Minireview

Penicillin-binding protein (PBP) inhibitor development: A 10-year chemical perspective

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Impact Statement

β-lactam antibiotics, that inhibit bacterial growth and cell wall formation by targeting penicillin-binding proteins (PBPs), have been successfully used for decades. However, the emergence of resistance to these antibiotics presents a clear challenge. Here we review the literature covering research into novel PBP inhibitors through the last 10 years, including compounds that are active against major bacterial pathogens.

Abstract

Bacterial cell wall formation is essential for cellular survival and morphogenesis. The peptidoglycan (PG), a heteropolymer that surrounds the bacterial membrane, is a key component of the cell wall, and its multistep biosynthetic process is an attractive antibacterial development target. Penicillin-binding proteins (PBPs) are responsible for cross-linking PG stem peptides, and their central role in bacterial cell wall synthesis has made them the target of successful antibiotics, including β-lactams, that have been used worldwide for decades. Following the discovery of penicillin, several other compounds with antibiotic activity have been discovered and, since then, have saved millions of lives. However, since pathogens inevitably become resistant to antibiotics, the search for new active compounds is continuous. The present review highlights the ongoing development of inhibitors acting mainly in the transpeptidase domain of PBPs with potential therapeutic applications for the development of new antibiotic agents. Both the critical aspects of the strategy, design, and structure–activity relationships (SAR) are discussed, covering the main published articles over the last 10 years. Some of the molecules described display activities against main bacterial pathogens and could open avenues toward the development of new, efficient antibacterial drugs.

Keywords: Penicillin-binding protein, inhibitors, substrate analogs, β-lactams, non-β-lactam inhibitors

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Introduction

Bacterial cell wall formation is a complex, highly regulated process that is key for cellular survival and morphogenesis. Peptidoglycan (PG), a key component of the cell wall, is a heteropolymer that surrounds the bacterial membrane, offering protection from osmotic lysis and serving as a binding platform for virulence factors and adhesins.[1,2] Its central role in bacterial growth has made the biosynthetic machinery of the PG the target of successful antibiotics, including β-lactams, that have been used worldwide for decades. PG biosynthesis occurs during the cell division phase, where septum and polar caps are synthesized, and in non-spherical bacteria also during the elongation phase, where growth occurs along the longitudinal axis of the cell.[3] Proteins involved in these processes are associated with the divisome and/or the elongasome (or Rod complex) and their inhibition can lead to impaired cell growth and often cell lysis and death.[4,5]

Assembled PG is composed of polymerized disaccharide subunits, N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), cross-linked by stem peptides (Figure 1). PG biosynthesis is a multistep process that is initiated in the cytoplasm, where the Lipid II building block is synthesized in a stepwise fashion, after which it is flipped toward the outside of the inner membrane. In the periplasm, penicillin-binding proteins (PBPs) and shape, elongation, division, and sporulation (SEDS) proteins incorporate Lipid II into the growing peptidoglycan layer through glycosylation (where GlcNAc and MurNAc disaccharide units are polymerized) and transpeptidation (stem peptide cross-linking). SEDS and PBPs have been reported to both be able to catalyze glycosyltransfer, but only PBPs are able

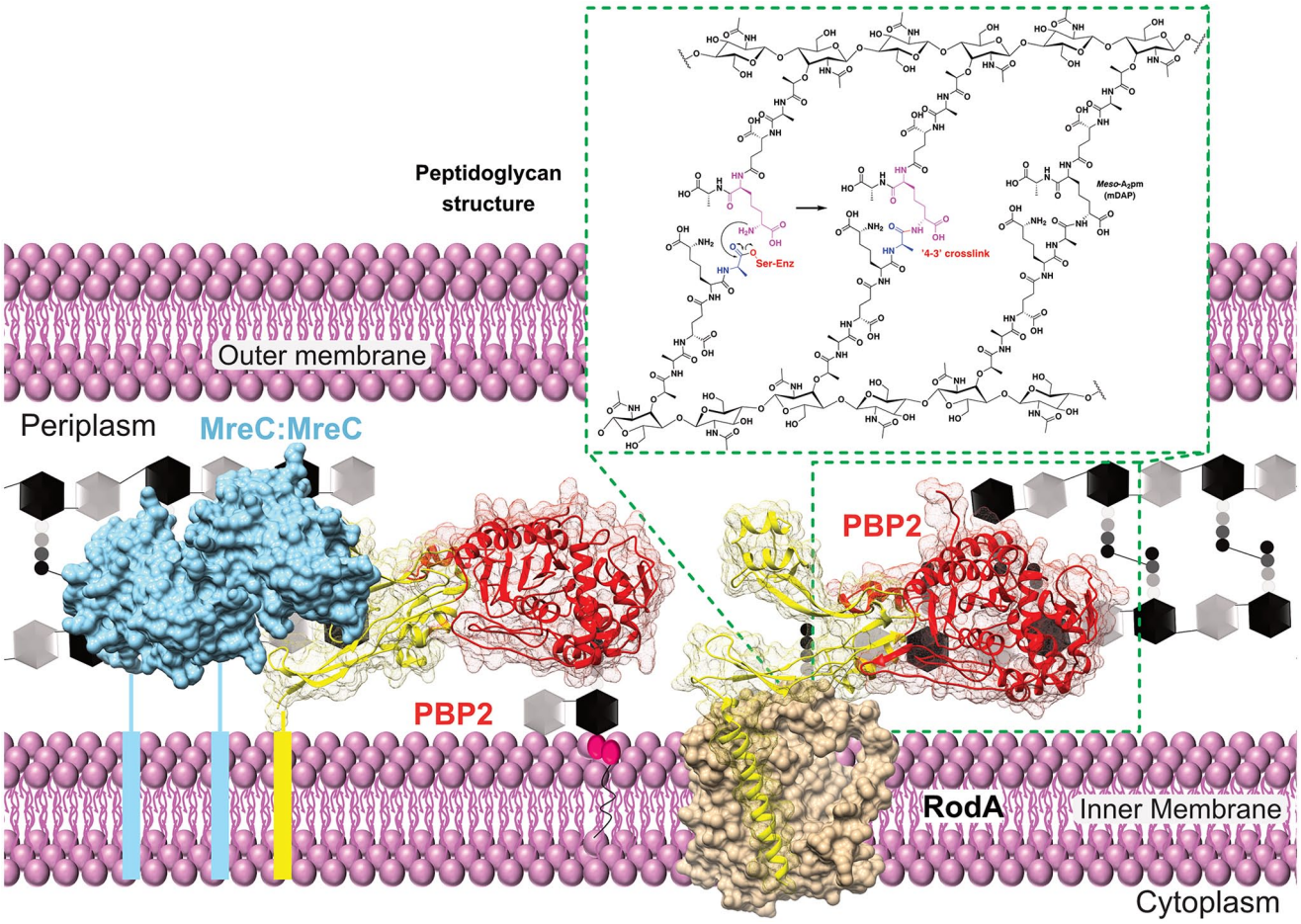

Figure 1. Schematic representation of the PG biosynthesis steps occurring in the Gram-negative periplasm, and complexes formed by PBP2. The active site serine of PBP2 recognizes the terminal D-alanine, D-alanine moiety of the stem peptide, forming an acyl–enzyme complex by a nucleophilic addition reaction. This is followed by the elimination of the last D-alanine residue of the chain, regenerating the carbonyl group. Subsequently, the nucleophilic attack at the carbonyl group of the acyl–enzyme complex is performed by the lateral amino group at position (3) of an adjacent chain, followed by the elimination of the enzyme that generates the classical "4–3" cross-link. During cell wall elongation, the PBP2-RodA complex (PDB ID: 6PL5)[12] catalyzes glycosylation (RodA) and transpeptidation (PBP2) of the Lipid II building block to generate a new peptidoglycan layer. PBP2 can be activated upon binding to an MreC dimer (PDB ID: 5LP5)[10] and could potentially bind to both RodA and MreC concomitantly. Green dotted box: chemical details of the transpeptidation reaction catalyzed by PBP2, whose N-terminus is in yellow and C-terminal (transpeptidase) domain is shown in red. The C55 lipid carrier is indicated in pink, with MurNAc in black and GlcNAc in gray.

to catalyze transpeptidation reactions.[2,4–7] It is precisely this transpeptidation reaction that is inhibited by β-lactam antibiotics; blocking peptide cross-linking eventually leads to weakening of the peptidoglycan and cell lysis.[8,9]

Different organisms can display diverging numbers of PBPs. *Escherichia coli*, for example, has 12 PBPs, while the 7 PBPs of *Bacillus subtilis* are also involved in sporulation and the oval-shaped *Streptococcus pneumoniae* has 6 PBPs. High molecular mass PBPs can either catalyze both glycosyltransfer and transpeptidation (class A), or only the transpeptidation (TP) reaction (class B).[8] The TP active site is located in the C-terminal domain of the molecule, while the N-terminus of class B PBPs is involved in binding to protein partners[10–12] (Figure 1).

During the transpeptidation reaction, the PBP active site recognizes the terminal D-alanine, D-alanine moiety of the stem peptide, forming an acyl–enzyme complex. The nucleophilic attack at the carbonyl group of the penultimate D-alanine by the lateral amino group at position (3) of an adjacent chain generates a classical "4–3" cross-link (Figure 1). β-lactam antibiotics structurally resemble the D-alanine, D-alanine dipeptide, and thus also form an acyl–enzyme complex with the active site serine of PBPs; however, the relative stability of this complex to nucleophilic attack is key for its inhibitory effect.[9,13,14]

Since the discovery of penicillin, β-lactams have been widely used and remain among the most important small molecules in clinical use, representing more than 50% of all prescribed antibiotics.[15] Notably, many PBPs are also involved in the development of resistance toward these drugs in a number of pathogens.[16] β-lactamases, enzymes produced by several infectious bacterial strains, have also been shown to play important roles in antibiotic resistance. Class A, C, and D β-lactamases are serine-based enzymes that perform inactivation in a two-step process.[17,18] The first step is the formation of the acyl–enzyme complex by the acylation of the catalytic serine through the nucleophilic attack of its hydroxyl group. Subsequently, the complex formed undergoes a second nucleophilic attack where the nucleophile is a water molecule, leading to hydrolysis and opening the β-lactam ring. Class B β-lactamases are metallo-enzymes that employ $Zn^{2}+$ or other metal ions in the reaction with the β-lactam substrate.[18] The metal stabilizes the hydroxide

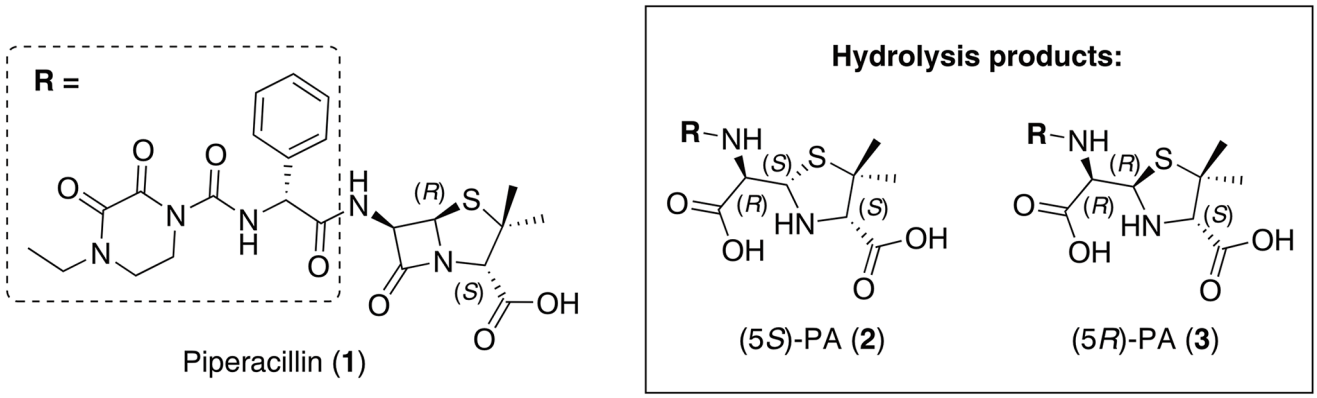

Figure 2. Structure of piperacillin (**1**, PIP) and the products of the hydrolysis and epimerization reactions (5*S*)-penicilloic acid ((5*S*)-PA, **2**) and (5*R*)-penicilloic acid ((5*R*)-PA, **3**).[29]

anion formed by a water molecule and coordinates its direct addition to the carbonyl group of the substrate, opening the β-lactam ring.[18] Upon completion of these reactions, β-lactams can no longer effectively target and inhibit PBPs.[16,17]

Nevertheless, PBPs have remained attractive targets for inhibitor development due to the essential role they play in bacterial growth and survival and their location on the outside of the cell membrane.[19] This review provides an update on the development of inhibitors acting mainly in the transpeptidase domain of PBPs with potential therapeutic applications for the development of new antibiotic agents. Both the critical aspects of the design and structure–activity relationships (SAR) are discussed, covering the main published articles within the last 10 years.

Substrate analog inhibitors

β-lactams. Structural biology has been extensively employed to characterize complexes between PBPs and β-lactam analogs with the objective of understanding catalytic details, resistance mechanisms, and developing novel ligands.[8,20–28] In these structures, the nucleophilic serine is covalently associated to the β-lactam carbonyl as an ester. Despite these efforts, until recently no reports had been published relating complex formation between a PBP and a ligand product resulting from hydrolysis by a β-lactamase. However, recent crystallographic studies found that an epimerized hydrolysis product of piperacillin (**1**, PIP), (5*S*)-penicilloic acid ((5*S*)-PA, **2**, Figure 2), can bind to PBP3 from *Pseudomonas aeruginosa*.[29] When PBP3 was co-crystallized with piperacillin, the electron density map revealed that the nucleophilic serine was not linked to the carbonyl group of the β-lactam as an ester, as expected. Instead, a noncovalent complex with 5S-stereochemistry was observed.[29] The noncovalent binding mode of the (5*S*)-PA epimer in the crystal structure indicated an apparent preference for this epimer rather than the (5*R*)-PA epimer (**3**, Figure 2) formed through hydrolysis and epimerization reactions. Using NMR spectroscopy, authors verified that, in solution, PBP3 first catalyzes the hydrolysis of piperacillin into (5*R*)-PA, which is slowly converted into (5*S*)-PA in a non-enzymatic fashion.[29] In the absence of PBP3, low levels of the hydrolyzed (5*R*)-PA were observed. Competition assays revealed that in contrast to piperacillin (IC_{50} of 166 ± 62 nM), both (5*R*)-PA and (5*S*)-PA are weak PBP3 inhibitors (IC_{50} of 196 ± 28 and 126 ± 18 μM, respectively), which may reflect the noncovalent nature of the complex formed. Despite that, these results provide new insights into the manner of penicilloic acid binding to PBP3 and could be used to guide the design of new PBP inhibitors.

Due to their complex cell wall architecture, Gram-negative pathogens present notable challenges toward the discovery of new antibacterial agents.[30] Resistance in Gram-negative organisms is multicausal and includes reduction of outer membrane permeability, caused by porin deficiency, as well as β-lactamase production and degradation of target affinity through the introduction of mutations in PBPs.[31] Cefiderocol (**4**), formerly known as S-649226, is a siderophore with a cephalosporin core (**5**, Figure 3) developed by Shionogi & Co., Ltd.[32–34] to treat infections caused by carbapenem-resistant Gram-negative bacteria. The compound has already been approved in the United States and Europe for the treatment of infections in adults.[35] Regarding the mechanism, the cephalosporin moiety of cefiderocol binds primarily to PBP3, as is the case for other cephalosporins, while the catechol moiety contributes to the formation of a chelated complex with ferric iron that facilitates the crossing of the outer membrane using the receptor-mediated bacterial iron transport system.[33] Bacterial iron transport systems accelerate and enhance the influx of cefiderocol into the periplasmic space, thereby enhancing its antimicrobial activity.[33]

Another example of a compound that can overcome the penetration barrier presented by the Gram-negative outer membrane and the action of β-lactamases is the tricyclic β-lactam **6** (Figure 3).[31] This compound emerged from the SAR evaluation of compound **7** (Figure 3).[36] Compound **6** shows antibacterial activity against several clinical isolates, solid therapeutic efficacy in the neutropenic mouse lung infection model, and a low frequency of production of spontaneous resistant mutants.[31] The presence of a sulfoxide group at the core structure with the γ-lactone ring was essential for the generation of potent antibacterial activities against several β-lactamase-producing strains.

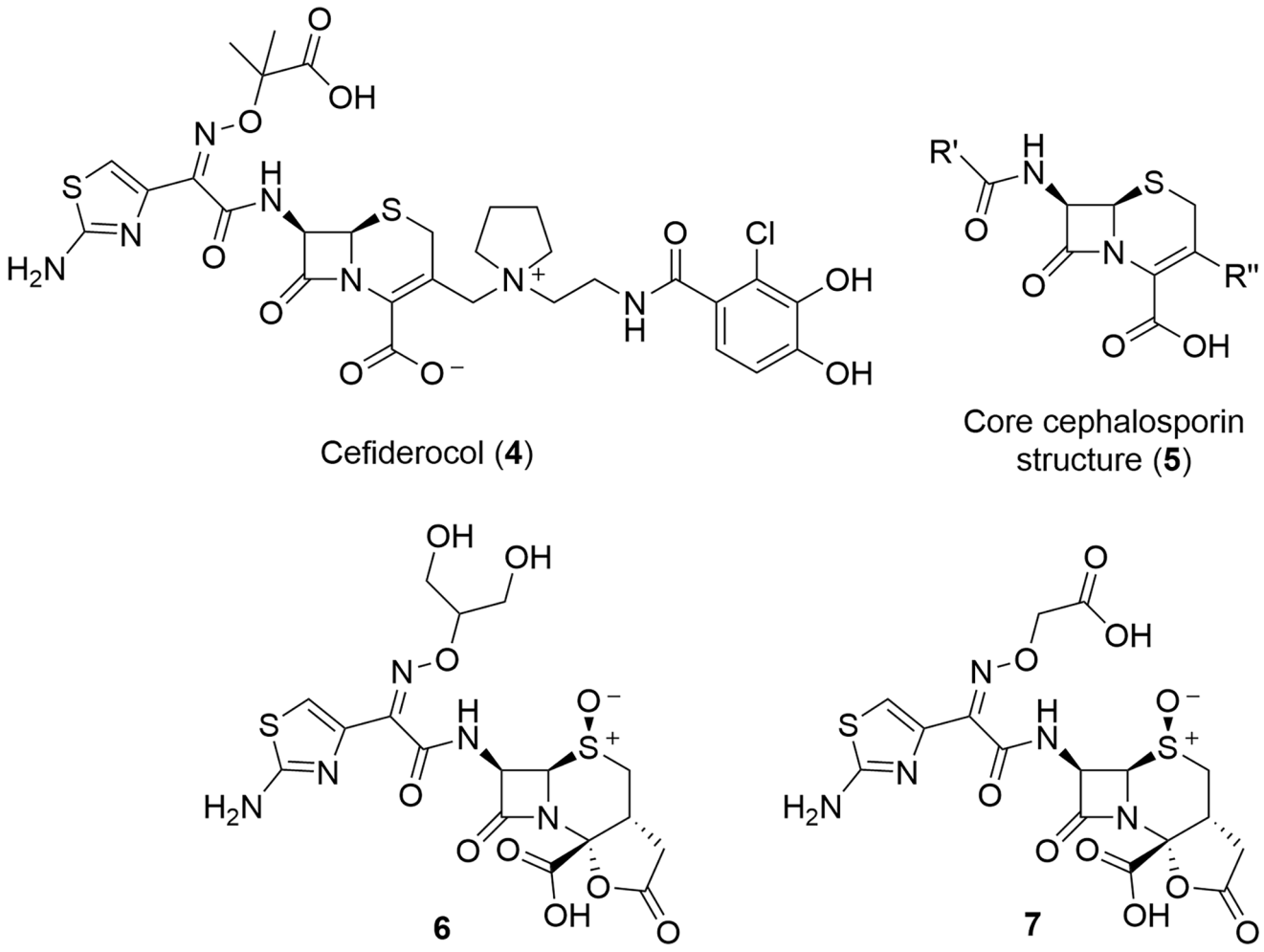

Figure 3. Cefiderocol (**4**), core structure of cephalosporin (**5**),[32–34] and compounds **6**[31] and **7**.[36]

Monobactams also target PBPs from Gram-negative species, such as *P. aeruginosa* PBP3.[37] Aztreonam (ATM, **8**, Figure 4) was the first clinical monobactam antibiotic successfully used to treat infections caused by Gram-negative bacteria.[38] Nonetheless, ATM has some limitations against *P. aeruginosa*, possibly due to its poor outer membrane permeability, β-lactamase susceptibility, and high propensity for efflux.[30,39] However, ATM has become an ideal starting point for structure optimization viewing the development of new monobactam antibiotic candidates due to its simple structure and clinical success. Structural analyses reveal ample space between the oxime-linked group in monobactams and the active site of PBP3, which can accommodate various substituents with different polarities and sizes to increase activity.[37]

Inspired by ATM's properties and chemical structure, 34 new monobactam derivatives were synthesized with nitrogen-based groups on the oxime side chain and were evaluated for their antibacterial activities using a phenotypic screening assay.[37] Hydrophobic unsaturated aliphatic chains, phenyl and amino groups, carboxyl as well as siderophore catecholate fragments were evaluated. These compounds had their minimum inhibitory concentration (MIC) determined against bacterial strains including drug-resistant *E. coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *P. aeruginosa* and drug-susceptible *Enterobacter cloacae* and *Enterobacter aerogenes* from the ATCC collection and clinical isolates from Chinese hospitals.[37] Most of the compounds exhibited reduced antibacterial potencies compared with the lead ATM, except for compounds **9** and **10**, both of which bear unsaturated allyl and propargyl groups (Figure 4). These compounds showed comparable potencies against *E. coli* (MICs of 0.25–0.5 μg mL^{-1}) and higher potency against *A. baumannii* and *E. cloacae* when compared to values obtained with ATM, with no cytotoxicity effects up to a concentration of 30 μg mL^{-1}.[37]

Isolated from *Nocardia uniformis* in the late 1970s,[40] the first monobactam described as an antibacterial agent was Nocardicin A (**11**, Figure 4). Although it originally had limited antibacterial activity, its promising potential inspired the synthesis of a new nocardicin-like analogs library.[41] First, an *in silico* design was performed to construct a virtual combinatorial library of novel monocyclic β-lactams that identified 64 hits from a virtual screening campaign. Then, the compounds had their potential activity against PBP5fm from *Enterococcus faecium* evaluated via *in silico* covalent docking studies. Subsequent to their synthesis and stereochemical determination, 30 selected compounds were submitted to a PBP binding and competition assay. It was

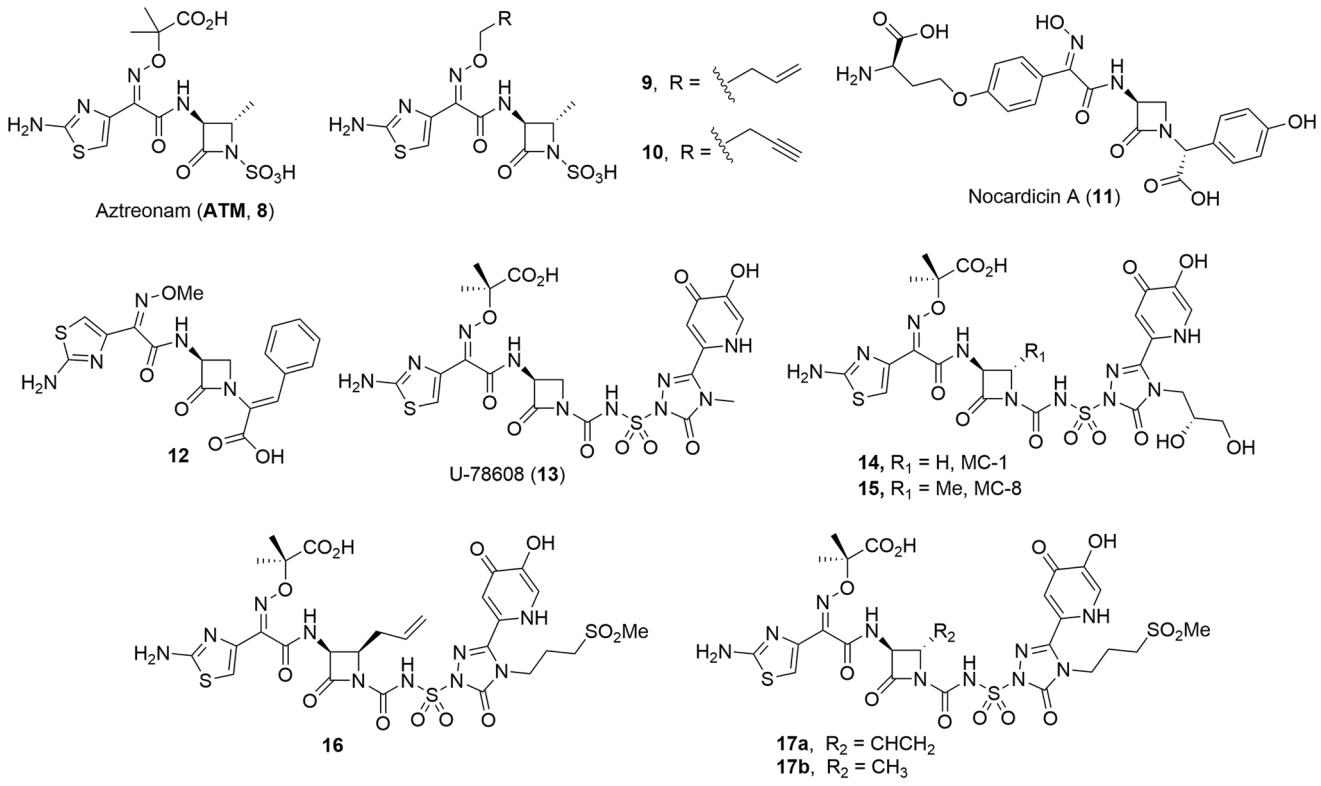

Figure 4. Examples of monobactams: Aztreonam (ATM, **8**),[37,38] derivatives **9** and **10**,[37] Nocardicin A (**11**),[40] benzylidene derivative **12**,[41] U-78608 (**13**),[42] MC-1 (**14**), and MC-8 (**15**),[43] compounds **16**, **17**, and **18**.[30]

observed that an adequate distance between the last functional group and the β-lactam core is essential to mimic the D-alanyl-D-alanine terminus of the natural substrate of the PBPs. Purified PBP3 from *E. coli* K12, PBP5fm from *E. faecium* D63r, and R39 DD-carboxypeptidase from *Actinomadura* spp. were then incubated with the compounds, and their residual activities were determined by labeling the free enzymes with BOCILLIN FL, a fluorescent derivative of penicillin V. The benzylidene derivative (**12**, Figure 4) showed a promising inhibitory potential of PBP3 with 28% residual activity and an IC_{50} value of 130 μM. Weak inhibition was observed of the other two enzymes, with 88% and 62% residual activities for PBP5fm and R39, respectively. The inhibitory activity of β-lactamases in the presence of the compounds was also determined and compound **12** inhibited the catalytic activity of the P99 class C β-lactamase by about 40%.

Monobactams U-78608 (**13**, Figure 4),[42] MC-1 (**14**, Figure 4), and MC-8 (**15**, Figure 4)[43] are known examples of monocyclic β-lactams that include iron-chelating siderophore groups within their structures.[30] Despite the potent inhibition of *P. aeruginosa* PBP3 and the resistance to hydrolysis by β-lactamases,[44] these compounds present poor hydrolytic stability and high human plasma protein binding.[43] In 2015, aiming to improve the activity of some monobactam derivatives, Murphy-Benenato and co-workers[30] developed a SAR study focused on increasing permeation by changing the siderophore mimics. Analogs with modifications on the side chains of the triazole, the iron-chelating group, the aminothiazole, and the 4-position of the β-lactam core were synthesized. Antibacterial activity against *P. aeruginosa*, PBP3 acylation rates, and physicochemical properties were assessed for all analogs. In general, the analogs demonstrated MICs toward *P. aeruginosa* ranging from 0.13 to 8 μg mL^{-1}, significant variability of PBP3 acylation rate constants (5.9×10^3 to 6.2×10^5 $M^{-1} s^{-1}$, depending on the size of the side chain on the β-lactam core), and good hydrolytic stability. Co-crystal structures of *P. aeruginosa* PBP3 with compounds **16**, **17a**, and **17b** (Figure 4) indicated that all three compounds were covalently bound to Ser294 of PBP3 with similar binding modes.[30] Some observed key differences between the structures of the allyl analog **16** and the methyl and vinyl analogs **17a** and **17b** included the absence of a salt bridge between Arg489 and the oxyiminopropylcarboxylic acid and a flipped conformation of the urea of the acylsulfonamide. Currently, **17a** is an example of a monobactam candidate in a preclinical stage of investigation.[37]

In some cases, an adopted strategy to overcome bacterial resistance is the combination of drugs with different modes of action.[31] Combinations approved in some countries include AVYCAZ® (ceftazidime, **18**, CAZ, and Avibactam, **19**, AVI, a β-lactamase inhibitor based on a diazabicyclooctane pharmacophore)[45] and VABOMERE® (meropenem, **20**, MER, and vaborbactam, **21**, VAB, a β-lactamase inhibitor based on a cyclic boronic acid pharmacophore)[46] (Figure 5). The combination of ATM (**8**, Figure 4) and AVI (**19**) has completed a phase II clinical trial for the treatment of infections caused by resistant Gram-negative bacteria.[47]

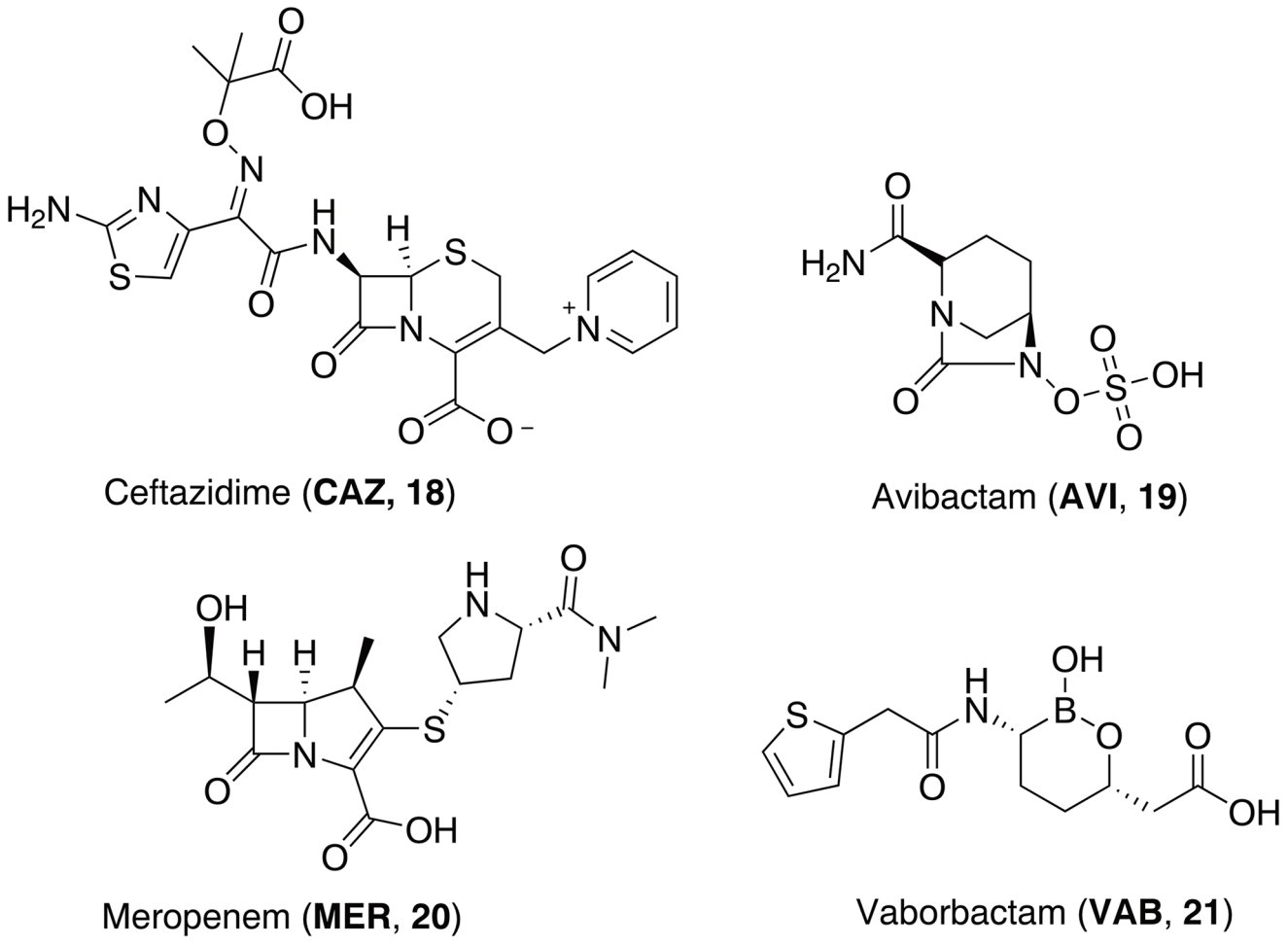

Figure 5. Antibiotic combinations in use: AVYCAZ® (ceftazidime, **18**, CAZ, and Avibactam, **19**, AVI)[45] and VABOMERE® (meropenem, **20**, MER, and vaborbactam, **21**, VAB).[46]

Antibiotic and adjuvant combinations can result in four possible effects: synergy, additivity, antagonism, and autonomy.[48] Synergy is the most desirable interaction and occurs when the effect of two drugs in combination is significantly greater than that of either drug alone.[48] Additivity is the sum of the effects of each drug, with the premise that they do not interact with each other. When a combination has less effect than either drug alone, they are considered antagonistic.[48] If the observed effect is equal to that of the most active drug, it is autonomous.[48] The effect of the combination can be determined through the measurement and calculation of the fractional inhibitory concentration (FIC) (see reference Kalan and Wright[48] for more details). An FIC index of $\leqslant 0.5$ indicates synergy and a value $\geqslant 4.0$ indicates antagonism. Additivity and autonomy are the intermediate values and cannot always be distinguished. They can be classified as "no interaction." Synergistic studies of compounds **9** and **10** (Figure 4) with AVI (**19**) were performed and showed FIC values $\leqslant 0.5$ against two drug-resistant *K. pneumoniae* strains.[37] These combinations show significantly reduced MIC values of up to 8-fold and 256-fold, respectively, in a ratio of 1:16 (samples **9** or **10** in combination with AVI).

Dual therapy with β-lactams is a poorly explored treatment option for multidrug-resistant infections. To evaluate different β-lactam combinations, 28 dual combinations of β-lactams derived from eight approved drugs from different β-lactams classes were tested.[39] The *in vivo* efficacy of each combination was compared with its constituent monotherapies against *Galleria mellonella* larvae infected with antibiotic-resistant strains of *P. aeruginos*a NCTC13437. The most potent dual combinations identified were MER/ATM (**20**/**8**) and MER/CAZ (**20**/**18**). The central hypothesis of this type of approach is that a broader spectrum of PBPs can be inhibited by combining β-lactams with different PBP affinity profiles.[39] While β-lactams confer antibiotic activity through PBP inhibition, individual antibiotics differ in their affinities for each one of the PBPs. In addition, an alternative hypothesis is that one of the components can bind preferentially, or with greater affinity, to β-lactamases, thus sequestering the hydrolytic capacity of the enzyme and allowing the other β-lactam component to bind to its PBP(s) target(s) more effectively.[39]

Non-β-lactam inhibitors. Lactivicins (**22**, Figure 6), a class of PBP inhibitors of microbial origin,[49] are non-β-lactam compounds that display cycloserine (five-membered lactam) and γ-lactone (five-membered cyclic ester) rings in their structures. X-ray crystallographic data support the covalent mechanism of action of lactivicins wherein the carbonyl of

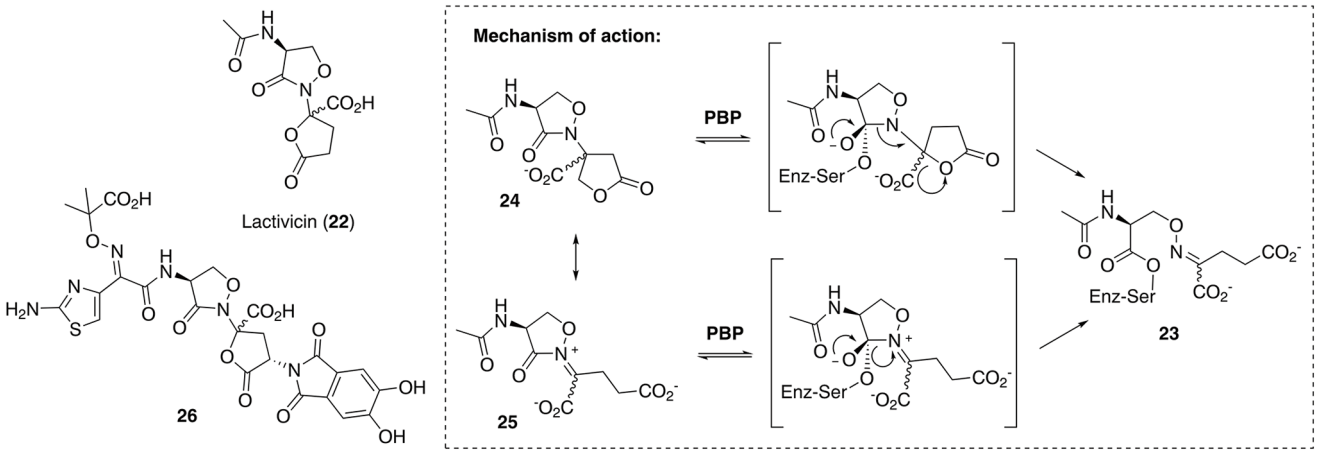

Figure 6. Lactivicin (**22**), its acyl–enzyme complex (**23**),[22] and phthalimide-conjugated lactivicin analog **26**.[50] Lactivicins are known to undergo ring-chain tautomerization (**24** to **25**) leading to equilibration of the γ-lactone chiral center. Both structures lead to **23**.

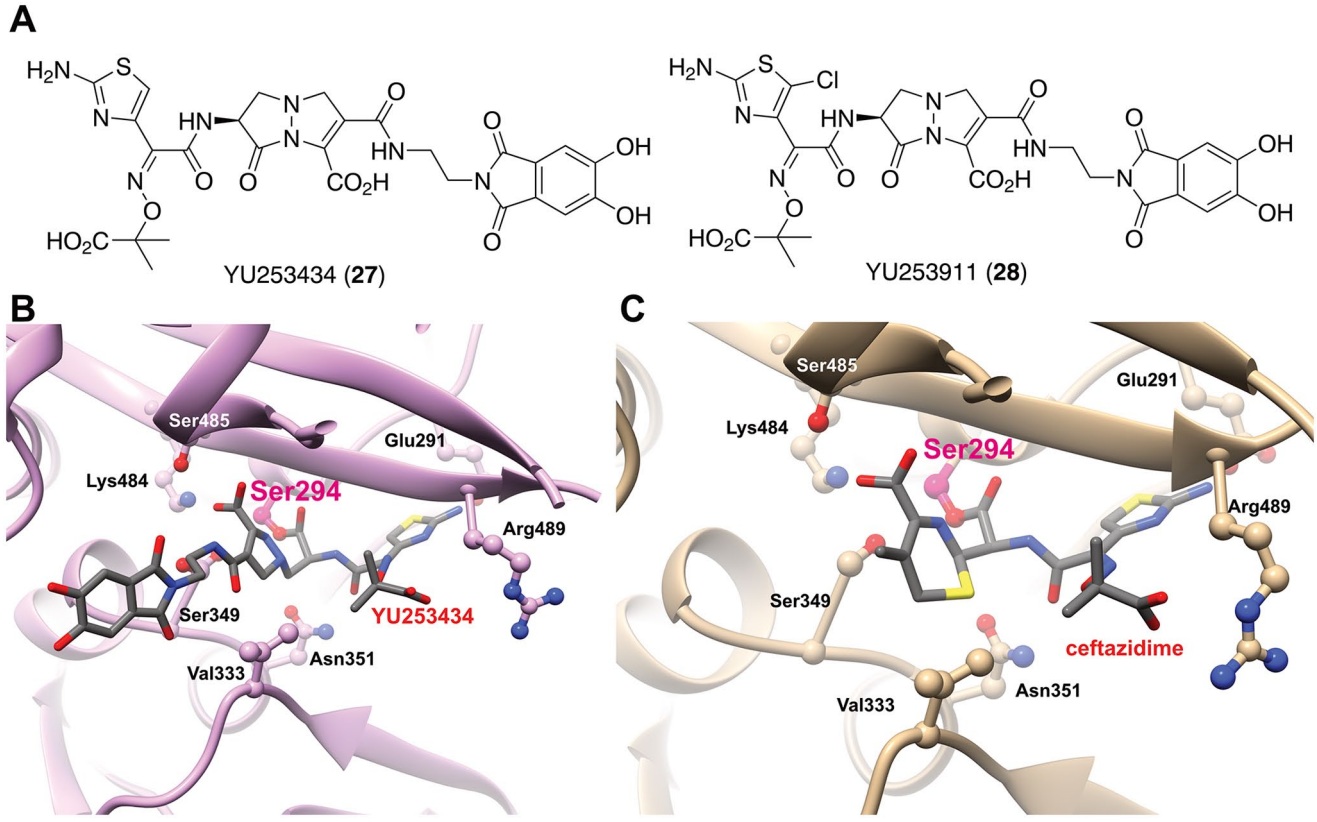

Figure 7. (A) γ-Lactam pyrazolidinone YU253434 (**27**)[52] and YU253911 (**28**).[53] (B) Crystal structure of *P. aeruginosa* PBP3 in complex with YU253434 (**27**) (PDB ID: 3PBO)[51] and (C) CAZ (**18**) (PDB ID: 3PBO),[41] zooming in on the active site and the vicinity of catalytic Ser294. In both structures, note that the aminothiazole moieties are in similar positions.

the cycloserine ring reacts with the active site serine hydroxyl to form a covalent bond that is analogous to that formed by a typical β-lactam (**23**, Figure 6).[22] Nucleophilic substitution at the carbonyl group of an amide usually occurs in a stepwise manner initiated by the formation of a tetrahedral intermediate as the rate-determining step. The presence of electron-withdrawing substituents, such as the appended lactone moiety and the cycloserine ring oxygen, provides sufficient stability for the transition state in the reaction with the PBP. In addition, lactivicins are known to undergo ring-chain tautomerization (**24** to **25**) leading to equilibration of the γ-lactone chiral center.[22]

The synthesis of small molecules that mimic siderophores, leading to an active cellular uptake strategy, has also been applied to lactivicins. To date, crystal structures of several lactivicin sideromimic analogs bound to PBP3 and PBP1a from *P. aeruginosa* have been described, together with their reactivities.[50] Among them, compound **26** (Figure 6) was identified

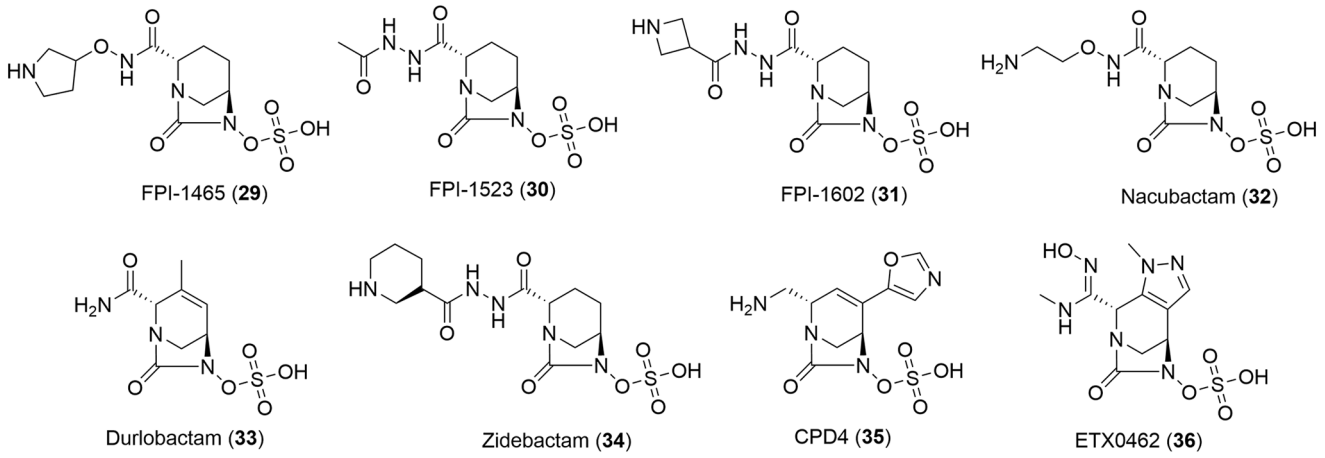

Figure 8. PBP inhibitors possessing a diazabicyclooctane (DBO) core scaffold: FPI-1465 (**29**),[54] FPI-1523 (**30**),[54] FPI-1602 (**31**),[54] Nacubactam (**32**),[56] Durlobactam (**33**),[57] Zidebactam (**34**),[58] CDP4 (**35**),[21] and ETX0462 (**36**).[59]

as a novel phthalimide-conjugated lactivicin analog exhibiting both the lowest MICs and IC_{50} values toward PBPs in the evaluated series (IC_{50} of 0.030 μM and 0.046 μM for PBP1a and PBP3, respectively). Compound **26** was identified after a design effort that aimed to explore potential intermolecular noncovalent aryl–aryl interactions between groups linked at the α-position in the lactone and residues Tyr503, Tyr532, and Phe533 in the PBPs. Crystallographic studies revealed a covalent acyl–enzyme interaction between *P. aeruginosa* PBP1a and **26**, with continuous electron density between the hydroxyl group of the active site Ser461 and the lactivicin acyl carbon. Furthermore, compound **26** appears to use a broader set of siderophore receptors for its uptake and has low susceptibility to β-lactamases, which provides a unique efficacy against Gram-negative bacteria.[50]

Molecules carrying the γ-lactam pyrazolidinone chemical scaffold also irreversibly inhibit PBPs by acylating the catalytic serine. Pyrazolidinones are poorly hydrolyzed by all four classes of β-lactamases.[51] YU253434 (**27**, Figure 7(A)) is a pyrazolidinone containing a dihydroxyphthalimide siderophore mimetic. In 2020, YU253434 was synthesized, and its antimicrobial activity was compared to that of current β-lactam antibiotics against clinical isolates of *P. aeruginosa*, *K. pneumoniae*, and *E. coli*.[51] The compound's PBP binding characteristics were visualized in a 2.4 Å crystal structure in complex with *P. aeruginosa* PBP3 where the catalytic Ser294 was acylated (Figure 7(B)). Comparing these structures and that of CAZ (**18**) (Figure 7(C)) covalently bound to PBP3,[52] it is noted that the aminothiazole moieties are in a similar position in both, making almost identical interactions. The following year, the synthesis of YU253911 (**28**, Figure 7(A)) was described.[53] This molecule is a chloroaminothiazole analog that displays enhanced potency against *Acinetobacter* spp. and *P. aeruginosa* (MIC of 0.5 μg mL^{-1}), as well as improved pharmacokinetic properties when compared to previously described pyrazolidinones.[53]

As mentioned before, avibactam (**19**) is a reversible, covalent inhibitor possessing a diazabicyclooctane (DBO) core scaffold.[54] AVI displays potent inhibitory activity against class A and C and some class D serine β-lactamases (SBLs),[45] through a mechanism that occurs via a reversible active site serine acylation, thus differing from the irreversible mechanism of action of β-lactam-based inhibitors.[55] Evidence suggests that by following regioselective ring opening of the five-membered cyclic urea of AVI, a major stable carbamoyl acyl-enzyme complex is formed.[45] β-lactam inhibitors, on the other hand, can undergo a series of fragmentation reactions after acyl–enzyme formation which lead to the destruction of the β-lactam scaffold.[45] Furthermore, the crystal structure of CTX-M-15 complexed to AVI revealed that the complex is stabilized by several interactions with residues in the active site which appear to be optimized for binding of the open ring form.[45] These findings corroborate the evidence that AVI is more efficient than β-lactam-based β-lactamase inhibitors since inhibition requires fewer avibactam molecules per β-lactamase (10–20 times less), compared to other β-lactam inhibitors.[45]

Synthetic modifications in the AVI core led to the development of derivatives with a broader spectrum of activity and resistance to β-lactamases, among other favorable properties. Three examples of these derivatives, FPI-1465 (**29**, Figure 8), FPI-1523 (**30**, Figure 8), and FPI-1602 (**31**, Figure 8), synthesized by Fedora Pharmaceuticals, were evaluated against *P. aeruginosa*, *E. coli*, and *Enterobacter* spp.[54] FPI-1602 (**31**) displayed antimicrobial activity against *P. aeruginosa* (PAO1, MIC of 2 μg mL^{-1}), *E. coli* (GN688 and GN610, MICs of 0.5 and 2 μg mL^{-1}, respectively), and *Enterobacter cloacae* (GN574 and 579, MICs of 1 and 2 μg mL^{-1}, respectively). In a gel-based competition assay using BOCILLIN FL and purified *E. coli* PBP1a, PBP1b, PBP2, and PBP3, it was observed that all the derivatives tested displayed preferential inhibition of PBP2. FPI-1523 (**30**, $IC_{50} = 3.2 \pm 0.4$ μM) and FPI-1602 (**31**, $IC_{50} = 3.6 \pm 0.3$ μM) displayed more potent inhibition than FPI-1465 (**29**, $IC_{50} = 15 \pm 1$ μM) and AVI ($IC_{50} = 63 \pm 6$ μM).[54] The crystal structure of *E. coli* PBP1b complexed to FPI-1465 (**29**) was solved to 2.85 Å and confirmed the covalent acylation of the catalytic serine.[54] Subsequent to this publication, several DBO-based β-lactamase inhibitors were described

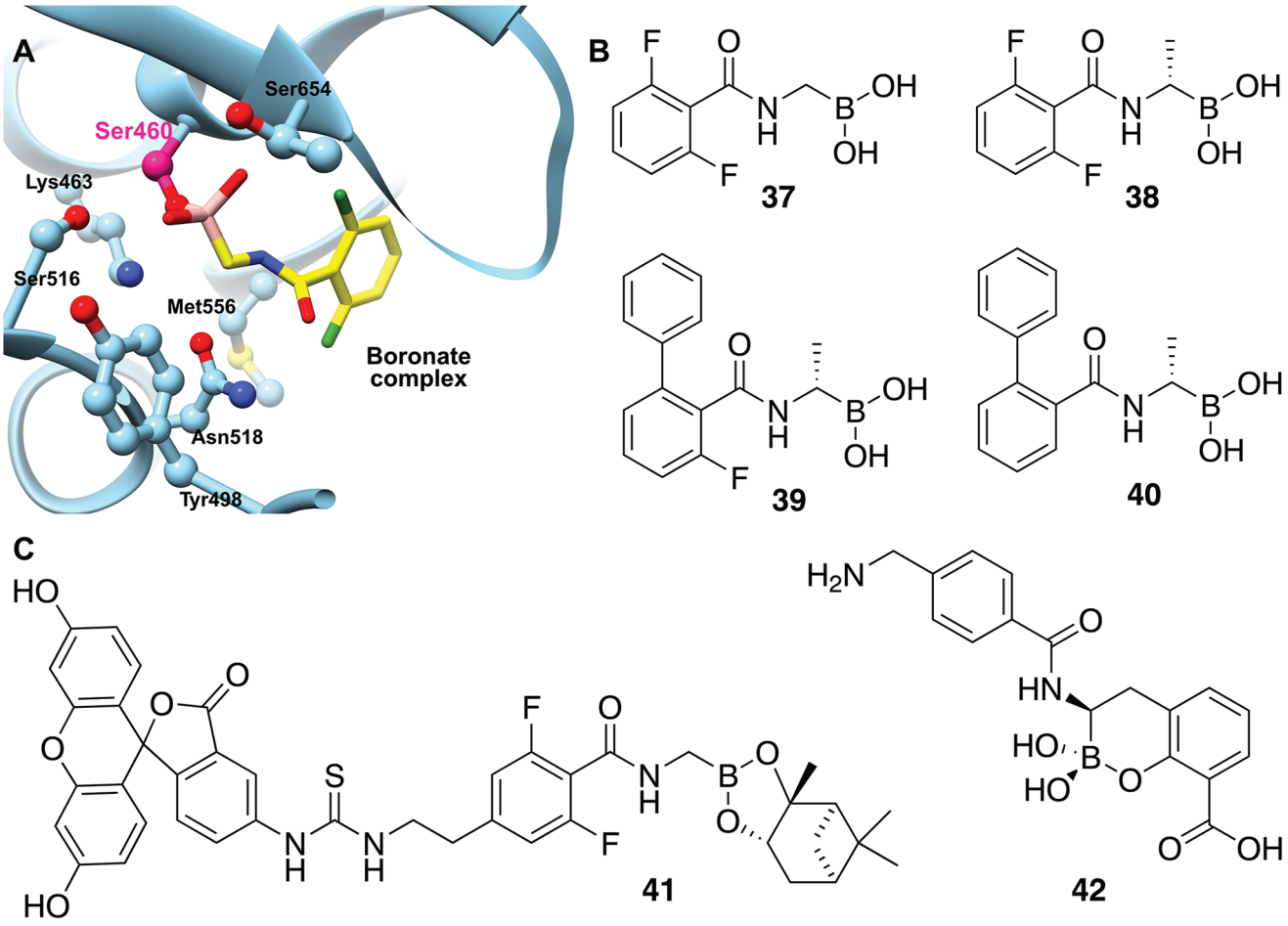

Figure 9. (A) Active site of PBP1b from *S. pneumoniae* bound to boronic acid **37** (PDB ID: 2Y2K).[25] (B) Boronic acid PBP inhibitors: compounds **37**, **38**, **39**, and **40**.[25] (C) Structure of fluorescein labeled-compound **41**[60] and cyclic boronate **42**.[61]

as PBP inhibitors, such as Nacubactam (**32**, Figure 8),[56] Durlobactam (**33**, Figure 8),[57] Zidebactam (**34**, Figure 8),[58] CDP4 (**35**, Figure 8),[21] and ETX0462 (**36**, Figure 8).[59]

Other inhibitors

Boron compounds. Boronic-acid-based inhibitors are organoboranes that act as Lewis acids and inhibit nucleophilic enzymes, including PBPs and β-lactamases.[25] In its trivalent form, the boron atom has an electron-deficient nature, allowing it to form reversible covalent "tetrahedral" adducts with nucleophilic molecules.[60] In 2011, Contreras-Martel *et al.*[25] identified boronic-acid-based PBP inhibitors by employing PBP1b from *S. pneumoniae* as a model enzyme. The evaluation of high-resolution crystal structures of PBP1b bound to a number of boronate-based inhibitors, including compound **37** (Figure 9(A)), allowed the verification that the Oγ from Ser460 makes a covalent bond with the boron atom, forming a tetrahedral complex. In the structure, the acetamido side chain of **37** is positioned similarly to what is observed in β-lactam acyl–enzyme complexes (Figure 9(A)). Notably, boronic acid **37** (Figure 9(B)) was the most active inhibitor tested, presenting an IC_{50} of 6.9 ± 0.1 μM against PBP1b. Antibacterial activity of the compounds was also evaluated with compounds **38**, **39**, and **40** (Figure 9(B)) showing the most promising results against Gram-positive species, including methicillin-resistant *S. aureus* (MRSA).

The ability of boronic acids to covalently bind to PBPs indicated that they could be promising labeling reagents for fluorescence polarization (FP)-based assays for PBPs and SBLs.[60] An example of a labeled molecule used in FP-based assays with PBPs is BOCILLIN FL.[61] However, the irreversible nature of the BOCILLIN-PBP bond limits its applicability for the study of weakly binding compounds. Aiming to develop an alternative assay based on reversibly binding reagents, Inglis and co-workers evaluated boronic-acid-based compounds[60] using a design based on crystal structures of PBP1b. It was observed that the replacement of the aromatic ring by a linker in the para position connecting the fluorescein group could be accommodated in the active site with the fluorescein group remaining largely exposed to the solution. Fluorescein-labeled compound **41** was synthesized by coupling a (–)-(pinanediolboronate)amine derivative with fluorescein-5-isothiocyanate (Figure 9(C)). Compound **41** binds to three different PBPs with modest affinity ($K_d = 4$–12 μM) and more tightly to the TEM1 ($K_d = 109$ nM). Evaluation of the efficiency of compound **41** in competitive binding assays against compound **37** showed that these compounds competed for binding to TEM1 with an EC_{50} value of approximately 900 nM. These results indicated

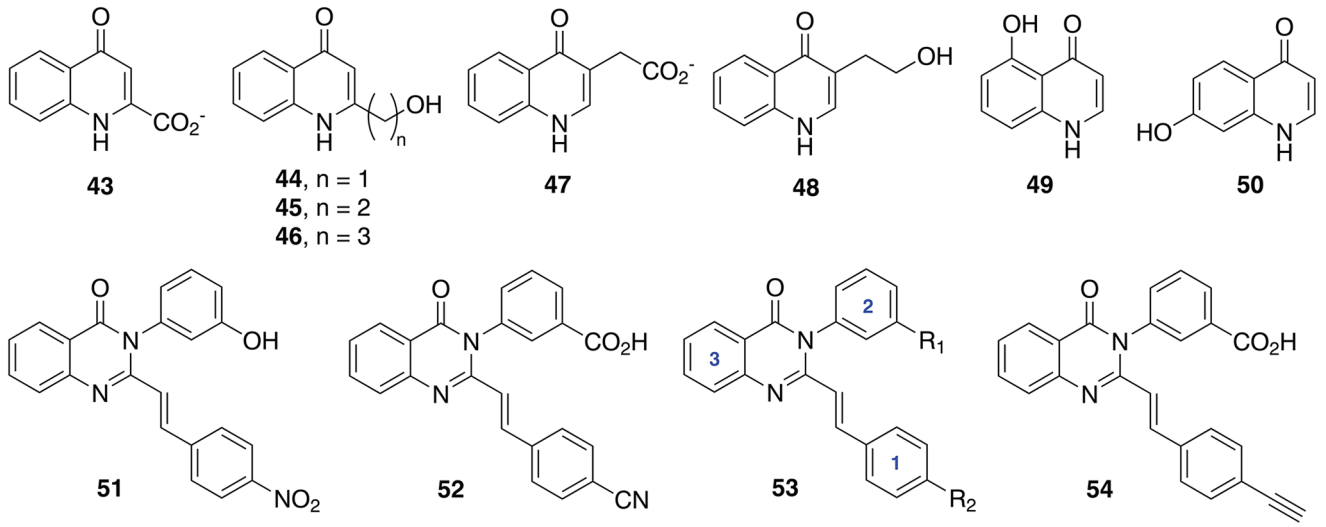

Figure 10. Compounds with PBP inhibition activity: 4-quinolones **43–50**,[65] quinazolinones **51**, **52**,[63] and **54**.[66]

that fluorescent boronic acids can serve as reversibly binding tracers in FP-based assays with PBPs and SBLs and potentially with other related enzymes.[60]

Cyclic boronates can potently inhibit both SBLs and metallo-β-lactamases (MBLs) by mimicking the common high-energy tetrahedral intermediate.[62] These compounds also strongly inhibit the non-essential PBP5 from *E. coli* through the same mechanism of action, leading to a residual activity that is lower than 1% at 10 μM.[62] SBLs and PBPs are evolutionarily and mechanistically related, but MBLs are distinct and constitute a heterogeneous group.[62] Compound **42** (Figure 9(C)) was identified as a potent inhibitor of all three enzyme classes *in vitro* (IC_{50} = 1.6 nM for PBP5, IC_{50} = 3.0 nM for TEM-1, IC_{50} = 3.0 and 29.0 nM for VIM-2 and NDM-1, respectively), but did not display antibacterial activity on its own. When employed in combination, it reduced the MIC of MER (**20**, Figure 5) for strains producing NDM-1 at the concentration of 10 μg mL^{-1}. Cyclic boronates are a promising class of compounds for the development of inhibitors for both MBLs and SBLs and, also, for direct inhibition of PBPs.

Heterocyclic compounds. Over the years, β-lactams have been the antibiotics of choice for treating *S. aureus* infections.[63] However, these molecules became obsolete with the emergence of MRSA. Clinical resistance to β-lactam antibiotics by MRSA is based on the acquisition of the *mecA* gene, which encodes PBP2a.[64] Strategies including computational fragment-based approaches were employed in the search for small molecules that could noncovalently bind within the PBP active site, including studies based on compounds with heterocyclic cores that differed from β-lactams. Investigations using docking studies suggested that 4-quinolones were good binding candidates. Competition assays using BOCILLIN FL in the presence of PBPs from *E. coli* were used to measure dissociation constants (K_i)s.[65] Derivatives **43**–**50** (Figure 10) were able to bind to PBPs with different affinities: K_i between 27 ± 4 and 510 ± 40 μM to PBP1a/1b; K_i between 26 ± 8 and 250 ± 30 μM to PBP2; K_i between 27 ± 7 and 120 ± 80 μM to PBP3; K_i between 3.1 ± 0.8 and 55 ± 10 μM to PBP4; K_i between 220 ± 80 and 520 ± 160 μM to PBP5/6. However, no antimicrobial activity was observed.[65]

A high-throughput *in silico* screening was subsequently performed, leading to 118 high-rankers that were tested for antibacterial activity against *E. coli* and the ESKAPE panel of bacteria (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species).[63] This evaluation indicated that quinazolinone **51** (Figure 10) displayed a MIC of 2 and 16 μg mL^{-1} against *S. aureus* ATCC 29213 and *E. faecium*, respectively. Aiming to improve this *in vitro* potency, 80 analogs of quinazolinone **51** were synthesized and screened for *in vitro* antibacterial activity and other desired properties.[63] Quinazolinone **52** (Figure 10) emerged from these studies with a MIC of 2 μg mL^{-1} against MRSA NRS70 and showed efficacy in the mouse peritonitis model of infection with a terminal half-life of 20 h and an oral bioavailability of 50%.[63] The crystal structure of the complex between PBP2a and quinazolinone **52** revealed density for the compound within a secondary, potential allosteric site of PBP2a at 60 Å from the transpeptidase active site. The binding of ligands at the allosteric site of PBP2a has been suggested to lead to the opening of the active site, enabling the inhibition of peptidoglycan synthesis.[63] Subsequently, SAR for the 4(3 H)-quinazolinone core with 77 analogs was investigated by introducing variations on the rings 1, 2, and 3 at the core structure (**53**, Figure 10).[66] This evaluation led to the discovery of compound **54** (Figure 10), which presents MICs of 0.03 μg mL^{-1} (*S. aureus* ATCC 29213), 0.06 μg mL^{-1} (*S. aureus* ATCC 27660 and VRS1), 0.06 μg mL^{-1} (*S. aureus* NRS119), 0.03 μg mL^{-1} (*S. aureus* VRS2) and better efficacy than compound **52** in the mouse neutropenic thigh infection model.[66] Although the quinazolinone by itself is bacteriostatic *in vitro*, compound **54** presents bactericidal synergy when used in combination with PIP (**1**) and/or tazobactam (a β-lactamase inhibitor).[67] Compound **54** also binds to the allosteric site of PBP2a, as demonstrated by X-ray crystallography, and to PBP1.[67]

Another class of non-β-lactams that inhibits MRSA PBP2a are the oxadiazoles.[64,68] *In silico* screening of the

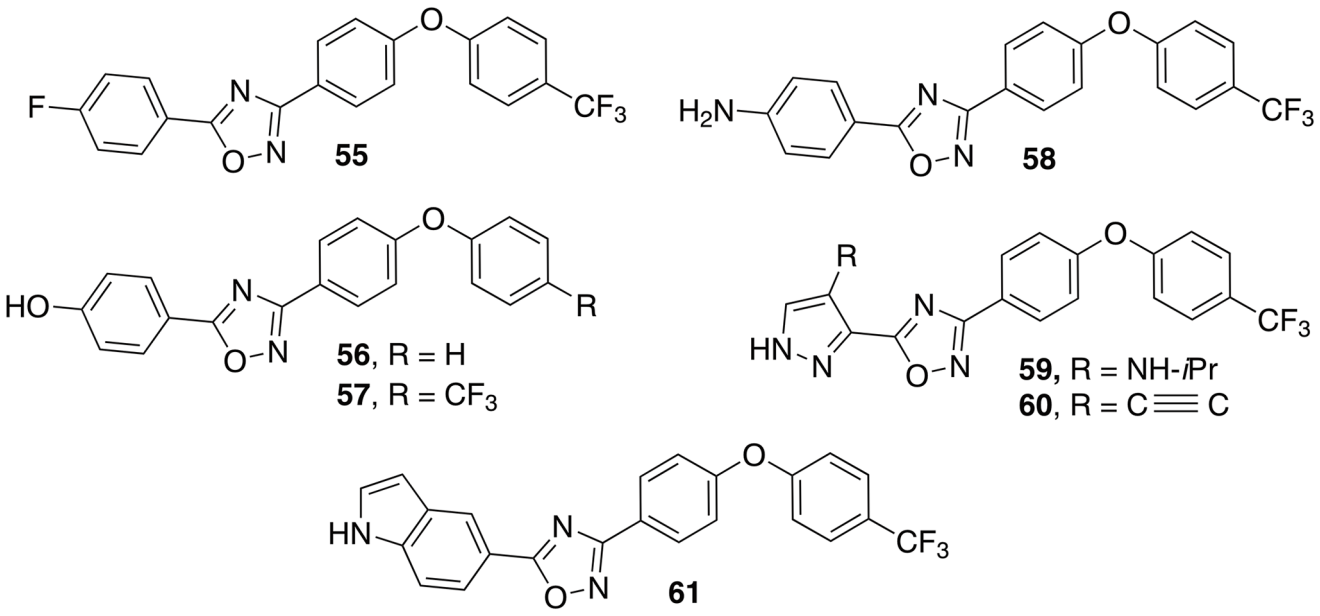

Figure 11. Examples of oxadiazole derivatives with antibacterial activity: compounds **55**–**58**[64] and **59**–**61**.[68]

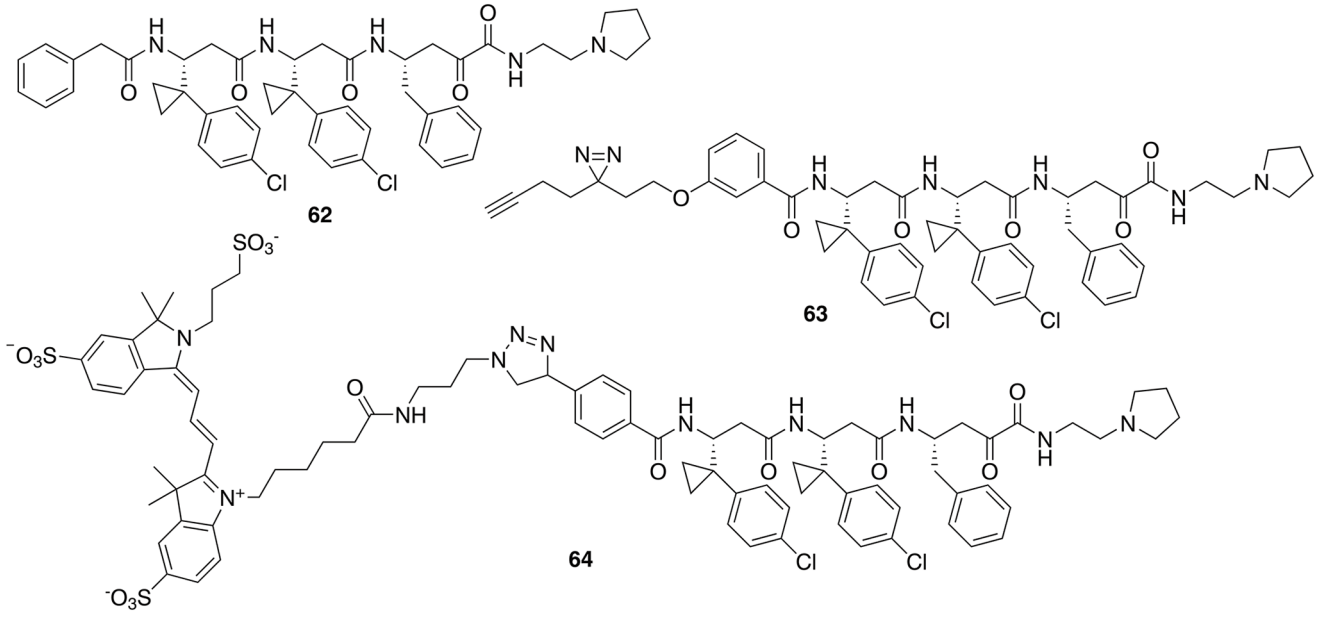

Figure 12. Lead β-peptide oligomer **62** and its labeled modified compounds **63** and **64.**[69]

ZINC database led to the selection of 29 compounds that were tested for antibacterial activity against *E. coli* and other ESKAPE pathogens.[64] Compound **55** (Figure 11) emerged from the screening as a possible inhibitor and inspired the synthesis of a library with 370 derivatives that were evaluated against the same panel. Compounds **56**–**58** (Figure 11) presented MIC values against *S. aureus* (including MRSA) and vancomycin-resistant *E. faecium* in the range of 1–2 μg mL^{-1}. MIC values indicate that PBPs other than PBP2a are likely inhibited by oxadiazoles since not all bacteria tested express PBP2a.[64] The chemical structural space of oxadiazole **57** was also explored by syntheses of 120 derivatives.[68] Although some compounds are potently active *in vitro* against bacteria, they are also cytotoxic. For example, pyrazole derivatives **59** and **60** (Figure 11) were the most active (MICs of 0.50 and 0.25 μg mL^{-1}, respectively) against *S. aureus* ATCC 29213, but also exhibit toxicity to mammalian cells.[68] Replacement of the pyrazole group by an indole circumvented the toxicity issues while maintaining the antibacterial activity. Compound **61** (Figure 11) showed good efficacy *in vivo* (MIC 4 μg mL^{-1}) with a long half-life, a high volume of distribution, low clearance, and 97% oral bioavailability.[68] These studies indicated that the discovery of new classes of compounds may be critical to future success in the treatment of MRSA infections since only linezolid and tedizolid are presently orally approved bioavailable treatments.

Carbonyl compounds. In 2019, Stepek *et al.*[69] produced a library of β-peptide oligomers through synthetic fermentation using an α-ketoacid-hydroxylamine (KAHA) amide-forming ligation strategy with a mixture of 3 α-ketoacid initiators, 24 isoxazolidine monomers with different side chains, and 4 terminators. Phenotypic screening followed by SAR studies led to the identification of peptide **62** (Figure 12), which was able to inhibit the growth of *B. subtilis* with a MIC of 5.7 μg mL^{-1} and with low toxicity (IC_{50} above 100 μg mL^{-1}) against HEK293 cells. Using a chemical proteomic approach, PBP4 from *B. subtilis* was identified as its target through the use of a photoaffinity-labeled variant of compound **62** containing a diazirine and an alkyne group (**63**, Figure 12) and evaluated by quantitative mass spectrometry. When conjugated to a cyanine-3 probe and tested in microscale thermophoresis (MST) with PBP4 from *B. subtilis*, compound **64** (Figure 12) showed high affinity for the protein with a dissociation constant (K_d) of 364 ± 19.1 nM.[69] This work showed that synthetic fermentation can be a rapid and useful tool for phenotypic screening through the preparation and evaluation of thousands of molecules on a live organism. It also describes a pipeline where synthetic fermentation cultures could be used for biological evaluation after dilution without any purification step.

Conclusions and future perspectives

Since the discovery of β-lactams, several other classes of compounds with antibiotic activity have been discovered identified and used to save millions of lives. As pathogens inevitably become resistant to antibiotics, the search for new active compounds is under permanent pressure. In this search several strategies are used, but essential proteins for bacterial wall synthesis, such as PBPs, remain excellent targets. As observed, strategies aimed at the search for new antibiotics starting from well-known chemical classes by medicinal chemistry are among the most used approaches. However, with the advancement of *in silico* technologies, high-throughput screening methods, new labeling techniques, and a better understanding of protein structures using artificial intelligence (AI) strategies, the development of new PBP inhibitors with good *in vivo* efficacy could become a reality. An interdisciplinary approach will be essential for overcoming the challenges of the antibiotic crisis.

AUTHORS' CONTRIBUTIONS

All authors contributed to the planning and writing of the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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