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A Comparative Study of Various Synthesis and Activities of Inhibitors of Metallo-B-Lactamase: A Review

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Abstract – Resistance to b-lactam antibiotics is mediated primarily by enzymes that hydrolytically inactivate the drugs by one of two mechanisms: serine nucleophilic attack or metaldependent activation of a water molecule. Serine b-lactamases are countered in the clinic by several codrugs that inhibit these enzymes, thereby rescuing antibiotic action. The synthesis enabled confirmation of the stereochemical configuration of the compound and offers a route for the synthesis of derivatives in the future of particular concern is the metallo-β-lactamases (MBLs), which are a family of di-zinc containing metalloenzymes capable of hydrolyzing a very broad range of common β-lactam antibiotics. MBLs are not inhibited by clavulanic acid, a drug commonly co-administered with β-lactam antibiotics as an inhibitor of other serine β -lactamases. The aim of the research is to design, dock (computer calculation of an optimum fit of a molecule into an enzyme active site to find a possible docking geometry and energy), synthesize and assay of potent and selective inhibitors of MBLs (IMP-1).

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INTRODUCTION

β-Lactam antibiotics are the most widely used group of antimicrobial drugs in the clinic today. The penicillins, cephalosporins, carbapenems, and monobactams comprise a family of bactericidal antibiotics that share the b-lactam ring that is the reactive moiety of this class of drugs. Resistance to β-lactams can occur through altered antibiotic uptake or efflux, or the synthesis of insensitive target proteins (the cellwall biosynthetic enzymes that cross-link peptidoglycans), but it is a large group of hydrolytic enzymes, the β-lactamases, that represent the principal mode of resistance and drug failure in the clinic. b-Lactamases are subdivided by their use of one of two general chemical mechanisms that result in hydrolytic ring-opening reactions. Serine b-lactamases (SBLs) use an active-site serine residue in a covalent capture mechanism that results in the formation of a transient acyl enzyme intermediate, followed by hydrolytic release of the inactive product. Metallo- β-lactamases (MBLs) on the other hand employ active-site metals (one or two Zn2+ centers) to activate an active-site water molecule that acts as a nucleophile in b-lactam ring opening.

To overcome the emergence of b-lactamases in pathogenic bacteria, new derivatives of b-lactam antibiotics have been regularly introduced to the market over the past decades, thus resulting in several generations of drugs with improved pharmacological profiles and ability to evade resistance. This strategy, while highly successful, is proving increasingly challenging to pursue with effectiveness. A parallel approach has been the coformulation of β-lactam drugs with inhibitors of b-lactamases. This method has also proven to be highly effective. The introduction of β-lactamase inhibitors clavulanic acid, tazobactam, sulbactam, and recently avibactam in various coformulations with penicillins and cephalosporins has extended the lifetime and clinical efficacy of several drugs. This approach so far has focused on the SBLs, which have been the most prominent b-lactamases in pathogenic bacteria. Over the past several years, however, MBLs have increased in frequency and concern. MBLs, unlike most SBLs, can inactivate essentially all penicillins, cephalosporins, and carbapenems, thereby threatening the majority of clinically used antibiotics. In particular, the emergence and widespread global distribution of Gramnegative pathogens harboring the NDM-1 MBL has proven to be a grave cause of concern. There is a growing clinical need for inhibitors of MBLs that can be given as codrugs.

We have recently shown that the fungal natural product aspergillomarasmine A (AMA, Figure 1) is a potent inactivator of MBLs. AMA operates by a zinc-chelation mechanism resulting in an inactive enzyme. NDM and VIM MBLs are particularly sensitive to the action of AMA. Furthermore, AMA in combination with meropenem successfully cured mice infected with a

lethal dose of Klebsiella pneumonia harboring the NDM-1 MBL, thus demonstrating the potential of AMA as a candidate MBL inhibitor that could find use as a codrug with b-lactam antibiotics.

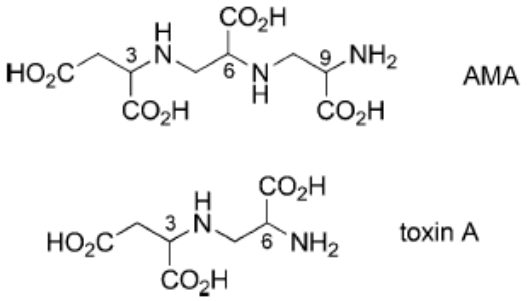

Figure 1. Aspergillomarasmine A (AMA) and toxin A.

We obtained AMA through fermentation of a producing strain of Aspergillus versicolor. A study of AMA in the past revealed a lack of clarity on the absolute configuration of the natural product. In the original 1965 description of the discovery of AMA, the stereochemical assignment was laspartic acid, d-aminopropionic acid, and d-aminopropionic acid (ldd configuration at carbon atoms 3, 6, and 9, respectively, Figure 1). In 1979, the structure of toxin A (equivalent to AMA lacking the N-terminal aminopropionic acid, Figure 1) was assigned the ld configuration (at C3 and C6, respectively). Later, in 1991, this assignment was corrected to ll through chemical synthesis and feeding experiments. Access to analogues of AMA has also been limited owing to the reliance on available products of fermentation. We have developed a synthetic strategy towards AMA to enable the synthesis of derivatives possessing the AMA scaffold and to confirm the absolute configuration.

Retrosynthetic analysis of AMA suggests the compound is derived from an aspartic acid moiety coupled to two activated serine fragments. Experiments by Haenni et al. and Friis et al. demonstrated that the aspartic acid residue has the l configuration; therefore, we prepared four stereoisomers of AMA to definitively assign the absolute configuration of the natural product. The synthesis takes advantage of two successive reactions with the aziridine derived from either d- or lserine. By the use of either antipode of the N-tritylated methyl ester of serine and the procedure described by Zwaneburg and co-workers, azidines l-2 and d-2 were prepared (Figure 2).

To facilitate efficient ring opening, it was necessary to replace the trityl group with a more electron withdrawing protective group. An o-nosyl group was introduced (to yield l-3 or d-3) by an in situ “one-pot” strategy to avoid isolation of the intermediate free aziridine, which is reported to be very unstable. The nucleophilic ring-opening reaction of aziridines l-3 and d-3 with the free base of l-aspartic acid di-tert-butyl ester in dry tetrahydrofuran gave the protected a,b-diamino derivatives ll-4 and ld-4, respectively.

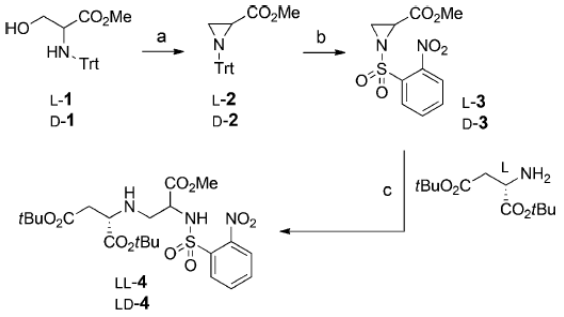

Figure 2. a) MsCl, triethylamine, THF, 65^{0}C, 60 h, 90%; b) TFA, CH_2Cl_2/MeOH, 0^{0}C, 30 min, then o-NsCl, room temperature, 16 h, 71%; c) l-aspartic acid di-tert-butyl ester, THF, RT, 16 h, 80%. Ms=mesyl, Ns=nosyl, THF=tetrahydrofuran, TFA=trifluoroacetic acid, Trt=trityl.

The o-nosyl group was removed by the use of a solution of thiophenol and diisopropylethylamine in acetonitrile to yield either LL-5 or LD-5 (Figure 3). The second serine equivalent could then be introduced once again by treatment of the a,b-diamino derivative LL-5 with the o-nosyl-protected aziridine L-3 to yield LLL-6. Similarly, LD-5 was treated with d-3 to give yield LDD-6, LL-5 was treated with D-3 to give LLD-6, and LD-5 was treated with L-3 to give LDL-6. The deprotection sequence took advantage of the trimethyltin hydroxide protocol described by Nicolaou et al. for the hydrolysis of the methyl esters (to give LLL-7, LDD-7, LLD-7, and LDL-7), followed by removal of the o-nosyl group as described above (to give LLL-8, LDD-8, LLD-8, and LDL-8). Finally, cleavage of the tert-butyl ester groups with trifluoroacetic acid in dichloromethane yielded LLL-AMA, LDD-AMA, LLD-AMA, and LDL-AMA. Interestingly, under these conditions, small amounts of the cyclic anhydro-AMA were produced. Anhydro-LLL-AMA and anhydro-LDD-AMA were isolated and characterized.

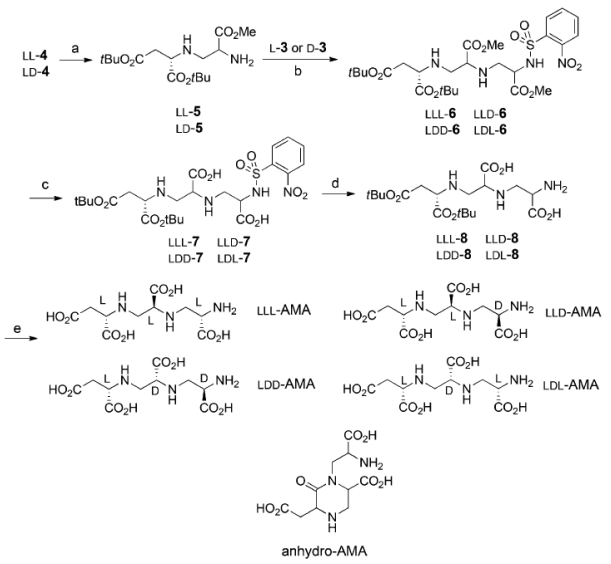

Figure 3. a) PhSH, DIPEA, acetonitrile, room temperature, 1 h, 80%; b) THF, 30%; c) Me_3SnOH (6 equiv), DCE, 80^{0}C, 3 h, 82%; d) PhSH, DIPEA, acetonitrile, room temperature, 5 h, 50%; e) TFA/CH2Cl2 (1:1), 48C, 24 h, 23%. DCE=dichloroethane, DIPEA=diisopropylethylamine.

The optical rotation of the natural AMA isolated from Aspergillus versicolor showed an $[\alpha]_D^{20}$ value of -48^0. The synthetic samples showed values of -47^0 (LLL-AMA), +7^0 (LDD-AMA), -13^0 (LLD-AMA), and -19^0 (LDL-AMA).

Examination of the NMR spectra of these compounds afforded further evidence that the natural product has the LLL configuration, as the 1H NMR spectra of the synthetic and the natural sample were identical. Furthermore, a 1H NMR spectrum obtained after the mixing of LLL-AMA with natural AMA (in a 1:1 ratio) showed only one set of peaks (see the Supporting Information).

Before the early 20th century, treatments for infections were based primarily on folk medicines. Many ancient cultures used specially selected mold and plant material extracts against infectious diseases. Synthetic antibiotics and development of antibacterial agents began in Germany with Paul Ehrlich in the late 1880s. The observations by many researchers in the early 20th century about the inhibition of microbial growth by the extracts of other microorganisms led to the discovery of natural antibiotics. The term 'antibiosis', meaning "against life," was first coined by the French bacteriologist Jean Paul Vuillemin for this phenomenon exhibited by these early antibacterial drugs. Paul Ehrlich screened hundreds of dyes against various organisms and finally discovered a medicinally useful drug, the synthetic antibiotic Salvarsan (Figure 4) which is now called arsphenamine.

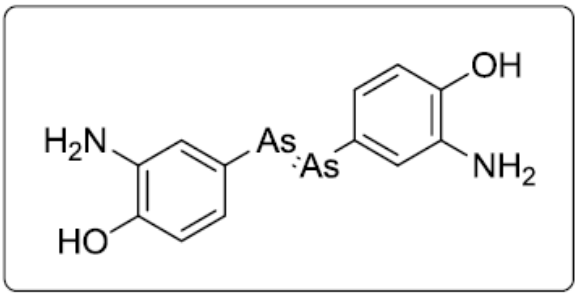

Figure 4: The chemical structure of Salvarsan.

In 1928, Alexander Fleming noticed that a mold growth (Penicillium notatum) over the culture of many petri plates with infectious microbes killed those cultures completely. Fleming postulated that the effect was due to an antibacterial compound named as penicillin, which could be further exploited for chemotherapy.

The first commercially available antibiotic, Prontosil 4 was developed by a research team led by Gerhard Domagk in 1932. This initiated a new era of antibacterial agents. Later, Penicillin G (Figure 5) was purified in 1942 by Florey and Chain showed a potent antibacterial activity against a wide range of bacteria. This discovery led to a renewed interest in the search of antibiotic compounds with similar efficacy and safety.

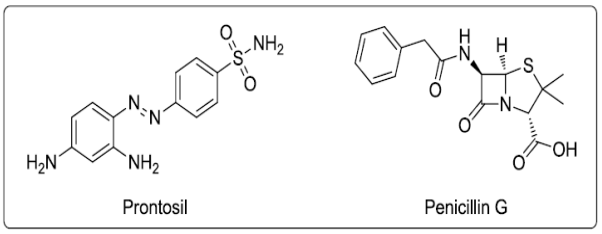

Figure 5: The chemical structures of Prontosil and Penicillin G.

Since their discovery, antibiotics have become the first line treatment for bacterial infections. The use of antibiotics is not monitored strictly in many parts of the world and more than 50% of antibiotics are used without prescription worldwide. Due to the clinical overuse of antibiotics, many bacteria have developed resistance against several antibiotics. This poses a significant threat to public health, and hence necessitates the discovery of new classes/generations of antibiotics.

There are commonly two kinds of bacteria which are known as Gram-positive and Gram-negative bacteria. The difference between the two classes of bacteria is the structures of their cell walls. The Gram-positive bacteria membrane has three layers whereas the Gram-negative bacteria have more complex cell wall structure with five layers. Gram-negative bacteria are usually more resistant against antibiotics because

they have additional membrane (lipopolysaccharide) which poses a major barrier for some antibiotics to penetrate (Figure 6).

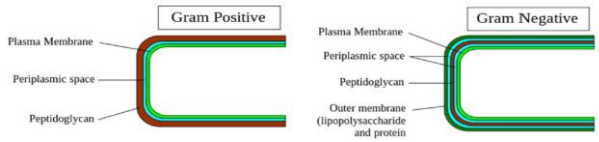

Figure 6: The membrane structures of Gram-positive and Gram-negative bacteria.

B-LACTAM ANTIBIOTICS RESISTANCE

As all projects in the following Chapters deal with the hydrolysis of β-lactam antibiotics by bacteria, the discussion has only been limited to β-lactam antibiotics resistance. Bacteria have several mechanisms to attain resistance against ¥â-lactam antibiotics. These include a) mutations to the active site of penicillin-binding-protein to prevent drug binding, b) modification of the cell wall to prevent drug entry and assist active removal of antibiotic compounds, and c) producing the class of enzyme known as ¥â-lactamase, which includes serine ¥â-lactamases and metallo-¥â-lactamase (MBLs) which hydrolyze the ¥â-lactam ring of drug compound, thereby inactivating them. One solution for the last mechanism is co-administration of antibiotics with ¥â-lactamase inhibitors. These inhibitors prevent the metabolism of penicillins and spare their antibacterial action by inhibiting ¥â-lactamase.

For many years, antibiotics have been used extensively by physicians throughout the world and hence many bacteria have developed resistance against several agents of this class of drugs. Due to this, many infections which were treated effectively in the past have become life-threatening. This problem of resistance was overcome by including some new agents along with penicillin (sulbactam with ampicillin) which were able to inhibit the ¥â-lactamase enzymes. Later, these agents were also found ineffective and there was a need to discover new classes of ¥â-lactamase inhibitors.

The aim of this research is to investigate, design, dock (using computer modeling software to calculate optimized geometries and binding energies of molecules within the enzyme.s active site), synthesize and screen new compounds against metallo-¥â-lactamase (MBLs).

CLASSIFICATION OF β-LACTAMASES

â-Lactamases are enzymes produced by bacteria which are responsible for bacterial resistance to β-lactam antibiotics. The β-lactamases act by cleaving the lactam ring and deactivating the molecule's antibacterial properties (Figure 7).

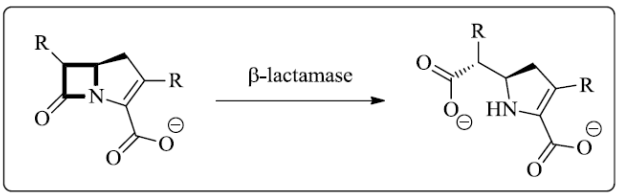

Figure 7: Hydrolysis of antibiotics by β-lactamase enzyme.

The β-lactamase enzyme was first identified in *Escherichia coli* by Abraham and Chain in 1941. However, the group was not successful in isolating the enzyme until four years later when a β-lactamase enzyme was successfully isolated by Kirby from *Staphylococcus aureus.* There are different classification schemes for β-lactamase enzymes which are based on amino acid sequence homology, substrate and inhibitor profiles or functional characteristics.

The classification proposed by Ambler divides β-lactamases into zinc-containing enzymes (class B) and serine β-lactamases (classes A, C, D) based on amino acid sequence homology (Figure 8). The crystal structures of some enzymes are available in Protein Data Bank (PDB).

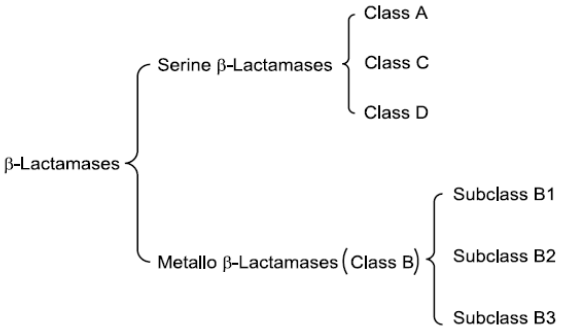

Figure 8: Ambler's classification of β-lactamases.

1. Serine β-Lactamases

Serine β-Lactamase Classes – Table 1 summarizes all three classes of serine β-lactamases (A, C and D) enzymes and their substrates published by Bush *et al.* and was current as of 2010.

Bush-Jacoby Group (2010)	Class	Enzyme	Substrate(s)
1	C	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1	Cephalosporins
1e	C	GC1, CMY-37	Cephalosporins
2a	A	PC1	Penicillins
2b	A	TEM-1, TEM-2, SHV-1	Penicillins, first and second generation of cephalosporins
2be	A	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1	Extended-spectrum cephalosporins, monobactams
2br	A	TEM-30, SHV-10	Penicillins
2ber	A	TEM-50	Extended-spectrum cephalosporins, monobactams
2c	A	PSE-1, CARB-3	Carbenicillin
2ce	A	RTG-4	Carbenicillin, cefepime
2d	D	OXA-1, OXA-10	Cloxacillin
2de	D	OXA-11, OXA-15	Extended-spectrum cephalosporins
2df	D	OXA-23, OXA-48	Carbapenems
2e	A	CepA	Extended-spectrum cephalosporins
2f	A	KPC-2, IMI-1, SME-1	Carbapenems

Table 1: Classification schemes for serine β-lactamases, expanded from Bush *et al.*

Mechanism of Serine â-Lactamases - Serine â-lactamases inactivate â-lactam antibiotics by the formation of an acyl enzyme intermediate, which is produced by nucleophilic attack on the â-lactam by the hydroxyl group of a serine residue. In this proposed mechanism, Glu166 participates in activating a water molecule for both acylation (a) and deacylation (c) steps.

Inhibitors of Serine β-Lactamases - Approximately 50% of available antibiotics in the market are β-lactams. Effective inhibitors have been discovered for a number of serine β-lactamases such as clavulanic acid, sulbactam and tazobactam. Clavulanic acid is a commercially and clinically available inhibitor for serine β-lactamases which is co-administrated with antibiotics such as amoxicillin (typically sold as Co-amoxiclav).

2. Metallo-β-Lactamases

In contrast to serine β-lactamases, MBLs use at least one but more commonly two Zn2+ ions in their active site to catalyze the hydrolysis of β-lactam rings. In 2009, a new carbapenem-resistant *Klebsiella pneumoniae* strain was found in New Delhi (India) by a group of researchers led by Yong. It was later named as NDM-1. This posed a new challenge to develop new MBL inhibitors as none of the antibiotics were effective against NDM-1.

Metallo-β-Lactamase Subclasses - MBLs are classified into three subclasses (B1, B2 and B3). This division is based on substrate selectivity and amino acid sequence, particularly the amino acid ligands that chelate the Zn2+ ions. Table 2 summarizes various MBLs according to their subclasses.

Subclass	Enzyme[a]	Organism[b]	Genbank accession number	PDB code[c]
B1	BcII	*Bacillus cereus*	M11189	1BMC
	CcrA	*Bacteroides fragilis*	M63556	1ZNB
	IMP-1	*Pseudomonas aeruginosa*	M4PZF0	1JJT
		Serratia marcescens,	S71932	1DD6
	IMP-4	*Enterobacter cloacae*	YP_006965428	
	VIM-2	*Pseudomonas aeruginosa, Acinetobacter baumanii*	AF191564	2YZ3, 1K02
	VIM-4	*Pseudomonas aeruginosa, Acinetobacter baumanii*	AY135661	2WHG
	VIM-7	*Pseudomonas aeruginosa, Acinetobacter baumanii*	AM778842	2Y87
	BlaB	*Chryseobacterium meningoseptica*	AF189298	1M2X
	SPM-1	*Pseudomonas aeruginosa*	AY341249	2FHX
	NDM-1	*Klebsiella pneumonia, Escherichia coli*	JN420336	3Q6X, 3SPU,3RKJ
	VIM-1	*Pseudomonas aeruginosa, Acinetobacter baumanii*	T18050	
	GIM-1	*Pseudomonas aeruginosa*	AJ620678	
	SIM-1	*Acinetobacter baumanii*	AY887066	
	DIM-1	*Pseudomonas stutzeri*	GU323019	
	TMB-1	*Achromobacter xylobacter*	FR771847	
	Bla2	*Bacillus anthracis*	CP001598	
	KHM-1	*Citrobacter freundii*	AB364006	
B2	CphA	*Aeromonas hydrophila*	X57102	1X8G, 3F9O
	Sfh-1	*Serratia fonticola*	AF197943	3SD9
	ImiS	*Aeromonas veronii*	Y01415	
B3	L1	*Stenotrophomonas maltophilia*	AB294542	1SML
	FEZ-1	*Legionella gormannii*	Y17896	1K07
	BJP-1	*Bradyrhizobium japonicum*	NP772870	3LVZ45
	AIM-1	*Pseudomonas aeruginosa*	AM998375	4AWY
	THIN-B	*Janthinobacterium lividum*	CAC33832	
	GOB-1	*Chryseobacterium meningoseptica*	ABO21417	
	CAU-1	*Caulobacter crescentus*	CAC87665	
	CAR-1	*Erwinia caratovora*	Q6D395	
	SMB-1	*Serratia marcescens*	AB636283	
	POM-1	*Pseudomonas otitidis*	ADC79555	
	CRB11	*Uncultured bacterium*	ACS83724	

[a] Enzyme variants are not included in this table unless an X-ray structure of the variant is available. For example, there are many variants of IMP-1 that are not listed in the table.
[b] The strains listed represent the original strain(s) for which the enzyme was identified and does not include the bacteria to which the gene has spread.
[c] Protein Data Bank codes are listed for all enzymes for which X-ray structures have been determined.

Table 2: Classification of Metallo-β-lactamases.

Metallo-β-Lactamase Structures - A typical metallo β-lactamase has been shown in Figure 9. The picture below illustrates a surface picture of IMP-1. The active site is in the region which is shown by the yellow dotted line. For creation of this picture, IMP-1 (PDB Code: 1JJT) was downloaded and chain B was deleted as the topologies of the active sites of both chain A and chain B are identical then the surface was created with Maestro software. The active site of this enzyme contains two zinc atoms which are responsible for hydrolysis of β-lactam rings. However, they are not shown in this picture as single atoms are too small to be visible at this scale. In all chapters for showing the better results of docking, the size of both zinc atoms has been increased in the region of the active site of IMP-1.

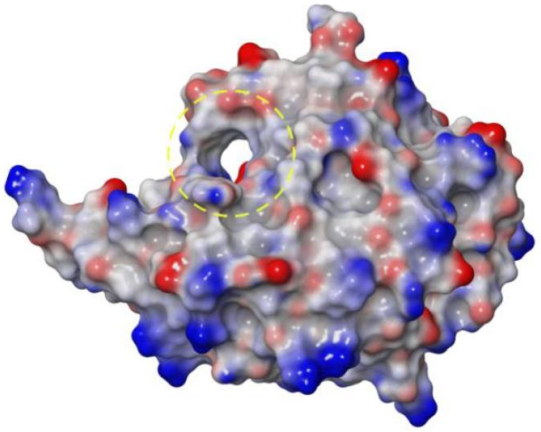

Figure 9: Representative typical active-site pocket of MBL (IMP-1).

CONCLUSION

â-Lactam antibiotics make up more than 50% of all commercially prescribed antibiotics for treatment of bacterial infections. The â-lactam ring is part of the structure of several antibiotic families; the principal â-lactam antibiotics are carbapenems, cephalosporins, penicillins, and monobactams. The general mechanism of action of â-lactam antibiotics is inhibition of peptidoglycan synthesis which constitutes a major portion of bacterial cell wall synthesis. The attachment of â-lactam antibiotics to penicillin-binding protein leads to the inhibition of transpeptidase (a bacterial enzyme that cross-links the peptidoglycan chains to form rigid cell walls) which eventually leads to the death of bacteria. Bacteria have several mechanisms to attain resistance against â-lactam antibiotics.

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