TRABALLO FIN DE GRAO

GRAO EN QUÍMICA

UNIVERSIDADE
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Facultade de Química

Gold-promoted cascade reactions in biological media

Curso 2023/24

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FACULTADE DE QUÍMICA

José Luis Mascareñas Cid, titor e docente do Departamento de Química Orgánica, e María Tomás Gamasa, cotitora e docente do Departamento de Química Orgánica , autorizan a presentación do Traballo de Fin de Grao da alumna Ximena Sánchez Iglesias na convocatoria de xullo do curso 2023-2024, o cal foi realizado baixo a súa dirección no Centro Singular de Investigación en Química Biolóxica e Materiales Moleculares da Universidade de Santiago de Compostela (CiQUS).

E para que así conste asinamos o presente informe en Santiago de Compostela o 30 de xuño de 2024.

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ABBREVIATIONS

allyl	prop-2-en-1-yl	NADH	nicotinamide adenine dinucleotide (reduced)
BSA	bovin serum albumin	NMR	nuclear magnetic resonance
^{13}C-NMR	carbon NMR	NTF_2	bis(trifluoromethylsulfonyl)amide
CuAAC	copper-catalyzed azide-alkyne cycloaddition	OSCs	organic solar cells
d	doublet	OTf	trifluoromethanesulfonate
dba	dibenzylideneacetone		
DEPT-135	distortionless enhancement by polarization transfer	PBS	phosphate-buffered saline
δ	chemical shift	PhSH	phenylthiol
DMEM	Dulbecco's Modified Eagle Medium	ppm	parts per million
DMSO	dimethyl sulfoxide	PTA	1,3,5-triaza-7-phospaadamantane
e.g.	*exempli gratia*	r.t.	room temperature
eq.	equivalents	Rf	retention factor
Et_3N	triethylamine	ROS	reactive oxygen species
EtOAc	ethylacetate	s	singlet
GSH	L-glutathione	SM	starting material
^{1}H-NMR	proton NMR	SPAAC	strain-promoted azide-alkyne cycloaddition
HPLC-MS	high performance liquid chromatography – mass spectrometry	TMB	1,3,5-trimethoxybenzene
HSA	human serum albumin	THF	tetrahydrofuran
IED-DA	inverse electron-demand Diels-Alder reaction	TLC	thin layer chromatography
J	coupling constant	t	triplet
JohnPhos	2-(di-tert-butylphosphino)biphenyl	t_r	retention time
λ	wavelength	UV	ultraviolet
m	multiplet	vol	volume
MeCN	acetonitrile	w/o	without

ABSTRACT

In recent years, the possibility of introducing abiotic reactions inside living systems in a biocompatible way has aroused great interest, thanks to the multiple applications in chemical biology and biomedicine that could lead to the development of new therapies and treatments. Within these reactions, the use of transition metals as catalytic structures can open the door to new reactivities to be carried out within biological systems. Among them, gold, a precious metal long considered highly inert, has begun to gain importance in the field of Bioorthogonal Chemistry.

In this Final Degree Project, it is proposed the development of a gold-promoted double cyclization through a cascade mechanism within biological media. Thus, the study involves the optimization of the transformation towards biological conditions, that is, high dilution, water and air compatibility, as well as the tolerance to the presence of different biomolecules and complex media. We have obtained promising results suggesting the viability of these cascade processes in biological environments and even in live mammalian cells (A549). This strategy constitutes an important new approach to build up high complexity molecules from simple compounds in a straightforward, elegant and bioorthogonal manner in living cells.

RESUMEN

En los últimos años, la posibilidad de introducir reacciones abióticas dentro de sistemas vivos de manera biocompatible ha despertado un gran interés, gracias a las múltiples aplicaciones en química biológica y biomedicina que podrían conducir al desarrollo de nuevas terapias y tratamientos. Dentro de estas reacciones, el uso de metales de transición como estructuras catalíticas permite abrir la puerta a nuevas reactividades que llevar a cabo dentro de sistemas biológicos. Entre ellos, el oro, un metal precioso que durante mucho tiempo ha sido considerado inerte, ha comenzado a ganar relevancia en el campo de la Química Bioortogonal.

En este Trabajo de Fin de Grado se propone el estudio de una reacción de doble ciclación promovida por oro a través de un mecanismo en cascada en el interior de medios biológicos. Así, se ha optimizado la transformación para alcanzar condiciones biológicas; esto es, altas diluciones y compatibilidad con el aire y el agua, así como tolerancia a la presencia de diferentes biomoléculas y medios complejos. Los resultados obtenidos son prometedores, sugiriendo la viabilidad de estos procesos en cascada, no solo en medios biológicos, sino también en el interior de células de mamífero (A549). Esta estrategia constituye un importante nuevo enfoque para la construcción de moléculas de alta complejidad a partir de compuestos sencillos de una manera simple, elegante y bioortoogonal dentro de células vivas.

RESUMO

Nos últimos anos, a posibilidade de introducir reaccións abióticas dentro de sistemas vivos de maneira biocompatible despertou un gran interese, grazas ás múltiples aplicacións en química biolóxica e biomedicina que poderían conducir ao desenvolvemento de novas terapias e tratamentos. Dentro destas reaccións, o uso de metais de transición como estruturas catalíticas permite desenvolver novas reactividades dentro de sistemas biolóxicos. Entre eles, o ouro, un metal precioso que durante moito tempo foi considerado inerte, comezou a gañar relevancia no campo da Química Bioortogonal.

Neste Traballo de Fin de Grao proponse o estudo dunha reacción de dobre ciclación promovida por ouro a través dun mecanismo en cascada no interior de medios biolóxicos. Así, optimizouse a transformación para alcanzar condicións biolóxicas; isto é, altas dilucións e compatibilidade co aire e auga, así como tolerancia á presencia de diferentes biomoléculas e medios complexos. Os resultado obtidos son prometedores, suxerindo a viabilidade destes procesos en cascada, non solo en medios biolóxicos, senón tamén no interior de células de mamífero (A549). Esta estratexia constitúe un importante novo enfoque para á construción de moléculas de alta complexidade a partir de compostos sinxelos dunha maneira simple, elegante e bioortoogonal dentro de células vivas.

1. INTRODUCTION

Nature has historically served as a source of inspiration for science. The complex network of chemical transformations that makes life possible constitutes the basis of Chemical Biology, which applies chemical knowledge to the exploration of living systems, the development of techniques for the synthesis and modification of biomolecules, as well as the design of new chemical processes based on biology.

1.1 Bioorthogonal chemistry

Bioorthogonal chemistry, defined as reactions that do not disrupt the intrinsic biochemical processes that take place inside living systems,[1] was first described by Carolyn R. Bertozzi in 2003. These chemical reactions, characterized by a good biocompatibility and high selectivity, must tolerate physiological conditions of pH and temperature.[2] Nevertheless, developing new bioorthogonal transformations in complex living systems is really challenging, due to the networks of interconnected metabolites, ions and biopolymers responsible for the array of cellular processes that happen within cells, that create an intricate media to conduct non-natural reactions.[3]

The rise of bioorthogonal transformations in recent years has been possible due to their impact in multiple fields. It can be implemented in medicinal chemistry (e.g. biomedical imaging or biotherapeutics), materials science (e.g. polymer synthesis or energy storage materials), basic research (e.g. protein modification and synthesis) and biotechnology.[4,5] This opens the possibility of evolving efficient methods to perform genetic code expansion, drug labeling identification and delivery, metabolic engineering and different types of bioconjugation.[5] It was this significant impact what earned Bertozzi (ft. Meldal and Sharpless) the Nobel Prize in 2022, “for the development of click chemistry and bioorthogonal chemistry”.

However, compared to traditional organic synthesis, where concentration of reactants, solvents and temperature can be modulated, air and moisture can be eliminated and catalyst can be added, if necessary, bioorthogonal reactions face an uncontrollable environment.[4]

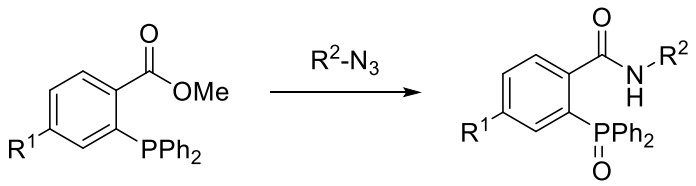
Thus, to successfully carry out a chemical reaction under physiological conditions, some requirements should be followed:

- *Chemoselectivity.* Reactions must be selective for a certain functional group and inert to the vast compendium of chemical functionalities found *in vivo*,[1] avoiding competing side reactions engaging endogenous biological reactants.[4]
- *Biocompatibility*. Living environments imply aqueous media and an elevated control of pH and temperature, to avoid performing toxic reactions that could interfere in the current function of the medium.[5]

- *Reaction rate.* It is important to optimize the kinetics of the reactions with the objective of reducing the concentrations used *in vivo*, considering that a great part of these reactions follow second-order rates, dependent on concentration of the two reactants.[1]

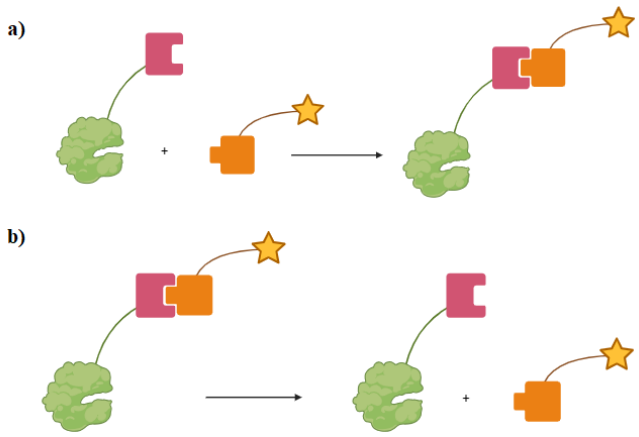
Analyzing these characteristics, such as selectivity and functional tolerance, it is noted that bioorthogonal chemistry is, although conceptually different, quite close to click chemistry in its attributes, being fairly common for a bioorthogonal reaction to meet the requirements of the latter.[4]

Studying its history, the beginnings of bioorthogonal chemistry can be linked to protein bioconjugation in the pursue of a selective method for monitoring biochemical processes within cells.[1] In this research, the first approaches to bioorthogonal reactions were condensations between aldehydes/ketones and hydrazides, hydrazine or aminooxy compounds, where selectivity issues were notable. Thus, the first success in the search for bioorthogonality was achieved with azides, which demonstrated to be inert substrates under biological environments. They debuted in the Staudinger ligation,[6] a transformation of azides into amides by reaction with arylphosphine derivatives (Scheme 1).[4]



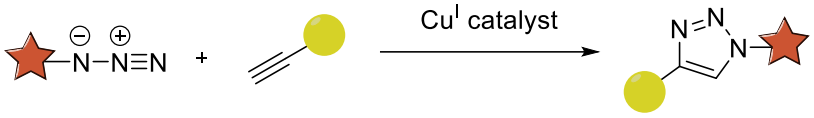
Scheme 1. Staudinger ligation.

From then on, an important set of bioorthogonal strategies where developed, classified in two families of processes: bioorthogonal bond-forming reactions and bioorthogonal bond-cleavage reactions.



Scheme 2. Representative: (a) ***bioorthogonal bond-forming reaction****, (b)* ***bioorthogonal bond-cleavage reaction****.*

Within the first type of transformations, graphically represented in Scheme 2a, it is worth highlighting the well-known copper-catalyzed alkyne azide cycloaddition (CuAAC),[7,8] reported by Sharpless and Meldal (Scheme 3).



Scheme 3. Copper-catalyzed alkyne azide cycloaddition.

Essentially, the reaction consists of a [3+2] addition between terminal alkynes and azides, to regioselectively afford 1,4-disubstituted-1,2,3-triazoles. It shows high selectivity, great kinetics and bioorthogonality, though it must be said that its use, mostly related to the modification of cell surface glycans and proteins,[9] is limited by the toxicity of copper and ascorbate (used to obtain Cu^{I} from a Cu^{II} source), able to generate ROS (reactive oxygen species).[10] However, many alternatives have been described to avoid these issues, both in the modification of azides[11] and the ligands to protect the copper complex.[12,13]

In the following years, more biofriendly alternatives to the CuAAC reaction have been developed, such as the metal free cycloaddition with strained cycloalkynes (SPAAC),[14] or the inverse electron-demand Diels-Alder[15] (IED-DA).[2,4] The expansion of this field has also allowed to develop new procedures, such as palladium-catalyzed cross-couplings and ruthenium-catalyzed cross-metathesis reactions, among other examples.[2]

The second group of bioorthogonal transformations involves bond-breaking reactions (Scheme 2b). Typical synthetic methods to cleave chemical bonds use harsh conditions based on acidic-basic, nucleophilic or redox reagents not always compatible with physiological conditions. An alternative approach takes advantage of the great reactivity of the organometallic chemistry. Towards this idea, many bond breaking bioorthogonal reactions promoted by transition metal catalysts have already been described and incorporated to the armory of bioorthogonal chemistry.[2]

Along the years, the field of biorthogonal organometallic chemistry has acquired a synthetic aim with numerous examples of intracellular bond-forming processes in live cells.

1.2 Organometallic catalysis in biological media

Metabolism is sustained by myriads of enzymes that work to conduct properly each reaction that takes place within living systems. Many of these biocatalysts have metal ions embedded in their active sites, taking part as redox centers or Lewis acids, such as Fe, Mn, Zn or Mg.[16] It is interesting to notice that natural metalloenzymes do not use second or third row transition metal catalysts, being limited their reactivity mainly to redox processes or acid-base transformations. Whereas the use of these metalloenzymes in synthetic chemistry together with the development of artificial ones has received a lot of attention, the exploration of artificial transition metal catalysis inside live cells have been unconsidered for a long time.

Considering the high and broad reactivity of these precious metals (such as typical organometallic mechanisms involving oxidative addition, β-elimination, transmetalations, etc.), it is not surprising that efforts in the last decade have been focused on broadening the range of reactions that might happen inside biological media.[17]

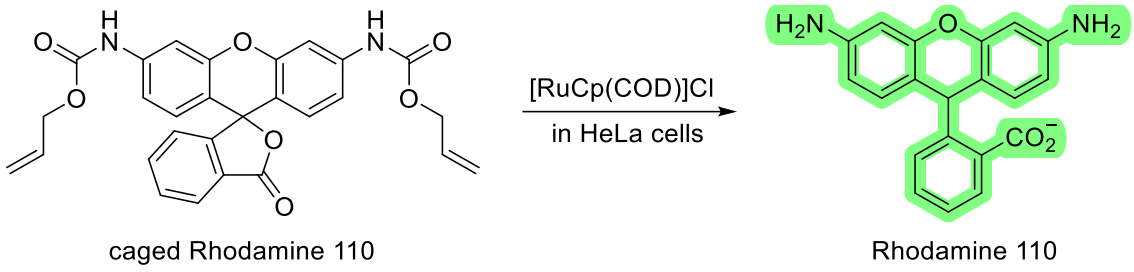
However, as in any bioorthogonal reaction, parameters like cell uptake, transport and toxicity must be considered to value the difficulty of the task. Moreover, numerous biomolecules (such thiols, amines and other nucleophiles, as well as redox species) can kill the catalytic activity, a critical aspect in these processes. Besides, in many cases it is not clear if these reactions within living systems are truly catalytic, since turnover has not been fully demonstrated.[10]

Accordingly, most of these transformations have been restricted to the use of palladium, ruthenium and copper complexes, although chemistry based on other metals like gold has been gaining relevance in recent years.[18]

1.2.1 Ruthenium; roots of bioorthogonal organometallic chemistry

In 1985 was reported one of the first examples of metal-catalyzed reactions in biological settings: the hydrogenation of carbon double bonds in fatty acids using hydrogen gas performed within mesophyll protoplasts with a ruthenium catalyst.[19]

Another important advance in the field came from Meggers group, who developed deallylation reactions propelled by ruthenium(II) complexes.[20] They performed the deprotection of bis-allylcarbamate caged rhodamine 110, liberating the highly fluorescent rhodamine in the interior of HeLa cells (Scheme 4).



Scheme 4. Deallylation reaction promoted by ruthenium(II) catalyst proposed by Meggers.

This work, considered key in the emergence of the field, would be updated a few years later with new Ru(II) catalysts, requiring in both cases the use of the toxic PhSH as an external nucleophile.[21] They could solve this problem introducing Kitamura-type ruthenium(IV) precatalysts with quinoline ligands,[22] using biocompatible GSH as nucleophile.[23]

At the same time, Mascareñas et al. reported that stated Ru promoted uncaging reaction could be used to achieve the activation of DNA-binding compounds inside living mammalian cells.[24] The same group demonstrated the feasibility of performing uncaging reactions in the mitochondria of cells, simply by tuning the ruthenium complexes with targeting moieties, and applied this organelle-selective activity to the uncaging of bioactive compounds.[25]

1.2.2 Palladium

Palladium chemistry has a long history. Its widely demonstrated catalytic power, tuneability and transformative potential make very appealing its use in biological media.[17] Thus, despite this type of reagents are very sensitive to the intricate biological milieu, there are some examples that demonstrate their intracellular performance.[26] For instance, in 2014, a depropargylation and deallylation reaction mediated by homogeneous palladium catalyst was reported by Chen, who proved the efficiency of $Pd(dba)_2$ and $allyl_2Pd_2Cl_2$ on promoting these chemical uncagings for protein activation in living cells.[27] Likewise, the group of Bradley improved the internalization of palladium catalysts by synthesizing Pd-carbene complexes linked to penetrating peptides.[28]

On the other side, the bioorthogonal version of classical cross-coupling reactions such as Suzuki-Miyaura[29] or Sonogashira have been used to modify the cell-surface of *Escherichia coli*,[30,31] or even the surface of mammalian cells.[32]

1.3 Gold reactivity in biological environments

1.3.1 Historical review

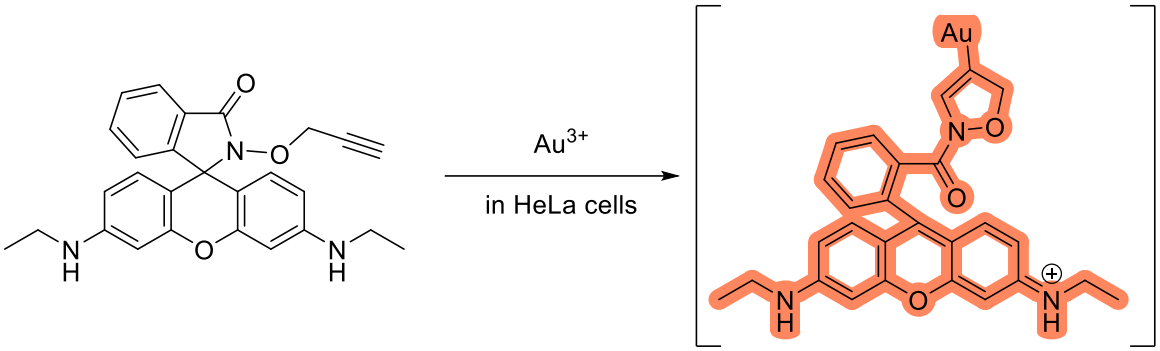
Traditionally, the use of gold as a catalyst has been avoided due to the assumption of its elevated price, as well as its inertness (it possesses the highest normal potential of all metals, with 1.48 V).[33] However, in recent years, gold and gold salts have been widely applied not just in catalysis, but also in the creation of new nanomaterials.

It must be taken into account that gold properties are influenced by its electronic and geometric disposition, as well as bonded ligands and counter anions.

Reactivity of gold complexes is mainly based upon the activation of C-C multiple bonds for a nucleophile attack, thanks to the strong carbophilicity of gold ions.[34] These reactions are isohyptic, since gold does not change its oxidation state in the catalytic cycle,[33] chemoselective and modifiable by tuning the ligands. Thus, gold complexes have mainly participated in C-H activations, alkyne, alkene and nitrile hydrations, enyne cycloisomerizations, polymerizations and hydroaminations.

However, considering its bioorthogonal application, speciation should be considered due to the cytotoxicity of gold cations, which can cause damage to the kidney, liver and peripheral nervous system.[35] For example, Au^{3+} ions can bind strongly to DNA and promote its cleavage.[36]

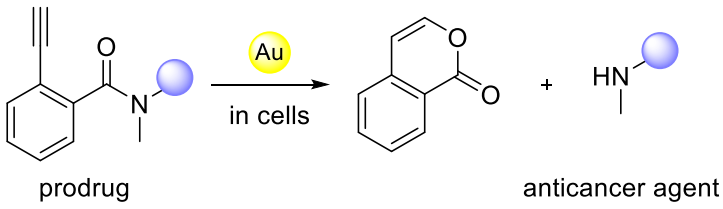
Indeed, the origins of gold chemistry in the biooorthogonal field lie in the development of fluorescence sensors for Au^{3+} ions.[37] First examples appeared in 2009, from Jou and Yang groups, based on the use of fluorogenic probes like rhodamine-alkyne derivatives for the detection of Au^{3+} species, even in live cells (Scheme 5).[36,38]



Scheme 5. Gold-catalyzed cyclization of rhodamine substrate by Yang et al.

One year later, Kim and coworkers published a study where the propargylamide-derived rhodamine was substituted by an apo-coumarin structure. The study was centered in organic solvents, with an isolated example in HaCaT cells.[39] Also to detect the presence of gold salts, the groups of Patra[40] and Ahn[41] presented the cyclization of 2-ethynylbenzoic acid derivatives to form fluorescent isochromene scaffolds inside A549 and HeLa cells, respectively.

Aside from all these biosensing applications, gold catalysis has also been used for the uncaging of drugs within living systems. In this context, the group of Tanaka developed a method to unmask amines based on the activation of alkynes through gold-catalysis (Scheme 6), which could lead to the activation of anticancer pharmacological agents.[42]

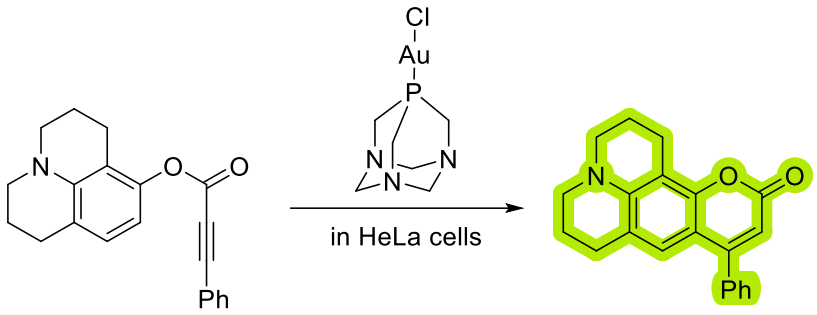


Scheme 6. Tanaka's unmask of amines by gold catalysis.

In a similar carbocyclization approach but with a synthetic perspective, in 2018, Mascareñas' group took up the mantle of the pro-coumarin substrate to perform an Au(I)-conducted (more tolerant to air and moisture) hydroarylation, obtaining a highly fluorescent aminocoumarin product.

In this research, gold(I) chloride complexes were designed under [AuCl(L)] structures, alluding to the ligands' capacity to modulate solubility, cell uptake, reactivity, toxicity and conjugation. Meanwhile, the presence of the chloride ion allows water to behave as a chloride scavenger and solvating agent. This activation triggers the reactivity of the gold, while avoiding the use of external chloride removers like silver(I) salts.

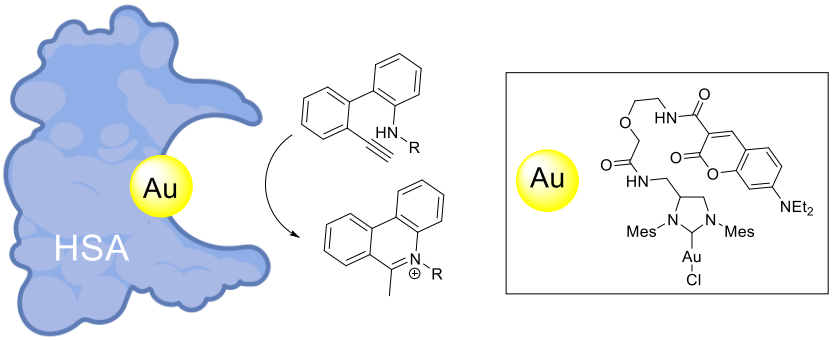
Thereby, this study showed the capacity of gold(I) chlorides such as [Au(PTA)Cl], (PTA = 1,3,5-triaza-7-phospaadamantane), to perform mild intramolecular alkyne hydroarylations in aqueous media, and even in HeLa cells (Scheme 7).[18]



Scheme 7. Hydroarylations inside cancer cells promoted by gold complexes.

The same authors also reported, for the first time, on the concurrent performance of two different artificial metal promoted reactions simultaneously in a selective manner inside living cells, in a way that resembles to the complex networks that occur in metabolism.[18]

Three years after, in 2021, the group of Tanaka presented and intramolecular hydroamination of alkynes using gold(I) carbene catalysts, for the obtention of bioactive 5-methylphenatrinium skeletons, typical chemotherapy prodrugs (Scheme 8). To allow biocompatibility, they developed an artificial metalloenzyme based on the HSA-coumarin interaction, protecting the catalytic activity of the bonded gold with the protein structure. This strategy is introduced on the path of obtaining controlled generation of drugs starting from inactive substrates. In this case, it was probed against cancer cells (A549), with promising results.[43]



Scheme 8. Tanaka's intramolecular hydroamination of alkynes with a gold(I) catalyst, inside A549 cells.

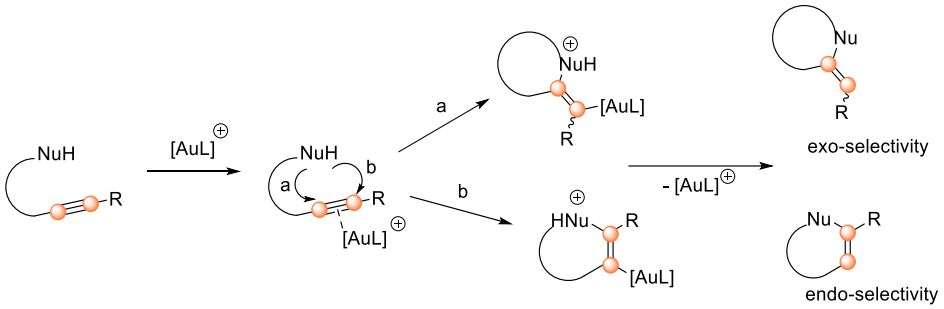
The same group demonstrated the use of gold-promoted procedures inside living mice. Keeping the therapeutic applicability as objective, Tanaka and coworkers investigated the adaptation and deployment of organ-targeting glycoalbumins like biocompatible metal carriers. With this intention, they used the coumarin-BSA interaction to situate gold reagents inside specific organs, forming a glyco-coumarin-Au complex able to promote catalytic amide bond formation between fluorescent propargyl ester probes and amines situated on tissue surface proteins.[44]

All these examples show the great potential of gold chemistry for the development of medical and therapeutic applications, such as intracellular drug activation. Exemplary therapies could be the design of cancer therapeutic metalloenzymes that could be uncaged in their active form, and release at targeted organs.[44] In contrast, the field of gold synthetic bioorthogonal reactions is almost unexplored.

1.3.2 Cycloisomerization of alkynes and cascade reactions

One of the most promising gold-promoted reactions to be used in bioorthogonal chemistry is, as mentioned before, the gold-catalyzed cycloisomerization of alkynes. The intrinsic inertness and metal coordination ability of alkynes make this functionality a perfect group to perform bioorthogonal metal-promoted reactions.[17]

A simplified mechanism for the gold-promoted cycloisomerization can be descripted based on the intramolecular nucleophilic addition to gold-activated alkynes (Scheme 9).[45]



Scheme 9. Mechanism of gold-catalyzed cycloisomerization.

The first step consists of the coordination of the metal to the alkyne, based on its carbophilicity. This activates the triple bond, making it the perfect target to undergo a nucleophilic attack. The cyclization can happen in both alkyne carbons, giving rise to the creation of the product by exo or endo-selectivity, once the deprotonation and the release of the catalyst occurs.

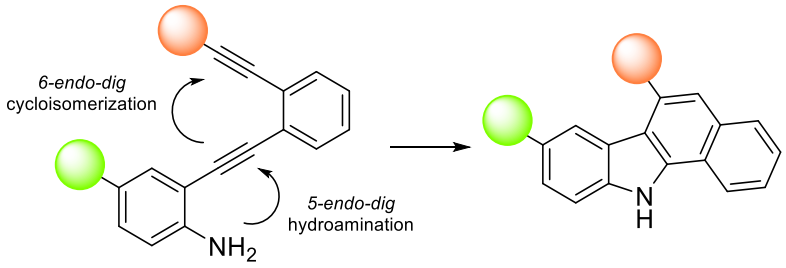
In recent years, this reactivity was extended to cascade reactions, which allow an increase in molecular complexity in a simpler manner, while reducing waste product formation.[46] This type of chemical transformations, described as those that take place through several reactive intermediates in consecutive reactions able to create multiple bonds, show two main benefits: great complexities and efficiency in one step, along with lower environmental impact.[47]

Gold-catalyzed cascade processes, due to the metal alkynophilicity already commented, are a perfect approach for the build-up of complex and cyclic molecules, including natural products such as carbazoles.

1.3.3 Synthesis and relevance of carbazoles

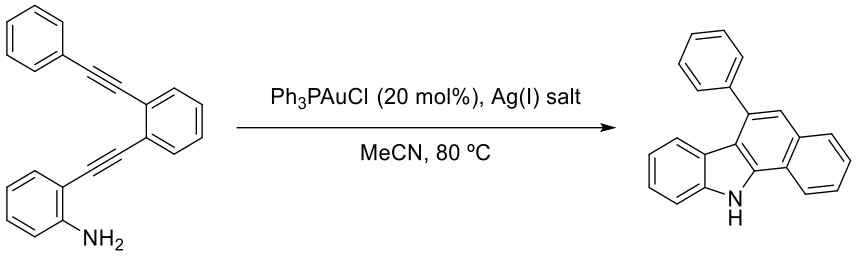
Aryl-annulated carbazoles are examples of compounds with diverse biological activity.[48] Especially, aryl- and heteroaryl-annulated[a]carbazoles display antitumor[49,50] and antimicrobial[51] activity, as well as application on light-emitting diodes,[52] fluorescence reactants,[53] chemoreceptors,[54] donor-acceptor-donor materials[55] and even in organic solar cells (OSCs).[56]

Having highlighted the importance of carbazoles, it becomes evident the interest in developing methodologies for their synthesis. One approach could be a cascade strategy based on a gold-catalyzed *5-endo-dig* hydroamination followed by a *6-endo-dig* cycloisomerization (Scheme 10).[48]



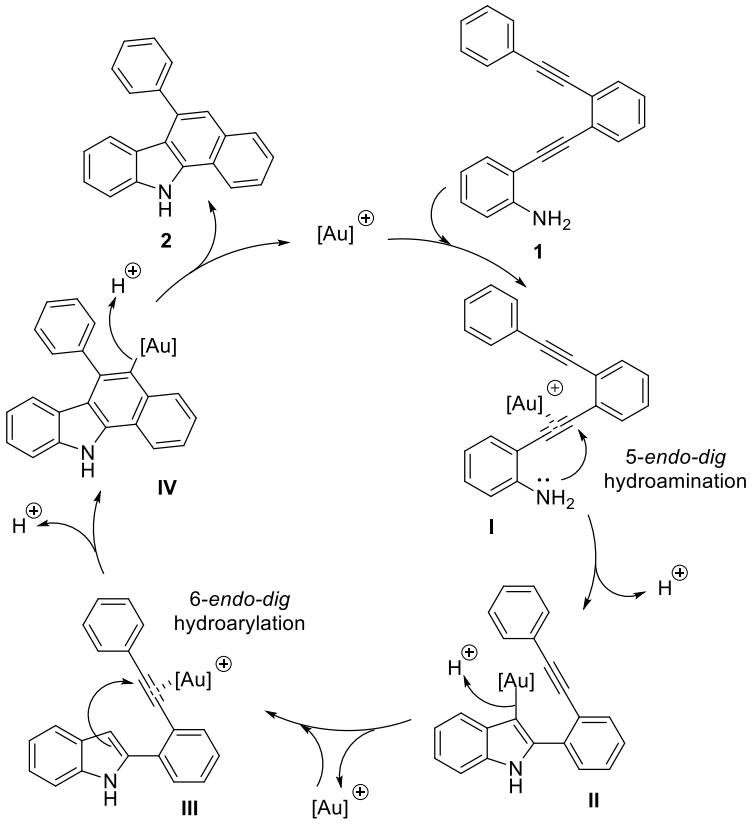
Scheme 10. Strategy followed by cascade reactions to create heteroaryl-annulated[a]carbazoles.

Following this strategy, Hirano et al. reported the reaction indicated in Scheme 11, proposing the mechanism shown in Scheme 12.[57]



*Scheme 11. Synthesis of 6-phenyl-11H-benzo[a]carbazole **2** in an organic solvent, reported by Hirano et al.*

The reaction starts with the activation of the alkyne situated between the two arenes of the substrate (**1**) by the gold(I) complex (**I**), promoting the 5-*endo-dig* hydroamination and yielding the indolylgold intermediate **II**. The first cyclization product, **III,** is generated by the subsequent proto-deauration. Further activation of the second alkyne by the catalyst leads to *6-endo-dig* cyclization at the C-3 position of the indole, producing the rearomatization to give the arylgold intermediate **IV**. The last step involves proto-deauration of **IV**, resulting in the formation of the carbazole **2** and release of the active catalyst.



Scheme 12. Mechanism proposed by Hirano et al. for cascade cyclization with carbazole formation.

It can be observed that this procedure is favored by the *ortho* substitution of the arylalkyne and the nucleophilic group, forcing proximity of both reactants.[47]

The mechanism is supported by checking the reaction of a plausible intermediate (similar to intermediate **III** lacking the gold atom), which when treated under the standard reaction conditions affords the same carbazole.[57]

2. OBJECTIVES AND WORK PLAN

The present work, in the framework of Final Degree Project, aims to broaden the scope of intracellular bioorthogonal reactions utilizing gold catalysts. We proposed the development of new biocompatible gold-promoted cascade reactions in biological or even cellular environments. Specifically, we set out these goals:

(a) Synthesis of bioactive skeletons such as carbazoles, by the intramolecular gold-promoted double cyclization of designed substrates through a cascade process.
(b) Optimize the reaction towards biocompatible conditions.
(c) Confirm the tolerance of the reaction to different biological media and biomolecules.
(d) Translate the reaction into live mammalian cells.

In the consecution of these objectives, the following plan was proposed:

MONTH	TASK
November	Bibliographical research and familiarization with the lab
December	Synthesis and characterization of reactant **1**
January	Synthesis and characterization of product **2**
February	Screening of gold catalysts
March	Optimization of *in vitro* conditions
April	Refinement of the final conditions
May	Biological media screening and biocompatibility study with biomolecules Memory writing
June	Experiments in live mammalian cells Memory writing

3. RESULTS AND DISCUSSION

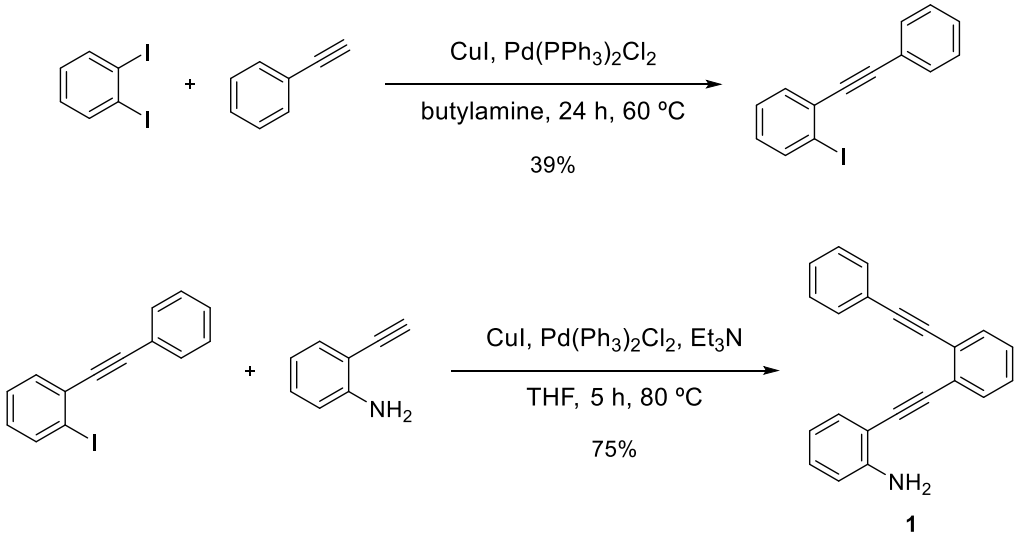
Introducing a new chemical transformation into a living system requires to follow certain steps to ensure the viability of the process, that include the study of the reaction and the optimization of the conditions to guarantee its compatibility to aqueous and complex biological media. Finally, the viability of the reaction in living cells can be explored.

The present work was developed under the direct supervision of last year PhD student Cinzia D'Avino.

3.1 Preliminary assays for a gold-cascade synthesis of carbazoles

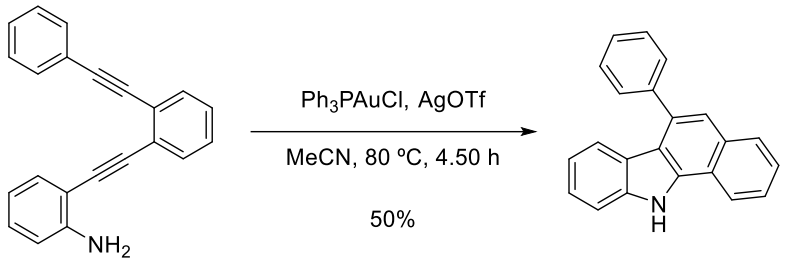
Aiming to study new gold-promoted bioorthogonal cascade reactions, we selected the one described in organic solvents by Hirano *et al* (Scheme 11).[48]

The substrate **1** was prepared through a two-step synthesis, starting from commercial reagents. A Sonogashira coupling between o-diiodobenzene and phenylacetylene using bis(triphenylphospine)palladium(II) and copper(I) iodide, in butylamine, provided 1-iodo-2-(phenylethynyl)benzene in 39% yield. This compound was subjected to a second Sonogashira coupling with 2-ethynylaniline, this time in THF and Et_3N, obtaining 2-((2-phenylethynyl)phenyl)ethynyl)aniline (**1**) in 75% yield. The overall yield of this synthesis was 29%.



*Scheme 13. Synthetic route of 2-((2-phenylethynyl)phenyl)ethynyl)aniline **1.***

Once obtained compound **1**, the gold-promoted cyclization was attempted following Hirano's organic reaction conditions. Thus, this aniline derivative was treated with chloro(triphenylphosphine)gold(I), using AgOTf as chloride scavenger in MeCN and stirred for 4.5 hours. The product **2** was formed in 50% yield, although in a lower efficiency to the reported by Hirano (81%, in only 1.5 hours).

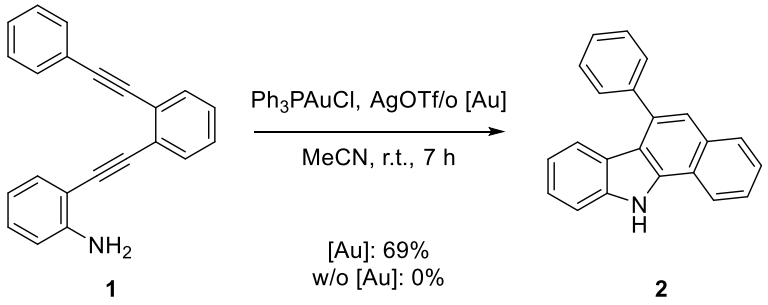


*Scheme 14. Synthesis of 6-phenyl-11H-benzo[a]carbazole **2** under Hirano's conditions.*

To translate organic reactions to biological media it is mandatory to perform the transformations at physiological temperature, that is, 37 ºC. Thus, we explored the reaction at a lower temperature. When the reaction was carried out at room temperature (25 ºC) and time was rise to 7 hours, a 69% yield was obtained, similar to the 70% reported in bibliography.

In this case, the yield of **2** was determined by ^{1}H-NMR using 1,3,5-trimethoxybenzene (TMB) as internal standard.

Importantly, it was also confirmed that the reaction is unviable without the catalyst.

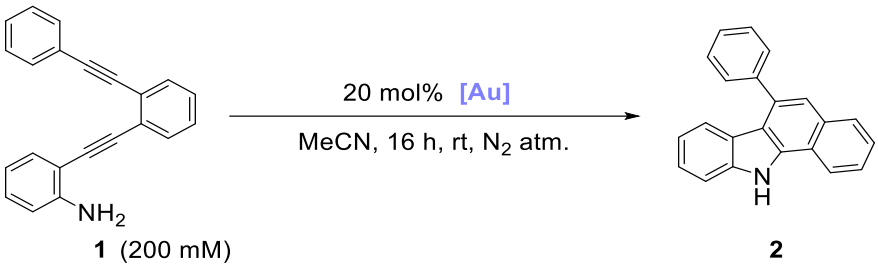


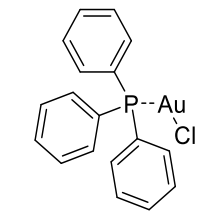
Scheme 15. Cyclization performed at r.t. with and without catalyst. [Au] = Ph_3PAuCl.

3.2 *In vitro* optimization

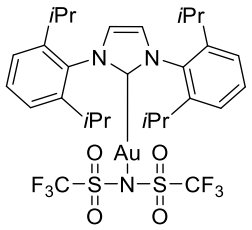
3.2.1 Screening of other reactions conditions

The first study undertaken was the selection of the gold catalyst using different gold complexes.

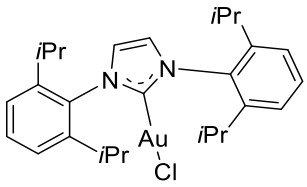




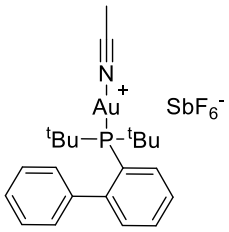
0%



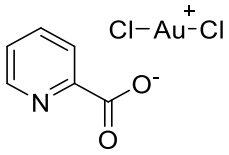
96%



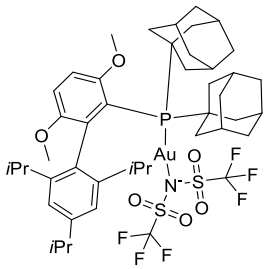
0%



99%



85%



75%

AuCl

23%

$AuCl_3$

55%

$HAuCl_4 \cdot 3H_2O$

66%

w/o

0%

*Scheme 16. Catalyst screening for the synthesis of carbazole **2**.*[a]

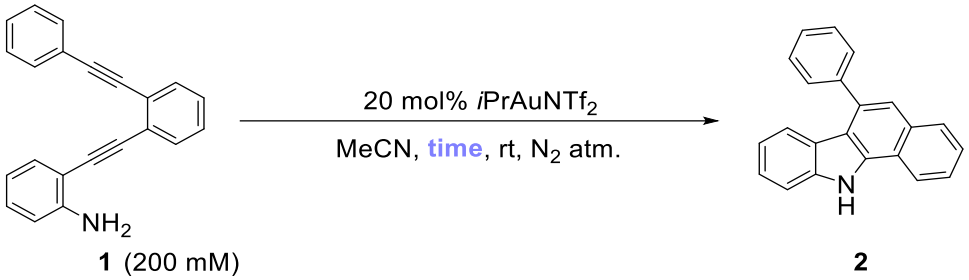
*[a]Conditions: 200 mM of **1** (0.12 mmol), 20 mol% Au catalyst, in MeCN (0.59 mL), overnight, under N_2 atmosphere at room temperature. [b]Yields were determined by ^{1}H-NMR using TMB as internal standard.*

Important information can be extracted from this study. First, the reaction with PPh_3AuCl without silver salt led to the recovery of compound **1**, confirming the need of the chloride scavenger. This was also observed with *i*PrAuNTf$_2$ and *i*PrAuCl (96% and 0%, respectively). Moreover, gold(I) complexes showed a better performance compared to the gold(III) salts.

In this screening, two catalysts, JohnPhos and *i*PrAuNTf$_2$, show excellent yields, above 90%. Since the first complex presents low cell-uptake and high cytotoxicity (as previously found by the group), the studies continued with *i*PrAuNTf$_2$.

Using this complex, it was found that high yields can be obtained only after 10 min of reaction (Table 1, entry 3).

*Table 1. Time screening for the synthesis of carbazole **2**.[a]*



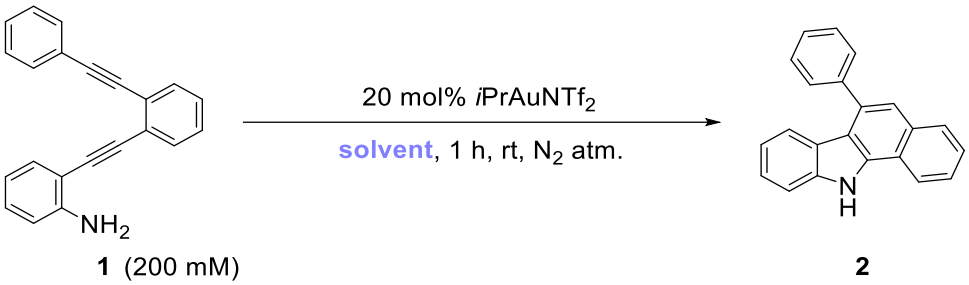
1 (200 mM) **2**

Entry	Time	Yield (%)[b]
1	7 hours	97
2	1 hour	98
3	10 min	99

*[a]Conditions: 200 mM of **1** (0.12 mmol), 20 mol% iPrAuTf2, in MeCN (0.59 mL), under N_2 atmosphere at room temperature. [b]Yields were determined by ^{1}H-NMR using TMB as internal standard.*

In the next set of experiments, the performance of the reaction in different solvents together with the water compatibility was studied. The results, shown in Table 2, demonstrated that an organic media was not necessary, obtaining comparable yields when an aqueous milieu was used (entry 3). In this case, the reactions were stirred for 1 h to ensure the total consumption of substrate under the new reaction media. However, it should be pointed out the insolubility of the substrate and product in H_2O, using the term *on water* to refer to the heterogeneous character of the reaction.

*Table 2. Solvent screening for the synthesis of carbazole **2**.[a]*



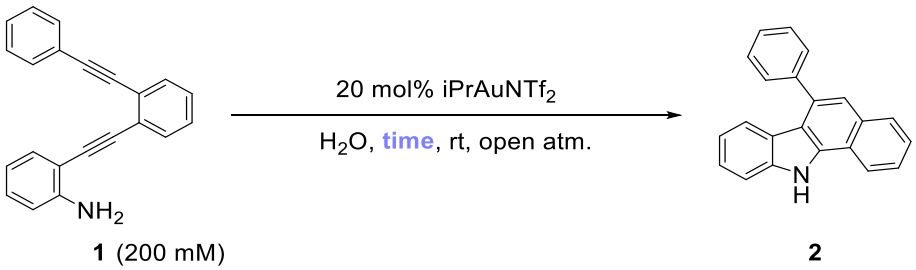
1 (200 mM) **2**

Entry	Solvent	Yield (%)[b]
1	MeCN	98
2	DMSO	100
3	H_2O	93

*[a]Conditions: 200 mM **1** (0.12 mmol), 20 mol% iPrAuTf2, in different solvents (0.59 mL), one hour and under N_2 atmosphere at r.t. [b]Yields were determined by ^{1}H-NMR using TMB as internal standard.*

Once the water compatibility was confirmed, the reaction was tested in an atmosphere open to air, essential to the development of future *in cellulo* experiments. Simultaneously, time was overlooked again, due to the latter changes. The results are summarized in Table 3.

*Table 3. Time and atmosphere screening for the synthesis of carbazole **2**.[a]*



1 (200 mM) **2**

Entry	Time	Yield (%)[b]
1	10 min	96
2	30 min	94
3	1 hour	89
4	24 hours	89

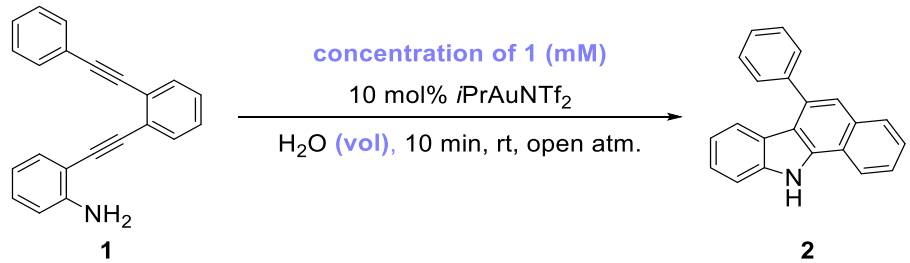
*[a]Conditions: 200 mM **1** (0.12 mmol), 20 mol% iPrAuTf$_2$, in H$_2$O (0.59 mL) under open atmosphere, r.t. [b]Yields were determined by ^{1}H-NMR using TMB as internal standard.*

These results again revealed the fast kinetics of the reaction, as well as the tolerance to oxygen and humidity. Consequently, from now on, all the experiments are performed open to air, unless otherwise noted.

The next question raised is whether the catalyst loading and starting material concentration could be reduced, since *in cellulo* experiments are highly sensitive to elevated amounts of reagents, producing stress, behavior alteration or even cytotoxicity.

Firstly, the reaction performed under optimized conditions, that is, at 200 mM scale of substrate, in water, r.t., 10 min, but decreasing the gold loading to 10 mol%, led to 91% yield (Table 4, entry 1). Subsequently, the influence of the concentration of substrate was studied. As shown in the table, the molarity could be reduced up to 40 times, to 5 mM of starting material, keeping the yields above 80% (entries 3, 5, 7 and 9). The reaction also took place at 1 mM, with an acceptable 65% (entry 11). Importantly, no product was detected in the absence of the gold complex (entries 2, 4, 6, 8 and 10).

*Table 4. Screening of catalyst loading and concentration of **1** for the synthesis of carbazole **2**.[a]*



1 **2**

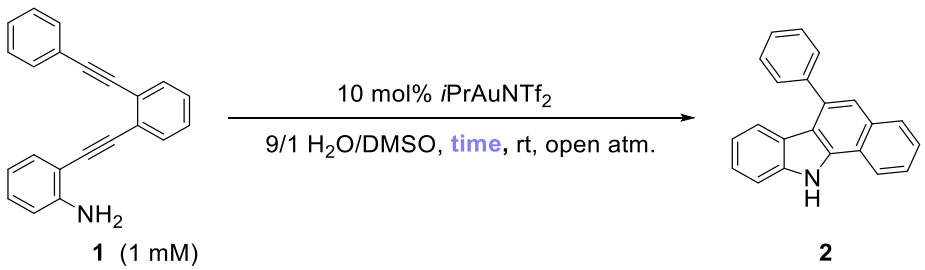
Entry	[1] (mM)	mmol SM	Vol (mL)	Catalyst	Yield (%)[b]
1	200	0.12	0.59	Yes	91
2	200	0.12	0.59	-	0
3	100	0.034	0.34	Yes	88
4	100	0.034	0.34	-	0
5	50	0.034	0.68	Yes	83
6	50	0.034	0.68	-	0
7	10	0.034	3.4	Yes	87
8	10	0.034	3.4	-	0
9	5	0.034	6.8	Yes	87
10	5	0.034	6.8	-	0
11	1	0.034	34.1	Yes	65
12	1	0.034	34.1	-	0

*[a]Conditions: Different concentration of **1** (0.12 mmol at 200 mM and 0.034 mmol in the following), 10 mol% iPrAuTf$_2$, in H_2O (0.59 mL in 200 mM to 34.1 mL in 1 mM) under open atmosphere, r.t. [b]Yields were determined by ^{1}H-NMR using TMB as internal standard.*

During this experimentation, it became evident the necessity of reducing the reaction volume to be able to finally transfer the reaction into living systems. For this, the operational protocol was changed, requiring the preparation of stock solutions from both the substrate and the catalyst. To achieve it considering the lack of solubility of the reactants in water, it was proposed the addition of DMSO. Thus, stock solutions of compound **1** and the gold catalyst in DMSO were prepared, and then added to the water used as reaction media, in a Schlenk tube, in a proportion 9 to 1 (H_2O/DMSO).

Again, a time screening was developed in the aim of a wider understanding of the transformation taking place. As indicated in Table 5, the yields after 10 min were remarkable.

*Table 5. Time screening for the synthesis of carbazole **2** at 1 mM scale.[a]*

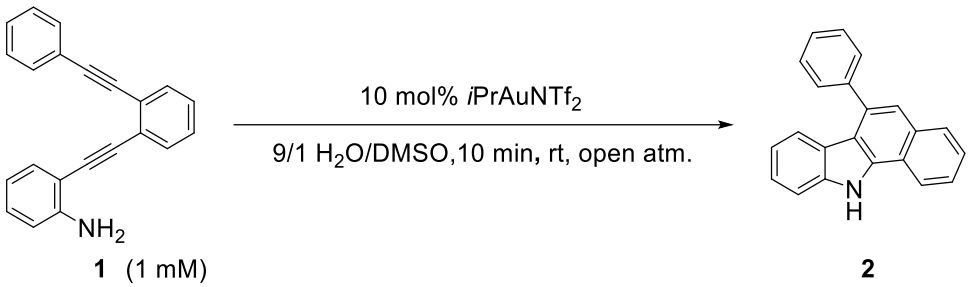


1 (1 mM) **2**

Entry	Time	Yield (%)[b]
1	10 min	74
2	30 min	71
3	1 hour	77

[a]*Conditions: 1 mM of* ***1*** *(0.0058 mmol), 10 mol% iPrAuTf₂, in 9/1 H₂O/DMSO (5.8 mL) under open atmosphere, r.t.*
[b]*Yields were determined by* [1]*H-NMR using TMB as internal standard.*

Bearing in mind all this, it was determined that the final optimized conditions in aqueous media are those indicated in Scheme 17.



Scheme 17. Final optimized conditions for the synthesis of carbazole ***2*** *in aqueous media.*

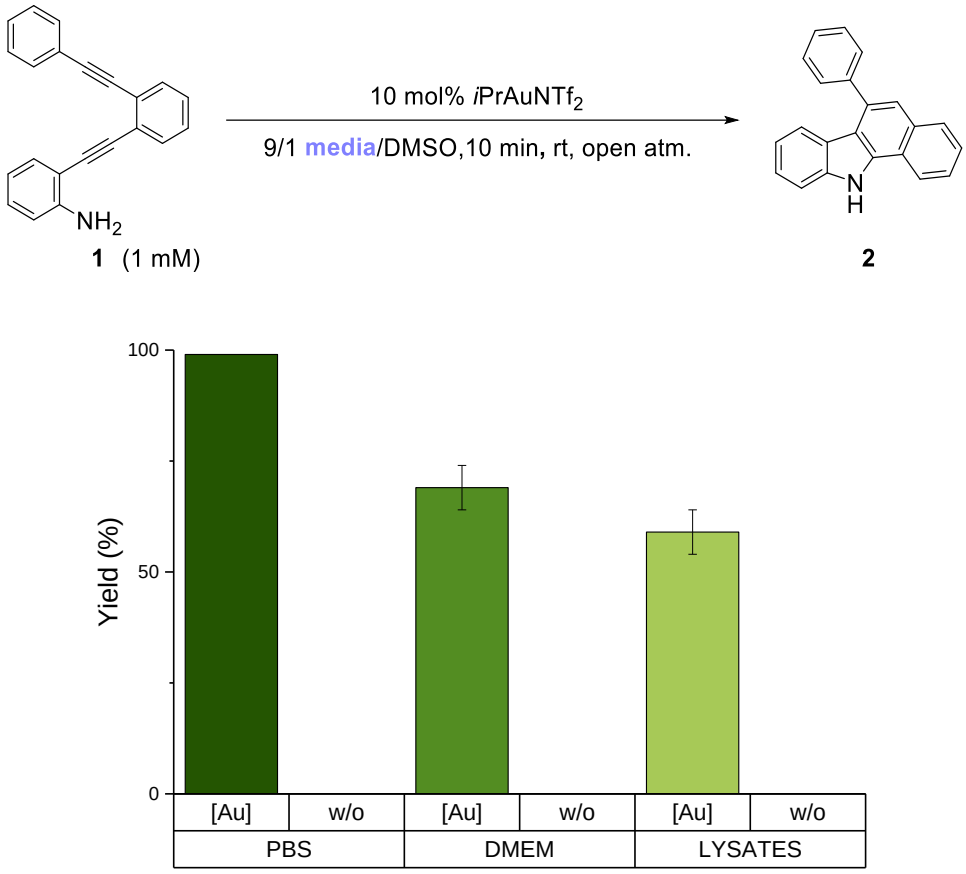
3.2.2 Reaction in biorelevant media

When a bioorthogonal research is being conducted, the analysis of the reaction within biological milieu is an interesting *in vitro* study, since the increasing complexity of the media can enlighten about the viability of the process.

The following mediums were tested:

- PBS (phosphate buffered saline solution, pH 7.4). Solution of inorganic salts (NaCl, KCl, Na_2HPO_4 and KH_2PO_4). It is commonly used for washes, dilutions and to maintain the integrity of tissues and cells in biological and biochemical investigations.
- DMEM (Dulbecco's Modified Eagle Medium). Cell culture medium designed for eukaryotic cells' growth, containing nutrients and different growth factors. In its composition there are diverse amino acids, vitamins, inorganic salts and other components like phenol red, sodium pyruvate and dextrose.
- Cellular lysates. Extraction of cellular content resuspended in PBS. Concentration given in mg protein/mL PBS.

Firstly, the reaction was performed in the presence of PBS, observing the formation of the product in an excellent yield (99%). In DMEM the reaction was also effective, although the yield decreased to 69%. Remarkable, the reaction is also possible in presence of cellular lysates, leading to a 59% yield of the product.

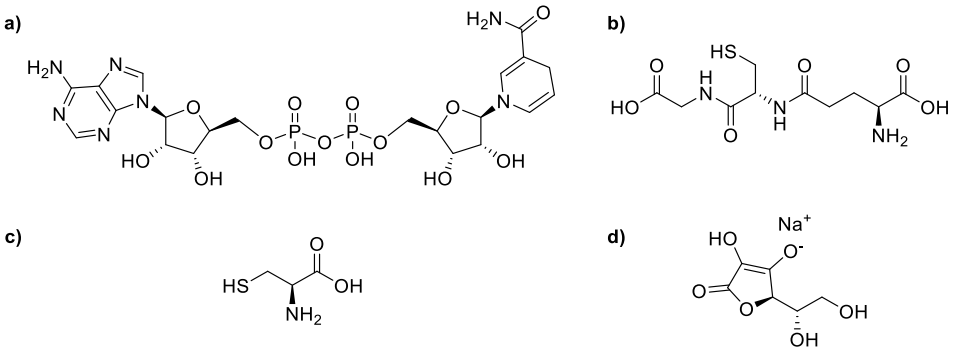


Scheme 18. Bar graphic showing the results of the reaction in different biological media, with and without [Au]. Conditions: 1 mM of ***1*** *(0.0034 mmol), 10 mol% iPrAuTf₂, in 9/1 media/DMSO (3.4 mL) under open atmosphere, r.t. Yields were determined by* ^{1}*H-NMR using TMB as internal standard.* PBS *1x: 136.9 mM of NaCl, 2.68 mM of KCl, 10.14 mM of* Na_2HPO_4 *and 1.76 mM of* KH_2PO_4*. Lysates: 2.05 mg protein/mL of sample.*

These results allow to conclude that the reaction is highly orthogonal, tolerating the presence of different type of salts and molecules. Considering the outcome of the experimentation, the next studies were carried out in PBS, where the effect of the studied biomolecules can be easily spotted.

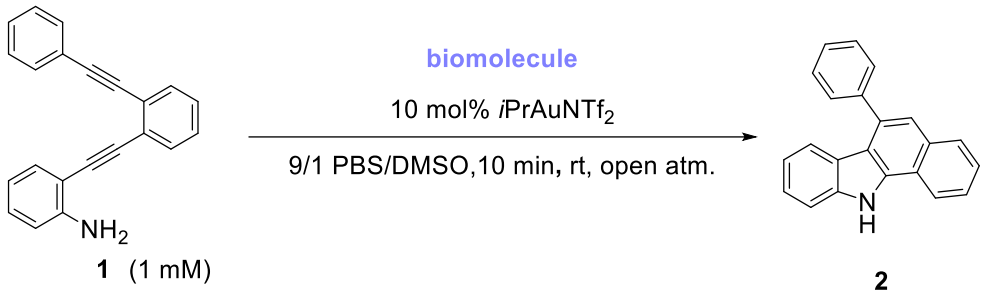
3.2.3 Compatibility with specific biomolecules

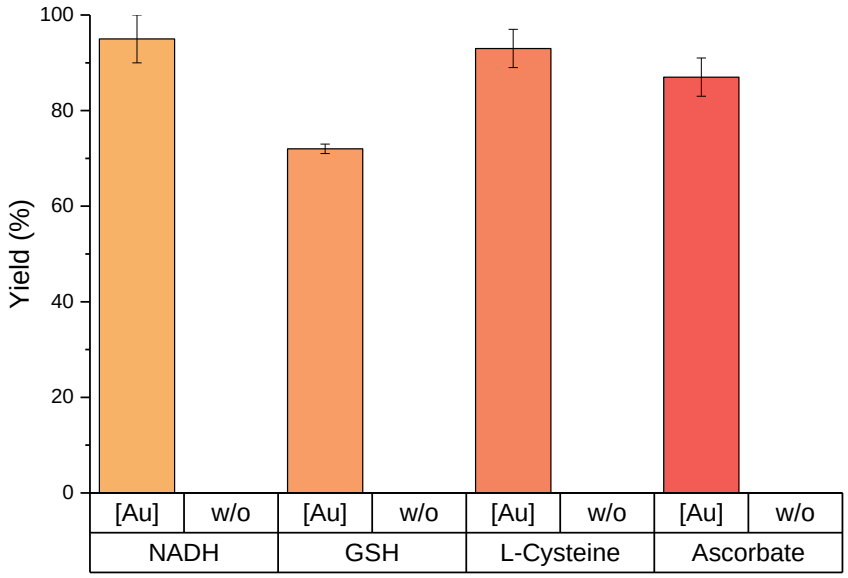
As previously stated, the complex network of biomolecules that exist within cells is an aspect to take into consideration when an abiotic reaction is intended to be introduced in cellular environments. Therefore, the performance of the reaction in the presence of some representative biomolecules was next investigated.



*Scheme 19. Structures of the biomolecules used: (a) **NADH**; (b) **GSH**; (c) **L-cysteine**; (d) **sodium ascorbate**.*

The entities studied were NADH, GSH, L-cysteine and ascorbate (vitamin C), typical reducing species ordered from highest to lowest reducing power inside biological conditions. They were used in a 1 mM concentration (in a ratio 1:1 to substrate **1**). The results are indicated in the next Scheme 20.





Scheme 20. Bar graphic showing the results of the reaction in the presence of different representative biomolecules.

*Conditions: 1 mM of **1** (0.0034 mmol), 1 eq of biomolecule, 10 mol% iPrAuTf$_2$, in 9/1 H$_2$O/DMSO (3.4 mL) under open atmosphere, r.t. Yields were determined by ^{1}H-NMR using TMB as internal standard.*

As can be deduced, the reaction tolerates very well the presence of these biomolecules. Considering the previous 99% yield of the transformation in PBS media (Scheme 18), it is noticeable that the effect of adding NADH, L-cysteine and sodium ascorbate goes almost unnoticed, slightly decreasing the yield to 95%, 93% and 87%, respectively. GSH, for its part, reduces the efficiency to 72%, demonstrating again the high performance of the reaction. This is somehow surprising, known the thiophilicity that gold species present and the ability of thiols to inhibit gold activity. Thus, it is stablished that the transformation, and therefore, both the starting material and the gold catalyst, are tolerant to the existence of typical biological molecules in the reaction media.

Besides, it is noteworthy that no one of the species studied can carry out the process on their own, needing the addition of the gold catalyst to promote the chemical transformation.

3.3 Fluorescence studies

When studying bioorthogonal reactions *in cellulo*, it is crucial to have a method capable of determining whether the transformation occurs within the cell or not. There are two main options: HPLC-MS spectrometry (where yield can be measured) or fluorescence microscopy (qualitative results based on the fluorescence of the product). Moreover, fluorescence microscopy allows a real time monitoring of the process in live cells.

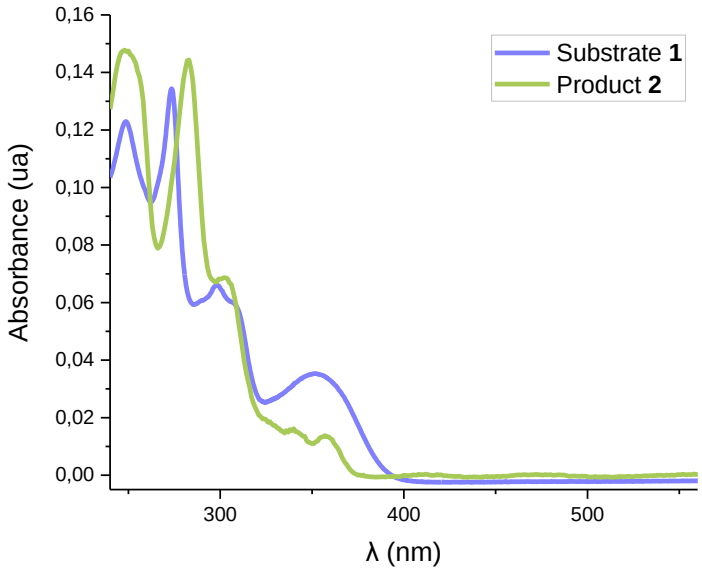
With the intention of developing a qualitative analysis of the carbazole synthesis inside cells, a preliminary fluorescence study was conducted to determinate if the reaction could be followed by fluorescence microscopy.

3.3.1 UV study

Fluorescence is a physical phenomenon involving the absorption of light by the electrons of a substance, causing them to be excited to a higher energy level. The return of the electrons to their original energy state by non-radiative deactivation processes produces the release of protons of lower energy, emitting light.

Due to this, it is essential to examinate the absorption spectrum of the substances involved in the reaction, with the aim to use the wavelength of maximum absorption to excite the sample. Thus, both the starting material and the product were subjected to UV spectroscopy, from 240 to 800 nm. Both substances ceased to show absorbance beyond 400 nm. Therefore, the results are amplified in Scheme 21.

It can be observed three main peaks of maximum absorption of the product (251, 283 and 358 nm) and other four related to the substrate (249, 274, 298 and 354 nm).

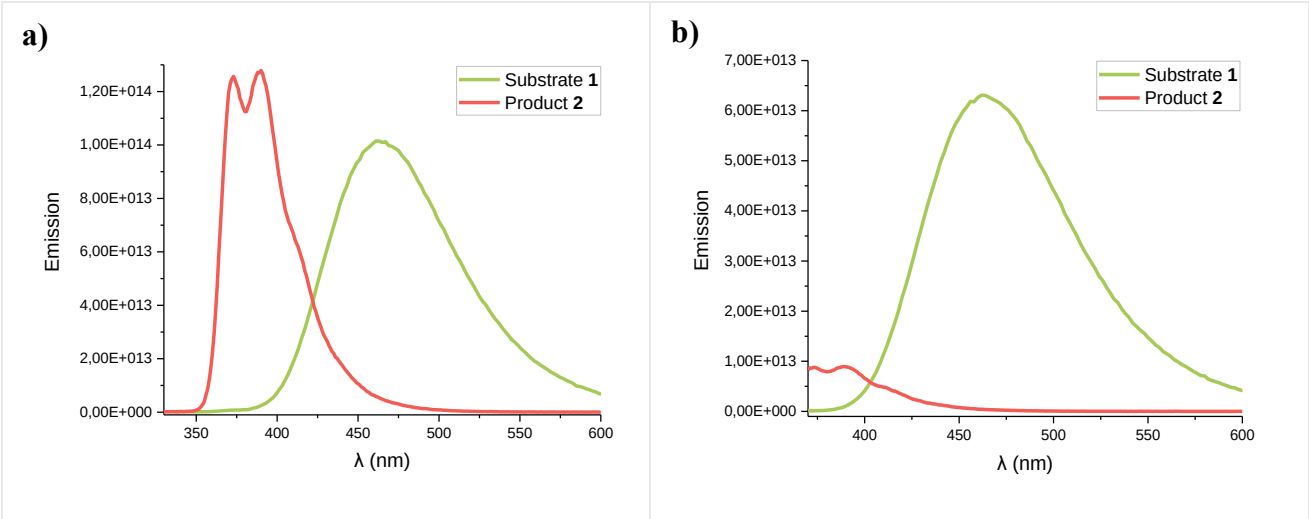


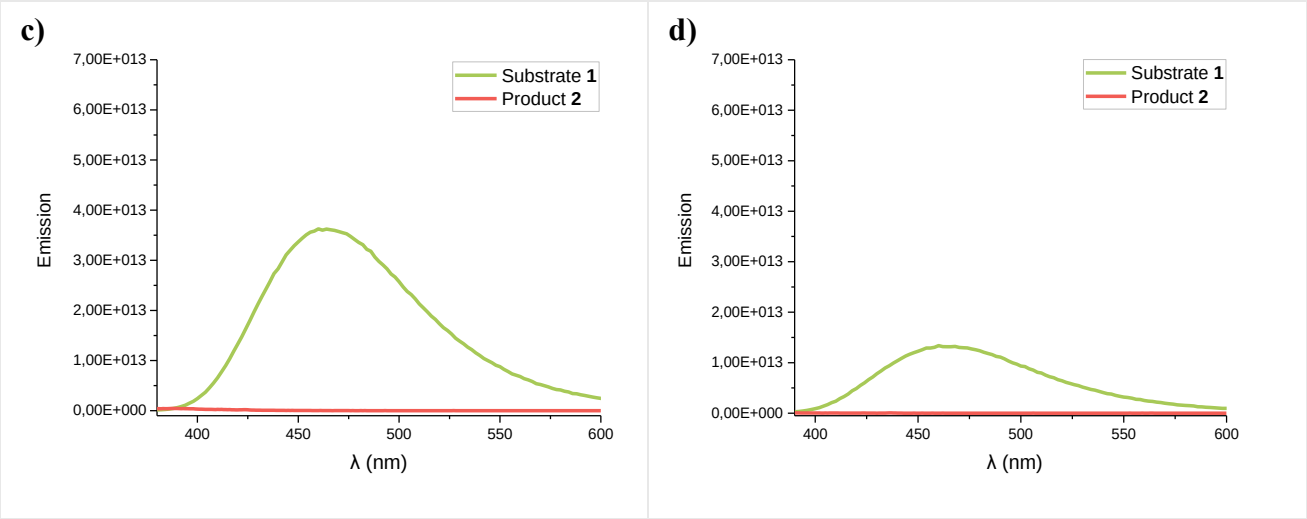
*Scheme 21. Absorption spectra of substrate **1** and product **2** in the visible region.*

3.3.2 Fluorescence study

Once determined the wavelength of maximum absorption, the next step was to study the fluorescent emission spectra, using the state wavelength to excite the sample. It was chosen 283 nm to illustrate the emission differences between both substances, since it is the wavelength that shows more disparity between substrate and product (Scheme 22a). However, as the final aim of this study is to determinate whether fluorescence microscopy can be used to follow the reaction, the wavelengths analyzed are those within the range available at the microscope. These are: 365, 375, 385, 405, 488 and 561 nm.

Exciting the samples at this wavelengths, six spectra were obtained. Considering the results, it has been decided to show only those wavelengths where there are relevant emissions.





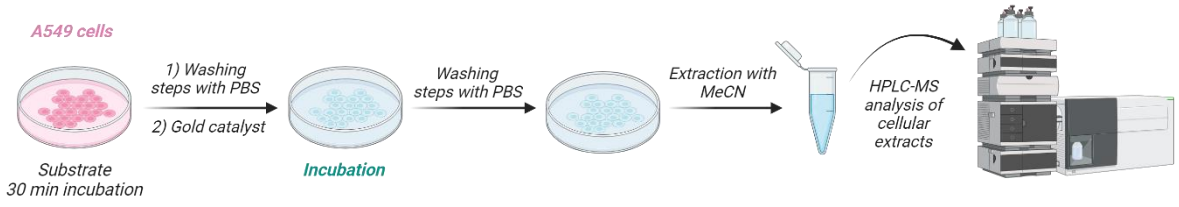
*Scheme 22. Emission spectra of the substrate **1** and product **2** (10 μM) at different excitation wavelengths: (a) 283 nm; (b) 365 nm; (c) 375 nm; (d) 385 nm.*

The latest results suggest that fluorescence is not the best option to monitor the process. Therefore, HPLC-MS was used.

3.4 *In cellulo* experiments

In order to evaluate the viability of the reaction within living environments, A549 cells, a typical lung cancer cell line, were selected for the assays. We used different conditions of concentrations (of both substrate **1** and catalyst), times and several gold complexes/salts that demonstrated catalytic activity in the *in vitro* experiments.

All the *in cellulo* experimentation was performed following the next procedure (Scheme 23). Cells were incubated at 37 ºC for 30 min with the substrate **1** and then washed with PBS. Once the excess of starting material was retired, the gold stock solution was added to the plate, and then incubated for a certain time. After the reaction time, the cellular content was extracted with MeCN and analyzed by HPLC-MS to determinate the product formation.

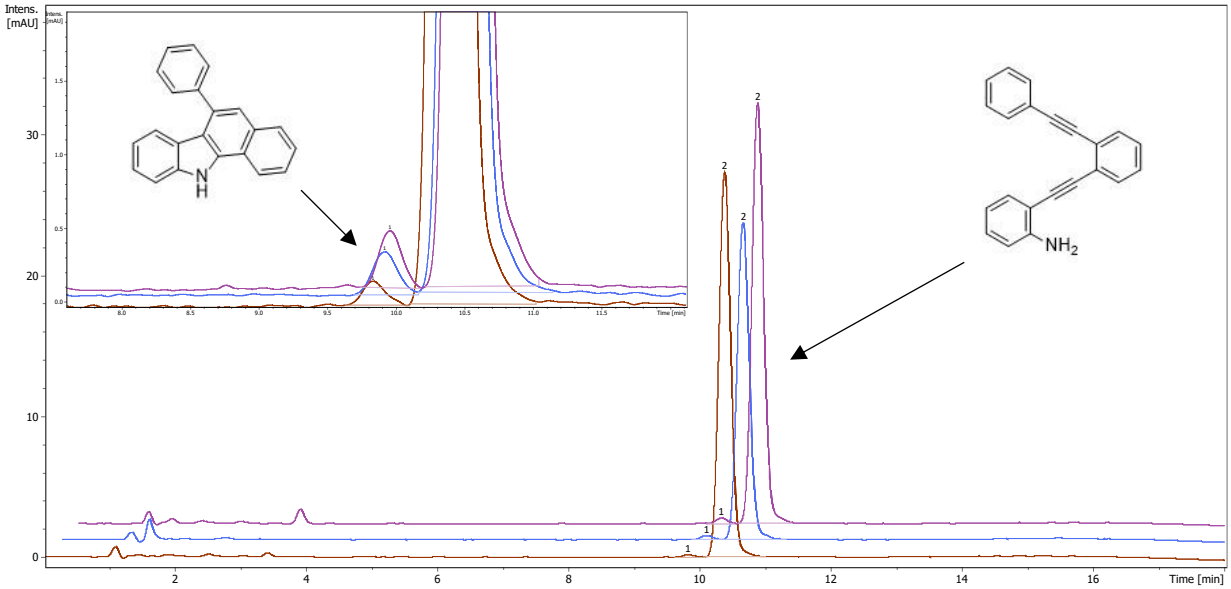


Scheme 23. General protocol for in cellulo experimentation.

After treatment of cells with 50 μM of *i*PrAuNTf$_2$ for 6 h, the morphology of cells changed, what suggested that at these concentrations this gold complex is toxic. However, product **2** could be detected inside cells after 3 h of treatment by HPLC-MS. Besides, a preliminary test with two gold salts, $AuCl_3$ and $NaAuCl_4 \cdot 2H_2O$, was also carried out, but they did not give the product.

With these outcomes in mind and considering the slight cytotoxicity of *i*PrAuNTf$_2$, the next optimization was centered in reducing the toxicity of the treatment. Thus, either shorter incubation times (1 hour, with the exact same concentrations) or lower catalyst loading (3 hours, with 25 μM of *i*PrAuNTf$_2$) were tested. Successfully, in both experiments, the product could be detected by HPLC-MS in the MeCN extraction. Besides, the assays corroborated a better cell-uptake and higher cell survival.

Finally, it was carried out a time screening study, using a concentration of 50 μM of **1** and 25 μM of *i*PrAuNTf$_2$. The results obtained by HPLC-MS, shown in Scheme 24, demonstrate the catalytic activity inside the cells. Besides, as it can be seen in Table 6, the area of the product peak (directly related with the concentration of the substance inside the MeCN extractions, and therefore, inside the cells) shows the correlation between the efficiency of the transformation and the time of incubation, achieving higher amounts of product with longer times.



*Scheme 24. HPLC analysis of the MeCN cell extractions for the reaction in 1 hour (red), 2 hours (blue) and 3 hours (purple). Legend: (**1**) product, (**2**) substrate.*

Table 6. Time screening inside living cells. Relation between incubation time and area of the product peak

Entry	Time (h)	Product area
1	1	1.9090
2	2	3.7985
3	3	4.5588

However, further optimization of the conditions should be considered in the future, being the current results a perfect starting point for the refinement of the reaction within cells. In addition, for future experiments, a calibration line must be plotted to be able to quantify the reaction.

3.5 Experimental procedures

3.5.1 General information for in vitro experiments

The different reactants used were purchased from Sigma Aldrich, Alfa Aesar, Strem, Merck, BLD Pharmatech, Fisher, Apollo Scientific and Panreac. All of them were used without further purification.

Solvents are of reagent grade, and dry solvents were acquired from Sigma Aldrich, employed without further purification. When water was used, it was obtained from an integrated Millipore Milli-Q water purification system. The phosphate-buffered saline solution (PBS) was diluted 10 times with water prior to use from the solution 10x previously prepared following standard procedures. The DMEM used was obtained from ThermoFisher Scientific.

Chemical procedures that required inert conditions were performed in a Schlenk line with standard techniques. Solvent additions and liquid operations were carried out with plastic syringes and needles from Braun. Dryings were done with sodium sulfate anhydrous. TLC were achieved in 2.5x5 cm aluminum plates with silica gel Merck 60 F_{254}, using UV light (λ = 254 and 365 nm) for their visualization. The silica gel used in column chromatography is from ThermoFisher Scientific Silicagel, 60A.

Vacuum concentration required the used of *Büchi R-210* rotary evaporator equipped with a V-850 vacuum regulator, V-700 vacuum pump and B-491 thermostatic bath. Subsequently, samples were submitted to the vacuum line.

NMR spectra were recorded on a *VARIAN Mercury-300* and an *Agilent VNMRS-300 spectrometers* (300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR and DEPT-135) and were analyzed with MestreNova® processing program. Chemical shifts (δ) were expressed in part per million (ppm) downfield from tetramethylsilane, calibrated in base of the residual solvent peak (d-chloroform δ = 7.260 ppm (^{1}H); $CDCl_3$ δ = 77.2 (^{13}C)). Multiplicity was expressed as follows: singlet (s), doublet (d), triplet (t), multiplet or unsolved signal (m), and combinations of these. Coupling constants (J) were expressed in Hertz (Hz). Multiplicities in ^{13}C were determined by DEPT-135.

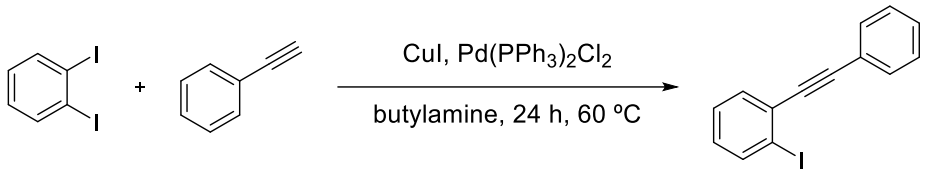
UV measurements were performed with *Jasco V-770 spectrophotometer.* The fluorescence study was made with an *Edinburgh FS5 spectrofluorometer* thermostated cell compartment at 20 ± 0.5 °C, using 1 cm quartz cells. The settings were the following: increment 0.5 nm, averaging time 0.1 s, excitation slit width 1.5 nm, emission slit width 1.5 nm.

HPLC analysis was performed on a *THERMO Ultimate 3000* coupled to a *Bruker AmaZon SL ion trap LC-MS* with a flow rate of 0.35 mL/min at room temperature. The initial conditions for the solvent system were isocratic with H_2O/MeCN (45:55), maintained 10 minutes.

They were followed by a gradual change of 5 min to H_2O/MeCN (5:95). The latest conditions were kept 1 min. Then, settings were returned to the initial ones (45:55) for 2 min. The chromatogram was recorded using a UV detection at λ = 300 nm.

3.5.2 Synthesis of 1-iodo-2-(phenylethynyl)benzene

Procedure adapted from Hirano et al.[48]



Bis(triphenylphosphine)palladium(II) dichloride (182.5 mg, 0.26 mmol, 2.5 mol%) and copper(I) iodide (49.3 mg, 0.26 mmol) were dissolved in butylamine (20 mL) in a purged round flask under nitrogen atmosphere. Phenylacetylene (1.2 mL, 11.2 mmol, 1.06 eq) and 1,2-diiodobenzene (1.4 mL, 10.6 mmol, 1 eq) were added to the stirred solution, again under N_2 flow. The mixture was stirred for 24 hours at 60 ºC under argon, showing the TLC (silica gel, n-Hexane/EtOAc 20:1) complete disappearance of the starting material.

The mixture was cooled to room temperature, quenched by addition of saturated aqueous NH_4Cl and washed three times with brine. The organic layer was dried over Na_2SO_4, filtrated and concentrated under vacuum. The residue was purified by column chromatography on silica gel with n-hexane/ethyl acetate 20:1 to afford 1-iodo-2-(phenylethynyl)benzene (1.15 mg, 36%) as a pale yellow oil.

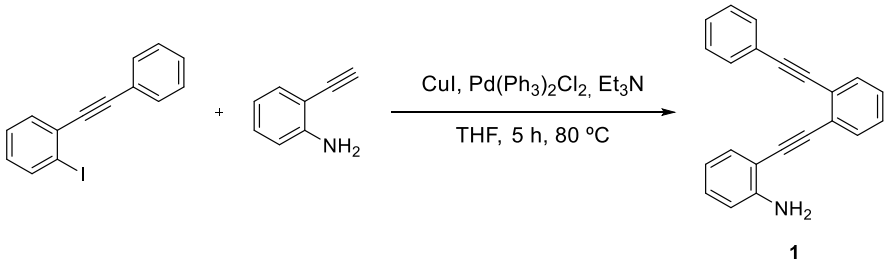
^{1}H-NMR (300 MHz, d-chloroform): δ 7.88 (d, ^3J = 8.0 Hz, 1H), 7.63-7.58 (m, 2H), 7.53 (d, ^3J = 7.7 Hz, 1H), 7.40-7.29 (m, 4H), 7.01 (t, ^3J = 7.7 Hz, 1H); **^{13}C-NMR** (75 MHz, d-chloroform): δ 138.9 (CH_{Ar}), 132.6 (CH_{Ar}), 131.8 (2xCH_{Ar}), 130.0 (C_{Ar}), 129.5 (CH_{Ar}), 128.8 (CH_{Ar}), 128.5 (2xCH_{Ar}), 128.0 (CH_{Ar}), 123.1 (C_{Ar}), 101.3 (C_{Ar}), 93.2 (C_{alk}), 91.8 (C_{alk}).

Rf: 0.47 (hexane/ethyl acetate 20:1).

Data in accordance with the literature.[58]

*3.5.3 Synthesis of 2-{[2-(phenylethynyl)phenyl]ethynyl}aniline **1***

Procedure extracted from Hirano et al.[48]



1

A mixture of 1-iodo-2-(phenylethynyl)benzene (1.15 mg, 3.77 mmol, 1.0 eq), copper(I) iodide (35.7 mg, 0.050 eq) and bis(triphenylphosphine)palladium(II) dichloride (131.6 mg, 5 mol%) were purged in a round flask and subjected to nitrogen atmosphere. Once they were dissolved with Et_3N (10.2 mL), a solution of ethynylaniline (470.0 mg, 4.012 mmol) in THF (3.9 mL) was added to the mixture, that was stirred at 80 ºC for 5.0 h under argon with reflux.

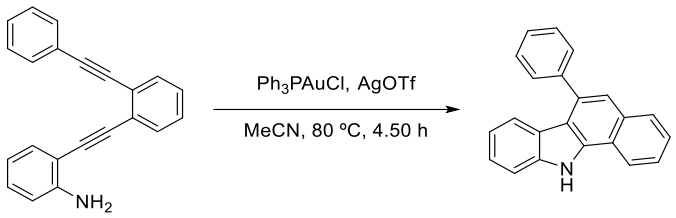
The mixture was cooled to room temperature, quenched by addition of saturated aqueous NH_4Cl and extracted three times with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, filtrated and concentrated under vacuum. The product 2-((2-(phenylethynyl)phenyl)ethynyl)aniline (**1**) was purified by column chromatography on silica gel with hexane/EtOAc 4:1 as a pale yellow solid (843.5 mg, 76%).

^{1}H-NMR (300 MHz, d-chloroform)**:** δ 7.63-7.53 (m, 4H), 7.42-7.30 (m, 6H), 7.14 (t, ^3J = 7.7 Hz, 1H), 6.70 (t, ^3J =8.9 Hz, 2H), 4.43 (s, 2H); **^{13}C-NMR** (75 MHz, d-chloroform): δ 148.4 (C_{Ar}), 132.3 (CH_{Ar}), 132.1 (CH_{Ar}), 132.0 (2xCH_{Ar}), 131.6 (CH_{Ar}), 130.1 (CH_{Ar}), 128.7 (CH_{Ar}), 128.5 (2xCH_{Ar}), 128.3 (CH_{Ar}), 127.9 (CH_{Ar}), 126.0 (C_{Ar}), 125.2 (C_{Ar}), 123.2 (C_{Ar}), 117.8 (CH_{Ar}), 114.2 (CH_{Ar}), 107.8 (C_{Ar}), 93.9 (C_{alk}), 93.2 (C_{alk}), 90.6 (C_{alk}), 89.0 (C_{alk}).

R_f: 0.36 (hexane/AcOEt 8:2); 0.37 (hexane/toluene 2:1). **$C_{22}H_{15}N$ [M^+]:** 294.23. **t_r**: 10.5 min. Data in accordance with the literature.[48]

*3.5.4 Synthesis of 6-phenyl-11H-benzo[a]carbazole **2***

Procedure adapted from Hirano et al.[48]



1 **2**

A mixture of chloro(triphenylphosphine)gold(I) (2.9 mg, 0.0059 mmol, 5 mol%) and silver trifluoromethanesulfonate (1.5 mg, 0.0059 mmol, 0.05 eq) were dissolved in acetonitrile (0.59 mL) in a purged Schlenk tube under argon atmosphere. Once 2-((2-(phenylethynyl)ethynyl)aniline (35.2 mg, 0.12 mmol, 1.0 eq) was added, the mixture was stirred at 80 ºC until TLC (silica gel, n-hexane/EtAOc 9:3) showed complete conversion of the starting material (4.5 h).

After cooling the Schlenk to room temperature, the mixture was diluted with ethyl acetate, washed three times with saturated aqueous NH_4Cl and brine, dried over Na_2SO_4, filtered and concentrated under vacuum.

The product **2** was purified by column chromatography (silica gel) with n-hexane/toluene (2:1), obtaining 6-phenyl-11H-benzo[a]carbazole as pale orange solid (17.6 mg, 50%*).

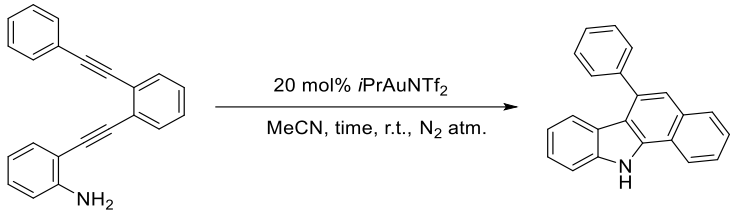
^{1}-H-NMR (300 MHz, d-chloroform): δ 8.91 (s, 1H), 8.17 (d, ^{3}J= 7.8 Hz, 1H), 8.01 (d, ^{3}J= 7.9 Hz), 1H), 7.70 (d, ^{3}J = 7.4 Hz, 2H), 7.64-7.51 (m, 7H), 7.45 (d, ^{3}J = 8.1 Hz, 1H), 7.37 (t, ^{3}J = 7.6 Hz, 1H), 7.05 (t, ^{3}J = 7.6 Hz, 1H). **^{13}C-NMR** (75 MHz, d-chloroform): δ 141.4 (C_{Ar}), 138.9 (C_{Ar}), 136.8 (C_{Ar}), 135.4 (C_{Ar}), 132.3 (C_{Ar}), 129.5 (2xCH_{Ar}), 129.1 (CH_{Ar}), 128.5 (2xCH_{Ar}), 127.7 (CH_{Ar}), 125.8 (CH_{Ar}), 125.6 (CH_{Ar}), 124.8 (CH_{Ar}), 124.1 (C_{Ar}), 122.3 (CH_{Ar}), 121.1 (CH_{Ar}), 120.5 (CH_{Ar}), 120.4 (C_{Ar}), 119.8 (CH_{Ar}), 117.0 (C_{Ar}), 111.0 (CH_{Ar}).

Rf: 0.42 (hexane/ethyl acetate 9:3); 0.29 (hexane/toluene 2:1). **$C_{22}H_{15}N$ [M^{+}]:** 294.24. **t_r**: 9.9 min.

Data in accordance with the literature.[59]

* Yield slightly overrated due to remaining toluene solvent. The further purification for the isolation of the product was performed via HPLC-preparative (60-95% of MeCN in water for 32 min).

3.5.5 Representative procedure for reactions at 200 mM scale using iPrAuNTf$_2$ as complex and MeCN as solvent (section 3.2.1)

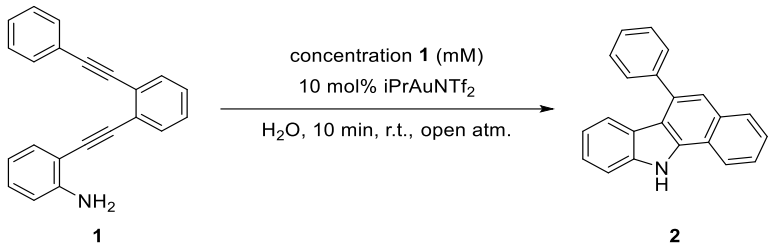


1 (200 mM) **2**

Aniline **1** (35.2 mg, 0.12 mmol, 1.0 eq) and *i*PrAuNTf$_2$ (20.8 mg, 0.024 mmol, 20 mol%) were added to a Schlenk tube and subjected to a purge by three vacuum-nitrogen cycles.* Then, MeCN (0.59 mL) was added to the mixture, which was magnetically stirred at room temperature overnight. Over time, the solution turned yellow. After approximately 16 hours, the mixture was diluted with ethyl acetate, washed with saturated aqueous NH_4Cl and brine, dried over Na_2SO_4, filtered and concentrated under vacuum. A yield of 96% was determined by ^{1}H-NMR with TMB as internal standard. The starting material conversation was complete.

** When the reactions are performed open to air, both compound **1** and catalyst are added to a Schlenk tube in open atmosphere.*

*3.5.6 Representative procedure for the study of the dilution (section 3.2.1)**



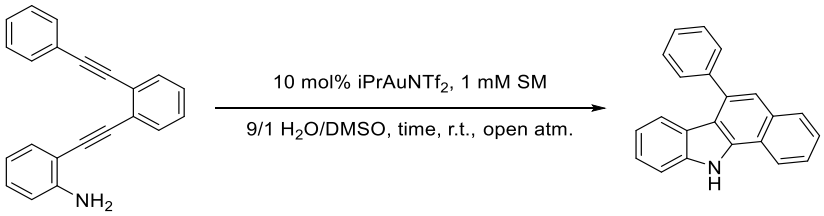
1 **2**

Aniline **1** (10.0 mg, 0.034 mmol, 1.0 eq) and *i*PrAuNTf2 (3.0 mg, 0.0035 mmol, 10 mol%) were stirred in a round flask with water (0.34 mL) at room temperature under open atmosphere for 10 min.

The mixture, a cloudy suspension that turns yellow over time, was diluted with ethyl acetate, washed with saturated aqueous NH_4Cl and brine, dried over Na_2SO_4, filtered and concentrated under vacuum. A yield of 88% was determined by ^{1}H-NMR with TMB as internal standard. The starting material conversation was complete.

** Alterations: concentration of **1** was gradually reduced and, therefore, volume of water was increased.*

3.5.7 Representative procedure for reactions at 1 mM scale using stock solutions in DMSO (section 3.2.1)*



1 **2**

A stock solution of *i*PrAuNTf$_2$ (2.0 mM) in DMSO and another one of SM **1** (20 mM), again in DMSO, were previously prepared to add to water (5.2 mL) in a Schlenk tube. Both solutions were pipetted with a volume of 290 µL, first the substrate followed by the catalyst. Due to the insolubility of the starting material, a suspension was formed. It was stirred at room temperature for 10 minutes.

The mixture was extracted with ethyl acetate three times, dried over Na_2SO_4 and then filtered with Florisil® (60-100 mesh) to eliminate the gold catalyst. Then, the solution was concentrated under vacuum. A yield of 74% was determined by ^{1}H-NMR with TMB as internal standard. The starting material conversation was complete.

** Times were screened.*

*For the study of the biocompatibility, a third solution of 1.0 eq of a biomolecule was prepared in PBS (0.1 mL) and added before compound **1** and catalyst to the reaction media (3.0 mL).*

3.5.8 Internal standard method for yield quantification

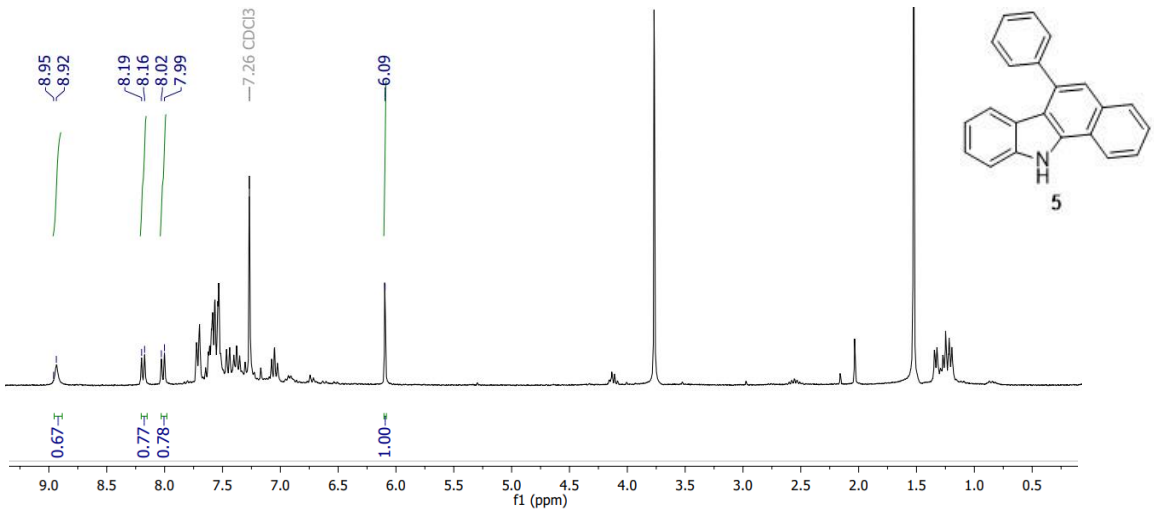
Nuclear magnetic resonance (NMR) spectroscopy allows elucidation of small and macro molecules, as well as quantification of signals thanks to the proportionality between the resonance line and the number of resonant nuclei.[60]

This enables the utilization of an internal standard to analyze reaction crudes, allowing the easy obtention of the transformation yield by comparing a well-known peak of the internal standard with one of the product whose integration have been previously identified. This way, an ideal internal standard would be one highly purified, stable, chemically inert and soluble in the NMR solvent used.[61]

In this research it was used 1,3,5-trimethoxybenzene (TMB), where its aromatic peak (δ= 6.09 ppm) can be used to determine the yield of the reaction. The comparation was made based on the mean of three signals of the product: two aromatic H from the carbazole moiety (δ = 8.17 ppm, d, 1H; δ = 8.01 ppm, d, 1H) and the H from the NH group (δ = 8.91 ppm, s, 1H).

The known amount of TMB added was calculated in base of a 1:1 relation between the investigated signals, assuming a100% yield. To simplify the calculations, a factor of 1/3 was employed. This adjustment accounts for the fact that the utilized peak of TMB integrates for three protons (3H), while the corresponding peaks from the product integrate for one proton (1H). Thus, for the experiments performed in Table 5 (Entry 1):

$$Mass_{TMB} = 0.006\ mmol\ product \cdot \frac{1\ mmol\ TMB}{1\ mmol\ product} \cdot \frac{1\ H}{3\ H} \cdot \frac{168.19\ mg\ TMB}{1\ mmol\ TMB} = 0.34\ mg\ TMB$$



Scheme 25. Example of yield determination by internal standard of 1,3,5-trimethoxybenzene. Spectrum corresponding to procedure described in Table 5.

The determined amount of internal standard would be added to the crude with the previous preparation of stock solutions.

3.5.9 General information for in cellulo experiments

All steps were performed on a sterile clean bench *Tesltar AV-100* at room temperature. Solutions stored in a fridge were pre-warmed in a water bath (37ºC) before use.

A549 cell cultures were incubated in DMEM (Dulbecco's modified Eagle's medium) acquired from Sigma-Aldrich, which was enriched with penicillin (100 units/mL), streptomycin (100 units/mL) and glutamine 5 mM, all from *Invitrogen*. Cells were seeded in well plates at the specified concentration two days prior to treatment. Proliferating cultures were maintained in an incubator at 37 ºC, 5% CO_2 and 95% humidity.

3.5.10 Representative procedure for in cellulo reactions

To perform the different experiments, 1.5 million cells per plate (100 nm) were seeded two days before treatment. In the beginning of the experimentation, the growing media was retired prior to the addition of the solutions previously prepared in DMEM. Treatment of the cells was initiated with the first one containing the aniline **1** (100 or 50 μM, final volume of 3 mL), for 30 minutes, using freshly prepared stock solution (10 mM in DMSO). Once the time is over, the media was aspired and cells were washed twice with PBS (3 mL). Then, the second DMEM solution was added, containing *i*PrAuNTf$_2$ (50 and 25 μM, final volume of 3 mL), from again freshly prepared stock solution (10 mM in DMSO).

After 3 hours, the media was collected and kept for posterior analysis, as well as the 2 new washes with PBS (3 mL). All these fractions were lyophilized and filtered by HPLC filter before being subjected to HPLC-MS. Finally, cells were extracted with MeCN (1 mL) three times. The portions were concentrated under vacuum, agitated by vortex and subjected to sonication for 5 min. Then, they were filtered with HPLC filters and analyzed by HPLC-MS to determine the product **2** formation.

CONCLUSIONS

In the present Final Degree Project, a new bioorthogonal reaction has been described based on the intramolecular double cycling promoted by gold of an aniline by means of a cascade mechanism. Its original parameters, based on organic synthesis, have been optimized, modifying catalysts and their loads, times, concentrations, solvents and atmosphere, finally determining that the reaction can be carried out in mild conditions. The perfected parameters allow the chemical transformation to be carried out in 9/1 H_2O/DMSO, in 10 minutes, with a load of 10 mol% of *i*PrAuNTf$_2$ as a catalyst, with a substrate concentration of 1 mM, at room temperature and in an open atmosphere.

It has also been shown that the reaction is perfectly compatible with more complex media, obtaining good results in both PBS, DMEM and cell lysates. At the same time, the formation of carbazole from aniline has been found to be perfectly tolerant to the presence of biomolecules such as NADH, GSH, L-Cysteine or sodium ascorbate, which act with very little inhibition in *in vitro* tests. Finally, preliminary studies have been developed on the adaptation of the reaction to the interior of cellular media, using the A549 line for these *in cellulo* analyses, demonstrating its correct functioning and bioorthogonality.

These results could open the way to the development of bioorthogonal reactions for the formation of structures of great molecular complexity through cascade mechanisms, acting in a simple and sustainable way. This could lead to the *in situ* formation of cyclic and heterocyclic structures of interest to Medicinal Chemistry, highlighting for example the antimicrobial and antitumor action of annulated derivatives of carbazoles.

In this way, it is interesting to contemplate the future optimization of preliminary assays in cells, improving the conditions and carrying out quantitative studies that allow a better understanding of the reaction, as well as carrying it out in different cell lines to observe its effects. On the other hand, the future modification of the final structure of the cyclization is proposed with the aim of making it fluorescent, facilitating its *in vivo* study through fluorescence microscopy.

CONCLUSIONES

En el presente Trabajo de Fin de Grado, se ha descrito una nueva reacción bioortogonal basada en la doble ciclación intramolecular promovida por oro de una anilina mediante un mecanismo en cascada. Se han optimizado sus parámetros originales, fundamentados en la síntesis orgánica, modificando catalizadores y sus cargas, tiempos, concentraciones, disolventes y atmósfera, determinando finalmente que la reacción se puede realizar en condiciones suaves.

Los parámetros perfeccionados permiten llevar a cabo la transformación química en 9/1 H_2O/DMSO, en 10 minutos, con una carga de 10 mol% de *i*PrAuNTf$_2$ como catalizador, con una concentración de sustrato de 1 mM, a temperatura ambiente y en atmósfera abierta.

También se ha demostrado que la reacción es perfectamente compatible con medios más complejos, obteniendo buenos resultados tanto en PBS, DMEM como en lisados celulares. Al mismo tiempo, la formación del carbazol a partir de la anilina ha resultado ser perfectamente tolerante a la presencia de biomoléculas como NADH, GSH, L-Cisteína o ascorbato sódico, las cuales actúan con una muy pequeña inhibición en ensayos *in vitro*. Por último, se han desarrollado estudios preliminares sobre la adaptación de la reacción al interior de medios celulares, utilizando la línea A549 para estos análisis *in cellulo*, demostrando su correcto funcionamiento y bioortogonalidad.

Estos resultados podrían abrir el paso al desarrollo de reacciones bioortogonales de formación de estructuras de gran complejidad molecular a través de mecanismos en cascada, actuando de manera sencilla y sostenible. Esto podría dar lugar a la formación *in situ* de estructuras cíclicas y heterocíclicas de interés para la Química Médica, destacando por ejemplo la acción antimicrobiana y antitumoral de los derivados anillados de los carbazoles.

De esta manera, es interesante contemplar la optimización futura de los ensayos preliminares en células, mejorando las condiciones y realizando estudios cuantitativos que permitan comprender mejor la reacción, así como llevarla a cabo en diferentes líneas celulares para observar sus efectos. Por otro lado, se propone la futura modificación de la estructura final de la ciclación con el objetivo de hacerla fluorescente, facilitando su estudio *in vivo* a través de microscopía de fluorescencia.

CONCLUSIÓNS

No presente Traballo de Fin de Grado, describiuse unha nova reacción bioortogonal baseada na dobre ciclación intramolecular promovida por ouro dunha anilina mediante un mecanismo en cascada. Optimizáronse os seus parámetros orixinais, fundamentados na síntese orgánica, modificando catalizadores e as súas cargas, tempos, concentración, disolventes e atmosfera, determinando finalmente que a reacción pódese realizar en condicións suaves. Os parámetros perfeccionados permiten levar a cabo a transformación química en 9/1 H_2O/DMSO, en 10 minutos, cunha carga de 10 mol% de *i*PrAuNTF$_2$ como catalizador, cunha concentración de substrato de 1 mM, a temperatura ambiente e atmosfera aberta.

Tamén demostrouse que a reacción é perfectamente compatible con medios máis complexos, obtendo bos resultados tanto en PBS, DMEM como en lisados celulares. Ao mesmo tempo, a formación do carbazol a partir da anilina resultou ser perfectamente tolerante á presencia de biomoléculas como NADH, GSH, L-Cisteína ou ascorbato sódico, os cales actúan cunha moi pequena inhibición en ensaios *in vitro*. Por último, desenvolvéronse estudos preliminares sobre a adaptación da reacción ao interior de medios celulares, utilizando a liña A549 para estes análises *in cellulo*, demostrando o seu correcto funcionamento e bioortogonalidade.

Estes resultados poderían abrir o paso ao desenvolvemento de reaccións bioortogonais de formación de estruturas de gran complexidade molecular a través de mecanismos en cascada, actuando de maneira sinxela e sostible. Isto podería dar lugar á formación *in situ* de estruturas cíclicas e heterocíclicas de interese para a Química Médica, destacando por exemplo a acción antimicrobiana e antitumoral dos derivados anulados dos carbazoles.

Desta maneira, é interesante contemplar a optimización futura dos ensaios preliminares en células, mellorando as condicións e realizando estudos cuantitativos que permitan comprender mellor a reacción, así como levala a cabo en diferentes liñas celulares para observar os seus efectos. Por outro lado, proponse a futura modificación da estrutura final da ciclación co obxectivo de facela fluorescente, facilitando o seu estudo *in vitro* a través de microscopio.

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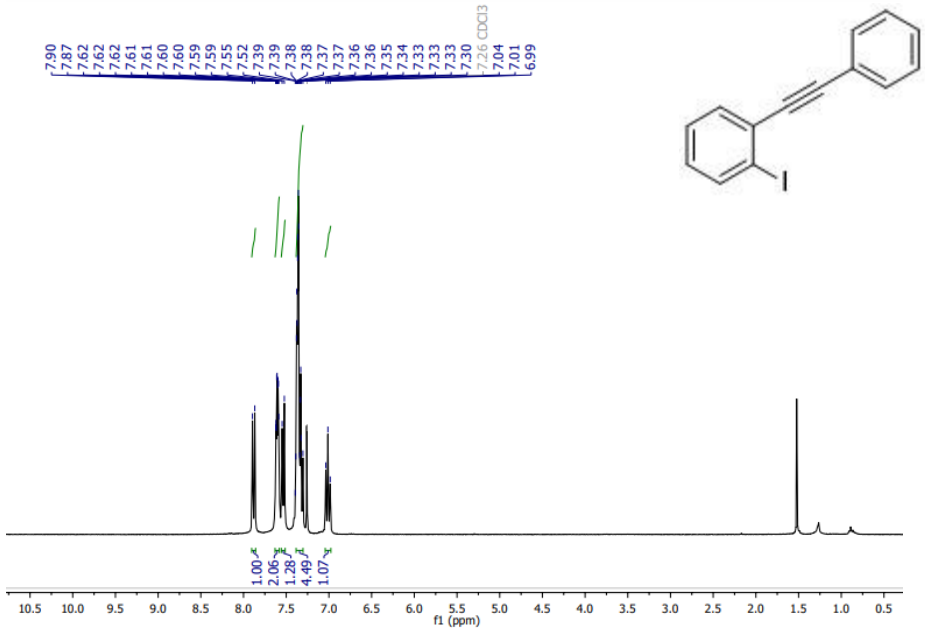
ANNEX: CHARACTERIZATIONS

1-iodo-2-(phenylethynyl)benzene

^{1}H-NMR



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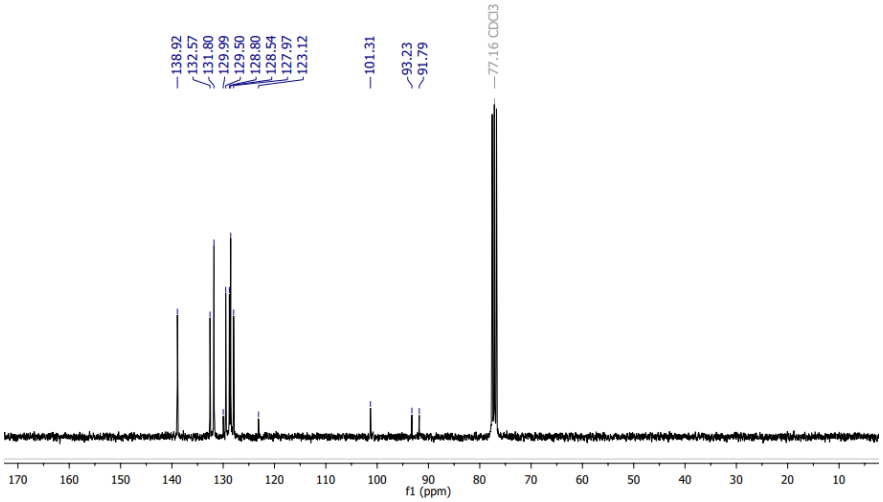
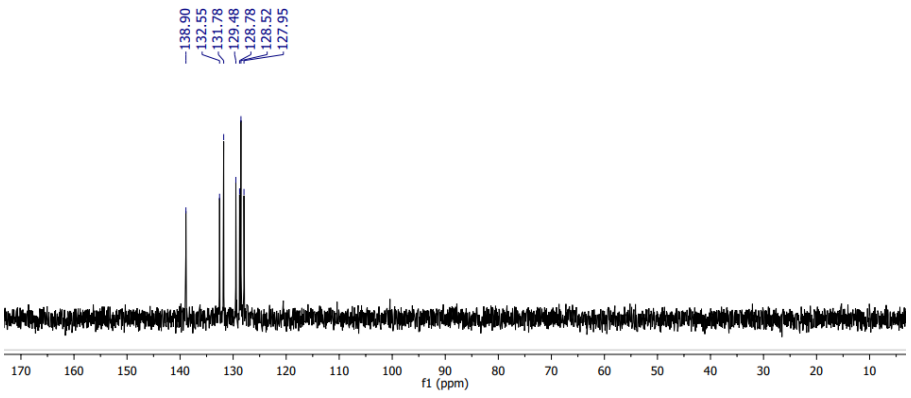
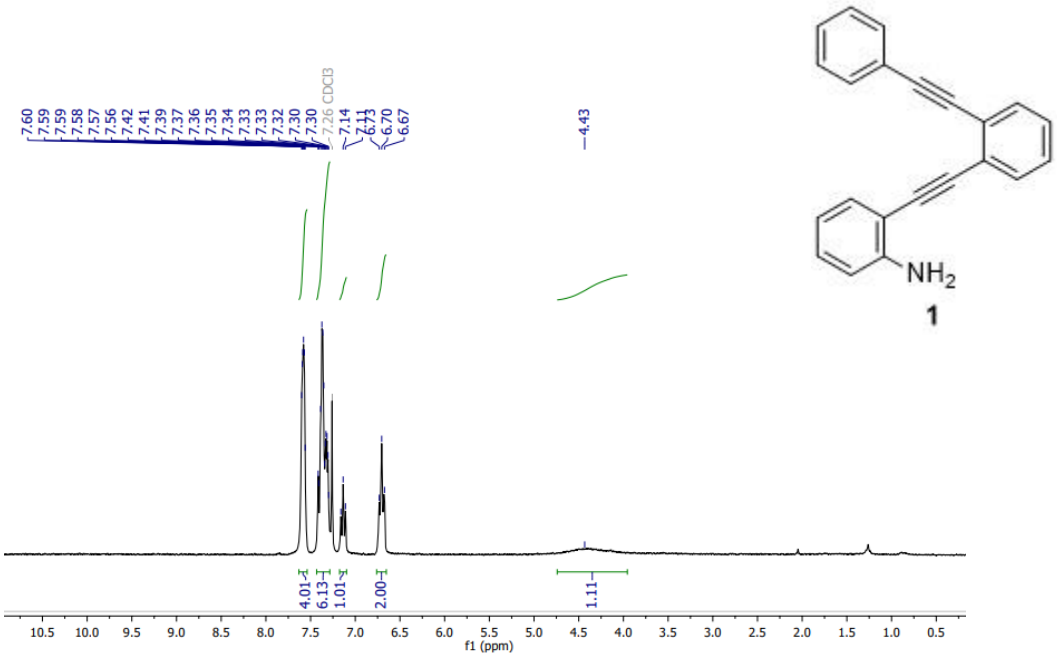


DEPT-135



2-((2-(phenylethynyl)phenul)ethynyl) ***1***

^{1}H-NMR



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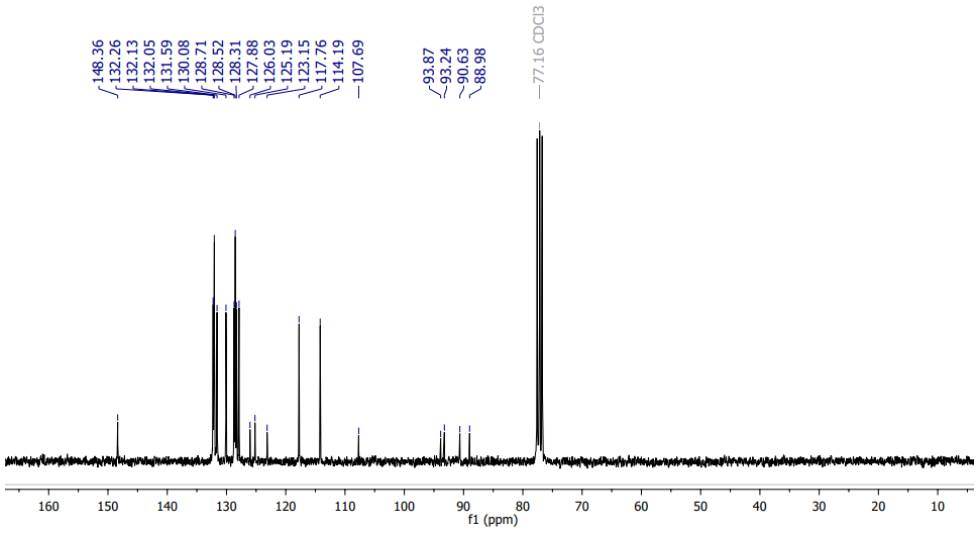

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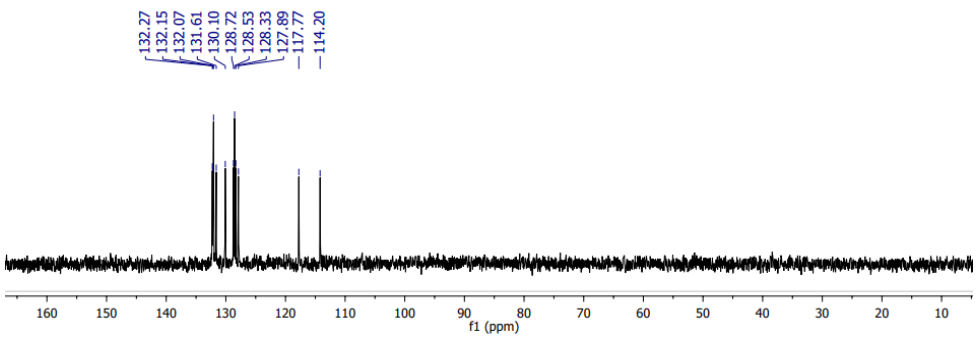

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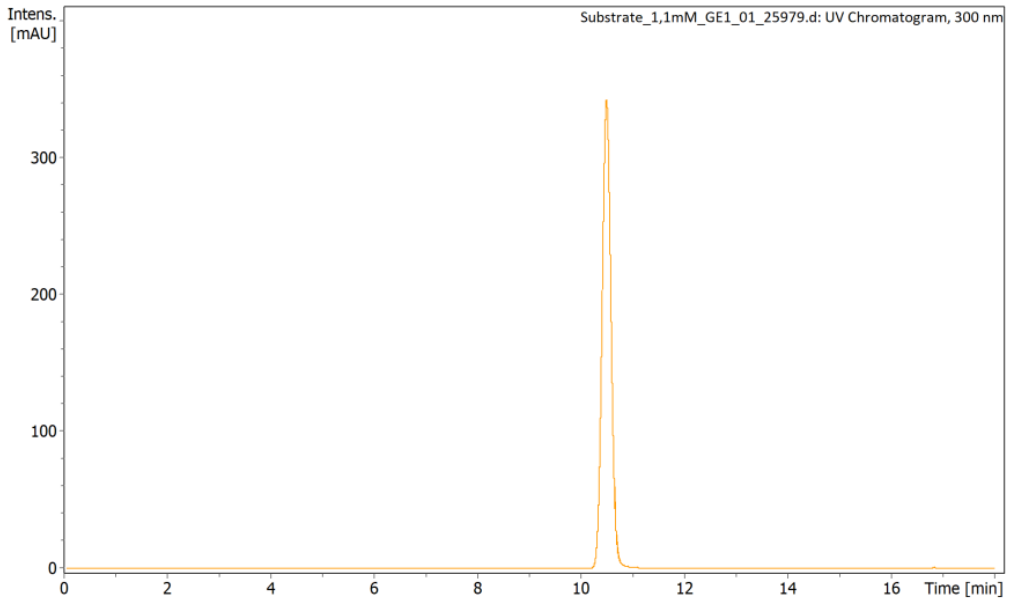
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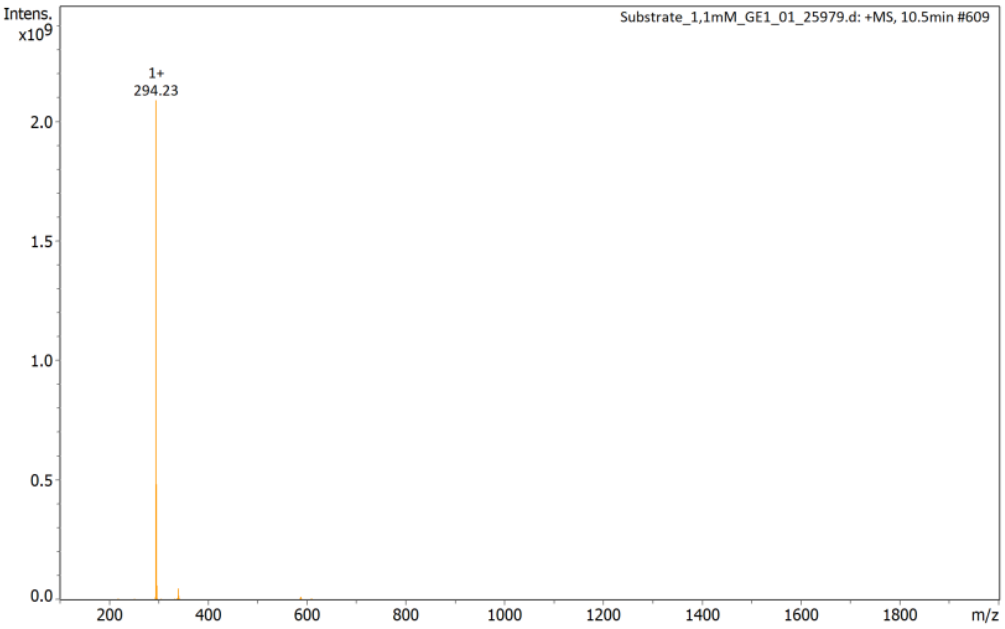
100
0
0
2
4
6
8
10
12
14
16
Time [min]


Intens.
x10^9
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1+
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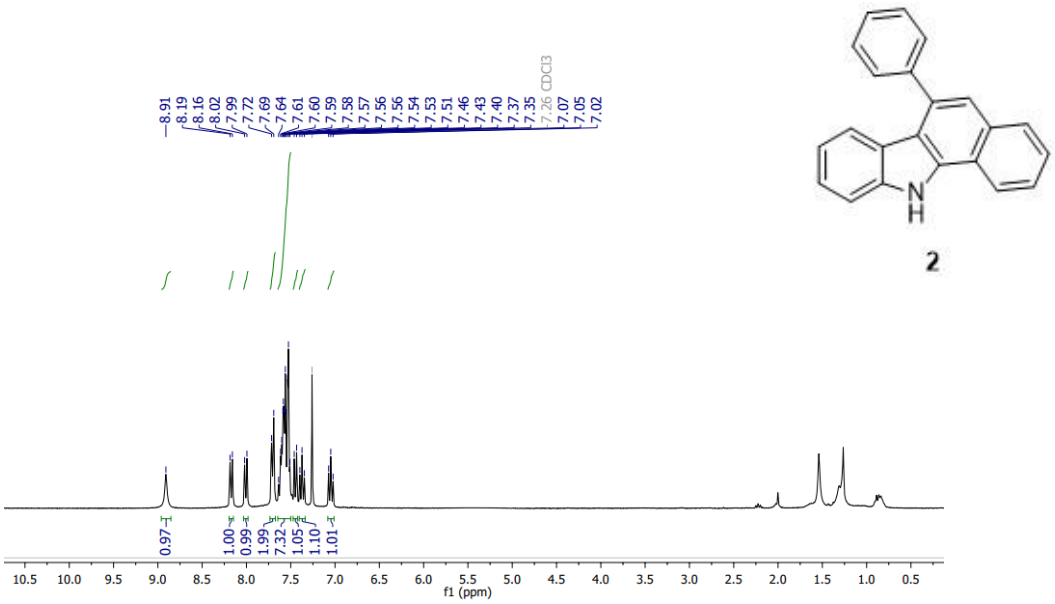
2.0
1.5
1.0
0.5
0.0
200
400
600
800
1000
1200
1400
1600
1800
m/z

6-phenyl-11H-benzo[a]carbazole ***2***

^{1}H-NMR



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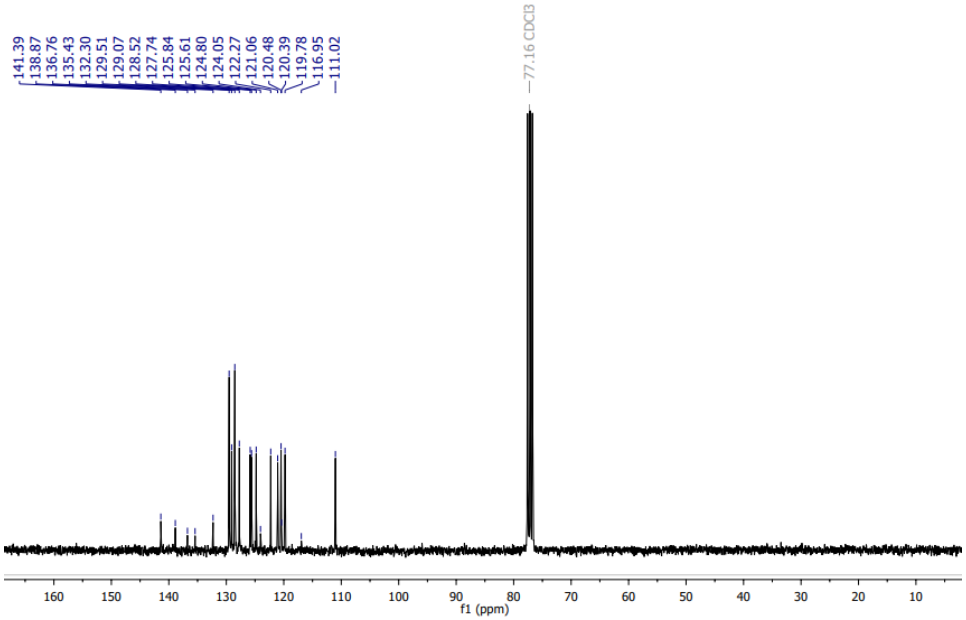
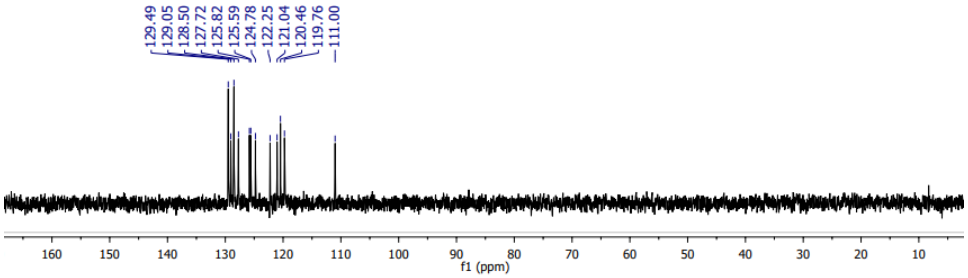


DEPT-135



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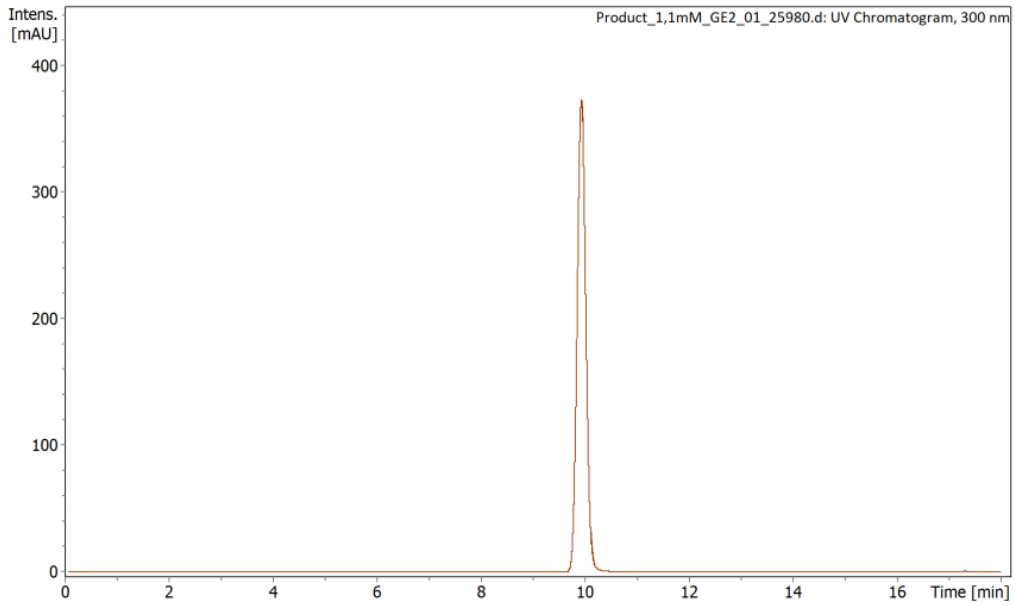
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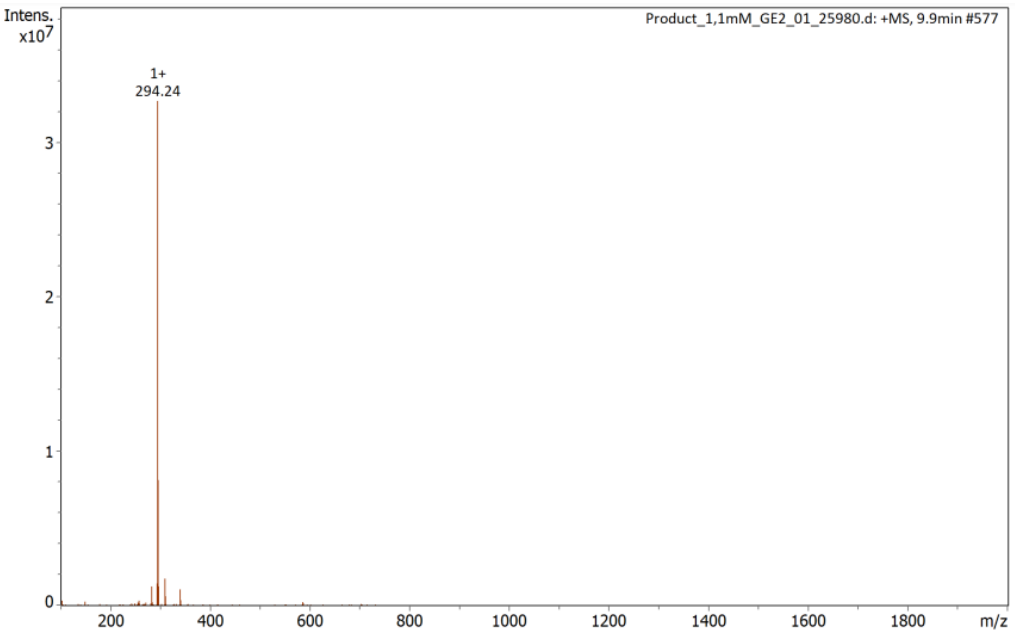
4
6
8
10
12
14
16
Time [min]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3
2
1
0
200
400
600
800
1000
1200
1400
1600
1800
m/z