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β-Lactam antibiotics and β-lactamases: historical perspectives and a review of β-lactamase inhibitors derived from natural products

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The history of β-lactam antibiotics and, subsequently, β-lactamase inhibitors highlight the indispensable role of natural products in modern medicine. The isolation and testing of β-lactam bearing natural products led to world-changing therapeutic breakthroughs, yielding efficacious, safe, and cost-effective medicines that are still widely used today. The scientific triumph of β-lactams was, however, followed by a period of reduced research into new antibiotics, during which time bacterial pathogens acquired multiple mechanisms of resistance. The available evidence suggests that this situation is not irretrievable – whereas insufficient medicinal chemistry is currently being carried out to enable a renaissance in antibiotic development, our molecular understanding of antimicrobial modes of action and resistance mechanisms has improved dramatically. The history of β-lactams and many other drug classes suggests that natural product-based approaches will be critical in the future. Here, we summarise the history of β-lactams, focusing on natural product science. We then examine historical and recent efforts to identify new types of β-lactamase inhibitors using natural product-based screening approaches. We conclude by providing a perspective on how we can most efficiently discover β-lactamase inhibitors from microbial and plant-derived natural products.

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1. Introduction

The rise of bacterial antimicrobial resistance (AMR) presents major global health and economic challenges.[1–7] According to estimates for 2021, bacterial AMR was directly responsible for ~1.14 million fatalities and was associated with an additional

~4.71 million fatalities globally. Amongst the World Health Organisation regions, the South Asian and sub-Saharan African regions were the most affected, accounting for ~47% of global fatalities due to bacterial AMR in 2021.[3] If left unchecked, AMR is predicted to cause 8.2 million global fatalities annually by 2050.[3] In addition to its toll in human lives, the financial costs of AMR are substantial. In China, the economic cost attributed to AMR was estimated to be US$77 billion in 2017, while the United States of America is reported to be losing US$55 billion annually due to AMR.[4,6,7] The World Bank projects that AMR will lead to a 1.1–3.8% reduction in annual global gross domestic product by 2050.[5] The adverse economic effects of AMR will most likely disproportionately impact low-to middle-income countries, further widening the inequality gap between developed and developing countries.

The increase in antibiotic resistance rates over time has been a major driver of the current AMR crisis.[8] Of particular concern are the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), which, along with *Mycobacterium tuberculosis*, present a significant public health challenge.[3,9,10] The ESKAPE pathogens now possess a plethora of intrinsic and acquired mechanisms that enable resistance to multiple antibiotic classes. The increasing resistance to carbapenems (*e.g.*, imipenem, meropenem, tebipenem, and ertapenem), which have often been considered the last line of defence against infections caused by multidrug-resistant pathogens, highlights the AMR crisis. Carbapenem-resistant strains of *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* are now widespread globally.[11] Many ESKAPE pathogens have also

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developed resistance to extended-spectrum cephalosporins, fluoroquinolones, and aminoglycosides.[3] The pan-resistance of many ESKAPE pathogens contributes significantly to AMR by substantially limiting treatment options against them, compromising infection management, and increasing the risk of morbidity and mortality.[3,9,10] The severity of the AMR threat is evident: in 2021, an estimated 683 000 deaths were attributed to infections caused by antibiotic-resistant ESKAPE pathogens alone.[3]

Since the mid-20th century, when the major antibiotic classes were discovered, there has been a dearth in the discovery of new antibiotic classes.[10,12] Contributing factors to this lack of innovation include scientific and technical challenges, limited economic incentives, the efficacy and low costs of existing drugs, and regulatory hurdles.[1,13] The recent lack of new antibiotic classes has exacerbated the global AMR crisis, underscoring the need for a multifaceted solution that extends beyond developing novel antibiotics to include agents that can reverse existing drug resistance. The efficacy of agents targeting the underlying mechanisms of resistance is demonstrated by pioneering 20th-century research that led to β-lactamase inhibitors that protect β-lactam antibiotics, an approach that has the potential to be more widely employed.[14] There is, however, a need for a deeper understanding of both direct and indirect resistance mechanisms to develop new medicines best suited to address the challenge of AMR.

The development and spread of antibiotic resistance results from multiple processes operating at different levels. It is important to distinguish between intrinsic insensitivity to antibiotics, wherein certain bacteria are naturally shielded from antibiotics, for example due to innate cell wall impermeability, and acquired resistance mechanisms that emerge under selective pressure. At the cellular level four major direct mechanisms of acquired resistance have been identified: limiting drug uptake, modifying drug targets, drug efflux, and drug inactivation.[15,16] In Gram-negative *Enterobacter* species, the reduced uptake of imipenem and cephalosporins is achieved by changes in the structures and numbers of porin channels in the outer-membrane, thereby limiting antibiotic entry into cells.[15,16] In Gram-positive bacteria, target modification is a widely used mechanism of drug resistance. For example, mutations or changes in the number of penicillin-binding proteins (PBP) reduce the effectiveness of β-lactam antibiotics. Similarly, drugs that disrupt nucleic acid synthesis, such as fluoroquinolones, are rendered ineffective by target modification.[9,15,16] Most bacteria intrinsically encode (or can acquire) genes for efflux pumps that expel toxic substances out of cells, including antibiotics. Lastly, bacteria can acquire resistance by inactivating drugs *via* their enzyme catalysed degradation or modification. The latter mechanism is employed by a broad spectrum of bacteria, most famously exemplified by the β-lactamases that inactivate β-lactams by catalysing their hydrolysis, yielding inactive β-amino acids.[16] Enzymes that degrade other classes of antibiotics, including tetracyclines, colistin, and fosfomycin, have been identified.[17–20]

Amongst the available antibiotic classes, the β-lactams are arguably the most important currently in use, constituting approximately 60–65% of the total antibiotic market.[21] Increasing resistance to β-lactams, primarily due to β-lactamase activity, poses a critical challenge to these once-considered miracle drugs.[3] To combat β-lactamase-mediated resistance, one established strategy is to use compounds that inhibit β-lactamases. Natural products are a reliable source of lead compounds for drug discovery,[22] including clinically relevant β-lactamase inhibitors. This statement is strikingly exemplified by the discovery and development of the bacteria-derived compound clavulanic acid ~50 years ago. Use of clavulanic acid in combination with a β-lactam antibiotic, in particular amoxicillin, is still increasing.[22]

In this review, we provide a perspective on β-lactams and β-lactamases, with an emphasis on the past and likely future roles of natural product science in both discovering new antibiotics

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and combating their emerging resistance. We conclude by offering thoughts on future avenues to find novel β-lactamase inhibitors from natural product sources.

2. β-Lactams and β-lactamases

2.1 Discovery of β-lactams

The serendipitous observation of the antibacterial activity of penicillin(s) by Alexander Fleming at St. Mary's Hospital, London, heralded the dawn of the modern antibiotic era (Fig. 1 and 2). Fleming observed that alcohol extracts of *Penicillium* mould possessed antibacterial activity against *Staphylococcus* cultures.[23] Fleming, however, failed to isolate the active ingredient from the crude extracts, a challenge subsequently addressed in part by Ernst Chain, Guy Newton, Edward Abraham and others working at the University of Oxford in the 1940s. Abraham's proposal that the core penicillin structure comprised a 4-membered cyclic amide fused to a thiazolidine ring was subsequently validated by crystallography performed by Dorothy Crowfoot Hodgkin (Fig. 1).[24]

Clinical trials using partially purified penicillin began in late 1941, though were hampered by the low quality of (impure) penicillin obtained at the time. The apparent low toxicity of the purified molecule(s) encouraged further trials. By 1945, large-scale production of penicillin in the United States of America and elsewhere had begun, primarily through batch fermentation involving *Penicillium chrysogenum*; the addition of corn-steep liquor resulted in the production of the breakthrough drug benzylpenicillin (penicillin G),[25,26] which was widely used across war-torn Europe.[27] Notwithstanding the success of penicillin, bacterial resistance emerged in the late 1940s (Fig. 2). Soon thereafter, Chain and Abraham discovered the first β-lactamase and purified the enzyme from *E. coli*, highlighting the role of enzymes in mediating antibiotic resistance.[28] By the early 1950s, clinical reports began to emerge on streptococcal resistance to penicillin, prompting research into β-lactamase-stable penicillin analogues as well as new classes of β-lactams and β-lactamase inhibitors.[29]

In the quest for new antibiotics to combat resistance, the discovery of cephalosporins was a significant breakthrough.

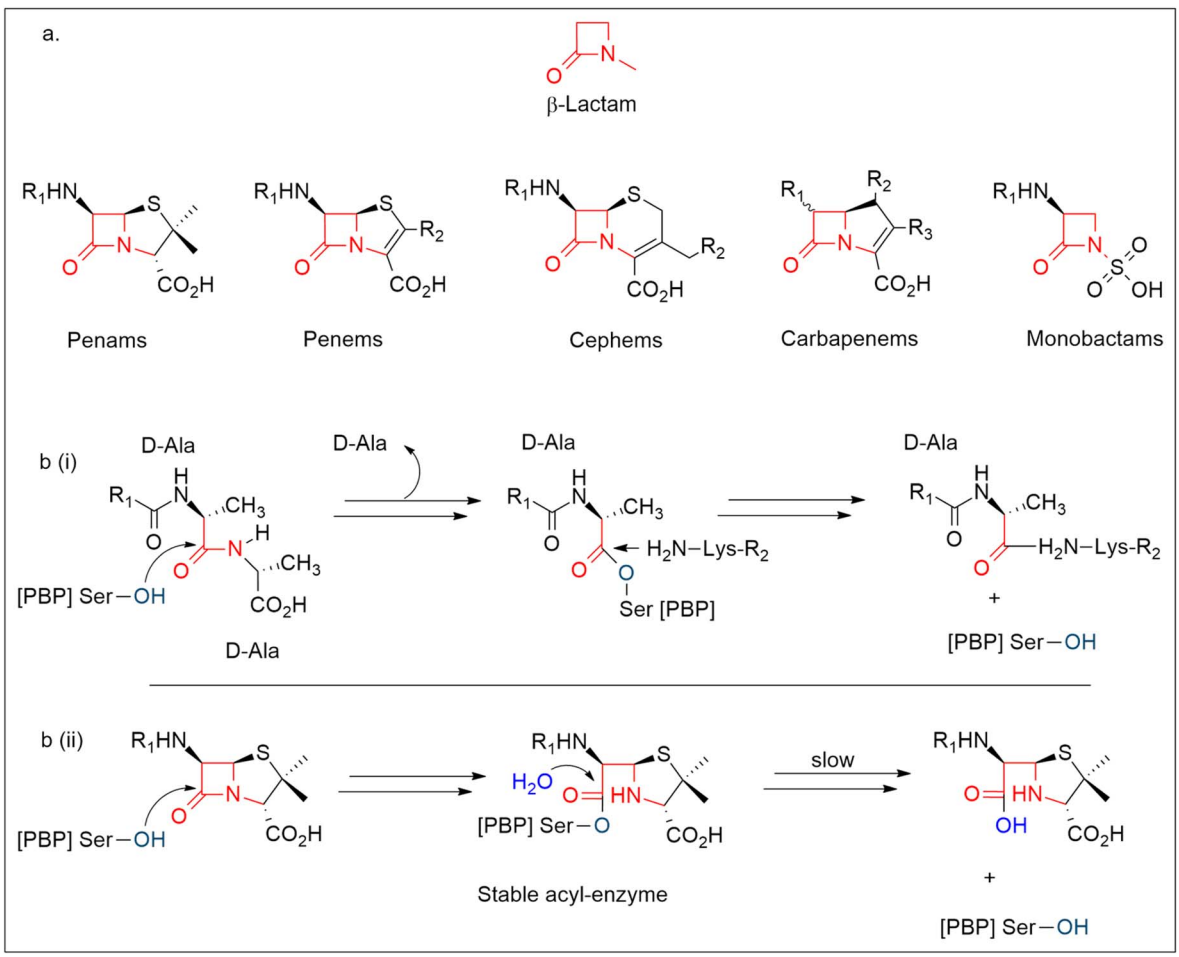


Fig. 1 Structural classes of clinically used β-lactams and their interaction with penicillin-binding proteins (PBP). (a) Core structures of the major β-lactam classes: penams, penems, cephems, carbapenems, and monobactams. (b) Mechanistic comparison of PBP-mediated transpeptidation and inhibition: (i) penicillin-binding proteins catalyse the reaction of a C-terminal D-Ala–D-Ala unit with a PBP nucleophilic serine to produce D-Ala and an acyl–enzyme complex, which undergoes cross-linking with a Lys-sidechain; (ii) β-lactams react with the nucleophilic serine of the PBPs, forming a stable acyl–enzyme complex that undergoes slow hydrolysis, rendering it functionally irreversible. Nucleophilic serine-β-lactamases employ a related mechanism, though their acyl–enzyme complexes normally undergo efficient hydrolysis (see Fig. 7).

Giuseppe Brotzu, at the Hygiene Institute in Cagliari, Italy, isolated an antibiotic-producing strain of *Cephalosporium acremonium* from seawater near the outlet of a sewage pipe.[30] Importantly, Brotzu demonstrated the antibacterial activity of fermentation extracts of *C. acremonium* in patients with a variety of infections, including typhoid fever, where penicillin had failed. Brotzu sent his strain to Oxford, where Abraham and Newton purified two β-lactams: the first was a penicillin and the second was cephalosporin C (Fig. 2 and 3).[31,32] They assigned the structure of cephalosporin C through chemical analysis,[31,33] a proposal validated through X-ray crystallography in 1961.[34] This work marked the first report of a natural β-lactam with a fused ring structure different from that of the penicillins: in cephalosporins, the β-lactam is fused to a six-membered dihydrothiazine ring (Fig. 1).

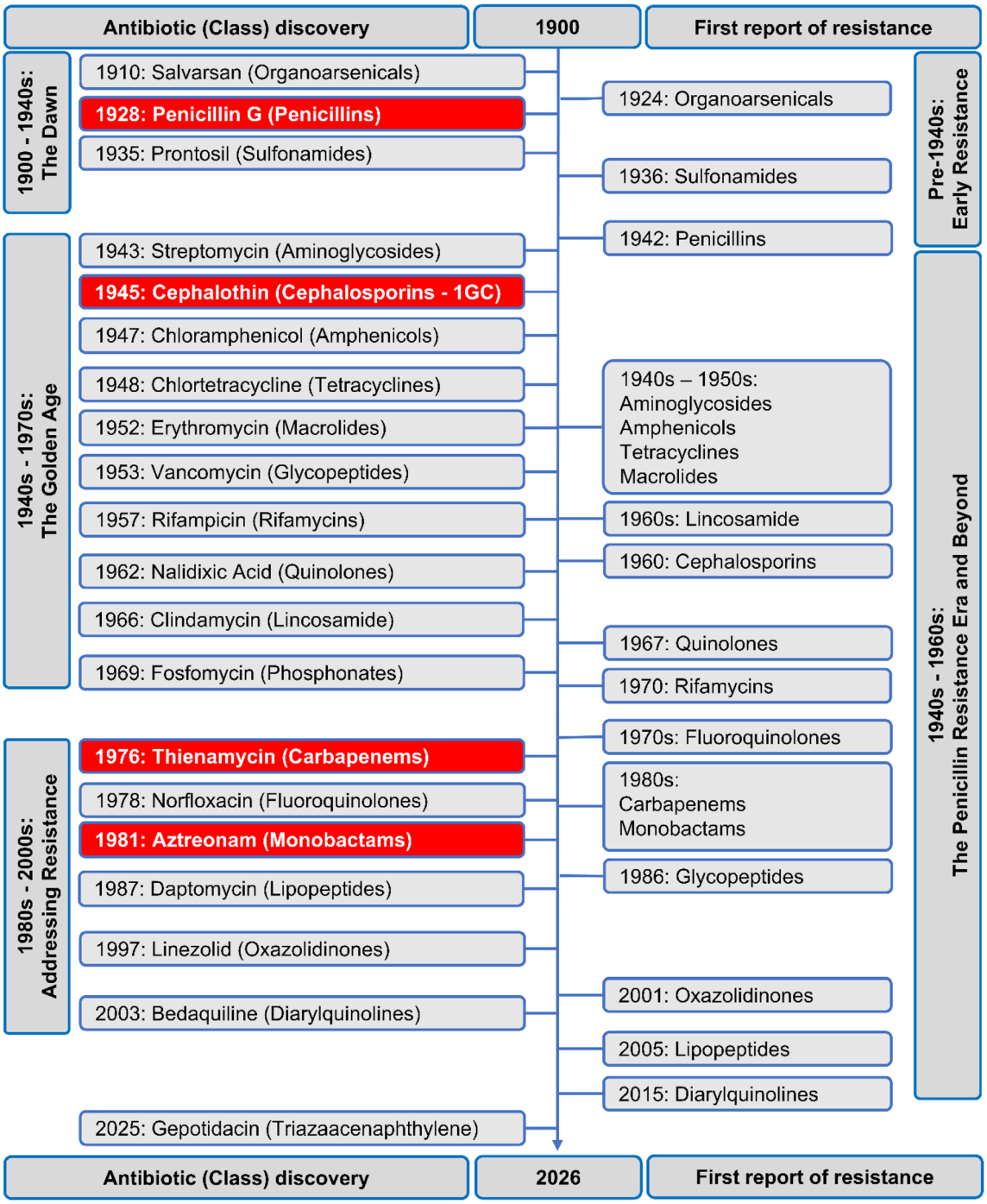


Fig. 2 Historical timeline showing dates of reports concerning discovery of major clinically used antibiotic classes from 1900 to 2025 and the subsequent emergence of resistance. This figure highlights the relationship between antibiotic discovery and identification of resistance, emphasising the importance of identifying strategies to combat resistance during antibiotic development. 1GC – 1st generation cephalosporins.

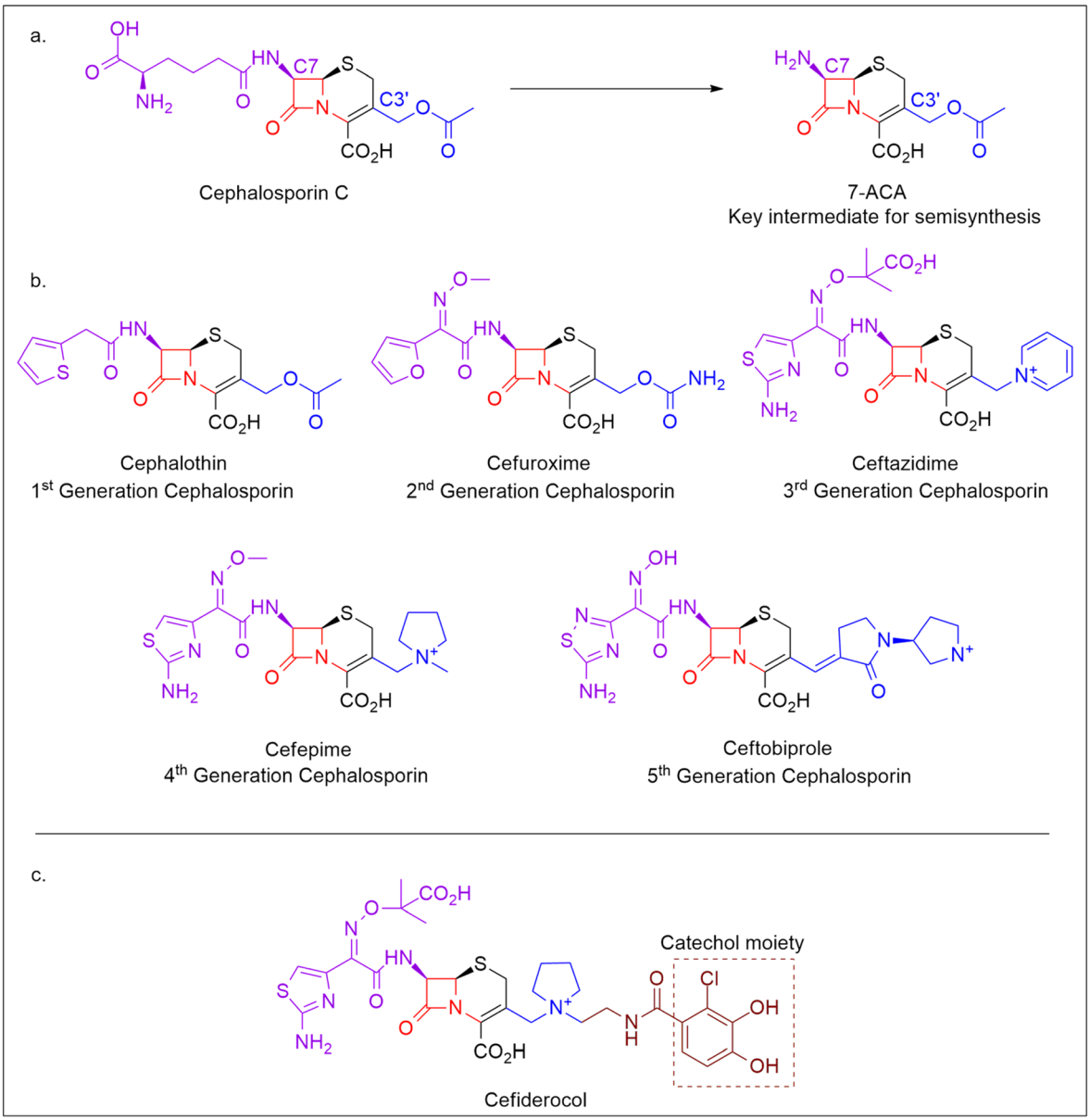


Fig. 3 Evolution of cephalosporins. (a) Development of cephalosporins from natural cephalosporin C, which is converted to 7-amino-cephalosporinic acid (7-ACA), a precursor for semisynthesis. (b) 1st–5th generation semisynthetic derivatives with expanding Gram-negative coverage and improved β-lactamase stability. (i) 1st generation cephalosporins: spectrum of activity – excellent Gram-positive activity, but limited activity against Gram-negative bacteria; β-lactamase stability – poor stability against extended serine-β-lactamases (ESBLs) and many other β-lactamases. (ii) 2nd generation cephalosporins: spectrum of activity – improved Gram-negative activity; β-lactamase stability – increased stability against some β-lactamases. (iii) 3rd generation cephalosporins: spectrum of activity – enhanced Gram-negative and reduced Gram-positive activity with some central nervous system penetration; β-lactamase stability – stable against many serine-β-lactamases but not ESBLs or AmpC. (iv) 4th generation cephalosporins: spectrum of activity – broader Gram-negative and Gram-positive activity; β-lactamase stability – enhanced penetration and stability against AmpC and many ESBLs. (v) 5th generation cephalosporins: spectrum of activity – retains broad Gram-negative coverage and is the first cephalosporin active against methicillin-resistant *S. aureus*; β-lactamase stability – some activity against ESBL producers, however, not stable against all carbapenemases.[42] (c) Cefiderocol is a recently developed siderophore-bearing broad-spectrum cephalosporin.[43,44] Cefiderocol possesses a cephalosporin core with the C7 sidechain of ceftazidime and a C3′ sidechain, related to that of cefepime, but modified by conjugation to a catechol.[43] The catechol group enables cefiderocol to exploit the iron/siderophore transport system to penetrate the bacterial outer membrane and reach the periplasmic space of many Gram-negative bacteria.[45]

Although displaying less pronounced activity than some penicillins, cephalosporin C was found to be relatively stable to contemporary serine-β-lactamases, consistent with Brotzu's microbiological observations.[35,36] Subsequently, the core scaffold of the cephalosporins, 7-aminocephalosporanic acid, was prepared from cephalosporin C (Fig. 3),[37,38] thereby enabling the development of semisynthetic cephalosporins with modified C7 sidechains. There are two positions in the

cephalosporin C molecule from which extensive modifications have been made, namely the C7 amide derivatives (prepared *via* C7 amines) and variation of the C3′ group. The production of many semisynthetic derivatives over the years has led to the development of the different generations of cephalosporins (Fig. 3).[39,40] The most recently approved cephalosporin is cefiderocol (approved in 2019), for which initial resistance has, unfortunately, already been reported in carbapenem-resistant Gram-negative pathogens.[41] Overall, the semisynthesis approach with cephalosporins effectively demonstrates the power of combining natural product science, medicinal chemistry, and clinically relevant microbiology to combat AMR.

Although new antibiotic classes emerged (Fig. 2), the penicillins and cephalosporins remained the most important antibiotic classes through the 1960s and early 1970s. It was during this period that insight into the mechanism of action of penicillins emerged. Tipper and Strominger proposed that penicillin inhibits bacterial cell wall biosynthesis by covalently binding to the nucleophilic active site serine residue of bacterial transpeptidases, commonly referred to as PBPs. Penicillin was proposed to structurally mimic the D-Ala–D-Ala moiety of peptidoglycan precursors; however, unlike the acyl–enzyme complex derived from the reaction of the transpeptidase nucleophilic serine with D-Ala–D-Ala, the complex formed from the reaction of penicillin is inert to transpeptidation (it can undergo slow hydrolysis), thus preventing crosslinking of peptidoglycans.[46,47] Such inhibition of transpeptidase compromises the integrity of bacterial cell walls, leading to osmotic lysis (Fig. 1).

By the late 1960s, researchers at Beecham Pharmaceuticals had identified a series of β-lactamase inhibitors and, at least partially, β-lactamase-resistant β-lactams. Amongst these were the olivanic acids, a type of carbapenem, which were isolated from fermentation broths of *Streptomyces olivaceus*.[48] It is important to note that by this time, it had been revealed that multiple bacteria (and fungi) produce multiple β-lactams,[49,50] especially those of the *Streptomyces* genus.[51] About the same time, progress on β-lactamase-resistant β-lactams was being made by the Merck group, which was screening fermentation broths of *Streptomyces cattleya* for inhibitors of cell wall biosynthesis, leading to the discovery of several representatives of the thienamycin family of carbapenems (Fig. 1, 2, and 4).[52] The carbapenems were shown to contain a β-lactam ring fused to an unsaturated five-membered ring in which a CH_2 group replaces the sulphur present in the thiazolidine ring of penicillin (Fig. 1 and 4).[53] The carbapenems also differ from penicillin and cephalosporins in the nature and stereochemistry of the sidechains linked to the β-lactam ring, in particular by the replacement of the C6/C7 penicillin amides with the C6 hydroxyethyl group of the clinically used carbapenems.[54]

Thienamycin was found to display exceptionally high *in vitro* activity against a wide range of bacteria, including some strains of *P. aeruginosa* and, importantly, manifested substantial resistance to hydrolysis by contemporary serine-β-lactamases.[55] However, its instability both *in vitro* and *in vivo* rendered thienamycin unsuitable for clinical use.[56] To improve on thienamycin, chemists at Merck synthesised an *N*-formimidoyl derivative (imipenem) with improved *in vitro* and *in vivo* shelf life (Fig. 4). However, like all studied natural carbapenems, imipenem is a substrate for renal dehydropeptidase (DHP-I) which limited its *in vivo* activity.[57] Cilastatin is a competitive inhibitor of DHP-I; in 1985, a 1 : 1 combination of imipenem and cilastatin was launched under the brand name Primaxin®.[58]

Subsequent work at Merck revealed that synthetic carbapenems with a 1-β-methyl substituent were stable towards DHP-I and had a potency similar to that of imipenem, paving the way for a second generation of semisynthetic carbapenems such as meropenem and ertapenem (Fig. 5).[59] The carbapenems became a commonly used last line of defence against bacterial infections where other β-lactams had failed. By the late 1990s, a few reports of carbapenem resistance in *K. pneumoniae* emerged; however, it was not until the 2000s that reports of widespread, major carbapenem resistance emerged, involving different Gram-negative bacteria (Fig. 3).[60,61]

The search for β-lactamase-resistant β-lactam compounds led to the discovery of another important β-lactam class, the monocyclic monobactams (Fig. 1, 2 and 5). The nocardicins were the first monocyclic β-lactam antibiotics to be discovered, being identified from fermentation broths of *Nocardia uniformis* subsp. *tsuyamanensis* by the Takeda company.[62] Subsequently, the monobactams were isolated from *Agrobacterium radiobacter* and characterised by researchers at Squibb.[63] They were found to inhibit certain nucleophilic serine-β-lactamases and were

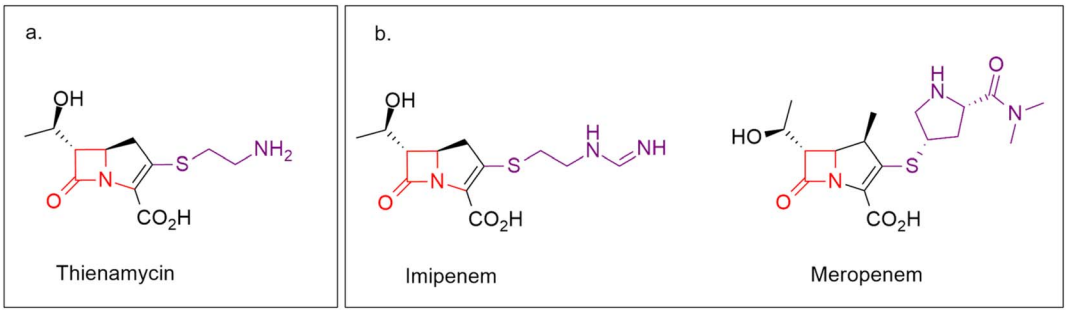


Fig. 4 Natural carbapenems inspired the development of clinically used carbapenems produced by total synthesis. (a) Thienamycin, a natural carbapenem, was not suitable for clinical use. (b) Synthetic versions of clinically used carbapenems.

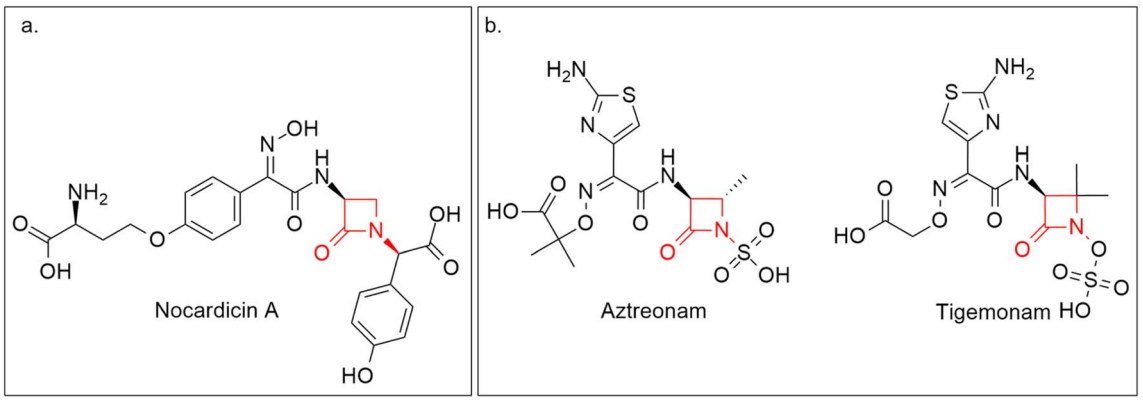


Fig. 5 Selected structures of monocyclic β-lactams. (a) Natural (nocardicin A) and (b) synthetic (clinically used aztreonam and tigemonam) monobactams. Monobactams lack an additional ring system fused to the β-lactam ring.

subsequently found to be surprisingly stable against the class B metallo-β-lactamases. The efficiency and low toxicity of the monobactams, in particular aztreonam, means that they are now widely used in human and animal medicine.[64] However, as previously alluded to, bacterial pathogens have responded to the challenges posed by β-lactams by evolving β-lactamases that circumvent their lethality, including those targeting monobactams.

Like the carbapenems, the clinically used monobactams, of which aztreonam is the most important, are produced by total synthesis rather than direct fermentation or semisynthesis, as is the case for penicillins and cephalosporins. The carbapenem and monobactam classes of β-lactams thus serve as excellent examples of how natural product structures can inspire the development of synthetic derivatives suited for clinical use.

2.2 Discovery of β-lactamases

Although other β-lactam resistance mechanisms (*e.g.*, efflux pumps, PBP modification) are important, from a clinical perspective, the most prevalent response to date by bacteria to β-lactam antibiotic pressure is likely through the production of β-lactamases (Fig. 6).[65,66] Following the pioneering work of Chain and Abraham,[28] >2000 unique β-lactamases have been identified (Fig. 6).[67] β-Lactamases are localised within the periplasmic space,[46,68] though some β-lactamases act extracellularly.[69,70]

β-Lactamases are classified using two principal systems, namely (i) the activity-based Bush–Jacoby–Medeiros system and (ii) the Ambler system.[71] The Ambler classification system is the most widely adopted, including in this review. The Ambler system categorises β-lactamases into four classes, A, B, C, and D (Table 1), primarily based on specific amino acid sequences and their β-lactam hydrolysis mechanisms.[72,73] The Ambler class A, C, and D β-lactamases employ a nucleophilic serine residue in catalysis and are hence termed serine-β-lactamases. By contrast, the Ambler class B β-lactamases are metallo-hydrolases and hence are termed metallo-β-lactamases.[73] Serine-β-lactamases are structurally related to the PBPs and share a Ser–xxx–xxx–Lys active site motif.[74] β-Lactam inactivation by serine-β-lactamases proceeds *via* nucleophilic attack by the serine residue, forming an acyl-enzyme (ester) intermediate, which undergoes hydrolysis. In contrast, metallo-β-lactamases instead utilise a metal-activated (likely Zn^{2+}) water nucleophile to drive the hydrolytic reaction (Fig. 7).[75]

2.2.1 Serine-β-lactamases. The Ambler class A serine-β-lactamases include clinically important and structurally diverse serine-β-lactamase subfamilies, including the earliest discovered TEM (Temoniera; first identified in *E. coli*), SHV (sulfhydryl variant; identified in *K. pneumoniae*), CTX-M (cefotaximase, active against cefotaxime), and KPC (*K. pneumoniae* carbapenemase) β-lactamases (Fig. 6).[76,77] Gram-positive bacteria produce several serine-β-lactamases, such as BCL-1 (*Bacillus* spp.), PC1 (*Staphylococcus* spp.), and BlaL/BlaU (*Streptomyces* spp.). BlaC from *M. tuberculosis* is also a class A serine-β-lactamase.

The active site of class A serine-β-lactamases is bordered by an α-helical domain, an α/β domain, and an Ω-loop.[78] The hydrolytic activity of class A serine-β-lactamases ranges from narrow-spectrum to broad-spectrum and includes carbapenem-hydrolysing activity, making this class particularly concerning.[79] Serine-β-lactamase catalysis proceeds *via* a canonical two-step acylation–deacylation process, which involves the nucleophilic serine (Ser70), a hydrolytic water, and an active site lysine (Lys73) and glutamic acid (Glu166) residues, the latter of which act as the general acid/base machinery for catalysis.[65] Class A serine-β-lactamases such as TEM and SHV can be potently inhibited by the 'classical' serine-β-lactamase inhibitors, namely clavulanic acid, tazobactam, and sulbactam.[80,81] Other class A serine-β-lactamases, especially the highly evolved and disseminated KPCs, evade such inhibitors. These, however, are susceptible to new generation serine-β-lactamase inhibitors such as avibactam, relebactam, and vaborbactam.[82,83]

Class C β-lactamases mainly comprise chromosomally encoded cephalosporinases and are widespread in Gram-negative bacteria. The best characterised class C serine-β-lactamases are the AmpC enzymes from *Enterobacteriaceae*, whose expression is induced by β-lactams. Class C β-lactamases are widely disseminated across the chromosomes of bacterial pathogens,

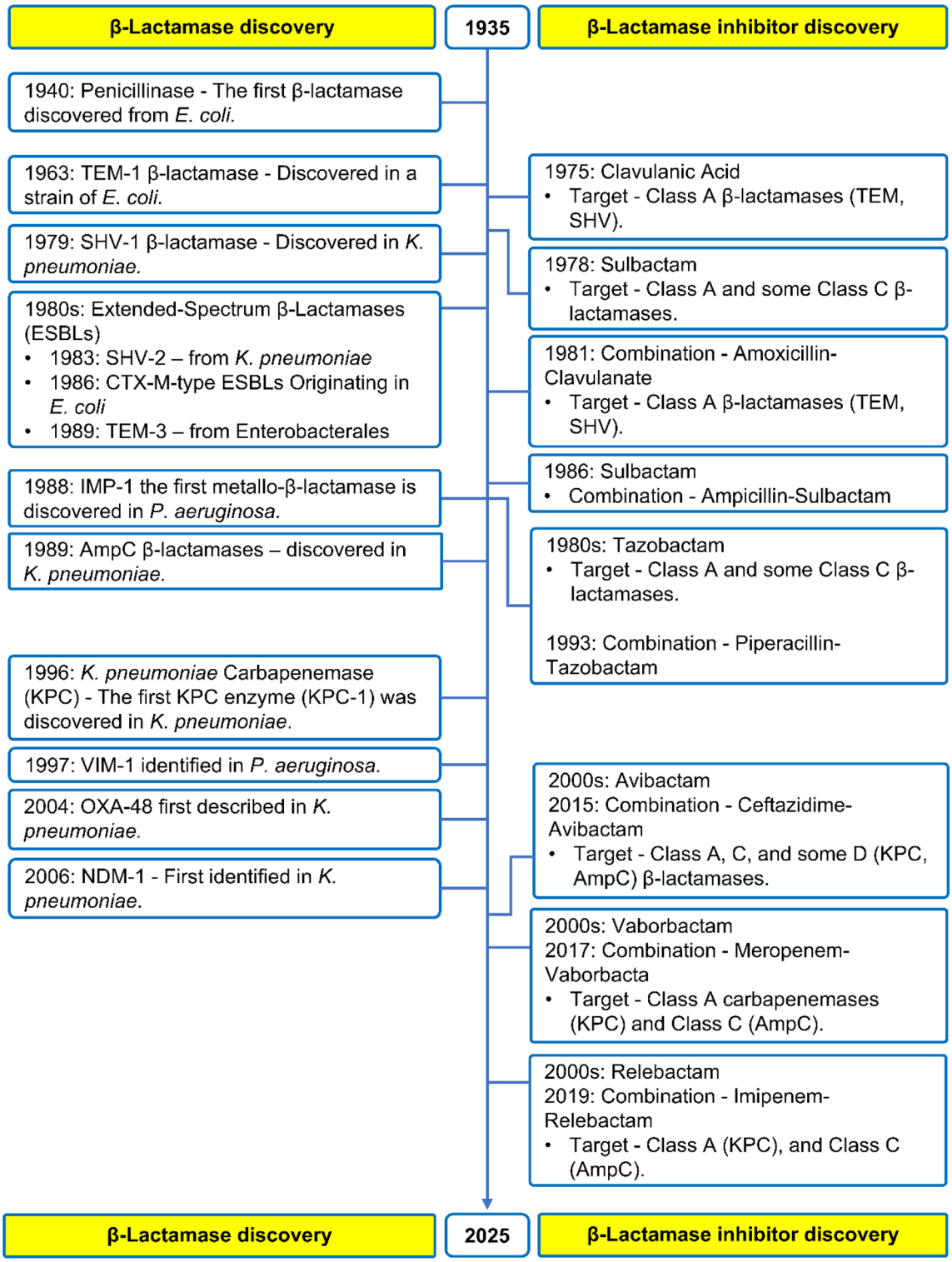


Fig. 6 Historical timeline showing milestones in the discovery of β-lactamases and β-lactamase inhibitors. Note that not all the β-lactamase inhibitors are β-lactams.

including *Citrobacter freundii* (CMY enzymes) and *A. baumannii* (ADC enzymes).[84–87] Class C serine-β-lactamases also occur in plasmids harboured, *inter alia*, in *K. pneumoniae* (ACT-1) and *Salmonella* Enteritidis (DHA-1). Their three-dimensional structures are related to class A serine-β-lactamases, and they follow the two-stage acylation–deacylation mechanism. Unlike the class A active sites, those of the class C enzymes appear more open, and the general acid/base residues differ, involving Tyr150 and Lys67.[65,87]

Table 1 Different Ambler β-lactamases classes (A–D) and examples of clinically used β-lactamase inhibitors active against them

Class	Mechanism	Clinically relevant example	Clinically relevant inhibitors[a]
Class A	Serine-β-lactamase	KPC, CTX-M, SHV, and TEM	Clavulanic acid, sulbactam, tazobactam, avibactam, relebactam, and vaborbactam
Class B	**Metallo-β-lactamase**	**NDM, VIM, and IMP**	**None**
Class C	Serine-β-lactamase	AmpC and CMY	Avibactam
Class D	Serine-β-lactamase	OXA-10, OXA-23, and OXA-48	Avibactam and relebactam

[a] Note that in most, but not all, cases β-lactamase inhibitors have no or little intrinsic antibacterial activity, with carbapenems being both antibiotics and inhibitors of some serine-β-lactamases.

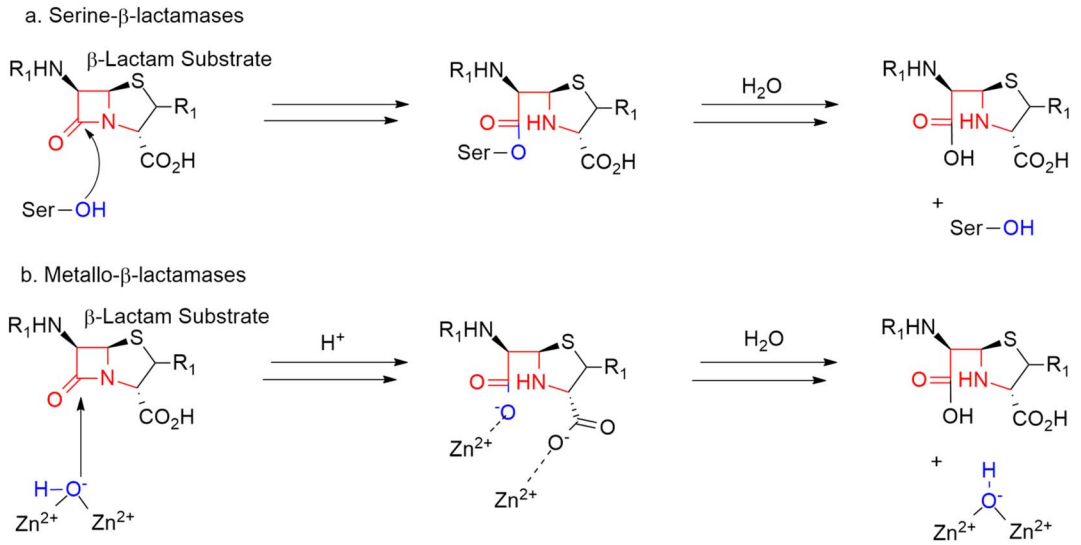


Fig. 7 Outline of β-lactam hydrolysis mechanisms as catalsyed by: (a) nucleophilic serine-β-lactamases, involving a two step reaction with acylation and deacylation steps, and (b) metallo-β-lactamases, which employ a Zn^{2+} activated H_2O nucleophile for β-lactam hydrolysis.

Resistance mediated by the class D serine-β-lactamases, also known as oxacillinases (OXAs), is particularly concerning, especially with respect to carbapenem (and cephalosporin) resistance in Gram-negative bacteria, including *A. baumannii* and *Enterobacteriaceae*.[88,89] From a mechanistic perspective, the OXAs differ from the class A and C serine-β-lactamases in that they employ a carboxylated lysine residue as the general base for the acylation and deacylation steps. Additionally, they also appear to have a greater number of hydrophobic residues at their active sites, which distinguishes them structurally to class A and C serine-β-lactamases.[65] The OXAs are both chromosomally and plasmid encoded, with the OXA-48 group being reported in multiple plasmids and in some Gram-positive pathogens.[90] They are also strongly resistant to inhibitory molecules. This is especially critical, as the increasing prevalence of OXAs amongst opportunistic pathogens renders clinical therapies ineffective.[91] There is an urgent need to develop effective inhibitors of class D serine-β-lactamases.

2.2.2 Metallo-β-lactamases. The class B metallo-β-lactamases are subdivided into the B1, B2, and B3 subfamilies. The B1 and B3 metallo-β-lactamases use two Zn^{2+} ions, whereas the B2 metallo-β-lactamases use only one Zn^{2+} ion. Interestingly, a variant of the class B1 New Delhi Metallo-β-lactamase (NDM), namely NDM-15, has evolved to function effectively as a mono-Zn^{2+} enzyme.[92] For a substantial period following the identification of the first class B metallo-β-lactamases from *P. aeruginosa* in 1966, the metallo-β-lactamases were not clinically relevant. This situation has now changed, with the B1 subfamily metallo-β-lactamases becoming increasingly clinically important, particularly with respect to carbapenem resistance. The NDM, IMP (Imipenemase), and VIM (Verona integron-encoded metallo-β-lactamase) B1 metallo-β-lactamases are widespread,[93,94] though not yet as prevalent as the serine-β-lactamases.[94] Nevertheless, metallo-β-lactamases are a highly active, broad-spectrum group of enzymes that are responsible for antibiotic resistance in some of the most serious chronic nosocomial infections caused by both Gram-positive and Gram-negative pathogens.[95] Furthermore, metallo-β-lactamases are encoded on conjugative plasmids and are widespread amongst ESKAPE pathogens.[96]

Metallo-β-lactamases are characterised by a His/Asn116–xxx117–His118–xxx119–Asp120–His/xxx121 motif that forms a metal-binding centre located at the interface of the two β-sheets that comprise the core of the protein.[97] Class B1 metallo-

β-lactamases (NDM, VIM, IMP) possess a binuclear Zn^{2+} centre made up of tri-His (3H) ($Zn^{2+}1$) and Cys–His–Asp ($Zn^{2+}2$) metal-binding sites. The two Zn^{2+} ions at the metal sites are bridged by the 'hydrolytic' water molecule/hydroxide ion, with an additional water molecule bound to $Zn^{2+}2$.

In the presently small B2 metallo-β-lactamase sub-group, which utilises one Zn^{2+} ion for catalysis, the first conserved His of the defining motif is replaced by an Asn. This substitution disrupts coordination at one of the two potential Zn^{2+} binding sites, resulting in binding of only a single metal ion. The B3 metallo-β-lactamases, like the B1 metallo-β-lactamases, utilise two Zn^{2+} ions. However, in the B3 metallo-β-lactamases, the $Zn^{2+}2$ coordinating Cys residue is replaced by another residue, which varies amongst B3 enzymes, with a His121 acting as the metal co-ordinating residue in some metallo-β-lactamases.[65,96,98,99]

The differences in structures and detailed mechanisms of the metallo-β-lactamases is striking. Moreover, these enzymes are resistant to all currently available serine-β-lactamase inhibitors,[95] something that is a cause for serious concern. A positive note, however, is that bicyclic boronates, including compounds in clinical development, inhibit both serine-β-lactamases and, albeit less efficiently, metallo-β-lactamases.[96,97]

The history of β-lactams offers a clear example of how the discovery of a groundbreaking new therapy is followed by a period of intense effort and productivity, leading to multiple widely used medicines. This remarkably productive period was followed by a phase of reduced innovation in antibiotic research, during which time bacterial pathogens acquired multiple resistance mechanisms.[99–102] The available evidence suggests that this situation is not irretrievable. Although relatively little recent medicinal chemistry has been applied to the rational improvement of β-lactams, with the work of Shionogi in Japan being an exception, our molecular understanding of resistance has improved dramatically as a consequence of new methods.[81,97,98,103–108] The history of β-lactams as outlined above suggests that natural product-based approaches will be critical in the future. Below, we summarise work, both historical and recent studies aimed towards the identification of new types of β-lactamase inhibitors, beginning with an overview of the evolution of screening approaches used in the discovery of natural product-based inhibitors.

2.2.3 Evolution of β-lactamase inhibitor discovery strategies. The threat posed by β-lactamase mediated resistance prompted efforts focused on the discovery and development of β-lactamase inhibitors, with work beginning in earnest in the 1960s. Pioneering work in this area was undertaken by researchers at Beecham Pharmaceuticals in the UK.[109] They implemented a phenotypic screening strategy centred on functional reversal of β-lactam resistance. By screening microbial fermentation products for their ability to resensitise β-lactam-resistant Gram-negative bacteria to penicillin, this approach directly linked screening outcomes to clinically meaningful antibacterial activity.[109] This strategic screening program was remarkably successful, directly leading to the landmark discovery of clavulanic acid, the first clinically successful natural product β-lactamase inhibitor.

In parallel with the industry efforts at Beecham Pharmaceuticals, academic researchers at the Kitasato Institute in Japan, developed a systematic screening program to identify penicillinase inhibitors from microbial metabolites.[110,111] This approach was significant as it represented an early example of a target-based screen, *i.e.* specifically isolating the enzyme of interest rather than relying solely on whole-cell interactions. By focusing on enzyme inhibition as a primary selection criterion, the Kitasato programme expanded the conceptual framework for inhibitor discovery and enabled the identification of microbial metabolites with direct β-lactamase inhibitory activity.[110,111]

Through the late 20th century and into the 21st century, screening approaches for β-lactamases inhibitors evolved, shifting towards high throughput screening. Leading this transition was Pfizer, which sought to efficiently discover novel antibiotics from both fermentation extracts and synthetic libraries in a cost-effective manner.[112] This strategy has subsequently been adopted across both industry and academic research programs, in a manner enhanced by the availability of purified recombinant β-lactamases enabling screening against multiple targets.

As metallo-β-lactamases emerged as an increasing clinical threat, screening approaches required further adaptation to address the unique requirements of their zinc-dependent catalysis. In this context, academic researchers, have played pivotal roles in developing medium-to-high throughput phenotypic and target-based assays tailored to identifying metallo-β-lactamase inhibitors.[113,114] Contributions from small companies and academics have been important at a time where the major pharmaceutical industry has largely exited the discovery space for new antibiotics and antibiotic potentiators.

β-Lactamase inhibitor screening has increasingly been informed by high-resolution structural and computational approaches.[115–117] Techniques such as X-ray crystallography, complemented by molecular dynamics simulations, have enabled direct visualisation of enzyme–inhibitor complexes, providing detailed insights into binding modes, metal coordination, and conformational dynamics.[115–117] This structure-guided paradigm supports rational inhibitor design, allowing for the systematic modification of molecular scaffolds to optimise binding affinity, pharmacokinetic properties, and stability against enzymatic degradation. These approaches collectively have been particularly instrumental, as exemplified by the discovery of vaborbactam[118] and other cyclic boronates,[119,120] as well as the identification of the aspergillomarasmine A[113] and indole-2-carboxylate[121] scaffolds as potent metallo-β-lactamase inhibitors.

In summary, β-lactamase inhibitor screening has evolved from foundational phenotypic assays into a sophisticated, often structure-guided, paradigm. Modern discovery pipelines now rely on integrated assay cascades that can systematically filter vast natural product libraries, moving from high-throughput primary screens to precise hit validation and rational optimisation. This structured approach efficiently manages the complexity of screening campaigns, allowing researchers to prioritise the most promising scaffolds for further

development. Having summarised the methodological frameworks, the following section reviews the landmark and recent discoveries of natural product β-lactamase inhibitors identified from microbial and plant-based sources.

3. β-Lactamase inhibitors from natural products

Natural products, in particular secondary metabolites,[122] have had a central role in the development of modern antibiotics and β-lactamase inhibitors. This section further describes the demonstrated and potential contribution of microbial and plant-derived natural products to the discovery of β-lactamase inhibitors (Tables 2 and 3).

3.1 β-Lactamase inhibitors from microorganisms

An influential search for β-lactamase inhibitors was conducted by researchers at the Kitasato University in Japan during the early 1970s.[110,111] Recognising penicillinases as a key driver of penicillin resistance, they developed an innovative screening method to identify microbial metabolites that inhibit these enzymes. Through systematic screening of bacterial and fungal filtrates, they identified four *Streptomyces* strains with penicillinase-inhibition activity, including *Streptomyces gedanensis* ATCC 4880, which produced a potent inhibitor designated KA-107. KA-107 was isolated and, whilst lacking intrinsic antibacterial activity, was found to synergistically restore the efficacy of penicillin G, ampicillin, and phenethicillin against a resistant *S. aureus* strain (FS-1277). Notably, growth inhibition was only observed when KA-107 was co-administered with a penicillin, supporting its mode of action as a β-lactamase inhibitor, a proposal supported by optical density-based growth curves, which demonstrated prolonged suppression of bacterial growth when both agents were present.[110,111]

Shortly after the groundbreaking work in Japan, studies on clavulanic acid, isolated from fermentation broths of *S. clavuligerus*,[139] were reported (as described in Section 2.2.3; Fig. 8a). Clavulanic acid belongs to the clavam family of β-lactams, which are characterised by a fused bicyclic β-lactam and oxazolidine ring system, with the (3*R*,5*R*) stereochemistry, which confers potent inhibition of some serine-β-lactamases, while exhibiting weak antibacterial activity. In contrast, clavams (such as clavaminic acid, a biosynthetic precursor of clavulanic acid) with the (3*S*,5*S*)-stereochemistry, can exhibit intrinsic antibacterial activity.[140] The structure of clavulanic acid was elucidated using spectrophotometric methods and X-ray crystallography.[141] Its discovery was revolutionary because at the time of its clinical introduction, there was no available agent that restored the activity of any antibiotic against drug-resistant bacteria.

Clavulanic acid works as a "mechanism based" inhibitor of serine-β-lactamase: it initially reacts to form an acyl–enzyme complex, which can either undergo hydrolysis (turnover) or oxazolidine ring-opening, leading to stable acyl–enzyme complexes (Fig. 8a).[142] Clavulanic acid was used clinically for the first time in combination with the penicillin antibiotic amoxicillin (marketed as Augmentin), thereby protecting the latter against β-lactamases.[109] Clavulanic acid shows strong potency against the class A serine-β-lactamases such as TEM-1, TEM-2, and SHV-1, as well as some ESBLs, including TEM-3 and SHV-2

Table 2 Microbial-derived natural products with reported β-lactamase inhibitory activities[a]

Compound	Source organism	Target β-lactamase	IC_{50}[b] (μM)	pIC_{50}[c]
Clavulanic acid	*S. clavuligerus*	TEM-1	0.09	7.05 (ref. 123)
		SHV-1	0.03	7.52 (ref. 123)
		OXA-1	1.8	5.74 (ref. 123)
		OXA-4	8.4	5.08 (ref. 123)
MM4550	*S. olivaceus*	R-TEM	0.001	9.00 (ref. 124)
MM13902	*S. olivaceus*	R-TEM	0.05	7.30 (ref. 124)
MM17880	*S. olivaceus*	R-TEM	0.03	7.52 (ref. 124)
SB236049	*C. funicola*	*B. cereus* II	0.7	6.15 (ref. 125)
		CfiA	2.0	5.70 (ref. 125)
		IMP-1	151	3.82 (ref. 125)
SB236050	*C. funicola*	*B. cereus* II	256	3.59 (ref. 125)
SB238569	*C. funicola*	*B. cereus* II	19	4.72 (ref. 125)
AMA	*A. versicolor*	NDM-1	4.0	5.40 (ref. 113)
		VIM-2	9.6	5.02 (ref. 113)
9-HHIA	*Aspergillus* sp.	IMP-1	50.5	4.30 (ref. 126)
10-HHIA	*Aspergillus* sp.	IMP-1	31.6	4.50 (ref. 126)
Emerione A	*E. nidulans*	NDM-1	12.1	4.92 (ref. 127)
Asperfunolone A	*Aspergillus* sp.	NDM-1	36.2	4.44 (ref. 127)
Holomycin	*S. clavuligerus*	NDM-1 (nitrocefin)	0.153	6.81 (ref. 128)
		NDM-1 (imipenem)	0.11	6.96 (ref. 128)

[a] Plant species indicated as "Source organisms" that have been identified through literature searches as either the original sources from which the compounds were first isolated or as well-established producers of these compounds. [b] IC_{50} values in the table have been harmonised to a consistent unit, μM. Those previously reported as μg mL^{-1} were converted to μM units using the following formula $IC_{50}\ (\mu M) = \frac{IC_{50}\ (\mu g\ mL^{-1}) \times 1000}{\text{molecular weight}\ (g\ mol^{-1})}$. [c] IC_{50} values were converted to pIC_{50} using the following formula: $pIC_{50} = -\log_{10}(IC_{50}[M])$.

Table 3 Plant-derived natural products with reported β-lactamase inhibitory activities[a]

Compound	Source organism	Target β-lactamase	IC_{50}[b] (μM)	pIC_{50}[c]
SB-202742	*S. mombin*	TEM-1	13.5	4.87 (ref. 129)
		OXA-1	215.25	3.67 (ref. 129)
		P99	44.56	4.35 (ref. 129)
Rosmarinic acid	*R. officinalis*	VIM-2	41.5	4.38 (ref. 130)
Salvianolic acid A	*S. miltiorrhiza*	VIM-2	27.2	4.57 (ref. 130)
		NDM-1	160.3	3.79 (ref. 130)
Salvianolic acid	*S. miltiorrhiza*	TEM-1	189	3.72 (ref. 130)
Hesperidin	*Citrus* sp.	NDM-1	3.35	5.48 (ref. 131)
Diosmin	*Citrus* sp.	NDM-1	20.7	4.68 (ref. 131)
Rutin	*S. japonica*	NDM-1	15.5	4.81 (ref. 131)
Stevioside	*Stevia rebaudiana*	NDM-1	214.1	3.67 (ref. 131)
Magnolol	*Magnolia officinalis*	NDM-1	24.3	4.61 (ref. 132)
Pterostilbene	*Pterocarpus* sp.	NDM-1	60	4.22 (ref. 133)
Embelin	*E. ribes*	NDM-1	2.1	5.68 (ref. 134)
		VIM-1	∼200	3.70 (ref. 134)
		IMP-1	∼100	4.00 (ref. 134)
Carnosic acid	*R. officinalis*	NDM-1	27.07	4.57 (ref. 135)
α-Mangostin	*G. mangostana*	OXA-48	0.52	6.28 (ref. 136)
		NDM-1	25.43	4.59 (ref. 136)
Quercetin	*S. japonica*	OXA-48	1.47	5.83 (ref. 137 and 138)
		NDM-1	5–10	5.00–5.30 (ref. 137 and 138)
Fisetin	*Fragaria* × *ananassa*	OXA-48	0.47	6.33 (ref. 138)
Luteolin	*Reseda luteola*	OXA-48	0.55	6.26 (ref. 138)
3′,4′,7-Trihydroxyflavone	*Medicago sativa*	OXA-48	1.89	5.72 (ref. 138)
Apigenin	*Matricaria chamomilla*	OXA-48	3.64	5.44 (ref. 138)
Kaempferol	*Delphinium consolida*	OXA-48	4.54	5.34 (ref. 138)
Isoquercitrin	*Cercis canadensis*	OXA-48	11.55	4.94 (ref. 138)
Chrysin	*Camellia sinensis*	OXA-48	108	3.97 (ref. 138)
Galangin	*Alpinia officinarum*	OXA-48	216	3.67 (ref. 138)
Taxifolin	*Pseudotsuga taxifolia*	OXA-48	3.22	5.49 (ref. 138)
Naringenin	*Citrus* × *paradisi*	OXA-48	68.1	4.17 (ref. 138)

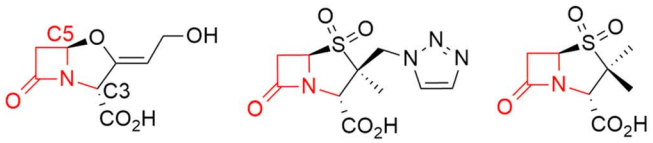
[a] Plant species indicated as "Source organisms" that have been identified through literature searches as either the original sources from which the compounds were first isolated or as well-established producers of these compounds. [b] IC_{50} values in the table have been harmonised to a consistent unit, μM. Those previously reported as μg mL^{-1} were converted to μM units using the following formula $IC_{50}\ (\mu M) = \frac{IC_{50}\ (\mu g\ mL^{-1}) \times 1000}{\text{molecular weight}\ (g\ mol^{-1})}$. [c] IC_{50} values were converted to pIC_{50} using the following formula: $pIC_{50} = -\log_{10}(IC_{50}[M])$.

(Fig. 8a). It has mixed activity against class C and D serine-β-lactamases, and it is inactive against class B metallo-β-lactamases. Despite these limitations, clavulanic acid remains widely used in clinical practice. Moreover, it has served as a structural scaffold that inspired the development of other clinically important, mechanistically related β-lactamase inhibitors, namely, tazobactam and sulbactam, both of which are prepared by semisynthesis from penicillin precursors (Fig. 8a).[123]

More recently, new classes of synthetic serine-β-lactamase inhibitors have been developed that do not contain a β-lactam, but which react with the nucleophilic serine (Fig. 8b). Although synthetic, these inhibitors mimic the mechanisms used by natural products to block β-lactamases. Avibactam was the first member of the diazabicyclooctane class of reversibly reacting broad-spectrum serine-β-lactamase inhibitors to be approved for clinical use. Avibactam is clinically used in combination with the cephalosporin ceftazidime.[143] Its development was groundbreaking because it demonstrated the viability of β-lactamase inhibition by a non-β-lactam scaffold. Variants of avibactam have been developed, some of which exhibit antibacterial activity.[143] After a long development period, boronates have also emerged as clinically used inhibitors of serine-β-lactamases and, in some cases, metallo-β-lactamases (Fig. 8b). These compounds react with serine-β-lactamases and bind to metallo-β-lactamase to give complexes that mimic the high-energy tetrahedral intermediates during β-lactam catalysis.[144,145] To date, vaborbactam, a monocyclic boronate, is the only clinically approved β-lactamase inhibitor of this class. However, bicyclic boronates, such as taniborbactam, with a broader spectrum of activity, will likely be approved in the near future.[119,146]

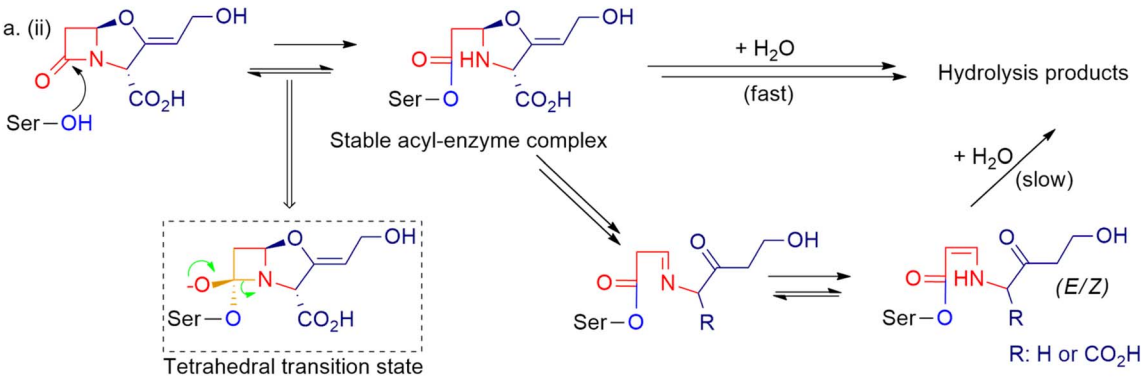
The olivanic acids, a carbapenem subclass, were discovered alongside work that led to the discovery of clavulanic acid.[139] Three compounds, designated MM 4550, MM 13902, and MM 17880, were isolated from *S. olivaceus* ATCC 31126 (Fig. 9a)[124,139] and share a common (5*R*),(6*R*),(8*S*)-stereochemistry. In *in vitro* antibacterial studies, all three compounds displayed marked activity against both Gram-positive and Gram-negative bacteria, including *S. aureus*, *E. coli*, *K. aerogenes*, *Proteus* spp., and *P. aeruginosa*, many of which were β-lactamase producers. MIC values ranged from 0.05 to 500 μg mL^{-1}, depending on the

a. (i)



	Enzyme	Clavulanic Acid	Tazobactam	Sulbactam
		$IC_{50}\mu M$ (pIC_{50})		
Class A	TEM-1	0.09 (7.05)	0.04 (7.40)	6.10 (5.21)
	SHV-1	0.03 (7.52)	0.14 (6.85)	17.00 (4.77)
	TEM-3	0.03 (7.52)	0.01 (8.00)	0.03 (7.52)
	SHV-5	0.01 (8.00)	0.08 (7.10)	0.63 (6.20)
	TEM-10	0.03 (7.52)	0.08 (7.10)	0.34 (6.47)
	SHV-3	0.04 (7.40)	0.10 (7.00)	2.70 (5.57)
	TEM-6	0.12 (6.92)	0.17 (6.77)	0.45 (6.35)
	TEM-7	0.10 (7.00)	0.18 (6.74)	0.62 (6.21)
	TEM-9	0.29 (6.54)	0.34 (6.47)	0.90 (6.05)
	TEM-5	0.03 (7.52)	0.28 (6.55)	1.20 (5.92)
	TEM-2	0.18 (6.74)	0.05 (7.30)	8.70 (5.06)
Class D	OXA-1	1.58 (5.80)	1.40 (5.85)	4.70 (5.33)
	OXA-2	1.40 (5.85)	0.01 (8.00)	0.14 (6.85)
	OXA-4	8.40 (5.08)	5.60 (5.25)	16.00 (4.80)
	OXA-5	3.10 (5.51)	0.25 (6.60)	18.00 (4.74)
	OXA-7	0.36 (6.44)	0.61 (6.21)	40.00 (4.40)

a. (ii)



b.

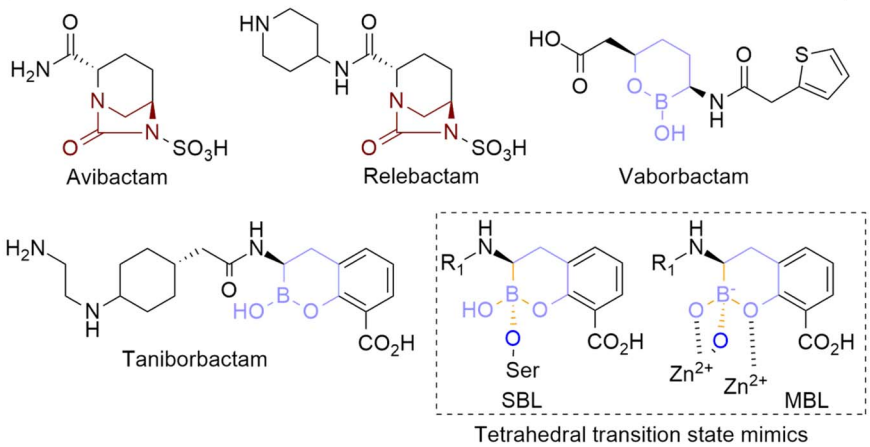


Fig. 8 Structural classes and mechanisms of β-lactamase inhibitors. (a) (i) IC_{50} values (μM) of classical β-lactamase inhibitors, clavulanic acid and related compounds tazobactam and sulbactam, against selected class A (TEM and SHV variants) and class D (OXA-type) serine-β-lactamases (data are from Brown *et al.*[139]). Clavulanic acid and tazobactam exhibit potent inhibition against class A enzymes with sub-micromolar IC_{50} values. Tazobactam is somewhat more active than clavulanic acid for a few class D β-lactamases, notably the OXA-2 and OXA-5. Note sulbactam and tazobactam are not naturally occurring compounds and are prepared from penicillin precursors. (a) (ii) Schematic illustration of the mechanism of clavulanic acid against serine-β-lactamase: the initially formed acyl–enzyme complex either undergoes hydrolysis or fragmentation of its oxazolidine ring to give stable acyl–enzyme complexes, including *E*/*Z*-enamines (with *trans*/*E*-enamines being observed). (b) Structures of more recently developed non-β-lactam β-lactamase inhibitors. Representative structures of diazabicyclooctane inhibitors (avibactam, relebactam) and boronic acid-based inhibitors (vaborbactam, taniborbactam), the latter of which react with both serine-β-lactamases and metallo-β-lactamases to give complexes proposed to mimic the tetrahedral transition state(s).

strain and compound, with MM 13902 and MM 17880 consistently demonstrating lower MICs across several isolates. In β-lactamase inhibition assays, the olivanic acids inhibited a wide range of serine-β-lactamases in cell-free systems (with IC_{50} values ranging from 0.5 to 75 ng mL^{-1}; with pIC_{50} values ranging from 6.72 to 9.01), supporting dual roles as antibiotics and serine-β-lactamase inhibitors. In these β-lactamase inhibitory assays, MM 4550 consistently emerged as the most potent inhibitor, particularly where penicillin was used as the substrate.[124] Mechanistic studies have revealed that the carbapenems, including the olivanic acids, initially form a Δ^2-pyrroline acyl–enzyme complex, which can either undergo hydrolysis or tautomerisation to give an epimeric Δ^1-pyrroline form(s), which is inhibitory (Fig. 9a).[147]

Olivanic acids were also isolated from a different microbial species: Umezawa *et al.*[150] discovered two β-lactamase inhibitors, MC696-SY2-A and MC696-SY2-B, produced by cultures of *S. fulvoviridis* MC696-SY2; the former of these was shown to be identical or very similar to MM 4550 (Fig. 9a).[151] The compounds were identified using iodometric titration, an assay whereby β-lactamase-mediated hydrolysis of penicillin releases penicilloic acid, which reduces iodine to iodide, thereby decreasing the intensity of the characteristic blue starch–iodine complex.[152]

Okamura *et al.*[153] isolated a carbapenem with a C6 ethyl group (PS-5) (Fig. 9b), in studies searching for antibiotics by screening filtrates from soil-isolated *Streptomyces* species. PS-5 was isolated and its structure was elucidated using spectroscopic methods.[153] PS-5 demonstrated notable intrinsic antibacterial activity against *S. aureus* (MIC = 0.16 μg mL^{-1}) and *Diplococcus pneumoniae* (MIC 0.02 μg mL^{-1}). Importantly, at low concentrations PS-5 was shown to enhance the activity of ampicillin and cephaloridine against a β-lactamase producing *Proteus vulgaris* strain.[153] Mechanistic studies by Fukagawa *et al.*[154] established PS-5's mode of β-lactamase inhibition through the application of a newly developed computer-assisted UV-spectrophotometric assay, which analysed reaction progress curves using integrated Michaelis–Menten kinetics rather than traditional initial-velocity methods. These studies revealed that PS-5 was highly resistant to hydrolysis by the *Bacillus licheniformis* 749/C β-lactamase, and that its inhibition followed first-order kinetics. The rate of enzyme inactivation was shown to be both concentration- and time-dependent, although a small fraction of enzymatic activity invariably remained, suggesting that PS-5 may act through a reversible mechanism.[154]

Proteins can also be β-lactamase inhibitors: the β-lactamase inhibitory protein (BLIP), was isolated from *S. clavuligerus* by Doran *et al.*[155] and was shown to inhibit β-lactamases through non-covalent interactions. Mechanistically, BLIP acts as a tight-binding competitive inhibitor, occluding the β-lactamase active site.[156] Following this foundational discovery, Kang *et al.*[157] isolated a homologous protein, BLIP-I, from *Streptomyces exfoliatus* SMF19. BLIP-I exhibits 38% sequence identity with BLIP and is a potent inhibitor of the TEM-1 β-lactamase with a K_i of 0.047 nM. Site-directed mutagenesis studies, particularly at residue Asp49, showed that specific sidechain interactions were critical for maintaining tight binding to TEM-1, with the D49A mutation resulting in a 200-fold loss of inhibition potency.[157] Further expanding the BLIP-family, Lim *et al.*[158] reported on the discovery of BLIP-II, which is structurally distinct from both BLIP and BLIP-I. Despite lacking sequence similarity to BLIP, BLIP-II binds to TEM-1 with sub-nanomolar affinity, primarily through hydrophobic contacts at its apical β-turn regions, forming a sterically occlusive interaction that blocks substrate access to the TEM-1 active site. Kinetic analyses revealed that BLIP-II binding was tight and slowly reversible, requiring approximately 1 hour to achieve complete inhibition, indicating a structurally rigid yet adaptable inhibitory interface.[158]

The emergence of metallo-β-lactamases has prompted investigations aimed at discovering natural product-based inhibitors for them. A pioneering study was conducted by Payne *et al.*,[125] who identified metallo-β-lactamase inhibitors from the fungal strain *Chaetomium funicola*. Through targeted natural product screening, three structurally related tricyclic metabolites, SB236049, SB236050, and SB238569, were purified (Fig. 10a). Amongst these, SB236049 demonstrated the most potent activity against the *Bacteroides fragilis* CfiA (IC_{50} = 0.7 μM; pIC_{50} = 6.15) and *B. cereus* II (IC_{50} = 0.3 μM; pIC_{50} = 6.52) metallo-β-lactamases, while also inhibiting *P. aeruginosa* IMP-1 (IC_{50} = 151 μM; pIC_{50} = 3.82). Comparative analysis confirmed that all three compounds were inactive against the serine-β-lactamase P99, highlighting their selectivity towards metallo-β-lactamases.

Mechanistic studies revealed that SB236049, SB236050, and SB238569 are competitive inhibitors of the CfiA, *B. cereus* II, and IMP-1 metallo-β-lactamases.[125] Importantly, their inhibition activities remained constant across a range of Zn^{2+} concentrations, excluding nonspecific Zn^{2+} chelation as their mode of action. Detailed kinetic evaluation of SB238569 provided K_i values of 3.4 μM for CfiA, 17 μM for IMP-1, and 79 μM for *B. cereus* II. Crystallographic studies of SB236050 bound to CfiA showed that the inhibitor forms polar interactions with Lys184, Asn193, and His162, and makes a critical π-stacking interaction with Trp49 in the "flap" domain, which adopts a closed conformation over the active site upon inhibitor binding.[125]

When combined with meropenem, SB236050 and SB238569 exhibited promising antibacterial synergy.[125] Use of 8 μg mL^{-1} of these inhibitors resulted in an eight to sixteen-fold reduction in the meropenem MIC (<4 μg mL^{-1}) against clinical isolates of *B. fragilis* producing CfiA. However, no synergy was observed against IMP-1-producing *P. aeruginosa*, likely due to the inhibitors' poor outer membrane penetration rather than a lack of enzymatic inhibition.[125] Collectively, this study demonstratied that selective, competitive inhibition of metallo-β-lactamases by natural products is feasible.

King *et al.*[113] screened 500 natural product extracts combined with a sub-lethal concentration of meropenem against an efflux-deficient *E. coli* strain engineered to express NDM-1 and exhibit enhanced permeability to small molecules. From this cell-based screen, one hit extract from the fungus *Aspergillus versicolor* was identified that demonstrated reproducible activity in restoring meropenem activity against an NDM-1 producing *E. coli* strain. Using a bioactivity-guided isolation approach, the active compound was identified as the non-ribosomal peptide aspergillomarasmine A (AMA) (Fig. 10b).[113] Aspergillomarasmine A

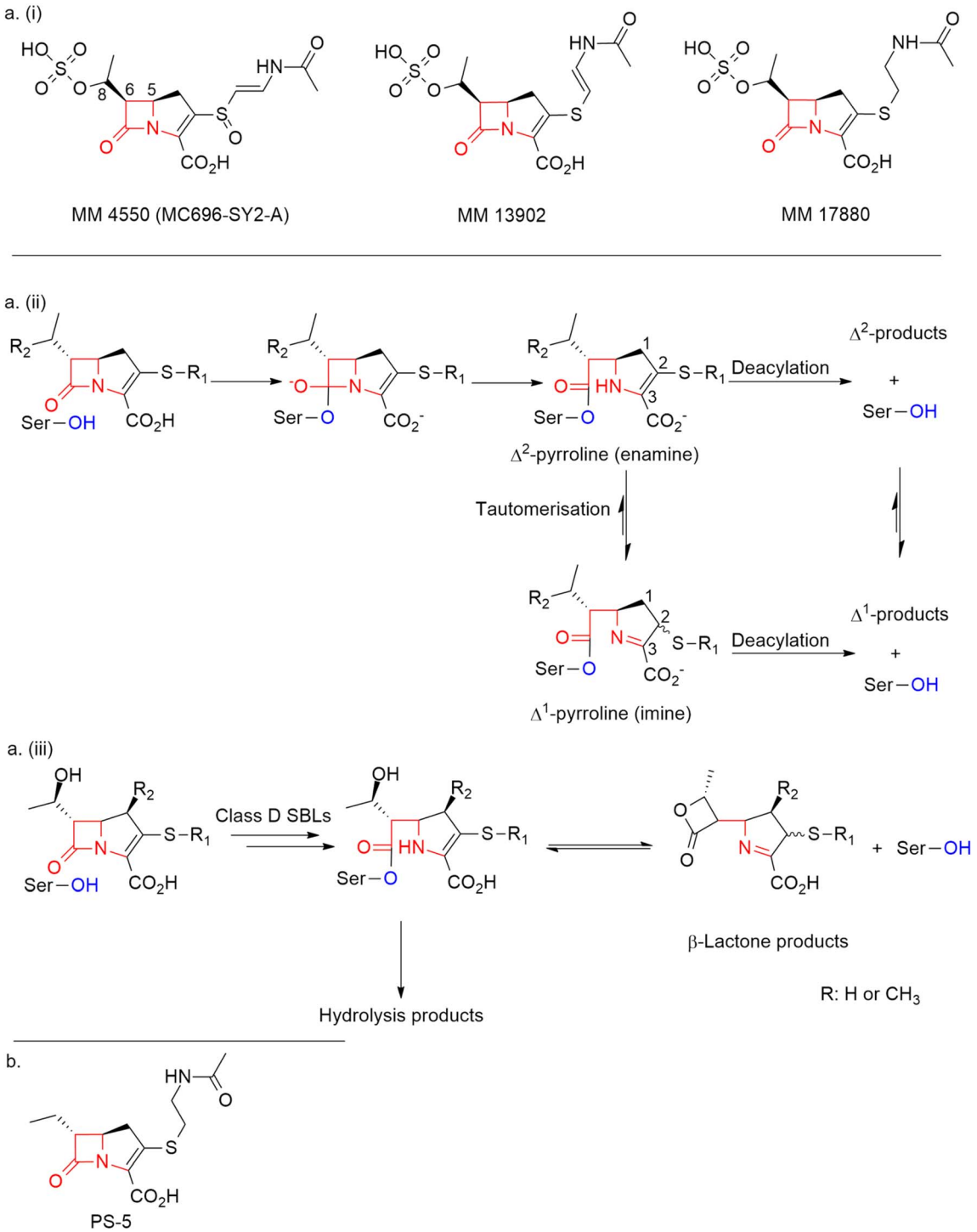


Fig. 9 Structures of microbial-derived olivanic acids, a carbapenem sub-type of serine-β-lactamase inhibitors, and the proposed outline mechanism of action of carbapenems. (a) (i) The olivanic acid compounds MM 4550, MM 13902, and MM 17880, which were isolated from *S. olivaceus*. MM 4550 was also isolated from *S. fulvoviridis* and named MC696-SY2-A. (a) (ii) Proposed pathway for inhibition of serine-β-lactamases by carbapenems. The acyl-enzyme initially forms as a Δ^2-pyrroline, which can tautomerise (that is, the double bond migrates from C2═C3 to C2═N) to give a more stable Δ^1-pyrroline (imine) complex, which is proposed to be the inhibited enzyme species.[147] (a) (iii) Note that in the case of the OXA serine-β-lactamases (class D SBLs), deacylation of carbapenem-derived acyl–enzyme complexes to give tautomeric lactone products can occur.[148,149] Not all potential products are shown. (b) Structure of the serine-β-lactamase inhibitor PS-5 isolated from a *Streptomyces* species.

demonstrated potent *in vitro* concentration-dependent inhibition of NDM-1, with an IC_{50} of 4.0 μM ($pIC_{50} = 5.40$), and an IC_{50} of 9.6 μM ($pIC_{50} = 5.02$) for VIM-2 inhibition. However, AMA was poorly active against IMP-7 and inactive against serine-β-lactamases such as TEM-1, CTX-M-15, KPC-2, and OXA-48. Mechanistic studies indicated that AMA acts as a selective Zn^{2+}

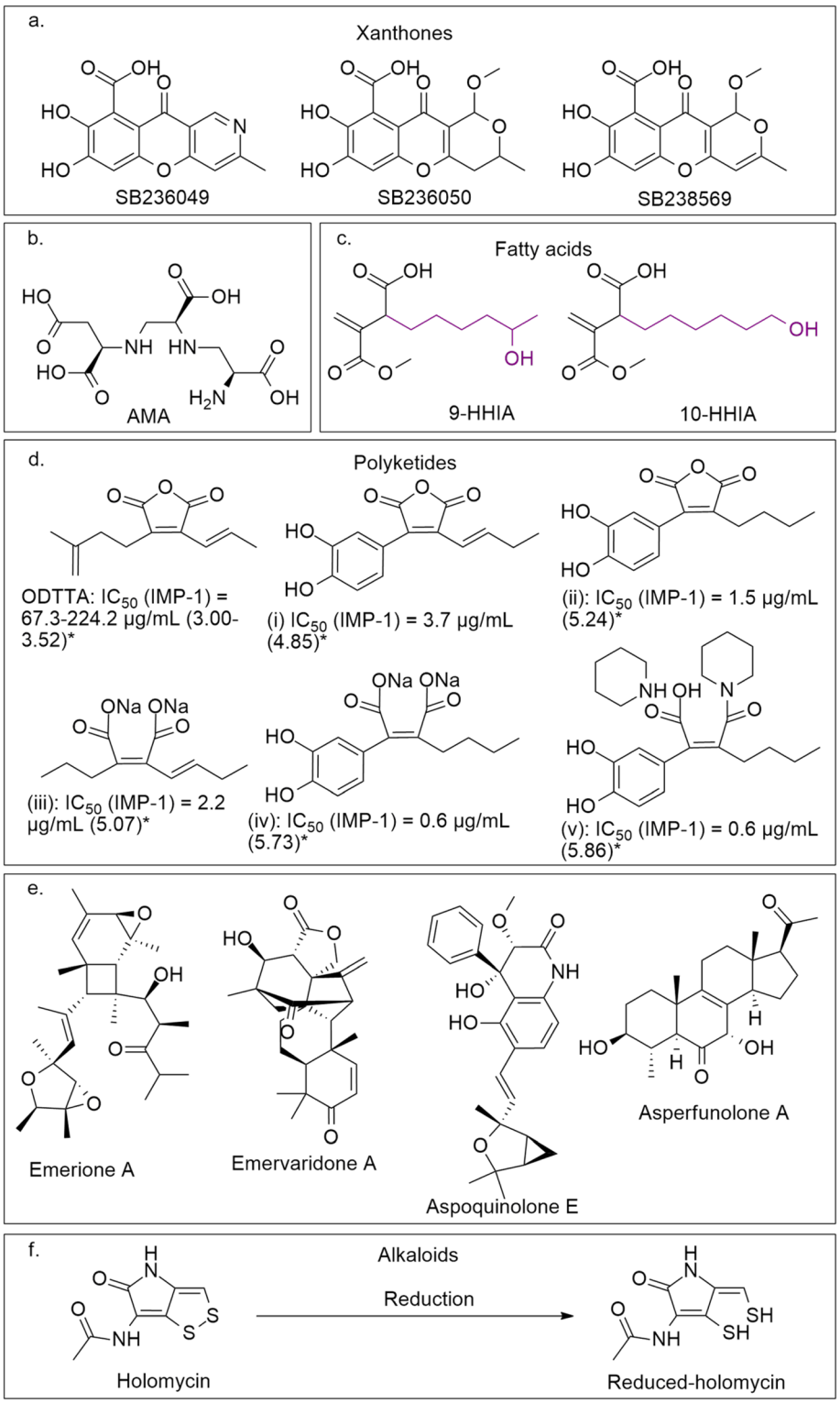


Fig. 10 Representative structures of microbe-derived metallo-β-lactamase natural inhibitors. (a) Tricyclic xanthone polyphenolic compounds, SB236049, SB236050, and SB238569, are reported to inhibit metallo-β-lactamases. (b) Aspergillomarasmine A (AMA), a non-ribosomal peptide Zn^{2+} chelator, acts a metallo-β-lactamase inhibitor. (c) Hydroxylated fatty acids. (d) The polyketide ODTAA and a series of derivative compounds [(i) to (v)]. * = pIC_{50} values (e) Meroterpenoids and quinolone alkaloids. (f) The dithiolopyrrolone-type alkaloid holomycin and its reduced form (red-holomycin).

chelator, removing the essential metal ions from the metallo-β-lactamase active site rather than directly interacting with the enzyme–substrate complex. This metal depletion mechanism was validated through independent approaches, including Zn^{2+} titration, inductively coupled plasma mass spectrometry, and time-dependent kinetic assays.[159,160] Detailed equilibrium

dialysis and NMR analyses further confirmed that AMA efficiently removes Zn^{2+} from both high- and low-affinity binding sites of NDM-1 and VIM-2, whereas IMP-7 exhibited partial resistance likely due to its tighter Zn^{2+} binding.[113,159] Aspergillomarasmine A counteracted resistance and fully restored the efficacy of meropenem, both *in vitro* and *in vivo*. This study marked the first report of a compound with *in vitro* and *in vivo* activity against metallo-β-lactamases.[113] Combination therapies of AMA with meropenem and the serine-β-lactamase inhibitor avibactam have shown promise for treating infections caused by bacterial strains co-expressing multiple β-lactamases, thereby extending the clinical potential of AMA.[161]

Another *Aspergillus* species, *Aspergillus* sp. OPMF00815, has served as a source of metallo-β-lactamase inhibitors, namely the hydroxyhexylitaconic acids.[126] Wachino *et al.*[126] conducted an extensive cell-based screen of 5488 microbial extracts leading to the identification of 16 samples. The 16 active extracts were further evaluated against IMP-1, with five showing >50% inhibition. Two extracts, F2400 and F1765, derived from an *Aspergillus* species, emerged as the most active (>70% inhibition) against IMP-1. These two extracts synergistically enhanced meropenem activity in cell-based assays, resulting in a 32-fold reduction in MIC. Focusing on sample F2400, the researchers successfully isolated two active compounds, 9-hydroxyhexylitaconic acid (9-HHIA) and 10-hydroxyhexylitaconic acid (10-HHIA) (Fig. 10c). Both these compounds demonstrated moderate inhibition of IMP-1, with IC_{50} values of 50.5 μM (pIC_{50} = 4.30) and 31.6 μM (pIC_{50} = 4.50), respectively; they showed moderate activity against VIM-2, but no activity against NDM-1 and SMB-1.[126] Encouragingly, both compounds restored meropenem efficacy in a dose-dependent manner against an IMP-1-producing *E. coli* transformant strain and two clinical isolates of *E. coli* and *K. pneumoniae*. Kinetic studies revealed the mechanism of action to be substrate-competitive, rather than due to Zn^{2+} chelation. Structural studies showed that these compounds interacted with the Zn^{2+} ions in the active site, forming a stable non-covalent complex. *In silico* docking studies further supported these findings, implying hydrophobic interactions between the elongated methylene sidechains of HHIAs and the hydrophobic loop regions surrounding the IMP-1 active site, which are absent in less selective inhibitors.[126]

Fungal compounds have proven to be worthy candidates in the search for metallo-β-lactamase inhibitors. From a virtual docking screen of ~1000 fungal compounds, four promising candidates, asperfunolone A, aspoquinolone E, emervaridone A, and emerione A, were identified with good docking scores against NDM-1 (Fig. 10d).[127] Of these, emerione A exhibited the best docking score (−39.5 kcal mol^{-1}) and the most potent *in vitro* activity (IC_{50} = 12.1 μM; pIC_{50} = 4.92) against NDM-1, closely followed by asperfunolone A (IC_{50} = 36.2 μM; pIC_{50} = 4.44), while the other two compounds did not demonstrate significant inhibition. Emerione A, a methylated polyketide, was observed to form interactions within the NDM-1 active site, including π–π stacking with Phe70 and direct coordination with the catalytic Zn^{2+} ions, suggesting a mixed inhibition mechanism involving both direct enzyme binding and partial metal chelation in solution. Emerione A and asperfunolone A showed weak antibacterial activity against *E. coli* and *K. pneumoniae*, with MIC values of 32 and 64 μM, respectively. Importantly, both emerione A and asperfunolone A restored the activity of meropenem against an NDM-1 producing *E. coli* strain. Emerione was the only compound able to resensitise NDM-1 producing *K. pneumoniae* strains to meropenem *in vitro*.[127]

In an extensive study, 6130 microbial broths were screened using a paper disk method. An extract of the fungus *Paecilomyces* (FKI-6801) was found to resensitise an IMP-1 producing *E. coli* clinical isolate to meropenem *in vitro*.[162] The active compound in the extract was isolated and identified as 3*Z*,5*E*-octa-3,5-diene-1,3,4-tricarboxylic acid-3,4-anhydride (ODTAA) (Fig. 10e). ODTAA was weakly active against IMP-1 with an estimated IC_{50} between 67 and 224 μg mL^{-1} (pIC_{50} between 3.00 and 3.52). Recognising the need for potency optimisation, the authors successfully accomplished the total synthesis of ODTAA in nine steps. Subsequent medicinal chemistry efforts introduced a catechol moiety to the maleic anhydride group and another in which the maleic anhydride was converted to a monamide. These efforts improved inhibition of IMP-1 with IC_{50} values of 0.55 and 0.56 μg mL^{-1} (pIC_{50} values of 5.73 and 5.86) being obtained. Moreover, synthetic derivatives (such as i, ii, iii, iv, and v in Fig. 10e) exhibited improved membrane permeability and restored the antibacterial activity of meropenem against IMP-1-producing *E. coli* and *P. aeruginosa*. This study highlights how integrating natural product discovery with strategic medicinal chemistry can significantly improve lead molecule efficacy, thus opening the prospect of developing clinically viable metallo-β-lactamase inhibitors.[162]

In addition to having served as a source of antibiotics and serine-β-lactamase inhibitors, *S. clavuligerus* has also yielded a metallo-β-lactamase inhibitor.[128] Holomycin, a dithiolopyrrolone antibiotic, and more specifically its reduced form (Fig. 10f), inhibits metallo-β-lactamase NDM-1 with an IC_{50} of 110 nM (pIC_{50} = 6.94). Mechanistic studies revealed that holomycin acts as a prodrug: it enters bacterial cells as the disulfide form and undergoes intracellular reduction to form the active ene-dithiol, reduced-holomycin, which exhibits strong Zn^{2+} binding properties, thereby disrupting metal homeostasis by chelating essential Zn^{2+} ions from bacterial metalloenzymes.[128] Spectrophotometric and mass spectrometric assays show reduced-holomycin binds zinc ions with high affinity, forming a stable 2 : 1 ligand-to-metal complex. The chelation mechanism was shown to be reversible as NDM-1 activity could be restored by the addition of excess Zn^{2+}.[128]

The work on holomycin is further evidence that *S. clavuligerus* stands out as a natural source of antibiotics as well as serine-β-lactamase and metallo-β-lactamase inhibitors, including both small molecules and proteins. It is reasonable to hypothesise that undiscovered organisms possess similar biosynthetic capabilities and could contribute to the discovery of new types of β-lactamase inhibitors.

3.2 β-Lactamase inhibitors from plants

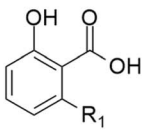
The successful discovery of β-lactamase inhibitors from microorganisms has motivated the search for similar

compounds in plants. Both past and ongoing plant-derived natural product screening efforts, using enzyme- and phenotypic-based assays to find extracts, fractions, and compounds that inhibit β-lactamases.

Coates *et al.*[129] reported on the bioassay-guided isolation of SB-202742 (Fig. 11), a serine-β-lactamase inhibitor identified from *Spondias mombin*, making it one of the first purified plant-derived β-lactamase inhibitors. This anacardic acid displayed reversible, non-competitive inhibition against several serine-β-lactamases, including TEM-1 (IC_{50} = 5 μg mL; pIC_{50} = 4.87), OXA-1 (IC_{50} = 79.7 μg mL; pIC_{50} = 3.67), and P99 (IC_{50} = 16.5 μg mL; pIC_{50} = 4.34), amongst others.[129] However, SB-202742 was inactive against a *S. aureus* Russell produced β-lactamase (IC_{50} > 567 μg mL; pIC_{50} < 2.82). Disappointingly, SB-202742 was unable to restore amoxicillin activity against various β-lactamase-producing bacteria, despite encouraging enzyme activity. The authors hypothesised the lack of cellular activity could be due to the inability of SB-202742 to traverse the bacterial cell wall. Interestingly, SB-202742 showed promising activity against a *Staphylococcus* species (MIC ranging from 2 to 32 μg mL^{-1}) and exceptional activity against *Streptococcus faecalis* (MIC = 0.25 μg mL^{-1}).[129] Building on this study, analogues of SB-202742 (Fig. 11, compounds a to d) were prepared and screened against a range of serine-β-lactamases providing interesting SAR data.[163] Their results showed that the length of the alkyl sidechain strongly influenced activity, with longer chains conferring greater activity. It was observed that saturated sidechains conferred better activity in comparison to unsaturated ones. Many of the analogues showed improved activity compared to SB-202742, except against *S. aureus* Russell where they were also inactive. While the analogues were orders of magnitude less active than clavulanic acid for most β-lactamases, they were comparatively more active against the class C β-lactamases. As with SB-202742, none of the analogues showed any synergistic antibacterial activity with amoxicillin.[163]

Denny *et al.*[164] showed the inhibition of a partially purified L-1 metallo-β-lactamase from *Stenotrophomonas maltophilia* by the flavonoid galangin (Fig. 12a). Activity of galangin against L1 was concentration dependent between 5 and 50 μg mL^{-1}. The inhibition of the L-1 metallo-β-lactamase was not reversed by the addition of excess Zn^{2+}, suggesting a non-chelating mechanism. Quercetin was also screened and showed inhibition of the L-1 metallo-β-lactamase, although this was less pronounced than that of galangin. In phenotypic assays, galangin did not have synergistic activity with imipenem, with the authors speculating that this could be due to limited cell-penetration of the compound.[164] Interestingly, eight years following this study, galangin was isolated from rhizomes of the plant *Alpinia officinarum* along with two other flavonoid compounds, namely kaempferide and kaempferide-3-*O*-β-D-glucoside (Fig. 12a).[165] While galangin showed inherent antibacterial activity, it also inhibited a penicillinase I from *B. cereus* and another penicillinase IV from *E. cloacae*. Galangin and two other test polyphenols, quercetin and baicalein (Fig. 12a), were shown to have synergistic activity with a wide range of β-lactams against clinical isolates of a penicillin-resistant *S. aureus* strain.[165]

Further reports have emerged regarding the metallo-β-lactamase-inhibiting properties of polyphenols, particularly flavonoids (Fig. 12–14). Shi *et al.*[131] used an *in silico* strategy to evaluate polyphenols against NDM-1. From a list of 22 prioritised compounds, six were screened *in vitro* against NDM-1. The flavonoid hesperidin was the most active, with an IC_{50} of 3.4 μM (pIC_{50} = 5.47). This was followed by the compounds diosmin (IC_{50} = 20.7 μM; pIC_{50} = 4.68) and rutin (IC_{50} = 15.5 μM; pIC_{50} = 4.81) (Fig. 12a). Stevioside had the weakest activity (IC_{50} = 214.1 μM; pIC_{50} = 3.67), and one compound, ginsenoside (Rg3), was inactive (Fig. 12b).[131] Using the virtual tool IFPTarget, Yu *et al.*[130] identified the polyphenol rosmarinic acid as a putative inhibitor of VIM-2. *In vitro* enzymatic assays confirmed its competitive reversible inhibition of VIM-2, with an IC_{50} of 41.5 μM (pIC_{50} = 4.38), and no Zn^{2+} chelation mechanism was implicated.[130] However, rosmarinic acid was weakly active against NDM-1 and TEM-1 at 600 μM (Fig. 13a). Nonetheless, three structurally related compounds, namely salvianic acid, caffeic acid, and salvianolic acid A (Fig. 13a), were evaluated, with the latter compound emerging as the most promising,



	IC_{50}μg/mL* (pIC_{50}) (Class A, C and D)		
	TEM-1 (A)	P99 (C)	OXA-1 (D)
SB: R_1 = $C_{17}H_{30}$ (17:3)	5 (4.87)	17 (4.34)	80 (3.67)
a: R_1 = $C_{17}H_{35}$ (17:0)	1.9 (5.30)	1 (5.58)	29 (4.11)
b: R_1 = $C_{15}H_{31}$	0.8 (5.64)	1.4 (5.40)	25 (4.14)
c: R_1 = $C_{10}H_{21}$	12 (4.37)	150 (3.27)	360 (2.89)
d: R_1 = C_8H_{17}	52 (3.85)	150 (3.39)	Inactive
Clavulanic acid	0.029 (6.84)	160 (3.10)	0.35 (5.76)

Fig. 11 Structure–activity relationship analysis of SB-202742 (SB) and its analogues against β-lactamases. Analogues of the plant-derived anacardic acid SB-202742 were assessed for inhibitory activity against class A (TEM-1), C (P99), and D (OXA-1) serine-β-lactamases. Activity correlated with sidechain length and saturation: longer, saturated alkyl chains (*e.g.*, a and b) showed improved potency, while shorter chains (c and d) reduced activity, with d inactive against OXA-1. Note that the numerical notation given in parentheses (*e.g.*, 17 : 3 and 17 : 0) follows the standard format *C* : *D*, where *C* represents the number of carbon atoms in the fatty acid chain (17) and *D* denotes the number of carbon–carbon double bonds (3 and 0). *IC_{50} has been reported in μg mL^{-1} which we believe to be the correct unit, as opposed to the mg mL^{-1} units provided by the authors.

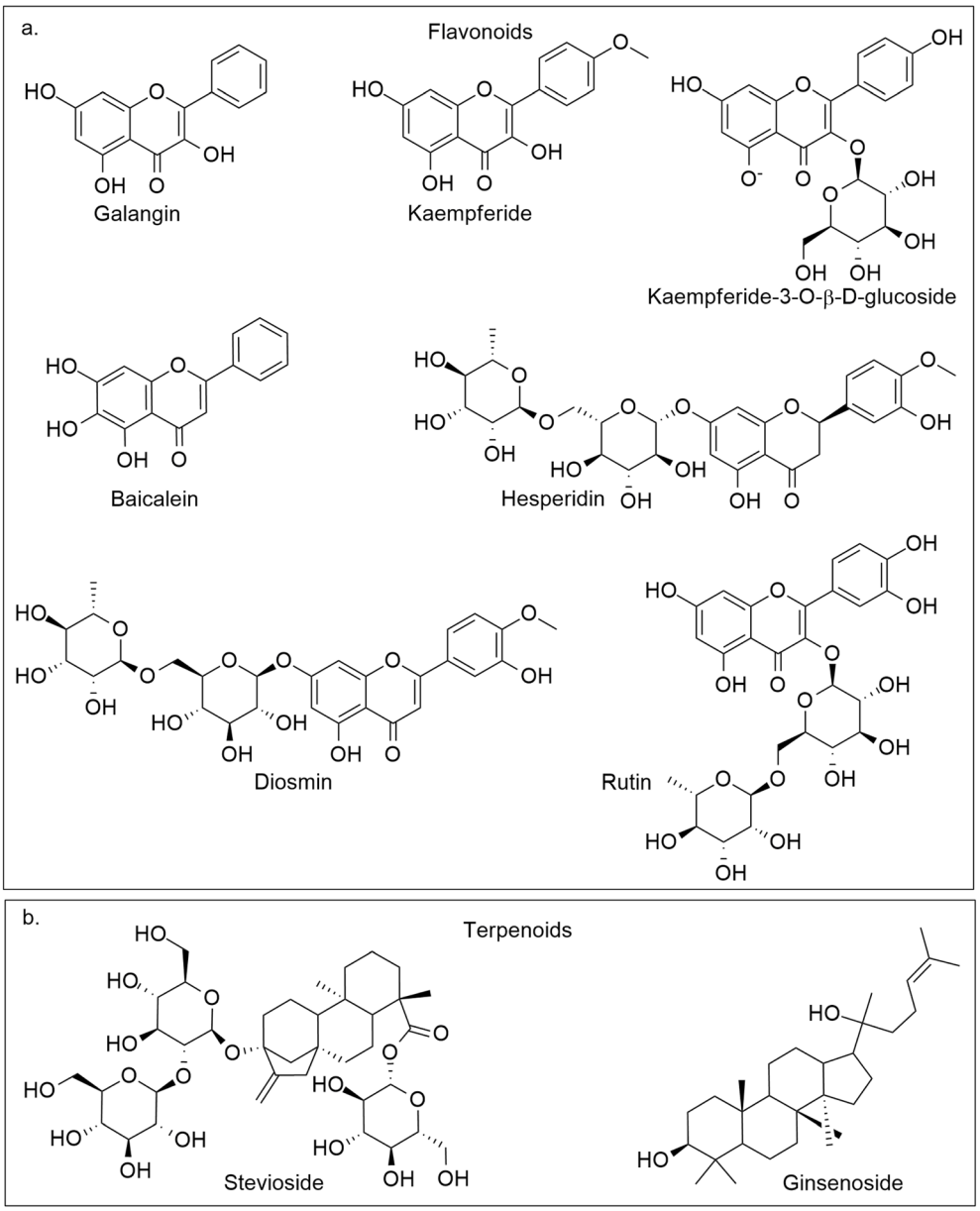


Fig. 12 Plant-derived flavonoids and terpenoids evaluated for β-lactamase inhibition.

showing activity against VIM-2 (IC_{50} = 27.2 μM; pIC_{50} = 4.57), NDM-1 (IC_{50} = 160.3 μM; pIC_{50} = 3.80), and TEM-1 (IC_{50} = 34.3 μM; pIC_{50} = 4.47).[130] In a cell-based assay using an *E. coli* strain expressing VIM-2, salvianolic acid A and rosmarinic acid were able to reduce the MIC of meropenem by 2 to 4-fold at non-lethal concentrations.[130]

Magnolol, a biphenolic natural product isolated from the bark of *Magnolia officinalis*, was identified as an NDM-1 inhibitor (Fig. 13b). Liu *et al.*[132] demonstrated that magnolol moderately inhibits NDM-1 activity (IC_{50} = 6.47 μg mL; pIC_{50} = 4.61) and restored the efficacy of meropenem against an NDM-1-producing *E. coli* strain, reducing MIC values by up to four-fold. While magnolol showed no intrinsic antibacterial activity, its combination with meropenem resulted in complete bacterial clearance within hours *in vitro*. Molecular modelling and mutagenesis studies indicated that magnolol binds directly to the active site (residues Val73, Lys211, Gly219, and His250), primarily *via* hydrophobic interactions and a stabilising hydrogen bond with Ser217, thereby preventing substrate access.[132] Importantly, unlike classical metal chelators, magnolol did not disrupt Zn^{2+} coordination,[132] possibly reducing the risk of off-target toxicity. Previous toxicological evaluations suggest that the magnolia bark extract (containing 94% magnolol) is well tolerated *in vivo*.[166,167] These findings highlight magnolol as a safe, natural product-derived lead with potential for development as an adjuvant β-lactamase inhibitor.

The polyphenols ellagic acid and urolithin were also evaluated against the serine-β-lactamase CTX-M-15 (Fig. 13b).[168] Ellagic acid inhibited CTX-M-15, while urolithin showed only weak activity. The authors hypothesised that ellagic acid's

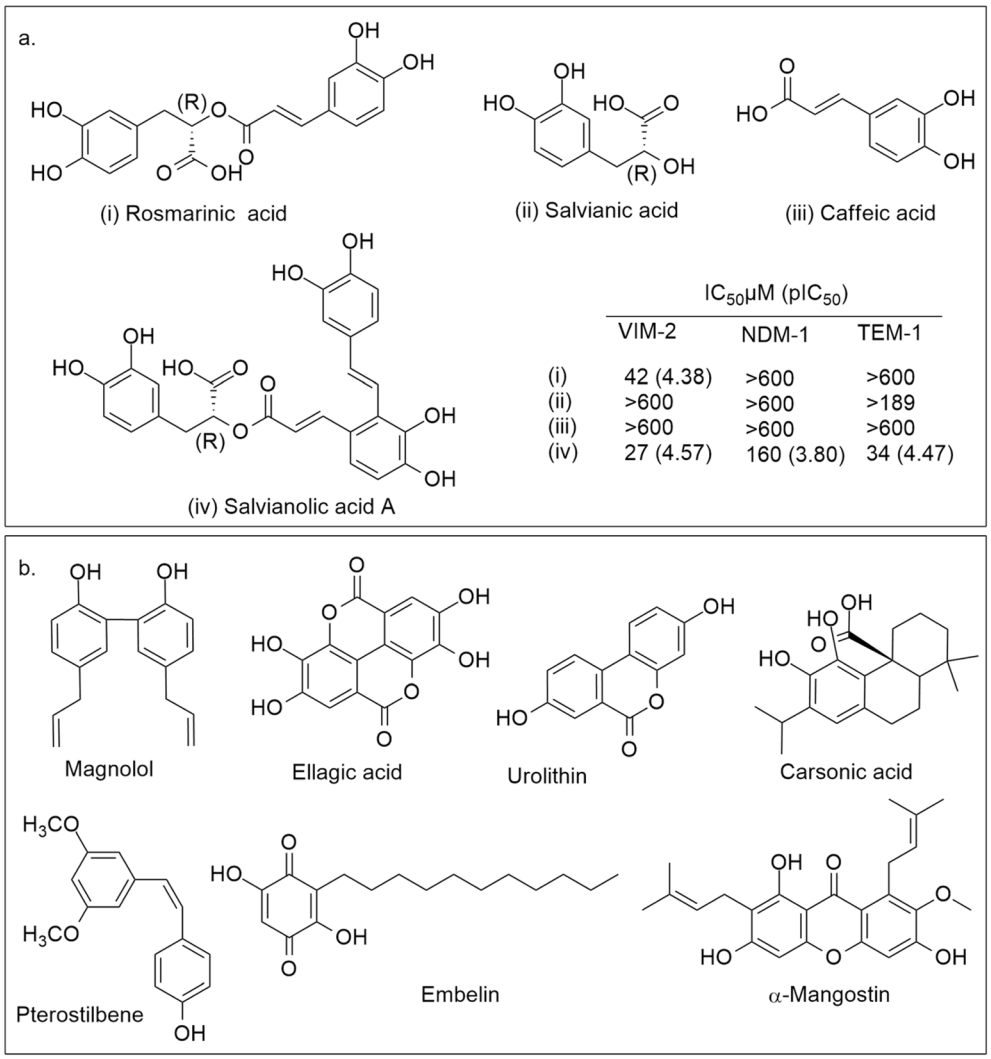


Fig. 13 Plant-derived natural products evaluated for β-lactamase inhibition. (a) Phenolic acids: rosmarinic acid (i), salvianic acid (ii), caffeic acid (iii), and salvianolic acid A (iv), which inhibit metallo-β-lactamases and serine-β-lactamases (see IC_{50} and pIC_{50} values). (b) Other phenol derivatives identified as β-lactamase inhibitors: magnolol, ellagic acid, urolithin, and carnosic, the stilbenoid pterostilbene, the tricyclic xanthone, α-mangostin, and the benzoquinone embelin.

mechanism involved reaction with the nucleophilic serine residue. Ellagic acid was inactive against NDM-1 at concentrations up to 10 mM.[168]

The polyphenol pterostilbene (Fig. 13b) also shows activity against NDM-1,[133] with an IC_{50} of 15.4 μg mL^{-1} ($pIC_{50} = 4.22$).[133] Cell-based assays revealed that pterostilbene alone has minimal antibacterial activity against NDM positive *E. coli* and *K. pneumoniae* strains; in combination with meropenem, pterostilbene showed synergy against the NDM positive isolates, significantly lowering its MIC. Encouragingly, synergistic activity of pterostilbene and meropenem was observed in *in vivo* models: a murine thigh model of *E. coli* infection and a mouse pneumonia model of *K. pneumoniae* infection. In both cases, the pterostilbene–meropenem combination significantly reduced the bacterial burden and improved survival rates compared to the single agent treatments.[133] Embelin (Fig. 13b), a hydroxyl-substituted benzoquinone from *Embelia ribes*, along with curcumin and resveratrol, emerged as promising hits from a primary screen of an in house collection of natural product extracts and chemicals.[134] Embelin emerged as the most active of these compounds with an IC_{50} of 2 μM ($pIC_{50} = 5.7$) against NDM-1 and further showed moderate activity against VIM-1 ($IC_{50} = 200$ μM; $pIC_{50} = 3.70$) and IMP-1 ($IC_{50} = 100$ μM; $pIC_{50} = 4.00$). Embelin restored the activity of various antibiotics, namely meropenem, imipenem, biapenem, cefepime, ceftazidime, and cefradine, in a dose-dependent manner by 2 to 512-fold against an NDM-1-producing *K. pneumoniae* strain, with the most significant improvement observed with the carbapenems.[134] Alone, embelin did not exhibit activity against *K. pneumoniae* at concentrations up to 128 μg mL^{-1}. Furthermore, embelin was able to restore meropenem sensitivity against NDM-1 positive clinical isolates of *E. coli* (17 isolates), *K. pneumoniae* (15 isolates), and *A. baumannii* (14 isolates). This was at concentrations at which embelin alone did not show antibacterial activity against these isolates.[134]

Carnosic acid, a natural diterpene found in *Salvia rosmarinus*, was identified as a promising allosteric inhibitor of NDM-1 (Fig. 13b).[135] The identification of carnosic acid, made through virtual screening, was validated using enzymatic assays, demonstrating an IC_{50} of 27.1 μM ($pIC_{50} = 4.57$) against NDM-1. Although carnosic acid itself lacked intrinsic antibacterial activity, it partially restored the efficacy of meropenem against an NDM-1 producing *E. coli* strain, resulting in a 4-fold reduction of the antibiotic's MIC. Mechanistic investigations, including molecular docking and 100 ns molecular dynamics simulations, indicated that carnosic acid may bind to a novel allosteric site on NDM-1, involving Phe46, Tyr64, Leu65, Asp66, and Thr94, and both hydrophobic interactions and hydrogen bonds. Carnosic acid binding is proposed to induce conformational changes in the active site region, leading to a loss of catalytic activity without direct chelation of the Zn^{2+} ions. Fluorescence quenching assays and binding energy calculations supported these findings. Mutagenesis of key residues (F46A, L65A, T94A) diminished binding affinity and inhibitory potency, confirming the specificity of the allosteric interaction. This study introduced an innovative paradigm in which a natural molecule achieves non-competitive, allosteric inhibition of NDM-1, contrasting with earlier active-site-binding inhibitors and potentially reducing the likelihood of resistance development. Given its low toxicity and broad availability, carnosic acid represents a promising lead scaffold for the development of allosterically binding metallo-β-lactamase inhibitors.[135]

Building on the success of their earlier study, Cheng *et al.*[136] evaluated 500 flavonoids for inhibition of the class D serine-β-lactamase OXA-48. Six compounds emerged as promising candidates (IC_{50} ranging from 0.52 to 52.7 μM; pIC_{50} ranging from 4.28 to 6.28). Amongst them, α-mangostin, a tricyclic xanthone (a sub-group of polyphenols), emerged as the most active and was subsequently prioritised for further studies (Fig. 13b). The authors screened α-mangostin against a panel of β-lactamases (at 50 μM) followed by dose response investigations against NDM-1, giving an IC_{50} value of 25.3 μM ($pIC_{50} = 4.60$).[136] Thus, α-mangostin dual activity against both the serine-β-lactamases and metallo-β-lactamases with selectivity towards OXA-48. Kinetic studies showed that α-mangostin was a non-competitive inhibitor of OXA-48. Using jump dilution and equilibrium distillation assays, the authors demonstrated that α-mangostin is a reversible inhibitor of OXA-48. Thermal shift assays showed that α-mangostin binds to and destabilise OXA-48, resulting in a decrease in the melting temperature.[136]

Quercetin is a flavonoid that has gained significant interest over the years for its β-lactamase inhibition properties (Fig. 14). In one study, it was observed that phenols with a keto group on the *ortho*-position were effective inhibitors of NDM-1: inhibition was improved by the presence of a second ketone substituent, particularly if it was either an aromatic or enolic ketone.[137] Using a chemoinformatic approach, 222 molecules bearing the minimum required moieties were identified and screened *in vitro* against NDM-1 using a miniaturised assay. Quercetin (71% inhibition at 50 μM), along with two other flavonoids, morin (78% inhibition at 80 μM) and myricetin (85% inhibition at 50 μM), were identified as the most active inhibitors against NDM-1. Myricetin ($IC_{50} = 3.3$ μM; $pIC_{50} = 5.48$) emerged as the most active from full-dose response studies, closely followed by quercetin, whose IC_{50} could only be estimated to be between 5 and 10 μM (pIC_{50} between 5.00 and 5.30) due to its limited solubility.[137] To address solubility issues, quercetin analogues were synthesised by adding either a methyl or an isopropyl group to increase their hydrophobicity. The addition of the latter functional groups resulted in a mixture of two analogues with improved activity (86% inhibition at 50 μM) compared to their parent, quercetin. Both quercetin and myricetin (at 100 μg mL^{-1}) showed synergistic activity with imipenem, significantly reducing its MIC by 4-fold (quercetin) and 16-fold (myricetin) (Fig. 14) against an NDM-1-expressing *E. coli* strain.[137] However, only myricetin showed synergy with imipenem against *K. pneumoniae*, reducing the MIC of the carbapenem 16-fold. NMR studies showed that the three flavonoids are specific inhibitors of NDM-1. Active site residues involved in binding with the flavonoids were identified as tryptophan 93 (W93), histidine 122 (H122), glutamine 123 (Q123), aspartic acid 124 (D124), asparagine 220 (N220), and histidine 250 (H250), as well as the two Zn^{2+} ions.[137]

Another study[138] employed an OXA-48 producing, efflux-deficient, and outer-membrane permeable *E. coli* construct to screen 150 polyphenols (at 40 μg mL^{-1}) for their ability to enhance the activity of piperacillin (at 128 μg mL^{-1}) against the construct strain. Fifteen percent of the compounds were excluded because they showed antibacterial activity against the construct in the absence of piperacillin. Quercetin emerged as the only compound that restored antibiotic activity without showing bactericidal activity alone. Motivated by these findings, the authors investigated 15 other flavonoids together with quercetin in an enzyme-based assay against OXA-48. Eight out of the 16 compounds showed >50% inhibition against OXA-48 (at 50 μM). The eight compounds were fisetin (90% inhibition), quercetin (87%), luteolin (81%), 3′,4′,7-trihydroxyflavone (80%), apigenin (77%), kaempferol (76%), taxifolin (76%), and isoquercitrin (61%) (Fig. 14). The three most active compounds were fisetin ($IC_{50} = 0.47$ μM; $pIC_{50} = 6.33$), luteolin ($IC_{50} = 0.55$ μM; $pIC_{50} = 6.26$) and quercetin ($IC_{50} = 1.47$ μM; $pIC_{50} = 5.83$).[138] Structure–activity relationship studies showed that hydroxylation in the core structure of flavonoids is important for inhibition of OXA-48. It was observed that the presence of a hydroxyl group in the A and B rings improved potency. Kinetic studies showed quercetin to be a non-competitive and reversible inhibitor of OXA-48. Using combination assays, quercetin (64 μg mL^{-1}) reduced the MIC of piperacillin 8-fold against an *E. coli* construct. Comparatively, quercetin was only able to reduce the MIC of imipenem 2-fold against the same *E. coli* strain.[138] Synergy between piperacillin and quercetin was further confirmed in an *in vivo* study, in which the drug combination significantly reduced bacterial load in the spleen and liver of mice infected with an OXA-48-producing *E. coli* strain. The two compounds alone did not significantly reduce the bacterial burden in these organs. Overall, the results of these two studies showed that quercetin has dual activity against both serine-β-lactamases and metallo-β-lactamases,

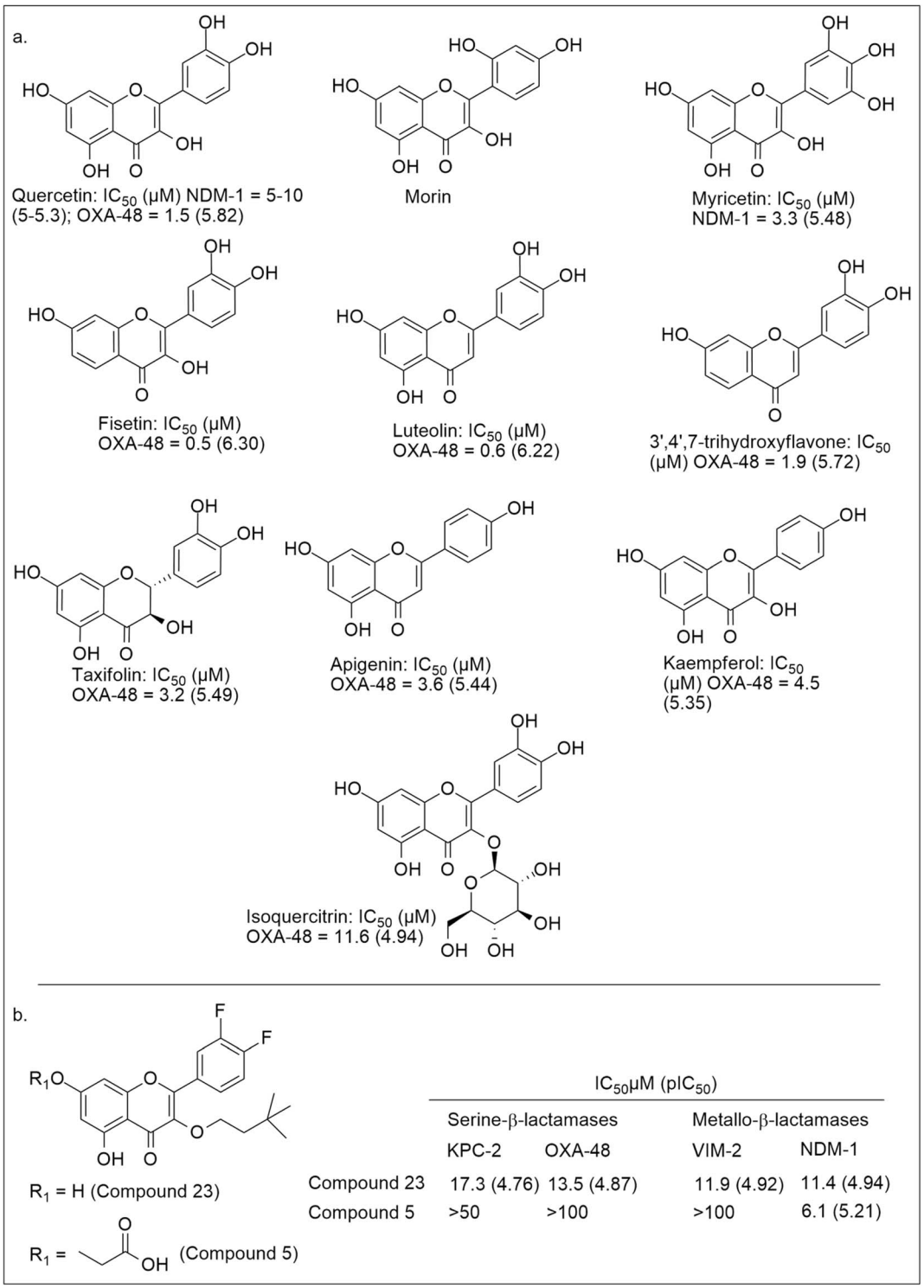


Fig. 14 Flavonoid-based β-lactamase inhibitors and optimisation of the quercetin scaffold. (a) Chemical structures of representative flavonoids reported to inhibit clinically relevant β-lactamases, highlighting quercetin and closely related flavonols and flavones. (b) Optimisation *via* 7-*O* substitution (R_1) on a fluorinated, 3-*O*-substituted quercetin framework. The structures depict variation at the 7-*O* position (R_1=H *vs.* an ionisable carboxylate-containing substituent) and the corresponding inhibitory potencies (IC_{50}, μM) against representative serine-β-lactamases (KPC-2, OXA-48) and metallo-β-lactamases (VIM-2, NDM-1).

a trait that few compounds have been observed to possess thus far,[138] with bicyclic boronates, *e.g.* taniborbactam, being another example.[119]

Recent work has focused on transforming quercetin into a potent medicinal chemistry lead. In 2024, Lee *et al.*[169] reported on the development of 3-*O*-substituted quercetin derivatives

designed to simultaneously inhibit two critical resistance mechanisms in Gram-negative *Enterobacteriaceae*, namely RND-type efflux pumps (such as AcrAB-TolC) and broad-spectrum carbapenemases. Molecular docking studies revealed that the introduction of an ethylene-linked sterically demanding group at the 3-OH position allows these molecules to occupy novel binding sites in both targets. Notably, compound 23, featuring a *tert*-butyl group, exhibited pan-carbapenemase inhibitory activity against KPC-2, OXA-48, VIM-2, and NDM-1, while also potently inhibiting efflux. This dual action successfully rescued the activity of meropenem in clinical multidrug-resistant strains and showed efficacy in a CRE-infected mouse model.[169]

This optimisation strategy was further refined in 2025 by addressing the challenges of periplasmic accumulation and outer membrane penetration in *P. aeruginosa*. Recognising that the highly hydrophobic nature of the earlier 3-*O*-alkyl derivatives limited their efficacy to toxic concentrations, the researchers introduced polar substituents at the 7-OH position.[170] This led to the identification of 7-*O*-carboxylic acid-substituted 3-*O*-alkyl difluoroquercetin, which enhanced periplasmic concentrations through better utilisation of porin pathways. This compound demonstrated potent synergism with aztreonam, a monobactam typically stable to metallo-β-lactamases but vulnerable to other β-lactamases and efflux. By simultaneously inhibiting NDM-1 and the mexAB-oprM efflux system, this compound sensitized 96% of carbapenemase-producing *P. aeruginosa* isolates to aztreonam at clinically relevant concentrations, marking a significant advancement in the development of non-toxic multidrug-resistant reversal agents.[170]

The chromone scaffold is ubiquitous in nature, occurring predominantly in plants and to a lesser extent in other natural sources.[171,172] It has recently been recognised as a promising pharmacophore for metallo-β-lactamase inhibition (Fig. 15). Christopeit *et al.*[173] reported 3-formylchromone [Fig. 15a(i)] as a reversible covalent inhibitor of NDM-1, forming a Schiff base with Lys224 in the active site [Fig. 15a(ii)]. Although its potency was moderate (K_I = 580 nM),[173] this study established chromones as viable warheads for metallo-β-lactamase inhibition, providing mechanistic validation through mass spectrometry, mutagenesis, and covalent docking studies. Subsequent optimisation by Liang *et al.*[174] led to 7-substituted 3-formylchromones, including the benzo[*b*]thiophene derivative, CS-23, which irreversibly inhibited NDM-1 *via* Lys211 modification and displayed nanomolar potency (IC_{50} = 36 nM; pIC_{50} = 7.44; K_I = 4.7 nM) [Fig. 15a(iii)]. Importantly, CS-23 synergises strongly with meropenem, reducing its MIC value against an NDM-1-producing *E. coli* strain by 32-fold, resulting in a full restoration of the carbapenem's efficacy.[174]

The volatile oil component houttuynin, a naturally occurring aldehyde from *Houttuynia cordata*, and its sodium houttuyfonate derivatives have been developed as partially reversible covalent NDM-1 inhibitors (Fig. 15b.).[175] These compounds couple covalent lysine reaction with zinc coordination and hydrophobic interactions, yielding low micromolar activity (IC_{50}s 1–14.2 μM; pIC_{50}s 4.85–6.00) and potentiating meropenem activity against resistant strains (Fig. 15b).[175] Collectively, these findings reveal that natural products not only provide privileged scaffolds, but can also be harnessed to generate covalent inhibitors of metallo-β-lactamases, representing a novel paradigm in the fight against these enzymes.

4. Future perspectives

Collectively, the foundational and subsequent studies reviewed here highlight the rich potential of microorganisms and plants as reservoirs of β-lactamase inhibitors. Systematic screening, bioassay-guided fractionation, and medicinal chemistry-driven optimisation have yielded promising scaffolds, however, persistent challenges, including bacterial cell penetration, metabolic stability, and target selectivity, continue to hinder the translational progress of many natural product classes. Historically, natural product discovery efforts progressed from actinomycete-derived serine-β-lactamase inhibitors to fungal[113,125] and plant-derived metabolites with activity against metallo-β-lactamases. Building on this trajectory, the field is now converging on the ambitious goal of identifying broad-spectrum inhibitors capable of simultaneously neutralising multiple β-lactamase classes, a critical objective in remedying antimicrobial resistance.

Within this context, a careful examination of the history of β-lactamase inhibitor discovery provides valuable guidance for future research directions, highlighting a vast but largely unexplored chemical space. For instance, although >400 000 naturally occurring compounds are catalogued,[176,177] only a small fraction have been investigated for β-lactamase inhibition. Accordingly, more strategic prioritisation of natural compounds is warranted, particularly guided by historical insights into chemical classes that have repeatedly demonstrated efficacy. Amongst these, flavonoids represent a promising class of β-lactamase inhibitors, especially highly hydroxylated derivatives containing catechol moieties. However, their polarity limits membrane permeability, particularly in Gram-negative bacteria, and some are substrates of bacterial efflux pumps. Medicinal chemistry approaches, such as methylation of hydroxyl groups, prenylation, or halogenation, can enhance lipophilicity and membrane permeability.[178–180] Alternative strategies include glycosylation to improve solubility and hybridisation with antibiotic warheads, to leverage on the dual mechanisms of action. Moreover, to avoid altering the physicochemical profile of the flavonoid scaffold, nanotechnology-based delivery systems warrant exploration to improve cellular uptake.[179]

While flavonoids offer a strong foundation, the discovery landscape of naturally occurring β-lactamase inhibitors is expanding beyond this class of molecules to include tricyclic phenolic compounds and proteins. Tricyclic phenolic compounds, whether fungal or plant-derived,[125] have demonstrated notable activity, suggesting that these frameworks represent an underexplored chemical space for further β-lactamase inhibitor development. In parallel, naturally produced protein β-lactamase inhibitors (BLIP, BLIP-I, and BLIP-II), demonstrate exceptional potency against β-lactamases, but their application is limited by their size, poor uptake,

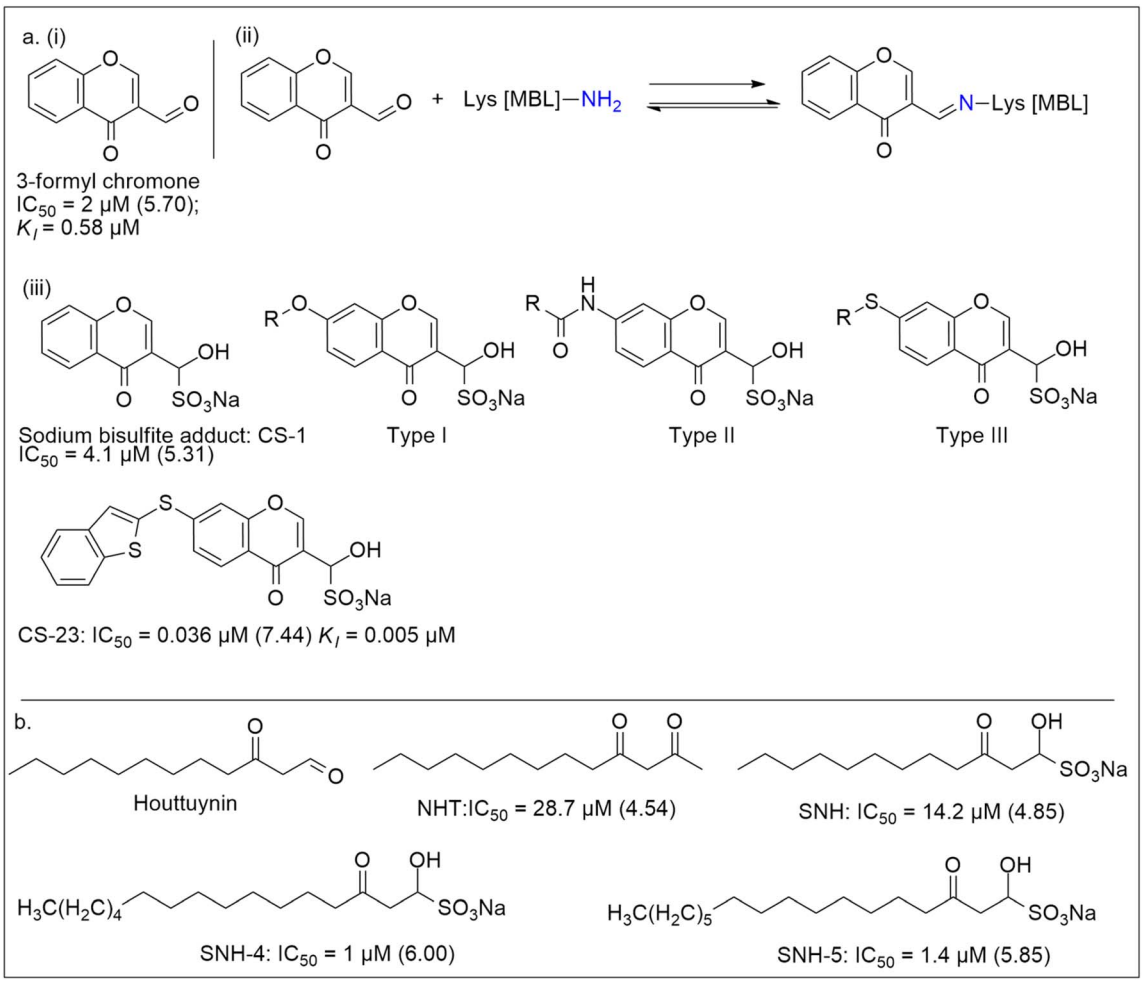


Fig. 15 Structures and inhibitory activities of selected natural product-derived covalently reacting inhibitors of NDM-1. (a) Chromone-based inhibitors: (i) 3-formylchromone, the first reported reversible covalent inhibitor of NDM-1, forms a Schiff base with Lys residues in the NDM-1 active site (IC_{50} = 2 μM; pIC_{50} = 5.70; K_I = 0.58 μM). (ii) Proposed mechanistic scheme showing Schiff base formation between 3-formylchromone and the amino group of the active site Lys. (iii) Representative 7-substituted chromone derivatives and their sodium bisulfite adducts, including type I–III analogues, with enhanced potency. The benzo[*b*]thiophene derivative CS-23 emerged as a potent irreversible inhibitor of NDM-1 (IC_{50} = 0.036 μM; pIC_{50} = 7.44; K_I = 0.005 μM). (b) Houttuynin-derived inhibitors: the sodium houttuyfonate derivatives (SNH series) exhibit partially reversible covalent inhibition of NDM-1. Optimised homologs (*e.g.*, SNH-4, IC_{50} = 1 μM, pIC_{50} = 6.00; SNH-5, IC_{50} = 1.4 μM, pIC_{50} = 5.85) exhibit significantly greater potency than the houttuynin analogue NHT (IC_{50} = 28.7 μM; pIC_{50} = 4.54).

proteolytic instability, and the potential for adverse immune reactions. Consequently, this creates an opportunity to explore engineered protein–protein interaction inhibitors and novel peptide mimics of the BLIPs. In this context, cyclic peptides, potentially identified through phage-display or RNA display type approaches,[181] represent a compelling yet underexplored frontier for β-lactamase inhibitors. Unlike linear peptides, cyclic structures offer enhanced stability and high selectivity, a strategy already showing success in targeting undruggable sites in oncology.[182] The advantage of protein mimicking cyclic peptides is that they will retain binding specificity with improved pharmacokinetics and synthetic tractability, thereby enhancing their viability as drug candidates. However, this increased stability of cyclic peptides must be balanced against potential toxicity risks associated with prolonged clearance half-lives.

Complementing these molecular design strategies is the need for broader bioprospecting, with relatively few plant and microbial species having been systematically explored as sources of β-lactamase inhibitors. In this regard, priority groups that merit further investigation include taxa such as *Brassicaceae* lineage[183] and the *Salvia* genus, which have yielded compounds such as rosmarinic acid and carnosic acid.[130,135] Similarly, from a microbial perspective, although *Streptomyces* and various fungi are prolific sources of natural products, many species remain unexplored, thereby motivating broader screening of Actinomycetes and bioremediation-associated fungi,[184,185] given their capacity to produce metal-chelating secondary metabolites relevant to metallo-β-lactamase inhibition. To further expand this chemical space, attention should turn to marine derived natural products, where the marine environment harbours an extraordinary biodiversity, yielding structurally unique

metabolites.[186] However, translating these discoveries is often constrained by production challenges, as large-scale synthesis is often seen as a bottleneck in advancing these complex molecules through drug discovery pipelines. In this context, advances in biocatalysis hold promise in enabling exploitation of the biosynthesis of complex natural products,[187,188] including those of marine origin.[189] In parallel, the integration of synthetic biology and natural biosynthesis offers an opportunity to enable production of modified natural products with enhanced pharmacological properties. While synthetic biology tools have been developed for engineering microbial biosynthetic pathways, their application to plants and other producer organisms is an emerging frontier with potential to expand the arsenal of accessible β-lactamase inhibitor chemotypes. Beyond marine systems, underexplored terrestrial organisms such as lichens, which produce unique compounds,[190] warrant systematic evaluation.

To effectively operationalise these discovery efforts, innovative methodologies for the isolation and identification of β-lactamase inhibitors from complex natural product extracts are required. Integration of metabolomics tools such as Global Natural Products Social Molecular Networking (GNPS),[191] alongside advanced annotation tools such as SIRIUS,[192] DreaMS,[193] and ChemEmbed,[194] is significantly accelerating the annotation and prioritisation of bioactive metabolites. These modern techniques enable the rapid distinction of novel compounds from known chemical space, reduce redundancy in discovery, and facilitate a more targeted approach to natural product drug discovery.

Building further on these integrated approaches, the introduction of *in silico* tools has substantial potential to advance the field, including by enabling the efficient identification and optimisation of natural product β-lactamase inhibitor hit compounds. To date, most virtual screening efforts have focused on targeting the active sites of β-lactamases; expanding into allosteric site targeting may be a productive approach. Allosteric sites in β-lactamases have been recognised since 2004,[195] yet relatively few inhibitors have been identified that exploit such sites.[196,197] Recent studies have demonstrated the feasibility of such an approach, with carnosic acid emerging as the first reported natural product to inhibit NDM-1 *via* an allosteric mechanism.[135] Given this proof of concept, there is substantial merit in pursuing the discovery of additional natural allosteric inhibitors to diversify therapeutic strategies against β-lactamase-mediated resistance further. Combination therapies of traditional and allosteric inhibitors could have a synergistic effect, helping to overcome antibiotic resistance.[198] One important issue that should be considered with respect to binding at non-essential allosteric sites is whether resistance will efficiently emerge, something that has been an issue with allosteric drugs used for cancer treatment.[199]

5. Conclusion

In conclusion, the history of the development of β-lactam antibiotics and β-lactamase inhibitors highlights the value of natural products as a fertile source of bioactive scaffolds. Natural products have yielded structurally diverse transpeptidase and β-lactamase inhibitors operating *via* a range of inhibition mechanisms, including competitive and non-competitive inhibition, Zn^{2+} ion chelation, covalent reaction, and allosteric modulation. Despite decades of progress, however, the vast diversity of plant and microbial taxa remains largely unexplored in the search for novel antibiotics and β-lactamase inhibitors. We have outlined a framework for prioritising and investigating such resources to identify β-lactamase inhibitors. When integrated with medicinal chemistry and structural biology approaches, natural product-derived candidates hold significant promise for advancement along the drug discovery pipeline toward preclinical development.

6. Author contributions

Phanankosi Moyo – conceptualisation, writing – original draft, supervision, and writing – review and editing. Ikhane O. Albert, Neo Hlungwani, Perfoy Lumu, and Ndivhuwo Kevin Khorommbi – writing – original draft, and writing – review and editing. Thulani Sibanda, Karina Calvopina Tapia, Gurleen Kaur, Nana Kwaku Buabeng, Cynthia A. Danquah, Sekelwa Cosa, George Siegwart, and Lyndy J. McGaw – writing – review and editing. Christopher J. Schofield – conceptualisation, supervision, and writing – review and editing. Vinesh J. Maharaj – conceptualisation, supervision, and writing – review and editing.

7. Conflicts of interest

There are no conflicts of interest to declare.

8. Data availability

No primary research results, software or code are included, and no new data were generated or analysed as part of this review.

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