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Overcoming Resistance to β-Lactam Antibiotics

Roberta J. Worthington and **Christian Melander***
Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695

Abstract

β-Lactam antibiotics are one of the most important antibiotic classes but are plagued by problems of resistance and the development of new β-lactam antibiotics through side chain modification of existing β-lactam classes is not keeping pace with resistance development. In this perspective we summarize small molecule strategies to overcome resistance to β-lactam antibiotics. These approaches include the development of β-lactamase inhibitors and compounds that interfere with the ability of the bacteria to sense an antibiotic threat and activate their resistance mechanisms.

Drug-resistant bacterial infections are a continually escalating problem that significantly threatens public health and new approaches to combat them are sorely needed. This synopsis presents a brief background to the β-lactam class of antibiotics and details the mechanisms by which bacteria exhibit resistance. We discuss the various strategies that have been investigated to overcome resistance to β-lactams, focusing on the most successful strategy to-date - the use of β-lactamase inhibitors, and on more recent work involving the use of small molecules to interfere with pathways that allow bacteria to sense and respond to the presence of antibiotics, including the development of the 2-aminoimidazole (2-AI) class of compounds as suppressors of β-lactam resistance.

β-Lactams are one of the three largest classes of antibiotics[1] and the history and mechanism of action of these antibiotics has been extensively reviewed.[2] β-Lactams have been amongst the most successful drugs for the treatment of bacterial infections caused by numerous species for the past 60 years,[3] and represent (as of 2004) over 65% of the world antibiotic market[4] but have been plagued by the problem of increasing clinical resistance. β-Lactams exert their antibiotic effects by mimicking the natural D-Ala-D-Ala substrate of the family of enzymes known as penicillin-binding proteins (PBP), which are responsible for cross-linking the peptidoglycan component of the bacterial cell wall.[5] β-Lactam antibiotics form an acyl-enzyme complex with PBPs, as evidenced by the crystal structure reported by Lee et al. of a cephalosporin derivative bound to a bifunctional carboxypeptidase/transpeptidase from *Streptomyces sp.* strain R61,[6] thereby inhibiting their transpeptidation activity and disrupting the integrity of the cell wall, which ultimately results in cell lysis.

There are several classes of β-lactam antibiotics including: penicillins, cephalosporins, carbapenems and monobactams (Figure 1). The first β-lactam antibiotic to be introduced to the clinic was penicillin G in the early 1940s, and by 1944 reports of penicillin-resistant *Staphylococcus aureus* began to emerge, due mainly to the production of β-lactamases, enzymes that inactivate the antibiotic by hydrolyzing the β-lactam core.[7] The subsequent isolation of 6-amino-penicillanic acid (6-APA) in 1959 allowed the development of numerous semi-synthetic penicillins such as methicillin that were stable to attack by staphylococcal β-lactamases as a result of steric protection of the β-lactam ring.[8] However methicillin-resistant *S. aureus* (MRSA) isolates were observed within two years of

* *Corresponding author*: ccmeland@ncsu.edu.

introduction to the clinic,[9] due to production of an alternative penicillin-binding protein (PBP2a) that is resistant to inhibition by currently available β-lactam antibiotics. This resistance is due to limited accessibility of the antibiotics to the active site, which results in a reduced rate constant for acylation (3–4 orders of magnitude) as compared to other PBPs, and an increased dissociation constant for the preacylation complex.[10,11] In contrast to the low accessibility of the PBP2a active site to β-lactam antibiotics, the native peptidoglycan substrate is still able to access the active site, believed to be a result of conformational changes brought about by allosteric binding of peptidoglycan to the enzyme, resulting in effective peptidoglycan cross-linking and subsequent cell-wall viability.[11]

Early penicillins also exhibited little activity against Gram-negative pathogens, which was overcome by the development of aminopenicillins that were active against *Escherichia coli, Shigella,* and *Salmonella* species but not *Pseudomonas aeruginosa* or *Klebsiella* species.[12] Replacement of the amino group of aminopenicillins with a carboxyl group, giving rise to the carboxypenicillins, delivered β-lactams that were effective against *P. aeruginosa* as a result of their low affinity for the AmpC β-lactamase. However, as is continually observed following the introduction of any new antibiotic, resistant strains were soon isolated.

The cephalosporin class of β-lactam antibiotics, discovered in the late 1940s, is stable to the staphylococcal β-lactamase, which was a clinical problem early on, and several generations of semi-synthetic cephalosporins have been developed. Early cephalosporins proved useful for the treatment of infections caused by Gram-negative bacteria, with the exception of *P. aeruginosa*,[8] while third-generation cephalosporins, including cefoperazone and ceftazidime were used successfully for many years to treat infections caused by *P. aeruginosa.*

The discovery of both the carbapenem and monobactam classes of β-lactam antibiotics, which resulted in the introduction of imipenem in 1985 and aztreonam in 1986, gave rise to increased therapeutic options for bacterial infections that had become recalcitrant to treatment with other β-lactams. Carbapenems have in the past been reserved for the most difficult infections caused by Gram-negative bacteria,[13] however resistance is now widespread in Enterobacteriaceae, *P. aeruginosa* and *Acinetobacter* spp., mostly due to the increasing prevalence of carbapenemases.

Despite intensive medicinal chemistry campaigns to modify β-lactam antibiotics, several bacterial strains have developed resistance to every β-lactam antibiotic (and every antibiotic in general) introduced to the clinic. Resistance to β-lactam antibiotics predominantly occurs through one of two mechanisms: 1) the production of β-lactamases, which is the most common resistance mechanism in Gram-negative bacteria, or 2) the production of an altered PBP with a lower affinity for most β-lactam antibiotics.

There are two structural types of β-lactamases: 1) serine β-lactamases[14], and 2) metallo-β-lactamases (MBL). Important serine-β-lactamases include extended-spectrum β-lactamases (ESBL) that hydrolyze later-generation cephalosporins, and carbapenemases such as *Klebsiella pneumoniae* carbapenemases (KPC) that hydrolyze carbapenem antibiotics, in addition to later generation cephalosporins. MBLs are Zn (II) dependent enzymes that can accommodate most β-lactams in their active site and will hydrolyze almost all β-lactam antibiotics including carbapenems. Recent international dissemination of Gram-negative bacteria harboring plasmid encoded MBLs including the New-Delhi metallo-β-lactamase (NDM-1), has increased the clinical importance of this class of β-lactamases.[15,16] High-level β-lactam resistance can be conferred by the acquisition of plasmids containing numerous resistance genes, including multiple β-lactamases of different classes, which can be rapidly disseminated amongst many bacterial species, making some bacteria resistant to virtually all known β-lactam antibiotics.[17]

In MRSA, the key resistance determinant is the production of PBP2a, which, as mentioned earlier, is much less efficiently inhibited by β-lactam antibiotics compared to native *S. aureus* PBPs. PBP2 and PBP2a function together to provide the transpeptidation and transglycosylation activities necessary to crosslink peptidoglycan and construct a functional cell wall even in the presence of β-lactam antibiotics.[18]

Based upon these resistance mechanisms, there are essentially two options to allow the continued employment of β-lactam antibiotics: 1) design new β-lactam antibiotics that are not affected by the above mentioned bacterial resistance mechanisms, or 2) combine current β-lactam antibiotics with a drug that disables the resistance mechanisms. While the development of new β-lactam antibiotics continues to be explored,[19] the fact remains that no new β-lactam class has been discovered in over thirty years and new β-lactam derivatives based upon existing β-lactam scaffolds comprise only a minority of the new antibiotics in clinical development.[20] It is therefore expected that combination therapy will account for the most common use of β-lactam antibiotics in the future.

Arguably, the most successful approach employed to date to extend the utility of β-lactam antibiotics is to pair the antibiotic with an inhibitor of β-lactamase activity. One such example is clavulanic acid, which was isolated from *Streptococcus clavuligerus* in 1972.[21] Clavulanic acid contains a β-lactam core but possess limited antibiotic activity and is instead a potent inhibitor of many serine β-lactamases, binding to the active site of the enzyme and exhibiting concentration-dependent competitive inhibition. Clavulanic acid forms an acyl enzyme complex that can either rearrange to form a stable enamine intermediate, thereby transiently inhibiting enzyme activity or can acylate a second active site nucleophile to irreversibly inactivate the enzyme.[22,23] In 1984 clavulanic acid entered into clinical use paired with amoxicillin and marketed as Augmentin, which became the first β-lactam antibiotic/β-lactamase inhibitor combination used to treat bacterial infections. Augmentin was the best-selling antibiotic in 2001, demonstrating the effectiveness of the β-lactamase inhibitor approach in clinical settings.[24]

Many β-lactamases are not inhibited by currently available inhibitors,[25,26] however several newer β-lactamase inhibitors are currently being developed (Figure 2) that have demonstrated promising *in vitro* inhibition of ESBLs, including the imidazole-substituted 6-methylidene-penem compound BLI-489[27] **1** and the tricyclic carbapenem LK-157 **2**.[28] The diazabicyclooctane (DBO) class of β-lactam inhibitors, first developed in the mid-1990s by Hoechst Marion Roussel (now part of Sanofi-Aventis) has proven a useful source of non-β-lactam derived inhibitors. DBO β-lactamase inhibitors inhibit serine β-lactamases including ESBLs and KPCs but are not active against MBLs. Two DBOs, MK-7655 **3**, and Avibactam (NXL104, AVE1330A) **4**, are in clinical development. MK-7655 increases the susceptibility of AmpC- and KPC-producing *P. aeruginosa* and *K. pneumoniae* isolates to imipenem,[29] and a concise scalable synthesis of this compound was recently reported in 12 steps with an overall yield of 10%.[30] Avibactam has been shown to restore the activity of ceftazidime against a number of Gram-negative bacteria including ESBL producing *K. pneumoniae* and *E. coli*,[3] and has demonstrated efficacy in animal models and in phase II clinical trials.[31] Like other β-lactamase inhibitors, Avibactam covalently binds the enzyme in a reversible manner and unlike β-lactam derived inhibitors is not susceptible to hydrolysis once bound to the enzyme, instead deacylaction of the inhibitor/enzyme complex regenerates intact Avibactam.[31] Additionally, the off-rate for deacylation is slow (0.045 min^{-1} for TEM-1, corresponding to a half-life of enzyme recovery of 16 min)[31] and Avibactam does not induce β-lactamase production as is the case for clavulanic acid.[3]

Due to increasing prominence of MBLs in MDR infections, there have been significant efforts to design compounds that are able to inhibit MBLs. As mentioned above, MBLs are

not inhibited by any clinically available β-lactamase inhibitors and the design of drugs that inhibit these enzymes is particularly challenging, due to both the structural diversity of MBLs and the necessity to avoid the development of compounds that indiscriminately chelate metal ions, which would result in unwanted effects on other metalloenzymes.[32] Using a coordination chemistry based approach, Chen et al. recently identified two low micromolar MBL inhibitors, 2-phenyl-4,5-dihydrothiazole-4-carboxylic acid **5** and 2-(3-aminophenyl)-4,5-dihydrothiazole-4-carboxylic acid **6**. Compound **5** inhibited the *P. aeruginosa* MBL IMP-1 with an IC_{50}value of 5.5 μM, and compound **6** inhibited the *Bacillus anthracis* MBL Bla2 with an IC_{50}value of 4.9 μM; however no compound was identified that effectively inhibited both MBLs.[13] The inhibitor cocktail BAL30367, which is a triple combination of the siderophore monobactam BAL19764 **7**, a bridged monobactam BAL29880 **8**, and clavulanic acid[33] has shown good *in vitro* activity in restoring the activity of β-lactam antibiotics against MBL producing Enterobacteriaceae. Other classes of MBL inhibitors include the methylcarbapenems J-110,441 **9**[34] and J-111,225 **10**,[35] which have been shown to lower imipenem MICs against *P. aeruginosa*, and the mercaptomethyl penicillinate **11**,[36] which increases the susceptibility of MBL producing *P. aeruginosa* to piperacillin.

Current clinical strategies to restore activity to β-lactam antibiotics are for the most part restricted to combining the antibiotic with a β-lactamase inhibitor. However, it is clear that alternative approaches to overcome β-lactam resistance are necessary and recent approaches include targeting other steps in cell wall-biosynthesis and interference with bacterial mechanisms to sense the antibiotic threat and activate resistance mechanisms. Representative examples of these approaches are discussed below.

Many of these alternative strategies to potentiate the effects of β-lactam antibiotics involve targeting another step of cell-wall biosynthesis, either with a compound that is not microbicidal when administered alone, or with another antibiotic that when in combination with the β-lactam antibiotic displays synergistic effects. For example, several clinically available antibiotics that act through inhibition of early steps in cell wall biosynthesis such as fosfomycin, bacitracin, and glycopeptides exhibit synergy with β-lactam antibiotics against MRSA when administered sub-inhibitory concentrations. For example, in the case of fosfomycin, minimum inhibitory concentrations (MICs) of methicillin are reduced by up to 128-fold (from 800 μg/mL to 6 μg/mL) in the presence of 25% the MIC of fosfomycin.[37]

Several other compounds that inhibit one of the many proteins involved in cell-wall biosynthesis have been investigated for the potential to increase bacterial susceptibility to β-lactam antibiotics, predominantly in MRSA (Figure 3). FtsZ, a guanosine triphosphatase (GTPase) involved in cell division was identified as being a β-lactam susceptibility determinant in MRSA, most likely due to its role in the recruitment of proteins required for peptidoglycan synthesis.[38] A known inhibitor of FtsZ, PC190723 **12** that has potent antibacterial activity against MRSA and *Bacillus subtilis* (MICs of 0.5 to 1 μg/ml),[39] was shown to act synergistically with β-lactam antibiotics against a large number of MRSA strains. PC190723 causes a marked change in both FtsZ and PBP2 localization, such that a much lower amount of β-lactam is required to inactivate the residual correctly located (and therefore functional) PBP2. The combination of PC190723 and imipenem was also synergistic *in vivo* and was shown to be efficacious in a murine thigh model of MRSA infection.[38]

GlmS, a glucosamine-6-phosphate synthase involved in the first step of the peptidoglycan precursor synthesis was identified through a chemical genetic screen as being a possible drug target to potentiate the effects of β-lactam antibiotics in MRSA. This confirmed by the fact that the known GlmS inhibitor Nva-FMDP **13** exhibits synergistic activity with a broad

range of β-lactam antibiotics against diverse methicillin-resistant staphylococci including MRSA-COL and MRSA USA300.[18]

SpsB is an essential cell surface signal peptidase that plays a role in protein translocation across the cytoplasmic membrane in *S. aureus*.[40] The natural products krisynomycin **14** and actinocarbasin **15**, identified through a high-throughput screening campaign as potentiating the activity of imipenem against MRSA, were subsequently shown to be potent inhibitors of SpsB (IC_{50} values of 120 and 50 nM respectively).[41] Synthesis of a number of derivatives of actinocarbasin[42] led to the development of the structurally simplified analogue M131 **16**, which has an IC_{50} value of 10 nM against SpsB and restored imipenem susceptibility (MIC ≤ 4 μg/mL) to MRSA-COL (imipenem MIC alone 16–32 μg/mL) at a concentration of 0.125 μg/mL. M131 was also shown to synergize with imipenem *in vivo* using two murine models of MRSA infection. The authors posited that the activity of these compounds is a result of the obstruction of the secretion of proteins involved in cell wall biosynthesis that are essential for β-lactam resistance in MRSA.[41]

The natural product tunicamycin **17**,[43] and the antiplatelet drug ticlopidine (Ticlid) **18**,[44] which inhibit the *N*-acetylglucosamine-1-phosphate transferase TarO, an enzyme involved in the synthesis of the cell-wall component teichoic acid both increase the susceptibility of MRSA to β-lactam antibiotics. Tunicamycin lowers the oxacillin MIC from 25 to 0.4 μg/mL at a concentration of 0.08 μg/mL while Ticlid lowers the cefuroxime MIC against several MRSA stains including a USA300 strain by up to 64- fold.

Cyslabdan **19**, a diterpene produced by *Streptomyces* sp. K04-0144, potentiates imipenem activity against MRSA. The molecular target of cyslabdan was revealed using a pull-down assay with a biotinylated analogue of cyslabdan to be FemA,[45] a protein involved in the formation of the pentaglycine bridge component of peptidoglycan.[46] Cyslabdan was shown to inhibit the enzymatic activity of FemA *in vitro* and displayed negligible inhibition of the functionally related proteins FemB and FemX. Cyslabdan causes an accumulation of nonglycyl and monoglycyl murein monomers in MRSA call wall peptidoglycan, further suggesting that its mode of action of β-lactam resistance suppression is though inhibition of pentaglycine biosynthesis.[45]

The sesquiterpene farnesol **20** has also been shown to increase the susceptibility of MRSA to β-lactam antibiotics and was shown to reduce C_{55} lipid carrier synthesis through the malevonate pathway, resulting in a subsequent reduction in murein monomer precursor transport across the cell membrane.[47]

An alternative approach to re-sensitizing resistant pathogens to the effects of β-lactam antibiotics involves interfering with the pathways by which bacteria sense the presence of antibiotics and activate their resistance mechanisms. For example the expression of both β-lactamase and PBP2a in MRSA is inducible upon exposure to β-lactams, controlled by the *bla* and *mec* regulatory systems respectively,[16] while expression of chromosomally encoded AmpC β-lactamase is induced upon exposure to β-lactams in many Gram-negative bacteria including *P. aeruginosa*.[48] Induction of AmpC expression in *P. aeruginosa* upon exposure to β-lactams is controlled by metabolites of the peptidoglycan-recycling pathway, which is comprised of a number of proteins. Genetic inactivation of several of these proteins, including the *N*-acetyl-β-D-glucosoaminidase NagZ and the inner membrane permease AmpG results in increased susceptibility to β-lactam antibiotics in *P. aeruginosa*, and these proteins were therefore proposed as potential targets for small-molecule inhibitors to enhance β-lactam efficacy.[48] The known *N*-acetyl-β-D-glucosoaminidase inhibitor PUGNAc **21**, which has been shown to inhibit NagZ from *Vibrio cholerae*,[49] increased the susceptibility of AmpC hyperproducing strains of *P. aeruginosa* to ceftazidime.[50] PUGNAc

is unfortunately also a potent inhibitor of human *N*-acetyl-β-D-glucosoaminidases, limiting its potential for use in a clinical setting. A medicinal chemistry program implemented to develop second-generation inhibitors with enhanced specificity for NagZ resulted in the identification of EtBuPUG **22** that exhibits over 100-fold selectivity for NagZ over the human enzyme.[49]

Our group has spent several years developing simplified, synthetically accessible 2-AI derivatives of the marine natural products oroidin and bromoageliferin as anti-biofilm compounds (Figure 4).[51] More recently, attention has turned to the investigation of the ability of a subset of these compounds to potentiate the effects of β-lactam antibiotics against both Gram-positive and Gram-negative bacteria. The first report of suppression of antibiotic resistance by a member of the 2-AI class was in 2010,[52] in which compound **23** was documented to lower the MICs of penicillin G and methicillin against MRSA by eight-fold and four-fold respectively, and the MIC of imipenem against several strains of multi-drug resistant *Acinetobacter baumannii* by four to eight-fold. Several derivatives of compound **23**, which contain a substituent at the 4-position, were able to lower the MIC of oxacillin against MRSA by four-fold.[53] The related 2-AI **24** was shown to suppress resistance of MRSA to oxacillin[54] and further development of this scaffold by substitution and each position around the 2-AI heterocycle[54–56] revealed that derivatization at the 1-position resulted in the greatest increase in activity, leading to the identification of compound **25**, which potently suppressed resistance of multiple clinically-derived MRSA strains to oxacillin by upwards of 512-fold at 5 μM. Mechanistic studies with this compound using a panel of knockout strains revealed that the resistance suppression activity was dependent upon the presence of the *vraRS* genes, which encode for the VraRS two-component system.[54] Bacterial two-component systems are sensory systems that regulate the expression of specific genes in response to external stimuli and are involved in the regulation of diverse bacterial behaviors, including antibiotic resistance.[57] In MRSA the VraSR system is induced by exposure to several cell-wall acting antibiotics including β-lactams, glycopeptides, and bacitracin[58] and upon induction upregulates the expression of a number of genes known as the cell-wall stress stimulon (CWSS), which include genes encoding for PBP2 in addition to the other cell-wall synthesis enzymes MurZ and SgtB.[59] This results in increased resistance to most VraSR inducing agents. MRSA mutants that are deficient in the VraSR two-component systems are treatable with an oxacillin regimen *in vivo*, whereas the wild-type MRSA strain is recalcitrant to oxacillin treatment, thus suggesting that targeting this system with small-molecule inhibitors is a viable strategy for potentiating oxacillin activity.[60] Another 2-AI **26**, suppresses resistance in Gram-negative bacteria, lowering the MICs of an NDM-1 producing strain of *K. pneumoniae* to imipenem and meropenem,[61] and the mechanistic basis of this activity is currently under investigation. Similarly to the activity of the 2-AI class of molecules upon β-lactam resistance in MRSA, the antipsychotic phenothiazine thioridazine **27** (Figure 4) also suppresses resistance of MRSA to oxacillin[62,63] and dicloxacillin[64] and it has been shown that the oxacillin-induced transcription of several genes belonging to the VraSR regulon is reduced in the presence of thioridazine,[63] as is transcription of *mecA* and expression of PBP2a.[62]

In conclusion, the β-lactam class of antibiotics has arguably been one of the most important and successful drug classes for the last seven decades and, while not the sole solution, can continue to play a valuable role in the fight against infections caused by pathogenic bacteria. Unfortunately, the development of new β-lactam antibiotics will most likely not be sufficient to keep pace with continually evolving bacterial resistance. Therefore, adjuvant approaches to restore the effectiveness of these antibiotics, such as those discussed in this perspective, will most likely play a pivotal role in our evolving approach to the treatment of multi-drug resistant bacterial infections.

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Biographies

Roberta Worthington obtained her PhD from the University of Manchester studying modified nucleic acids before completing a post doc in medicinal chemistry at the Wolfson Institute for Biomedical Research, University College London. She is currently a Research Assistant Professor at North Carolina State University where her research focuses on small molecule control of antibiotic resistance in pathogenic bacteria.

Christian Melander obtained his B.S. degree in chemistry from UC Davis and a PhD from Columbia University. After postdoctoral positions at Caltech and The Scripps Research Institute, he joined the faculty in the department of chemistry at North Carolina State University where he is currently an Associate Professor. Research in the Melander group focuses mainly on infectious disease, with a focus on the development of small molecules to control pathogenic bacterial behavior.

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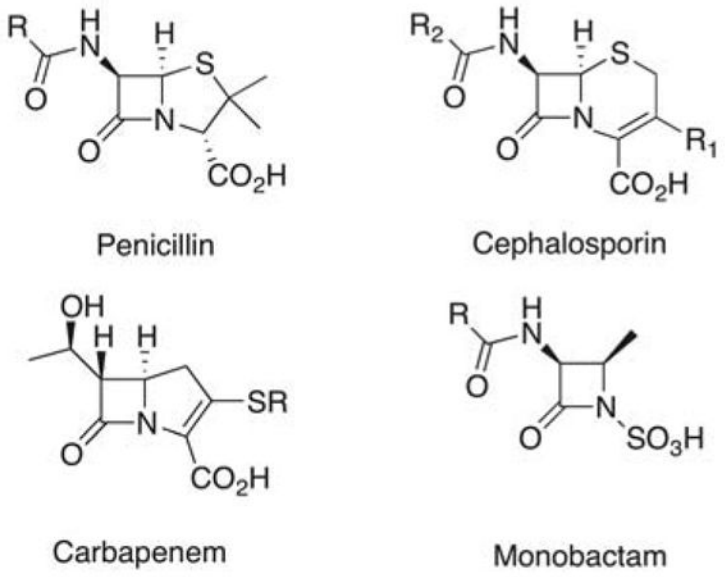

Figure 1.
Structures of the different β-lactam antibiotic classes

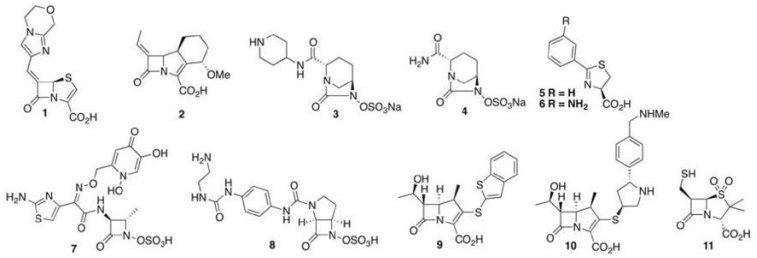

Figure 2.
New β-lactamase inhibitors

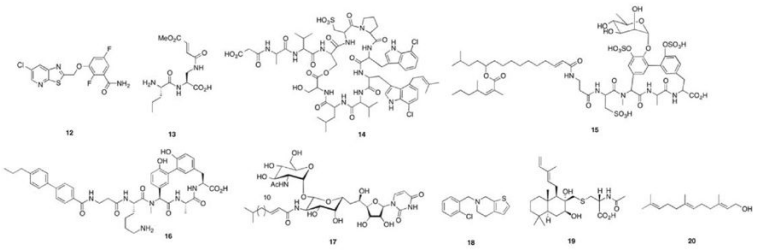

Figure 3.
Compounds that potentiate β-lactam antibiotic activity by interfering with cell wall biosynthesis.

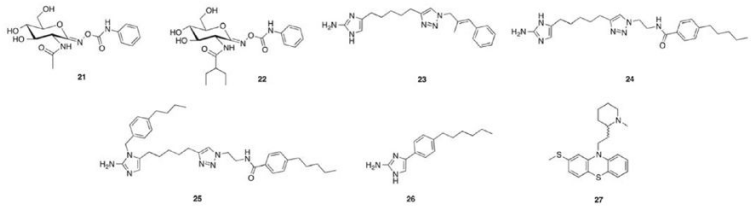

Figure 4.
Compounds that potentiate β-lactam antibiotic activity by affecting antibiotic resistance activation pathways