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Focused Review

Metal binding and structure–activity relationship of the metalloantibiotic peptide bacitracin

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Abstract

Bacitracin is a widely used metallopeptide antibiotic produced by *Bacillus subtilis* and *Bacillus licheniformis* with a potent bactericidal activity directed primarily against Gram-positive organisms. This antibiotic requires a divalent metal ion such as Zn^{2+} for its biological activity, and has been reported to bind several other transition metal ions, including Mn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+}. Despite the widespread use of bacitracin since its discovery in the early 1940s, the structure–activity relationship of this drug has not been established and the coordination chemistry of its metal complexes was not fully determined until recently. This antibiotic has been suggested to influence cell functioning through more than one route. Since bacterial resistance against bacitracin is still rare despite several decades of widespread use, this antibiotic can serve as an ideal lead for the design of potent peptidyl antibiotics lacking bacterial resistance. In this review, the results of physical (including NMR, EPR, and EXAFS) and molecular biological studies regarding the synthesis and structure of bacitracin, the coordination chemistry of its metal derivatives, the mechanism of its antibiotic actions, its influence on membrane function, and its structure and function relationship are discussed.

Keywords: Antibiotic; Bacitracin; Function; Metal; Peptide; Structure

1. Introduction

Peptide antibiotics form a unique group of 'bio-active molecules' [1–5]. Many peptide antibiotics have novel structural motifs, such as cyclic structures, contain uncommon amino acids, especially D-form amino acids, and are often further modified (such as in β-lactam antibiotics) and conjugated with sugars, lipids, and other molecules. Depending on their amino acid components and their conjugates, the mechanisms of their actions may vary dramatically [6]. Examples of peptide antibiotics include some well known or commonly used drugs [7], such as polymyxin, amphomycin, actinomycin, gramicidin, vancomycin, penicillin, cephalosporin, and bacitracin.

Bacitracin was first discovered in 1943 and named after a culture of bacillus and the last name of a 7-year-old American girl, Margaret Tracey, from whose wounds the *Bacillus* was isolated [8,9]. It is a potent peptide antibiotic of narrow spectrum directed primarily against Gram-positive cocci and bacilli, including *Staphylococcus*, *Streptococcus*, and *Clostridium difficile* as well as some Archaebacteria such as *Methanobacterium*, *Mathanococcus*, and *Halococcus* [7–13] and the oyster-infecting *Perkinsus marinus* [14]. This antibiotic has also been shown to exhibit an interesting metal-dependent, particularly Cu^{2+} ion, inhibition toward the growth of the mold *Neurospora crassa* [15,16]. It is one ingredient in several commercially available topical 'triple antibiotic' ointments (along with neomycin and polymyxin B) such as Polysporin® and Neosporin® that are used to prevent infections in minor cuts and burns [17]. Although this antibiotic has been generally considered safe for topical use, it has recently been found in a few cases to generate delayed hypersensitivity, acute IgE-mediated allergic reactions, and even life-threatening anaphylaxis [18–22]. Bacitracin is not administered systemically as it is nephrotoxic (toxic to kidney cells), and is used only as a last resort. It is considered safe when taken orally as the gastrointestinal tract does not absorb significant amounts of the drug [10,17] (however, the absorption was found significant in rainbow trout [23]). Bacitracin has thus been used for the treatment of gastrointestinal infections (such as antibiotic-

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associated colitis and diarrhea caused by *C. difficile* [24–26] and was found to be as effective as vancomycin [27]), vancomycin-resistant *Enterococcus faecium* [28] (however, a recent study shows its inefficiency [29]), and intestinal infections by *Entamoeba histolytica* [30]. It has been widely utilized as an animal feed additive to improve animal body weight and to prevent diseases in farm animals [31–33]. Consequently, bacitracin is important in both the pharmaceutical and livestock industries and is produced in large quantities throughout the world.

Bacitracin has been known to bind divalent metal ions since the early stage of its study [34]. In the past several years, the metal binding property of different congeners of bacitracin has also been studied with potentiometric and spectroscopic techniques. The structures of bacitracin and its metal complexes have emerged by the use of several physical methods, including NMR, ^{1}EPR, UV/VIS spectroscopy, mass spectroscopy, X-ray crystallography, and EXAFS. As the antibiotic activities of bacitracin and its several congeners have been obtained, the relationship between the structure and function of this peptide antibiotic and the role of metal ion in this antibiotic have emerged. Bacterial resistance against antibiotics has become a threatening health issue in recent years. However, bacterial resistance of bacitracin is still scarce despite its wide use in the past several decades. Thus, it can serve as a potential lead for design of potent antibiotic metallopeptides and analogues devoid of bacterial resistance for combating bacterial infection.

2. Bacitracin congeners and biosynthesis of bacitracin

Bacitracin is produced as a mixture of closely related congeners by *Bacillus subtilis* or *Bacillus licheniformis* [35–39] (Fig. 1). Bacitracin is very soluble in water, methanol, and dimethyl sulfoxide, soluble in ethanol, and slightly soluble in acetone, benzene and ether, and is almost insoluble in chloroform [10]. Acidic and neutral aqueous solutions of bacitracin are relatively stable while solutions above pH 9 degrade rapidly at room temperature.

Soon after its discovery, the mixture of crude bacitracin was separated into several components by the use of the counter current distribution (CCD) technique [40–42]. In the 1970s, HPLC began to replace the old CCD method for bacitracin purification [43]. As a result, the 'pure CCD fractions' of the minor bacitracin components B, C, D, E, and F were all shown to be mixtures of two or more different peptides. Eventually, over 30 different minor components were separated from the crude mixture and analyzed by the use of fast atom bombardment tandem mass spectrometry and electrospray ionization mass spectrometry to establish their structures [44–49]. A recent capillary chromatographic study further isolated bacitracin into more than 50 peaks [50]! Several different nomenclatures have been used to classify the bacitracin congeners. In this review we follow the nomenclature by Ikai et al. [48].

Bacitracins B_1, B_2, and B_3 have the same sequence as bacitracin A_1, except that Ile-1, Ile-5, and Ile-8, respectively, are substituted by a Val (Fig. 1). Similarly, substitution of Val for Ile at positions 1, 5, and 8 with different combinations affords bacitracins D and E (Fig. 1; Table 1). In addition to these congeners, a few modified bacitracin derivatives have also been prepared chemically or isolated from the crude mixture [51–54]. For example, the biologically inactive bacitracin F can be obtained by air oxidation of bacitracin A_1 in slightly alkaline aqueous solutions, in which the aminomethylene-thiazoline moiety is converted into a keto-thiazole moiety (Fig. 1) [51,52]. Similarly, oxidation of bacitracins B and D produces the corresponding keto-thiazole-containing bacitracins H and I (Fig. 1; Table 1). Desamido bacitracin is prepared by hydrolyzing Asn^{12} in 0.1 N NaOH. Bacitracin F and desamido bacitracin A_1 have been characterized with UV/VIS [51], NMR (see next section), and electrospray ionization mass spectroscopy [53]. Bacitracin A_2 is prepared by acid isomerization of the L-Ile^{1} amino group in 3% acetic acid solution [55] to give a D-allo-Ile^{1} N-

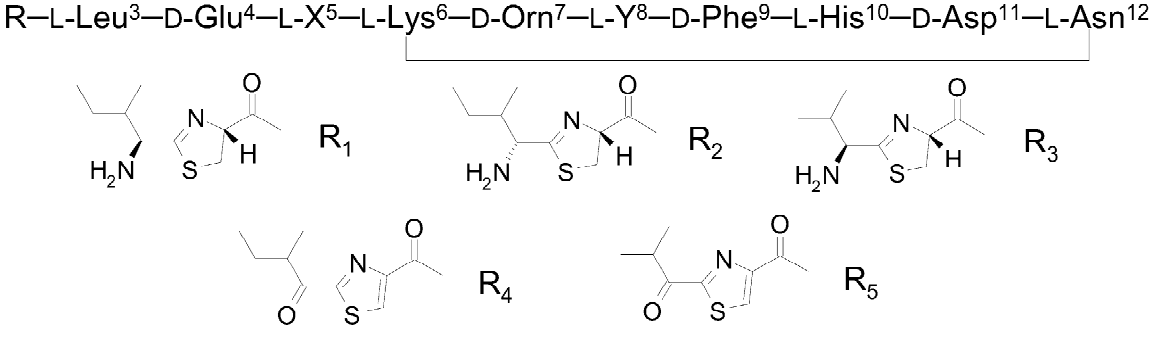

Fig. 1. Schematic structure of bacitracin congeners. The thiazoline ring at the N-terminus in bacitracin is formed by the condensation of the carbonyl group of L-Ile^{1} or L-Val^{1} with the methylthiol group of L-Cys^{2}. The amino acid sequences and N-terminal structures of bacitracins A, B, and D are shown in Table 1. Bacitracin E contains Val^{5}, Val^{8}, and R^{3}; bacitracin H_1: Ile^{5}, Ile^{8}, and R_5; H_2: Val^{5}, Ile^{8}, and R_4; H_3: Ile^{5}, Val^{8}, and R_4; I_1: Val^{5}, Ile^{8}, and R_5; I_2: Ile^{5}, Val^{8}, and R_5; and I_3: Val^{5}, Val^{8}, and R_4. The activities of the latter minor congeners have not been extensively investigated.

Table 1
Bacitracin congeners[a] and antibiotic activities (Act.)

Congener	MW	X^5	Y^8	R	Act.[b]	Act.[c]	Act.[d]	Act.[e]	Act.
A_1	1422.7	L-Ile	L-Ile	R_1	0.39	3.13	6.25	1e-7	79[f]
A_2	1422.7	L-Ile	L-Ile	R_2	–	–	–	–	23[g]
B_1	1408.7	L-Ile	L-Ile	R_3	0.78	12.5	25	2e-7	–
B_2	1408.7	L-Val	L-Ile	R_1	0.78	12.5	25	2e-7	–
B_3	1408.7	L-Ile	L-Val	R_1	0.78	6.25	25	2e-7	–
D_1	1394.6	L-Val	L-Ile	R_3	3.13	25	>25	–	–
D_2	1394.6	L-Ile	L-Val	R_3	3.13	25	>25	–	–
D_3	1394.6	L-Val	L-Val	R_1	1.56	12.5	>25	–	–
F	1419.6	L-Ile	L-Ile	R_4	>25	>25	>25	5e-5	–
Desamido[h]	1423.6	L-Ile	L-Ile	R_1	–	–	–	7e-4	–

[a] See Fig. 1 for X^5, Y^8, and R substitutions within the bacitracin molecule. The amino acid sequences and N-terminal structures of bacitracins E, H, and I are described in Fig. 1 caption.
[b] Minimal growth inhibitory concentration (MIC, μg/ml) versus *Micrococcus luteus* ATCC 9341 [48].
[c] MIC (μg/ml) versus *Staphylococcus aureus* IFO 12732 [48].
[d] MIC (μg/ml) versus *Bacillus cereus* ATCC 11778 [48].
[e] MIC (M) (not completely purified) versus *Micrococcus lysodeikticus* [124].
[f] Commercial bacitracin (mixture) has 58.6 U/mg of activity [48].
[g] The antibiotic activities (U/mg) reported by Craig et al. [55] were for partially purified bacitracin samples. A significant amount of A_1 was still present in the A_2 sample. Therefore, the actual activity for A_2 should be significantly less.
[h] Desamido bacitracin has the same structure as bacitracin A_1 with an Asp substituted for Asn^{12}. It has a negligible biological activity [124].

terminus (Fig. 1), and was referred to as a 'low potency' bacitracin. This isomer has virtually identical ^{1}H NMR and mass spectra as bacitracin A_1 [53]. Bacitracins B account for ~30% of the mass of the crude bacitracin mixture, while bacitracins D–I are only found in trace amounts. It is also known that bacitracins A and B account for ~95% of the biological activity of the crude bacitracin mixture [56]. A summary of the primary structures and the antibiotic activities of the congeners is shown in Table 1.

The biosyntheses of many peptides and polyketides and their hybrid conjugates follows a non-ribosomal pathway catalyzed by large clusters of peptide and ketide synthetases and peptide/ketide 'hybrid' synthetases, respectively [57–62]. The genes of the synthetases/synthases of peptides and polyketides from a few microorganisms have recently been analyzed and cloned and the synthetase further studied [63–70], including the synthetase of bacitracin [71–83]. For example, the cyclic heptapeptide microcystin, a protein phosphatase inhibitor and a hepatotoxin produced by cyanobacteria, is synthesized by a synthetase complex encoded by a gene cluster with three open reading frames and some common conserved amino acid sequence motifs with other peptide synthetases such as an ATP-binding domain [65]. Another cyanobacterial hepatotoxin cylindrospermopsin and the antitumor drug bleomycin (a natural peptide-ketide hybrid) from *Streptomyces* have been verified to be produced by synthetase clusters comprised of polyketide synthase and peptide synthetase modules [66,84,85]. The studies of several peptide and polyketide synthetases and their hybrids, including crystallographic studies of the adenylation domain and an NMR study of a peptidyl carrier domain [86–88], have greatly enhanced our understanding of the structure and mechanism of this superfamily of 'mega enzymes' and have also allowed us to gain further insight into the action of bacitracin synthetase. Since these and other synthetase complexes possess enzymatic activities toward the syntheses of secondary metabolites [65–67], they thus are potential targets for drug discovery in the production of potential bio-active peptides and polyketides as well as their hybrids [89,90].

Like those structurally diverse peptides and polyketides, bacitracin congeners are also non-ribosomal products of a large peptide synthetase complex [71–83]. The structure and mechanism of bacitracin synthetase resemble those of other peptide and polyketide synthetases, which are comprised of a multi-domain modular structure for the catalysis of the initiation of the synthesis via ATP-activating formation of thioester linkage to the enzyme, elongation mediated by condensation of the thioester-linked amino acid and/or peptide on the peptide carrier domain following a mechanism not yet fully understood, and termination of the peptide or polyketide chain by a thioesterase domain via transfer of the final product to a serine in the thioesterase followed by hydrolysis [90,91]. The reactant amino acids or carboxylates are specifically recognized and covalently linked to the different domains before being transferred to an intermediate peptide or polyketide chain. Changing of the stereochemistry is carried out by epimerization domains in the enzyme complex.

Bacitracin synthetase has been known to comprise of a complex modular structure as in the case of other peptide/polyketide synthetases since early studies of this enzyme [71–80]. This enzyme catalyzes an ATP-dependent synthesis of bacitracin, starting from the N-terminus based on the observation of a few N-terminal peptidyl intermediates such as Ile–Cys, Ile–Cys–Leu, Ile–Cys–Leu–Glu, and

several other N-Ile-containing peptides [92]. The role of ATP has been suggested to be involved in the formation of the labile aminoacyl adenosine intermediates. As in the case of other non-ribosomal peptide/polyketide biosyntheses, the synthesis of bacitracin has been suggested to involve thioester-linkages based on the observation of covalent peptide-enzyme complexes [92]. The thiazoline ring in bacitracin has been suggested to be synthesized at the stage of Ile–Cys formation on the basis of the detection of the oxidized thiazole product of the Ile–Cys intermediate [93,94]. The thiazoline ring and analogous thiazole ring are found in a number of peptide antibiotics and siderophores such as bleomycin, which are synthesized with a similar mechanism [84,95,96]. An early study revealed that the activity of bacitracin synthetase is affected by the divalent metal ions Mg^{2+}, Mn^{2+}, Fe^{2+}, and Co^{2+} (Zn^{2+} was not checked) as well as bacitracin [97], suggesting that bacitracin and metal ions may exhibit feedback control of the synthetase.

The bacitracin synthetase opron contains the genes *bacA*, *bacB*, and *bacC*, which have been recently cloned and determined to encode three products: BA1 of 598 kDa, BA2 of 297 kDa, and BA3 of 723 kDa [83]. BA1 contains five modules to incorporate the first five amino acids, an epimerization domain attached to the fourth module for the inclusion of D-Asp4, and a cyclization domain for the formation of the thiazoline ring between Ile^1 and Cys^2; BA2 is comprised of two modules and an epimerization domain for D-Orn^6 incorporation; and BA2 contains five modules for the addition of Ile^8–Asn^{12} with two epimerization domains and the thioesterase domain, consistent with previous studies [71–80]. A disruption of the *bacB* gene results in a bacitracin-deficient mutant, confirming the involvement of this gene in bacitracin synthesis [83]. Moreover, the expression of a foreign bacitracin synthetase in a host *B. subtilis* results in the production of bacitracin at high level, confirming the functional role of bacitracin synthetase and its association with bacitracin self-resistance genes [98].

3. Structure of bacitracins

Since the discovery of bacitracin in 1943 by Meleney and Johnson, researchers have been trying to establish the structure of this natural product [8,9]. However, pure components such as the most potent bacitracin A_1 could not be completely purified for structural analysis. The bacitracin mixture was first separated into three components by the use of the CCD technique [40], which were determined to contain Ile, Leu, Glu, Lys, Orn, Phe, His, and Cys by means of amino acid analysis using starch column chromatography. The presence of D-amino acids was also noted at that time and the presence of a thiazoline ring in bacitracin A was postulated [99]. The CCD technique was further improved in the early 1950s [41,42], allowing the separation of the crude bacitracin into ten components which included bacitracins A, B, C, D, E, F, and G named after the order of separation. Bacitracin F was observed to contain an unusual chromophore with a broad absorption at 288 nm, which was eventually identified as a thiazole ring [51,52].

Evidence for a cyclic heptapeptide structure in bacitracin A was provided at the early stage in the structural study of bacitracin [100–102]. Following this lead, several partial structures of this peptide were published [103–105]. The development of liquid chromatographic techniques has allowed the isolation of pure components [43–45], and their structures determined with mass spectroscopic methods [44–48]. The first 'accepted structure' for bacitracin A_1 was proposed in the mid-1960s [106], ~20 years after its discovery and approval as a certifiable antibiotic. Bacitracin A_1 has been determined to be a cyclic dodecapeptide by means of chemical, spectroscopic, and crystallographic techniques. It contains an unusual thiazoline ring formed by the condensation of the Ile^1 carboxyl group with the –SH and $-NH_2$ groups of Cys^2, a cyclic heptapeptide structure formed via an amide linkage between the side chain ϵ-NH_2 of Lys^6 and the C-terminus of Asn^{12}, and four D-amino acids including D-Glu^4, D-Orn^7, D-Phe^9, and D-Asp^{11}. These unusual structural features may protect this peptide from degradation by proteases [107]. Bacitracin A_1 was chemically synthesized in 1996 which confirmed the structure of this antibiotic peptide produced by microorganism [108], and opened the door to preparation of other isomers/analogues and congeners of this antibiotic for further investigation of its structure–activity relationship and rational design of peptide antibiotics for combating bacterial infections.

The first ^{1}H NMR spectrum for bacitracin A_1 was obtained in 1972 (cf. Fig. 2) [109]. Several more NMR studies quickly followed, including a tritium exchange study [110], a ^{1}H NMR relaxation study [111], and a ^{13}C NMR study [112]. The full assignment of the ^{1}H NMR signals of bacitracin was not achieved, however, until the early 1990s by the use of modern 2D NMR techniques [113,114]. The results from the 2D NMR studies indicated that the 'tail' of the bacitracin peptide (Ile^1 to Glu^4, Fig. 1) bends toward the seven-membered ring, placing the thiazoline ring, Glu^4, and His^{10} in close proximity to form a potential metal binding site in solution. The amino acids side chains of Phe^9 and Ile^8 are close to Leu^3 on the basis of nuclear Overhauser effect (NOE) measurements.

A few other congeners have also been identified and characterized with NMR techniques. For example, bacitracin F shows dramatic changes of the signals upon oxidation of the original thiazoline ring into a thiazole ring (labeled signals of bacitracin A_1 in Fig. 2A) [53,115], and desamido-bacitracin can be conclusively identified by the disappearance of the two characteristic amido-NH_2 signals of Asn^{12} at 7.55 and 6.90 ppm (marked signals in Fig. 2B) [53,115].

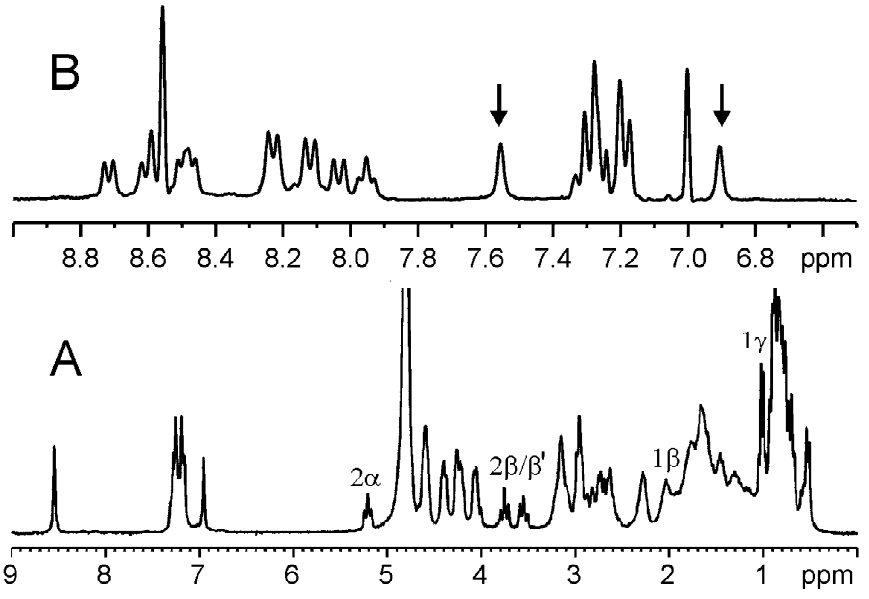

Fig. 2. Proton NMR spectrum (250 MHz) of (A) bacitracin A1 in D_2O at pH meter-reading 5.4 and (B) the down-field region of the spectrum of bacitracin A_1 in H_2O at pH 5.0. The oxidation of the thiazoline ring causes a dramatic change of the signals due to the first two amino acids (marked signals in spectrum A). The arrows in spectrum B indicate the solvent exchangeable signals of Asn^{12} side-chain $CONH_2$ protons in bacitracin A_1, which disappear upon hydrolysis of Asn^{12} into Asp^{12} [53].

Although the ^1H NMR and mass spectra of the A_2 congener are identical to those of the A_1 congener, the ^1H NMR spectra of the Co^{2+} complexes of these two congeners are quite different which reflects their different metal binding properties and coordination chemistry (Section 7).

4. Metal binding and coordination chemistry

Bacitracin has been known to bind several divalent metal ions to form 1:1 complexes [34]. An order for metal binding affinity of divalent metal ions has been established as $Cu^{2+}>Ni^{2+}>Co^{2+}\sim Zn^{2+}>Mn^{2+}$ [116]. The biological activity of this peptide antibiotic has also been determined to be associated with divalent metal ions [15,16,117–119]. Bacitracin was first suggested to bind metal via its His^{10} imidazole and the thiazoline ring, but not the carboxyl groups. The thiazoline ring nitrogen or sulfur and the amino group of Ile^1 were also suggested to be involved in metal binding based in part on a proton release titration [120].

The involvement of His^{10} imidazole and thiazoline nitrogen or sulfur in metal binding was also determined by the use of ^1H NMR and optical rotary dispersion spectrometry [121]. The histidine was determined to bind to the metal via its imidazole ϵ-nitrogen based on the equal downfield shifts noted for the 2-CH and 4-CH imidazole ring protons upon Zn^{2+} binding. Again, there was no evidence for or against the participation of other groups in metal binding. The involvement of His^{10} imidazole ring and the carboxylate groups of Asp^{11} and Glu^4 in metal ion was also proposed on the basis of an analysis of ^{13}C NMR spectra of the peptide upon its binding with Cu^{2+} and Mn^{2+} in the pH range of 6–8 [122]. The thiazoline ring was suggested to bind to Mn^{2+} at pH ~6.6. Later studies [123–125] indicated that divalent metal ions such as Zn^{2+} were bound to bacitracin through the δ-N of the imidazole ring of His^{10}, the sulfur atom of the thiazoline ring, and the Glu^4 carboxylate. No evidence for the binding of Asp^{11} was found, and the participation of the Ile^1 terminal amino group was not conclusive.

A few metal complexes of bacitracin have been studied with other physical methods. Mn^{2+}-, Co^{2+}-, and Cu^{2+}-bacitracins were studied by means of EPR spectroscopy [126]. The Mn^{2+} complex does not bind to the drug at pH 5.2, and the Co^{2+} complex exhibits only broad EPR features at 77 K attributable to the fast relaxing $S=2/3$ Co^{2+} center. The Cu^{2+}-bacitracin complex at pH 5.2 affords a slightly rhombic EPR spectrum with large copper hyperfine coupling constants of $A_z=534$ MHz and a clear nitrogen superhyperfine coupling, consistent with a tetragonally distorted geometry with two coordinated nitrogens and two coordinated oxygens [127]. From which, a distorted octahedral coordination was concluded with His^{10}, Glu^4, Asp^{11}, and the thiazoline ring nitrogen atom bound to the metal ion. A possible coordination of the metal to the sulfur of the thiazoline ring can be ruled out since this coordination would afford much smaller g and A values [127]. A recently EXAFS study of Zn-bacitracin suggested that the first coordination sphere of the metal is comprised of three N-containing ligands and one O-containing ligand, which have been assigned to the N-terminal amino group,

the imidazole of His^{10}, the thiazoline nitrogen, and the carboxylate of Glu^4 [128]. The Ile^1-NH_2 amino group was suggested to be close to the metal; however, whether or not it was coordinated to the metal could not be concluded. Sulfur binding to the metal was ruled out in this study, and has also been excluded in a later ab initio study of chemical shift effects of the ^1H NMR features [129].

5. Cell wall synthesis and the mechanisms of bacitracin action and resistance

The bacterial cell wall is a good target for therapeutic agents. Gram-positive bacteria typically have thick cell walls composed of a unique polysaccharide called peptidoglycan (as much as 90%; but ~10% in Gram-negative bacteria) that is not present in eukaryotic cells [130]. Therefore, the inhibition of the biosynthesis of the peptidoglycan layer should in principle affect only the bacterial cells, and not the animal cells. This is the basis for the selective toxicity against microorganisms by those drugs that inhibit the synthesis of the bacterial cell wall, such as bacitracin [131] and the β-lactam antibiotics (penicillins and cephalosporins). The influence of bacitracin on cell wall synthesis was established by early studies, including accumulation of cell-wall intermediates, inhibition of amino acids incorporation into cell wall, and cell lysis [132–134].

The peptidoglycan layer is constructed from repeating disaccharide units of *N*-acetylglucosamine (GlcNAc) and peptidyl *N*-acetylmuramic acid (MurNAc) connected by β-1,4-glycosidic bonds [130,135]. The peptide includes L-Ala, D-Glu, L-Lys, and one or two D-Ala are attached to MurNAc via an *O*-lactyl group at the carbon-3-hydroxyl position. During the peptidoglycan synthesis (Fig. 3), the UDP-MurNAc-pentapeptide conjugate is attached to the luminal side of the endoplasmic reticulum (ER) membrane via undecaprenyl (or bactoprenyl) monophosphate. Addition of GlcNAc via a β-1,4-glycosidic bond forms a disaccharide complex, β-1,4-GlcNAc-(3-pentapeptidyl-MurNAc)-*O*-PO_2^--*O*-PO_2^--*O*-undecaprenyl. At last, the disaccharide is released from the long-chain undecaprenol and incorporated into the peptidoglycan layer by a β-1,4-glycosidic bond. Cross-strand linking between the side chain $-NH_2$ of Lys^3 with D-Ala^4 (while D-Ala^5 is lost during the cross link) also occurs at this stage and imparts strength and rigidity to the cell wall. The peptidylsugar-carrying molecule is released at the last stage as undecaprenyl pyrophosphate, which is dephosphorylated to regenerate its monophosphate form by a membrane-bound pyrophosphatase. The monophosphate form then binds to another UDP-MurNAc-pentapeptide to begin a new cycle of peptidoglycan biosynthesis. This biosynthetic pathway is analogous to that of glycoproteins in eukaryotes wherein the undecaprenol analogue, dolichol phosphate instead serves as the sugar-carrying molecule [135,136].

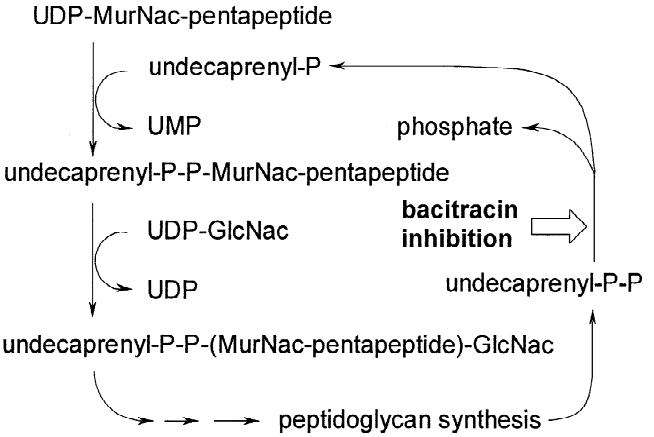

Fig. 3. The pathway of the biosynthesis of cell wall and bacitracin inhibition of this process. The structure of the fundamental unit of the peptidoglycan layer consists of GlcNAc and MurNAc linked by a β-1,4-glycosidic bond. The amino acids may vary in Gram-positive bacteria, e.g. the D-Glu can be replaced by a D-Gln or a D-Glu–Gly, and the L-Lys can be replaced by meso- or L,L-diaminopimelic acid, L-ornithine, L-α,α-diaminobytyric acid, L-homoserine, L-Glu, or L-Ala. The GlcNAc-MurNAc disaccharides are usually cross-linked to other disaccharide units via their peptide side chains, usually between L-Lys^3 and D-Ala^4. During the last stage of peptidoglycan synthesis, undecaprenyl pyrophosphate is released and eventually hydrolyzed by a pyrophosphatase. The tight binding of bacitracin with undecaprenyl pyrophosphate prevents the recycling of this sugar carrier, thus inhibiting cell wall synthesis.

Metallobacitracin has been shown to bind very tightly to the long-chain C_{55}-isoprenol (i.e. undecaprenyl) pyrophosphate, with a formation constant of $K_f = 1.05 \times 10^6\ M^{-1}$ for the Co^{2+} complex [137,138]. Since the hydrolysis of the long-chain undecaprenyl pyrophosphate into monophosphate is considered an essential step in peptidoglycan synthesis, the above observation thus suggests that metallobacitracin may interfere with cell wall synthesis by tight binding to undecaprenyl pyrophosphate which prevents its hydrolysis (Fig. 3). As a result, undecaprenyl monophosphate becomes much less available for binding to the UDP-MurNAc-pentapeptide complex to initiate the second stage of the peptidoglycan biosynthesis. Hence, the biosynthesis of the bacterial cell wall is inhibited. This mechanism has also been suggested to inhibit the synthesis of the peptidylglycan-like pseudomurein in *Methanobacterium* spp. [12].

From the studies discussed above, it can be expected that a malfunctioning of the synthesis of undecaprenyl phosphate in bacteria may result in higher bacitracin susceptibility as recently observed [139] since the recycling of the sugar-carrying phosphate can be easily blocked with a much lower concentration of bacitracin. A later study found that bacitracin can also inhibit the transfer of GlcNAc from UDP-GlcNAc to isoprenyl-pyrophosphate-GlcNAc to yield isoprenyl-pyrophosphate-$(GlcNAc)_2$ in yeast [140], thus also inhibiting the subsequent transfer of $(GlcNAc)_2$ to proteins. A previous study revealed that bacitracin could inhibit the formation

of dolichyl-pyrophosphate-GlcNAc, but with little influence on the formation of the dolichyl pyrophosphate-$(GlcNAc)_2$ and the dolichyl phosphates of mannose and Glc [141,142]. This study suggested that bacitracin may inhibit the enzyme involved in the synthesis of dolichyl-pyrophosphate-GlcNAc rather than a direct binding to the pyrophosphates. A recent revisit of this inhibition has resulted an opposite result in which bacitracin was found to stimulate the formation of dolichyl-pyrophosphate-GlcNAc, but to inhibit the formation of dolichyl pyrophosphate-$(GlcNAc)_2$ [143]. Further investigations seem required to clarify this controversy in order to provide further insight into bacitracin action and polyprenyl biosynthetic pathway.

A recent study found that those antibiotics that inhibit the late stages of peptidoglycan biosynthesis cause a surprising side-effect by inducing vancomycin resistance in *Enterococcus faecium* [144]. It was found that exposure to bacitracin led to the synthesis of the lactate-containing UDP-MurNAc-pentadepsipeptide precursor that is required for vancomycin resistance. Bacitracin thus can serve as a model system for the understanding of 'induced drug resistance'. The resistance of a few exopolysaccharide-secreting Gram-negative bacteria, *Xanthomonas campestris*, *Sphingomonas* strains S-88 and NW11, and *Escherichia coli* K-12, toward bacitracin is found to be simply due to a termination of the synthesis of the exopolysaccharide [145].

The transport of a wide range of molecules and ions, such as amino acids, oligopeptides, sugars, fatty acids, and macromolecules, across cell membrane is associated with an ATP-binding cassette (ABC) transporter [146,147], which is also involved in multi-drug resistance in cancer cells and microorganisms [148–150]. An ABC transporter system has been revealed to be involved in bacitracin resistance [151,152] and collateral detergent sensitivity [153] which has been determined to be composed of two hydrophobic proteins, a diffusion channel and an ATP-binding moiety. Moreover, amplification of the bacA gene has been concluded to result in bacitracin resistance in *E. coli* [154]. Mutations on the *bacA* gene were also found to increase bacitracin susceptibility and change in virulence in *Streptococcus pneumoniae* and *S. aureus* [155]. Despite the above observations and extensive use since its discovery, bacitracin in general has not raised significant resistance.

6. Interactions with proteins and effects on cell membrane function

A few crystal structures have been resolved for bacitracin A_1 upon its binding to the serine proteases thermitase and savinase [156,157]. The groups involved in metal binding, including the thiazoline ring, Glu^4, and His^{10}, are well separated in these structures. Consequently, no metal is found in bacitracin upon its binding to the proteases. Bacitracin binds to savinase with a 2:2 ratio, in which each bacitracin binds to the catalytic site of one savinase and to the substrate binding site of another savinasae with the active sites of the two protease molecules facing each other. The structure of the protease molecules and their active sites are not affected by bacitracin binding. The D-Glu^4 side chain of bacitracin is H-bonded to savinase via $N_\epsilon H$ of His^{64} and $O_\gamma H$ and the main chain NH of Ser^{221} of the catalytic triad, and the oxy-anion hole-forming side chain of Asn^{155}. On one bacitracin molecule, D-Phe^9 fits into the substrate binding site of savinase which is significantly different from the other bacitracin molecule, where the D-Phe^9 side chain is not located in the substrate-binding site. Instead, His^{10} forms a weak H-bond with Ser^{130}. These crystal structures show that bacitracin molecule is quite flexible and can easily adopt different configurations upon interacting with biomolecules. To date, no crystal structures of bacitracin and its metal complexes have been published.

Bacitracin is known in general to inhibit proteases as in the crystallographic study described above and in several other cases [158–164], which is not related to their metal-binding properties. This antibiotic also inhibits metallopeptidases, presumably owing to its metal binding capability [165–167]. Bacitracin was reported to be an inhibitor of a metallo-insulinase [168]. However, a later study revealed that the most potent fractions in the bacitracin mixture for insulinase inhibition have no antibiotic activity and have molecular mass about twice that of bacitracin A [169]. Whether this inhibitor is a derivative of bacitracin or a compound unrelated to bacitracin awaits further structural characterization. The protease-binding property of bacitracin allows the use of this antibiotic for protease purification by means of bacitracin-affinity chromatography [170–172].

In addition to binding to proteases, bacitracin can also interact with membrane-bound proteins which has significant influence on the function of cell membrane. Bacitracin is known to strongly inhibit the membrane-bound glucosyltransferase system [173], and can inhibit protein disulfide isomerase (PDI) [174–176]. The inhibition of PDI on cell membrane by bacitracin can have a profound influence on cell function, including inhibition of platelet activation by standard agonists (but not platelet activation by peptide activators) [177], drug sensitivity of B-CLL cells [178], inhibition of HIV infection [179], inhibition of the reduction of activation of diphtheria toxin [176], inhibition of assembly of fibronectin and its inherent PDI activity [180], and selective inhibition of β1 and β7 integrin-mediated adherence of lymphoid cells to collagen, fibronectin, and/or laminin [181]. Whether or not these PDI inhibition activities are metal-dependent has not been investigated in the above studies.

The interaction of bacitracin with cell membrane can have a profound effect and change the morphology,

structure, and permeability of cell membranes and protoplasts [134,182–186] as well as artificial lipid vesicles or liposomes [187], which have been determined to be metal-dependent. Bacitracin noticeably increases the toxic effect of several divalent metal ions of the bacitracin-producing *Bacillus* and the mold *N. crassa* which has been suggested to be attributed to the increase in metal uptake due to this antibiotic [16,188]. This antibiotic can also inhibit the binding of ^{125}I-α_2-macroglobulin to plasma membrane [189] and was found to inhibit the insulin imprinting of *Tetrahymena* [190].

Some early studies showed that metallobacitracin can bind to a few isoprenyl phosphates and pyrophosphates [11,137,138], which reflects the membrane-binding capability of this antibiotic. The small dissociation constant K_d of 3.7×10^{-6} M for bacitracin binding to cells or protoplasts of *Micrococcus* [185] is consistent with the magnitude of the binding of this antibiotic to oligoisoprenyl pyrophosphate in vitro [137,138]. Bacitracin also exhibits a cation-dependent inhibition of nitrendipine binding to rat brain and cardiac membranes to different extents (IC_{50} = 400 and 4600 μg/ml, respectively), suggesting that brain and cardiac dihydropyridine-binding sites are either different or situated in different membrane environments [191].

7. Insight into structure–antibiotic relationship

The paramagnetic Co^{2+} complexes of several bacitracin congeners have recently been studied by means of 1D and 2D NMR techniques, including nuclear relaxation, chemical exchange techniques (i.e. difference spectra and EXSY) and a unique exchange-based (bond) correlation spectroscopy (exCOSY) [53,115]. Paramagnetic metal complexes are characteristic in showing far-shifted fast-relaxing NMR signals in a large spectral window which can be assigned to protons near the metal, resulting from both contact and dipolar shift mechanisms [192–195]. In the exCOSY spectra, only the peaks that undergo chemical exchange with the irradiated hyperfine shifted signal are detected. Thus, the clarity of an exCOSY spectrum is dramatically improved relative to a 'regular' COSY spectrum, which allows conclusive assignment of the hyperfine-shifted signals to be accomplished [53,115]. The Co^{2+} complexes exhibit many well resolved isotropically shifted ^{1}H NMR signals in a large spectral window (~200 ppm, Fig. 4). The hyperfine-shifted ^{1}H NMR signals of Co^{2+}-bacitracin A_1 have been completely assigned (as labeled in Fig. 4) which shows that the peptide binds to Co^{2+} via the His^{10} imidazole ring N_ϵ, the thiazoline nitrogen, and the Glu^4 carboxylate to form a labile complex in aqueous solutions at pH 5.4 [115]. The N-terminal amino group does not bind to Co^{2+} under the experimental conditions. In addition, there is no evidence to support the binding of the Asp^{11} carboxylate in contrast to the conclusion from the EPR study discussed above [126].

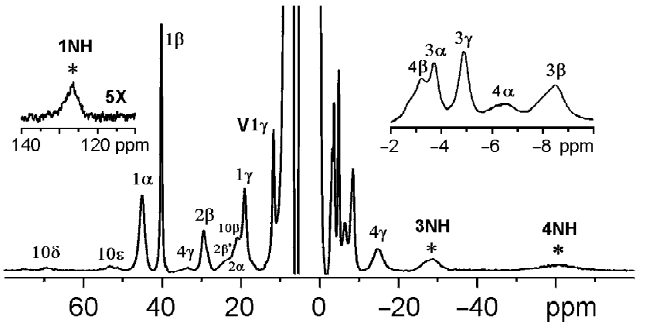

Fig. 4. ^{1}H NMR spectrum (250 MHz at ambient temperature) of the Co^{2+} complex of bacitracin mixture at pH 5.4. The hyperfine-shifted signals have been fully assigned as indicated. The peak at 11.7 ppm corresponds to the Val^1 γ protons of bacitracin B_1 in the mixture (cf. Fig. 6).

A structural model of Co^{2+}-bacitracin A_1 in mildly acidic aqueous solution was built by the use of the proton–Co^{2+} distances (r_{H-M}) obtained from T_1 values ($\propto r_{H-M}$) as restraints (Fig. 5) [192–195]. The structure indicates that the 'tail' of the bacitracin peptide (Ile^1 to Glu^4) wraps around the Co^{2+} ion with only a single group, the imidazole ring of His^{10}, bridging the metal ion to the cyclic heptapeptide moiety. The metal-binding ligands include the His^{10} imidazole, the Glu^4 carboxylate, and the thiazoline ring nitrogen with five or six coordination spheres *in solution* under the experimental conditions. This NMR study indicates that the metal coordination in solution may be different from the tetrahedral geometry observed in the recent EXAFS study in the solid state [128]. Although Co^{2+} and Zn^{2+} complexes are considered isostructural and Co^{2+} can be substituted for Zn^{2+} in biomolecules without affecting the structure and activity in almost all cases, whether or not Co^{2+}-bacitracin retains the same coordination chemistry as Zn^{2+}-bacitracin awaits further study of the structure of the latter complex.

The side chains of Phe^9 and Ile^5 in Co^{2+}-bacitracin are found to be close to each other and may form a flexible hydrophobic pocket [115], rendering it possible for the complex to bind with the hydrocarbon chain of isoprenyl pyrophosphates. This structure is quite different from that of the metal-free bacitracin determined previously by the use of NMR in which the side chain of Leu^3 was found to be close to those of Ile^8 and Phe^9 in one case at pH 3.2 [113] and backbone Leu^3 proton was found to be close to those of His^{10}, and Asn^{12} in another case at pH 4.85 [114], suggesting that a rearrangement of the hydrophobic moieties in bacitracin has occurred upon metal binding. These results suggest that it is important to study the metal-bound form of metal-dependent biomolecules because of the potential structural difference between the metal-free and metal-bound forms. Significant metal-induced structural change of antibiotic was also noticed from previous NMR [196] and crystallographic [197] studies of another metal-binding antibiotic streptonigrin. Nevertheless, this anti-

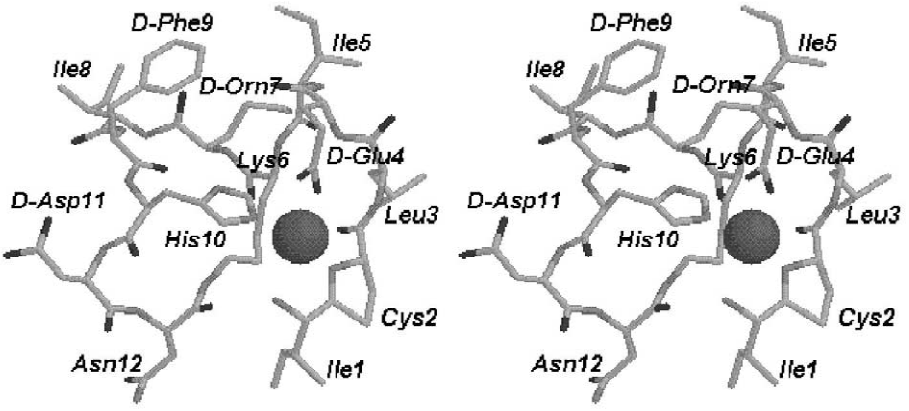

Fig. 5. Stereo views of Co^{2+}-bacitracin A_1 complex in H_2O at pH 5.0 produced by the use of relaxation time-generated distance constraints. The model shows that Phe^9 and Ile^5 are close to each other (~2.8 Å). A H-bond may be formed between the amino group of Ile^1 and the carbonyl of Asn^{12}.

biotic is found in all the NMR studies to adopt a configuration with the 'tail' of the first five amino acids folded to close proximity with the cyclic peptide structure.

The studies of Co^{2+} binding properties of several different bacitracin congeners indicate that the antimicrobial activities of these congeners correlate directly with their metal binding mode [115]. The isotropically shifted ^1H NMR spectral features of the high-potent bacitracin congeners, including bacitracins A_1, B_1, and B_2, are virtually identical (spectra A and B, Fig. 6) [115]. Since the isotropically shifted ^1H NMR signals can be attributed to the coordinated ligands and protons in close proximity to the metal, the observation indicates that the metal binding environments of these different metallobacitracin congeners are virtually identical. On the other hand, the bacitracin congeners with low antibiotic activities show different spectra from those of the active ones. For example, the protons due to Glu^4 are not observed in Co^{2+}-bacitracin A_2 and both Glu^4 and the thiazole ring are not detected in the Co^{2+} complex of bacitracin F (spectra C and D, Fig. 6), which reflects that these groups are not involved in Co^{2+} binding. This observation indicates that an appropriate metal binding, which involves Glu^4 and the thiazoline ring, is important for this peptide antibiotic to function properly. It is also interesting to note that the inversion of the stereochemistry of only one amino acid (L-Ile^1 to D-allo-Ile^1) in bacitracin A_2 that is not directly involved in metal binding can bring about such a significant change in the metal binding site and a dramatic decrease in the antibiotic activity.

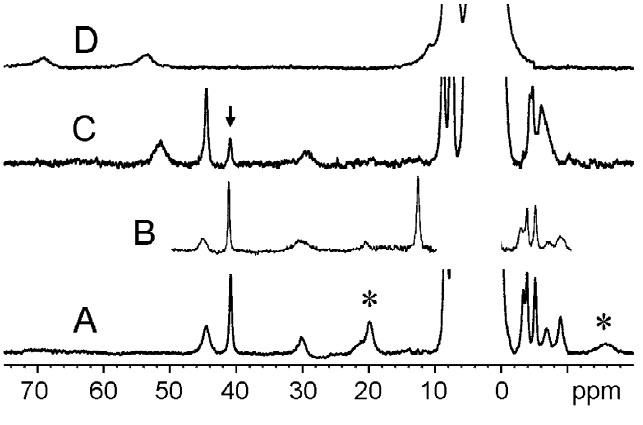

Fig. 6. Hyperfine-shifted ^1H NMR spectra (250 MHz at 298 K) of the Co^{2+} complexes of (A) bacitracin A_1, (B) bacitracin B_1, and (C) bacitracin A_2 in D_2O at pH meter-reading 5.4, and (D) bacitracin F at pH meter reading 7.5 in which only His^{10} signals are observed. Bacitracin B_2 shows identical hyperfine-shifted features as bacitracin A_1. The asterisked signals in A are attributable to Glu^4 which are not detected in Co^{2+}-bacitracin A_2 (C). The arrow in C indicates trace amount of A_1 that cannot be fully separated from A_2.

Metallobacitracin is known to bind long-chain isoprenol pyrophosphates which serves as the inhibition mechanism for bacterial cell wall synthesis as described in Section 5. The zinc form of this metalloantibiotic has also been determined to bind to phosphate ($K = 483\ M^{-1}$) and pyrophosphate (10,400 M^{-1}) as well as a few of their derivatives of long-chain alcohols, including isopentenyl pyrophosphate (8090 M^{-1}), farnesyl phosphate (5590 M^{-1}), farnesyl pyrophosphate ($8.3\times10^5\ M^{-1}$), and C55-isoprenyl pyrophosphate ($1.05\times10^6\ M^{-1}$ with Co^{2+}-bacitacin) [11,137,138]. However, structural information about these ternary complex has never been presented. The binding of several metal complexes of bacitracin with pyrophosphate follows the order $Zn^{2+} \gg Cd^{2+} > Mg^{2+} > Ni^{2+} > Co^{2+} > Hg^{2+} > Cu^{2+} \gg$ metal-free form [11]. The reason why the order does not follow the spectrochemical series for ligand-binding, particularly Cu^{2+}, awaits future exploration. The results corroborate a proposed mechanism for the action of this antibiotic, in which the binding of metallobacitracin to undecaprenyl pyrophosphate inhibits the synthesis of cell wall as well as the *N*-glycosylation process of luminal proteins [131]. Whether or not the different metallobacitracins can bind other phosphate and pyrophosphate-containing biomolecules and exhibit influ-

ence on a broad range of biological processes involving bioenergetics and some other metabolic pathways awaits future exploration.

8. Concluding remarks

Peptide and peptide-containing antibiotics have very diverse structures and functions that are associated with their significantly different molecular mechanisms of action, including inhibition of the synthesis of DNA, protein, and cell wall of microorganisms, and disruption of microbial membrane integrity [1–5]. Due to the emergence of bacterial resistance towards many of the commonly prescribed antibiotics, the development of new antibiotics is urgent. A recent publication indicates that the emergence of resistance against antibiotic peptides is less than against conventional antibiotics [198]. Bacitracin thus can serve as a prototype for the better understanding of the structure and function relationship of antibiotic peptide and as a lead drug for future rational design of antibiotic peptides. The available genes of bacitracin synthetase and other peptide and polyketide synthetases [63–83] afford us the tools for preparation of different congeners of peptide and polyketide as well as their hybrids which may serve as antibiotics for combating bacterial infections. Moreover, the success of chemical synthesis of bacitracin [108] also provides us with the means for this challenging task. In recent years, several families of metallopeptides have been designed as model systems for the exploration of metalloprotein structure and function [199–201]. Understanding of the structure and function relationship of the antibiotic peptide metallobacitracin may also point a new direction for the rational design of metallopeptides as potential models for metalloproteins.

9. Abbreviations

ABC	ATP-binding cassette
CCD	counter current distribution
COSY	(bond) correlation spectroscopy
EPR	electron paramagnetic resonance
ER	endoplasmic reticulum
EXAFS	extended X-ray absorption fine structure
exCOSY	exchange-based COSY
EXSY	exchange spectroscopy
GlcNAc	*N*-acetylglucosamine
HIV	human immunodeficiency virus
MIC	minimal growth inhibitory concentration
MurNAc	*N*-acetylmuramic acid
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
Orn	ornithine
PDI	protein disulfide isomerase
UDP	puridine diphosphate
UV/VIS	ultraviolet and visible absorption spectroscopy

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