Bioorganic Chemistry 150 (2024) 107528

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Short Review

Chemical sensors for the early diagnosis of bacterial resistance to β-lactam antibiotics

Rafael Canabal, Concepción González-Bello *

Centro Singular de Investigación en Química Biolóxica e Materiais Moleculares (CiQUS), Departamento de Química Orgánica, Universidade de Santiago de Compostela, Jenaro de la Fuente s/n, 15782 Santiago de Compostela, Spain

ARTICLE INFO

Dedicated to Mrs. Concepción Bello Valiña on the occasion of her 80th birthday.

Keywords:
Antibiotic resistance detection
Colorimetric sensors
Fluorescent sensors
Carbapenemases
Enzyme substrates

ABSTRACT

β-Lactamases are bacterial enzymes that inactivate β-lactam antibiotics and, as such, are the most prevalent cause of antibiotic resistance in Gram-negative bacteria. The ever-increasing production and worldwide dissemination of bacterial strains producing carbapenemases is currently a global health concern. These enzymes catalyze the hydrolysis of carbapenems – the β-lactam antibiotics with the broadest spectrum of activity that are often considered as drugs of last resort. The incidence of carbapenem-resistant pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and carbapenemase or extended spectrum beta-lactamase (ESBL)-producing Enterobacterales, which are frequent in clinical settings, is worrisome since, in some cases, no therapies are available. These include all metallo-β-lactamases (VIM, IMP, NDM, SMP, and L1), and serine-carbapenemases of classes A (KPC, SME, IMI, and GES), and of classes D (OXA-23, OXA-24/40, OXA-48 and OXA-58). Consequently, the early diagnosis of bacterial strains harboring carbapenemases is a pivotal task in clinical microbiology in order to track antibiotic bacterial resistance and to improve the worldwide management of infectious diseases. Recent research efforts on the development of chromogenic and fluorescent chemical sensors for the specific and sensitive detection and quantification of β-lactamase production in multidrug-resistant pathogens are summarized herein. Studies to circumvent the main limitations of the phenotypic and molecular methods are discussed. Recently reported chromogenic and fluorogenic cephalosporin- and carbapenem-based β-lactamase substrates will be reviewed as alternative options to the currently available nitrocefin and related compounds, a chromogenic cephalosporin-based reagent widely used in clinical microbiology laboratories. The scope of these new chemical sensors, along with the synthetic approaches to synthesize them, is also summarized.

1. Bacterial resistance to β-lactam antibiotics

According to the World Health Organization (WHO), Centers for Disease Control and Prevention (CDC) and European Centre for Disease Prevention and Control (ECDC), the emergence and worldwide spread of multidrug-resistant bacteria is dangerously compromising our ability to cure bacterial infections [1–4]. These pathogens are also limiting the success of much of the recent progress in modern medicine since antibiotics play a pivotal role in other therapies. Thus, these drugs are essential therapeutic tools to successfully overcome common secondary infections resulting from common life-saving procedures, such as chemotherapy, dialysis, surgery, organ transplantation, etc. The impact that this scenario has on healthcare-associated patients, the older population, or those with a compromised immune system is a matter of great concern given the lack of efficient therapies in certain cases. In particular, the treatment of infections caused by Gram-negative bacteria, such as the critical priority pathogens highlighted by the WHO, carbapenem-resistant *Pseudomonas aeruginosa*, carbapenem-resistant *Acinetobacter baumannii* and carbapenemase or extended spectrum beta-lactamase (ESBL)-producing Enterobacterales, is getting more and more challenging. These bacteria are resistant to carbapenems, which are often considered as the antibiotic of last resort, and are frequently found in hospitalized patients fitted with invasive devices or exposed to extended antibiotic regimens. It has been estimated that infections caused by multidrug-resistant pathogens were directly responsible for 1.27 million deaths and contributed to 4.95 million worldwide in 2019. In addition, they also represent related costs of over 1.5 billion € annually in healthcare expenses and productivity losses [5].

Bacterial resistance to antibiotics is a global health challenge resulting from the combination of several factors, including: (i) the

* Corresponding author.
E-mail address: concepcion.gonzalez.bello@usc.es (C. González-Bello).

https://doi.org/10.1016/j.bioorg.2024.107528
Received 17 March 2024; Received in revised form 30 May 2024; Accepted 3 June 2024
Available online 4 June 2024

inappropriate and excessive use of these drugs in medicine, veterinary medicine, and agriculture over the years; (ii) the limited development of novel antibiotics by the pharmaceutical industry since the 1990 s; and (iii) the lack of innovative strategies in the development of antibiotics, which have mainly focused on optimizing previous scaffolds that inhibit the same type of bacterial targets against which resistance mechanisms have spread worldwide [6–8]. All these factors have contributed to stimulating the intrinsic evolutionary character and adaptability of bacteria to survive in hostile environments to the present alarming levels [9].

Bacteria become drug resistant by developing challenging and smart mechanisms that are increasingly difficult to combat. The most relevant mechanisms are: (i) drug excretion by activation of efflux pumps that are able to remove antibiotics from the periplasmic space to the external environment; (ii) a reduction of drug uptake by implementing chemical modifications of the bacterial outer membrane; (iii) a reduction of antibiotic efficacy by introducing changes to the target amino acid sequence that alter drug binding and/or adduct formation; and (iv) production of drug-inactivating enzymes, which results in inactivated forms of the antibiotic [10–12]. Of these, the production of β-lactamase enzymes that catalyze hydrolysis of the β-lactam ring in β-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems), thus preventing the inhibition of its therapeutic target (PBP, Penicillin-Binding Proteins), represents the most prevalent cause of antibiotic resistance in Gram-negative bacteria (Fig. 1) [13–15]. Their increasing incidence and continuing evolution are a direct consequence of natural selection by exposure to β-lactam agents. Since this type of antibiotic still remains at the forefront of antimicrobial chemotherapy, representing about 70 % of antibiotics prescribed in the clinic and having proven to be safe and effective over more than 50 years, the impact of evolved pathogens harboring more challenging and sophisticated β-lactamases significantly narrows the therapeutic options and, in some cases, solutions are not available [16].

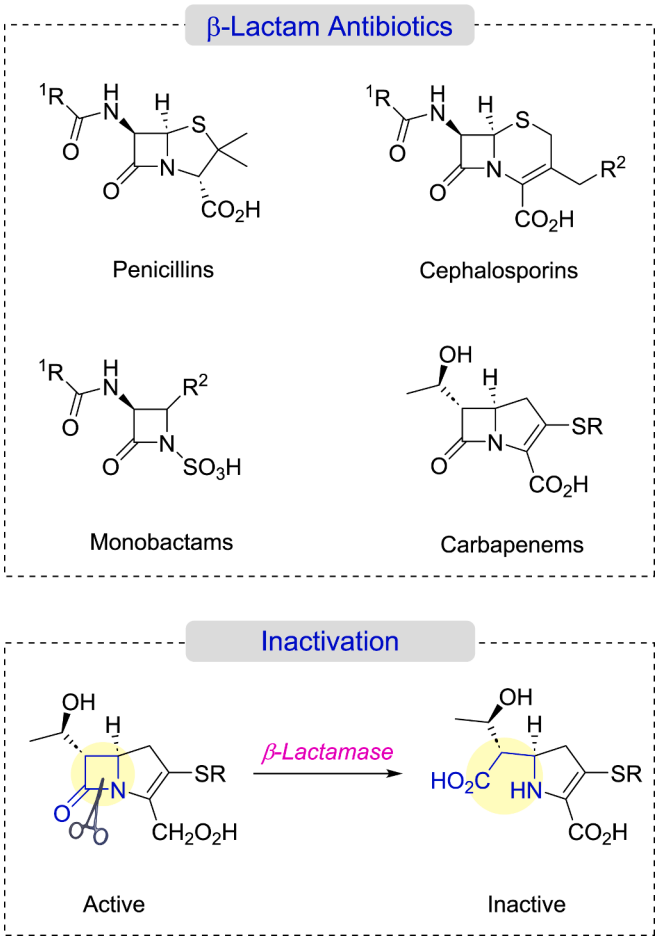


Fig. 1. Chemical structure of β-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems) and schematic representation of their inactivation catalyzed by β-lactamase enzymes.

To overcome this problem, the development of novel antibiotics with alternative mechanisms of action is an important task, which is currently performed mainly by small biotechnology companies, academic research groups or large public–private partnerships such as CARB-X (Combating Antibiotic Resistant Bacteria Biopharmaceutical Accelerator; https://carb-x.org/). Although bacteria will always develop some resistance, it is necessary to achieve optimal mechanisms to keep this exponential increase under control. As such, the rapid and sensitive diagnosis of the presence of multidrug-resistant strains is needed to track the development of bacterial resistance and to improve the worldwide management of infectious diseases. This diagnosis will both guide the physician's treatment decisions and avoid inappropriate prescription of ineffective antibiotics or the use of unnecessary ones. Nowadays, the early diagnosis of bacterial pathogens that are resistant to carbapenems is a crucial task in the practice of clinical microbiology [17]. These compounds have the broadest spectrum of activity and resistance to β-lactamases of all β-lactam antibiotics and are therefore considered drugs of restricted use, either alone or in combination with others, for difficult-to-treat infections, especially in a clinical setting. In this review, recent efforts to develop chemical probes for the early detection of β-lactamase production in multidrug-resistant pathogens in microbiology laboratories will be discussed. In particular, recently reported chromogenic and fluorogenic cephalosporin- and carbapenem-based β-lactamase substrates will be reviewed, including the scope of the diagnosis along with the synthetic approaches for their preparation.

2. Challenges in β-Lactamase detection

According to the Beta-Lactamase Database (BLDB; http://www.bldb.eu/; last updated 08 March 2024), around 8200 β-lactamases have been identified to date. They are typically classified into four groups (A–D) based on their sequence identities [18]. β-Lactamases of classes A (approx. 24 %) and C (approx. 47 %) are the most numerous group of enzymes, followed by classes D (approx. 16 %) and B (approx. 11 %). All of them constitute a highly diverse group of enzymes in terms of efficacy and specificity due to differences in the architecture and topology of the catalytic center and adjacent sites, as well as their mechanism of action (Fig. 2). An analysis of the three-dimensional structures available in the Protein Data Bank (PDB) for enzymes of classes A–D, both as wild-type form and bound or covalently modified by ligands (substrates or inhibitors), clearly reveals these differences (Fig. 2A). Thus, factors such as accessibility, topology, hydrophobicity of key recognition sites, along with the presence (class B enzymes) or absence (enzymes of classes A, C and D) of catalytic anions, which are key for catalysis and binding, can explain the diversity in specificity and catalytic efficacy. Enzymes of classes A, C and D, namely serine-β-lactamases, hydrolyze β-lactam antibiotics in an acylation/deacylation-based process using a catalytic serine residue as the reactive nucleophile (Fig. 2B), whereas no enzyme adducts are involved for class B enzymes (metallo-β-lactamases). Instead, a Zn(II)-bound hydroxide anion is used as a nucleophile to give an anionic intermediate after cleavage of the β-lactam C—N bond. Metallo-β-lactamases are divided into three subclasses: B1 and B3 are mainly di-zinc-dependent enzymes, while B2 are mono-zinc-dependent hydrolases.

The aforementioned features result in a great variation in substrate specificity and hydrolytic capacity, even within the same class (Fig. 3). Class A enzymes (known primarily as penicillinases) are a good example since they hydrolyze from penicillins to narrow- and extended-spectrum cephalosporins up to carbapenems. Of these, the most challenging class A enzymes are carbapenemases KPC, SME, IMI, NMC-A, and GES-like,

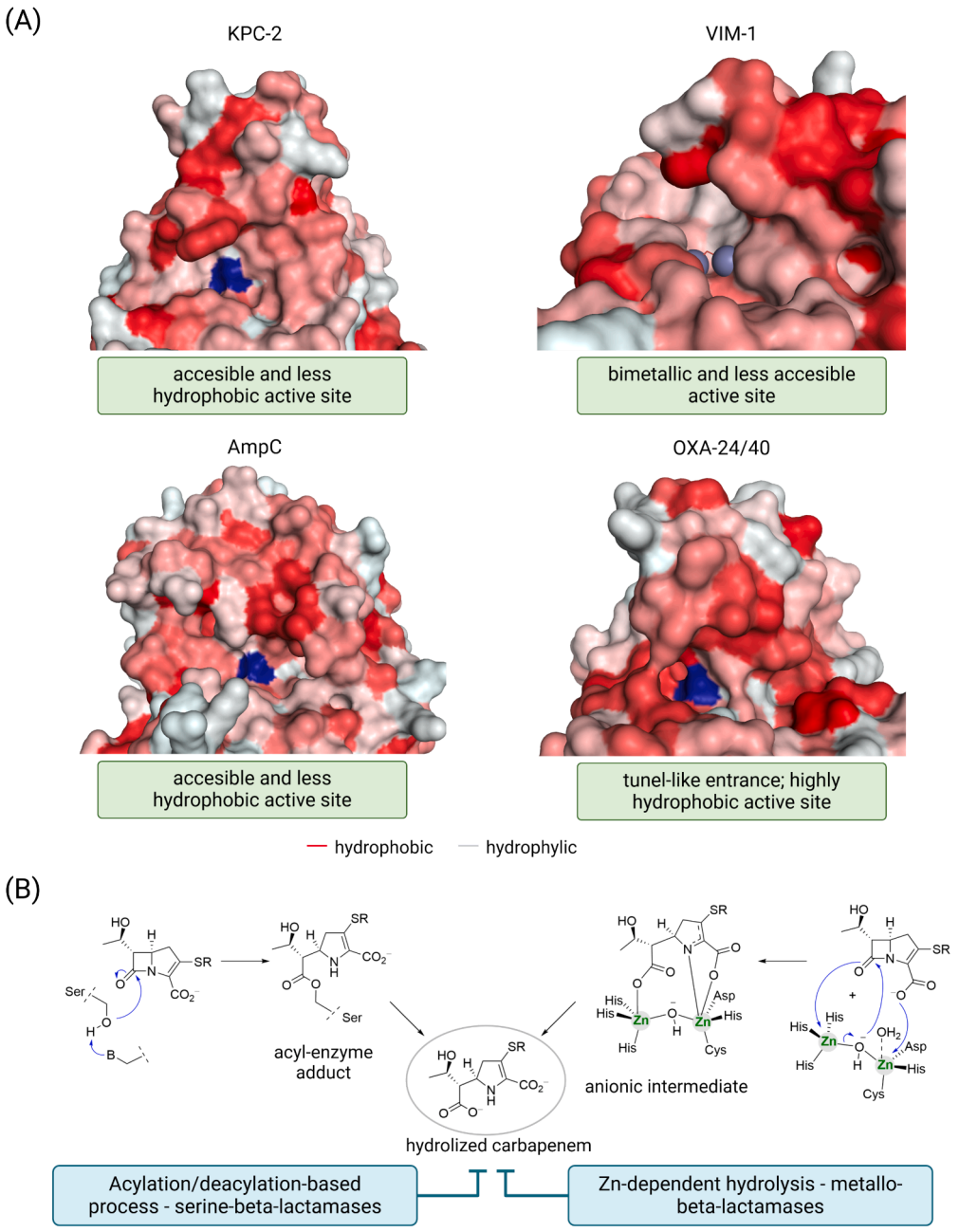


Fig. 2. (A) Close-up view of the active site of representative examples of classes A (KPC2, PDB ID 4ZBE, [19]), B (VIM-1, PDB ID 5N5I, [20]), C (AmpC, PDB ID 4HEF, [21]) and D (OXA-24/40, PDB ID 3FV7, [22]). The hydrophobic surface is shown in red. The catalytic serine residue is highlighted in blue and Zn^{2+} ions are shown as spheres. Note the different architectures and hydrophobicity of the enzyme active site for each example, as well as their overall arrangements. (B) Schematic representation of carbapenem hydrolysis catalyzed by serine-β-lactamases, which involves the formation of an acyl-enzyme adduct and subsequent hydrolysis, and metallo-β-lactamases, which implies the nucleophilic attack of a stabilized hydroxide anion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

which deactivate carbapenems. Class B enzymes (metallo-β-lactamases) show an extremely broad-spectrum substrate specificity. Thus, except for monobactams, such as aztreonam, virtually all β-lactam antibiotics are substrates for classes B1 and B3, while B2 enzymes specifically deactivate carbapenems. Metallo-β-lactamases such as IMP (B1), VIM (B1), NDM (B1), CphA (B2), and L1 (B3) currently represent a huge risk to hospitalized patients because they can potentially confer extremely broad-spectrum resistance to β-lactam antibiotics and no inhibitors of these enzymes have yet been approved by the US Food and Drug Administration (FDA). Cephalosporins are the preferred substrate for class C enzymes (cephalosporinases). Of these, the chromosomal class C β-lactamase (AmpC, encoded by *ampC* gene), also known as PDC (for *Pseudomonas*-derived cephalosporinase), is particularly challenging in clinical isolates of *P. aeruginosa*. The basal expression of AmpC is generally low, but high expression levels can be induced following exposure to some β-lactam antibiotics, thus making this bacterium intrinsically resistant to these drugs. Finally, class D enzymes, which are also known as oxacillinases (OXA), are the most genetically and biochemically diverse β-lactamases [23,24]. In general, they are characterized by their outstanding ability to hydrolyze cloxacillin/oxacillin along with carbenicillin. A large group of OXA-type enzymes have also evolved to hydrolyze expanded-spectrum cephalosporins (e.g., cefotaxime, ceftazime, and cefepime), such as OXA-14 and OXA-15. Among class D enzymes, the most dangerous group in terms of epidemiology

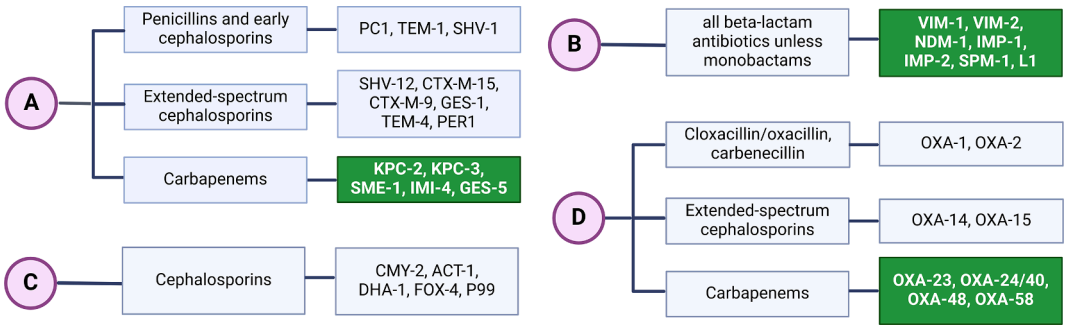


Fig. 3. Classification of β-lactamases (classes A–D), including distinctive substrates for each class and relevant enzyme(s). The most challenging carbapenemases produced by multidrug-resistant gram-negative pathogens, which are the worst-case scenario in clinical settings, are highlighted with a green shadow. Early cephalosporins correspond to the first developed drugs such as cefazolin and cephalexin, extended-spectrum cephalosporins include fourth- and fifth-generation derivatives such as ceftolozane. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and incidence is the carbapenem-hydrolyzing class D β-lactamases, which are a further expansion of the substrate profile of class D enzymes to include carbapenem antibiotics. Except for OXA-48, the majority of class D carbapenemases have been identified in clinical isolates of *Acinetobacter* spp., notably in *Acinetobacter baumannii* [25,26]. OXA-23, OXA-24/40 and OXA-58 are widely dispersed in clinical isolates of *A. baumannii*. OXA-48 is the most remarkable class D carbapenemase in *Klebsiella pneumoniae* and is also spread among other members of Enterobacterales. These enzymes are characterized by using a carbamylated lysine residue as catalytic residue and having a highly hydrophobic active site (Fig. 2A).

The most widely employed methods for the detection of β-lactamases in clinical microbiology laboratories are currently phenotypic techniques [27–32]. These include tests that imply bacterial culture for 12–24 h or even longer periods for slow growing bacteria, such as modified carbapenem inactivation method (mCIM) [33,34], Modified Hodge Test (MHT) [35], and the Double-Disk Synergy Test (DDST) [36]. For rapid diagnosis, qualitative colorimetric tests, such as the Carbapenemase Nordmann-Poirel (Carba NP) test [37,38] and its variants or β CARBA® test (Bio-Rad, Marnes-la-Coquette, France) are very useful [39,40]. Carba NP is the only carbapenemase-specific chromogenic assay for the rapid detection of carbapenemases implemented by the Clinical and Laboratory Standards Institute (CLSI) (~2h). It is based on the hydrolysis of imipenem by a bacterial lysate, which is detected by a change in pH, using phenol red as an indicator, which turns from red to orange/yellow. β CARBA® is a point of care test (~30 min) based on the color change due to the hydrolysis of a chromogenic substrate (unpublished) in the presence of active carbapenemase activity. All these colorimetric phenotypic methods are rapid, affordable and easy to implement, but usually show poor specificity and sensitivity, especially for carbapenemase detection, thus sometimes offering false-negative results [41–44].

Molecular tests, such as polymerase chain reaction (PCR)-based methods and real-time quantitative reverse transcriptase PCR (rt qRT-PCR), which are based on detection of the mRNA expression of carbapenemase-encoding genes [45], or proteomics-based methods (MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) [46,47], are important alternative options that circumvent some of the diagnostic limitations of phenotypic techniques [48]. These technologies provide a precise and accurate diagnosis of carbapenemase activity, but fail to detect unknown carbapenemase genes or enzymes not included in the search test. In addition, their implementation as part of the daily routine practice of microbiology services is not universal given the high costs and need for sophisticated instrumentation and trained laboratory personnel. Furthermore, immunochromatographic lateral flow assays based on monoclonal antibodies generated by immunization of mice have emerged as a cost-effective method for the sensitive and specific detection of the five most relevant carbapenemases, namely OXA-48 (class D) and KPC (class A), VIM, IMP and NDM (classes B) [49–53]. However, as noted previously, their main disadvantage is that they are unable to detect novel carbapenemases produced by the pathogen.

In recent years, a great deal of effort has been devoted to the development of chemical sensors with an ultrabroad spectrum of activity for clinical microbiology use. In addition to allowing the accurate monitoring of carbapenemase production in life-threatening pathogens, the objective of these studies is to develop specific and sensitive reagents for biochemical purposes, such as determination of the kinetic profile of new isolated serine- and metallo-carbapenemases resulting from bacterial evolution and the inhibitory potency of novel β-lactamase inhibitors under development, which is a blooming strategy in antibacterial drug discovery [13]. The marked differences between β-lactamases, particularly in their substrate profile, makes this goal a difficult task. Research efforts in this regard will be discussed in the following sections.

3. Cephalosporin-based reagents

The development of chemical probes based on the cephalosporin scaffold is the most developed and explored area, partly due to its more friendly chemistry and the intrinsically higher stability of cephalosporins compared with carbapenems. The most relevant examples of chromogenic and fluorogenic cephalosporin-based substrates are discussed below.

3.1. Chromogenic reagents

Nitrocefin, which was developed by Glaxo Laboratories Ltd. in 1971, was the first commercially available chromogenic cephalosporin that could be used to detect bacterial strains producing β-lactamase enzymes in biological samples (Fig. 4) [54,55]. The remarkable colorimetric change from yellow to deep red when its β-lactam ring is hydrolyzed by β-lactamase enzymes explains the success of this reagent, which is used in microbiology laboratories worldwide. Nitrocefin has an (*E*)-2,4-dinitro-1-(prop-1-en-1-yl)phenyl group in the C3 position that is responsible for the colorimetric change. The assay usually involves the detection of a bathochromic shift from around 380 nm to around 485 nm in the UV–Vis spectrum caused by extension of the conjugation chain upon β-lactam hydrolysis, which is quantified by measuring the absorption at 485 nm ($\varepsilon_{485\ nm} = 15600\ M^{-1}\ cm^{-1}$; ε = molar extinction coefficient).

Nitrocefin is an expensive reagent largely due to its great demand and issues associated with its preparation. The synthetic approaches described to date incorporate the chromogenic group in nitrocefin in a similar manner to the original work of the Glaxo researchers, in other words via a Wittig reaction between a phosphonium salt and 2,3-dinitrobenzaldehyde [55]. One of the main problems with this approach is the

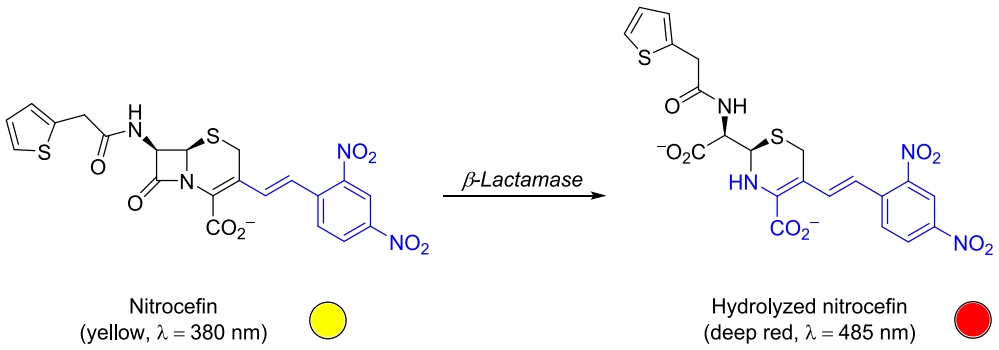
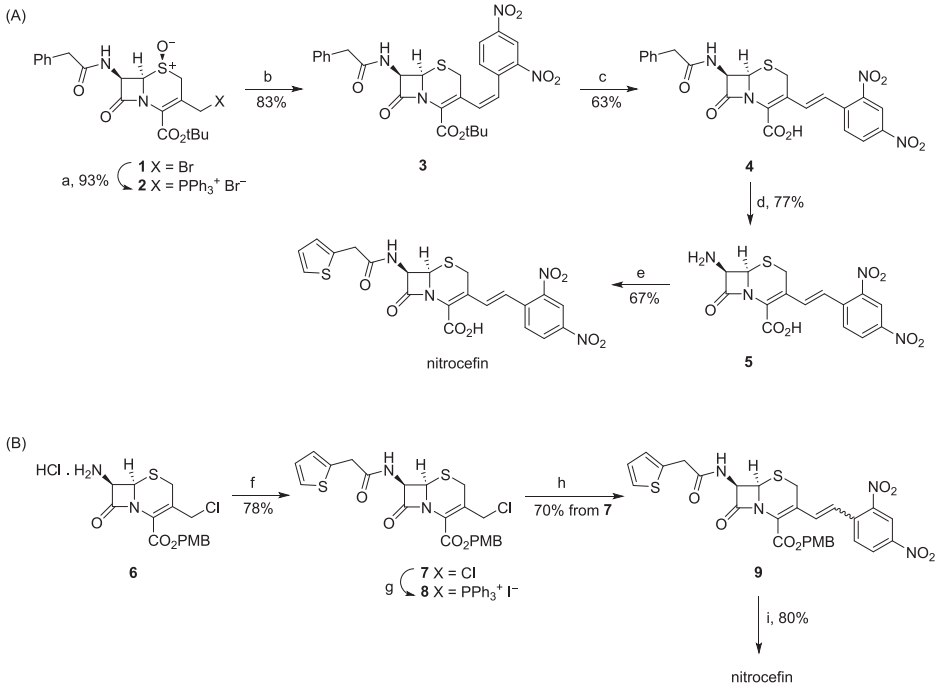


Fig. 4. Hydrolysis of nitrocefin catalyzed by β-lactamases. The moiety responsible for the color change is highlighted in blue. The wavelength in the UV–Vis spectrum usually employed to monitor the process is also indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

intrinsic reactivity of cephalosporins to undergo undesirable isomerization of the dihydrothiazine ring after protection of the carboxylate group [56]. To avoid this problem, Barendse et al. reported the use of commercially available sulfoxide **1**, which allows a multigram-scale synthesis of nitrocefin in five steps in a 25 % overall yield (Scheme 1A) [57]. In this case, the 2-thienylacetyl side chain is introduced in the final stages of the synthesis, performing the most difficult transformations (deprotection of the ester group with concomitant *Z/E* isomerization) with 2-phenylacetyl derivatives such as compounds **2**–**4**. Subsequently, Lee et al. [58] reported an improved version of the Glaxo process in which the use of potassium trimethylsilanolate as base was shown to be key for both acylation to give amide **7** and for the Wittig reaction between the phosphonium salt **8** and 2,4-dinitrobenzaldehyde (Scheme 1B). This synthetic approach also facilitates clean conversion of the major *Z* isomer into the desired *E* isomer while avoiding decomposition processes resulting from prolonged treatments with TFA or Lewis acids, such as $TiCl_4$ and $SnCl_4$, directed to promote *Z/E* isomerization. Smooth deprotection/isomerization is achieved by performing the TFA deprotection in 15 min at 0 °C, followed by stirring a 10 % DMSO in chloroform solution of the reaction mixture for 24 h. As a result, nitrocefin is obtained in a three-step synthesis from commercially available chloride **6** in 44 % overall yield.

The success of nitrocefin led to further studies aimed at developing structurally similar chromogenic cephalosporins. Examples include CENTA^{TM} and PADAC®, which were developed by researchers from Hoechst AG (Germany), in which, unlike nitrocefin, hydrolysis of the β-lactam ring releases the probe into the medium (Fig. 5). CENTA^{TM} provides a color change from light yellow (characteristic band at λ = 340 nm) to chrome yellow, which can be quantified by measuring the absorption in the UV–Vis spectrum at 405 nm associated with the generation of 2-mercapto-5-nitrobenzoic acid [59,60]. PADAC® (pyridine-2-azo-*p*-dimethylaniline cephalosporin) is a purple reagent (characteristic band λ = 570 nm) which turns light yellow (λ = 465 nm) upon hydrolysis due to the release of (*E*)-*N,N*-dimethyl-4-(pyridin-4-yldiazenyl)aniline [61,62]. CENTA^{TM} and PADAC® are used less often than nitrocefin since either the color change is less pronounced (yellow



Scheme 1. *Reagents and conditions:* (a) Ph_3P, MeOAc, MeOH, Δ. (b) 1. PCl_3, CH_2Cl_2, –10 °C; 2. 2,4-dinitrobenzaldehyde, $NaHCO_3$, CH_2Cl_2, RT. (c) $TiCl_4$, CH_2Cl_2, 0 °C. (d) Penicillin acylase, 32 °C, pH 7.5. (e) 1. 1,3-Bis(trimethylsilyl)urea, CH_2Cl_2, Δ; 2. 2-thiopheneacetyl chloride, –10 °C. (f) KOTMS, 2-thiopheneacetyl chloride, CH_3CN/CH_2Cl_2, 0 °C to RT. (g) NaI, Ph_3P, butanone, RT, dark. (h) 1. KOTMS, CH_3CN, –10 °C, dark; 2. 2,4-dinitrobenzaldehyde, CH_2Cl_2, –10 °C to RT. (i) 1. TFA, anisole, CH_2Cl_2, 0 °C, dark; 2. DMSO/CH_2Cl_2.

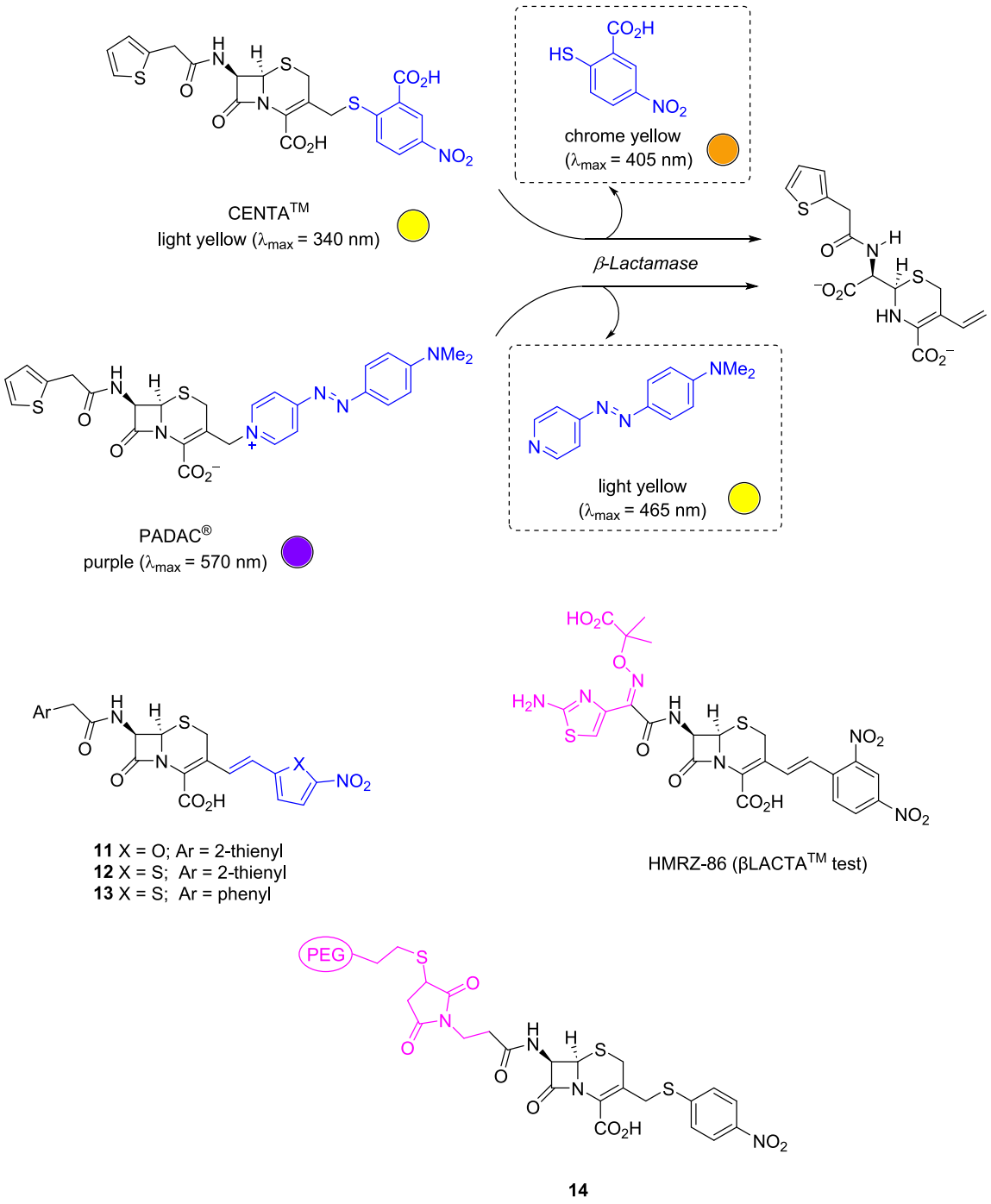


Fig. 5. Chromogenic cephalosporin-based reagents related to nitrocefin. HMRZ-86 is the main reactant in the βLACTA™ test. For CENTA™ and PADAC®, the wavelength in the UV–Vis spectrum usually employed for monitoring the process is also indicated.

range) or they are less reactive.

Ghavami et al. [63] described three nitrocefin analogs, compounds **11**–**13**, in which the dinitrophenyl group in nitrocefin was replaced by 5-nitrofuran-2-yl and 5-nitrothiophen-2-yl groups (Fig. 5). For compound **13**, the thiophen-2-yl substituent of nitrocefin was also substituted by a phenyl group, providing a synthetically more accessible derivative. Upon enzymatic hydrolysis, compounds **11**–**13** provide a color change from yellow to purple, along with a large bathochromic shift in the UV–Vis spectrum from about 426, 437 and 434 nm to about 498, 529 and 520 nm, respectively. These compounds, which also have a high molar extinction coefficient thus providing an excellent signal-to-noise ratio for β-lactamase detection, were assayed against enzymes of classes A (KPC-2), B (VIM-2, LMP-1, NDM-1, SPM-1, L1), C (GC-1) and D (OXA-10). In general, the catalytic efficiency (k_{cat}/K_m) of these compounds against the latter enzymes is comparable with nitrocefin.

For point-of-care diagnosis, disposable and inexpensive devices (paper-based, discs) containing the principal reagent of CENTA™, PADAC®, and nitrocefin, which do not require instrumentation or trained laboratory personnel for analysis, have been developed [64]. Furthermore, Chantemesse et al. [65] reported an amperometric assay based on the electrochemical detection of the β-lactamase activity using nitrocefin as substrate. This disposable carbon screen-printed sensor allowed the rapid and quantitative detection of *Escherichia coli* strains harboring extended spectrum β-lactamases (ESBLs) in urban

wastewaters by detecting the oxidation peak of the hydrolyzed nitrocefin (~0.2 V *vs* Ag/AgCl). ESBLs are enzymes that confer resistance to penicillins, cephalosporins, and the monobactam aztreonam. In addition, Alkekhia et al. [66] also demonstrated the utility of a poly(ethylene glycol) (PEG) polymer matrix for similar purposes by linking a derivative structurally related to the main reagent of CENTA™ via its C6 position (**14**; Fig. 5). Similarly, Hanaki et al. developed HMRZ-86, in which the ^{1}R side chain of the nitrocefin cephem core was replaced to increase susceptibility to third-generation cephalosporins (Fig. 5).[67,68] This compound is now commercialized as the βLACTA™ test (Bio-Rad), a new chromogenic method that has been shown to be useful for the detection of ESBL-producing bacteria, but not for bacterial strains harboring plasmid-mediated or chromosomally mediated AmpC enzymes [69,70]. The test is suitable for either isolated Enterobacterales colonies or bacterial pellets from positive blood cultures or urine.

3.2. Fluorogenic reagents

Several fluorogenic cephalosporin-based substrates for β-lactamase activity detection based on fluorescence activation upon hydrolysis of the β-lactam ring have been reported. In most cases, the fluorescent probe, which is covalently linked to the side chain in the ^{2}R position of the cephem core, is released into the medium to activate the fluorescence signal. The most relevant examples are summarized in Table 1.

Gao et al. described a fluorogenic cephalosporin-based biosensor (compound **15**) based on release of the phenolic dye resorufin, which fluoresces at 585 nm when excited at 550 nm, from a vinylogous cephalosporin (Table 1) [71]. The sulfoxide form of the cephalosporin was used to improve the stability of the compound in living media, as well as its *Z*-isomer, which is the major product in the Wittig coupling reaction employed, thus avoiding tricky *Z/E* isomerization process for this type of scaffold. Introduction of the resorufin moiety at the cephem core provides up to a 153-fold enhancement in the fluorescence intensity, thus facilitating detection of the production of β-lactamases at concentrations of less than 500 fM. The utility of compound **15** for imaging β-lactamase gene expression was demonstrated using TEM-1 (class A enzyme) as a biosensor.[71].

Cephalosporin **16** functionalized with a caged 3,7-diesterphenoxazine probe as a precursor of the fluorescent resorufin was reported by Xie et al. [72] The process requires sequential activation mechanisms for fluorescence detection, starting with hydrolysis of the β-lactam ring, followed by cleavage of the acetyl groups catalyzed by esterase enzymes, and final oxidation of the reduced resorufin with H_2O_2 (1 mM). The authors proved the utility of the assay for the efficient detection of bacterial strains producing TEM-1 (class A), KPC-3 (class A) and IMP-1 (class B) enzymes in 2 h.

The fluorogenic compound **17**, which enables the detection of *Mycobacterium tuberculosis* and bacillus Calmette–Guérin (BCG) *in vitro* and in living mice, has also been described (Table 1) [73]. This assay is based on specific detection of the chromosomal BlaC enzyme encoded by the *blaC* gene, which makes these bacteria intrinsically resistant to cephalosporin antibiotics and is an excellent biomarker for tuberculosis (TB) diagnosis. By taking advantage of the flexibility of the BlaC Ω-loop, which contains the residue E166 involved in hydrolysis of the enzyme adduct (deacylation process), the authors designed specific modifications at the C6 position in the cephalosporin scaffold. The introduction of a methoxy group with an *S* configuration at the latter position proved to be key for achieving an 8900-fold selectivity for BlaC over TEM-1, which was supported by resolution of the three-dimensional structure of the E166A BlaC variant enzyme covalently modified by **17** (PDB 3VFF, 2.78 Å). To monitor the process by fluorescence (λ_{exc} = 490 nm and λ_{em} = 535 nm), the TokyoGreen probe was incorporated into the ^{2}R group of the cephem core. Further optimization of **17** by introduction of a cyclopropyl group at the *C*2 position of the cephem core allowed the identification of compound **18**, with a 120,000-fold selectivity for BlaC enzyme over TEM-1 (Table 1) [74]. The specificity of compound **18** for BlaC expressing bacteria is excellent since no fluorescence signal is observed in incubation assays with other β-lactamase-expressing bacteria (10^{6} colony forming unit per milliliter, CFU mL^{-1}), specifically producing KPC, AmpC and SHV-18 β-lactamase enzymes. Compound **18** allows the detection of TB-positive samples with 90 % sensitivity and TB-negative samples with 73 % specificity. Subsequent optimization of compound **18** led to cephalosporin **19**, which showed a good diagnosis profile since it: (i) efficiently discriminates live from dead BCG; (ii) shows high specificity for *M. tuberculosis* over other bacterial species, including 43 non-TB mycobacteria; and (iii) is able to image *M. tuberculosis* in patients' sputum and BCG phagocytosis in real time [75]. Assuming one copy of BCG in the loading mixture, a detectable concentration of 100 CFU/mL could be detected by cephalosporin **19**.

TokyoGreen caged by dinitrophenyl arylation was also employed in cephalosporin **20** for fluorophore-releasing detection (Table 1) [76]. In this case, fluorescence activation was promoted by the thiophenyl group released upon hydrolysis, which undergoes a nucleophilic substitution reaction to produce fluorescence activation [84]. Compound **20** showed good selectivity for metallo-β-lactamases, specifically VIM-27, IMP-1 and NDM-1, over other carbapenemases such as KPC-3 (class A) and OXA-48 (class D) and ESBL, such as TEM-1 and BlaC (class A). This compound demonstrated ability to detect metallocarbapenemases in carbapenem-resistant Enterobacterales clinical isolates (10^{5} CFU mL^{-1}).

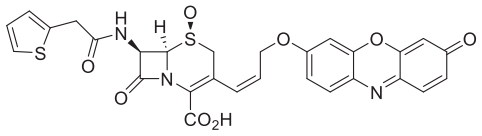
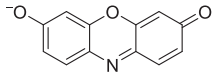
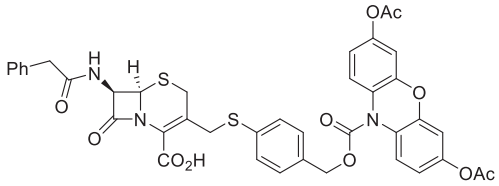
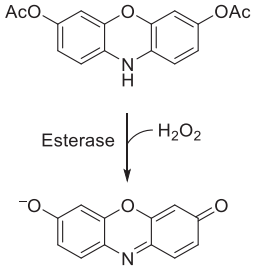
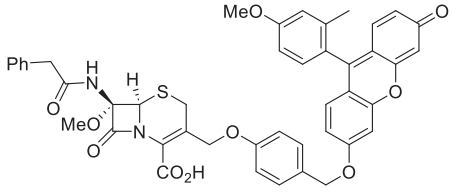
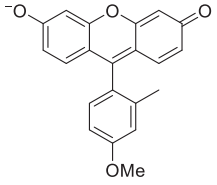
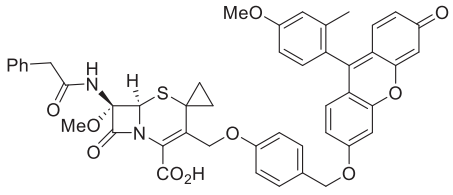
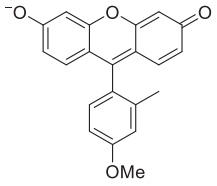
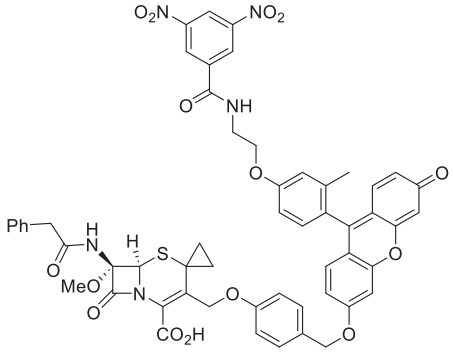
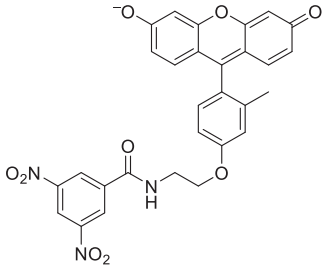
The first bioluminogenic substrate (compound **21**) for β-lactamase detection *in vivo* was reported by Yao et al. (Table 1) [77]. The basis of this assay is use of the light-emitting enzyme firefly luciferase, which catalyzes the oxidation of D-luciferin into oxyluciferin and offers huge sensitivity in small living systems. To this end, the authors introduced the firefly luciferase substrate D-luciferin at the ^{2}R position of the cephem core via an ether linkage, which is released upon β-lactam ring opening. Compound **21** was tested to image the activity of TEM-1 β-lactamase *in vivo*. Further functionalization of compound **21** with a DABCYL quencher in the ^{1}R group of the cephalosphorin core, compound **22**, improves the signal-to-background ratio (>1200-fold) of the detection by a relevant reduction of the initial background emission [78]. The assessment of the catalytic efficacy of several β-lactamases of clinical relevance, specifically KPC-3, TEM-1 and BlaC (Class A), IMP-1 (Class B), AmpC (Class C), and OXA-48 (Class D) against this compound, revealed that **22** is a good substrate for the tested enzymes of classes A and B (range 10^{4} to 10^{6} M^{-1} s^{-1}), but less efficient for AmpC and OXA-48 (range 10^{2} to 10^{3} M^{-1} s^{-1}). This bioluminogenic reagent allows the identification the β-lactamase production at the femtomolar level, detecting 10^{2} to 10^{3} CFU mL^{-1} of β-lactamase-producing Enterobacterales in urine samples within 30 min.

Furthermore, inspired by the chemical structure of ceftazidime, Thai et al. developed a pyridyl BODIPY derivative **23** that can be used to detect strains harboring ESBL resistant to cefotaxime and ceftazidime (third-generation cephalosporins), such as CTX-M−14, CTX-M−15, SHV-28, TEM-1, and CTX-M−9 (Table 1) [79]. A 2.5-fold increase in the intrinsic fluorescence intensity of **23**, which was quantified at 512 nm, was obtained upon reaction with these enzymes.

After analysis of the main structural differences between carbapenems and cephalosporins, Shi et al. designed the fluorogenic cephalosphorin **24**, which contains a 7-hydroxycoumarin moiety as fluorescent probe and is detected after hydrolysis (Table 1) [80]. For the design, it was considered that carbapenems have a carbon atom in their five-membered ring replacing the sulfur atom present in penicillins and cephalosporins [85]; and, unlike penicillins and cephalosporins, also have an *S*-configuration at the ^{1}R group, which prevents hydrolysis by most β-lactamases (except carbapenemases). Compound **24** showed good specificity and sensitivity for the detection of Enterobacterales strains producing carbapenem-resistant β-lactamase enzymes, specifically IMP-1, VIM-27 and NDM-1 (class B) and KPC-3 (class A). The detection limit of compound **24** for all these carbapenemases showed to be as low as 5 fmol.

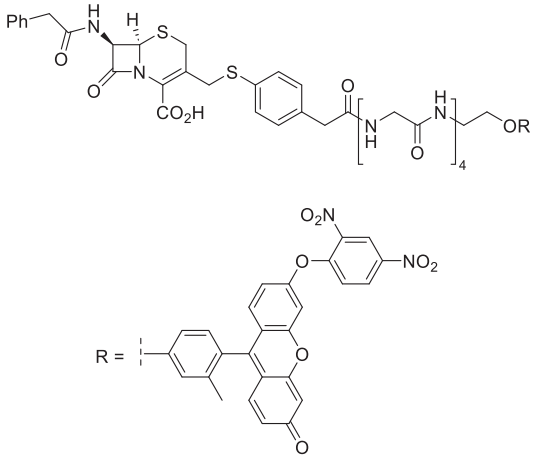
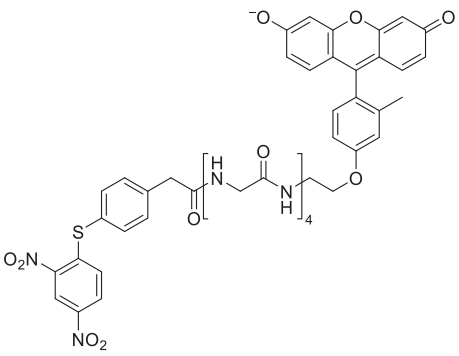
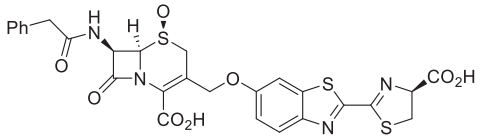
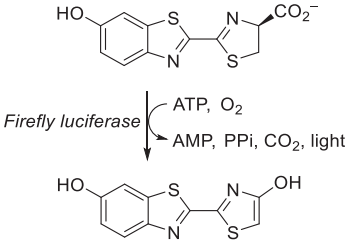
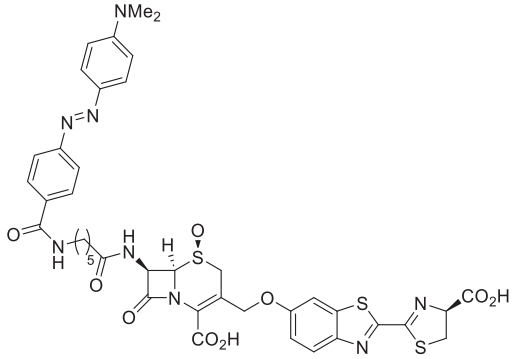
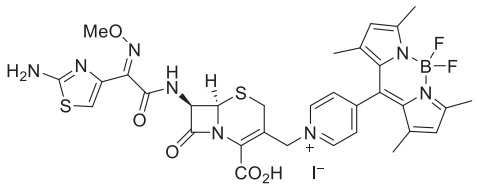
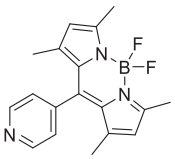
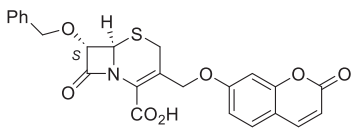
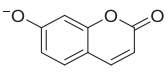
Compound **25**, which is covalently modified with a highly sensitive

Table 1
Examples of fluorogenic cephalosporine-based reagents in which the released probe is detected.

Cephalosporin reagent	Species or process detected	Assay method, type of β-lactamase detected, refence
 **15**		Fluorescence, TEM-1, [71]
 **16**		Fluorescence; assay also involving oxidation with H_2O_2, TEM-1, [72]
 **17**		Fluorescence, TEM-1, BlaC, [73]
 **18**		Fluorescence, TEM-1, BlaC, [74]
 **19**		Fluorescence, TEM-1, BlaC, [75]

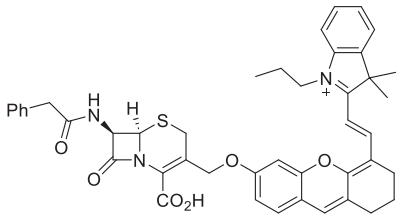
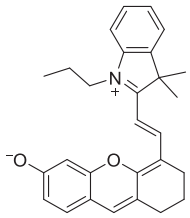
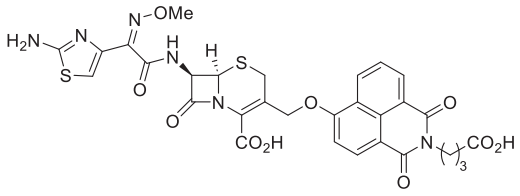
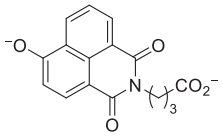
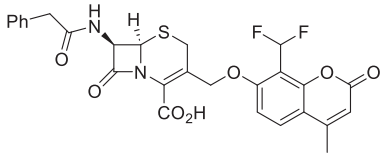
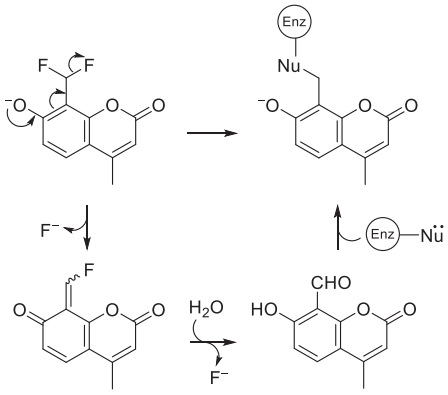
(*continued on next page*)

Table 1 (*continued*)

Cephalosporin reagent	Species or process detected	Assay method, type of β-lactamase detected, refence
 **20**		Fluorescence, VIM-27, IMP-1, NDM-1, [76]
 **21**		Bioluminescence, TEM-1, [77]
 **22**		Bioluminescence, KPC-3, TEM-1, BlaC, IMP-1, [78]
 **23**		Fluorescence, TEM-1, SHV-12, CTX-M−14, SHV-28, [79]
 **24**		Fluorescence, IMP-1, VIM-27, NDM-1, KPC-3, [80]

(*continued on next page*)

Table 1 (*continued*)

Cephalosporin reagent	Species or process detected	Assay method, type of β-lactamase detected, refence
 **25**		Fluorescence, [81]
 **26**		Fluorescence, CTX-M−9, TEM-3, VIM-1, NDM-1, [82]
 **27**		Fluorescence, TEM-1, VIM-1, NDM-1, [83]

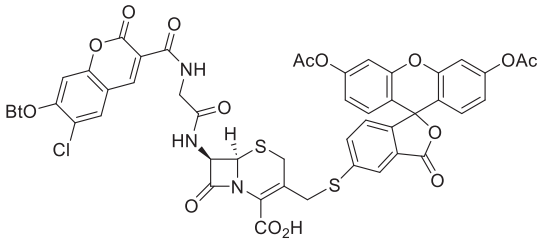
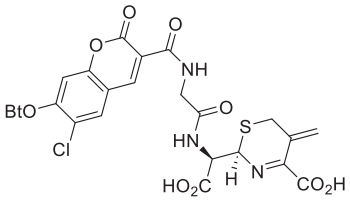
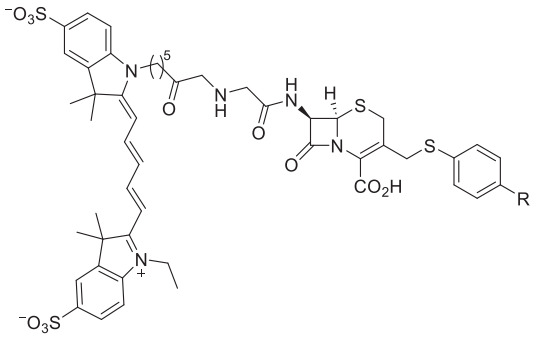
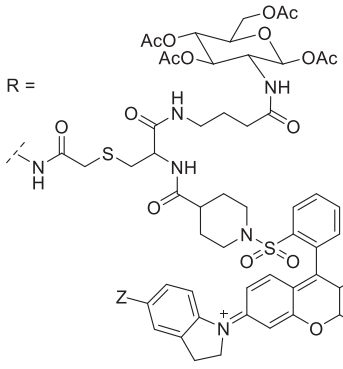
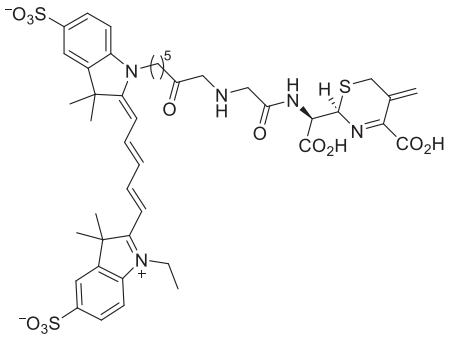
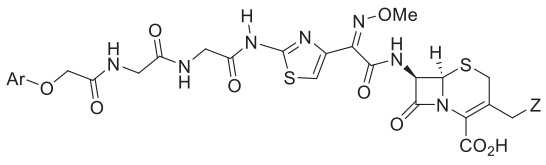
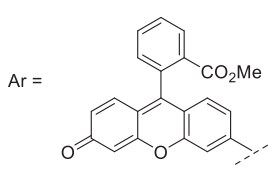
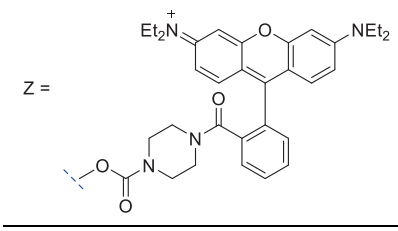
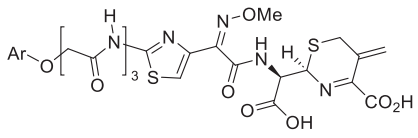
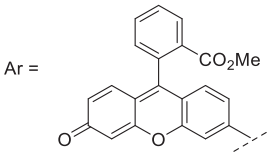
near-infrared fluorescent probe that emits at 707 nm when excited at 680 nm, has been used to detect and quantify different expression levels of β-lactamase production in methicillin-resistant and penicillin-resistant strains of *Staphylococcus aureus* with a detection limit of 0.02 nM (Table 1) [81]. A ratiometric fluorescent probe (compound **26**) for the selective detection of bacterial strains producing ESBL, including CTX-M−9−expressing *Enterobacter cloacae*, TEM-3-expressing *E. coli*, multidrug-resistant *A. baumannii*, VIM-1-expressing *Klebsiella pneumoniae*, and NDM-1 expressing *K. pneumoniae*, has been described by Mao et al. [82] The detection limits of compound **26** for the NDM-1, KPC-3, VIM-27, and IMP-1 enzymes were 0.4, 0.5, 0.6, and 0.9 pM, respectively.

The fluorogenic suicide substrate **27** was developed to detect and label bacterial strains producing β-lactamase enzymes with a fluorescent probe (Table 1) [83]. This process implies initial hydrolysis of the β-lactam ring in **27**, which triggers the release of a fluorescent precursor of a quinone-methide species. The latter intermediate is mainly hydrolyzed by the water solvent to generate another electrophilic species—an aldehyde—which reacts with a nucleophilic residue of the target. The authors validated their proposal in assays with multidrug-resistant strains of *A. baumannii* and *K. pneumoniae* producing VIM-1 and NDM-1 enzymes. No details of the enzyme sites undergoing covalent modification(s) at a molecular level, or the type of residues involved, were disclosed.

Approaches intended to quantify formation of the hydrolyzed cephalosporin have also been described (Table 2). Thus, Zlokarnik et al. developed compound **28** to visualize gene expression in single living mammalian cells by using TEM-1 β-lactamase—a class A enzyme—as a reporter [87]. The assay is based on the fact that TEM-1 β-lactamase is normally secreted into the periplasmic space but accumulates in the bacterial cytoplasm when its signal sequence is genetically deleted [88]. The authors designed an ester proform that is converted into its active species by esterase hydrolysis in the cytosol. Cephalosporin **28** and its active form are non-fluorescent compounds because the two fluorophores—a donor (7-hydroxycoumarin derivative) and an acceptor (fluorescein)—in its structure overlap sufficiently to allow efficient fluorescence resonance energy transfer (FRET). This effect is destroyed upon hydrolysis and the release of fluorescein into the medium. As a result, the hydrolyzed cephalosporin is detected by its strong fluorescence signal at 447 nm when excited at 409 nm, thus allowing real-time gene expression determination. Galarneau et al. [89] showed that this *in vitro* assay can also be used to quantify protein–protein interactions. Shao et al. [90] also applied FRET strategies to develop fluorogenic cephalosporin-based reagents by incorporating the fluorophore (fluorescein, water-soluble Cy3 and near-infrared Cy5.5) and the quencher [DABCYL (4-([4-(dimethylamino)phenyl]azo)benzoic acid), BHQ2 (black hole quencher-2) and BHQ3 (black hole quencher-3) derivatives] at the ^{2}R group of the cephem core. These reagents are essential assay components for the GeneBLAzer™ technology for sensing gene expression in living mammalian cells using fluorescence microscopy.

A near-infrared fluorogenic cephalosporin **29** for imaging β-lactamase expression in living mammalian cells was developed by Xing et al. (Table 2).[91,92] The authors designed a non-fluorescent substrate by

Table 2
Examples of fluorogenic cephalosporin-based reagents in which the hydrolyzed cephalosporin is detected.

Cephalosporin reagent	Species or process detected	Assay method/type of β-lactamase detected/reference
 **28**		Fluorescence TEM-1 [87,89]
  **29** Z = H, SO_3^-		Near-infrared fluorescence TEM-1 [91–93]
 **30**  	 	Ratiometric fluorescence TEM-1, NDM-1, AmpC, ESBL [94]

introducing a fluorescent and a quencher group into the scaffold. Thus, compound **29** contains a fluorogenic probe at the ^{1}R position, which emits at 670 nm when excited at 650 nm, and a quencher group (QSY21) at the ^{2}R position linked to the cephem core via a thiol moiety, which is readily released upon hydrolysis. The latter group is also functionalized with a peracetylated D-glucosamine group to facilitate cell internalization. The hydrolysis of **29** catalyzed by a β-lactamase enzyme triggers release of the quencher group into the medium, thus giving a fluorescent hydrolyzed cephalosporin. It is important to highlight that the sulfoxide form of the cephalosporin was employed to improve the stability of the compound, which also proved to be useful for real-time imaging of pulmonary infections caused by *M. tuberculosis* and BCG, as well as rapid quantification in living mice without needing to sacrifice them [93]. The assay is based on detection of the chromosomal BlaC enzyme.

Alternatively, Zhang et al. developed a fluorescent cephalosporin (compound **30**) with a single emission peak at 590 nm when excited at 440 nm, which is achieved using the FRET approach (Table 2) [94]. In the presence of β-lactamase enzyme, one of the probes in **30** is released into the medium, thus cancelling the FRET effect. As a result, the enzyme activity is quantified by monitoring the ratio of fluorescence intensity at two wavelengths. Compared to assaying absolute fluorescence intensity, this ratiometric approach provides more accurate and reliable sensing of the enzyme activity. Compound **30** proved to be useful for selectively detecting two clinically important β-lactamases, namely NDM-1, which is a metallo-β-lactamase, and AmpC, which is a chromosomal class C β-lactamase encoded by the *ampC* gene, which can be induced in most clinical isolates by some of the β-lactam antibiotics themselves.

4. Carbapenem-based reagents

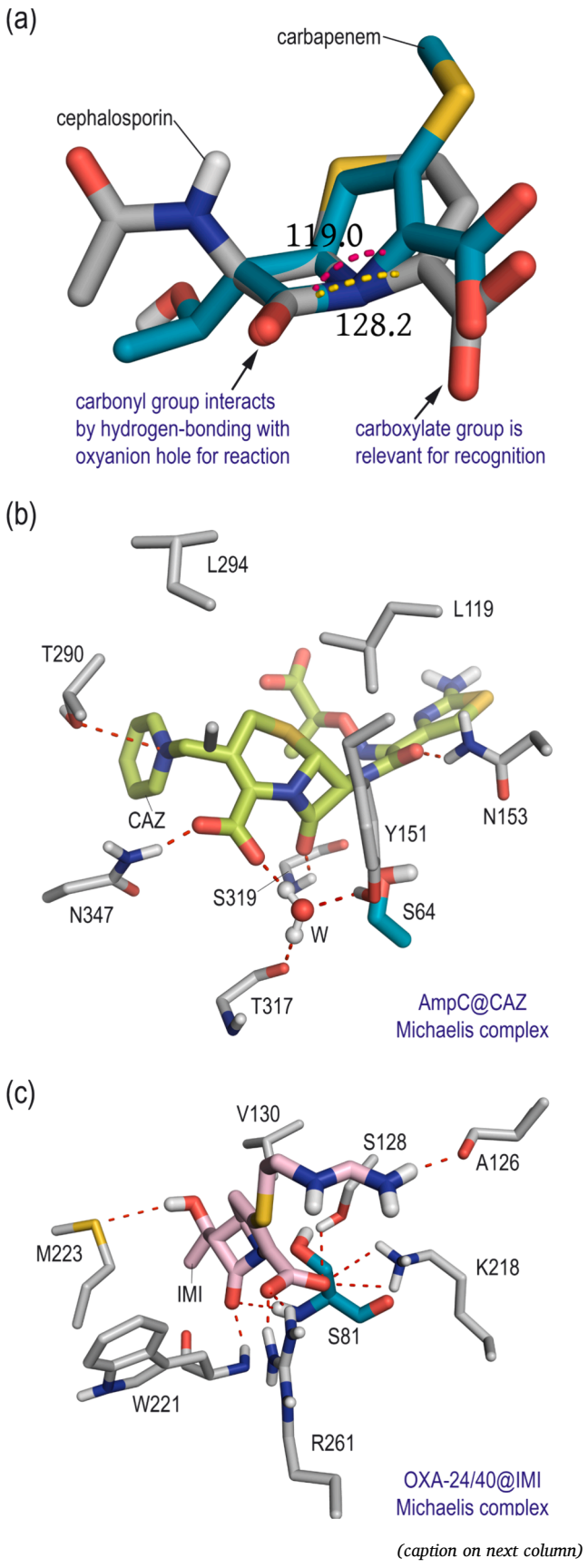
Carbapenemases are enzymes that hydrolyze nearly all β-lactam antibiotics, including carbapenems. Most carbapenemases are plasmid-mediated and are frequently found in life-threatening pathogens such as multidrug-resistant *A. baumannii*, *P. aeruginosa* and Enterobacterales. Infections caused by bacteria that produce this type of enzymes are of great concern as they are seriously compromising the use of carbapenems, which are considered to be the antibiotics of last resort [95]. A comparative analysis of the overall geometry of carbapenems and cephalosporins highlights important differences between both substrates. Thus, superposition of the minimum-energy conformation of cephalosporin (^{1}R = Me, ^{2}R = H) and carbapenem cores (R = Me) by molecular modelling calculations (Gaussian 09 W; [96]) revealed the more pronounced envelope-like conformation of carbapenems, with an angle between the rings of around 109° compared with around 128° for cephalosporins (Fig. 6a). As a result, the position of the carboxylate group, which is a key group for binding and recognition, is also displaced. Both features, along with the opposite stereochemistry of the β-lactam ring substituent, would induce differences in the recognition pattern (mainly carboxylate and carbonyl groups) and their reactivity against nucleophiles [serine residues (serine-β-lactamases) or hydroxide anion (metallo-β-lactamases)] in addition–elimination reactions that lead to hydrolysis (Fig. 6b and 6c). As such, for the specific detection and quantification of carbapenemase activity, chromogenic/fluorogenic probes based on the carbapenem scaffold appear to be the best option. Recent research efforts to develop carbapenem-based devices for the specific detection of carbapenemases are discussed below.

Mao et al. reported a carbapenem derivative modified with a BODIPY moiety (compound **31**) the UV–Vis spectrum of which shows a broad absorption band centered at 544 nm in sodium phosphate buffer pH 7.4 and is mainly non-fluorescent (Fig. 7).[99] In the presence of the metallocarbapenemase IMP-1, intermediate I is initially detected by UV–Vis spectroscopy but rapidly degrades to give BODIPY species **32** (detailed structure not disclosed). Overall, and after incubation for about 40 min, the color of the solution changes from purple to dark purple and finally pink, along with a 200-fold increase in the green fluorescence signal at 512 nm (λ_{exc} = 365 nm), which corresponds to species **32**. Carbapenem **31** also proved to be specific for other carbapenemases such as VIM-27 (class B), NDM-1 (class B), KPC-3 (class A) and OXA-48 (class D), with a relative selectivity between them: IMP-1, NDM-1 ≫ VIM-27, KPC-3 ≫ OXA-48. Only negligible fluorescence was detected upon incubation with ESBLs, such as TEM-1, TEM-3 and CTX-M−9, which are enzymes that confer resistance to penicillins, cephalosporins, and the monobactam aztreonam. Furthermore, high sensitivity was achieved with compound **33** upon replacing the BODIPY moiety in **31** by a phenyl group *para* substituted with an electron-withdrawing group (−NMe_3^+; Fig. 7) [100]. Compound **33** allows the highly sensitive detection of metallo-carbapenemases NDM-1, IMP-4 and VIM-27, as well as the serine-carbapenemases KPC-3 and OXA-48, exhibiting a color change from pale-yellow to red. The specificity of **33** for NDM-1 and IMP-4 was found to be very high, followed by KPC-3 and VIM-27, although it is less sensitive for OXA-48. In addition, after loading this chemical probe into a portable paper chip in combination with quantitative analysis via a smartphone, the presence of carbapenemases in diverse clinical sputum samples from sepsis patients with lung infections can be quickly diagnosed.

Inspired by the high sensitivity and specificity of compound **33** for carbapenemase detection, the 4-(NMe_3^+)phenyl group was also incorporated in the cephalosporin core, compounds **34**–**36**, to explore their specificity in the detection of other β-lactamase subtypes in clinical samples, including broad-spectrum β-lactamase (BSBL), extended-spectrum β-lactamase (ESBL), and AmpC (Table 3). To this end, two ^{1}R groups in the cephalosporin structure, as well as the additional introduction in C6 of a methoxy group with *S* configuration, were studied. While compound **34** detects all the β-lactamases explored, compound **36** specifically reacts with strains producing AmpC and carbapenemases. Compound **35** has an intermediate reactivity being substrate for all the type of β-lactamase tested unless BSBL. A sensor device created with these reagents is able to detect β-lactamase-producing bacteria in 100 clinical samples with 100 % accuracy within 0.25–3 h, enhancing the accuracy of the diagnosis from 48 % to 83 %, and further from 50.6 % to 97.6 % after removal of fungal interference. This sensor detects multidrug-resistant isolates with the lowest detectable concentration at 10^4 CFU mL^{-1}, which is about 1–2 orders of magnitude lower than paper-based nitrocefin reagent [64].

Alternatively, carbapenemase-based substrates linked to a probe via an ether bond susceptible to undergo a conjugated elimination to deliver the sensing species to the media have been described (Fig. 8). Thus, compound **29**, in which the 7-hydroxycoumarin probe is linked to the carbapenem core via an ether linkage, showed good specificity for metallo-carbapenemases, specifically VIM-27, NDM-1, and IMP-1, but failed to detect KPC-3 and OXA-48 enzymes, which are serine-carbapenemases of classes A and D, respectively [86]. Further studies varying the spacer between the carbapenem core and the coumarin probe (benzyl ethers, allyl carbamates, allyl ethers, and secondary amines) led to the discovery of compound **30**, which has a 4-hydroxybenzyl alcohol as spacer [102,103]. The latter spacer was found to be the best option for providing a good chemical sensor for diagnosis in terms of stability, rapid fluorescence signal and high sensitivity.[102] Indeed, compound **30** exhibited a more than 10-fold higher sensitivity for carbapenemase detection than that obtained with the Carba NP test, providing 100 % sensitivity in detecting carbapenemases KPC-2 and NDM-1. When comparing compounds **29** and **30**, it is worth highlighting the relevant sensitivity improvement for **30** as regards detecting OXA-48 (66.7 %, class D) and KPC-2 (100 %, class A).

To further improve the sensitivity of this type of chemical sensor against class D carbapenemases such as OXA-48, Ma et al. developed compound **31** (Fig. 8).[104] Unlike the previous examples, the carbapenem core in **31** lacks the *S*-methyl group in the five-membered ring, like imipenem, feropenem and panipenem. It was considered that the steric hindrance introduced by this methyl group was, to a significant extent, responsible for their more limited effectiveness, which is supported by the two orders of magnitude higher catalytic efficiency of



(caption on next column)

Fig. 6. (a) Overlapping of the minimum-energy conformation of cephalosporin (^{1}R = Me, ^{2}R = H) and carbapenem cores (R = Me) calculated using the AM1 method in the Gaussian 09 W program. (b) Binding mode of ceftolozane (CAZ, 3er generation cephalosporin) in the active site of AmpC for the acylation reaction (Michaelis complex) obtained by molecular dynamics simulation studies. [97] (c) Binding mode of imipenem (IMI) in the active site of OXA-24/40 for the acylation reaction (Michaelis complex) obtained by molecular docking.[98] The positions of the catalytic serine (blue) and relevant side chain residues are shown and labelled. Relevant hydrogen-bonding interactions are shown as red dashed lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

OXA-48 against imipenem and panipenem compared to meropenem, ertapenem or doripenem, which bear this *S*-methyl group [105,106]. A comparison of the available crystal structures for OXA-48 inhibited by imipenem (PDB ID 6P97, 1.80 Å, [107]), faropenem (PDB ID 6PSG, 2.13 Å, [108]), meropenem (PDB ID 6ZRP, 1.74 Å, [109]), and ertapenem (PDB ID 6ZRJ, 1.94 Å,[110]) (acyl-enzyme adducts), also reveals distinct interactions with key residues involved in recognition and binding, specifically R250 (helix α10) and T209 (motif III; K208, T209, G210, Y211), as well as differences in the arrangement of the carbapenem side chains (Fig. 9) [111,112]. The side chain of the modified carbapenems lacking the *S*-methyl group establishes a salt bridge between the carboxylate group and the guanidinium group in R250. In contrast, for those carbapenems bearing a methyl group, the carboxylate group is only anchored to the bottom part of the active site via one of its oxygen atoms. Compound **31**, which includes a resorufin probe linked to the carbapenem core via a longer spacer than for compound **30**, was found to have excellent sensitivity against metallo-carbapenemases NDM-1 and IMP-1, around 10-fold lower for VIM-2 and KPC-2, and around 100-fold lower for OXA-48, but in all cases in the picomolar range.

The synthetic approaches most frequently employed in the preparation of carbapenem-based chemical sensors are exemplified by the synthesis of compounds **26**, **29** and **30** (Scheme 2). The chemical probes are introduced via Stille cross-coupling reaction between triflate **34** and an organotin compound ($Bu_3SnCH = CH_2$, Bu_3SnCH_2OH) using $Pd_2(dba)_3$ as catalyst, followed by subsequent transformation of the resulting new functionality (compounds **35** and **38**). Triflate **34** is readily prepared in three steps from commercially available diazo derivative **32**: (i) TBS-protection of the free hydroxyl group; (ii) Rh-catalyzed intramolecular cyclization of the protected diazo dicarbonyl compound; and (iii) triflate formation. The BODIPY moiety is introduced by way of a Heck coupling reaction between the conjugated ester **35** and iodide **47** using $Pd(OAc)_2$ as catalyst. Removal of the TBS group by treatment with $NH_4{\cdot}HF_2$, followed by deprotection of the ester group by reaction with Zn(0) in sodium phosphate buffer solution (pH 6) affords compound **26**. Final deprotection of the ester group is usually quite a tricky step due to the lability of carbapenems against hydrolysis in basic and acidic media, thus usually providing moderate or even low yields.

The coumarin moiety in compound **29** is introduced by Pd-catalyzed Trost allylic alkylation using carbonate **39**, followed by removal of the protecting groups in **40** (Scheme 2). Carbonate **39** can be prepared in three steps from the conjugated ester **35**: (i) dihydroxylation of the exocyclic alkene with oxidative cleavage using catalytic osmium tetroxide and $NaIO_4$; (ii) reduction of the resulting aldehyde **36**; and (iii) carbonate formation. Compound **30** can be prepared via a Mitsunobu reaction between phenol **46** and alcohol **38**, which was obtained directly from the triflate **34** via a Stille reaction using Bu_3SnCH_2OH and $Pd_2(dba)_3.CHCl_3$ as catalyst, and subsequent removal of the protecting groups.

Finally, Das et al. [113] reported a chemiluminescent assay based on compound **48** for the detection of carbapenemase activity by introduction of a phenoxy-dioxetane luminophore, which is suitable for use under physiological conditions, in the carbapenem (Fig. 10). This process relies on formation of the phenolate-dioxetane intermediate **I** upon

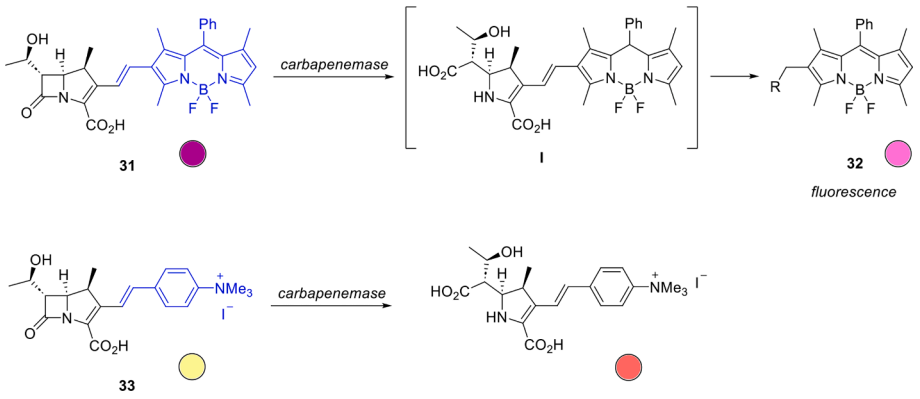
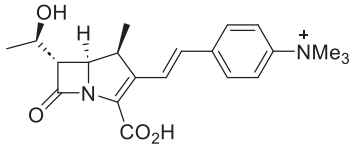
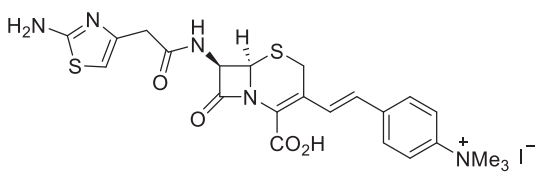
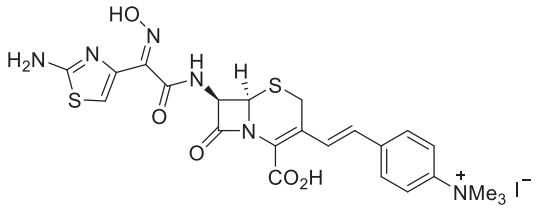
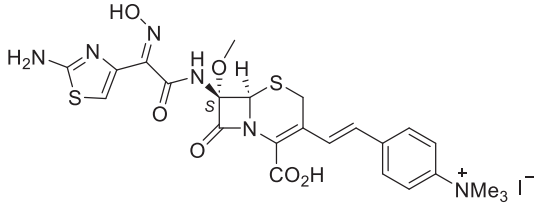


Fig. 7. Carbapenem-based reagents for carbapenemase detection in which the probe signal is detected upon hydrolysis of the carbapenem core either by fluorescence or color change. The chemical probes are highlighted in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Chromogenic reagents containing a 4-(NMe_3^+)phenyl group, compounds 33–36, and its ability to detect the production of different types of β-lactamase enzymes in clinical isolates.[101].

Compound	BSBL[a]	ESBL[b]	AmpC	Carbapenemase
 **33**	×	×	×	√
 **34**	√	√	√	√
 **35**	×	√	√	√
 **36**	×	×	√	√

[a]BSBL = broad-spectrum β-lactamase; [b]ESBL = extended-spectrum β-lactamase.

hydrolysis catalyzed by a carpenemase enzyme, which rapidly decomposes via a chemical excitation process to afford the excited benzoate ester **II**. Subsequent decay of **II** to its ground state releases energy in the form of green photon that it is measured as chemiluminescence.

Assays carried out with *P. aeruginosa* and *K. pneumoniae* strains harboring IMP-2 and KPC-2 enzymes, respectively, along with non-carbapenem resistant strains of *E. coli*, demonstrated the utility of compound **48** for carbapenemase detection. The sensitivity against IMP-

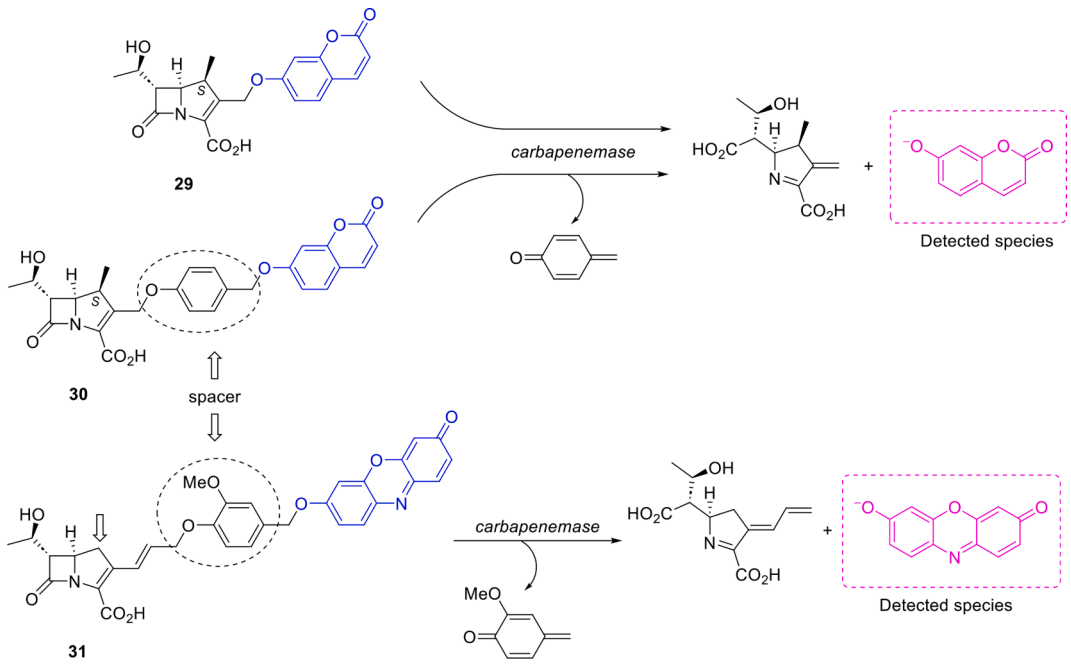


Fig. 8. Carbapenem-based reagents for carbapenemase detection in which the detected species is released to the medium. The chemical probes are highlighted in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2 (class B) was found to be higher than for KPC-2 (class A).

5. Outlook

The methods based on the use of chromogenic and fluorogenic substrates for the early detection of β-lactamase activity represents a great advance in the treatment of infectious diseases. Bypassing the need for time-consuming culture (petri discs, broth culture) or isolation (bacteria lysis) procedures prior to diagnosis facilitates the rapid selection of the most suitable therapy for each patient, thus avoiding the use of ineffective drugs or preserving the use of antibiotics of last resort. Inspired by the success of nitrocefin, a chromogenic cephalosporin-based substrate developed in the 1970s, alternative reagents using the same scaffold have been described. Most substrates reported are based on fluorescent detection, some of them on the hydrolyzed reagent, but mostly on release of the fluorescent form of the probe upon hydrolysis. In general, these fluorogenic reagents show outstanding selectivity and sensitivity for clinically relevant metallo-carbapenemases (VIM, NDM, IMP), while their efficacy against carbapenem-hydrolyzing class D enzymes, such as OXA-48, OXA-23 and OXA-24/40, is less remarkable. It is worth highlighting that metallo-carbapenemases are broad-spectrum hydrolases that transform virtually all β-lactam antibiotics, except monobactams, while carbapenems are the distinctive substrate for class D carbapenemases. Therefore, it seems that the use of cephalosporin scaffolds should not be a good option for detecting the latter enzymes, as seen for nitrocefin and related compounds/kits. Furthermore, in some of the fluorogenic reagents reported, larger and less affordable fluorescent probes were introduced to enhance the detection. However, these are bulky substituents, and expensive groups from a synthetic point of view, that need to be accommodated in the catalytic site for enzyme transformation, which might be a disadvantage for those enzymes with a more rigid or/and less accessible active site, such as OXA-23 or OXA-24/40. In these cases, design of the optimal spacer between the scaffold and the probe that is able to provide flexibility, as well as promote attractive interactions with the macromolecule, would be an important task to achieve a good diagnostic kit.

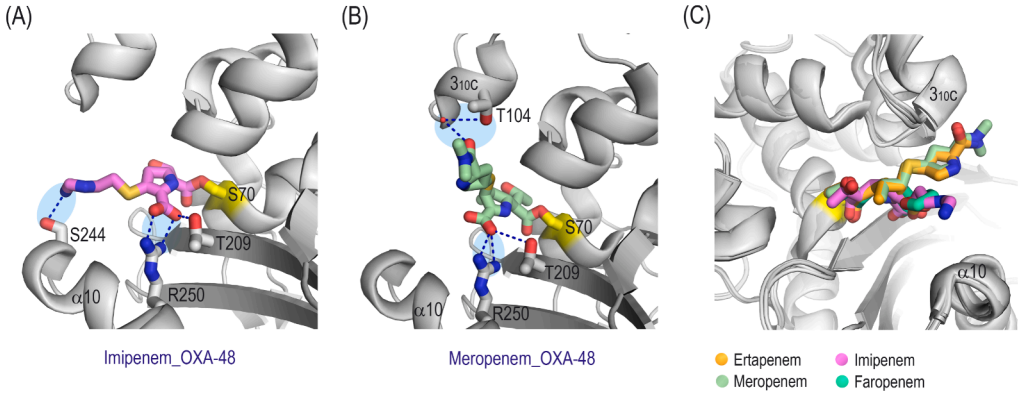
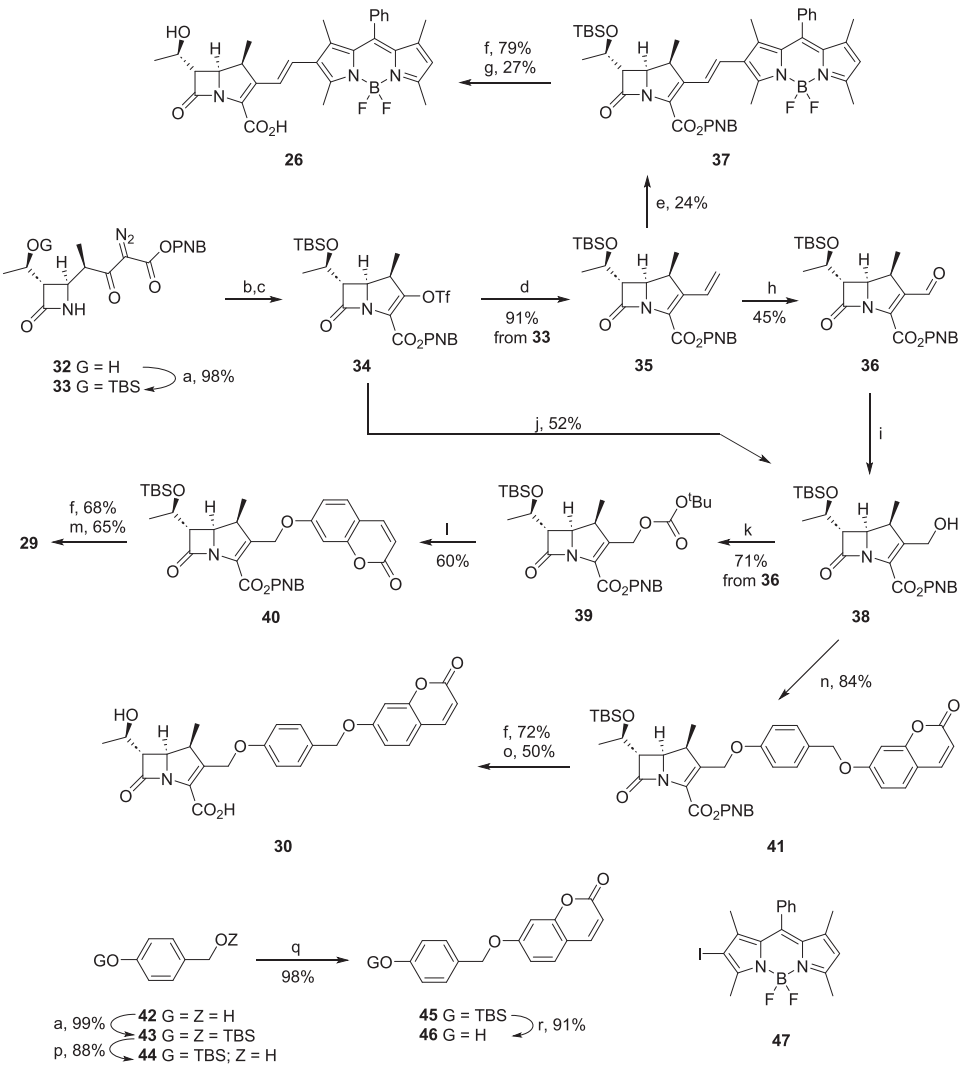


Fig. 9. (A,B) Detailed views of the active site of the OXA-48 acyl-enzyme complex after reaction with imipenem (A, PDB ID 6P97, 1.80 Å, [107]) and meropenem (B, PDB ID 6ZRP, 1.74 Å, [108]). Relevant hydrogen-bonding and electrostatic interactions between the ligands and the enzyme are shown as dashed lines (blue) and highlighted with a blue shadow. Relevant residues are shown and labeled. (C) Superposition of the crystal structure of OXA-48 covalently modified with ertapenem (PDB ID 6ZRJ, 1.94 Å, [109]), faropenem (PDB ID 6PSG, 2.13 Å, [110]), imipenem (panel A) and meropenem (panel B). The catalytic serine residue S70 is shown in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



Scheme 2. *Reagents and conditions*: (a) TBSCl, imidazole, DMF, RT. (b) $Rh_2(OAc)_4$, $ZnCl_2$, CH_2Cl_2, Δ. (c) 2,2,6,6-tetramethylpiperidine, DIPEA, Tf_2O, –50 °C. (d) $Bu_3SnCH=CH_2$, $Pd_2(dba)_3$, P(2-furyl)$_3$, $ZnCl_2$, *N*-methyl-2-pyrrolidone, 30 °C. (e) **47**, $Pd(OAc)_2$, P(*o*-Tol)$_3$, Et_3N, DMF, 80 °C. (f) $NH_4.HF_2$, *N*-methyl-2-pyrrolidone, DMF, RT. (g) Zn, THF/phosphate buffer (pH 6.0). (h) OsO_4, $NaIO_4$, H_5IO_6, THF/H_2O, RT. (i) BH_3·THF, −15 °C. (j) Bu_3SnCH_2OH, $Pd_2(dba)_3.CHCl_3$, P(2-furyl)$_3$, $ZnCl_2$, *N*-methyl-2-pyrrolidone, 70 °C. (k) isobutyl chloroformate, Py, CH_2Cl_2, 0 °C. (l) Umbelliferone, $Pd_2(dba)_3.CHCl_3$, $P(OEt)_3$, PhMe/THF, 30 °C. (m) H_2, Pt/C, THF/PBS (pH 7.0), RT. (n) 46, DIAD, PPh_3, PhMe, 0 °C. (o) H_2, Rh/C, 1 M $NaHCO_3$, THF/H_2O, RT. (p) NIS, MeOH, RT. (q) Umbelliferone, DIAD, Ph_3P, PhMe, RT. (r) AcOH, TBAF, THF, RT.

Although studies into the development of carbapenem-based reagents are still in their infancy, the diagnostic enhancement achieved by some of the reagents reported against OXA-48 enzyme are excellent. Finally, despite the greater synthetic difficulties of the chemistry of the carbapenem scaffold, mainly resulting from its greater sensitivity to hydrolysis, especially in the final deprotection stage of the carboxylate group, the advantages of this scaffold are evident. Although fluorogenic substrates are very sensitive reagents because of the fluorescence detection, the visual identification of β-lactamase activity by using chromogenic reagents, which do not require the use of sophisticated

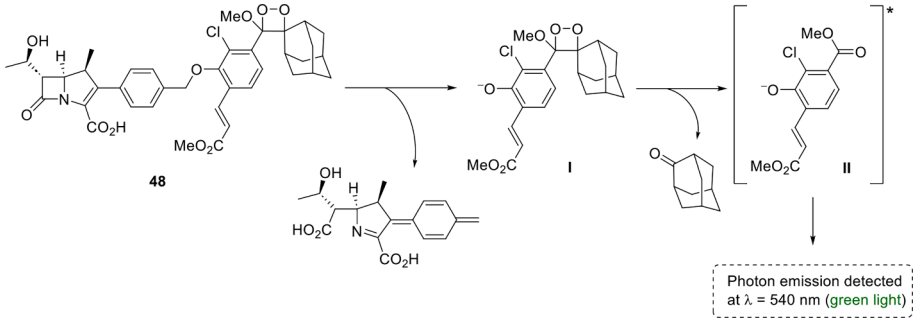


Fig. 10. Chemiluminescent carbapenem-based assay for detecting carbapenemase production. Schematic representation of the chemical excitation activation mechanism.

instrumentation and qualified personal, are more practical and particularly suitable for rapid point-of-care detection. In this context, the recent research efforts to provide chromogenic carbapenem-based reagents are excellent. As such, an exciting future is predicted for this type of reagent in terms of translation to the clinic.

CRediT authorship contribution statement

Rafael Canabal: Writing – review & editing. **Concepción González-Bello:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was funded by the Spanish State Agency of Research (PID2022-136963OB-I00/AEI/10.13039/501100011033, CG-B), the Xunta de Galicia [ED431C 2021/29 and Centro singular de investigación de Galicia accreditation 2023-2027 (ED431G 2023/03), CG-B], and the European Regional Development Fund (ERDF). We thank Dr. Jorge Arca-Suárez for helpful discussions on writing this manuscript.

References

[1] N. Gilbert, Four stories of antibacterial breakthroughs, Nature 555 (2018) S5–S7, https://doi.org/10.1038/d41586-018-02475-3.
[2] D.G.J. Larsson, C.-F. Flach, Antibiotic resistance in the environment, Nat. Rev. Microbiol. 20 (2022) 257–269, https://doi.org/10.1038/s41579-021-00649-x.
[3] R.E. Baker, A.S. Mahmud, I.F. Miller, M. Rajeev, F. Rasambainarivo, B.L. Rice, S. Takahashi, A.J. Tatem, C.E. Wagner, L.-F. Wang, A. Wesolowski, C.J.E. Metcalf, Infectious disease in an era of global change, Nat. Rev. Microbiol. 20 (2022) 193–205, https://doi.org/10.1038/s41579-021-00639-z.
[4] E.D. Brown, G.D. Wright, Antibacterial drug discovery in the resistance era, Nature 529 (2016) 336–343, https://doi.org/10.1038/nature17042.
[5] Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis, Lancet, 399 (2022) 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0.
[6] U. Theuretzbacher, Antibacterial innovation in European SMEs, Nat. Rev. Drug Discov. 15 (2016) 812–813, https://doi.org/10.1038/nrd.2016.229.
[7] G.D. Wright, The antibiotic resistome: the nexus of chemical and genetic diversity, Nat. Rev. Microbiol. 5 (2007) 175–186, https://doi.org/10.1038/nrmicro1614.
[8] A.Y.G. Yip, O.G. King, O. Omelchenko, S. Kurkimat, V. Horrocks, P. Mostyn, N. Danckert, R. Ghani, G. Satta, E. Jauneikaite, F.J. Davies, T.B. Clarke, B. H. Mullish, J.R. Marchesi, J.A.K. McDonald, Antibiotics promote intestinal growth of carbapenem-resistant *Enterobacteriaceae* by enriching nutrients and depleting microbial metabolites, Nat. Commun. 14 (2023) 5094, https://doi.org/10.1038/s41467-023-40872-z.
[9] V.M. D'Costa, C.E. King, L. Kalan, M. Morar, W.W.L. Sung, C. Schwarz, D. Froese, G. Zazula, F. Calmels, R. Debruyne, G.B. Golding, H.N. Poinar, G.D. Wright, Antibiotic resistance is ancient, Nature 477 (2011) 457–461, https://doi.org/10.1038/nature10388.
[10] C. Walsh, Molecular mechanisms that confer antibacterial drug resistance, Nature 406 (2000) 775–781, https://doi.org/10.1038/35021219.
[11] J.M.A. Blair, M.A. Webber, A.J. Baylay, D.O. Ogbolu, L.J.V. Piddock, Molecular mechanisms of antibiotic resistance, Nat. Rev. Microbiol. 13 (2015) 42–51, https://doi.org/10.1038/nrmicro3380.
[12] B. Khameneh, R. Diab, K. Ghazvini, B.S. Fazly Bazzaz, Breakthroughs in bacterial resistance mechanisms and the potential ways to combat them, Microb. Pathogen. 95 (2016) 32–42, https://doi.org/10.1016/j.micpath.2016.02.009.
[13] C. González-Bello, D. Rodríguez, M. Pernas, Á. Rodríguez, E. Colchón, β-Lactamase inhibitors to restore the efficacy of antibiotics against superbugs, J. Med. Chem. 63 (2020) 1859–1881, https://doi.org/10.1021/acs.jmedchem.9b01279.
[14] K. Bush, Past and present perspectives on β-lactamases, Antimicrob. Agents Chemother. 62 (2018) e01076–e11018, https://doi.org/10.1128/aac.01076-18.
[15] K. Bush, P.A. Bradford, Interplay between β-lactamases and new β-lactamase inhibitors, Nat. Rev. Microbiol. 17 (2019) 295–306, https://doi.org/10.1038/s41579-019-0159-8.
[16] K. Poole, Resistance to β-lactam antibiotics, CMLS, Cell. Mol. Life Sci. 61 (2004) 2200–2223, https://doi.org/10.1007/s00018-004-4060-9.
[17] M.S. Cole, P.V. Hegde, C.C. Aldrich, β-Lactamase-mediated fragmentation: historical perspectives and recent advances in diagnostics, imaging, and antibacterial design, ACS Infect. Dis. 8 (2022) 1992–2018, https://doi.org/10.1021/acsinfecdis.2c00315.
[18] T. Naas, S. Oueslati, R.A. Bonnin, M.L. Dabos, A. Zavala, L. Dortet, P. Retailleau, B.I. Iorga, Beta-lactamase database (BLDB) – structure and function, J. Enzyme Inhib. Med. Chem. 32 (2017) 917–919, https://doi.org/10.1080/14756366.2017.1344235.
[19] N.P. Krishnan, N.Q. Nguyen, K.M. Papp-Wallace, R.A. Bonomo, F. van den Akker, Inhibition of *Klebsiella* β-lactamases (SHV-1 and KPC-2) by avibactam: a structural study, PLoS One 10 (2015) e0136813.
[20] R. Salimraj, P. Hinchliffe, M. Kosmopoulou, J.M. Tyrrell, J. Brem, S.S. van Berkel, A. Verma, R.J. Owens, M.A. McDonough, T.R. Walsh, C.J. Schofield, J. Spencer, Crystal structures of VIM-1 complexes explain active site heterogeneity in VIM-class metallo-β-lactamases, FEBS J. 286 (2019) 169–183, https://doi.org/10.1111/febs.14695.
[21] S.D. Lahiri, S. Mangani, T. Durand-Reville, M. Benvenuti, F. De Luca, G. Sanyal, J. D. Docquier, Structural insight into potent broad-spectrum inhibition with reversible recyclization mechanism: avibactam in complex with CTX-M-15 and *Pseudomonas aeruginosa* AmpC β-lactamases, Antimicrob. Agents Chemother. 57 (2013) 2496–2505, https://doi.org/10.1128/aac.02247-12.
[22] G. Bou, E. Santillana, A. Sheri, A. Beceiro, J.M. Sampson, M. Kalp, C.R. Bethel, A. M. Distler, S.M. Drawz, S.R.R. Pagadala, F. van den Akker, R.A. Bonomo, A. Romero, J.D. Buynak, Design, synthesis, and crystal structures of 6-alkylidene-2′-substituted penicillanic acid sulfones as potent inhibitors of *Acinetobacter baumannii* OXA-24 carbapenemase, J. Am. Chem. Soc. 132 (2010) 13320–13331, https://doi.org/10.1021/ja104092z.
[23] E.-J. Yoon, S.H. Jeong, Class D β-lactamases, J. Antimicrob. Chemother. 76 (2020) 836–864, https://doi.org/10.1093/jac/dkaa513.
[24] F. Khan, B. Chaudhary, A.U. Khan, Class D type β-lactamases, in: M. Shahid, A. Singh, H. Sami (Eds.), β-Lactam Resistance in Gram-Negative Bacteria: Threats and Challenges, Springer Nature Singapore, Singapore, 2022, pp. 125–138.
[25] K. Bush, P.A. Bradford, Epidemiology of β-lactamase-producing pathogens, e00047-19, Clin. Microbiol. Rev. 33 (2020), https://doi.org/10.1128/cmr.00047-19.
[26] J.D.D. Pitout, G. Peirano, M.M. Kock, K.A. Strydom, Y. Matsumura, The global ascendency of OXA-48-type carbapenemases, e00102-00119, Clin. Microbiol. Rev. 33 (2019), https://doi.org/10.1128/cmr.00102-19.
[27] H. Leonard, R. Colodner, S. Halachmi, E. Segal, Recent advances in the race to design a rapid diagnostic test for antimicrobial resistance, ACS Sensors 3 (2018) 2202–2217, https://doi.org/10.1021/acssensors.8b00900.
[28] G. Bou, A. Fernández-Olmos, C. García, J.A. Sáez-Nieto, S. Valdezate, Métodos de identificación bacteriana en el laboratorio de microbiología, Enferm. Infecc. Microbiol. Clin. 29 (2011) 601–608, https://doi.org/10.1016/j.eimc.2011.03.012.
[29] O. del Corpo, J. Senécal, J.M. Hsu, A. Lawandi, T.C. Lee, Rapid phenotypic testing for detection of carbapenemase- or extended-spectrum ß-lactamase-producing Enterobacterales directly from blood cultures: a systematic review and meta-analysis, Clin. Microbiol. Infect. 29 (2023) 1516–1527, https://doi.org/10.1016/j.cmi.2023.09.007.
[30] Y.L. Lee, H.M. Chen, I.M. Hii, P.R. Hsueh, Carbapenemase-producing Enterobacterales infections: recent advances in diagnosis and treatment, Int. J. Antimicrob. Agents 59 (2022) 106528, https://doi.org/10.1016/j.ijantimicag.2022.106528.
[31] S. Ambretti, P. Gaibani, A. Berlingeri, M. Cordovana, M.V. Tamburini, G. Bua, M. P. Landini, V. Sambri, Evaluation of phenotypic and genotypic approaches for the detection of class A and class B carbapenemases in *Enterobacteriaceae*, Microb. Drug. Resist. 19 (2013) 212–215, https://doi.org/10.1089/mdr.2012.0165.
[32] S. Bernabeu, L. Dortet, T. Naas, Evaluation of the β-CARBA™ test, a colorimetric test for the rapid detection of carbapenemase activity in Gram-negative bacilli, J. Antimicrob. Chemother. 72 (2017) 1646–1658, https://doi.org/10.1093/jac/dkx061.
[33] K. van der Zwaluw, A. de Haan, G.N. Pluister, H.J. Bootsma, A.J. de Neeling, L. M. Schouls, The carbapenem inactivation method (CIM), a simple and low-cost alternative for the Carba NP test to assess phenotypic carbapenemase activity in gram-negative rods, PLoS One 10 (2015) e0123690.
[34] V.M. Pierce, P.J. Simner, D.R. Lonsway, D.E. Roe-Carpenter, J.K. Johnson, W. B. Brasso, A.M. Bobenchik, Z.C. Lockett, A. Charnot-Katsikas, M.J. Ferraro, R. B. Thomson Jr., S.G. Jenkins, B.M. Limbago, S. Das, Modified carbapenem inactivation method for phenotypic detection of carbapenemase production among *Enterobacteriaceae*, J. Clin. Microbiol. 55 (2017) 2321–2333, https://doi.org/10.1128/jcm.00193-17.
[35] K. Lee, Y. Chong, H.B. Shin, Y.A. Kim, D. Yong, J.H. Yum, Modified Hodge and EDTA-disk synergy tests to screen metallo-β-lactamase-producing strains of *Pseudomonas* and *Acinetobacter* species Clin, Microbiol. Infect. 7 (2001) 88–91, https://doi.org/10.1046/j.1469-0691.2001.00204.x.
[36] Y. Arakawa, N. Shibata, K. Shibayama, H. Kurokawa, T. Yagi, H. Fujiwara, M. Goto, Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds, J. Clin. Microbiol. 38 (2000) 40–43, https://doi.org/10.1128/jcm.38.1.40-43.2000.
[37] E. Literacka, M. Herda, A. Baraniak, D. Żabicka, W. Hryniewicz, A. Skoczyńska, M. Gniadkowski, Evaluation of the Carba NP test for carbapenemase detection in *Enterobacteriaceae*, *Pseudomonas* spp. and *Acinetobacter* spp., and its practical use in the routine work of a national reference laboratory for susceptibility testing, Eur. J. Clin. Microbiol. Infect. Dis. 36 (2017) 2281–2287, https://doi.org/10.1007/s10096-017-3062-0.

[38] L. Poirel, P. Nordmann, Rapidec carba NP test for rapid detection of carbapenemase producers, J. Clin. Microbiol. 53 (2015) 3003–3008, https://doi.org/10.1128/jcm.00977-15.
[39] Z. Lu, X. Wang, L. Ma, L. Dou, X. Zhao, J. Tao, Y. Wang, S. Wang, D. Liu, Y. Shen, X. Yu, W. Yu, L. Jia, Z. Wang, J. Shen, K. Wen, Carba PBP: a novel penicillin-binding protein-based lateral flow assay for rapid phenotypic detection of carbapenemase-producing Enterobacterales, J. Clin. Microbiol. 62 (2024) e0012023.
[40] S. Jenkins, N.A. Ledeboer, L.F. Westblade, C.A. Burnham, M.L. Faron, Y. Bergman, R. Yee, B. Mesich, D. Gerstbrein, M.A. Wallace, A. Robertson, K. A. Fauntleroy, A.S. Klavins, R. Malherbe, A. Hsiung, P.J. Simner, Evaluation of NG-Test Carba 5 for rapid phenotypic detection and differentiation of five common carbapenemase families: results of a multicenter clinical evaluation, e00344-00320, J. Clin. Microbiol. 58 (2020), https://doi.org/10.1128/jcm.00344-20.
[41] M. Sadek, L. Poirel, P. Nordmann, Rapid detection of carbapenemase-producing *Pseudomonas* spp. using the NitroSpeed-Carba NP test, Diagn. Microbiol. Infect. Dis. 99 (2021) 115280, https://doi.org/10.1016/j.diagmicrobio.2020.115280.
[42] L. Dortet, L. Poirel, P. Nordmann, Rapid detection of carbapenemase-producing *Pseudomonas* spp, J. Clin. Microbiol. 50 (2012) 3773–3776, https://doi.org/10.1128/jcm.01597-12.
[43] P. Nordmann, L. Poirel, L. Dortet, Rapid detection of carbapenemase-producing *Enterobacteriaceae*, Emerg. Infect. Dis. 18 (2012) 1503–1507, https://doi.org/10.3201/eid1809.120355.
[44] L. Dortet, L. Poirel, P. Nordmann, Rapid identification of carbapenemase types in *Enterobacteriaceae* and *Pseudomonas* spp. by using a biochemical test, Antimicrob. Agents Chemother. 56 (2012) 6437–6440, https://doi.org/10.1128/aac.01395-12.
[45] L. Miglietta, A. Moniri, I. Pennisi, K. Malpartida-Cardenas, H. Abbas, K. Hill-Cawthorne, F. Bolt, E. Jauneikaite, F. Davies, A. Holmes, P. Georgiou, J. Rodriguez-Manzano, Coupling machine learning and high throughput multiplex digital PCR enables accurate detection of carbapenem-resistant genes in clinical isolates, Front. Mol. Biosci. 8 (2021) 775299, https://doi.org/10.3389/fmolb.2021.775299.
[46] I. Burckhardt, S. Zimmermann, Using matrix-assisted laser desorption ionization-time of flight mass spectrometry to detect carbapenem resistance within 1 to 2.5 hours, J. Clin. Microbiol. 49 (2011) 3321–3324, https://doi.org/10.1128/jcm.00287-11.
[47] J. Hrabák, R. Walková, V. Studentová, E. Chudácková, T. Bergerová, Carbapenemase activity detection by matrix-assisted laser desorption ionization-time of flight mass spectrometry, J. Clin. Microbiol. 49 (2011) 3222–3227, https://doi.org/10.1128/jcm.00984-11.
[48] H. Savli, A. Karadenizli, F. Kolayli, S. Gundes, U. Ozbek, H. Vahaboglu, Expression stability of six housekeeping genes: A proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR, J. Med. Microbiol. 52 (2003) 403–408, https://doi.org/10.1099/jmm.0.05132-0.
[49] P. Bogaerts, A.S. Berger, S. Evrard, T.D. Huang, Comparison of two multiplex immunochromatographic assays for the rapid detection of major carbapenemases in Enterobacterales, J. Antimicrob. Chemother. 75 (2020) 1491–1494, https://doi.org/10.1093/jac/dkaa043.
[50] Y. Glupczynski, A. Jousset, S. Evrard, R.A. Bonnin, T.-D. Huang, L. Dortet, P. Bogaerts, T. Naas, Prospective evaluation of the OKN K-SeT assay, a new multiplex immunochromatographic test for the rapid detection of OXA-48-like, KPC and NDM carbapenemases, J. Anticrob. Chemother. 72 (2017) 1955–1960, https://doi.org/10.1093/jac/dkx089.
[51] D.W. Wareham, M.H. Abdul Momin, Rapid detection of carbapenemases in enterobacteriaceae: evaluation of the resist-3 O.K.N. (OXA-48, KPC, NDM) lateral flow multiplexed assay, J. Clin. Microbiol. 55 (2017) 1223–1225, https://doi.org/10.1128/jcm.02471-16.
[52] A. Saleh, S. Göttig, A.G. Hamprecht, Multiplex immunochromatographic detection of OXA-48, KPC, and NDM carbapenemases: impact of inoculum, antibiotics, and agar, e00050-00018, J. Clin. Microbiol. 56 (2018), https://doi.org/10.1128/jcm.00050-18.
[53] C. Greissl, A. Saleh, A. Hamprecht, Rapid detection of OXA-48-like, KPC, NDM, and VIM carbapenemases in Enterobacterales by a new multiplex immunochromatographic test, Eur. J. Clin. Microbiol. Infect. Dis. 38 (2019) 331–335, https://doi.org/10.1128/aac.1.4.283.
[54] C.H. O'Callaghan, A. Morris, S.M. Kirby, A.H. Shingler, Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate, Antimicrob. Agents Chemother. 1 (1972) 283–288.
[55] C.H. O'Callaghan, J.C. Clark, J. Kennedy, A. Montrose, S.M. Kirby, H. Iver, A.G. Long, A. Morris, A.H. Shingler, N.G. Weir, Test for β-lactamase activity using chromogenic cephalosporin compound, US Patent, US3830700A, priority data Jan 23, 1970.
[56] S. Mobashery, M. Johnston, Preparation of ceph-3-em esters unaccompanied by $\Delta^3 \rightarrow {}^{\Delta}2$ isomerization of the cephalosporin, J. Org. Chem. 51 (1986) 4723–4726, https://doi.org/10.1021/jo00374a045.
[57] N.C.M.E. Barendse, P.A.M. van der Klein, J. Verweij, H.A. Witkamp, W.J. van Zoest, E. de Vroom, Synthesis of the β-lactamase indicators cefesone and nitrocefin, Synthesis 2 (1998) 145–147, https://doi.org/10.1055/s-1998-2018.
[58] M. Lee, D. Hesek, S. Mobashery, A practical synthesis of nitrocefin, J. Org. Chem. 70 (2005) 367–369, https://doi.org/10.1021/jo0487395.
[59] C. Bebrone, C. Moali, F. Mahy, S. Rival, J.D. Docquier, G.M. Rossolini, J. Fastrez, R.F. Pratt, J.M. Frère, M. Galleni, CENTA as a chromogenic substrate for studying β-lactamases, Antimicrob. Agents Chemother. 45 (2001) 1868–1871, https://doi.org/10.1128/aac.45.6.1868-1871.2001.
[60] R.N. Jones, H.W. Wilson, W.J. Novick Jr., A.L. Barry, C. Thornsberry, *In vitro* evaluation of CENTA, a new β-lactamase-susceptible chromogenic cephalosporin reagent, J. Clin. Microbiol. 15 (1982) 954–958, https://doi.org/10.1128/jcm.15.4.677-683.1982.
[61] R.N. Jones, H.W. Wilson, W.J. Novick, In vitro evaluation of pyridine-2-azo-p-dimethylaniline cephalosporin, a new diagnostic chromogenic reagent, and comparison with nitrocefin, cephacetrile, and other β-lactam compounds, J. Clin. Microbiol. 15 (1982) 677–683, https://doi.org/10.1128/jcm.15.5.954-958.1982.
[62] S. Kobayashi, S. Arai, S. Hayashi, T. Sakaguchi, Simple assay of beta-lactamase with agar medium containing a chromogenic cephalosporin, pyridinium-2-azo-p-dimethylaniline chromophore (PADAC), Antimicrob. Agents Chemother. 32 (1988) 1040–1045, https://doi.org/10.1128/aac.32.7.1040.
[63] A. Ghavami, G. Labbé, J. Brem, V.J. Goodfellow, L. Marrone, C.A. Tanner, D. T. King, M. Lam, N.C.J. Strynadka, D.R. Pillai, S. Siemann, J. Spencer, C. J. Schofield, G.I. Dmitrienko, Assay for drug discovery: synthesis and testing of nitrocefin analogues for use as β-lactamase substrates, Anal. Biochem. 486 (2015) 75–77, https://doi.org/10.1016/j.ab.2015.06.032.
[64] K.E. Boehle, J. Gilliand, C.R. Wheeldon, A. Holder, J.A. Adkins, B.J. Geiss, E. P. Ryan, C.S. Henry, Utilizing paper-based devices for antimicrobial-resistant bacteria detection, Angew. Chem. Int. Ed. Engl. 56 (2017) 6886–6890, https://doi.org/10.1002/anie.201702776.
[65] B. Chantemesse, L. Betelli, S. Solanas, F. Vienney, L. Bollache, A. Hartmann, M. Rochelet, A nitrocefin-based amperometric assay for the rapid quantification of extended-spectrum β-lactamase-producing *Escherichia coli* in wastewaters, Water Res. 109 (2017) 375–381, https://doi.org/10.1016/j.watres.2016.11.066.
[66] D. Alkekhia, H. Safford, S. Shukla, R. Hopson, A. Shukla, β-Lactamase triggered visual detection of bacteria using cephalosporin functionalized biomaterials, Chem. Commun. 56 (2020) 11098–11101, https://doi.org/10.1039/d0cc04088f.
[67] H. Hanaki, R. Kubo, T. Nakano, M. Kurihara, K. Sunagawa, Characterization of HMRZ-86: a novel chromogenic cephalosporin for the detection of extended-spectrum β-lactamases, J. Antimicrob. Chemother. 53 (2004) 888–889, https://doi.org/10.1093/jac/dkh166.
[68] H. Hanaki, Y. Koide, H. Yamazaki, R. Kubo, T. Nakano, K. Atsuda, K. Sunakawa, Substrate specificity of HMRZ-86 for beta-lactamases, including extended-spectrum β-lactamases (ESBLs), J. Infect. Chemother. 13 (2007) 390–395, https://doi.org/10.1007/s10156-007-0563-2.
[69] A. Renvoisé, D. Decré, R. Amarsy-Guerle, T.D. Huang, C. Jost, I. Podglajen, L. Raskine, N. Genel, P. Bogaerts, V. Jarlier, G. Arlet, Evaluation of the βLacta test, a rapid test detecting resistance to third-generation cephalosporins in clinical strains of *Enterobacteriaceae*, J. Clin. Microbiol. 51 (2013) 4012–4017, https://doi.org/10.1128/jcm.01936-13.
[70] M.I. Morosini, M. García-Castillo, M. Tato, D. Gijón, A. Valverde, P. Ruiz-Garbajosa, R. Cantón, Rapid detection of β-lactamase-hydrolyzing extended-spectrum cephalosporins in *Enterobacteriaceae* by use of the new chromogenic βLacta test, J. Clin. Microbiol. 52 (2014) 1741–1744, https://doi.org/10.1128/jcm.03614-13.
[71] W. Gao, B. Xing, R.Y. Tsien, J. Rao, Novel fluorogenic substrates for imaging β-lactamase gene expression, J. Am. Chem. Soc. 125 (2003) 11146–11147, https://doi.org/10.1021/ja036126o.
[72] J. Xie, R. Mu, M. Fang, Y. Cheng, F. Senchyna, A. Moreno, N. Banaei, J. Rao, A dual-caged resorufin probe for rapid screening of infections resistant to lactam antibiotics, Chem. Sci. 12 (2021) 9153–9161, https://doi.org/10.1039/d1sc01471d.
[73] H. Xie, J. Mire, Y. Kong, M. Chang, H.A. Hassounah, C.N. Thornton, J. C. Sacchettini, J.D. Cirillo, J. Rao, Rapid point-of-care detection of the tuberculosis pathogen using a BlaC-specific fluorogenic probe, Nat. Chem. 4 (2012) 802–809, https://doi.org/10.1038/nchem.1435.
[74] Y. Cheng, H. Xie, P. Sule, H. Hassounah, E.A. Graviss, Y. Kong, J.D. Cirillo, J. Rao, Fluorogenic probes with substitutions at the 2 and 7 positions of cephalosporin are highly BlaC-specific for rapid *Mycobacterium tuberculosis* detection, Angew. Chem. Int. Ed. Engl. 53 (2014) 9360–9364, https://doi.org/10.1002/anie.201405243.
[75] Y. Cheng, J. Xie, K.H. Lee, R.L. Gaur, A. Song, T. Dai, H. Ren, J. Wu, Z. Sun, N. Banaei, D. Akin, J. Rao, Rapid and specific labeling of single live *Mycobacterium tuberculosis* with a dual-targeting fluorogenic probe, Sci. Transl. Med. 10 (2018) eaar4470, https://doi.org/10.1126/scitranslmed.aar4470.
[76] A. Song, Y. Cheng, J. Xie, N. Banaei, J. Rao, Intramolecular substitution uncages fluorogenic probes for detection of metallo-carbapenemase-expressing bacteria, Chem. Sci. 8 (2017) 7669–7674, https://doi.org/10.1039/C7SC02416A.
[77] H. Yao, M.-K. So, J. Rao, A bioluminogenic substrate for *in vivo* imaging of β-lactamase activity, Angew. Chem. Int. Ed. Engl. 46 (2007) 7031–7034, https://doi.org/10.1002/anie.200701931.
[78] T. Dai, J. Xie, J.A. Buonomo, A. Moreno, N. Banaei, C.R. Bertozzi, J. Rao, Bioluminogenic probe for rapid, ultrasensitive detection of β-lactam-resistant bacteria, Anal. Chem. 95 (2023) 7329–7335, https://doi.org/10.1021/acs.analchem.3c00478.
[79] H.B.D. Thai, J.K. Yu, B.S. Park, Y.-J. Park, S.-J. Min, D.-R. Ahn, A fluorogenic substrate of β-lactamases and its potential as a probe to detect the bacteria resistant to the third-generation oxyimino-cephalosporins, Biosens. Bioelectron. 77 (2016) 1026–1031, https://doi.org/10.1016/j.bios.2015.10.081.
[80] H. Shi, Y. Cheng, K.H. Lee, R.F. Luo, N. Banaei, J. Rao, Engineering the stereochemistry of cephalosporin for specific detection of pathogenic carbapenemase-expressing bacteria, Angew. Chem. Int. Ed. Engl. 53 (2014) 8113–8116, https://doi.org/10.1002/anie.201402012.

[81] L. Li, Z. Li, W. Shi, X. Li, H. Ma, Sensitive and selective near-infrared fluorescent off–on probe and its application to imaging different levels of β-lactamase in *Staphylococcus aureus*, Anal. Chem. 86 (2014) 6115–6120, https://doi.org/10.1021/ac501288e.
[82] W. Mao, X. Qian, J. Zhang, L. Xia, H. Xie, Specific detection of extended-spectrum β-lactamase activities with a ratiometric fluorescent probe, ChemBioChem 18 (2017) 1990–1994, https://doi.org/10.1002/cbic.201700447.
[83] W. Mao, L. Xia, Y. Wang, H. Xie, A self-immobilizing and fluorogenic probe for β-lactamase detection, Chem. Asian J. 11 (2016) 3493–3497, https://doi.org/10.1002/asia.201601344.
[84] S. Shaltiel, Thiolysis of some dinitrophenyl derivatives of amino acids, Biochem. Biophys. Res. Commun. 29 (1967) 178–183, https://doi.org/10.1016/0006-291X(67)90583-9.
[85] S.J. Coulthurst, A.M.L. Barnard, G.P.C. Salmond, Regulation and biosynthesis of carbapenem antibiotics in bacteria, Nat. Rev. Microbiol. 3 (2005) 295–306, https://doi.org/10.1038/nrmicro1128.
[86] W. Mao, Y. Wang, X. Qian, L. Xia, H. Xie, A carbapenem-based off–on fluorescent probe for specific detection of metallo-β-lactamase activities, ChemBioChem 20 (2019) 511–515, https://doi.org/10.1002/cbic.201800126.
[87] G. Zlokarnik, P.A. Negulescu, T.E. Knapp, L. Mere, N. Burres, L. Feng, M. Whitney, K. Roemer, R.Y. Tsien, Quantitation of transcription and clonal selection of single living cells with β-lactamase as reporter, Science 279 (1998) 84–88, https://doi.org/10.1126/science.279.5347.84.
[88] N. Kaderabkova, M. Bharathwaj, R.C.D. Furniss, D. Gonzalez, T. Palmer, D.A. I. Mavridou, The biogenesis of β-lactamase enzymes, Microbiology 168 (2022) 001217, https://doi.org/10.1099/mic.0.001217.
[89] A. Galarneau, M. Primeau, L.E. Trudeau, S.W. Michnick, Beta-lactamase protein fragment complementation assays as *in vivo* and *in vitro* sensors of protein protein interactions, Nat. Biotechnol. 20 (2002) 619–622, https://doi.org/10.1038/nbt0602-619.
[90] Q. Shao, Y. Zheng, X. Dong, K. Tang, X. Yan, B. Xing, A covalent reporter of β-lactamase activity for fluorescent imaging and rapid screening of antibiotic-resistant bacteria, Chem. Eur. J. 19 (2013) 10903–10910, https://doi.org/10.1002/chem.201301654.
[91] B. Xing, A. Khanamiryan, J. Rao, Cell-permeable near-infrared fluorogenic substrates for imaging β-lactamase activity, J. Am. Chem. Soc. 127 (2005) 4158–4159, https://doi.org/10.1021/ja042829+.
[92] B. Xing, A. Khanamiryan, J. Rao, Cell-permeable near-infrared fluorogenic substrates for imaging β-lactamase activity, J. Am. Chem. Soc. 128 (2006) 1394, https://doi.org/10.1021/ja054676b.
[93] Y. Kong, H. Yao, H. Ren, S. Subbian, S.L.G. Cirillo, J.C. Sacchettini, J. Rao, J. D. Cirillo, Imaging tuberculosis with endogenous β-lactamase reporter enzyme fluorescence in live mice, PNAS 107 (2010) 12239–12244, https://doi.org/10.1073/pnas.1000643107.
[94] J. Zhang, Y. Shen, S.L. May, D.C. Nelson, S. Li, Ratiometric fluorescence detection of pathogenic bacteria resistant to broad-spectrum β-lactam antibiotics, Angew. Chem. Int. Ed. Engl. 51 (2012) 1865–1868, https://doi.org/10.1002/anie.201107810.
[95] K.M. Papp-Wallace, A. Endimiani, M.A. Taracila, R.A. Bonomo, Carbapenems: past, present, and future, Antimicrob. Agents Chemother. 55 (2011) 4943–4960, https://doi.org/10.1128/aac.00296-11.
[96] M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G.A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H.P. Hratchian, A.F. Izmaylov, J. Bloino, G. Zheng, J.L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J.A. Montgomery Jr., J.E. Peralta, F. Ogliaro, M. Bearpark, J.J. Heyd, E. Brothers, K.N. Kudin, V.N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J.C. Burant, S.S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J.M. Millam, M. Klene, J.E. Knox, J.B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R.E. Stratmann, O. Yazyev, A.J. Austin, R. Cammi, C. Pomelli, J.W. Ochterski, R.L. Martin, K. Morokuma, V.G. Zakrzewski, G.A. Voth, P. Salvador, J.J. Dannenberg, S. Dapprich, A.D. Daniels, Ö. Farkas, J.B. Foresman, J.V. Ortiz, J. Cioslowski, D.J. Fox, Gaussian 09 Rev. D.01, in, Wallingford, CT, 2009.
[97] J. Arca-Suárez, J.C. Vázquez-Ucha, P.A. Fraile-Ribot, E. Lence, G. Cabot, M. Martínez-Guitián, C. Lasarte-Monterrubio, M. Rodríguez-Iglesias, A. Beceiro, C. González-Bello, F. Galán-Sánchez, A. Oliver, G. Bou, Molecular and biochemical insights into the *in vivo* evolution of AmpC-mediated resistance to ceftolozane/tazobactam during treatment of an MDR *Pseudomonas aeruginosa* infection, J. Antimicrob. Chemother. 75 (2020) 3209–3217, https://doi.org/10.1093/jac/dkaa291.
[98] D. Rodríguez, M. Maneiro, J.C. Vázquez-Ucha, A. Beceiro, C. González-Bello, 6-arylmethylidene penicillin-based sulfone inhibitors for repurposing antibiotic efficiency in priority pathogens, J. Med. Chem. 63 (2020) 3737–3755, https://doi.org/10.1021/acs.jmedchem.0c00127.
[99] W. Mao, L. Xia, H. Xie, Detection of carbapenemase-producing organisms with a carbapenem-based fluorogenic probe, Angew. Chem. Int. Ed. Engl. 56 (2017) 4468–4472, https://doi.org/10.1002/anie.201612495.
[100] W. Li, J. Wang, C. Li, Z. Zong, J. Zhao, H. Gao, D. Liu, Achieving ultrasensitive chromogenic probes for rapid, direct detection of carbapenemase-producing bacteria in sputum, JACS Au 3 (2023) 227–238, https://doi.org/10.1021/jacsau.2c00607.
[101] W. Li, J. Li, H. Xu, H. Gao, D. Liu, Rapid and visual identification of β-lactamase subtypes for precision antibiotic therapy, Nat. Commun. 15 (2024) 719, https://doi.org/10.1038/s41467-024-44984-y.
[102] J. Kim, Y. Kim, A.Z. Abdelazem, H.J. Kim, H. Choo, H.S. Kim, J.O. Kim, Y.J. Park, S.J. Min, Development of carbapenem-based fluorogenic probes for the clinical screening of carbapenemase-producing bacteria, Bioorg. Chem. 94 (2020) 103405, https://doi.org/10.1016/j.bioorg.2019.103405.
[103] H.S. Kim, J.O. Kim, J.E. Lee, K.G. Park, H.K. Lee, S.Y. Kim, S.J. Min, J. Kim, Y. J. Park, Performance of a novel fluorogenic assay for detection of carbapenemase-producing *Enterobacteriaceae* from bacterial colonies and directly from positive blood cultures, e01026-01019, J. Clin. Microbiol. 58 (2019), https://doi.org/10.1128/jcm.01026-19.
[104] C.-W. Ma, K.-K.-H. Ng, B.-H.-C. Yam, P.-L. Ho, R.-Y.-T. Kao, D. Yang, Rapid broad spectrum detection of carbapenemases with a dual fluorogenic-colorimetric probe, J. Am. Chem. Soc. 143 (2021) 6886–6894, https://doi.org/10.1021/jacs.1c00462.
[105] J.-D. Docquier, V. Calderone, F. De Luca, M. Benvenuti, F. Giuliani, L. Bellucci, A. Tafi, P. Nordmann, M. Botta, G.M. Rossolini, S. Mangani, Crystal structure of the OXA-48 β-lactamase reveals mechanistic diversity among class D carbapenemases, Chem. Biol. 16 (2009) 540–547, https://doi.org/10.1016/j.chembiol.2009.04.010.
[106] K.M. Papp-Wallace, V. Kumar, E.T. Zeiser, S.A. Becka, F. van den Akker, Structural analysis of the OXA-48 carbapenemase bound to a "poor" carbapenem substrate, doripenem, Antibiotics 8 (2019) 145. https://www.mdpi.com/2079-6382/8/3/145.
[107] C.A. Smith, N.K. Stewart, M. Toth, S.B. Vakulenko, Structural insights into the mechanism of carbapenemase activity of the OXA-48 β-lactamase, Antimicrob. Agents Chemother. 63 (2019) e01202–e01219, https://doi.org/10.1128/aac.01202-19.
[108] G. Tassone, F. De Luca, C. Pozzi, F. Di Pisa, M. Benvenuti, S. Mangani, J.D. Doquier, Crystal structure of class D β-lactamase OXA-48 in complex with meropenem, https://doi.org/10.2210/pdb6ZRP/pdb.
[109] G. Tassone, F. De Luca, C. Pozzi, F. Di Pisa, M. Benvenuti, S. Mangani, J.D. Doquier, Crystal structure of class D β-lactamase OXA-48 in complex with ertapenem, https://doi.org/10.2210/pdb6ZRJ/pdb.
[110] A. Akhtar, O.A. Pemberton, Y. Chen, Structural basis for substrate specificity and carbapenemase activity of OXA-48 class D β-lactamase, ACS Infect. Dis. 6 (2020) 261–271, https://doi.org/10.1021/acsinfecdis.9b00304.
[111] V. Stojanoski, L. Hu, B. Sankaran, F. Wang, P. Tao, B.V.V. Prasad, T. Palzkill, Mechanistic basis of OXA-48-like β-lactamases' hydrolysis of carbapenems, ACS Infect. Dis. 7 (2021) 445–460, https://doi.org/10.1021/acsinfecdis.0c00798.
[112] J. Chiou, Q. Cheng, P.T.-F. Shum, M.H.-Y. Wong, E.W.-C. Chan, S. Chen, Structural and functional characterization of OXA-48: insight into mechanism and structural basis of substrate recognition and specificity, Inter. J. Mol. Sci. 22 (2021) 11480. https://www.mdpi.com/1422-0067/22/21/11480.
[113] S. Das, J. Ihssen, L. Wick, U. Spitz, D. Shabat, Chemiluminescent carbapenem-based molecular probe for detection of carbapenemase activity in live bacteria, Chem. Eur. J. 26 (2020) 3647–3652, https://doi.org/10.1002/chem.202000217.