Published in final edited form as:
J Med Chem. 2010 April 22; 53(8): 3013–3027. doi:10.1021/jm9012938.

Evolving Carbapenemases: Can Medicinal Chemists Advance One Step Ahead Of The Coming Storm? (Perspective)

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Introduction

Carbapenems can be an effective treatment of infections with multidrug-resistant Gram-negative bacteria such as *Pseudomonas aeruginosa*,[1] *Acinetobacter* spp.,[2] *Klebsiella pneumoniae*,[3] and other Enterobacteriaceae.[4] They are semi-synthetic or synthetic β-lactam compounds that are distinguished from other β-lactam compounds such as penicillins and cephalosporins by the absence of a sulfur atom in the bicyclic core and a different stereochemistry at Cα of the β-lactam ring (in penicillins and carbapenems, this atom is usually referred to as C6; in cephalosporins as C7) (Figure 1). The most popular carbapenem antibiotics are imipenem[5, 6] (Merck, 1985), meropenem[7, 8] (Sumitomo Pharmaceuticals and AstraZeneca, 1996), ertapenem[9, 10] (Merck, 2005), and doripenem[11, 12] (Shionogi Co. and Johnson & Johnson, 2005) (Figure 2). All of these broad-spectrum drugs are used intravenously. Carbapenems are considered to be drugs of last resort due to the fact that they are not inactivated by and effectively inhibit many β-lactamases (most Ambler class A and C β-lactamases[13]), while these enzymes efficiently hydrolyze penicillins and cephalosporins. β-Lactamases hydrolyze the β-lactam ring of β-lactam antibiotics blocking peptidyltransferase (also referred to as penicillin binding protein or PBP) activity that is critical for the peptidoglycan biosynthesis of the bacterial cell wall.[14] β-Lactam antibiotics inhibit peptidyltransferase by forming a stable acyl-enzyme intermediate after an active-site serine of pepditytransferase cleaves the β-lactam ring through a nucleophilic attack.[15] Similar to peptidyltransferase, most β-lactamases contain an active site serine, which exerts a nucleophilic attack on and cleaves the β-lactam ring, resulting in turnover by the enzyme. These enzymes are referred to as serine β-lactamases (SBLs) and, based on sequence and structural homology, have been grouped into classes A, C, and D by Ambler.[13] CTX-M β-lactamases are a group of class A SBLs expressed by Enterobacteriaceae that confer resistance toward the third-generation cephalosporin cefotaxime (Figure 3).[16] As a consequence, carbapenems are frequently used to treat infections with Enterobacteriaceae expressing these enzymes. The increased use of carbapenems drives the emergence of carbapenem resistance mechanisms.

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Supporting Information Available: Additional Information on the Epidemiology of Clinically Important Carbapenemases. This material is available free of charge via the Internet at http://pubs.acs.org.

An increasing number of recent reports indicate that some β-lactamases can efficiently hydrolyze carbapenems. This alarming situation is made worse by the lack of new antibiotics at or near clinic that are active against resistant Gram-negative organisms, particularly nonfermenters such as *Pseudomonas aeruginosa and Acinetobacter baumannii*. Among SBLs, the most notable carbapenemases are variants of the OXA group (class D) and *Klebsiella pneumoniae* carbapenemases (KPCs, class A). Metallo-β-lactamases (MBLs), a separate class of enzymes (Ambler class B[13]), employ a water/hydroxide ion nucleophile activated by coordination to one or two Zn(II) ions and recognize a broad spectrum of β-lactams, including carbapenems. This perspective focuses on the MBLs of the IMP type (IMPs efficiently hydrolyze imipenem) and the VIM type (VIM represents Verona integron-borne metallo-β-lactamase), since these enzymes seem to be the clinically most important MBLs.[17]

We will (1) give a summary of these four important groups of carbapenemases, OXA, KPC, IMP, and VIM, including their epidemiology, structure, mechanism, and substrate specificity, (2) summarize approaches that have been undertaken to develop MBL inhibitors to reverse antibiotic resistance (potent SBL inhibitors such as clavulanic acid[18] are already in clinical use), and (3) propose a novel approach to efficiently screen for such drugs using the *Shape Signatures* algorithm.

Clinically Important Carbapenemases

The carbapenemases of the OXA, KPC, IMP, and VIM types are clinically important enzymes. They are all encoded on mobile genetic elements, located on plasmids or chromosomes, and are frequently isolated from patients suffering from antibiotic resistant infections.

OXA β-Lactamases

OXA β-lactamases are classified by a preference for the β-lactam antibiotic oxacillin (Figure 3). These enzymes are class D SBLs of about 28 kDa molecular weight[19] and exhibit an α/β protein fold. Several distinct lineages within the very divergent OXA group of enzymes have acquired the ability to hydrolyze carbapenems. Although relatively weak toward most carbapenem substrates compared to the KPC, IMP, and VIM enzymes discussed below, the activity of these enzymes is sufficient to confer carbapenem resistance. OXA carbapenemases are frequently found in *Acinetobacter* spp., in particular, in *Acinetobacter baumannii*. These Gram-negative bacteria colonize and infect patients, especially in intensive care units.[20] The first OXA β-lactamase variant with carbapenemase activity, OXA-23, was isolated from a patient in Edinburgh, UK, in 1985.[21] It was originally called ARI-1 and later renamed OXA-23 due to its sequence similarity to other OXA enzymes.[22] Today, this enzyme has been isolated in other European countries, but also in Africa, Asia, South America, and North America (See Supporting Information S2 for more details). More recently, the emergence of OXA-48 in Enterobacteriaceae has given rise to concern.[23] The OXA genes are very diverse; some are encoded on plasmids and some are chromosomal. Based on phylogenetic analysis, Barlow and Hall have suggested that they have been transferred from chromosomes to plasmids several times millions of years ago.[24]

To date, 162 variants of OXA β-lactamases have been documented, including a few redundant assignments.[25] Several of these also possess the ability to inactivate carbapenems and have been found world wide (See Supporting Information S2 for more details). An extensive review of this group including phylogenetic trees and activity profiles has been published by Walther-Rasmussen and Hoiby.[26] At the time of publication of that review (2006), 121 different OXA enzymes had been reported, of which 45 were known to have carbapenemase activity.

OXA carbapenemases confer only moderate resistance levels to pathogenic bacteria and their action is often complemented by other resistance mechanisms such as porin deficiencies and

efflux pump overexpression.[26] Other recent reviews on carbapenemases also include more detail on OXA β-lactamases.[27, 28] Overall, the diversity, global presence, and the fact that transfer to plasmids has probably occurred long ago suggest that the OXA enzymes are an evolutionarily established group of SBLs.

Klebsiella Pneumoniae Carbapenemases (KPCs)

While there are several class A SBLs with carbapenemase activity, *Klebsiella pneumoniae* carbapenemases (KPCs) are by far the most important in the clinic. These are enzymes of about 28.5 kDa molecular weight (calculated[29] for the mature proteins missing the N-terminal 24 residues) that also exhibit an α/β protein fold. Although the name suggests that they are specific to *Klebsiella pneumoniae* and foremost carbapenemases, enzymes of this group have also been found in other pathogenic bacteria, such as *Pseudomonas aeruginosa*,[30] *Serratia marcescens*,[31] and *Enterobacter* spp.,[32] and they can also inactivate cephalosporins such as cefotaxime (Figure 3).[27] The first KPC (originally named KPC-1) was found in a clinical isolate of *Klebsiella pneumoniae* in North Carolina in 1996.[33] Currently, nine KPC variants have been reported[25] and isolated world wide, most frequently in the United States and Israel (Figure 4 and Supporting Information S2-S3). The sequences of KPC-1 and KPC-2 (a point mutant of KPC-1) have been found to be identical after resequencing,[34] and we will refer to this enzyme as KPC-2. The other eight variants are labeled KPC-3 through KPC-10. All known KPCs deviate from KPC-2 by only up to a few amino acid substitutions (Figure 5), suggesting that they may be direct descendents of KPC-2 (See Supporting Information S2-S3 for more details).

In a review article published in 2007, Walther-Rasmussen and Hoiby included a section on KPC enzymes; at that time only four KPC variants were known.[35] The fact that KPC enzymes have spread and evolved to this degree in only thirteen years is alarming.

Metallo-β-Lactamases (MBLs)

β-Lactamases that employ one or two active site Zn(II) ions to catalyze the cleavage of β-lactams belong to the class B or metallo-β-lactamases (MBLs),[13] enzymes of about 25 kDa molecular weight. They all exhibit an αββα fold,[36] which includes a compact core of two β-sheets sandwiched by α-helices on either side and is now known as the metallo-β-lactamase fold.[37] The active site containing one or two Zn(II) ions is located at the edge of the two β sheets.[36] MBLs have been further divided into three subgroups, B1, B2, and B3, based on sequence, structure, and activity similarities.[38, 39] MBLs from subclass B1 and B3 possess a binuclear active site, which requires one or two Zn(II) ions for full activity. The Zn1 site is formed by three histidine residues, whereas the Zn2 site is formed by aspartic acid, cysteine/histidine, and a histidine residue. Subclass B2 enzymes are catalytically active with one Zn(II) ion binding to the Zn2 site. Enzymes of subclass B2 selectively hydrolyze carbapenems, whereas enzymes of subclasses B1 and B3 also hydrolyze penicillins and cephalosporins.[40] MBLs cannot inactivate aztreonam (Figure 3). MBLs are a relatively novel class of enzymes and new types and variants of known types are isolated from the environment and clinical isolates at an alarming pace. We will focus here on the clinically important IMP and VIM enzymes, which belong to subclass B1. The Zn1 binding site of B1 enzymes consists of His116, His118, and His196; the Zn2 binding site consists of Asp120, Cys221, and His263 (we use the standard numbering scheme for MBLs[38, 39] throughout this article). Mutations at position 120 have shown that the Zn2 binding site and with it the Zn1-Zn2 distance are quite flexible.[41]

IMP β-Lactamases

Together with other MBLs such as BcII (*Bacillus cereus* β-lactamase II),[42, 43] CcrA from *Bacteroides fragilis*,[44, 45] BlaB from *Chryseobacterium meningosepticum*,[46] VIM enzymes (see below), and SPM-1 (Sao Paulo metallo-β-lactamase 1) found in *Pseudomonas aeruginosa*,[47, 48] IMP enzymes form subclass B1. IMP-1 was originally isolated in 1991 from

a patient with a *Serratia marcescens* infection in Japan and characterized as a protein of 246 amino acids in length and molecular weight of about 30 kDa (25.1 kDa as determined by electrospray ionization mass spectrometry[49] and calculation[29]), encoded by the chromosomal bla_{IMP} gene of 39.4% GC content.[50] This GC content is much lower than the usual GC content of *Serratia marcescens* genes (56.2 to 58.4%)[51] and an extraneous origin of the bla_{IMP} gene has been suggested.[50] IMP-1 has also been isolated from *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Pseudomonas putida*, *Alcaligenes xylosoxidans*,[52] *Acinetobacter junii*,[53] *Providencia rettgeri*,[54] *Acinetobacter baumannii*,[55] and *Enterobacter aerogenes*,[56] usually on a mobile gene cassette inserted into an integron, either on the chromosome or on a plasmid.[57] After being isolated first in Japan,[50, 52] IMP-1 was isolated in several other Asian countries, in Europe, and in South America (See Supporting Information S3-S5 for more details). The global spread of IMP-1 and other IMP enzymes is depicted in Figure 6.

Crystal structures of IMP-1[41, 58-61] and the two D120A and D120E mutants[41] have been reported. They confirm the general structure of MBLs described above. Besides the Zn(II)-ligating residues, a characteristic residue present in all IMP enzymes and in most subclass B1 enzymes (not VIM enzymes) is Lys224, which interacts with the carboxylate group of a co-crystallized mercaptocarboxylate inhibitor[58] and supposedly the carboxylate at C3/C4 of penicillins and carbapenems/cephalosporins. A mechanism has been proposed by Wang *et al.* for nitrocefin hydrolysis by CcrA,[62] the active site of which is virtually identical to that of IMP-1[58, 63] and which shares 35.9% sequence identity with IMP-1.[50] According to this mechanism, the Zn(II) ions are coordinated by a bridging water/hydroxide molecule that performs a nucleophilic attack on the carbonyl carbon in the β-lactam ring of the substrate, resulting in cleavage of the amide bond and deactivation of the antibiotic. Relevant for inhibitor design, this mechanism differs from the one catalyzed by SBLs in that it lacks a covalent enzyme-substrate intermediate.[64]

To date, twenty six IMP variants have been isolated in countries across the globe, representing every continent except Africa and Antarctica (Figure 6). The phylogenetic relationship between these enzymes is shown in Figure 7. In the following, we will point out a few landmark enzymes; for a detailed account of all variants, refer to Supporting Information S3-S5.

IMP-2 was first isolated from *Acinetobacter baumannii* in Italy in 1997.[65] Its relatively low amino acid sequence identity (85%) to IMP-1, the difference between the gene cassettes carrying the IMP-1 and IMP-2 encoding genes, and the different geographic origins suggest different phylogenetic origins of IMP-1 and IMP-2.[65] The substrate spectrum of IMP-2 was overall similar to that of IMP-1 (penicillins, cephalosporins, and carbapenems, but not aztreonam), however, with significantly lower catalytic efficiencies toward ampcillin and cephaloridine and significantly higher catalytic efficiencies toward carbenicillin and meropenem[65] relative to IMP-1.[57] The phylogenetic tree in Figure 7 shows that IMP-1 and IMP-2 belong to two distinct groups of closely related enzymes (discussed in detail in the Supporting Information S3-S4).

IMP-4 was the first MBL to be discovered on the continent of Australia.[66] The first IMP enzyme to be discovered in the Americas was IMP-7, which was isolated from *Pseudomonas aeruginosa* in Canada in 1995 and 1996.[67] IMP-15 and IMP-18 are variants that were isolated in the United States. IMP-15 was recently isolated from *Pseudomonas aeruginosa* in Kentucky, USA, and it was likely imported from Mexico, where the patient was injured and initially treated.[68] IMP-18 was isolated from *Pseudomonas aeruginosa* in the United States,[69] Mexico,[70] and Puerto Rico.[71]

In summary, the group of IMP enzymes exhibits great diversity and an almost explosive discovery of new variants all around the globe. This group of enzymes is certainly one of the

most concerning threats to state-of-the-art antimicrobial chemotherapy. Some attempts to predict novel IMP variants that might evolve in the future have been undertaken. For instance, point mutants of IMP-1 harboring a N233A[72] and a F218Y[73] mutation, respectively, were found to exhibit enhanced catalytic efficiencies compared to IMP-1.

VIM β-Lactamases

VIM β-lactamases are another group of MBLs of subclass B1[39] of about 25 kDa molecular weight. The first enzyme of this group reported was isolated from *Pseudomonas aeruginosa* found in a patient at the Verona University Hospital in Northern Italy in 1997.[74] It was found that the gene encoding this enzyme was borne on an integron, which gave rise to its name Verona integron-borne metallo-β-lactamase 1 or VIM-1.[74] Apart from *Pseudomonas aeruginosa* in Italy, VIM-1 has been isolated from other organisms in other European countries and in Turkey, but to our knowledge not outside these countries (See Supporting Infromation S6 for more details). The global spread of VIM-1 and other VIM enzymes is depicted in Figure 8.

Twenty three VIM variants have been reported to date.[25] Their phylogenetic relationships are shown in Figure 9. VIM-2, the second VIM enzyme to be reported, was actually isolated prior to VIM-1 in 1996 in France, also from *Pseudomonas aeruginosa.*[75] Its amino acid sequence is 90% identical to that of VIM-1 and it also hydrolyzes all tested β-lactam antibiotics except the monobactam aztreonam.[75] Its activity toward cephalosporins is similar to and that toward penicillins and carbapenems higher than that of VIM-1.[76] Geographically, VIM-2 is more widely spread than VIM-1. Interestingly, and similar to the IMP enzymes, VIM-1 and VIM-2 also belong to clusters of closely related enzymes (Figure 9). VIM-2 is the only enzyme of the VIM group that has been crystallized, in complex with a mercaptocarboxylate inhibitor[77] and as the free enzyme both with an oxidized and reduced Cys221.[78] VIM-7 is the first VIM enzyme to be isolated in the United States. Its gene was found on an integron in a *Pseudomonas aeruginosa* isolate in 2001.[79] VIM-2 was not isolated there until 2003.[80] VIM-7 has less sequence identity (77%) to VIM-1 than any of the other variants up to VIM-6 (89 to 99%). It has therefore been suggested that VIM-7 has arisen independently in the United States rather than having been imported from Europe or Asia.[79] VIM-2, on the other hand, may have been disseminated to the United States from Jordan.[81] VIM-7 effectively hydrolyzes all tested β-lactam antibiotics except aztreonam.[76] Its activity toward cephalosporins is lower and that toward penicillins higher than that of VIM-2.[76] Its activity toward the two carbapenems imipenem and meropenem is similar to, but that toward ertapenem significantly higher than that of VIM-2.[76] It has been suggested that some of the changes in catalytic efficiencies may be due to a Y218F mutation.[76] Details on the occurence and relationship of all VIM variants can be found in the Supporting Information S6-S8.

As for the other carbapenemase groups described above, the occurrence of VIMs in different organisms, their global spread and the continued discovery of new variants in just a little more than a decade demonstrates their rapid dissemination and evolution. Figure 8 indicates that the VIM enzymes are so far the most globally spread MBLs.

Recent Approaches to Finding MBL Inhibitors

The structural complexity and heterogeneity of MBLs have posed challenges to finding a general inhibitor to treat infections caused by MBL-producing strains. A number of structures have been determined by X-ray crystallography for enzymes from all three subclasses B1-B3, summarized in Table 1, which provide important insights for understanding the catalytic mechanism, substrate selectivity and for guiding rational (computer-aided) inhibitor design.

A superposition of crystal structures of MBLs from all three subclasses is shown in Figure 10. Selected enzymes include VIM-2 (subclass B1, blue, PDB code 2YZ3[77]), IMP-1 (subclass B1, cyan, PDB code 1DD6[58]), CphA (subclass B2, orange, PDB code 2QDS[82]), and L1 (subclass B3, red, PDB code 2AIO[83]). This representation reveals that in addition to the metal binding sites, the MBL active site is characterized by two hypervariable and flexible loops. The first loop (loop1) connects β-strands 3 and 4, while the second loop (loop2) is positioned opposite to loop1 connecting β-strand 11 and alpha-helix 4. The X-ray crystal structures of MBLs co-complexed with hydrolyzed substrate/inhibitors depict extensive interactions with these loops, suggesting that they may play a key role in substrate recognition, binding, and catalysis. It is logical to infer that these loops would also bear equal significance in the binding affinity and specificity of small-molecule inhibitors. Interestingly, the amino acid side chains of Trp64, Val67, and Lys224 play key roles in enzyme-inhibitor interactions in co-crystals of the MBL IMP-1 and a succinic acid.[59] The residues Phe61 and Arg228 in VIM-2, Val67 and Lys224 in CphA, and Leu68 and Ser223 (Leu69 and Ser225 according to Garau *et al.*[39]) in L1 are anticipated to serve as important contact points to guide medicinal chemistry efforts toward the design of pan MBL inhibitors.

A wide array of computational methods such as molecular dynamics simulations, quantum mechanics calculations, and virtual ligand-receptor screening (“docking and scoring”) have shown utility in discerning of the catalytic mechanism of MBLs and modeling the structural and dynamic effects of substrate/inhibitor binding at the atomic level. Biologically relevant phenomena that are not easily observed experimentally, including the protonation state of the zinc-bound water/hydroxide or ligand, the active site hydrogen bonding network, the Zn1-Zn2 distance, and the catalytic reaction pathway, have been the focus of molecular modeling studies on MBLs with either one or two active-site Zn(II) ions.[84-93] For some binuclear B1 and B3 MBLs, the second Zn(II) ion in the Zn2 binding site serves to stabilize the build up of negative charge on the anionic N atom during hydrolysis of the peptide bond, thereby decreasing the activation free energy for nucleophilic attack. As a result, the overall catalytic efficiency of the binuclear enzyme is enhanced over the mononuclear variant. Results from these simulations suggested that separate catalytic mechanisms might apply to MBLs depending on differences in Zn(II) utilization, which is in agreement with experimental reports.[62]

There is growing concern about the rapid emergence of MBL expressing strains that exhibit a pan-resistant phenotype, particularly in the event that MBLs evolve into more efficient enzymes through mutation. Complementing experimental techniques, molecular modeling approaches have been employed to predict MBL evolution in order to effectively tackle MBL-conferred resistance to antibiotics.[94, 95] Using rankings derived from molecular dynamics simulations, the variations in observed catalytic efficiencies of two wild-type enzymes (IMP-1 and IMP-6) to four cephalosporins were successfully reproduced.[96] Later, the same approach was applied to computationally predict novel MBL point mutants that may cause problems if they evolve naturally. Five predicted variants from this study were characterized through *in vitro* experiments.[49] As predicted, a variant of IMP-6 with only a single-site mutation converted the tested antibiotics more efficiently than IMP-6. This case represents one example by which computational approaches have advanced our understanding of crucial aspects of MBL pharmacology.

To address the emerging threat to public health posed by MBL-conferred resistance to antibiotics, new and more effective MBL inhibitors are urgently needed. Several classes of MBL inhibitors have been reported in the literature, including biphenyl tetrazoles,[97] trifluoromethyl alcohol, ketone derivatives of L- and D-alanine,[98] thiols,[82, 99-102] thioester derivatives,[99, 103, 104] hydroxamates,[105, 106] cysteinyl peptides,[107] mercaptocarboxylates,[58, 99, 100, 108] sulfonylhydrazones,[109] 2,3-disubstituted succinic acids,[59] phthalic acid derivatives,[110] pyridine dicarboxylates,[111] and tricyclic natural compounds.[112] Representative compounds

from each class are shown in Figure 11. Most of these compounds exhibit appreciable inhibitory activity only on a limited number of MBLs; consequently, they are unsuitable for treating infections caused by pan-resistant bacterial strains. To our knowledge, there are no MBL inhibitors currently in late-stage clinical development. In the private sector, this drought in the antibiotic pipeline is largely driven by economic pressures. Antibiotics are considered financially unattractive. They are generally used for short periods of time and for a specific indication, and they are subject to antibiotic resistance due to overuse. Encouragingly, the situation is beginning to improve. Thanks largely to efforts by organizations such as the Bill & Melinda Gates Foundation, public-private partnerships, and the NIH, today major funding initiatives are directed toward antibiotic drug development. Clearly, sustained awareness and adequate financial resources will be essential to mount an appropriate response.

Studies on the X-ray crystal structures of inhibitor-MBL co-complexes are providing critical information on the binding modes of the inhibitors and their specific interactions with the active-site residues. The collection of those MBL inhibitors is summarized in Table 2 and they bind in a similar fashion within the MBL active site. In general, inhibitors of MBLs feature two functional groups: hydrophobic moieties which bind in a predominantly hydrophobic pocket around loop1, and a metal-ligating group that interacts with the Zn(II) ion(s). In some cases, interactions between inhibitors and MBLs are stabilized by hydrogen bonds or electrostatic interactions with conserved residues such as Lys224 in loop2. Insights gleaned from exploring the X-ray crystal structures of MBLs from the three subclasses with various inhibitors present opportunities to formulate new strategies for the rational design of potent, broad-spectrum inhibitors of MBLs.

Combining X-ray crystallographic and molecular modeling studies, Lienard and coworkers recently showed that several thiols provide broad-spectrum inhibition of MBLs with values of the inhibition constant in the sub-micromolar range against all three MBL subclasses.[82] These represent the first published MBL inhibitors that may serve as templates for further development toward clinically useful drugs with inhibitory activities against MBLs spanning all three subclasses. Interestingly, their analysis revealed that the same compound may adopt differential binding modes when interacting with MBLs from different subclasses. For example, Compound 2 utilized a thiol to coordinate the Zn(II) ion in IMP-1 from subclass B1 and the carboxylate group to form strong electrostatic interactions with the conserved Lys224 residue. On the contrary, in CphA (subclass B2), the thiol is hydrogen bonded with the side chains of neighboring residues while the carboxylate group is now interacting with the Zn(II) ion. These findings, together with structural data on new thiol inhibitors, provide an excellent basis for the design and development of more potent MBL inhibitors with broad-spectrum activities.

Virtual screening (VS) has been an effective computational approach that has become the mainstay in early-stage drug discovery at major pharmaceutical companies. The process involves the rapid *in silico* assessment of binding potentials for large libraries of chemical structures to biological macromolecular targets of interest, typically a protein receptor or enzyme. VS of ligand-receptor pairs requires three basic components: databases of chemical structures for screening; the structure of the target protein; and a computational algorithm to explore and assess (i.e., score) the binding mode(s) of the ligand within the protein's binding pocket. Commonly termed ligand-receptor “docking and scoring” by specialists in the field, VS has been widely adopted and adapted for multiple purposes including for prediction of ligand-protein affinities and interatomic interactions and for rapid screening of extensive chemical databases in search of prospective drug leads for many protein targets.[97] Computational modeling of the cationic Zn(II) metal center poses multiple challenges, including considerations of solvation effects, geometric structure, and charge parameterization particularly in the vicinity of metal ions.[113] To account for charge transfer in Zn(II) centers in

a docking study with CcrA, an empirical decrease of the formal charge of +2 of the Zn(II) ion by 0.2 per Zn(II) ligand in combination with a balancing change of charge of the ligands has been employed.[114] To account for distribution of the positive charge of the Zn(II) ion into the electron-deficient sp^3 orbitals, a cationic dummy atom approach[115] has been used in molecular dynamics simulations of IMP-1.[92] A thorough discussion of these issues is beyond the scope of this Perspective.

Olsen *et al.*[116] presented a successful application of docking and scoring to MBLs. In their study, the GOLD docking program provided the best overall performance in terms of low RMSDs between experimental and docked structures and good statistical correlations between the GOLD score and the corresponding experimental inhibitor activities. In a similar study, structure-based VS techniques were deployed against several metalloenzymes including CcrA. This process yielded five inhibitors which were later confirmed experimentally to possess low micromolar activities against this MBL from *Bacteroides fragilis*.[114] The results of these studies maintained that the docking and scoring approach is a reliable method for identification of potential new MBL inhibitors.

Shape Signatures: a Novel Approach to Finding MBL Inhibitors

Our particular interest is focused on finding MBL inhibitor templates that can be developed into broad-spectrum inhibitors for class B β-lactamases. Therefore, an appropriate VS scheme would require screening of the prospective inhibitors against a select array of MBL enzymes that includes at least one MBL from each subclass (e.g. IMP-1 and/or VIM-2 for B1, CphA for B2, and L1 for B3). The size, composition, and structural diversity of chemical databases for screening are important factors that influence the final outcome of a VS study. Recently, a fragment-based screening approach was proposed to prioritize a series of inhibitor fragments for AmpC, a Class A β-lactamase.[117] In this study, the workers observed that fragment-based screening explored a broader region of chemical space than screening of lead-like and drug-like databases. This study highlights the importance of input compound library preparation in the screening process.

To date there are few reports of VS studies targeting the class B β-lactamases (MBLs).[114] We propose a VS scheme, incorporating both traditional and novel computational approaches for the design and filtering of compound libraries, toward discovery of MBL inhibitors exhibiting broad-spectrum inhibitory activities against class B β-lactamases (Figure 12). In the process of designing libraries of MBL-directed compounds, we introduce a novel computational method, *Shape Signatures*, which rapidly compares molecules based on similarity in shape, polarity, and other bio-relevant properties.[118-124] The degree of similarity between a pair of molecules can be assessed by comparing their 1D *Signatures* (shape only) or 2D *Shape Signatures* (shape and surface charge distribution). This process is fast and efficient, and it eliminates tedious and subjective atom-based alignment of the molecules required in many traditional molecular modeling approaches. Unlike traditional quantitative structure-activity relationship (QSAR)-based approaches that require the user to compute and select hundreds of discrete descriptors for each molecule, the *Shape Signature* can be viewed as a very compact descriptor that encodes molecular shape and electrostatics in a single entity. Large *Shape Signature* databases of small-molecule compounds can be screened easily and rapidly using simple metrics to identify small molecules that are similar in shape and polarity, but not necessarily similar in chemical structure. Likewise, the *Shape Signatures* of small molecules will identify complementary protein receptor sites. Consequently, it is well-suited for diverse applications in virtual screening, drug discovery, and predictive toxicology. The *Shape Signatures* method excels at scaffold hopping (crossing chemical families); therefore, it is more likely to discover structurally diverse molecules that may show attractive bioactivity for the three subtypes of MBLs.

Shape Signatures for a small-molecule compound are probability distributions, expressed as histograms, derived from a ray-trace of the volume enclosed by the solvent-accessible surface of the molecule. They are rotationally-invariant descriptors that can be used to rapidly compare molecules in terms of shape similarity, and the method can easily accommodate additional biorelevant properties defined on the molecular surface, such as electrostatic charge. *Shape Signatures* is computationally fast, easy to use in that it avoids specification of complex structural queries or molecular alignment schemes, and can accommodate an almost limitless number of chemical compounds. The method thus lends itself to rapid comparison of large databases of chemical compounds with each other or with a known bioactive molecule of interest (the query compound).

Commonly used computational approaches include ligand-based drug design with pharmacophores, structure-based drug design (drug-receptor docking), QSARs, and quantitative structure-property relationships (QSPRs). Regulatory agencies as well as the pharmaceutical industry are actively involved in development of computational tools that will improve the speed and efficiency of drug discovery and development, decrease the use of animals, increase predictability, and decrease uncertainty.[125]

In the present case, our query compounds are the thiols which have already exhibited broad-spectrum inhibitory activity for the three MBL subclasses.[82] The initial aim is to prepare a balanced chemical database that is structurally diverse yet incorporates our current understanding of the structure-activity relationships of known MBL ligands. The database should reflect the key features and commonalities of existing MBL inhibitors as well as the valuable information presented by the available crystal structures of MBL-inhibitor complexes. This can be achieved by filtering the initial chemical database using MBL-binding features incorporated in separate pharmacophores derived from known inhibitors of each of the three subclasses. Pharmacophores represent the geometric arrangement of the minimal structural elements of ligands (hydrogen bond donors/acceptors, metal ligating moieties, and hydrophobic regions) that are deemed essential for binding to the target protein(s). Compounds matching at least one of the subclass pharmacophores would proceed to the next step for docking and scoring into the structural models of representative B1, B2, and B3 enzymes. The Zn(II) centers could be represented similar to the approaches mentioned above. The “hits” from this VS scheme would be ranked and prioritized based on their binding modes and docking scores, after which a subset is selected for further consideration in the drug discovery process. Adopting this screening process in concert with medicinal chemistry, structural analysis, methods in biochemistry and molecular biology, and pre-clinical evaluation, the authors have embarked on a strategy to discover pan MBL inhibitors that show promise for eventual applications in the clinic.

Concluding Remarks

There is an urgent need for pan MBL small molecule inhibitors that can be used in combination with currently approved antibiotics.[126] Extensive progress has been made in the discovery of specific, potent MBL inhibitors against *individual* enzymes; however, there is a paucity of inhibitors with clinical potential in the treatment of infections caused by a broad spectrum of bacterial pathogens. One major challenge is to find good inhibitors that can permeate through the bacterial envelope. Identifying inhibitors that act on MBLs *and* SBLs is an even bigger challenge due to the different catalytic mechanisms and active site shapes. *Shape Signatures* could assist the design of such inhibitors or at least point to whether such an inhibitor design is feasible or not. Human microflora itself has been recently shown to harbor a reservoir of antibiotic resistance genes,[127] which could be caused by constant exposure to low levels of antibiotics in food and drinking water.[128] This Perspectives article provides new tools for the

medicinal chemist for the design of β-lactamase inhibitors using a combination of virtual screening and molecular epidemiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by awards from Research Corporation for Science Advancement (to P.O. and J.H.T.), Kean University President Farahi (to J.H.T.), the Provost's Teacher-Scholar Program at Cal Poly Pomona (to P.O.), a Howard Hughes Medical Institute Research Apprenticeship (to K.T.D.), and an NIH R21-GM081394 grant from the National Institute of General Medical Sciences (to W.J.W.).

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Abbreviations

SBL	serine β-lactamase
MBL	metallo-β-lactamase
OXA	oxacillinase
KPC	*Klebsiella pneumoniae* carbapenemase
IMP	imipenemase
VIM	Verona integron-borne metallo-β-lactamase
VS	virtual screening
QSAR	quantitative structure-activity relationship
QSPR	quantitative structure-property relationship

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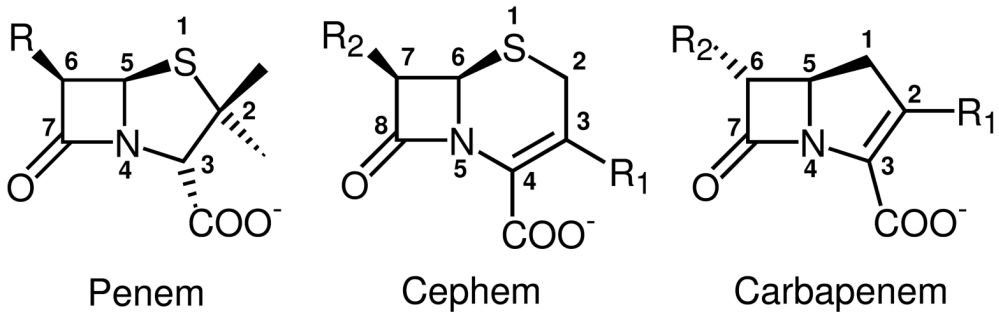


Figure 1.

Chemical structures of the bicyclic cores of different classes of β-lactam antibiotics. The penem core is found in penicillins and consists of a β-lactam ring fused with a tetrahydrodrothiazole ring. The cephem core is found in cephalosporins and consists of a β-lactam ring fused with a dihydrothiazine ring. The carbapenem core consists of a β-lactam ring fused with a dihydropyrrole ring. Heavy atoms of the bicyclic systems are numbered according to common use rather than according to the IUPAC nomenclature to facilitate comparisons between the different antibiotics. Note that the numbering of the R groups is arbitrary; here we started labeling R groups from the ones attached to the core atoms with the lowest number.

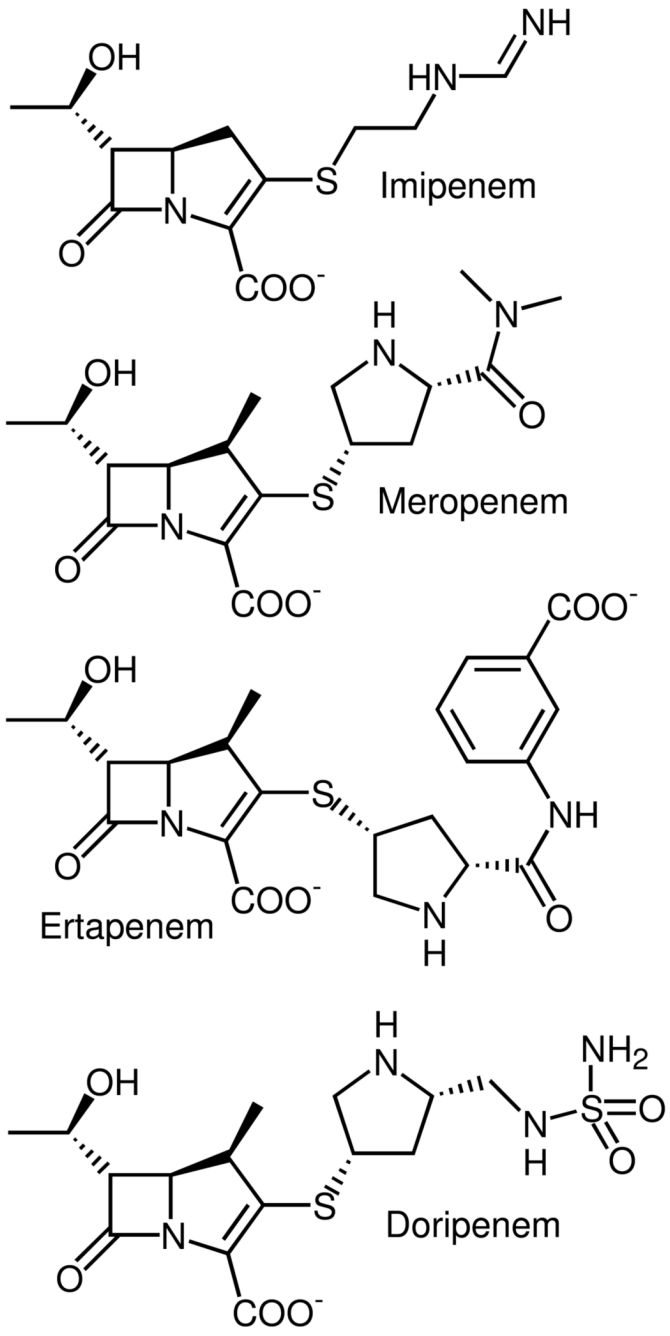


Figure 2.
Chemical structures of four commonly prescribed carbapenems: imipenem ((5*R*,6*S*)-3-[2-(aminomethylideneamino)ethylsulfanyl]-6-(1-hydroxyethyl)-7-oxo-1-azabicyclo[3.2.0] hept-2-ene-2-carboxylic acid), meropenem (3-[5-(dimethylcarbamoyl)pyrrolidin-2-yl] sulfanyl-6- (1-hydroxyethyl)-4-methyl-7-oxo- 1-azabicyclo[3.2.0] hept-2-ene-2-carboxylic acid); ertapenem ((4*R*,5*S*,6*S*)-3-[(3*S*,5*S*)-5-[(3-carboxyphenyl)carbamoyl]pyrrolidin-3-yl] sulfanyl-6-(1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid); and doripenem ((4*R*,5*S*,6*S*)-6-(1-hydroxyethyl)-4-methyl-7-oxo-3-[(3*S*,5*S*)-5-[(sulfamoylamino)methyl]pyrrolidin-3-yl]sulfanyl-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid).

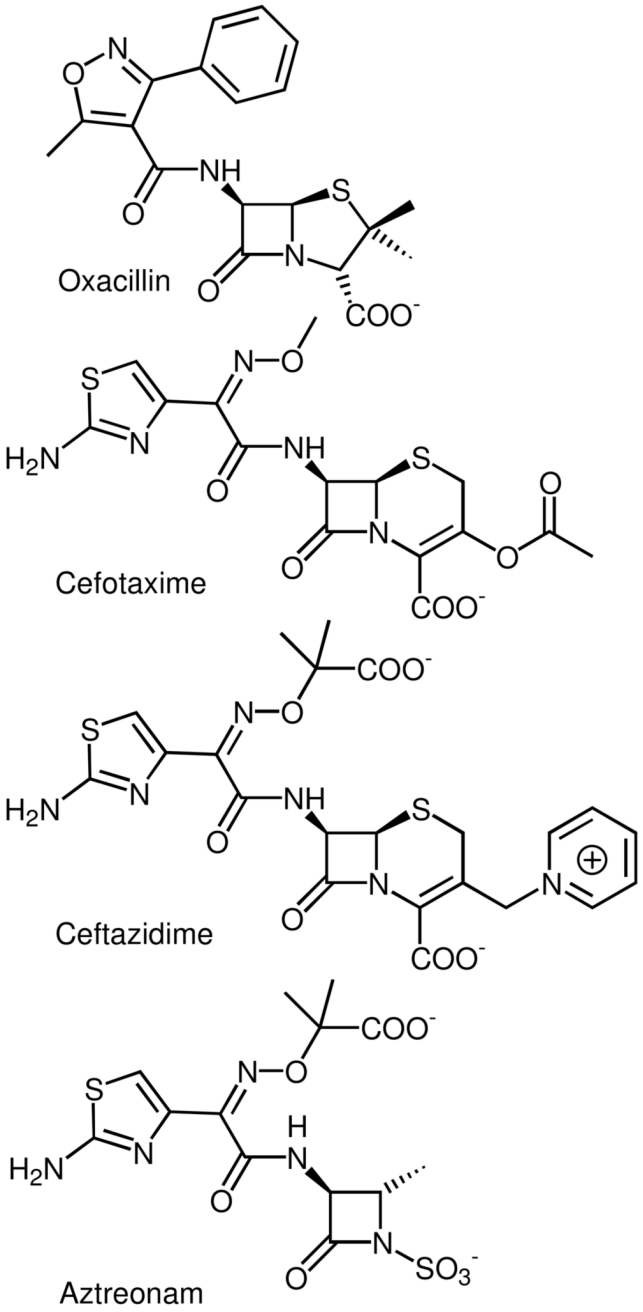


Figure 3.

Chemical structures of selected non-carbapenem β-lactam antibiotics in clinical use: oxacillin ((2*S*,5*R*,6*R*)-3,3-dimethyl-6-[(5-methyl-3-phenyl-1,2-oxazole-4-carbonyl)amino]-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid), a penicillin; cefotaxime ((6*R*,7*R*,*Z*)-3-(acetoxymethyl)-7-(2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid), a third generation cephalosporin; ceftazidime ((6*R*,7*R*,*Z*)-7-(2-(2-aminothiazol-4-yl)-2-(2-carboxypropan-2-yloxyimino)acetamido)-8-oxo-3-(pyridinium-1-ylmethyl)-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylate), another third generation cephalosporin; and aztreonam (2-(([(1*Z*)-1-(2-amino-1,3-thiazol-4-yl)-2-

([(2*S*,3*S*)-2-methyl-4-oxo-1-sulfoazetidin-3-yl]amino)-2-oxoethylidene]amino)oxy)-2-methylpropanoic acid), a monocyclic β-lactam or monobactam.

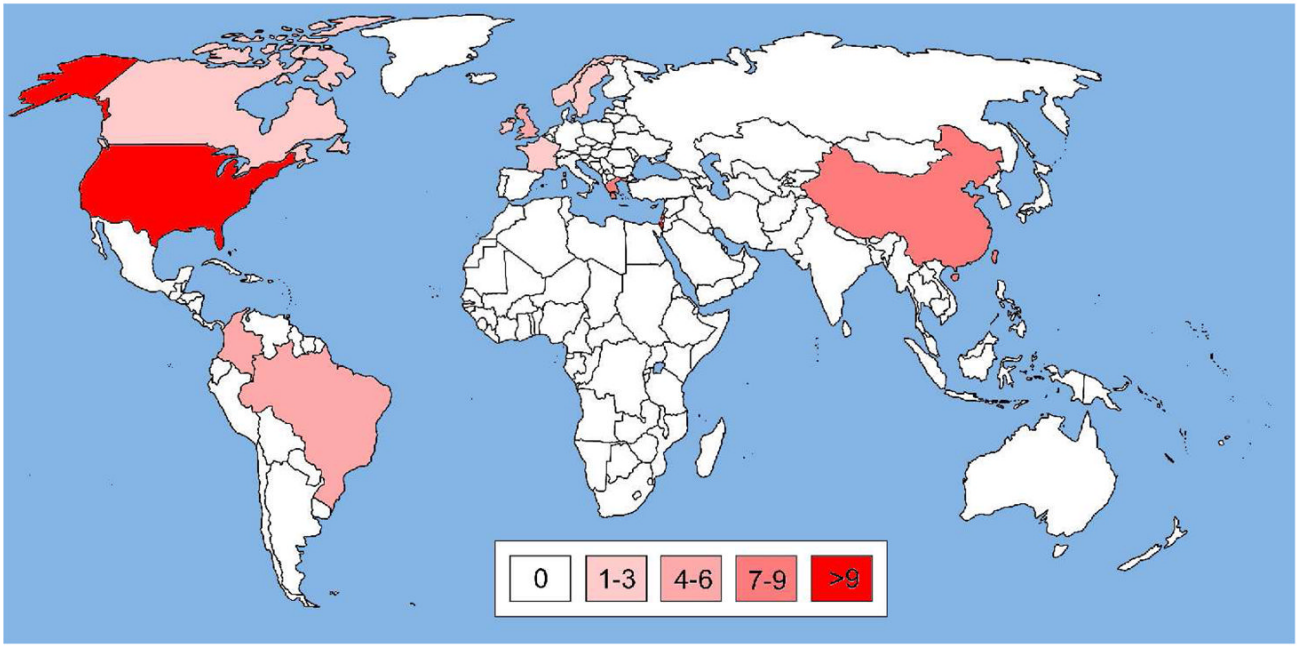


Figure 4.

World map illustrating the global spread of KPC enzymes. A blank world map was obtained from http://upload.wikimedia.org/ and countries with KPC occurences were colored in different opacities of red (symbolizing SBLs) according to the number of publications found on PubMed at http://www.ncbi.nlm.nih.gov/. Publications were retrieved using search strings such as “KPC-* United States” and titles and abstracts were checked for content. Only articles reporting occurences of KPCs were included, while review articles and reports restricted to computational and/or *in vitro* studies were excluded. Countries, for which ten or more publications with KPC reports were found, were colored in red with 100% opacity; those with fewer publications with lower opacities: 7-9 publications, 80%; 4-6 publications, 60%; 1-3 publications, 40%; no publications, white (see color code in the Figure). For more details see Supporting Information S2-S3.

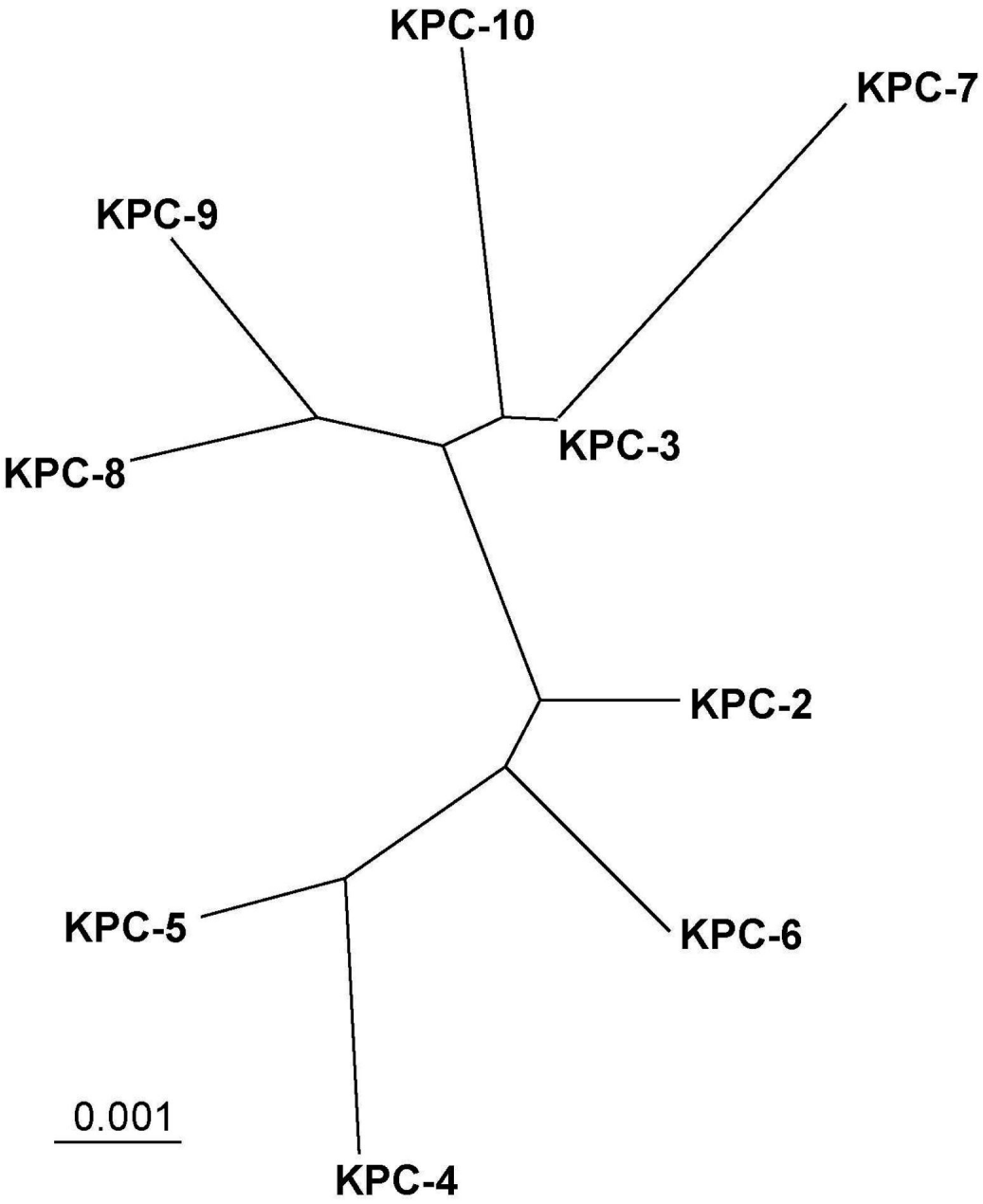


Figure 5.

Radial phylogenetic tree of currently known KPC enzymes. Amino acid sequences of KPC enzymes including the leader sequence were retrieved from GenBank at http://www.ncbi.nlm.nih.gov/and aligned using Clustal X Version 2.0.9[129] using default parameters. The phylogenetic tree was visualized using TreeView.[130] The bar at the lower left corner gives a measure for amino acid sequence diversity. For instance, two enzymes differing by only one of 293 amino acid residues share 99.66% sequence identity and differ by 0.34% (0.0034). The KPC-9 sequence was missing five and four residues at the N- and C-termini, respectively. Since these residues are 100% conserved in the other enzymes, we added the missing residues accordingly. For more details see Supporting Information S2-S3.

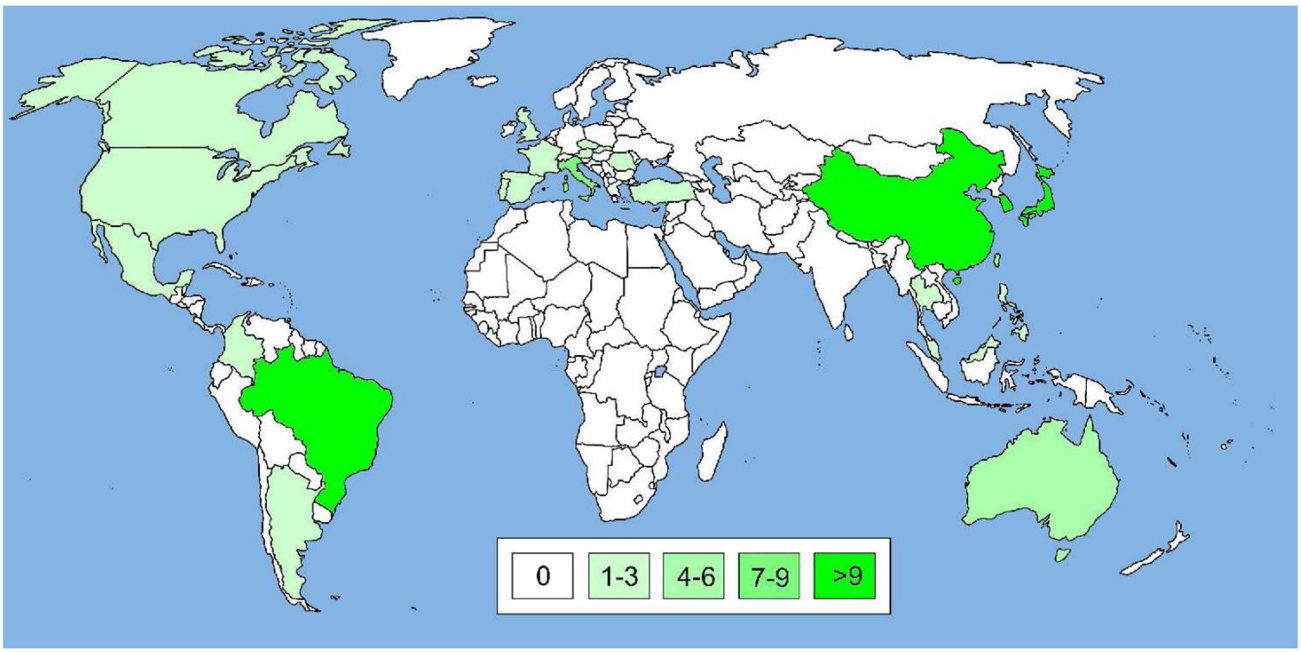


Figure 6.
World map illustrating the global spread of IMP enzymes. The map was prepared as described for Figure 4 except that IMP-specific search strings were used to retrieve articles and that countries with IMP occurence were colored in green, symbolizing MBLs. In some cases, where no published articles were available, GenBank entries were taken into account, e.g., for Thailand. For more details see Supporting Information S3-S5.

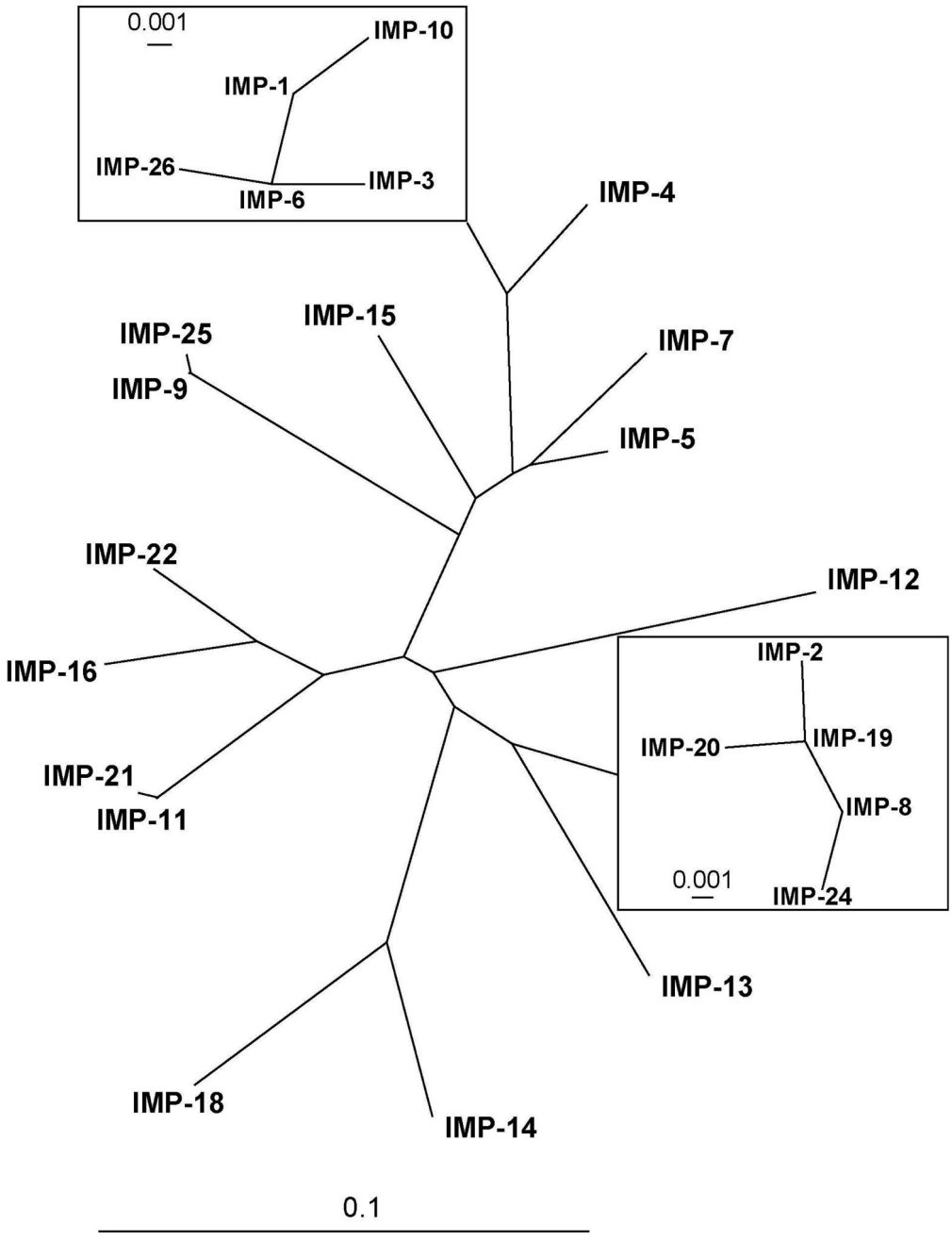


Figure 7.

Radial phylogenetic tree of currently known IMP enzymes, generated as described for Figure 5. Two clusters of closely related enzymes, the “IMP-1 cluster” and “IMP-2 cluster” are shown as insets (note the different scale of the sequence diversity measures). For more details see Supporting Information S3-S5.

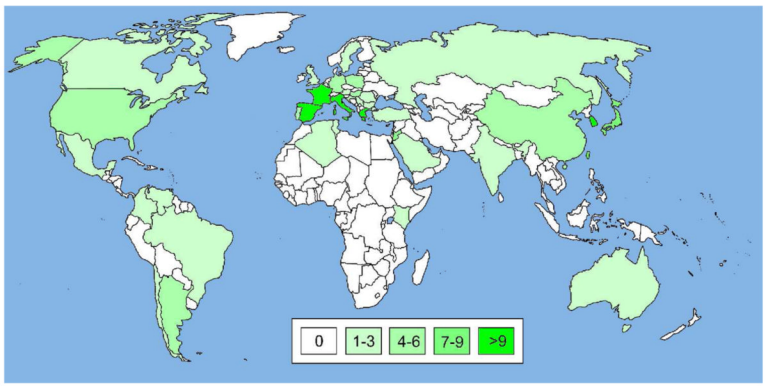


Figure 8.
World map illustrating the global spread of VIM enzymes. The map was prepared as described for Figure 6 except that VIM-specific search strings were used to retrieve articles. For more details see Supporting Information S6-S8.

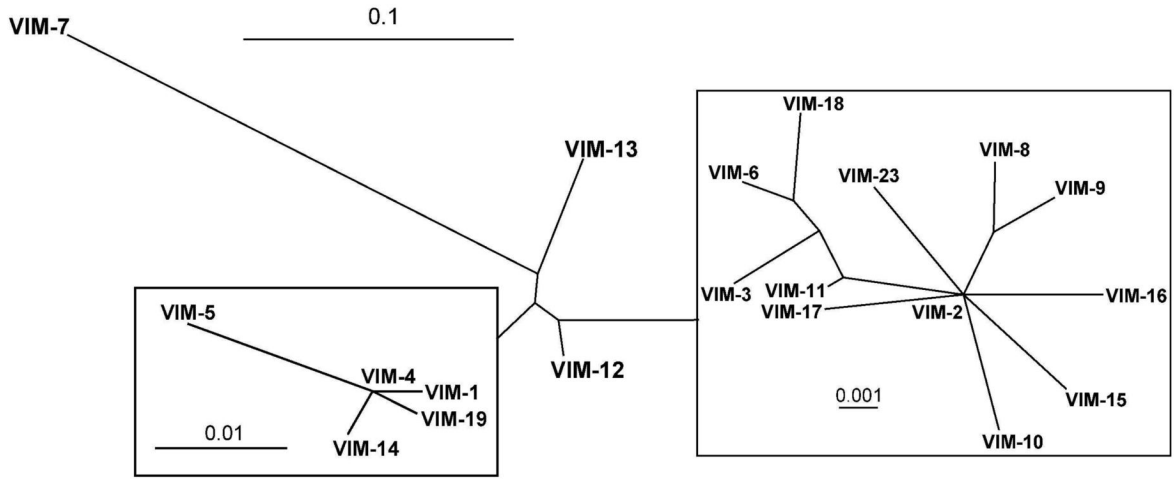


Figure 9.
Radial phylogenetic tree of currently known VIM enzymes, generated as described for Figure 5. For more details see Supporting Information S6-S8.

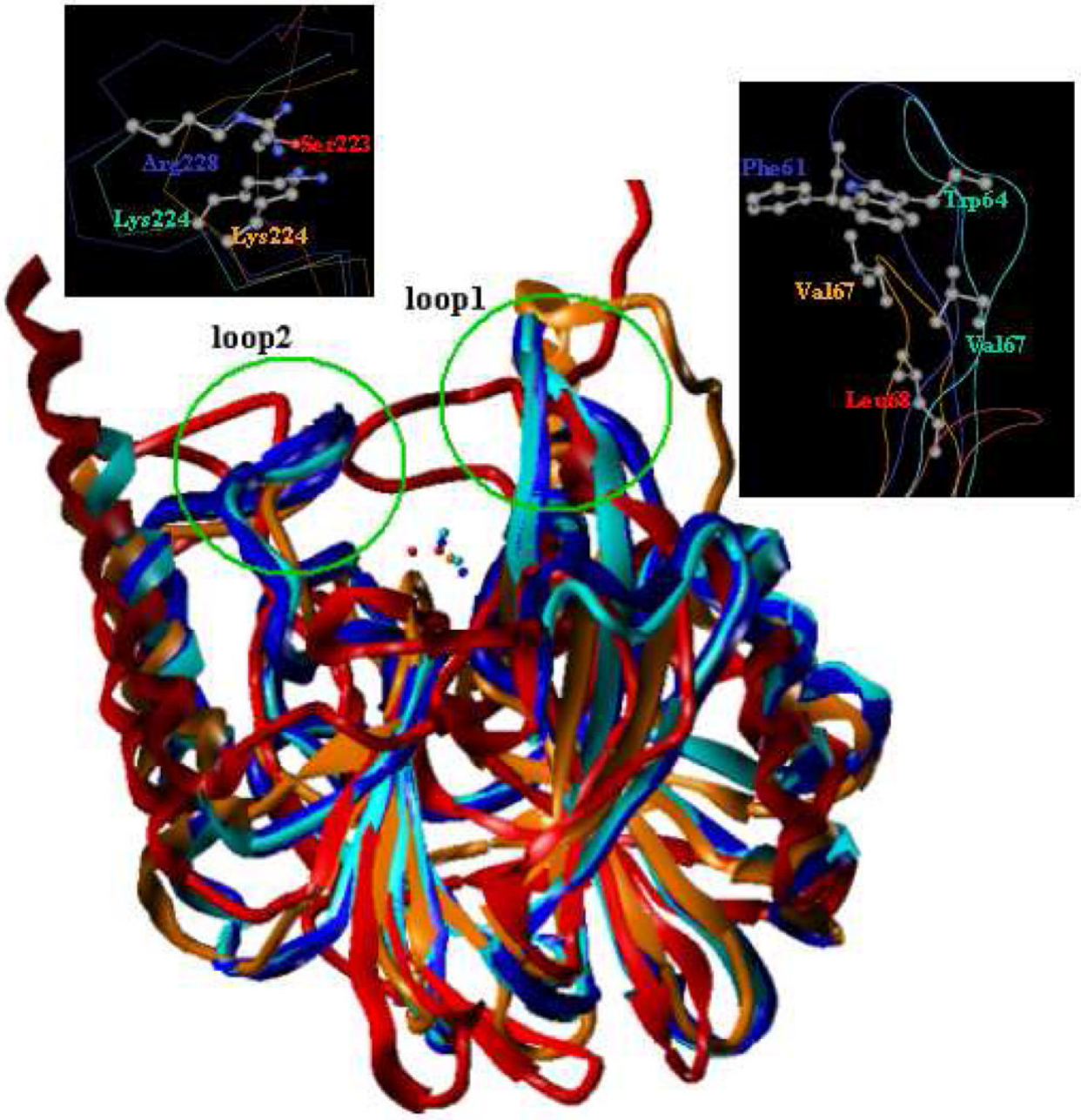


Figure 10.

Superimposition of crystal structures of MBLs from three subclasses. Selected enzymes include VIM-2 (subclass B1, blue, PDB code 2YZ3), IMP-1 (subclass B1, cyan, PDB code, 1DD6), CphA (subclass B2, orange, PDB code 2QDS), and L1 (subclass B3, red, PDB code 2AIO). Two flexible loops are highlighted by green circles. Important residues in the two loops are depicted and colored by atom type (carbon, grey; oxygen, red; nitrogen, blue). Clearly, residues in loop1 are hydrophobic, while those in loop2 possess mostly positively charged side chains.

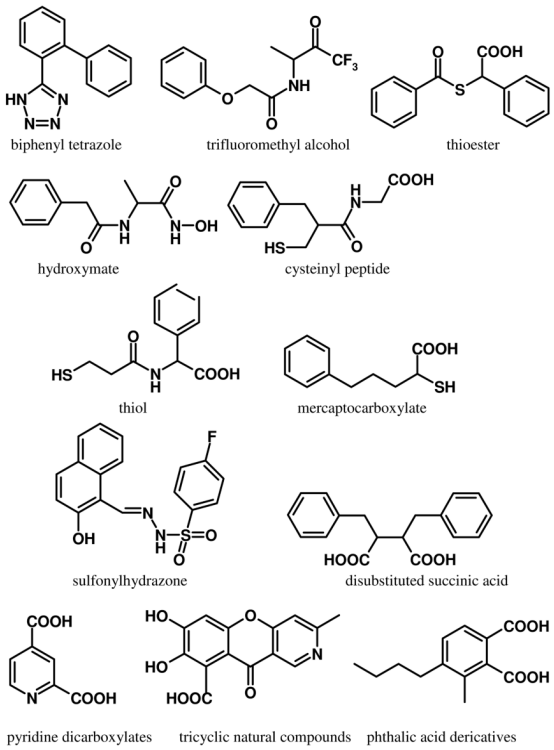


Figure 11.
Chemical structures of selected MBL inhibitors.

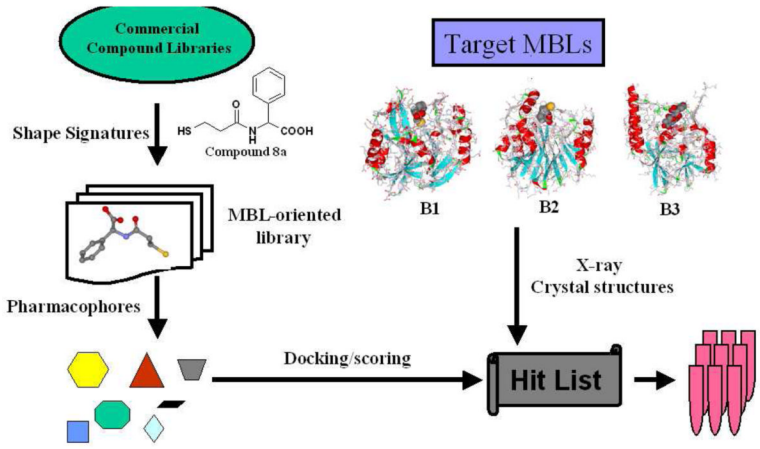


Figure 12.
Proposed VS scheme for the discovery of MBL inhibitors with broad-spectrum activities against B1, B2, and B3 subclasses.

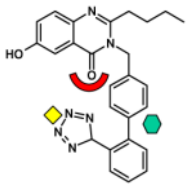
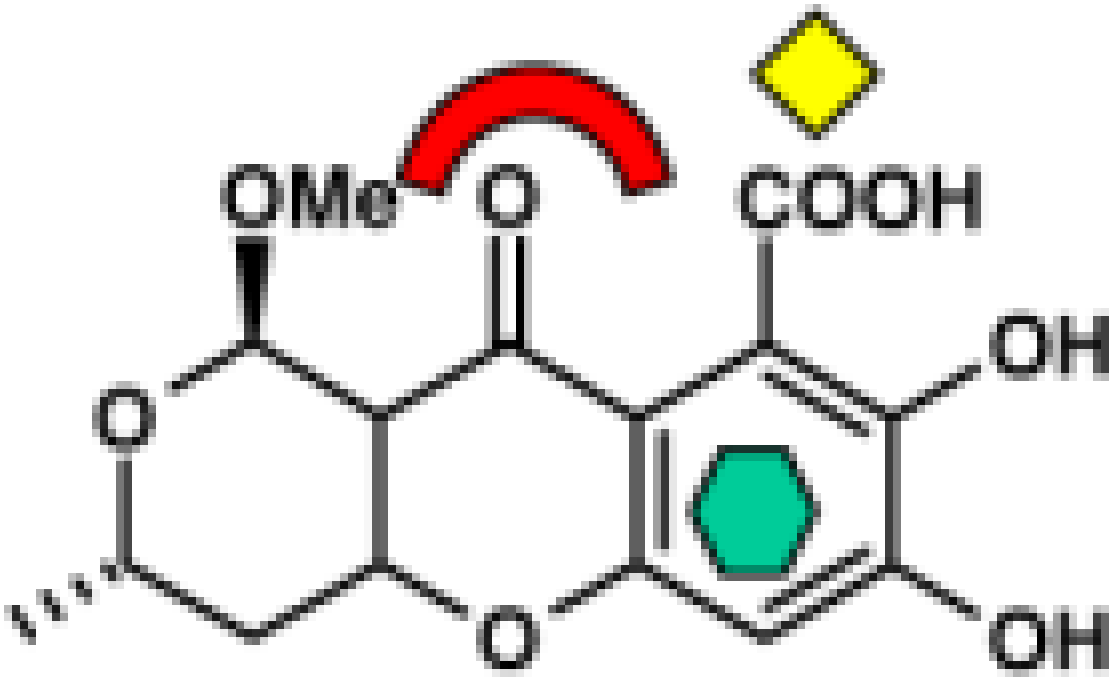
Table 1

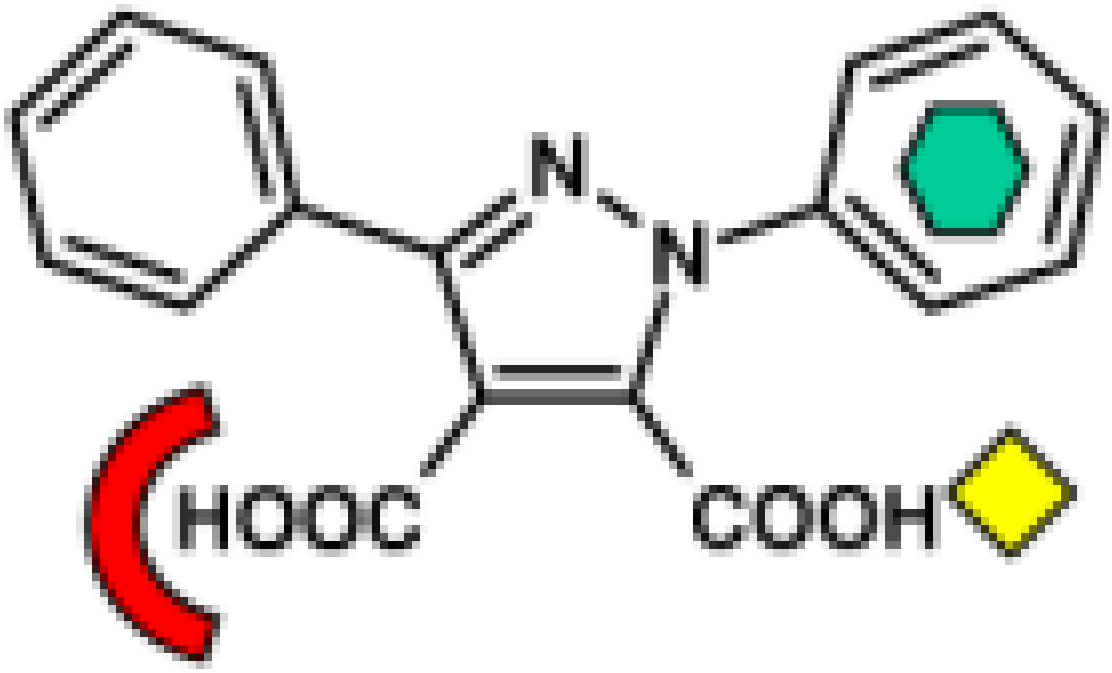
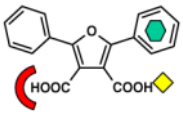
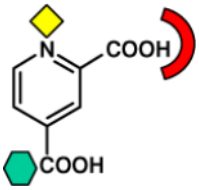
Summary of different MBLs, organisms from which they were isolated, their subclass, metal ions bound in the crystal structure, and the corresponding PDB entry codes.

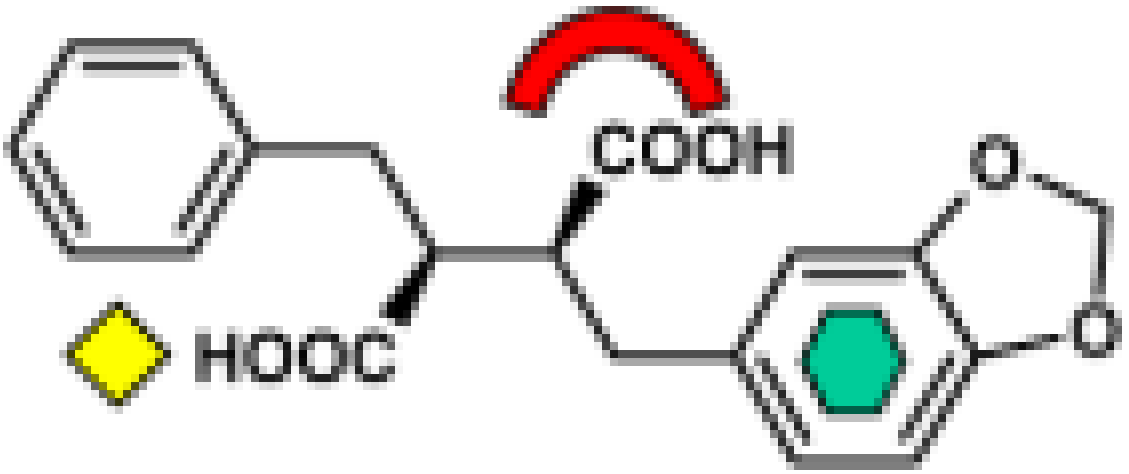
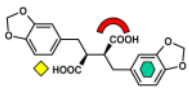
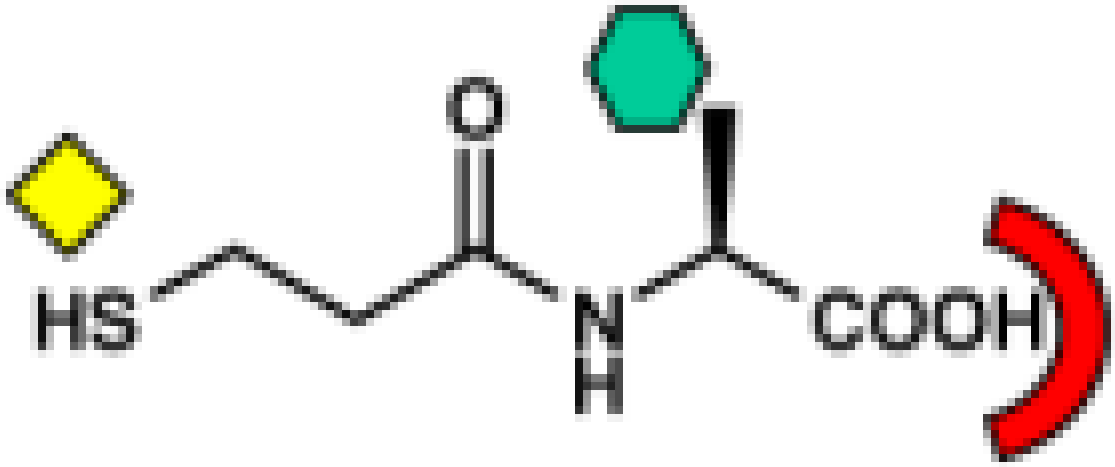
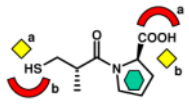
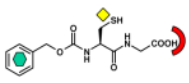
MBL	Organism	Subclass	Metal ions	PDB Entry Codes
BcII	*Bacillus cereus*	B1	2 Zn	2UYX, 2BFK, 2BFL, 2BFZ, 2BG2, 2BG6, 2BG7, 2BG8, 2BGA, 3FCZ, 1BC2, 1BVT
			2 Cd	1MQO
			1 Zn	2NZE, 2NZF, 2NXA, 2NYP, 1DXK, 2BC2, 3BC2, 1BMC
CcrA	*Bacteroides fragilis*	B1	2 Zn	1A8T, 2BMI, 1A7T, 1ZNB, 1KR3,1HLK
			2 Cd	2ZNB
			1 Zn, 1 Hg	3ZNB
IMP-1	*Serratia marcescens*	B1	2 Zn	2DOO, 1VGN, 1WUO, 1WUP
	Pseudomonas aeruginosa			1JJE, 1JJT, 1DDK, 1DD6
BlaB	*Elizabethkingia meningospetica*	B1	2 Zn	1M2X
SPM-1	*Pseudomonas aeruginosa*	B1	1 Zn	2FHX
VIM-2	*Pseudomonas aeruginosa*	B1	2 Zn	2YZ3, 1KO2, 1KO3
CphA	*Aeromonas hydrophila*	B2	2 Zn	3F9O, 3FAI
			1 Zn	1X8G, 1X8H, 1X8I, 2GKL, 2QDS
L1	*Stenotrophomonas maltophilia*	B3	2 Zn	2QDT, 2QDS, 2AIO, 2FU8, 2FU9, 2FM6, 2QIN, 2HB9, 2GFJ, 2GFK, 1SML
			2 Cu	2FU7
			1 Zn	2H6A
				2FU6
FEZ-1	*Fluoribacter gormanii*	B3	2 Zn	1L9Y, 1JT1, 1K07
BJP-1	*Bradyrhizobium japonicum*	B3	2 Zn	2GMN

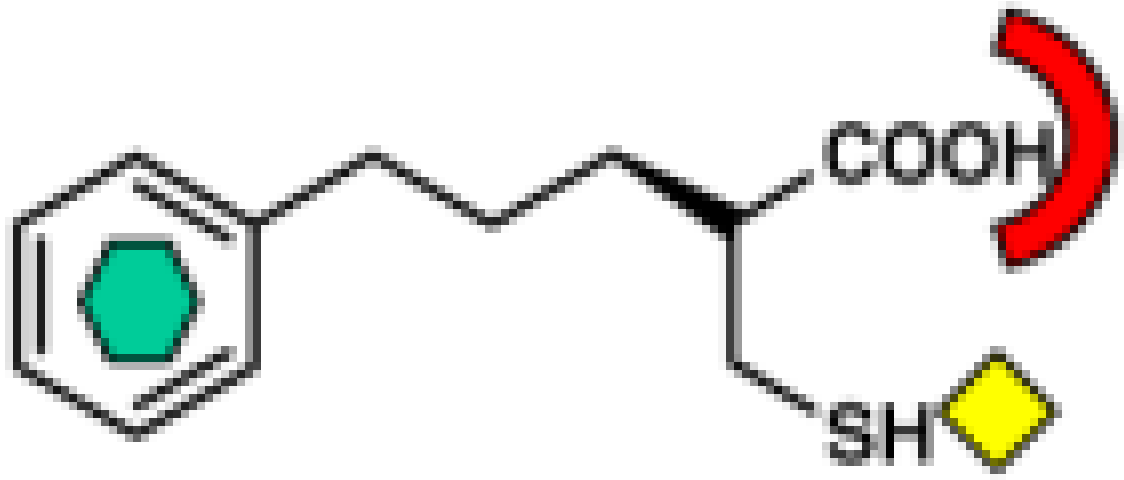
Table 2

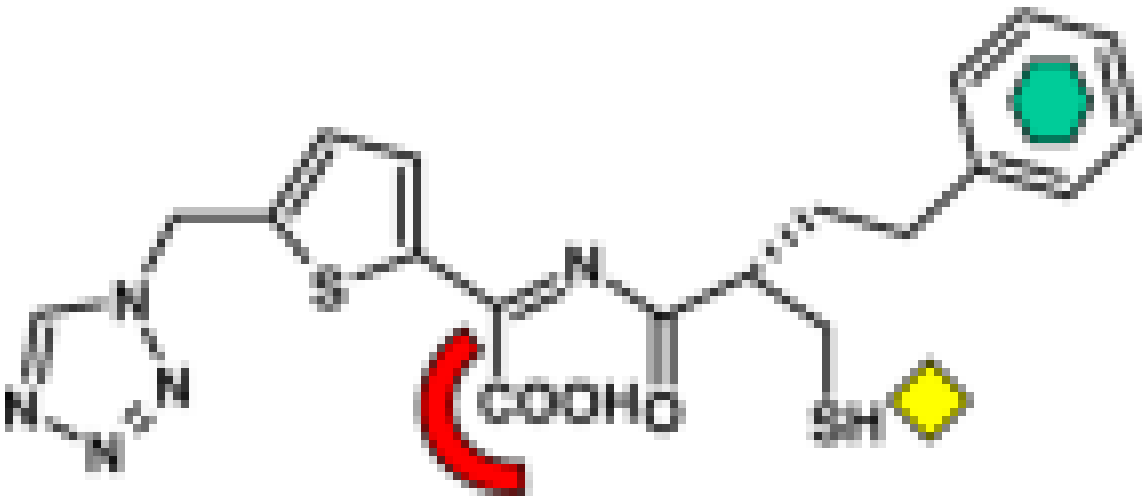
MBL inhibitors that were co-crystalized with the enzymes. Binding mode for each inhibitor is illustrated by three symbols (yellow diamond, metal-ligating group; green hexagon, hydrophobic group; red arch, electrostatically interacting group). **a** and **b** designate two different binding modes that were observed for the same inhibitor, depending on the different subclass of interacting MBLs.

Structural Class	Chemical Structure
Biphenyl tetrazole	
Tricyclic compounds	

Structural Class	Chemical Structure
Dicarboxylic acid	
Dicarboxylic acid	
Dicarboxylic acid	

Structural Class	Chemical Structure
Dicarboxylic acid	
Dicarboxylic acid	
Thiol	
Thiol	
Thiol	

Structural Class	Chemical Structure
Thiol	
Thiol	

Structural Class	Chemical Structure
Thiol	
Thiol