.3.5. 6-Bromo-8-hydroxy-3-(4′-methylphenyl)coumarin (**10**)

Yield 43%; mp 192–193 °C. ^{1}H NMR ($CDCl_3$): 2.35 (s, 3H, –CH_3), 7.19 (d, 1H, H-7, J = 2.3), 7.27 (d, 2H, H-3′, H-5′, J = 8.0), 7.40 (d, 1H, H-5, J = 2.2), 7.61 (d, 2H, H-2′, H-6′, J = 8.1), 8.10 (s, 1H, H-4), 10.80 (s, 1H, –OH). ^{13}C NMR ($CDCl_3$): 20.9, 115.52, 119.8, 120.3, 121.8, 127.8, 128.4, 128.8, 131.5, 138.4, 139.0, 141.0, 145.6, 159.2. MS m/z (%): 333 (17), 332 (98), 331 ([M+1]$^+$, 18), 330 (M$^+$, 100), 305 (13), 304 (77), 303 (22), 302 (79), 223 (12), 166 (14), 165 (30),

152 (30), 115 (14). Anal. Calcd for $C_{16}H_{11}BrO_3$: C, 58.03; H, 3.35. Found: C, 58.06; H, 3.37.

2.4. Voltammetric measurements

Voltammetric experiments were carried out using an Autolab PGstat 10 running with GPES 4.9 software, Eco-Chemie, Utrecht, The Netherlands. Measurements were carried out using a three-electrode system in a 0.5 mL one-compartment electrochemical cell (Cypress System Inc., USA). Glassy carbon electrode (GCE, d = 1.5 mm) was the working electrode, Pt wire the counter electrode and the Ag/AgCl (3 M KCl) reference electrode. The experimental conditions for cyclic voltammetry were scan rate 50 mV s^{-1}. For differential pulse (DP) voltammetry were: pulse amplitude 50 mV, pulse width 70 ms and scan rate 5 mV s^{-1}. For square wave (SW) voltammetry were: pulse of 50 mV, frequency of 10 Hz and a potential increment of 2 mV, corresponding to an effective scan rate of 20 mV s^{-1}.

The GCE was polished using diamond particles of 3 μm (Kemet, UK) before each electrochemical experiment. After polishing, it was rinsed thoroughly with Milli-Q water. Following this mechanical treatment, the GCE was placed in buffer supporting electrolyte and voltammograms were recorded until a steady state baseline voltammograms were obtained. This procedure ensured very reproducible experimental results.

3. Results

Relevant aspects concerning the electrochemistry of the hydroxylated 3-arylcoumarins were investigated using cyclic voltammetry (CV), differential pulse (DP) voltammetry and square wave (SW) voltammetry.

The effect on the oxidation potential caused by the presence of a halogen on the coumarin structure was investigated. Halogens, substituents that are more electronegative than carbon, will inductively pull the electron density out of the ring. Due to the lone electron pairs this compounds are able to donate electronic density stabilizing the reaction intermediates by resonance in the *ortho* and *para* positions.

The effect of an alkyl group, in this specific case a methyl group, in the oxidation potential of a compound was also studied. The alkyl groups are electron donating, activating the ring enabling faster reaction rates.

3.1. Cyclic voltammetry

The compound **6** was investigated by CV in pH 7.0, Fig. 1, showing on the first scan oxidation peak P_1, at E_{p1} = +0.65 V, corresponding with an irreversible reaction. The value of $|E_p - E_{p/2}|$ = +50 mV indicates that one electron is involved in the first oxidation process [39]. A reduction peak P_{2c}, on the reverse scan, appears at E_{p2c} = +0.05 V corresponding to the reduction of the oxidation products formed during the oxidation at peak P_1.

In the second scan at lower potentials a new oxidation peak P_{2a}, at E_{p2a} = +0.08 V, confirmed the reversibility of peak P_2, that occurs with the transfer of two electrons [40]. The same behavior was found for compounds **8** and **10**, both with only one oxidizable hydroxyl group on their structure.

CVs of compound **8** showed an identical behavior to compound **6**. That means that the presence of a weak electron donating substituent, in a different ring where the hydroxyl group is, does not influence the oxidation potential. The strong adsorption of the oxidation products, which blocked the electrode surface, was also observed for compounds **6**, **8** and **10**, and oxidation peak P_1 current always decreased in the second scan.

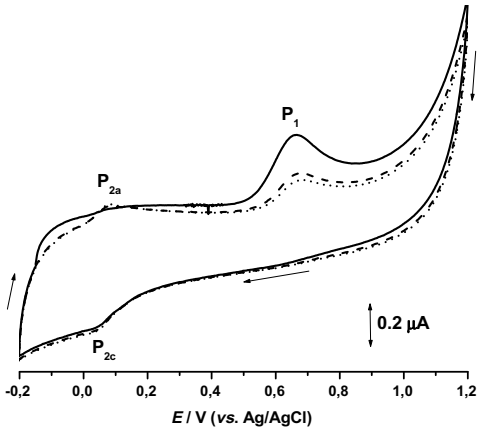


Fig. 1. CVs in 0.5 mM compound **6**, in 0.2 M phosphate buffer pH = 7.0: (—) first, (- - -) second and (•••) third scans. Scan rate 50 mV s^{-1}.

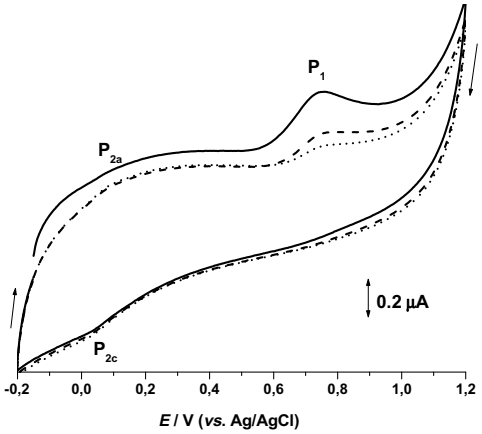


Fig. 2. CVs in 0.5 mM compound **10**, in 0.2 M phosphate buffer pH = 7.0: (—) first, (- - -) second and (•••) third scans. Scan rate 50 mV s^{-1}.

In the case of compound **10**, Fig. 2, the oxidation peak P_1 appears at a slightly higher potential, at E_{p1} = +0.71 V. This can be due to the presence of bromine, an electron withdrawing substituent which causes an acidity increment on the phenol moiety. The oxidation process corresponding to the peak P_1 is irreversible for compounds **6**, **8** and **10** and occurs with the transfer of one electron. It has been proved in this work that the oxidation of the hydroxyl group at the position C8 occurs irreversibly and it is formed an oxidation product which is oxidized at a lower potential in a reversible process.

The compounds **6**, **8** and **10** oxidation products are electroactive and reversibly oxidized at peak P_{2a}–P_{2c} with the transfer of two electrons. The compound **10** peak P_1 oxidation product, occurs at reversible peak P_{2a}–P_{2c}, E_{p2a} = +0.08 V and E_{p2c} = +0.05 V, and presented a very similar behavior to compound **6**, Fig. 1, showing that bromine does not interfere on the redox reaction of compound **10** oxidation product, with the oxidisable hydroxyl group and the

halogen in the *meta* position in the same ring. It can be remarked that compound **10** peak P_2 current was lower than compound **6** peak P_2 current, which could indicate that the different substituents do not affect the peak P_1 current, but affect the peak P_2 current.

The presence of electron withdrawing substituents, that deactivate the ring, causes an increase in acidity of the phenol. This effect involves charge, when the substituents are situated in the *ortho*- and *para*-positions, in respect to the hydroxyl group, due to conjugation of these positions with the group. This explains that the oxidation potential of compound **10** peak P_1 occurs at a slightly higher oxidation potential than the compound **6** peak P_1 that presents a not so acidic hydroxyl group. The CV study was done in the pH range between 4.5 and 8.5. The oxidation process is complex and pH-dependent, indicating that the oxidation of all compounds occurs associated with a proton transfer.

3.2. Differential pulse voltammetry

To clarify the mechanism of oxidation of this series of compounds DP voltammetry was used. Successive DP voltammograms in 0.5 mM compound **8**, in pH 4.5, acetate buffer showed the occurrence of one oxidation peak P_I, at E_{p1} = +0.80 V, and after the second scan peak P_2, at E_{p2} = +0.20 V, which corresponds to the oxidation of the peak P_I oxidation product, Fig. 3. The peak P_I width at half height was $W_{1/2}$ = 100 mV indicating an oxidation process with one electron involved [39].

The peak P_1 current decreased and peak P_2 current increased with increasing number of scans. This confirms that a strong adsorption of the oxidation product of compound **8** on the electrode blocks the electrode surface.

The same DP voltammetric behavior found for compound **8** was observed for compounds **6** and **10**, Fig. 4, but the compound **10** peak P_1 oxidation was for a higher potential than at compounds **6** and **8**, while compound **10** peak P_2 had the same peak potential as in compounds **6** and **8**, but compound **10** peak P_2 current was lower.

The DP voltammogram in compound **7**, that has two hydroxyl groups, presented two oxidation peaks due to two oxidizable hydroxyl groups, Fig. 5. In the first DP voltammogram the oxidation peak P_I width at half height was $W_{1/2} \sim 200$ mV corresponding to two oxidation peaks overlapped, that was confirmed after the baseline was subtracted in the DP voltammogram. The peaks overlap is due to the two similar hydroxyl groups in the compound **7**, the hydroxyl groups in positions C4′ and C8 of the 3-arylcoumarin, and the oxidation occurs at similar potentials, as they are both phenol groups.

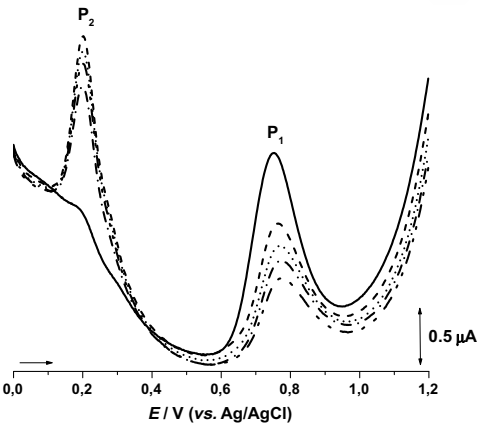


Fig. 3. Successive DP voltammograms (1 → 5) in 0.5 mM compound **8**, in 0.2 M acetate buffer pH = 4.5. Scan rate 5 mV s^{-1}.

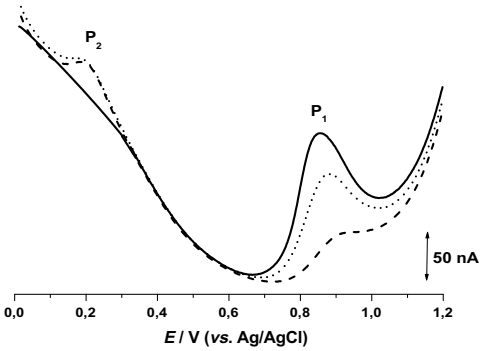


Fig. 4. DP voltammograms in 0.5 mM compound **10**, in 0.2 M acetate buffer pH = 4.5: (—) first, (•••) second and (- - -) third scans. Scan rate 5 mV s^{-1}.

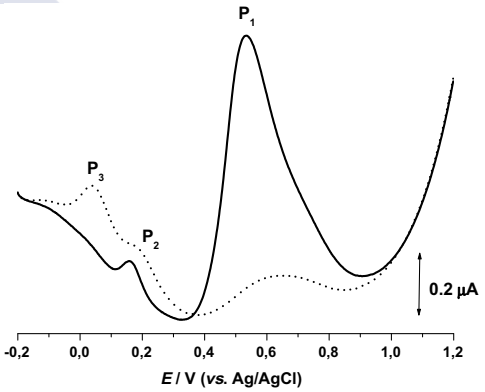


Fig. 5. DP voltammograms in 0.5 mM compound **7**, in 0.2 M phosphate buffer pH = 7.0: (—) first and (•••) second scans. Scan rate 5 mV s^{-1}.

The first scan DP voltammogram in compound **7** showed also another oxidation peak, peak P_2, at E_{p2} = +0.17 V, at a lower potential, corresponding to the oxidation of a catechol group. Although peak P_2 appears already on the first scan, peak P_2 is due to the oxidation product of peak P_1. This denotes that compound **7** was oxidized during its synthesis before the electrochemical experiences. The peak P_2 width at half height was $W_{1/2} \sim 60$ mV indicating that two electron are involved in the oxidation process, confirming that is a catechol moiety oxidation product of peak P_1 [39,40].

On the second scan, the oxidation product formed after P_1 oxidation, peak P_3, at E_{p3} = +0.05 V, occurs, as observed by CV, and is due to the oxidation of the hydroxyl group at position C8 in the P_1 oxidation product. This is confirmed by the appearance of peak P_3 only in the second scan and also at lower oxidation potential. The peak P_3 width at half height was $W_{1/2} \sim 60$ mV indicates that two electrons are involved on the oxidation of the hydroxyl group at position C8 oxidation product of compound **7**. The strong adsorption of the oxidation products, which blocked the electrode

surface, enabled to observe more easily on the second scan the overlap that occurs in P_1 corresponding to a transfer of one electron in each peak.

The DP voltammetric study was undertaken for compounds **6**, **8** and **10**, for a wide pH range and the potentials of peaks P_1, P_2 and P_3 were shifted to more negative values with increasing pH. The dependence of E_p vs. pH was linear the whole pH range studied and followed the relationships presented in Table 1. The E_p vs. pH, relationship enables the determination of the number of protons involved in the oxidation process. In all cases the oxidation potential was pH-dependent following a linear relationship with the slope of −0.059 V, which corresponds to a electron transfer reaction involving the same number of electrons and protons.

From Table 2, the transfer of one electron and one proton occurs for peak P_1, attributed to the hydroxyl group correspondent to a phenol group, whereas, oxidation product peaks P_2 and P_3, both occur with the transfer of two electrons and two protons.

Compound **9** shows a DP voltammogram very similar to that of compound **7**, Fig. 6. The oxidation peak P_1 observed is broad, indicating the presence of two hydroxyl groups that oxidize at similar potential. It also appears in the first scan a peak P_2 at lower potential. The peak P_3, a product of oxidation of P_1, appears in the second scan. A great decrease in peak P_1 current on the second scan indicates a strong adsorption of oxidation products formed on the electrode surface. The halogen group attached to the aromatic ring, with the hydroxyl group, has an electron withdrawing effect that makes the ring poorer in electron density. This causes a deactivating effect and a decreasing in the reactivity of the ring.

3.3. Square wave voltammetry

The advantages of SW voltammetry are greater speed of analysis, lower consumption of electro-active species in relation to DP voltammetry, and reduced problems with blocking of the electrode surface. A great advantage of the square-wave method is the possibility to see during one scan if the electron transfer reaction is reversible or not. Since the current is sampled in both the positive and the negative-going pulses, peaks corresponding to the oxidation or reduction of the electroactive species at the electrode surface are obtained in the same experiment.

The SW voltammetry conditions chosen led to well-defined SW voltammograms, and showed similar results to CV and DP voltammetry, i.e. the same oxidation peaks and oxidation products peaks, a strong adsorption on the second scan and the decrease of the oxidation potential with increasing pH.

Therefore, the irreversibility of P_1 and the reversibility of their oxidation product P_2, in compounds **6**, **8** and **10**, was confirmed by SW voltammetry, Fig. 7. Compounds **7** and **9** with two hydroxyl groups in their structure, presented the irreversible peak P_1 with overlapping, at E_{p1} = +0.85 V, and two reversible peaks, peak P_2, at E_{p2} = +0.39 V, due to oxidation during synthesis, and peak P_3, at E_{p3} = +0.21 V, Fig. 8, confirmed in the second scan after reversing the potential scan just before peak P_1 and obtaining P_3.

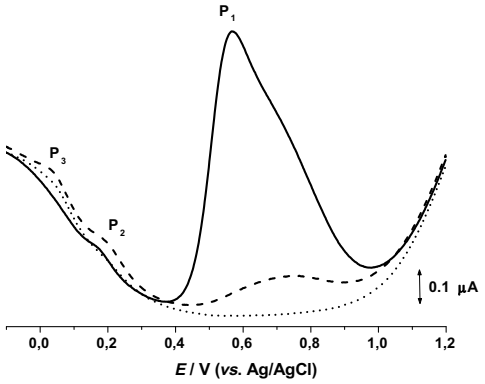


Fig. 6. DP voltammograms in 0.5 mM compound **9**, in 0.1 M phosphate buffer pH = 7.0: (—) first, (- - -) second and (•••) third scans. Scan rate 5 mV s^{-1}.

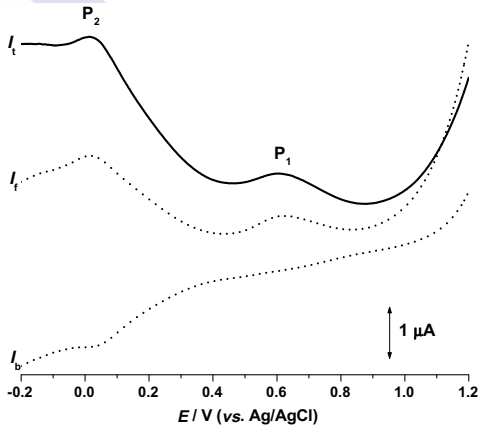


Fig. 7. Second SW voltammogram in 0.5 mM of compound **6**, in pH = 8.5, third scan. I_t – total current, I_f – direct current and I_b – forward current; f = 25 Hz, ΔE = 2 mV, v_{eff} = 50 mV s^{-1}.

4. Discussion

All described 3-arylcoumarins (compounds **1–10**) were efficiently synthesized, characterized and evaluated for their antioxidant functionality. Relevant aspects concerning the electrochemistry (compounds **6–10**) of this selected series of synthesised coumarin-resveratrol hybrids were obtained. It was found that the different substituents have only a slightly effect upon

Table 2
DP voltammetric data for compounds **6–10**.

	Peak P_1			Peak P_2			Peak P_3		
	E_p vs. pH	e^-	H^+	E_p vs. pH	e^-	H^+	E_p vs. pH	e^-	H^+
6	E_p = 0.98–0.057 pH pH	1	1	E_p = 0.47–0.056 pH pH	2	2	–	–	–
7	E_p = 0.96–0.061 pH	1	1	E_p = 0.61–0.059 pH	2	2	E_p = 0.48–0.059 pH	2	2
8	E_p = 1.02–0.059 pH	1	1	E_p = 0.46–0.060 pH	2	2	–	–	–
9	E_p = 1.03–0.060 pH	1	1	E_p = 0.62–0.058 pH	2	2	E_p = 0.46–0.059 pH	2	2
10	E_p = 1.08–0.060 pH	1	1	E_p = 0.43–0.057 pH	2	2	–	–	–

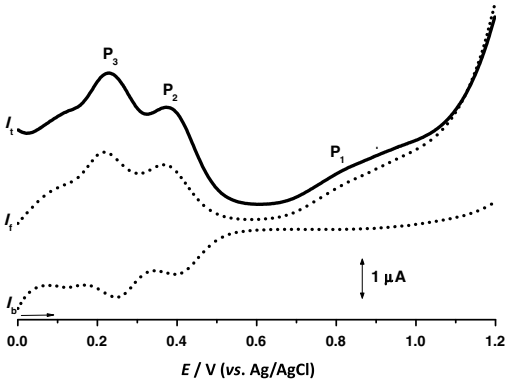


Fig. 8. Second SW voltammogram in 0.5 mM compound **9**, in 0.1 M acetate pH = 4.5 buffer, fourth scan. I_t – total current, I_f – forward current and I_b – backward current; f = 25 Hz, ΔE = 2 mV, v_{eff} = 50 mV s^{-1}.

reactivity of the selected coumarins. Also differences in the electroactive substituents on similar structures lead to characteristic differences in their voltammetric behavior.

Considering that the electrochemical behavior of these compounds depends on their structural characteristics, useful information on their antioxidant functionality can be drawn from the CV, DP and SW voltammograms for each hydroxylized compound performed at different pH values.

All synthetized compounds possess in common a hydroxyl group on position C8 and differ by the presence or absence of bromine, an electron withdrawing atom, in the benzene ring and the presence or absence of a hydroxyl strong electron donating group or a methyl weak electron donating group in the benzene ring linked to the position C3 of the coumarin structure. Electrochemical studies of this series of compounds allowed to investigate the influence on the oxidation potential of hydroxycoumarins of different substituents.

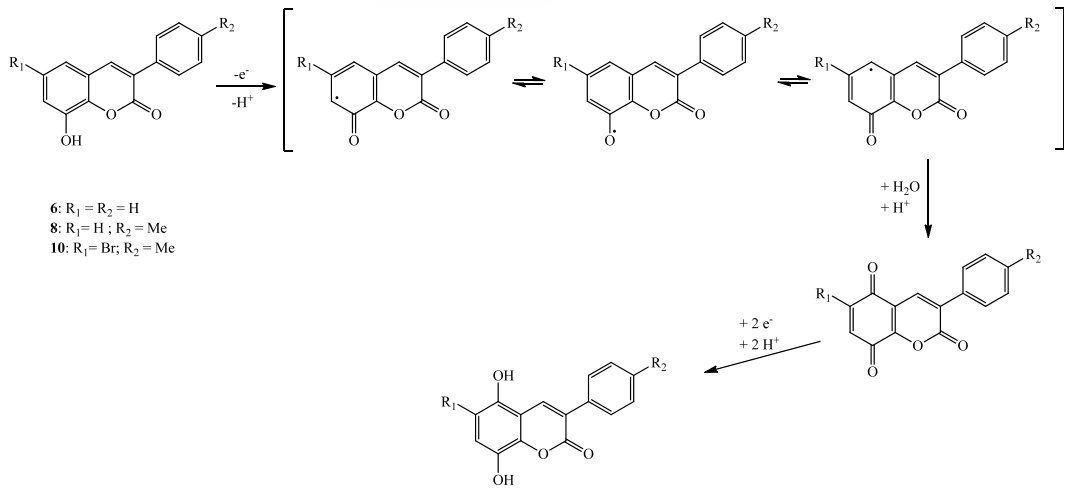
The results indicated that compounds **6**, **8** and **10** oxidation occurs in one step whereas compounds **7** and **9** oxidation occurs in two consecutive steps in for 4.5 < pH < 8.5. The irreversibility of all these steps was established by CV and SW voltammetry. The involvement of one electron and one proton in the oxidation steps was evaluated from the peak width at half height of the DP voltammograms and the slope of E_p vs. pH plot for each compound is in Table 1. The oxidation products oxidation peaks of each compound involved always two electrons and two protons.

Compounds **9** and **10** with bromine at position C6 on the coumarin moiety have the highest oxidation potentials on the series studied. This is due to the presence of an electron withdrawing substituent which causes an acidity increment on the phenol. Therefore, the reactivity of these compounds is different from the others. An oxidation mechanism was proposed for compounds **6**, **8** and **10**, Scheme 2.

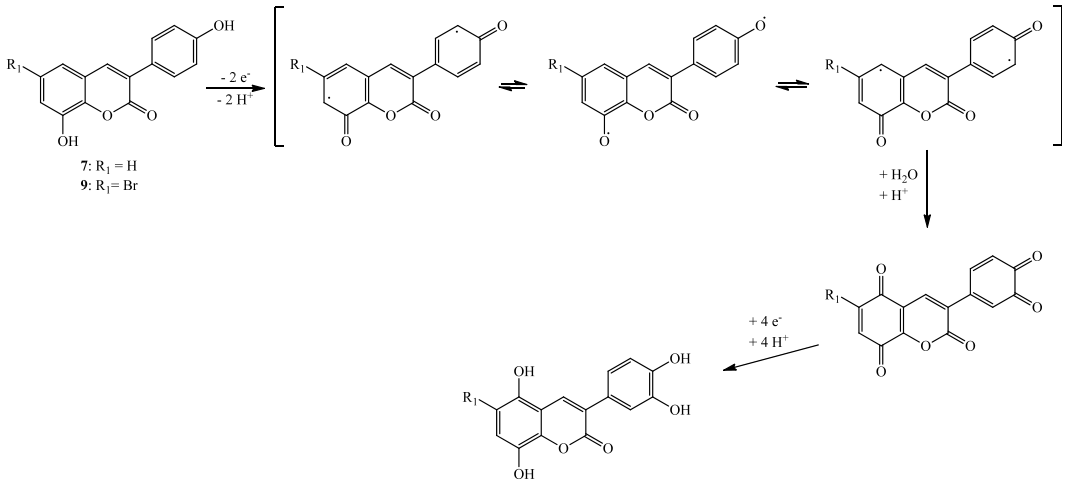
Cyclic voltammograms of compound **8** showed an identical behavior to compound **6**. That means that the presence of a methyl group weak electron donating substituent, in a different ring from the hydroxyl group, does not influence the reactivity of the compounds and the oxidation potential.

Compounds **7** and **9** besides the hydroxyl group on the position C8 have another hydroxyl group in position C4′, and this two oxidations potentials are very near and their peaks overlap. The oxidation mechanism occurs in two consecutive irreversible reactions with the transfer of an electron and a proton for the overlapped peaks in P_1.

The oxidation products formed after peak P_{1_a} identified as peak P_3, corresponds to the oxidation of the hydroxyl group on position C8, present in compounds **6**, **8** and **10**. The oxidation products formed after peak P_{1_c} identified as peak P_2, had a reversible oxidation process with transfer of two protons and two electrons with oxidation potential values with similar characteristics to a catechol group, corresponding to the oxidation of the hydroxyl group on position C8, present in compounds **7** and **9**. An oxidation mechanism was proposed for compounds **7** and **9**, Scheme 3.



Scheme 2. Proposed oxidation mechanism for compounds **6**, **8** and **10**.



Scheme 3. Proposed oxidation mechanism for compounds **7** and **9**.

5. Conclusions

The synthesis of ten new 3-arylcoumarins was carried out in an efficient, direct and versatile way using a Perkin reaction as key step. The ether derivatives had been hydroxylated, with good yields, giving the corresponding hydroxyl derivatives that were studied. All the new 3-arylcoumarins were electrochemically oxidized. The electrochemical data showed that all these novel coumarins can be oxidized at relatively low potentials, and the electrochemical results demonstrated that using electrochemical methods the electron transfer mechanisms of this new series of 3-arylcoumarins can be clarified. In compounds **6**, **8** and **10**, with only one oxidizable hydroxyl group on their molecule, the electrochemical behavior showed that one electron is involved in the oxidation process that occurs in one step. In compounds **7** and **9** the oxidation occurred in two consecutive steps. It was demonstrated than it was easier to oxidize the hydroxyl group at position C8 of the coumarin structure than the hydroxyl group at position C4′. As expected, the radical phenoxyl in C4′ is more stable. The experiments showed an irreversible reaction corresponding to the oxidation of the hydroxyl groups at positions C8 and C4′. This behavior, which is related with their molecular structure, clearly showed their good antioxidant properties.

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Remarkable antioxidant properties of a series of hydroxy-3-arylcoumarins ☆

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ABSTRACT

In the present work we synthesized a series of hydroxy-3-arylcoumarins (compounds **1**–**9**), some of them previously described as MAO-B selective inhibitors, with the aim of evaluating their antioxidant properties. Theoretical evaluation of ADME properties of all the derivatives was also carried out. From the ORAC-FL, ESR and CV data it was concluded that these derivatives are very good antioxidants, with a very interesting hydroxyl, DPPH and superoxide radicals scavenging profiles. In particular compound **9** is the most active and effective antioxidant of the series (ORAC-FL = 13.5, capacity of scavenging hydroxyl radicals = 100%, capacity of scavenging DPPH radicals = 65.9% and capacity of scavenging superoxide radicals = 71.5%). Kinetics profile for protection fluorescein probe against peroxyl radicals by addition of antioxidant molecule **9** was also performed. Therefore, it can operate as a potential candidate for preventing or minimizing the free radicals overproduction in oxidative-stress related diseases.

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1. Introduction

Phenolic compounds are bioactive substances widely distributed in the vegetable kingdom. Generally, this group of compounds has one or more aromatic rings in their structure and one or more hydroxyl groups. They have been described to act as natural antioxidants and their presence contributes to prevent or minimize several types of oxidative processes.[1] Due to their antioxidant activity their ingestion is correlated with interesting benefits to health. Therefore, the research and characterization of new bioactive phenolic substances from diet has been intensified in the last years, either for the development of nutraceutics or medicines.[1] Due to their antioxidant properties they can protect cells from the oxidative damage of the reactive oxygen species (ROS). In fact, the overproduction of free radicals have been related to cellular membrane and DNA damage, and indirectly with aging and oxidative-stress related diseases like cancer, cardiovascular and neurodegenerative pathologies.[2] Therefore, antioxidants are very important for protecting the organisms from oxidative disorders, in which ROS are also involved.[3,4] Antioxidants are capable of decrease or prevent oxidation processes through different mechanisms, such as scavenging free radicals, inhibition of pro-oxidant enzymes or chelation of transition metal ions.[5]

An increasing number of reports suggested the involvement of oxidative stress in neurodegenerative diseases (ND), where the increased formation of ROS can contribute to neuronal damage and cell death.[3,4]

Suggestion has been made that the etiology of Parkinson's (PD) and Alzheimer's (AD) diseases may be closely linked to biochemical changes resultant from this oxidative stress.[6–8] Dopamine (DA) auto-oxidation naturally produces oxidative species and may contribute to ND such as PD and ischemia/reperfusion-induced damage. Monoamine oxidase (MAO) enzyme (particularly MAO-B) is responsible for metabolizing DA and plays an important role in oxidative stress through altering the redox state of neuronal and glial cells, leading to neuronal death.[9] Consequences are an over-production of MAO and non-MAO initiated hydrogen peroxide (H_2O_2) by proliferated reactive microglia and inability of neurons to dispose of H_2O_2 and other recative species like peroxyl radicals.[10] H_2O_2 produces highly toxic ROS, namely hydroxyl

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radical, by Fenton reaction that is catalyzed by iron and neuromelanin.[11] Concerning the mechanism of the clinical efficacy of MAO-B inhibitors in PD, the inhibition of DA degradation (a symptomatic effect) and also the prevention of the formation of neurotoxic DA degradation products, that is, ROS and DA derived aldehydes have been speculated.[12] The neuroprotective effect of rasagiline, a well-known MAO-B inhibitor, might be explained through multiple mechanisms, possibly due to reduction of DA catabolism with a subsequent increased activity on dopaminergic D_2 receptors and suppressing the action of ROS as well.[13] So, the possible mechanism of neuroprotection of MAO-B inhibitors may be related not only to MAO-B inhibition but also to induction and activation of multiple factors related with oxidative stress and apoptosis.[14]

Coumarins are a family of compounds widely distributed in the nature.[15] Due to their structural features, and biological properties, namely anticancer, anti-inflammatory, antioxidant, antithrombotic, vasorelaxant, antiviral and enzymatic inhibition agents, they have been ascribed as important building blocks in Organic Chemistry and Medicinal Chemistry.[16–23]

Recently, it was shown by our group that 3-substituted aryl coumarins are potent and selective MAO-B inhibitors.[24–30] In addition, it has been found that hydroxycoumarins are antioxidants scavenging ROS and/or chelating transition metals, exhibiting tissue-protective properties.[6,31–33] The complementarity of these activities for 3-arylcoumarins was not previously studied and described. The versatility of the used reactions allowed obtaining a family of compounds with hydroxyl and/or methyl substituents in different positions of the molecule. The election of these derivatives has considered the previously MAO-B inhibitory pharmacological evaluation and the low cost of the commercial reagents to begin with. Also, the influence of the substituents in the desired activity was taken into account.

2. Materials and methods

2.1. Chemistry

Melting points were determined using a Reichert Kofler thermopan or in capillary tubes on a Büchi 510 apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX spectrometer at 300 and 75.47 MHz, respectively, using TMS as internal standard (chemical shifts in δ values, J in Hz). Mass spectra were obtained using a Hewlett–Packard 5988A spectrometer. Elemental analyses were performed using a Perkin–Elmer 240B microanalyser and were within ±0.4% of calculated values in all cases. Silica gel (Merck 60, 230-00 mesh) was used for flash chromatography (FC). Analytical thin layer chromatography (TLC) was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm).

2.1.1. General procedure for the preparation of methoxy-3-arylcoumarins

To a solution of the conveniently substituted *ortho*-hydroxybenzaldehyde (7.34 mmol) and the corresponding phenylacetic acid (9.18 mmol) in dimethyl sulfoxide (15 mL), *N,N′*-dicyclohexylcarbodiimide (11.46 mmol) was added. The mixture was heated at 110 °C for 24 h. Then, ice (100 mL) and acetic acid (10 mL) were added to the reaction mixture. After keeping it at room temperature for 2 h, the mixture was extracted with ether (3 × 25 mL). The organic layers were combined and washed with sodium bicarbonate solution (50 mL, 5%) and water (20 mL). Subsequently, the solvent was evaporated under vacuum and the dry residue was purified by flash chromatography (hexane/ethyl acetate 9:1), to give the desired methoxy-3-arylcoumarins.[23,28]

2.1.2. General procedure for the preparation of hydroxy-3-arylcoumarins

To a solution of a methoxy-3-arylcoumarin (0.50 mmol) in acetic acid (5 mL) and acetic anhydride (5 mL), at 0 °C, hydriodic acid 57% (10 mL) was added dropwise. The mixture was stirred under reflux, for 3 h. The solvent was evaporated under vacuum and the dry residue was purified by crystallization (CH_3CN).[23,28,34]

2.1.2.1. 3-(3′,4′-Dihydroxyphenyl)-6-methylcoumarin (4). Yield: 92%; mp 199–200 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.43 (s, 3H, –CH_3), 6.43 (s, 1H, H-2′), 7.01 (d, J = 7.1 Hz, 1H, H-5′), 7.14 (d, J = 7.0 Hz, 1H, H-6′), 7.28–7.32 (m, 2H, H-7, H-8), 7.57 (d, J = 2.2 Hz, 1H, H-5), 7.83 (s, 1H, H-4), 10.40 (s, 2H, –OH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 20.7, 100.1, 110.7, 111.6, 115.7, 119.4, 120.1, 127.3, 127.4, 132.1, 133.7, 138.8, 146.5, 146.8, 151.3, 161.6; EI MS m/z: 269 (13), 268 (M^+, 100), 241 (31), 240 (70), 239 (22), 165 (30), 125 (12), 111 (10); Anal. Calcd for $C_{16}H_{12}O_4$: C, 71.64; H, 4.51. Found: C, 71.60; H, 4.49.

2.1.2.2. 3-(3′,4′-Dihydroxyphenyl)-8-methylcoumarin (5). Yield: 85%; mp 205–206 °C; ^{1}H NMR (300 MHz, DMSO-$\boldsymbol{d_6}$): δ 2.50 (s, 3H, –$\boldsymbol{CH_3}$), 6.80 (d, $\boldsymbol{J}$ = 8.3 Hz, 1H, H-5′), 7.05 (dd, $\boldsymbol{J}$ = 8.2, $\boldsymbol{J}$ = 2.2 Hz, 1H, H-6′), 7.22 (d, $\boldsymbol{J}$ = 2.2 Hz, 1H, H-2′), 7.25 (d, $\boldsymbol{J}$ = 7.6 Hz, 1H, H-6), 7.44 (dd, $\boldsymbol{J}$ = 7.4, $\boldsymbol{J}$ = 1.0 Hz, 1H, H-7), 7.57 (dd, $\boldsymbol{J}$ = 7.6, $\boldsymbol{J}$ = 1.1 Hz, 1H, H-5), 8.09 (s, 1H, H-4), 9.09 (s, 1H, –OH), 9.24 (s, 1H, –OH); ^{13}C NMR (75 MHz, DMSO-$\boldsymbol{d_6}$): δ 14.9, 115.4, 116.0, 119.5, 119.9, 124.1, 124.6, 125.7, 126.1, 126.5, 132.2, 138.7, 144.8, 146.2, 150.9, 159.9; EI MS $\boldsymbol{m/z}$: 270 (12), 269 (76), 268 (M^+, 82), 241 (47), 240 (100) 239 (57), 211 (23), 166 (19), 165 (58), 152 (18), 139 (14), 125 (30), 111 (28), 82 (19); Anal. Calcd for $\boldsymbol{C_{16}H_{12}O_4}$: C, 71.64; H, 4.51. Found: C, 71.63; H, 4.49.

2.1.2.3. 3-(3′,5′-Dihydroxyphenyl)-8-methylcoumarin (6). Yield: 90%; mp 180–181 °C; ^{1}H NMR (300 MHz, DMSO-$\boldsymbol{d_6}$): δ 2.49 (s, 3H, –$\boldsymbol{CH_3}$), 6.70 (s, 3H, H-2′, H-4′, H-6′), 7.26 (t, $\boldsymbol{J}$ = 7.6 Hz, 1H, H-6), 7.48 (d, $\boldsymbol{J}$ = 7.8 Hz, 1H, H-7), 7.62 (d, $\boldsymbol{J}$ = 7.7 Hz, 1H, H-5), 8.11 (s, 1H, H-4), 10.27 (s, 2H, OH); ^{13}C NMR (75 MHz, DMSO-$\boldsymbol{d_6}$): δ 15.3, 103.3, 107.2, 119.6, 124.5, 125.1, 126.8, 127.2, 133.1, 136.6, 140.9, 151.6, 158.5, 160.0; EI MS $\boldsymbol{m/z}$: 269 (21), 268 (M^+, 100), 241 (12), 240 (63), 239 (26); Anal. Calcd for $\boldsymbol{C_{16}H_{12}O_4}$: C, 71.64; H, 4.51. Found: C, 71.60; H, 4.50.

2.1.2.4. 3-(3′,4′,5′-Trihydroxyphenyl)-8-methylcoumarin (7). Yield: 82%; mp 189–190 °C. ^{1}H NMR (300 MHz, DMSO-$\boldsymbol{d_6}$): δ 2.49 (s, 3H, –$\boldsymbol{CH_3}$), 6.95 (s, 2H, H-2′, H-6′), 7.20 (t, $\boldsymbol{J}$ = 7.4 Hz, 2H, H-6, H-7), 7.38 (d, $\boldsymbol{J}$ = 7.4 Hz, 1H, H-5), 7.79 (s, 1H, H-4), 10.55 (s, 2H, –OH), 10.60 (s, 1H, –OH); ^{13}C NMR (75 MHz, DMSO-$\boldsymbol{d_6}$DMSO-$\boldsymbol{d_6}$): δ 15.6, 106.1, 119.4, 124.2, 125.4, 125.7, 127.7, 130.4, 132.7, 135.6, 139.8, 146.7, 146.9, 160.6; EI MS $\boldsymbol{m/z}$: 285 (16), 284 (M^+, 100), 283 (84), 256 (32), 181 (10) 141 (10); Anal. Calcd for $\boldsymbol{C_{16}H_{12}O_5}$: C, 67.60; H, 4.25. Found: C, 67.61; H, 4.28.

2.2. Antioxidant assays

2.2.1. Oxygen radical antioxidant capacity-fluorescein (ORAC-FL)

The ORAC analyses were carried out on a Synergy HT multi detection microplate reader, from Bio-Tek Instruments, Inc. (Winooski, USA), using white polystyrene 96-well plates, purchased from Nunc (Denmark). Fluorescence was read from the top, with an excitation wavelength of 485/20 nm and an emission filter of 528/20 nm. The plate reader was controlled by Gen 5 software. The reaction was carried out in 75 mM sodium phosphate buffer (pH 7.4), and 200 μL final volume. FL (70 nM, final concentration) and hydroxy-3-arylcoumarin solutions in methanol with

a range of concentration between 0.3 and 2 μM were placed in each well of 96-well plate. The mixture was pre-incubated for 15 min at 37 °C, before rapidly adding the AAPH solution (18 mM, final concentration). The microplate was immediately placed in the reader and automatically shaken prior to each reading. The fluorescence was recorded every 1 min for 120 min. A blank with FL and 2,2′-azobis(2-methylpropionamidine)dihydrochloride (AAPH) using methanol instead of the antioxidant solution were used in each assay. Five calibration solutions using Trolox (0.5–2.5 μM) as antioxidant were also done. The inhibition capacity was expressed as ORAC values and is quantified by integration of the area under the curve (AUC_{NET}). All reaction mixtures were prepared in triplicate and at least three independent assays were performed for each sample. The area under the fluorescence decay curve (AUC) was calculated integrating the decay of the fluorescence where F_0 is the initial fluorescence read at 0 min and F is the fluorescence read at time. The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to the blank. Data processing was performed using Origin Pro 8 SR2 (Origin Lab Corporation, USA).

2.2.2. Hydroxyl radical scavenging assay using electron spin resonance (ESR)

Reactivity of all the hydroxy-3-arylcoumarin derivatives against the hydroxyl radical was investigated using the non-catalytic Fenton type method. ESR spectra were recorded in the X band (9.7 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 kHz field modulation, equipped with a high-sensitivity resonator at room temperature. Spectrometer conditions were: microwave frequency 9.81 GHz, microwave power 20 mW, modulation amplitude 0.91 G, receiver gain 59 db, time constant 81.92 ms and conversion time 40.96 ms. The scavenging activity of each derivative was estimated by comparing the DMPO-OH adduct signals in the antioxidant–radical reaction mixture and the control reaction at the same reaction time, and is expressed as scavenging percent of hydroxyl radical.

To prepare the samples, 150 μL of *N*,*N*-dimethylformamide (DMF) and 50 μL of NaOH (3 mM) were mixed, followed by the addition of 50 μL of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) spin trap (30 mM final concentration) and finally 50 μL of hydrogen peroxide 30%. The mixture was put in an ESR cell and the spectrum was recorded after five minutes of reaction. All the compounds were studied to 4 mM final concentration (300 μL final volume).

2.2.3. DPPH radical scavenging assay using ESR

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging capacity[35–38] of the hydroxy-3-arylcoumarin derivatives was determined by an ESR spectrometry method.[39–43] Each hydroxy-3-arylcoumarin solution was mixed with DPPH stock solution to initiate the antioxidant–radical reaction. All the reaction mixtures contained 1.0 mM of DPPH and 1.0 mM of the studied compound. The control solution was prepared in absence of the studied compounds. Both DPPH and compounds solutions were prepared in acetonitrile. ESR signals were recorded after five minutes of reaction. Spectrometer conditions were: microwave frequency 9.81 GHz, microwave power 20 mW, modulation amplitude 0.95 G, receiver gain 59 db, time constant 81.92 ms and conversion time 40.96 ms. The scavenging activity of each compound was estimated by comparing the DPPH signals in the antioxidant–radical reaction mixture and the control reaction at the same reaction time, and was expressed as scavenging percent of DPPH.

2.2.4. Superoxide antioxidant assay using cyclic voltammetry (CV)

Cyclic voltammetry (CV) measurements were performed in a Metrohm 693VA instrument with a 694VA stand convertor and a 693VA processor, at room temperature, using a three-electrode cell. A glassy carbon (GC) electrode presenting an area of 0.03 cm^2 was used as the working electrode. The electrode surface was polished to a mirror finish with alumina powder (0.3 and 0.05 μM) before use and after each measurement. Platinum wire was used as auxiliary electrode and silver-silver chloride (Ag/AgCl, 3 M KCl) of Metrohm Company with a plastic tip was used as a reference electrode. The CV experiments were carried out in dimethyl sulfoxide (DMSO) of analytical grade (Sigma–Aldrich) with 0.1 M of tetrabutylammonium perchlorate (TBAP) as supporting electrolyte. Superoxide anion radical was generated in DMSO containing TBAP 0.1 M. The scan rate was kept 30 mV/s and potential window was −1.0 to −0.0 V. The atmospheric solubility of oxygen in DMSO was 2.1 mM.[44] The final concentration of each derivative (30 μM) was achieved by additions of the corresponding aliquot of stock solution (10 mL final volume). Finally, the antioxidant activity was assessed from the change in the cathodic current of the voltammograms in absence and present of the derivatives, using pertinent mathematical formulations.

2.2.5. Data statistical analysis

Statistical analyses were conducted using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). The data are expressed as means ± SD. The experimental data were analyzed by one-way analysis of variance (ANOVA), and differences between groups were assessed using Tukey's post-test. The level of significance was set at $p < 0.05$, and all experiments were replicated 3 times.

3. Results

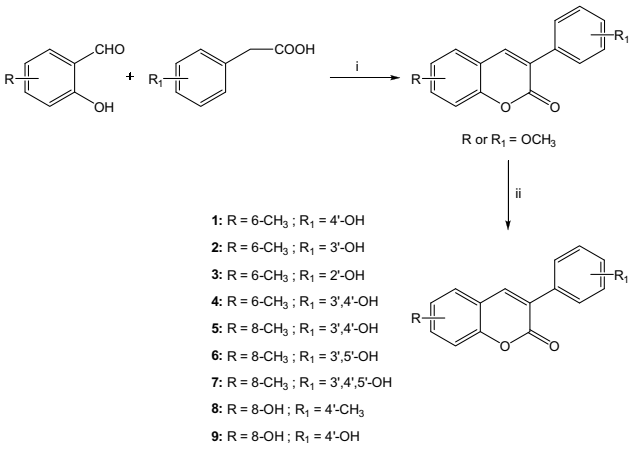
3.1. Chemical synthesis

Coumarin derivatives **1**–**9** were efficiently synthesized according to the protocol outlined in Scheme 1. The general reaction conditions and the characterization data of the new compounds were described in the experimental section. Perkin condensation of different *ortho*-hydroxybenzaldehydes with the adequate arylacetic acid, using *N*,*N*′-dicyclohexylcarbodiimide (DCC) as dehydrating agent,[28] afforded the methoxy-3-arylcoumarins. Hydroxyl derivatives were obtained from the above-mentioned methoxy substituted precursors by acidic hydrolysis, using hydriodic acid 57% in the presence of acetic acid and acetic anhydride.[28]

3.2. Antioxidant capacity assays

The evaluation of the antioxidant activity of the studied compounds was performed towards different types of reactive oxygen or nitrogen species—peroxyl, hydroxyl, superoxide and DPPH radicals. ORAC-FL, ESR and CV assays were the techniques used to obtain the desired results.

The peroxyl radical scavenging activity of the synthesized hydroxy-3-arylcoumarins was evaluated by the oxygen radical absorbance capacity (ORAC) method[45] This assay use a fluorescence-based technology (ORAC-FL) and allow obtaining a relative antioxidant index by using as reference trolox, a hydrosoluble vitamin E derivative. The exposition of the fluorophore, in this case fluorescein (FL) to the peroxyl radical lead to an oxidation process reflected as a decay of fluorescence emission through time. In ORAC assays, the loss of fluorescence of FL generally corresponds to an induction time and is reliant on antioxidant capacity of a compound. In fact, it refers to the time in which the FL is protected against the oxidative damage of peroxyl radicals and this behavior is associated to a competitive reaction between the radical and the antioxidant.[46,47] ORAC data take into account the induction time,



Scheme 1. Reagents and conditions: (i) DCC, DMSO, 110 °C, 24 h; (ii) HI 57%, AcOH, Ac_2O, reflux, 3 h.

Table 1
ORAC-FL index and hydroxyl, DPPH and superoxide radical scavenging data obtained for compounds **1**-**9**

Compd	ORAC-FL index	% Scavenging hydroxyl radicals[a]	% Scavenging DPPH radicals[a]	% Scavenging superoxide radicals[b]
1	6.1	100	27.7	17.4
2	6.7	5.2	3.4	17.5
3	8.4	100	10.6	25
4	5.3	6.05	28.3	28.9
5	5.7	100	66.7	77.3
6	5.5	75	11.2	22.6
7	5.3	100	100	76.5
8	6.3	16	28.6	17.4
9	13.5	100	65.9	71.5
Trolox	1.0	—	—	—
Quercetin	7.28[c]	—	20.0[d]	—
Catechin	6.76[c]	—	44.5[d]	—

[a] The scavenging activity of hydroxyl and DPPH radicals effect was calculated as follows: $[(A_0 - A_x)/A_0] \times 100$, where A_x and A_0 are the double-integral ESR for the first line of samples in the presence and absence of test compounds, respectively.

[b] The scavenging activity of superoxide radical effect was calculated as follows: $[(I\text{pc blank} - I\text{pc aox})/I\text{pc blank}] \times 100$, where Ipc blank is cathodic current in the absence of the studied compounds and Ipc aox is the cathodic current in the presence of the studied compounds.

[c] Data collected from Ref. 45.

[d] Data collected from Ref. 49.

initial rate and the range of total antioxidant inhibition in one value.[46]

Results expressed as ORAC-FL values are presented in Table 1. All the obtained ESR results for the% scavenging of the hydroxyl and DPPH radicals are also illustrated in Table 1. In addition, % of superoxide radical scavenging, performed by CV, are also represented in Table 1.

The ORAC-FL profile, intensity of fluorescence at 528 nm versus the incubation time was obtained for all derivatives. Compounds **3** and **9** (8.4 and 13.5, respectively) display the highest ORAC-FL indexes, comparing with the flavonoids quercetin and catechin that are very well known natural antioxidant compounds. Figure 1 shows the kinetic profile for protection of FL probe against peroxyl radicals obtained in presence of increasing concentrations of compound **9**. It was obtained a profile of fluorescence measure at 528 nm versus the incubation time at different concentration, for all derivatives.

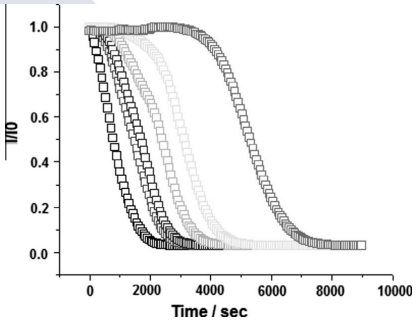


Figure 1. ORAC-FL profile (kinetic profile for protection FL probe against peroxyl radicals) for compound **9**.

In order to study the antioxidant reactivity of all the synthesized hydroxy-3-arylcoumarin derivatives towards hydroxyl radicals, a non-catalytic and competitive type Fenton system in which the DMPO spin trap was performed.[48] The ESR spin-trapping spectrum obtained in the control assay (DMPO+*N*,*N*-dimethylformamide + NaOH + H_2O_2) presents four hyperfine lines, due to the DMPO-OH adduct formation, as it is shown in Figure 2 (red line). For each putative antioxidant coumarin compounds ESR spectra were also acquired to check their capacity of scavenging hydroxyl radicals. The data obtained with compound **2** is depicted in Figure 2 (black line).

The intensity of the spectra decreases when the hydroxy-3-arylcoumarin derivatives were added into the system. For compound **9**, 100% of scavenging of hydroxyl radicals was obtained (Fig. 3—black line). This type of response was observed for all derivatives, reflecting different percentage of the hydroxyl radical scavenging activity (Table 1).

The stable free radical DPPH assay has been used for detecting the antioxidant activity in several chemical analyses.[35–38] Currently, DPPH assay is considered an easy and accurate method, appropriate for measuring the antioxidant capacity of fruits, vegetables, juices or extracts.[39] This is due to the electronic properties shared by DPPH and peroxyl radicals (the unpaired electron is delocalized through the pair of nitrogen or oxygen atoms, respec-

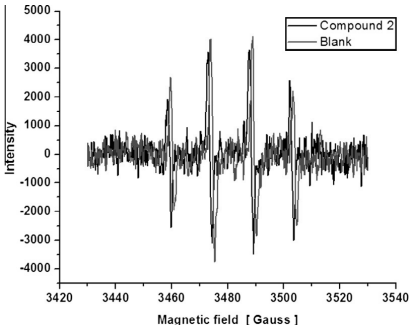


Figure 2. ESR spectra obtained for the control (adduct DMPO-OH without antioxidant molecule—red line) and for adduct DMPO-OH in the presence of compound **2** (black line).

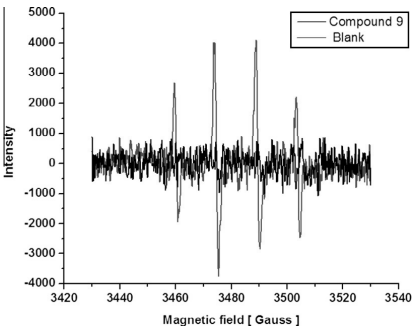


Figure 3. ESR spectra obtained for the control (adduct DMPO-OH without antioxidant molecule—red line) and for adduct DMPO-OH in the presence of compound **9** (black line).

tively), in such way that the reaction rate between DPPH and several antioxidants provides a good approximation for scavenging activities with lipid peroxyl radicals.[40,41] ESR spectra of DPPH in absence and presence of compounds **4**, **7** and **9** are showed in Figure 4.

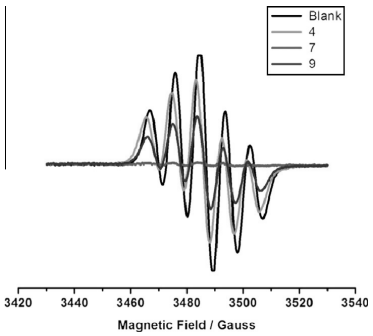


Figure 4. ESR signal from DPPH radical in absence (blank) and presence of compounds **4**, **7** and **9**.

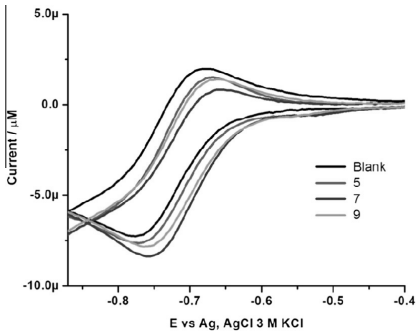


Figure 5. Cyclic voltammograms of superoxide radical in absence (blank) and presence of compounds **5**, **7** and **9** in DMSO + TBAP 0.1 M, on GC (working electrode) versus. Ag/AgCl, at room temperature, with scan rate of 30 mV/s.

Superoxide anion radical was generated by one electron reduction of the atmospheric molecular oxygen dissolved in DMSO at room temperature (25 °C). Then, the voltammetric performance of the nine compounds was studied, one by one, in DMSO and TBAP 0.1 M. The resultant CV responses for derivative **5**, **7** and **9**, and in absence of any derivative (blank) are represented in Figure 5.

3.3. Theoretical evaluation of ADME properties

In order to better understand the overall properties of the described compounds, the lipophilicity (expressed as the octanol/water partition coefficient and herein called log*P*), was calculated using the Molinspiration property calculation program.[50] The theoretical prediction of ADME properties (molecular weight, log*P*, number of hydrogen donors and acceptors) of all the compounds was carried out and is presented in Table 2.[51,52]

4. Discussion

In previous works, 3-arylcoumarin derivatives were described as potent and selective MAO-B inhibitors.[28] This family of compounds and their remarkable data on the selective inhibition of MAO-B isoenzyme and putative application for ND therapy were the inspiration for this work. In particular, compounds **1**–**3** were previously described as potent and selective MAO-B inhibitors, with IC_{50} values between 120 and 650 nM.[28] On the other hand the recent medicinal chemistry paradigms in the drug design, namely the rational discovery of multi-target drugs, as a promising strategy to combat this type of multifactorial diseases prompted us to look for other properties for this type of coumarins. Based on this data, a new family of derivatives sharing the same scaffold and type of substituents was designed and synthesized.

The evaluation of the antioxidant activity of the hydroxy-3-arylcoumarin compounds was performed towards different types of reactive oxygen or nitrogen species—peroxyl, hydroxyl, superoxide and DPPH radicals. ORAC-FL, ESR reactivity and CV assays were the techniques used to achieve the goals. From the obtained data, it was concluded that the antioxidant scavenging activity is related with the type of substituents presented in the 3-arylcoumarin skeleton.

Compound **9** was found to be the most interesting coumarin of the series. This compound has two hydroxyl groups in its structure, one at position 8 and another at position 4′ of the 3-arylcoumarin scaffold. The other compounds have structural combinations of two types of substituents (methyl and one, two or three hydroxyl groups). Compounds **1**, **3**, **5** and **7** have ORAC-FL values between

Table 2
Theoretical structural properties of the hydroxy-3-arylcoumarins derivatives **1**–**9**[a]

Compd	log*P*	Molecular weight	TPSA	*n*-OH acceptors	*n*-OHNH donors	Volume
1	3.68	252.27	50.44	3	1	224.57
2	3.66	252.27	50.44	3	1	224.57
3	3.89	252.27	50.44	3	1	224.57
4	3.19	268.27	70.67	4	2	232.59
5	3.17	268.27	70.67	4	2	232.59
6	3.11	268.27	70.67	4	2	232.59
7	2.88	284.27	90.90	5	3	240.61
8	3.92	252.27	50.44	3	1	224.57
9	2.99	254.24	70.67	4	2	216.03

[a] log*P*—octanol/water partition coefficient; TPSA–topological polar surface area; *n*-OH—number of hydrogen acceptors; *n*-OHNH—number of hydrogen bond donors. The data was determined with Molinspiration calculation software.[50]

5.3 and 8.4 and the higher scavenging activity towards hydroxyl radicals (100% − total scavenging). From these derivatives, compound **7** presented the best DPPH and superoxide radicals scavenging (100 and 76.5%, respectively). Compounds **1** and **3** have on their structure a methyl group at position 6 of the coumarin moiety and a hydroxyl group in the 3-aryl ring. The ORAC-FL indexes are 6.1 and 8.4, respectively, but the position of the hydroxyl group in the exocyclic aromatic ring seems to have no influence on the hydroxyl radical scavenging capacity (100%, in both cases). The DPPH radical scavenging values of these compounds are, respectively, 27.7% and 10.6% and superoxide radical scavenging values are, respectively, 17.4% and 25%. Their profiles are, therefore, very similar. Compounds **5** and **7** have a methyl group at position 8 of the coumarin moiety, and two or three hydroxyl groups, respectively, in the 3-aryl ring. Both ORAC-FL values (5.7 and 5.3, respectively), hydroxyl radical scavenging capacity (100%) and superoxide radical scavenging capacity (77.3% and 76.5%, respectively) are similar, proving that the presence of the extra hydroxyl group does not seem to significantly affect the peroxyl, hydroxyl and superoxide radicals scavenging activities. One the other hand, DPPH radical scavenging capacity is 66.7% for compound **5** and 100% for compound **7**. Compounds **2**, **4** and **8**, with ORAC-FL values between 5.3 and 6.7, presented the lowest hydroxyl scavenging capacity (between 5.2% and 16%). Compound **2** presented also the lowest DPPH radical scavenging capacity (3.4%) and compounds **1** and **8** the lowest superoxide radical scavenging capacities (17.4%). Comparing the data obtained for compound **4** (3′,4′-dihydroxy substituted) and for compound **1** (4′-hydroxy substituted), it can be concluded that the presence of two hydroxyl groups led to the decline in the ORAC-FL index (6.1–5.3) and the hydroxyl radical scavenging capacity (100%–6.05%). Comparing compound **4** with **2** (3′-hydroxy substituted), it is also noted a decrease in the ORAC-FL index (from 6.7 to 5.3), caused by the presence of two hydroxyl groups in the molecule. However, the hydroxyl radical scavenging capacity is almost the same (6.05% and 5.2%, respectively). Superoxide radical scavenging capacity is also similar (28.9 and 17.5, respectively). Compounds **4** and **5** have the same substitution pattern, changing only the position of the methyl group from 6 to 8. This modification strongly affects the hydroxyl radical scavenging capacity (from 6.05% to 100%), the DPPH radical scavenging capacity (from 28.3% to 66.7%) and superoxide radical scavenging capacity (from 28.9% to 77.3%). Compound **6**, with a methyl group at position 8 of the coumarin moiety and two hydroxyl groups at positions 3′ and 5′ of the 3-aryl ring, in spite of presenting an ORAC-FL value of 5.5 and low DPPH and superoxide radicals scavenging (11.2 and 22.6, respectively), evidence a significant tendency to scavenge hydroxyl radicals (75%).

As said before, the highest ORAC-FL values were found for compounds **3** and **9** (8.4 and 13.5, respectively). The results are very interesting comparing with the ORAC-FL values of catechin (6.76) and quercetin (7.28), very well known natural antioxidants. Comparing with trolox (ORAC-FL = 1.0), the interesting ORAC-FL values of compounds **3** and **9** make them promising antioxidant molecules. It is important to notice that compound **5**, **7** and **9** presented good trends against all the studied radicals.

From the obtained data, it is also remarkable that all the coumarin derivatives possess log*P* values compatible with those required to cross membranes. TPSA, described to be a predictive indicator of membrane penetration, is also found to be positive. In addition, it can be observed that no violations of Lipinski's rule (molecular weight, log*P*, number of hydrogen donors and acceptors) were found. This is important information about the promising potential of these derivatives.

All the synthesized compounds, in spite of presenting different chemical substituents, disclose interesting ORAC-FL values, in most cases accompanied by a remarkable ability to scavenge hydroxyl, DPPH and superoxide radicals. Therefore, the data acquired so far are relevant allowing proposing hydroxy-3-arylcoumarins as a valid scaffold for the design of novel antioxidants.

5. Concluding remarks

In conclusion, in the current work coumarins presenting very promising antioxidant profiles were described. Compound **9** proved to be the most interesting molecule of the whole series, with an ORAC-FL of 13.5, 100% of scavenging of hydroxyl radicals, 65.9% of scavenging of DPPH radicals and 71.5% of scavenging of superoxide radicals. This derivative has presented good antioxidant capacity towards different types of reactive oxygen or nitrogen species—peroxyl, hydroxyl, superoxide and DPPH radicals. Compound **3**, previously describe as very good selective MAO-B inhibitor, presented also a very interesting antioxidative profile. It is important to notice that compound **5** and **7** also presented good trends against all the studied radicals. In addition, it can be observed that no theoretical violations of Lipinski's rule were observed for all the studied derivatives. Therefore, the described compounds seem to present desirable ADME properties. Based on these results, it can be concluded that especially compounds **3** and **9** are potential candidates for a further optimization process and could be successfully employed in the prevention or minimization of the oxidative damage caused by overproduction of oxygen free radicals.

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New Insights into Functional and Structural Properties of 3-Arylcoumarins as an Interesting Scaffold in MAO-B Inhibition

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ABSTRACT: Design, synthesis, pharmacological evaluation and theoretical studies of a new series of halogenated 3-arylcoumarins were performed with the aim of finding new structural and biological features. This series displays several alkyl, hydroxyl, halogen and/or alkoxy groups in both benzene rings of the 3-arylcoumarin scaffold. Most of the studied compounds showed a high affinity and selectivity to the hMAO-B isoenzyme, with IC_{50} values on the low-nanoMolar (compounds **6**, **7**, **10**, **16**, **17**, **20** and **23**) and picoMolar range (compounds **5**, **8**, **9**, **11**, **12**, **14** and **15**). Most of the evaluated

compounds displayed higher MAO-B inhibitory activity and selectivity than selegiline. Coumarin **12** is the most active compound, being several times more active than selegiline and showing the highest hMAO-B specificity. To better understand the structure-activity relationships, docking experiments were carried out on hMAO structures. Finally, the prediction of passive blood-brain partitioning based on *in silico* derived physicochemical descriptors was performed.

KEYWORDS: Coumarin derivatives, Perkin reaction, Williamson reaction, hydrolysis reaction, monoamine oxidase inhibitors, docking studies, passive blood-brain partitioning model.

Introduction

Neurological diseases are considered as one of the important challenges in today's medicine because of the complexity, frequency of occurrence and progressive development of these pathologies. Among these, neurodegenerative disorders as Alzheimer´s disease (AD) and Parkinson´s disease (PD) play a major role.[1,2] PD is the second most common neurodegenerative disease in the western countries and up to now it cannot be cured. The disease is characterized by a loss of dopaminergic neurons in the *substancia nigra pars compacta* and other regions and the formation of intraneuronal aggregates known as Lewy bodies (LBs). LBs mainly consist of α-synuclein which is misfolded and fibrillated under pathogenic circumstances.[1] The loss of dopamine (DA) leads to a dysregulation of the motor circuits which result symptomatically in tremor, bradykinesia, rigidity and postural instability.[2] Additionally other neurotransmitter systems are affected, explaining the manifestation of symptoms like depression, sleeping abnormalities, anxiety and reduction of cognitive abilities.[2]

Unless a basal understanding of PD exists, in treatment, the missing DA is still replaced, without targeting the progress of neurodegeneration of dopaminergic and nondopaminergic neurons. L-dopa as a DA prodrug and drugs modifying the DA levels, as well as dopaminic receptor agonists, muscarinic receptor agonists, catecol-*O*-methyltransferase (COMT) inhibitors and selective monoamine oxidase

type B inhibitors (MAOI-B) are used in therapy.[1,3] Moreover, MAOI-B like selegiline show neuroprotective effects via a pathway unrelated to the MAO inhibition.[4] Therapy combining several drugs is common and is still widely used, but recently approaches have been done to combine different pharmacophoric features addressing several molecular targets in one molecule.[3] Novel strategies to treat PD also focus on the formation of α-synuclein fibrils and the different possibilities to maintain the native protein by considering chaperone activity,[5] fibril proteolysis via autophagic systems[6] and pathways of phosphorylation.[7]

MAO is an important flavin-containing enzyme which catalyzes the oxidative deamination of monoamines[8] and is located in the outer mitochondrial membrane of neurons and other cells.[9] These enzymes are responsible for catalyzing the oxidative deamination of neurotransmitters and dietary amines, regulating intracellular levels of biogenic amines in the brain and the peripheral tissues.[10] In humans, two subtypes of MAO, called MAO-A and MAO-B, exist. MAO-A catabolizes more favourable serotonin and norepinephrine, whereas the isoform B shows a higher affinity to phenylethylamine and benzylamine.[11,12] On the other hand, DA and tyramine are common substrates for both isoforms. The enzymatic isoforms have been identified on the basis of their amino acid sequences,[13] three-dimensional structure,[14] tissue distribution,[15] inhibitor selectivity[16] and substrate preferences.[17] Due to this, they play an important role in the inactivation of neurotransmitters and are targeted if a dysregulation of neurotransmitter levels, like in neurological disorders, occurs. The physiologic properties determine the clinical interest of MAO inhibitors. MAOI-A (clorgyline and moclobemide) are used in the treatment of depression and anxiety,[18] while MAOI-B (selegiline and rasagiline) have been applied to PD therapy (Figure 1).[19,20]

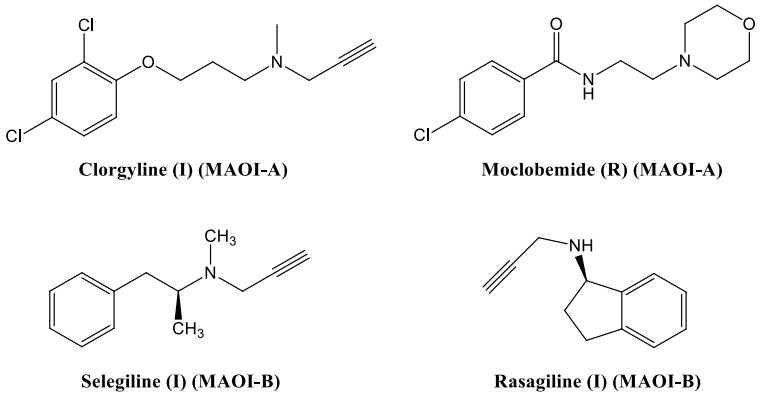


Figure 1. Chemical structure of MAOI-A (clorgyline and moclobemide) and MAOI-B (selegiline and rasagiline). I – irreversible. R – reversible.

In recent years, interest in selective hMAO-B inhibitors has significantly intensified due to the discovery that expression levels of this isoenzyme in neuronal tissue increase 4-fold with age, resulting in an increment of DA metabolism, as well as of production of hydrogen peroxide (H_2O_2), which cause oxidative stress, and may play a relevant role in the etiology of neurodegenerative diseases.[21] This research has resulted in a significant number of compounds with different structural scaffolds.[22-29] Additionally, the description of the crystal structure of the two MAO isoforms by Binda *et al.* has provided relevant information about the selective interactions and the pharmacophoric requirements needed for the design of potent and selective inhibitors.[30-34]

Due to the presence of MAO in the brain cells, MAOIs must be able to achieve and cross the blood-brain barrier (BBB), which prevents the access of polar molecules to the brain. The distribution of the compounds between blood and brain is a very important consideration for new drug candidates. The prediction of passive blood-brain partitioning based on *in silico* derived physicochemical descriptors it is an interesting tool.[35]

Due to the above described, in the last few years, a broad consensus has been reached concerning the necessity for the research of new, more potent and less toxic MAO-B selective inhibitors.

Coumarin is a natural molecule which can be found in several plants like tonka bean, vanilla grass and sweet woodruff.[36] Derivatives of this benzopyrone are of pharmaceutical interest because they have been showing different biological activities.[36] Works have been done that describe the coumarins as anticancer, anti-inflammatory, antimicrobial, cardioprotective, vasorelaxant and antioxidant agents.[36-46] Also, the coumarin nucleus has emerged in the 1990´s as a promising scaffold for MAO inhibitors.[47] Structure-selectivity relationship studies about coumarin derivatives suggested that selectivity was mainly determined by the nature of the linkage between the coumarin and the lipophilic aryl groups.[48] Furthermore, several coumarins showing a significant MAO inhibitory activity (Figure 2) have been reported and some of them are suggested as potential drugs against neurodegenerative diseases.[49-53]

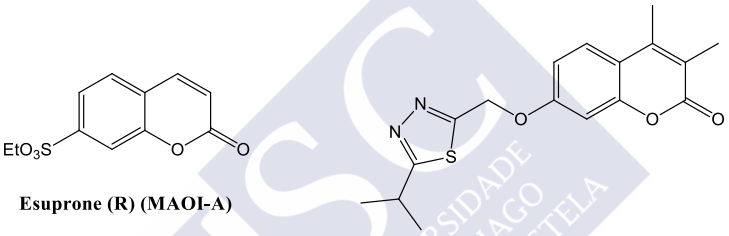


Figure 2. Structures of known coumarin-based MAOIs (esuprone and LU53439). R – reversible.

Our research group has been able to present 3-arylcoumarins (coumarin-stilbene derivatives - Figure 3) that show a high selectivity and affinity to MAO-B isoenzyme.[54-63] These results encouraged us to synthesize and study the MAO inhibitory activity of new analogues, in which a variety of groups with different size, electronic and lipophilic properties were introduced in both aromatic rings, in order to clarify the influence of the substitution pattern in the MAO inhibitory activity and selectivity of the 3-arylcoumarin skeleton.

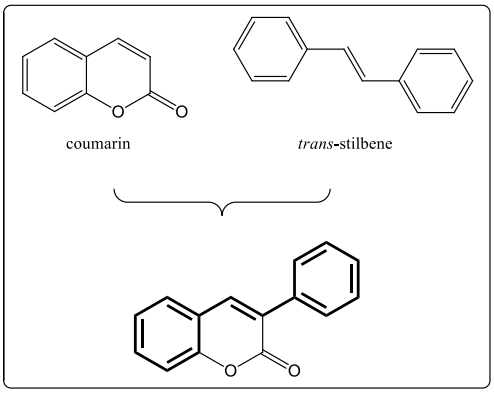


Figure 3. General chemical structure of synthesized stilbene-coumarin hybrid scaffold.

Based on the significant structural information currently available on MAOs,[64-67] and to analyze the structural requirements for MAO activity, docking experiments were carried out on hMAO-B and hMAO-A crystallographic structures. Considering the preliminary interesting results, our research group is still looking for more active and selective MAOIs.

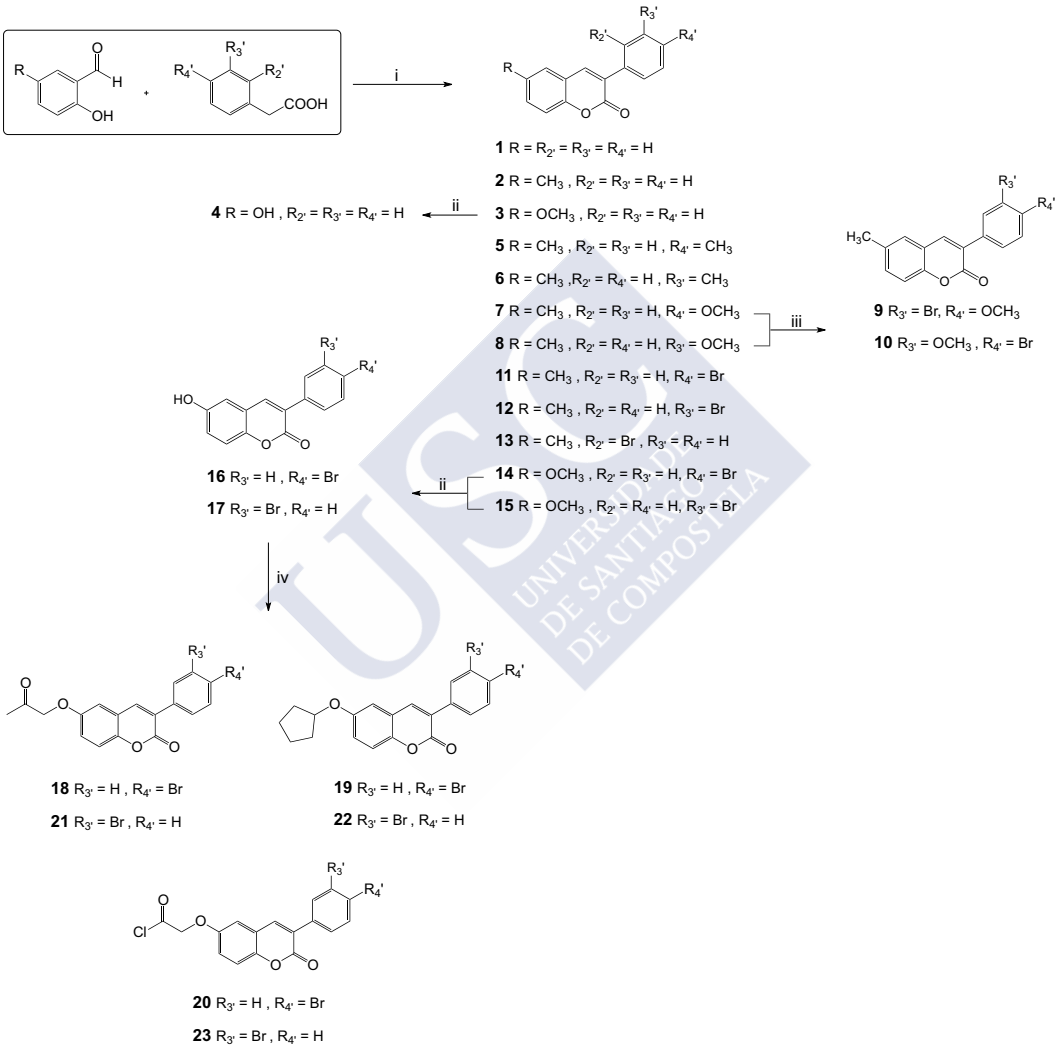
Results

Chemistry

The coumarin derivatives **1**-**23** were efficiently synthesized according to the protocol outlined in Scheme 1. The detailed chemical structures of the compounds are organized in Table 1. The general reaction conditions and the compounds characterization are described in the experimental section.

Perkin condensation of different *ortho*-hydroxybenzaldehydes with the adequate arylacetic acid, using *N,N'*-dicyclohexylcarbodiimide (DCC) as dehydrating agent,[55-57] afforded the 3-arylcoumarins **1**-**3**, **5**-**8** and **11**-**15**.[54,60,68] The treatment of the 4-(methoxyphenyl)-6-methylcoumarin **7** and 3-(methoxyphenyl)-6-methylcoumarin **8**,[57] with *N*-bromosuccinimide (NBS), in carbon tetrachloride (CCl_4), under reflux, using 2,2'-azobisisobutyronitrile (AIBN) as catalyst,[60] afforded the bromomethoxy derivatives **9** and **10**,[57] respectively. In addition, compounds **4**, **16** and **17** were obtained by acidic

hydrolysis of the respective methoxy derivatives **3**, **14** and **15**, using hydriodic acid 57% in the presence of acetic acid and acetic anhydride.[56] Finally, the Williamson reaction of hydroxycoumarins **16** and **17** with chloroacetone, 2-chloroacetyl chloride or cyclopentyl bromide,[57] gave the corresponding ethers **18**-**20** and **21**-**23**, respectively.



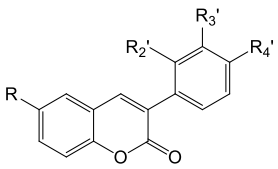
Scheme 1. Reagents and conditions: (i) DCC, DMSO, 110 ºC, 24 h; (ii) HI, AcOH, Ac_2O, reflux, 3 h; (iii) NBS, AIBN, CCl_4, reflux, 18 h; (iv) chloroacetone or cyclopentyl bromide or 2-chloroacetyl

chloride, K_2CO_3, acetone, reflux, 16-24 h.

Pharmacology

The biological evaluation of the test drugs on hMAO activity was investigated by measuring their effects on the production of hydrogen peroxide (H_2O_2) from *p*-tyramine (a common substrate for hMAO-A and hMAO-B), using the Amplex Red MAO assay kit (Molecular Probes, Inc., Eugene, Oregon, USA) and microsomal MAO isoforms prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for hMAO-A or hMAO-B (Sigma-Aldrich Química S.A., Alcobendas, Spain).[69] The production of H_2O_2 catalyzed by the two MAO isoforms can be detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a non-fluorescent and highly sensitive probe that reacts with H_2O_2 in the presence of horseradish peroxidase to produce a fluorescent product, resorufin. New compounds and reference inhibitors were unable to react directly with the Amplex Red reagent, which indicates that these drugs do not interfere with the measurements. On the other hand, in our experiments and under our experimental conditions, hMAO-A displayed a Michaelis constant (K_m) equal to 457.17 ± 38.62 μM and a maximum reaction velocity (V_{max}) in the control group of 185.67 ± 12.06 (nmol *p*-tyramine/min)/mg protein, whereas hMAO-B showed a K_m of 220.33 ± 32.80 μM and V_{max} of 24.32 ± 1.97 (nmol *p*-tyramine/min)/mg protein (n = 5). Most tested compounds concentration-dependently inhibited this enzymatic control activity (Table 1).

Table 1. Chemical Structure and hMAO-A and hMAO-B Inhibitory Activity *In vitro* of the Synthesized Derivatives **1**-**23** and Reference Compounds (Selegiline and Iproniazide)[a]



Compounds	R	R2'	R3'	R4'	IC_{50} hMAO-A	IC_{50} hMAO-B	S. I.[b]

1	H	H	H	H	*	11.81±0.80 μM	> 8.5[c]
2	CH_3	H	H	H	*	283.75±19.90 nM	> 352[c]
3	OCH_3	H	H	H	*	413.25 ± 60.50 nM	> 242[c]
4	OH	H	H	H	29.89 ± 2.02 μM	3.39 ± 0.23 μM	8.8
5	CH_3	H	H	CH_3	*	310.0±20.0 pM	> 333,333[c]
6	CH_3	H	CH_3	H	**	15.01±0.83 nM	> 6,667[c]
7	CH_3	H	H	OCH_3	*	13.05±0.90 nM	> 7,663[c]
8	CH_3	H	OCH_3	H	*	800.0±50.0 pM	> 125,000[c]
9	CH_3	H	Br	OCH_3	*	740.0±20.0 pM	> 135,870[c]
10	CH_3	H	OCH_3	Br	*	3.25±0.17 nM	> 31,250[c]
11	CH_3	H	H	Br	**	387.04±25.96 pM	> 258,371[c]
12	CH_3	H	Br	H	*	134.04±8.99 pM	> 746,045[c]
13	CH_3	Br	H	H	*	4.34±0.29 μM	> 23[c]
14	OCH_3	H	H	Br	*	320.0±0.17 pM	> 312,500[c]
15	OCH_3	H	Br	H	*	650.0±0.35 pM	> 153,846[c]
16	OH	H	H	Br	12.83±0.66 μM	32.0±1.7 nM	387
17	OH	H	Br	H	*	8.0±0.43 nM	> 12,500[c]
18	$C_3H_5O_2$	H	H	Br	*	**	-
19	C_5H_9O	H	H	Br	*	7.02±0.38 μM	> 14[c]
20	$C_2H_2O_2Cl$	H	H	Br	12.66±0.84 μM	7.01±0.47 nM	1,809

21	$C_3H_5O_2$	H	Br	H	*	1.48±0.079 μM	> 68[c]
22	C_5H_9O	H	Br	H	44.29±2.40 μM	1.79±0.096 μM	25
23	$C_2H_2O_2Cl$	H	Br	H	*	2.4±0.14 nM	> 37,037[c]
Selegiline	-	-	-	-	67.25±1.02 μM	19.60±0.86 nM	3,431
Iproniazide	-	-	-	-	6.56±0.76 μM	7.54±0.36 μM	0.87

[a] Each IC_{50} value is the mean ± S.E.M. from five experiments (n = 5). * Inactive at 100 μM (highest concentration tested), at higher concentrations the compounds precipitate. ** 100 μM inhibits the corresponding hMAO activity by approximately 40-45%, at higher concentrations the compounds precipitate. [b] Selectivity index: MAO-B selectivity ratios [IC_{50} (MAO-A)]/[IC_{50} (MAO-B)] for inhibitory effects of both new compounds and reference inhibitors. [c] Values obtained under the assumption that the corresponding IC_{50} against MAO-A is the highest concentration tested (100 μM).

Prediction of passive blood-brain partitioning

Compounds designed to be effective in the central nervous system (CNS) should be able to cross the BBB to reach the therapeutic target. It has been shown that compounds with logBB (logarithm of the ratio of the concentration of the compound in the brain and in the blood) <-1 are poorly distributed in the brain while molecules with logBB>0.3 can cross readily the BBB.[70,71] The distribution of the compounds **1-23** between the blood and the brain was calculated through a discriminant equation extracted from a model recently published.[35] The equation discriminates between compounds with logBB using a cut-off of 0.3 (see the equation in the Methods section). Topological polar surface area (TPSA) and the logarithm of octanol/water partition coefficient (logP(o/w)) were calculated and their values were substituted in the equation (1). If the result in the equation is >0 the compounds are

predicted to have a logBB≥0.3 which means that the compounds readily cross the BBB.[70] If the result is <0 the compounds could still cross the BBB but with logBB<0.3. Although two different models with cut-off of 0.3 and -1 were recently published,[35] only the equation using the threshold of 0.3 was used since all the compounds were predicted to cross readily the BBB (see Table 2). In order to better understand the overall properties of the described compounds, the theoretical prediction of other ADME properties (molecular weight, logS, number of hydrogen donors and acceptors) of all the studied compounds (**1**-**23**) was carried out and is presented in Table 2.

Table 2. Molecular Descriptors (TPSA and logP(o/w)) Included in Equation (1) and logBB Prediction for the Compounds Included in the Study. Other Calculated Theoretical Descriptors.

Compounds	logP(o/w)	TPSA	Value in equation (1)	logBB≥0.3	Other descriptors						
					a_acc	a_don	lip_ druglike	lip_ violation	logS	mr	Weight
1	3.881	26.300	0.927	+	1	0	1	0	-4.55	6.63	222.24
2	4.216	26.300	1.100	+	1	0	1	0	-5.02	7.08	236.27
3	3.874	35.530	0.668	+	2	0	1	0	-4.60	7.27	252.27
4	3.61	46.530	0.227	+	2	1	1	0	-4.19	6.76	238.24
5	4.514	26.300	1.254	+	1	0	1	0	-5.50	7.52	250.30
6	4.551	26.300	1.273	+	1	0	1	0	-5.50	7.52	250.30
7	4.172	35.530	0.822	+	2	0	1	0	-5.07	7.72	266.30
8	4.209	35.530	0.841	+	2	0	1	0	-5.07	7.72	266.30
9	5.005	35.530	1.252	+	2	0	1	0	-6.16	8.47	345.19
10	5.005	35.530	1.252	+	2	0	1	0	-6.16	8.47	345.19
11	5.014	26.300	1.512	+	1	0	1	0	-6.11	7.82	315.17
12	5.051	26.300	1.531	+	1	0	1	0	-6.11	7.82	315.17

13	5.012	26.300	1.511	+	1	0	1	0	-6.11	7.82	315.17
14	4.672	35.530	1.080	+	2	0	1	0	-5.69	8.01	331.16
15	4.709	35.530	1.099	+	2	0	1	0	-5.69	8.01	331.16
16	4.408	46.530	0.639	+	2	1	1	0	-5.28	7.50	317.14
17	4.445	46.530	0.658	+	2	1	1	0	-5.28	7.50	317.14
18	4.353	52.600	0.442	+	3	0	1	0	-6.03	9.02	373.20
19	6.209	35.530	1.873	+	2	0	1	1	-6.65	9.67	385.26
20	5.077	52.600	0.816	+	3	0	1	0	-6.84	9.06	393.62
21	4.39	52.600	0.462	+	3	0	1	0	-6.03	9.02	373.20
22	6.246	35.530	1.892	+	2	0	1	1	-6.65	9.67	385.26
23	5.114	52.600	0.835	+	3	0	1	0	-6.84	9.06	393.62

logP(o/w) = log of the octanol/water partition coefficient; TPSA = topological polar surface area; a_acc = number of hydrogen bond acceptor atoms; a_don = number of hydrogen bond donor atoms; lip_druglike = 1 if lip_violation < 2; lip_violation = number of violations of Lipinski's Rule; logS = log of the aqueous solubility (mol/L); mr = molecular refractivity; weight = molecular weight.

Docking Studies

Molecular docking calculations were performed to detect the most likely ligand-protein conformation for the 3-arylcoumarin derivatives. Following a similar docking protocol recently published,[57] the crystal structure of the hMAO-B in complex with 7-(3-chlorobenzyloxy)-4-carboxaldehyde-coumarin (PDB: 2V60)[67] was used to dock the compounds under study. Water molecules were deleted with the exception of the water molecule establishing a hydrogen bond with the crystallographic ligand. The docking simulations were carried out using QM-polarized ligand docking module in Maestro software (see Experimental section for a detailed description). To validate the docking protocol we used the co-

crystallized 7-(3-chlorobenzyloxy)-4-carboxaldehyde-coumarin (PDB: 2V60) as well as the crystallized ligands from other hMAO-B structures (PDB: 1OJ9, 1OJA, 1OJD, 2BK3, 2V5Z, 2V61, 2XFN, 3PO7, 4A79).[72] The RMSD (root mean square deviation) of the heavy atoms coordinates between the calculated and crystallized poses was evaluated and showed a value of 0.26 for the coumarin derivative **c17** in the hMAO-B 2V60 structure (see Table 3 and Figure 4a).

Table 3. RMSD Values Between the Most Stable Poses Calculated through QM-polarized Docking and Different Co-crystallized Ligands for hMAO-B.

PDB code	**RMSD** (root mean square deviation)
2V60	0.26
2XFN	0.77
1OJ9	0.85
1OJD	1.29
2V61	1.38
2V5Z	1.45
2BK3	1.51
1OJA	1.90
3PO7	2.66
4A79	2.68

Once the protocol was validated, molecular docking calculations were carried out also for the compounds of the synthesized series in the hMAO-B, although two of the most active compounds with substitutions at 6 position of the coumarin ring and *meta* or *para* positions in the 3-aryl ring were

considered representative of the study (compounds **12** and **14**, see Figure 4b-4d). The most favourable docking poses according to the energy score E_{model} were retrieved for all the compounds. As it was reported previously in this type of compounds,[57,61] docking simulations in hMAO-B showed that the coumarin ring is oriented toward the bottom of the binding pocket in 15 out of the 23 docked coumarin derivatives. This fact showed the preference of this type of compounds to adopt the described binding conformation (see Figure **4**). The phenyl ring in the benzopyrone system interacts with the FAD cofactor, Tyr60, Tyr398, Tyr435 and Phe343 through *van der Waals* and hydrophobic interactions. The 3-aryl fragment is directed towards the hydrophobic area in the entrance cavity, establishing for compound **12** (the best compound of the presented series) hydrophobic and *van der Waals* interactions mainly with residues Trp119, Leu164, Leu167, Phe168, Ile199 and Ile316. The analysis of the binding mode for the compounds **12** and **14** also showed that the ligand conformations are stabilized by a hydrogen bond between the Cys172 and the oxygen in the carbonyl group of the coumarin ring. However, the hydrogen bond angle is not optimum due to the Cys172 also interacts through an intramolecular hydrogen bond with Phe168. In both compounds, the Leu171 and the Ile199 establish an arene-H interaction with the coumarin ring and the 3-aryl ring respectively. The ligands also establish Coulomb interactions with the FAD cofactor and some residues, such as Glu84, Phe103, Leu171, Ile198, Ile199, Tyr326 and Tyr398. This fact supports the usefulness of the described protocol to analyze the binding mode of this type of coumarin derivatives. However, the most stable ligand conformation could be highly dependent on the nature of the substituents in the 3-aryl ring as well as the coumarin nucleus.

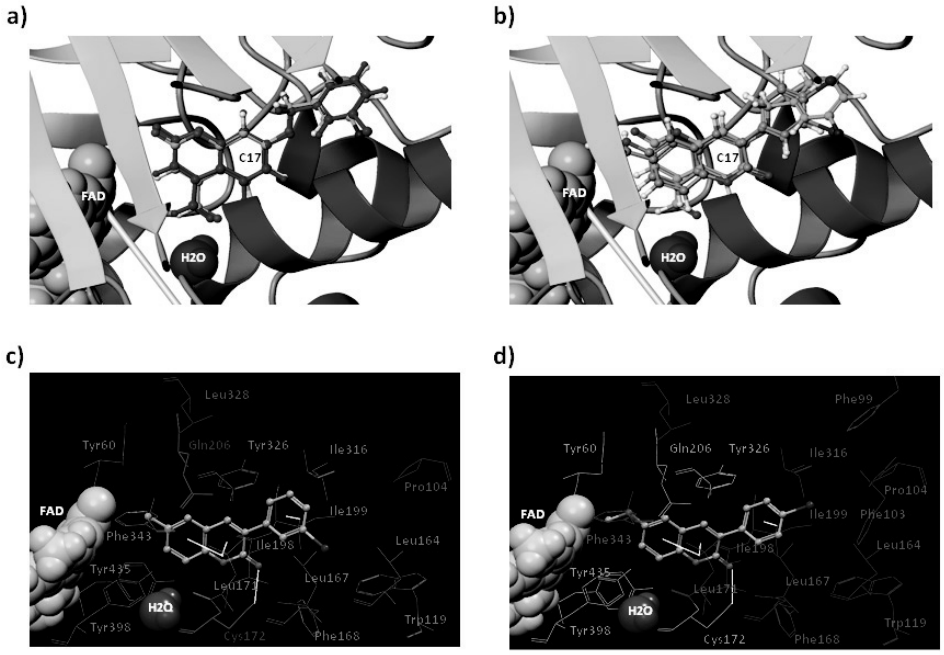


Figure 4. a) Comparison of the co-crystallized ligand **c17** (colored by element, green carbons) in the crystal hMAO-B (PDB: 2V60) and the most stable pose retrieved by the docking calculation (red color). The FAD cofactor is colored in yellow and the water molecule in blue. b) Comparison of the most stable binding modes for the compounds **12** and **14** (colored by element, grey carbons) against the co-crystallized compound **c17** in the crystal hMAO-B (colored by element, green carbons). c) Analysis of the most stable pose of the compound **12** into the hMAO-B pocket. The hydrogen bond between Cys172 and the oxygen in the carbonyl group along with the arene-H interactions with Leu171 and Ile199 are represented in white. Gln206 and Cys172 (violet) establish *van der Waals* interactions with the ligand. Leu328 and Pro104 (red color) interact with the compound **12** through hydrophobic forces. Residues that establish both hydrophobic and *van der Waals* interactions with the ligand are colored in orange. d) Most stable binding mode for the compound **14** in the hMAO-B. The hydrogen bond between Cys172

and the coumarin along with the arene-H interactions are represented in white. The binding pocket is colored according to the residue property: the hydrophobic residues are colored in red and the hydrophilic residues are colored in green.

In this article, we also studied hMAO selectivity using *in silico* evidences. We used the hMAO-A crystal structure 2Z5X to dock the compound **11** that showed low hMAO-A activity. Water molecules within 5 Å from the co-crystallized ligand were retained in the calculation. The RMSD between the theoretical and the crystallized conformation for the ligand harmine was 0.49 Å. The compound **11** was placed in a similar area as the co-crystallized ligand (see Figure **5a**). However, unlike the harmine ligand, the pose for compound **11** determined by docking did not show any hydrogen bond contacts with any water molecules. This fact could explain the difference of hMAO-A activity between both compounds. To further study the hMAO-A activity, compound **20** was also investigated by docking. No water molecules were retained in the cavity due to the larger size of the compound. The docking pose is in agreement with the previous calculation (see Figure **5b**). However, the oxyacetyl chloride chain at position 6 is placed deeply in the cavity. Although this conformation should have to displace some water molecules present in the crystal structure that establish an important H-bond network with the protein and this process could be energetically unfavourable, limiting the ligand binding potential, the carbonyl oxygen replaced a water molecule and established a H-bond with the Tyr197 that could stabilize the ligand conformation (see Figure **5b**).

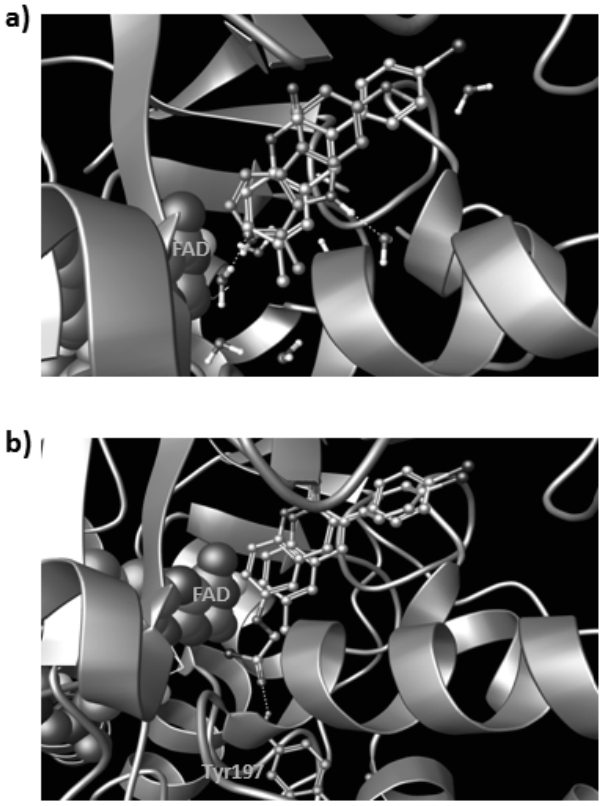


Figure 5. a) Comparison of the co-crystallized ligand harmine (colored by element, green carbons) in the hMAO-A (PDB: 2Z5X) and the pose retrieved by the docking calculation for compound **11** (pink carbons). The ligand harmine establishes H-bond interactions with two water molecules whereas compound **11** did not yield H-bond interactions. b) Comparison of the binding modes for the compounds **11** (pink carbons) and **20** (aquamarine carbons) in the hMAO-A. The carbonyl group in the oxyacetyl chloride chain at position **6** of the compound **20** establishes an H-bond with the residue Tyr197.

Discussion

All the described coumarins in this report (compounds **1-23**) were efficiently synthesized and evaluated

for their ability to inhibit the A and B isoforms of hMAO. The corresponding IC_{50} values and hMAO-B selectivity ratios [IC_{50} (MAO-A)]/[IC_{50} (MAO-B)] are shown in Table 1. The chemical structures of the newly designed compounds, as well as the biological and docking results, can help us with an interesting structure-activity relationship (SAR) study. A theoretical prediction, based on *in silico* derived physicochemical descriptors, of the BBB cross was carried out and the results also encouraged us to explore the potential of this chemical family as drug candidates. All the compounds **1-23** were predicted to readily cross the BBB according to the theoretical model. The model is very simple and explains the BBB crossing through passive diffusion without taking into account other phenomena, such as active transport mechanisms or the action of efflux pumps. Different authors developing QSPR models to evaluate the blood-brain partition coefficient have found the logarithm of the octanol/water partition coefficient and polar surface area as good descriptors to explain this property.[70,73,74] In addition, other molecular descriptors important in ADME properties, such as aqueous solubility, have been calculated. Although many drugs in the market have a logS value greater than -4 our compounds still present acceptable ranges of solubility with an average logS=-5.7. In addition, it can be observed that no violations of Lipinski's rule (molecular weight, logP, number of hydrogen donors and acceptors) were found for almost all the compounds (21 from 23). As MAOIs have to pass different membranes and reach the CNS, this supports the potential of these derivatives as drug candidates.

From the experimental results it can be observed that most of the tested compounds are selective inhibitors toward hMAO-B isoenzyme, with IC_{50} values in the low-nano and picoMolar range. In fact, only compound **18** proved to be inactive against both isoenzymes. Fourteen from the twenty-three tested compounds presented similar or better IC_{50} against hMAO-B than the selegiline (IC_{50} = 19.60 nM, reference hMAO-B inhibitor), with a much better selectivity profile. Thus, compounds **5**, **8**, **9**, **11**, **12**, **14** and **15** proved to inhibit selectively the B isoform of the hMAO enzyme, with extremely good inhibitory activities in the picoMolar range. Compound **1**, the 3-arylcoumarin skeleton, presented a hMAO-B activity (IC_{50} = 11.81 μM) in the same range of iproniazide (IC_{50} = 7.54 μM), being several

times more selective than this reference compound. This was the inspiration to continue with the synthesis of new derivatives presenting this scaffold. The presence of one methyl group at position 6 (compound **2**) increased the hMAO-B activity forty times, maintaining the excellent selectivity (IC_{50} hMAO-B = 284 nM). The presence of a methoxyl group at the same position afforded compound **3**, which showed similar MAOI-B activity to compound **2** (IC_{50} hMAO-B = 413 nM). The hydrolysis of that methoxyl group into a hydroxyl one (compound **4**) resulted in a significant loss of activity against hMAO-B (IC_{50} = 3.39 μM). Nevertheless, compound **4** was still better MAOI-B than the non-substituted 3-phenylcoumarin (compound **1**). According to this data, derivatives substituted at position 6 were explored in the current work. According to previous work,[54-61] it was the very interesting profile of compound **9**, with a methoxy group at position 4 and a bromine atom at position 3 of the 3-aryl group, which made us have a special interest for the bromine derivatives. Also based on previous work,[54-61] and in the IC_{50} values of the compounds **11**-**13** against hMAO-B enzyme, *meta* and *para* positions proved to be the most favourable points to substitute. Therefore, further experimental work was developed taking this information into account.

Four of the most active compounds are the new coumarin derivatives **11**, **12**, **14** and **15**, with IC_{50} against hMAO-B between 134 and 650 pM. Compounds **11** and **12** present a methyl group at position 6 of the scaffold, changing only the position of bromine atom linked in the 3-aryl ring, whereas compounds **14** and **15** have the same substitution pattern. In this case position 6 is substituted with a methoxy group, and the bromine atom is also presented respectively in *meta* and *para* positions of the 3-aryl ring. Comparing the presence of a methyl group (compounds **5** and **6**) or a methoxy group (compounds **7** and **8**) in the 3-aryl ring, keeping the same scaffold, with the compound **11** and **12**, presenting a bromine atom at the same positions, it can be observed a general improvement of the activity. So, we can infer that the presence of an electron withdrawing atom (bromine atom) instead of an electron donating group (methyl or methoxyl) at those positions is an important modification to improve the activity. Based on these new results obtained for compounds **11**-**12** and **14**-**15**, keeping the

bromine atom in *meta* and *para* positions, we decided to deeply explore the importance of the nature of the substitution at position 6. Eight new derivatives (compounds **16**-**23**) have been synthesized with the aim of introducing groups with different physicochemical properties at position 6 of the coumarin moiety.

Compounds **16** and **17**, obtained by hydrolysis of the 6-methoxy derivatives **14** and **15**, showed only a slight loss of activity. These compounds are still good candidates, with IC_{50} values against hMAO-B of 32 and 8 nM, respectively. Having these hydroxyl derivatives as starting materials, Williamson type reactions were carried out to obtain compounds **18**-**23**. The biological results did not significantly improved, but it was found some interesting data. Either the 6-(2-oxopropoxy) derivatives **18** and **21**, or the 6-(cyclopentyloxy) derivatives **19** and **22**, lost significantly the inhibitory activity and selectivity against hMAO-B. Compounds **20** and **23**, with only one chlorine atom more, respecting to compounds **18** and **21**, respectively, presented a very interesting activity profile, with activities against hMAO-B between 2 and 7 nM. Nevertheless, compound **20** presenting the bromine atom in *para* position, lost both potency and selectivity against this isoform.

To better understand the experimental results, and to support the structure-activity relationship study, docking calculations were performed. Based on the docking results, we have proposed a general binding conformation for the coumarins under study in the hMAO-B pocket that orientates the coumarin ring towards the FAD cofactor whereas the 3-aryl ring is directed to the entrance hydrophobic cavity. This fact is in accordance with the observation that small substituents at position 6 are better tolerated than bulky substituents. An increase of the size of the substituents at position 6 in the compounds **18-23** showed a loss of hMAO-B activity probably due to the lack of space in the binding cavity with a possible disruption of the proposed binding mode and possible shift of the coumarin nucleus towards the hydrophobic cavity. On the other hand, polar substituents with ability to establish hydrogen bonds, such as hydroxyl groups, could cause an opposite shift of the coumarin ring towards the FAD cofactor with the consequent disruption of some hydrophobic interactions between the 3-aryl ring and the

hydrophobic entrance cavity. In fact, docking analysis of the compounds **16** and **17** with a hydroxyl substituent at position 6 showed the possibility of establishing a hydrogen bond with the FAD cofactor favourable for the compound binding but causing at the same time a decrease in the hydrophobic interaction contribution by the 3-aryl fragment. Similar docking results could explain the different potency shown by compounds **1** and **2** against the hMAO-B (IC_{50} values are 11.81 and 0.284 μM respectively). The docking retrieved a pose for compound **1** slightly shifted towards the FAD that could diminish the contribution of the hidrophobic interactions to the binding with the entrance cavity. However, the coumarin ring for compound **2** is placed in a very similar manner as the compounds **12** and **14**, shown in Figure 4, that allows a better fit for the 3-aryl ring in the hidrophobic cavity.

The 3-aryl *ortho* bromine substituted derivatives have experimentally shown a decrease of activity since the *meta* or *para* bromine substituted compounds showed a better accommodation in the hydrophobic cavity.[57] In fact, the bromine atom in *ortho* showed a shorter distance to the carbonyl oxygen of the Phe168. Docking analysis suggests that polar substituents at *ortho* position of the 3-aryl ring, such as hydroxyl groups, could improve the hMAO-B activity compared to hydrophobic substituents. The compound **13** with bromine atom in *ortho* showed an IC_{50} = 4.34 μM whereas a similar compound with hydroxyl substituent was recently reported to have an improved activity (IC_{50} = 0.12 μM).[57] Contrarily, polar substituents at *meta* and *para* positions are not as appropriate for the activity as hydrophobic substitutions. This fact corroborates the information that was also recently reported by our research group.[57]

Selectivity of this type of compounds towards hMAO-B has also been studied showing the ability of the compounds to recognize the pocket in hMAO-B whereas a more limited binding has been found against the hMAO-A. On one hand, compound **11** did not show H-bond interactions with hMAO-A. On the other hand, and although compound **20** showed the capability of shifting some water molecules placed deeply in the cavity through the establishment of an H-bond with Tyr197, this overall process could energetically limit the ligand-protein binding. Both isoenzymes differ in some residues in the

pocket that affect selectively the binding of the studied coumarins.

Conclusion

In this study, a general and efficient synthesis of a new series of 3-arylcoumarins was developed using the Perkin, hydrolysis and the Williamson reactions. Determination of hMAO isoform activity was carried out and the majority of the compounds exhibited selectivity for the hMAO-B isoenzyme with high affinity, in the range of nano and picoMolar concentrations. Compound **12** is more than 6000 times more active and more than 200 times more selective than selegiline (reference compound) against MAO-B isoenzyme. Molecular docking studies were performed to establish the nature of the interaction between the studied compounds and hMAO enzymes leading to a rationalization of structure-activity relationship for the synthesized series. Additionally, prediction of blood-brain partitioning through a QSPR model showed the great potential of this type of compounds to cross the BBB and acting in the CNS. The results encourage to further explore the potential of this chemical family as drug candidates for the treatment of Parkinson's disease.

Experimental Section

Chemistry. Melting points were determined using a Reichert Kofler thermopan or in capillary tubes on a Büchi 510 apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX spectrometer at 300 and 75.47 MHz, respectively, using TMS as internal standard (chemical shifts in δ values, *J* in Hz). Mass spectra were obtained using a Hewlett Packard 5972-MSD spectrometer. Silica gel (Merck 60, 230–00 mesh) was used for flash chromatography (FC). Analytical thin layer chromatography (TLC) was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm). The purity of the compounds was assessed by high performance liquid chromatography (HPLC) coupled at diode array detector (DAD G1315D, Agilent Technologies) on an Agilent Technologies 1200 series equipped with a G1311A quaternary pump. Instrument software Agilent ChemStation for LC and

CE systems was used for data acquisition. Different analytical columns and mobile phases (all solvents were HPLC grade) were tested. The mobile phase was H_2O:CH_3CN=70:30 and an Eclipse xdb C18 column (5 μm particle size, 0.46 mm i.d., 25 cm length; Agilent Technologies) was used. The purity of the compounds was found to be ≥ 95%.

General Procedure for the Preparation of 3-Phenylcoumarins 1-3, 5-8 and 11-15. A solution of 2-hydroxy-5-methylbenzaldehyde/2-hydroxy-5-methoxybenzaldehyde (7.34 mmol) and the corresponding phenylacetic acid (9.18 mmol) in dimethyl sulfoxide (15 mL) was prepared. *N,N'*-dicyclohexylcarbodiimide (11.46 mmol) was added and the mixture was heated in an oil-bath at 110 ºC for 24 h. Ice (100 mL) and acetic acid (10 mL) were added to the reaction mixture. After keeping it at room temperature for 2 h, the mixture was extracted with ether (3 x 25 mL). The organic layer was extracted with sodium bicarbonate solution (50 mL, 5%) and then water (20 mL). The solvent was evaporated under vacuum and the dry residue was purified by FC (hexane/ethyl acetate 9:1).

3-(4-Bromophenyl)-6-methylcoumarin (11). Yield 62%; mp 197-198 ºC. ^{1}H NMR ($CDCl_3$) 2.44 (s, 3H, CH_3), 7.24 (dd, 1H, H-7, *J*=7.9, *J*=1.6), 7.36 (dd, 2H, H-5, H-8, *J*=7.9, *J*=1.7), 7.56-7.62 (m, 4H, H-2', H-3', H-5', H-6'), 7.78 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 20.8, 116.2, 119.2, 123.0, 127.0, 127.7, 130.1, 131.6, 132.8, 133.7, 134.3, 139.9, 151.7, 160.5. MS *m/z* 317 (18), 316 (99), 315 ($[M + 1]^+$, 19), 314 (M^+, 100), 288 (34), 287 (22), 286 (34), 285 (17), 179 (17), 178 (36), 152 (9), 118 (14), 89 (11), 76 (12). Anal. Calcd for $C_{16}H_{11}BrO_2$: C, 60.98; H, 3.52. Found: C, 61.00; H, 3.56.

3-(3-Bromophenyl)-6-methylcoumarin (12). Yield 65%; mp 159-160 ºC. ^{1}H NMR ($CDCl_3$) 2.45 (s, 3H, CH_3), 7.25-7.30 (m, 2H, H-7, H-8), 7.36 (dd, 2H, H-4', H-5', *J*=7.9, *J*=2.1), 7.56 (dd, 1H, H-6', *J*=7.8, *J*=2.2), 7.68 (d, 1H, H-5, *J*=2.0), 7.80 (s, 1H, H-4), 7.86 (t, 1H, H-2', *J*=1.8). ^{13}C NMR ($CDCl_3$) 20.8, 116.2, 119.1, 122.4, 127.2, 127.8, 129.9, 131.4, 131.7, 132.9, 134.3, 136.7, 140.4, 140.6, 151.7, 160.3. MS *m/z* 317 (18), 316 (99), 315 ($[M + 1]^+$, 19), 314 (M^+, 100), 286 (34), 285 (17), 179 (17), 178 (36), 152 (9), 118 (14), 89 (11), 76 (12). Anal. Calcd for $C_{16}H_{11}BrO_2$: C, 60.98; H, 3.52. Found: C,

60.94; H, 3.49.

3-(4-Bromophenyl)-6-methoxycoumarin (14). Yield 70%; mp 174-175 ºC. ^{1}H NMR ($CDCl_3$) 3.88 (s, 3H, OCH_3), 6.99 (d, 1H, H-5, *J*=2.8), 7.13 (dd, 1H, H-7, *J*=9.1, *J*=3.0), 7.29 (d, 1H, H-8, *J*=9.1), 7.54-7.60 (m, 4H, H-2', H-3', H-5', H-6'), 7.79 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 55.8, 109.9, 117.5, 119.5, 119.8, 123.2, 127.5, 130.1, 131.6, 133.6, 139.8, 148.0, 156.2, 160.4. MS *m/z* 333 (17), 332 (99), 331 ($[M + 1]^+$, 17), 330 (M^+, 100), 304 (14), 302 (14), 261 (11) 259 (11), 180 (12), 152 (55), 126 (20), 76 (11). Anal. Calcd for $C_{16}H_{11}BrO_3$: C, 58.03; H, 3.35. Found: C, 57.97; H, 3.30.

3-(3-Bromophenyl)-6-methoxycoumarin (15). Yield 72%; mp 151-152 ºC. ^{1}H NMR ($CDCl_3$) 3.91 (s, 3H, CH_3), 7.02 (d, 1H, H-5, *J*=2.9), 7.17 (dd, 1H, H-7, *J*=8.0, *J*=2.9), 7.34 (dd, 2H, H-4', H-5' *J*=10.0, *J*=3.9), 7.57 (dd, 1H, H-6', *J*=9.8, *J*=2.8), 7.70 (d, 1H, H-8, *J*=7.7), 7.82 (s, 1H, H-4), 7.78 (t, 1H, H-2', *J*=1.8). ^{13}C NMR ($CDCl_3$) 55.7, 110.0, 117.5, 119.7, 120.0, 122.5, 127.1, 127.3, 129.9, 131.4, 131.8, 136.7, 140.3, 148.1, 156.2, 160.3. MS *m/z* 333 (17), 332 (99), 331 ($[M + 1]^+$, 18), 330 (M^+, 100), 304 (14), 302 (14), 180 (14), 152 (44), 126 (16). Anal. Calcd for $C_{16}H_{11}BrO_3$: C, 58.03; H, 3.35. Found: C, 58.07; H, 3.40.

General Procedure for the Preparation of 3-(Bromomethoxyphenyl)coumarins 9 and 10. A solution of 3-(methoxyphenyl)coumarins (**7** or **8**, 3.76 mmol), NBS (4.51 mmol) and AIBN (cat.) in CCl_4 (5 mL) was stirred under reflux for 18 h. The resulting solution was filtered to remove the succinimide. The solvent was evaporated under vacuum and purified by flash chromatography (hexane/ethyl acetate 95:5) to obtain respectively compounds **9** and **10** in yields of 41% and 51%.[61]

General Procedure for the Preparation of Hydroxy-3-phenylcoumarins 4, 16 and 17. A solution of substituted 6-methoxy-3-phenylcoumarin (**3**, **14** or **15**, 0.50 mmol) in acetic acid (5 mL) and acetic anhydride (5 mL), at 0 ºC, was prepared. Hydriodic acid 57% (10 mL) was added dropwise. The mixture was stirred, under reflux temperature, for 3 h. The solvent was evaporated under vacuum and the dry residue was purified by CH_3CN crystallization to yield **4**, **16** and **17**, respectively.

3-(4-Bromophenyl)-6-hydroxycoumarin (16). Yield 85%; mp 224-225 ºC. ^{1}H NMR (DMSO-d_6) 7.04-

7.13 (m, 2H, H-5, H-8), 7.30 (dd, 1H, H-7, *J*=8.7, *J*=2.6), 7.63-7.74 (m, 4H, H-2', H-3', H-5', H-6'), 8.24 (s, 1H, H-4), 9.82 (s, 1H, OH). ^{13}C NMR (DMSO-d_6) 113.1, 117.3, 120.4, 120.5, 122.3, 126.1, 131.1, 131.6, 134.5, 141.4, 146.9, 154.3, 160.2. MS *m/z* 319 (19), 318 (99), 317 ([M + 1]$^+$, 19), 316 (M$^+$, 100), 290 (58), 288 (60), 181 (19), 153 (12), 152 (44), 126 (15), 118 (11), 76 (14). Anal. Calcd for $C_{15}H_9BrO_3$: C, 56.81; H, 2.86. Found: C, 56.77; H, 2.81.

3-(3-Bromophenyl)-6-hydroxycoumarin (17). Yield 89%; mp 219-220 ºC. ^{1}H NMR (DMSO-d_6) 7.06 (t, 2H, H-5, H-8, *J*=8.8), 7.28 (d, 1H, H-7, *J*=8.9), 7.42 (td, 1H, H-5', *J*=8.6, *J*=2.4), 7.61 (dd, 1H, H-4', *J*=8.0, *J*=1.3), 7.73 (dd, 1H, H-6', *J*=7.8, *J*=1.4), 7.93 (d, 1H, H-2', *J*=1.5), 8.26 (s, 1H, H-4), 9.80 (s, 1H, OH). ^{13}C NMR (DMSO-d_6) 113.2, 117.3, 120.3, 120.6, 121.9, 125.7, 128.0, 130.7, 131.5, 131.6, 137.6, 142.0, 146.9, 154.3, 160.2. MS *m/z* 319 (16), 318 (99), 317 ([M + 1]$^+$, 18), 316 (M$^+$, 100), 290 (55), 288 (57), 181 (27) 153 (17), 152 (50), 151 (13), 126 (16), 119 (17), 76 (14), 71 (15), 69 (12), 57 (24), 55 (16). Anal. Calcd for $C_{15}H_9BrO_3$: C, 56.81; H, 2.86. Found: C, 56.86; H, 2.90.

General Procedure for the Preparation of 6-(2-Oxopropoxy)-3-phenylcoumarins 18 and 21. To a suspension of anhydrous K_2CO_3 (**16** or **17**, 0.25 mmol) and the corresponding hydroxycoumarin (0.13 mmol), in anhydrous acetone (3 mL), the chloroacetone (0.25 mmol) was added. The suspension was stirred, at reflux temperature, for 16 h. The mixture was cooled and the precipitate was recovered by filtration and washed with anhydrous acetone (3 x 40 mL). The solvent was evaporated under vacuum and the dry residue was purified by FC (hexane/ethyl acetate 85:15) to obtain **18** and **21**, respectively.

3-(4-Bromophenyl)-6-(2-oxopropoxy)coumarin (18). Yield 81%; mp 157-158 ºC. ^{1}H NMR ($CDCl_3$) 2.35 (s, 3H, CH_3), 4.66 (s, 2H, CH_2), 6.98 (d, 1H, H-5, *J*=2.9), 7.20 (dd, 1H, H-7, *J*=9.1, *J*=2.9), 7.30 (d, 1H, H-8, *J*=9.1), 7.55-7.67 (m, 4H, H-2', H-3', H-5', H-6'), 7.79 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 26.6, 73.5, 111.1, 117.9, 119.8, 119.9, 123.4, 127.9, 130.1, 131.7, 133.4, 139.4, 148.6, 154.3, 160.2, 204.6. MS *m/z* 376 (11), 375 (72), 374 (100), 373 ([M + 1]$^+$, 73), 372 (M$^+$, 99), 332 (28), 331 (87), 330 (30), 329 (88), 302 (28), 301 (54), 245 (31), 164 (48), 163 (78), 152 (84), 151 (19), 126 (48). Anal. Calcd for $C_{18}H_{13}BrO_4$: C, 57.93; H, 3.51. Found: C, 57.87; H, 3.47.

3-(3-Bromophenyl)-6-(2-oxopropoxy)coumarin (21). Yield 83%; mp 167-168 ºC. ^{1}H NMR ($CDCl_3$) 2.30 (s, 3H, CH_3), 4.61 (s, 3H, CH_2), 6.94 (d, 1H, H-5, *J*=3.0), 7.15 (dd, 1H, H-7, *J*=9.1, *J*=2.0), 7.33 (dd, 2H, H-4', H-5', *J*=8.1, *J*=2.4), 7.53 (d, 1H, H-8, *J*=9.1), 7.66 (dd, 1H, H-6', *J*=8.2, *J*=2.3), 7.75 (t, 1H, H-2', *J*=2.5), 7.83 (s, 1H, H-4).^{13}C NMR ($CDCl_3$) 26.6, 73.5, 111.1, 117.9, 119.8, 119.9, 122.5, 127.2, 127.5, 130.0, 131.4, 132.0, 136.5, 139.9, 148.6, 154.3, 160.1, 204.5. MS *m/z* 375 (20), 374 (100), 373 ([M + 1]$^+$, 21), 372 (M$^+$, 100), 331 (43), 329 (43), 301 (15) 273 (10), 164 (17), 163 (31), 152 (30), 126 (13). Anal. Calcd for $C_{18}H_{13}BrO_4$: C, 57.93; H, 3.51. Found: C, 57.88; H, 3.47.

Preparation of 6-(2-Cyclopentyloxy)-3-phenylcoumarins 19 and 22. To a suspension of anhydrous K_2CO_3 (0.25 mmol) and the corresponding 6-hydroxycoumarin (**16** or **17**, 0.13 mmol), in anhydrous acetone (3.0 mL), the cyclopentyl bromide (0.25 mmol) was added. The suspension was stirred, at reflux temperature, for 24 h. The mixture was cooled and the precipitate was recovered by filtration and washed with anhydrous acetone (3 x 40 mL). The solvent was evaporated under vacuum and the dry residue was purified by FC (hexane/ethyl acetate 9:1) to obtain **19** and **22**, respectively.

3-(4-Bromophenyl)-6-(cyclopentyloxy)coumarin (19). Yield 63%; mp 164-165 ^{o}C. ^{1}H NMR ($CDCl_3$) 1.27-1.41 (m, 4H, 2xH-3'', 2xH-4''), 1.49-1.72 (m, 2H, 2xH-5''), 1.77-2.29 (m, 2H, 2xH-2''), 4.38 (m, 1H, H-1''), 6.96 (d, 1H, H-5, *J*=2.7), 7.15 (dd, 2H, H-7, H-8, *J*=9.1, *J*=2.7), 7.2-7.38 (m, 2H, H-2', H-6') 7.44-7.67 (m, 2H, H-3', H-5'), 7.76 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 24.0, 32.8, 80.1, 111.9, 117.5, 119.8, 120.9, 123.0, 123.1, 127.3, 130.2, 131.6, 133.7, 139.9, 147.8, 154.7. MS *m/z* 387 (6), 386 (25), 385 ([M + 1]$^+$, 6), 384 (M$^+$, 25), 319 (22), 318 (100), 317 (24), 316 (99), 290 (41) 288 (42), 181 (11), 152 (38). Anal. Calcd for $C_{20}H_{17}BrO_3$: C, 62.35; H, 4.45. Found: C, 62.40; H, 4.49.

3-(3-Bromophenyl)-6-(cyclopentyloxy)coumarin (22). Yield 65%; mp 228-229 ^{o}C. ^{1}H NMR ($CDCl_3$) 1.57-1.99 (m, 8H, 2xH-2'', 2xH-3'', 2xH-4'', 2xH-5''), 4.77 (m, 1H, H-1''), 6.95 (d, 2H, H-5, *J*=3.0), 7.05 (dd, 2H, H-7, H-8, *J*=8.8, *J*=2.7), 7.24-7.35 (m, 2H, H-4', H-5'), 7.53 (m, 1H, H-6'), 7.76 (s, 1H, H-4), 7.84 (t, 1H, H-2', *J*=2.0). ^{13}C NMR ($CDCl_3$) 24.0, 32.7, 80.1, 111.9, 117.5, 119.7, 121.0, 122.5, 127.0, 127.3, 129.9, 131.4, 131.7, 136.8, 140.4, 147.8, 154.7, 160.4. MS *m/z* 387 (11), 386 (45), 385 (M

+ 1]$^+$, 12), 384 (M^+, 45), 319 (19), 318 (100), 317 (22), 316 (100), 290 (27) 288 (27), 152 (11). Anal. Calcd for $C_{20}H_{17}BrO_3$: C, 62.35; H, 4.45. Found: C, 62.30; H, 4.39.

Preparation of 2-[(3-Penhylcoumarin-6-yl)oxy]acetyl chloride 20 and 23. To a suspension of anhydrous K_2CO_3 (0.25 mmol) and the corresponding 6-hydroxycoumarin (**16** or **17**, 0.13 mmol), in anhydrous acetone (3.0 mL), the 2-chloroacetyl chloride (0.25 mmol) was added. The suspension was stirred, at reflux temperature, for 24 h. The mixture was cooled and the precipitate was recovered by filtration and washed with anhydrous acetone (3 x 40 mL). The solvent was evaporated under vacuum and the dry residue was purified by FC (hexane/ethyl acetate 8:2) to obtain **20** and **23**, respectively.

2-[(3-(4-Bromophenyl)coumarin-6-yl)oxy]acetyl chloride (20). Yield 63%; mp 118-119 ^{o}C. ^{1}H NMR ($CDCl_3$) 5.52 (s, 2H, CH_2), 7.05-7.09 (d, 1H, H-5, *J*=2.5), 7.19 (d, 1H, H-8, *J*=8.3), 7.26 (dd, 1H, H-7, *J*=8.8, *J*=2.4), 7.60-7.72 (m, 4H, H-2', H-3', H5', H6'), 8.20 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 89.0, 113.1, 117.2, 120.4, 122.3, 126.1, 131.0, 131.4, 131.6, 134.4, 141.3, 146.8, 154.3, 160.2, 169.0. MS *m/z* 393 ([M + 1]$^+$, 10), 392 (M^+, 50), 319 (14), 318 (82), 317 (15), 316 (82), 297 (11) 295 (11), 290 (42), 288 (43), 216 (13), 214 (13), 181 (16), 172 (48), 171 (26), 170 (50), 169 (24), 152 (30), 126 (24), 118 (11), 90 (20), 89 (13). Anal. Calcd for $C_{17}H_{10}BrClO_4$: C, 51.87; H, 2.56. Found: C, 51.79; H, 2.52.

2-[(3-(3-Bromophenyl)coumarin-6-yl)oxy]acetyl chloride (23). Yield 66%; mp 157-158 ^{o}C. ^{1}H NMR ($CDCl_3$) 5.04 (s, 2H, CH_2), 7.05-7.08 (m, 1H, H-5, *J*=3.0), 7.25 (dd, 1H, H-7, *J*=8.0, *J*=3.5), 7.40 (d, 1H, H-8, *J*=8.0), 7.58-7.63 (m, 2H, H-4', H-5'), 7.72 (dd, 1H, H-6', *J*=7.6, *J*=1.2), 7.91 (d, 1H, H-2', *J*=1.3), 8.24 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) 65.5, 112.6, 113.2, 117.3, 120.2, 120.6, 121.9, 125.7, 128.1, 130.8, 131.7, 137.6, 142.0, 146.9, 154.3, 160.2, 169.5. MS *m/z* 393 ([M + 1]$^+$, 10), 392 (M^+, 50), 391 (17), 390 (86), 389 (17), 388 (86), 319 (16), 318 (99), 317 (29) 316 (100), 315 (13), 290 (53), 289 (19), 288 (54), 181 (21), 153 (17), 152 (60), 126 (23), 119 (16). Anal. Calcd for $C_{17}H_{10}BrClO_4$: C, 51.87; H, 2.56. Found: C, 51.82; H, 2.52.

Determination of hMAO Isoform Activity. Briefly, 0.1 mL of sodium phosphate buffer (0.05 M, pH

7.4) containing different concentrations of the test drugs (new compounds or reference inhibitors) in various concentrations and adequate amounts of recombinant hMAO-A or hMAO-B required and adjusted to obtain in our experimental conditions the same reaction velocity, i.e., to oxidize (in the control group) the same concentration of substrate: 165 pmol of *p*-tyramine/min (hMAO-A: 1.1 μg protein; specific activity: 150 nmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min/mg protein; hMAO-B: 7.5 μg protein; specific activity: 22 nmol of *p*-tyramine transformed/min/mg protein) were incubated for 15 min at 37 ºC in a flat-black-bottom 96-well microtest plate, placed in the dark fluorimeter chamber. After this incubation period, the reaction was started by adding (final concentrations) 200 μM Amplex Red reagent, 1 U/mL horseradish peroxidase and 1 mM *p*-tyramine. The production of H_2O_2 and, consequently, of resorufin was quantified at 37 °C in a multidetection microplate fluorescence reader (FLX800, Bio-Tek Instruments, Inc., Winooski, VT, USA) based on the fluorescence generated (excitation, 545 nm, emission, 590 nm) over a 15 min period, in which the fluorescence increased linearly.

Control experiments were carried out simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions of the vehicles. In addition, the possible capacity of the above test drugs to modify the fluorescence generated in the reaction mixture due to non-enzymatic inhibition (e.g., for directly reacting with Amplex Red reagent) was determined by adding these drugs to solutions containing only the Amplex Red reagent in a sodium phosphate buffer. To determine the kinetic parameters of hMAO-A and hMAO-B (K_m and V_{max}), the corresponding enzymatic activity of both isoforms was evaluated (under the experimental conditions described above) in the presence of a number (a wide range) of *p*-tyramine concentrations.

The specific fluorescence emission (used to obtain the final results) was calculated after subtraction of the background activity, which was determined from vials containing all components except the hMAO isoforms, which were replaced by a sodium phosphate buffer solution. In our experimental conditions, this background activity was practically negligible.

Prediction of passive blood-brain partitioning and calculation of molecular descriptors.

The procedure to calculate the theoretical logBB (the logarithm of the ratio of the concentration of the compound in brain and in blood) was described with more detail in a previous publication.[41] The ligands were prepared with LigPrep using Maestro 9.2 software[75,76] and the protonation state was established with Ionizer at pH 7. After calculating the atomic charges with the Gasteiger (PEOE) model, the descriptors TPSA (Topological Polar Surface Area) and logP(o/w) (log octanol/water partition coefficient) were calculated with MOE 2011.10.[77] The descriptors´ values were introduced in the following equation described by Vilar *et al*.:[41]

$$\log BBclass = 0.5159 \times \log P(o/w) - 0.0277 \times TPSA - 0.3462 \qquad (1)$$

$$N=307 \quad U=0.70 \quad F(2, 304)=63.79 \quad p<0.0001$$

where N is the number of compounds used in the training set of the model developed to extract the discriminant equation, U is the Wilks´statistic, F is the Fisher ratio and p is the significance level. If the result in the equation is >0 the compounds are predicted to have a logBB≥0.3 which means that the compounds readily cross the blood-brain barrier.[70] If the result is <0 the compounds could still cross the blood-brain barrier but with logBB<0.3.

Calculation of other molecular descriptors, such as pharmacophore atom type descriptors, atom counts and physical properties, were also carried out with MOE 2011.10.[77]

Molecular Docking Simulations.

Molecular docking simulations were carried out using Maestro software in the Schrödinger 2011 package.[76]

hMAO-B:

Ligand dataset preparation: The dataset was composed of 23 coumarin derivatives and different ligands belonging to hMAO-B crystal structures (PDB: 1OJ9, 1OJA, 1OJD, 2BK3, 2V5Z, 2V60, 2V61, 2XFN,

3PO7, 4A79).[72] Different protonation states at pH 7.0±2.0 using Epik and tautomers were generated with LigPrep module.[75] All the structures were optimized using OPLS_2005 force field.

Protein structure preparation: The crystal structure of the hMAO-B in complex with a coumarin derivative (PDB code: 2V60) was pre-processed using the Protein Preparation Workflow in Maestro software.[76] A water molecule establishing a hydrogen bond with the co-crystallized ligand was retained. Hydrogens were added through this procedure and minimized with the OPLS_2005 force field while heavy atoms were constrained. Hydrogen bonding optimization was carried out and included the reorientation of hydroxyl groups, the water molecule and amide groups of Asn and Gln residues. Protonation states of His, Asp and Glu residues were also optimized.

Receptor grid generation: A receptor grid was calculated using a *van der Waals* scaling factor of 1.0 with a partial charge cut-off of 0.25. The grid was centered in the co-crystallized coumarin derivative **c17** with an outer box length of 20 Å.

QM-polarized ligand docking procedure: In the first step of the QM-polarized docking,[78] the ligands were docked to the hMAO-B (2V60) using Standard Precision level (SP scoring function) of Glide[79] and three poses for each ligand were retained. Next, polarization of the ligand charges by the receptor was calculated. Quantum mechanical calculations using Jaguar with 6-31G*/LACVP* basis set, B3LYP density functional, and Ultrafine SCF accuracy level are carried out to determine the partial charges on the ligands atoms inside the protein pocket. In the third step, the ligands were redocked with the new charges using Extra Precision (XP) mode and three poses were retained for each ligand. Ligands *van der Waals* scaling was 0.8. The selection of the final pose was made taking into account the energy score E_{model} that combines the energy grid score, GlideScore and internal strain energy used in the conformational search.

hMAO-A: Similar treatment was carried out for the hMAO-A crystal structure with the ligand harmine (PDB code: 2Z5X).

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Table of Contents Graphic



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Synthesis and adenosine receptors binding affinities of a series of 3-arylcoumarins

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Abstract

Objectives In the present communication we report the synthesis, pharmacological evaluation, theoretical evaluation of ADME properties, and SAR study of a selected series of 3-arylcoumarins (compounds **1**-**9**). Adenosine receptors (ARs) binding activity and selectivity of the synthesized compounds **1**-**9** were evaluated in this study. Different substituents were introduced in both benzene rings of the evaluated scaffold, at positions 6 and 3' or 4' of the moiety. The lack of data on the 3-arylcoumarin scaffold encouraged us to explore the ARs' binding activity of a selected series of derivatives.
Methods A new series of coumarins (compounds **1-9**) were synthesized and evaluated by radioligand binding studies towards adenosine receptors.
Key findings Analyzing the experimental data it can be observed that neither the simple 3-arylcoumarin, nor the 4'-nitro derivatives presented detectable binding affinity for the evaluated receptors. Although, most of the other substituted derivatives have good binding affinity profiles, especially against the hA_1/hA_3 or only hA_3 AR.
Conclusions The most remarkable derivative is compound **2**, presenting the best affinity for hA_3 AR (K_i = 2,680 nM) and significant selectivity for this subtype.
Keywords 3-Arylcoumarins; adenosine receptors binding activity; ADME properties; SAR study.

Introduction

Adenosine receptors (ARs) are G-protein-coupled receptors that exert a huge number of physiological activities through activation of four different receptor subtypes: A_1, A_{2A},

A_{2B} and A_3.[1] ARs are distributed throughout a wide variety of tissues in mammalian systems and regulate diverse physiological functions by modulating cell signalling, being activated by the endogenous agonist adenosine and blocked by natural antagonists.[2,3] During the last years, the search for agonist and antagonist ligands binding to individual AR subtypes has been intensified, as the role of these receptors in drug discovery is continuously expanding.[4-6] Most of the AR antagonists are promising new drug candidates for a number of pathological processes such as inflammation (A_3),[5] heart and renal failure (A_1),[7] or neurological disorders like Parkinson´s [8,9] and Alzheimer's diseases (A_{2A} and/or A_1).[10] In particular A_3 AR, the most recently identified subtype, has been the subject of intensive research during the last decade as a potential therapeutic target in preventing ischemic damage in the brain and heart.[11] In addition, as the A_3 AR is expressed in relatively high densities in the lung, liver, neutrophils, macrophages and glial cells, it can encompass an important role in inflammation, immunosuppression and cell growth.[12] Therefore, A_3 AR ligands can have therapeutic potential against cancer, hepatitis, myocardial ischemia, rheumatoid arthritis or ophthalmic diseases.[13] More recently, an involvement of A_3 AR in neuroprotection was also proposed.[14] However, the mixed A_3-mediated protective/damaging effect remains enigmatic and has to be clarified. Therefore, additional studies must be performed to verify the modulating effect of the A_3 AR agonists and antagonists in neurodegenerative diseases. As a consequence, numerous A_3 AR agonists and antagonists have been developed and investigated for their potential therapeutic applications in diseases where the A_3 AR seems to have a positive role.

Of the large number of compounds exerting high potency and selectivity towards ARs, the xanthine and adenine scaffolds have been used to develop the so-called classical AR antagonists. In the search for nonclassical AR ligands, novel structures have recently been discovered. Examples are the quinolinone derivatives (Figure 1 – I), recognized as putative A_1, A_{2A} and A_{2B} AR ligands.[15] Based on these findings, and taken into consideration that the coumarin nucleus can be a non-nitrogen aromatic bioisoster scaffold, it was found to be relevant to explore it in the design of novel AR antagonists. Our interest in this new scaffold was also motivated by the structure similarity between the coumarin skeleton and chromone scaffolds (Figure 1 – II), previously described as AR antagonists that present a particular selectivity for the A_3 and A_{2A} AR subtypes.[16-18] Among them, our attention was focused on the 3-arylcoumarin ring system (Figure 1 – III), which has never been proposed as a scaffold for AR ligands. The recent findings, and the lack of data on the 3-arylcoumarin scaffold encouraged us to explore the ARs' binding activity of a selected series of derivatives.

(Figure 1)

Figure 1. Examples of nonclassical AR ligands. Quinolinone derivatives (I), chromone derivatives (II) and 3-arylcoumarin derivatives (III).

Coumarins are an important class of benzopyrone heterocyclic compounds, of natural or synthetic origin, associated with remarkable pharmacological activities. [19,20] Diverse

biological properties, such as antioxidant, anti-HIV, anticancer, anti-inflammatory, antimicrobial, vasorelaxant or enzymatic inhibitory agents, have been ascribed to this type of structure. [19-33] The fused heterocyclic framework of coumarins has been widely used as a archetype for the synthesis of a wide variety of analogues suitable to perform structure-activity relationship studies intended to improve their biological properties.[19,20] In the last few years our research group has been engaged in the synthesis of new biologically active coumarins. In particular, the 3-arylcoumarin scaffold has been a focal point of our recent studies demonstrating their importance as monoamine oxidase (MAO) inhibitors.[27-36] Different synthetic strategies can be used to obtain the arylcoumarin scaffold. The principal one is the construction of the coumarin nucleus by condensation: cyclization-type reactions. The classical Perkin condensation is perhaps the most direct and simple method known for the preparation of these molecules.[23] Some variations of this methodology are described in the chemistry section below.

In the present communication we report the synthesis, pharmacological evaluation, theoretical evaluation of ADME properties and SAR study of a selected series of 3-arylcoumarins (compounds **1**-**9**). Adenosine receptors (ARs) binding activity and selectivity of the synthesized compounds **1**-**9** were evaluated in this study.

Materials and Methods

Chemistry

Compounds **1**-**9** were efficiently synthesized according to the synthetic strategy outlined in Scheme 1. Perkin condensation of different commercially available *ortho*-hydroxybenzaldehydes with a conveniently substituted arylacetic acid, using *N,N'*-dicyclohexylcarbodiimide (DCC) as dehydrating agent,[28,29,32] afforded the 3-arylcoumarins **1**-**3**.[37-39] In fact, these reaction conditions have been also found to be appropriate when the reagents encompass in their structure methyl, methoxyl, ethoxyl or halogen groups. However, in the case of the hydroxyl substituents, these groups must be previously protected by acetylation. Therefore, compound **4** was obtained starting from the 2,5-dihydroxybenzaldehyde and the 4-methylphenylacetic acid, using potassium acetate in acetic anhydride, under reflux, for 16 hours. Acetylation of the hydroxyl groups and pyrone ring closure occurred simultaneously.[40] Nitro-substituted compounds **5** and **6**[41,42] were obtained via other modified Perkin conditions, using a substituted *ortho*-hydroxybenzaldehyde and 4-nitrophenylacetic acid, in presence of NaH in acetic anhydride, for 20 hours.[43] Compounds **7** and **8**[32] were obtained by acidic hydrolysis of the respective methoxy/acetoxy derivatives **3** and **4**, using hydriodic acid 57% in the presence of acetic acid and acetic anhydride.[29] Finally, the Williamson reaction of the hydroxycoumarin **8** with chloroacetone, under reflux of acetone for 16 hours, gave the corresponding ether **9**.[32]

(Scheme 1)

Scheme 1. *Reagents and conditions:* (a) DCC, DMSO, 110 ºC, 24 h; (b) CH_3CO_2K, Ac_2O, reflux, 16 h; (c) NaH, Ac_2O, r.t., 20 h; (d) HI, AcOH, Ac_2O, 110 ºC, 5 h; (e) chloroacetone, K_2CO_3, acetone, reflux, 16 h.

Melting points were determined using a Reichert Kofler thermopan or in capillary tubes on a Büchi 510 apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX spectrometer at 300 and 75.47 MHz, respectively, using TMS as internal standard (chemical shifts in δ values, J in Hz). Mass spectra were obtained using a Hewlett-Packard 5988A spectrometer. Elemental analyses were performed using a Perkin-Elmer 240B microanalyser and were within ±0.4% of calculated values in all cases. Silica gel (Merck 60, 230–00 mesh) was used for flash chromatography (FC). Analytical thin layer chromatography (TLC) was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm). The purity of compounds was assessed by high performance liquid chromatography (HPLC) coupled at diode array detector (DAD) on a Thermo Quest Spectrasystem (Thermo Fisher Scientific, Waltham, MA) equipped with a P4000 pump, an UV6000 UV-Vis diode array detector, and an SN4000 interface to be operated via a personal computer. Instrument software ChromQuest 5.0 (Thermo Fisher Scientific, Waltham, MA) was used for data acquisition. Different analytical columns and mobile phases (all solvents were HPLC grade) were tested. The mobile phase was H_2O:CH_3CN=70:30 and an Eclipse xdb C18 column (5 μm particle size, 0.46 mm i.d., 25 cm length; Agilent Technologies) was used. The purity of the compounds was found to be higher than 95%.

Synthetic methodologies

General procedure for the preparation of 3-phenylcoumarins **1**-**3**. A conveniently substituted *ortho*-hydroxybenzaldehyde (7.34 mmol) and the corresponding phenylacetic acid (9.18 mmol) were dissolved in dimethyl sulfoxide (15 mL). *N,N'*-Dicyclohexylcarbodiimide (11.46 mmol) was added, and the mixture was heated in an oil bath at 110 ºC for 24 h. After reaction, ice (100 mL) and acetic acid (10 mL) were added to the mixture. The reaction was kept at room temperature for 2 h, and then extracted with ether (3 x 25 mL). The organic layer was washed with sodium bicarbonate solution (50 mL, 5%) and then water (20 mL). The solvent was evaporated under vacuum, and the dry residue was purified by FC (hexane/ethyl acetate 9:1) to give the products **1** (mp 131-132 ºC)[37], **2** (mp 139-140 ºC)[38] or **3** (mp 98-99 ºC)[32,39] as beige powders, in yields of 70%, 65% and 75%, respectively.

Synthesis of 6-acetoxy-3-(4'-methylphenyl)coumarin (**4**). Compound **4** was synthesized under anhydrous conditions, using material previously dried at 60 ºC for at least 12 h and at 300 ºC during few minutes immediately before use. A solution containing anhydrous CH_3CO_2K (2.94 mmol), the 4-methylphenylacetic acid derivative (1.67 mmol) and the 2,5-dihydroxybenzaldehyde (1.67 mmol), in Ac_2O (1.2 mL) was prepared and refluxed (138 ºC) for 16 h. The reaction mixture was cooled, neutralized with 10% aqueous $NaHCO_3$, and extracted (3 x 30 mL) with EtOAc. The organic layers

were combined, washed with distilled water, dried (anhydrous Na_2SO_4), and evaporated under reduced pressure. The product was purified by recrystallization in EtOH and dried, to afford **4** as a beige powder.

Synthesis of 3-(4'-nitrophenyl)coumarins **5** and **6**. To a dry 100-mL round bottomed flask the conveniently substituted *ortho*-hydroxybenzaldehyde (42.5 mmol), 4'-nitrophenylacetic acid (42.5 mmol) and acetic anhydride (40.1 mL, 0.43 mol) were added. Then, sodium hydride (60% dispersion in mineral oil) (42.5 mmol) was added in small aliquots. After the dissolution of the reagents, an instantaneous (after 2-5 minutes) precipitation process was observed. The reaction mixture was stirred for 20 h and then 7 mL of water were added. After the addition of 43 mL acetic acid, the mixture was cooled to 4 ºC for 4 h. The resulting precipitate was filtered and washed with cold glacial acetic acid. The acetic acid was then removed as an azeotrope upon addition of 250 mL toluene and rotary evaporation to dryness. The process was repeated three times. The final residue was dried under vacuum and purified by FC (hexane/ethyl acetate 9:1) to give the products **5** (mp 100-101 ºC)[41] or **6** (mp 103-104 ºC)[42] as beige powders, in yields of 61% and 64%, respectively.

Synthesis of 6-hydroxy-3-phenylcoumarins **7** and **8**. A solution of the 6-methoxy/acetoxy substituted 3-phenylcoumarin (**3** or **4**, 0.50 mmol) in acetic acid (5 mL) and acetic anhydride (5 mL) at 0 ºC, was obtained. Hydriodic acid 57% (10 mL) was then added dropwise. The mixture was stirred at 110 ºC for 5 h. The solvent was evaporated under vacuum and the dry residue was purified by CH_3CN crystallization to give **7** or **8** (mp 214-215 ºC)[32] as beige powders, in yields of 55% and 61%, respectively.

Synthesis of 3-(4'-methylphenyl)-6-(2-oxopropoxy)coumarin (**9**). To a suspension of anhydrous K_2CO_3 (0.25 mmol) and the 6-hydroxy-3-(4'-methylphenyl)coumarin (**8**, 0.13 mmol), in anhydrous acetone (3 mL), chloroacetone (0.25 mmol) was added. The suspension was stirred, at reflux temperature, for 16 h. The mixture was cooled, and the precipitate was recovered by filtration and washed with anhydrous acetone (3 x 40 mL). The solvent was evaporated under vacuum, and the dry residue was purified by FC (hexane/ethyl acetate 85:15) to give compound **9** (mp 128-129 ºC)[32] as a beige powder, in a yield of 74%.

Biological assays

The adenosine binding affinity of compounds **1**-**9** for the human AR subtypes hA_1, hA_{2A}, hA_{2B} and hA_3, expressed in Chinese Hamster Ovary (CHO) cells, was determined in radioligand competition experiments.[44-47] In the binding affinity assay, it is measured the competition of ligands for specific binding of [^{3}H]CCPA binding at hA_1 ARs; specific binding of [^{3}H]NECA binding at hA_{2A} ARs; and specific binding of [^{3}H]HEMADO at hA_3 ARs. The results were expressed as K_i (dissociation constants), which were calculated with the program SCTFIT, and given as geometric means of at

least three experiments, including 95 % confidence intervals.[46] Due to the lack of a suitable radioligand for hA$_{2B}$ receptor in binding assay, the potency of antagonists at hA$_{2B}$ receptor was determined instead by inhibition of NECA-stimulated adenylyl cyclase activity with increasing concentrations of antagonist.[44,45] As a result, cAMP (cyclic adenosine monophosphate) production was inhibited in a concentration-dependent fashion, and IC_{50} and K_i values, respectively, were determined.

ADME properties

The preliminary data for an ADME profile analysis was calculated using the Molinspiration property calculation program.[48]

Statistical Analysis

K_i (dissociation constants) values (Table 1) are reported as geometric means of three independent experiments with each tested concentration of compounds measured in duplicates. K_i were calculated with the program SCTFIT. As an interval estimate for the dissociation constants 95% confidence intervals are given in parentheses.

Results

Structural Identification

6-Acetoxy-3-(4'-methylphenyl)coumarin (**4**): Yield 61%. Mp 146-147 ^{o}C; ^{1}H NMR δ ppm ($CDCl_3$-300 MHz): 2.38 (3H, s, CH_3), 2.44 (3H, s, CH_3), 7.24-7.32 (4H, m, H-2', H-3', H-5', H-6'), 7.40 (1H, dd, J = 8.7, 2.7 Hz, H-7), 7.63 (2H, d, J = 8.6 Hz, H-5, H-8), 7.77 (1H, s, H-4). ^{13}C NMR δ ppm ($CDCl_3$-75 MHz): 21.1, 21.3, 117.4, 119.9, 120.2, 124.6, 128.4, 129.1, 129.2, 131.5, 138.4, 138.4, 139.2, 146.7, 150.9, 169.3. MS (ESI): 295 ($M+H^+$, 20), 294 (M^+, 100). Anal. Calcd for $C_{18}H_{14}O_4$: C, 73.46; H, 4.79. Found: C, 73.41; H, 4.74.

6-Hydroxy-3-(3'-methylphenyl)coumarin (**7**): Yield 55%. Mp 132-3 ^{o}C; ^{1}H NMR δ ppm ($CDCl_3$-300 MHz): 2.24 (3H, s, CH_3), 6.97-7.29 (4H, m, H-5, H-7, H-4', H-6'), 7.31-7.33 (2H, m, H-2', H-5'), 7.48 (1H, d, J = 8.8 Hz, H-8), 8.11 (1H, s, H-4), 9.82 (1H, s, OH). ^{13}C NMR δ ppm ($CDCl_3$-75 MHz): 25.2, 113.2, 117.4, 120.4, 126.6, 127.5, 128.7, 129.8, 130.2, 135.4, 138.0, 141.1, 147.0, 154.5, 160.6, 169.8. MS (ESI): 253 ($M+H^+$, 15), 252 (M^+, 100). Anal. Calcd for $C_{16}H_{12}O_3$: C, 76.18; H, 4.79. Found: C, 76.15; H, 4.76.

Biological results

The data from radioligand binding experiments for compounds **1-9** is summarized in Table 1. Details for pharmacological experiments were previously described in materials and methods and in references 44-47.

Table 1. Affinity (K_i) to bind human A_1 and A_3 ARs expressed in CHO cells of coumarins **1-9**, in radioligand binding assays.

(Table 1)

One of the important requirements for a molecule to be a good drug candidate is the ability to cross membranes. Also, associated with this characteristic, a molecule must possess the appropriate ADME properties. To better understand the overall properties of the synthesized coumarins, preliminary data for an ADME profile analysis were carried out. The lipophilicity, expressed as the octanol/water partition coefficient (represented as logP), was calculated using the Molinspiration property calculation program (Table 2).[48] Therefore, a theoretical prediction of ADME properties of all compounds was carried out and is described by different parameters (Table 2).[49,50]

Table 2. Structural properties of the coumarin derivatives **1-9**.[a]

(Table 2)

Discussion

Compounds **1-9** were synthesized and tested for their *in vitro* binding affinity against human AR subtypes *h*A_1, *h*A_{2A}, *h*A_{2B} and *h*A_3, expressed in CHO cells. In the present communication, studies of the effect on the binding affinity by the presence of different substituents in the 3-arylcoumarin scaffold, at positions 3', 4' and 6, were performed. Since the 3-arylcoumarin scaffold never been described as AR ligand, we were encouraged to explore the ARs' binding activity of a selected series of derivatives. The experimental data show that all the 3-arylcoumarin derivatives have no detectable binding affinity to *h*A_{2A} and *h*A_{2B} ARs (K_i > 30,000 – 100,000 nM). Compound **1**, the non-substituted 3-arylcoumarin, presents no binding affinity for any of the evaluated receptors (K_i > 30,000 nM). The introduction of electron donating groups in both phenyl rings (methyl groups at positions 6 and 4') afforded compound **2**, the most interesting compound of this series with a K_i at the *h*A_3 AR of 2,680 nM. The structural modification of coumarin **1** results in significant affinity and selectivity for *h*A_3 ARs. The interesting result found for compound **2** was inspiring for the development of new *h*A_3 AR selective ligands. The changing of the methyl group of the phenyl substituent for a strong electron withdrawing nitro group led to compound **5**, which displays no binding affinity for any of AR subtypes. The presence of a nitro group at that position doesn't seem to be compatible with receptor binding, as confirmed by the data obtained with compound **6**. As the presence of a methyl group at position 4' seems to be important for the binding affinity, compounds **4**, **8** and **9** were synthesized and studied.
For compound **8**, with a hydroxyl group at position 6, a decrease in the selectivity was observed, although the affinity data for both *h*A_1 and *h*A_3 ARs were interesting (K_i =

7,900 and K_i = 4,910 nM, respectively). Compound **4**, with an acetoxy group in the same position of the coumarin skeleton, is a selective compound, with good binding affinity for the hA_3 AR (K_i = 7,280 nM). Finally, the presence of a 2-oxopropoxy group (compound **9**) in the previously mentioned position of the coumarin moiety led also to a noteworthy hA_3 AR ligand (K_i = 4,680 nM).

To complete the present study, the methyl group at 4' position (present in the best compound of the series-compound **2**) was transposed to position 3' (compounds **3** and **7**). In the case of compound **7**, as previously described for compound **8**, the presence of a hydroxyl group at position 6 has a strong influence on the selectivity and affinity towards hA_1 and hA_3 ARs (K_i = 9,250 nM and K_i = 14,600 nM, respectively). Compound **3** with a methyl group at position 3' of the 3-aryl ring is a selective hA_3 AR ligand (K_i = 5,050 nM), similar to the other non-hydroxylated 4'-methyl derivatives. In fact, the protection of the hydroxyl groups forming ether (compound **3**) or ester (compounds **4** and **9**) derivatives results in an increase in hA_3 selectivity.

From these preliminary results one can conclude that the presence of the methyl group in the aryl substituent will provide an important strategy to improve the AR binding affinity of coumarin-based ligands. In addition, chemical changes at position 6 are important for modulating the selectivity of compounds against hA_1/hA_3 or hA_3 ARs.

Moreover, due to the demonstrated importance of the 3-arylcoumarins as MAO inhibitors, these AR ligands can be further developed to give improvements in both ARs activity and ARs *vs* MAO selectivity. Also, since A_3 AR ligands are involved in neurodegenerative diseases, this study can be an encouragement for the development of *multitarget* drugs as AR ligands and MAO inhibitors. Compound **2**, presenting the better hA_3 affinity/selectivity of this series, was previously described as MAO-B selective inhibitor, with an IC_{50} of 308 pM. This compound is one of the best MAO-B inhibitors described by our research group. This is an additional data to consider this compound an excellent candidate for future studies in the field of neurodegenerative diseases.

Finally, analyzing the obtained theoretical results, it can be observed that no violations of Lipinski's rule (molecular weight, logP, number of hydrogen donors and acceptors) were found for the described coumarins. Also, TPSA, described to be a predictive indicator of membrane penetration, is also found to be positive for these drug candidates.

Conclusions

In the current study, a series of 3-arylcoumarin derivatives were synthesized and evaluated as putative adenosine receptor ligands. A detailed analysis of the experimental results allows us to conclude that the affinity and/or selectivity of the synthesized coumarins is influenced by the presence and the nature of the substituents at positions 3', 4' and 6 of the 3-arylcoumarin scaffold. The experimental data show that neither the simple 3-arylcoumarin nor the 4'-nitro derivatives presented detectable binding affinity for the evaluated receptors. Although, most of the other substituted derivatives have good binding affinity profiles, especially against the hA_1/hA_3 or only hA_3 AR. In

general, one can conclude that the presence of a methyl group at positions 3' or 4' of the 3-aryl ring is important for the binding affinity. Fulfilling this condition, the presence of a methyl, methoxy, acetoxy or 2-oxopropoxy groups at position 6 of the scaffold, results in a noticeable affinity and selectivity for hA$_3$ AR. The presence of a hydroxyl group at position 6 gives rise to non-selective ligands binding to both hA$_1$ and hA$_3$ ARs. This study demonstrates that 3-arylcoumarin is an interesting scaffold for the design of novel ARs ligands. Accordingly, compound **2** (K_i hA$_3$ AR = 2,680 nM), which is also a very interesting MAO-B selective inhibitor, can be considered the lead for the design and synthesis of new hA$_3$ AR coumarin-based selective ligands. This compound, as all compounds in this series, is in accordance with the Lipinski's rule. Therefore, the versatility of the synthetic routes and the substitution variability of these coumarins allow proposing them as privileged *multitarget* scaffolds for drug discovery in the field of neurodegenerative diseases.

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500 Table 1
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Compounds			hA_1	hA_3	Selectivity
	R	**R_1**	**K_i (nM)**	**K_i (nM)**	**hA_1/hA_3**
1	H	H	> 30,000	> 30,000	-
2	CH_3	4'-CH_3	> 30,000	2,680 (1,440-5,010)	> 11.2
3	OCH_3	3'-CH_3	> 30,000	5,050 (3,670-7,000)	> 5.9
4	$OCOCH_3$	4'-CH_3	> 30,000	7,280 (4,130-12,800)	> 4.1
5	CH_3	4'-NO_2	> 30,000	> 30,000	-
6	OCH_3	4'-NO_2	> 100,000	> 100,000	-
7	OH	3'-CH_3	9,250 (8,270-10,300)	14,600 (13,400-15,900)	0.6
8	OH	4'-CH_3	7,900 (4,080-15,300)	4,910 (3,970-6,070	1.6
9	OCH_2COCH_3	4'-CH_3	> 30,000	4,680 (3,870-5,660)	> 6.4

502 These results are average results of three experiments.

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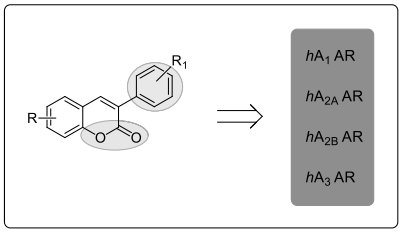
Table 2

Compounds			logP	Molecular weight (g/mol)	TPSA (Å^2)	n-OH acceptors	n-OHNH donors	Volume (Å^3)
	R	R_1						
1	H	H	3.74	222.24	30.211	2	0	200.00
2	CH_3	4'-CH_3	4.61	250.30	30.21	2	0	233.12
3	OCH_3	3'-CH_3	4.19	266.30	39.44	3	0	242.10
4	$OCOCH_3$	4'-CH_3	3.71	294.31	56.52	4	0	261.08
5	CH_3	4'-NO_2	4.12	281.27	76.03	5	0	239.89
6	OCH_3	4'-NO_2	3.73	297.27	85.27	6	0	248.88
7	OH	3'-CH_3	3.66	252.27	50.44	3	1	224.57
8	OH	4'-CH_3	3.68	252.27	50.44	3	1	224.57
9	OCH_2COCH_3	4'-CH_3	3.76	308.33	56.52	4	0	277.89

[a] TPSA, topological polar surface area; n-OH, number of hydrogen bond acceptors; n-OHNH, number of hydrogen bond donors. The data was determined with Molinspiration calculation software.[45]

Graphical Abstract

In the present communication we report the synthesis, pharmacological evaluation, theoretical evaluation of ADME properties and SAR study of a selected series of 3-arylcoumarins (compounds **1**-**9**). Adenosine receptors (ARs) binding activity and selectivity of the synthesized compounds **1**-**9** were evaluated in this study. Most of the substituted derivatives have good binding affinity profiles, especially against the hA_1/hA_3 or only hA_3 AR. The most remarkable derivative is compound **2**, presenting the best affinity for hA_3 AR (K_i = 2,680 nM) and significant selectivity for this subtype.



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6. Conclusiones

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✓ Effective and versatile synthetic methodologies for the preparation of large series of compounds were developed. The new compounds incorporate in a single structure the coumarin and stilbene cores, or the coumarin core and different groups such as esters, amides or carbamates. The methodologies for the synthesis of the coumarin and 3-arylcoumarin skeletons involved Perkin, modified-Perkin and palladium-coupling reactions. Modifications of these skeletons were carried out using simple and safe methods, involving different reactions such as bromination, amination, reduction, etherification, acylation, hydrolysis, etc..

✓ All the compounds were obtained in good yields, with the desired degree of purity and in sufficient quantity for the biological and/or physicochemical tests.

✓ All the compounds were characterized by melting point, mass spectra, ^{1}H NMR, ^{13}C NMR, DEPT and elemental analysis. IR and/or X-ray have further characterized some of the derivatives.

✓ Many of the tested compounds showed a high degree of potency and selectivity inhibiting *in vitro* the MAO-B isoform (IC_{50} in nano-picoMolar range), without modifying the enzymatic activity against MAO-A. In particular the 3-(3'-bromophenyl)-6-methylcoumarin proved to be more than 6000 times more active (IC_{50} = 134.04 pM) and over 200 times more selective than selegiline.

✓ Some of the derivatives have presented a moderate inhibitory activity against AChE. Others proved to be ligands of one or more of the four adenosine receptors. Other derivatives have also shown activity against tyrosinase. Particularly the 3-amino-7-hydroxycoumarin proved to inhibit the tyrosine 8 times more than the umbelliferone. Simple coumarins and 3-nitro-substituted-arylcoumarins presented antibacterial properties. In particular the 3-(3'-methylphenyl)-6-nitrocoumarin presented a MIC against *S. aureus* of 8 μg/mL. Many of the studied derivatives presented low oxidation potential, high ORAC-FL and an excellent ability to trap hydroxyl radicals. In particular, 3-(4'-

hydroxyphenyl)-8-hydroxycoumarin presented an ORAC-FL value of 13.5, in addition to a low oxidation potential and 100% of hydroxyl radicals trapping.

✓ All these studies are the result of interesting collaborations with different groups in different national and foreign universities. Without the joint effort these projects would not be possible and the results presented in this report have not been achieved. The work is necessarily interdisciplinary.

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7. Bibliografía

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