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Cyclic lipodepsipeptides: a new class of antibacterial agents in the battle against resistant bacteria

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Abstract

In order to provide effective treatment options for infections caused by multidrug-resistant bacteria, innovative antibiotics are necessary, preferably with novel modes of action and/or belonging to novel classes of drugs. Naturally occurring cyclic lipodepsipeptides, which contain one or more ester bonds along with the amide bonds, have emerged as promising candidates for the development of new antibiotics. Some of these natural products are either already marketed or in advanced stages of clinical development. However, despite the progress in the development of new antibacterial agents, it is inevitable that resistant strains of bacteria will emerge in response to the widespread use of a particular antibiotic and limit its lifetime. Therefore, development of new antibiotics remains our most efficient way to counteract bacterial resistance.

The rapid rise of multi-antibiotic-resistant bacteria has become a global public health problem [1–4]. Currently, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp. (**ESKAPE pathogens**) are causing serious concerns due to the rapid spread of multidrug-resistant strains [5,6]. Cautious use of existing antibiotics may slow further development of resistance, however, in order to provide effective treatment options for the future, discovery and development of new antibiotics is necessary, preferably with novel modes of action and/or belonging to novel classes of drugs.

The glycopeptide antibiotic vancomycin has long been considered as the last line of defense against bacterial strains that are resistant to most of other antibiotics [7]. However, the emergence of vancomycin-resistant bacterial strains, most notably in enterococcal and staphylococcal nosocomial pathogens, further emphasizes the need for the development of new and more potent antibiotics.

Due to their structural diversity and high potency, naturally occurring cyclic **depsipeptides**, which contain one or more ester bonds in addition to the amide bonds, have attracted a great deal of attention for discovery of new antibiotics [8–10]. The biosynthesis of these peptides proceeds nonribosomally and is catalyzed by a complex of multifunctional enzymes; termed non-ribosomal peptide synthases (NRPSs) [11,12]. NRPSs have unique modular structure in which each module contains the requisite domains for the recognition and activation of a

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single amino acid, generating huge structural and functional diversity of nonribosomal peptides [13]. Within this class of natural products, cyclic lipodepsipeptide daptomycin (Cubicin®, Cubist Pharmaceuticals, Inc., MA, USA) is already approved in the USA, EU and Canada for the treatment of complicated skin and soft tissue infections caused by Gram-positive bacteria, including multidrug-resistant *S. aureus, Streptococcus pyogenes, Streptococcus agalactiae* and *Enterococcus faecalis* [8,14–18]. Daptomycin is also approved in the USA for the treatment of *S. aureus* bloodstream infections (bacteremia), including right-sided infective endocarditis. Unfortunately, staphylococcal and enterococcal clinical isolates with a loss of daptomycin susceptibility *in vitro* have already been reported [19–21]. Even more alarming is the observed cross-resistance between daptomycin and vancomycin in *S. aureus* [22–24].

Ramoplanin (Nanotherapeutics Inc., FL, USA), a glycolipodepsipeptide [8,25–27], is another promising clinical candidate for treatment of infections caused by resistant Gram-positive bacteria. Ramoplanin is currently entering Phase III clinical trials for the treatment of *Clostridium difficile*-associated diarrhea. No evidence of ramoplanin-resistant *C. difficile* has been reported [28].

In the quest for novel antibiotics, fusaricidin or the LI-F family of naturally occurring anti-fungal antibiotics emerge as attractive candidates for the development of new antibacterial agents capable of reverting infections caused by multi-drug-resistant bacteria [8,29–32]. As with the previous two examples, these natural products demonstrated most promising activity against a variety of Gram-positive bacteria [30,31]. In contrast to daptomycin and ramoplanin, **fusaricidins/ LI-Fs** possess neutral peptide sequence and a single positively charged guanidinium group located at the termini of the lipidic tail. Synthetic modifications of the most active member of this class of natural products, fusaricidin A/LI-F04A, which includes incorporation of a somewhat simpler lipidic tail and substitution of an ester bond, with an amide bond afforded potent analogs with improved stability and greatly decreased nonspecific cytotoxicity [33]. Structural differences between the fusaricidin/ LI-F family of antibacterial agents and daptomycin or ramoplanin suggest that these antibacterial peptides exert their effects through different mode of action, and that they may be active against daptomycin- and ramoplanin-resistant Gram-positive strains.

There are numerous literature reports describing isolation, characterization and antimicrobial activities of cyclic lipodepsipeptides. Here, the focus is on daptomycin, ramoplanin and fucaricidin/LI-F cyclic lipodepsipeptides as an illustration of this class of antimicrobial peptides therapeutic potentials. The role and applications of these cyclic lipodepsipeptides from a new antibiotic discovery perspective will be discussed.

Daptomycin

In the 1980s, Lilly Research Laboratories (Surrey, UK) reported isolation of a series of novel antibiotics designated as A21978A, B, C, D and E from *Streptomyces roseosporus* NRRL 11379 culture broth (Figure 1; Table 1) [34,201]. One of the isolated components, A21978C, exhibited high activity against Gram-positive bacteria [35]. The HPLC analysis of A21978C demonstrated that this is a mixture of six compounds (C_0–C_5), out of which compounds **1–3** (A21978C_1–C_3) were most abundant.

Structural analysis revealed all compounds to be cyclic lipodepsipeptides comprising 13 amino acid residues, five of which were nonproteinogenic amino acids, such as L-ornithine (L-Orn), L-*threo*-3-methylglutamic acid, L-kynurenine (L-Kyn) and two D-amino acid residues. Initially, the configuration of Asn^2 was assigned as L-, and recently corrected to a D-configuration [36]. Ten amino acids form the depsipeptide macrocyclic ring with a

lactone linkage between the L-Kyn^{13} carboxyl group and the Thr^{4} hydroxyl group. The remaining tripeptide, Asp^{3}-Asn^{2}-Trp^{1}, is attached to the Thr^{4} carboxyl group. The amino acid sequence is conserved in all six $C_{0–5}$ compounds and the structural differences lie in the fatty acid chain attached to the Trp^{1} amino group. In the compounds **1–3**, the fatty acid chain was found to be *anteiso*-undecanoyl, *iso*-dodecanoyl and *anteiso*-tridecanoyl, respectively. A minor component of the complex, C_0, was found to have a mixture of both linear and branched decanoyl, whereas C_4 and C_5 both contain dodecanoyl fatty acids [37]. The member of the A21978C complex with the linear decanoyl fatty acid was named daptomycin **(4;** Figure 1; Table 1).

Comparison of A21978C$_1$ **(1)** activity with those of vancomycin, teichomycin and several β-lactam antibiotics, demonstrated that **1** has comparable activity to vancomycin against all streptococci and staphylococci tested, including methicillin-resistant *S. aureus* (MRSA) and penicillin-resistant pneumococci [38]. It was also found to be bactericidal against enterococci at concentrations close to MICs (2 μg/ml). A21978C$_1$ did not interact with penicillin-binding proteins of bacterial cell membranes nor did it interfere with the DNA, RNA or protein synthesis. However, **1** inhibited peptidoglycan biosynthesis of both *Streptococcus faecalis* and *S. aureus*. Even more interesting was the finding that the activity of this family of naturally occurring antibiotics strongly depends on the concentration of Ca^{2+} in the culture medium, whereas no increase in activity was observed if medium is supplemented with Mg^{2+}, Zn^{2+} or Ba^{2+} [38]. The maximum antibacterial potency of A21978C is reached at the Ca^{2+} concentration of 50 mg/l (1.25 mM); the concentration of ionized calcium normally found in human serum [39]. It has also been demonstrated that these antibiotics cause potassium release from *S.aureus*, suggesting that their bacterial target is the membrane and/or cell wall [40,41]. Altogether, the activity of A21978C antibiotics was proposed to be very complex, depending on the insertion of the antibiotic into the bacterial membrane. Studies performed by Lakey *et al.*, and more recently by Jung *et al.*, on daptomycins' interaction with phospholipid vesicles in the presence of Ca^{2+} suggested that charge neutralization through Ca^{2+} complexation facilitates penetration of the peptide into the membrane [42,43]. It was found that the extent of penetration into the lipid bilayer is inversely proportional to the length of the fatty acid side chain, indicating that the Ca^{2+}-dependent interaction is a specific interaction involving polar head groups of the phospholipids and the lipopeptide, with the role of the fatty acid chain being the most important for the initial antibiotic attachment to the membrane. However, the length of the fatty acid was reported to have a significant impact on peptide's nonselective toxicity. Peptides bearing longer side chain fatty acids exhibited higher toxicity, while the optimal balance between toxicity and antimicrobial activity was obtained with the A21978C derivative, compound **4,** containing a decanoyl chain, which was later named daptomycin [44,45]. In 1988, Huber *et al.* reported large quantity production of desired daptomycin by supplying decanoic acid during *S.roseosporus* fermentation, which, to this day, remains the commercial way of producing this antibiotic [46–48]. The lack of suitable solution or solid-phase synthetic approaches toward daptomycin and its analogs means chemoenzymatic methods are important alternatives to generate and scale up daptomycin derivatives for antibacterial screening and drug development. Chemoenzymatic methods usually combine standard solid-phase peptide synthesis with enzymatic peptide cyclization mediated through excised thioesterase domains from NRPSs [49,50]. Antibacterial activity studies of chemoenzymatically synthesized daptomycin derivatives revealed that four amino acids, Asp^{7}, Asp^{9}, L-3-MeGlu^{12} and L-Kyn^{13}, are important for the antibacterial potency of a peptide. Furthermore, derivatives containing different macrocyclic core size were prepared by shifting the Thr^{4} residue to position 3, 5 or 6 in the linear precursor generating eight-, nine- and 11-membered rings [50]. Genetic engineering of the NRPS in the daptomycin biosynthesis was further exploited for combinatorial biosynthesis of novel antibiotics. Baltz *et al.* combined NRPS module exchanges, NRPS subunit exchanges, inactivation of the

tailoring enzyme glutamic acid 3-methyltransferase and natural variation of the lipid tail to generate a library of daptomycin analogs [35,51]. These bioengineered daptomycin analogs include modifications at the L-Kyn^{13} position, and modifications of the daptomycin macrocyclic core at residues that are not conserved among structurally related lipopeptides. Some of these analogs were as active as daptomycin, demonstrating that the combinatorial biosynthesis is an effective tool to generate daptomycin analogs with significant structural diversity for further clinical evaluation.

A semisynthetic method was also successfully applied to modify the daptomycin amino acid core. In an attempt to enhance the potency of daptomycin, Parr *et al.* synthesized a series of N$^{\delta}$-modified L-Orn^{6} analogs using activated esters, anhydrides, and guanidinylating reagents [52]. Based on the MIC data, it was observed that the Orn^{6} amino group is not essential for the antibacterial activity of daptomycin. Some analogs maintaining a free amine in the Orn region demonstrated similar or slightly better antibacterial activity, and significantly different pharmacokinetic profiles than daptomycin [52].

Although it is well known that daptomycin exerts its mode of action on the bacterial cell membrane in Gram-positive bacteria, its precise mechanism of action is still not fully understood. Initially, Allen *et al.* demonstrated that daptomycin disrupts the bacterial membrane rendering it permeable, as demonstrated by the loss of intracellular potassium upon exposure of bacteria to the peptide. The fact that this antibiotic inhibited incorporation of [^{14}C] alanine and [^{14}C] diaminopimelic acid into the peptidoglycan of *S. aureus* and *Bacillus megaterium*, and the lack of any significant inhibition of protein, RNA, DNA or lipid biosynthesis indicated that the membrane is probably not a lethal target for this antibiotic. Instead, they suggested that daptomycin inhibits formation of peptidoglycan precursor, UDP-MurNAc-pentapeptide [53]. Silverman *et al.* proposed a multistep mode of action for daptomycin (Figure 2) [41]. According to the proposed mechanism, in the first step, daptomycin weakly binds to the cytoplasmic membrane and complex Ca^{2+}. This causes a conformational change and leads to the insertion of daptomycin into the plasma membrane and its subsequent oligomerization.

In the second step, daptomycin oligomers form a channel, through which intracellular potassium is lost, resulting in membrane depolarization and subsequent bacterial cell death. Recently, a more complex mode of action was proposed for daptomycin. Hancock and colleagues suggested that the bactericidal action of daptomycin is not exclusively a result of the membrane depolarization, but rather daptomycin's interaction with several bacterial components, such as the cell wall, various enzymes, RNA and DNA [43]. In 2008, Chopra and coworkers re-examined the daptomycin mode of action proposed by Silverman *et al.* by performing more detailed experiments on kinetics of membrane depolarization and loss of K^{+}, Mg^{2+} and ATP [54]. Obtained experimental data showed that daptomycin-induced efflux of Mg^{2+} and ATP occurs in conjunction with K^{+} leakage. Based on these findings, Chopra *et al.* proposed that the bactericidal activity of daptomycin is not simply a consequence of K^{+} efflux and probably involves more general disruption of the membrane.

Although Eli Lilly and Co. (IN, USA) began development of daptomycin in 1985, they later abandoned further pursuit due to Phase II clinical trial results demonstrating the occurrence of potential drug-induced myopathic events [55]. In 1997, Cubist Pharmaceuticals Inc. licensed daptomycin and re-instigated clinical development of the drug. To determine the dosing regimen with potentially lower muscle toxicity, two studies were conducted with dogs. Repeated intravenous administration every 24 h versus every 8 h for 20 days indicated that once-daily administration appeared to have minimized potential for daptomycin-induced skeletal-muscle effects [56]. Further clinical trials were conducted using a single, daily dose of the antibiotic. Daptomycin was approved in the USA in 2003 for the treatment of

complicated skin and skin structure infections caused by *S. aureus* (MSSA), MRSA, *S. pyogenes*, *S. agalactiae*, *Streptococcus dysgalactiae* subsp. *equisimilis* and *E. faecalis* (vancomycin-susceptible only) [17], and, in 2006, for the treatment of bacteremia and right-sided endocarditis caused by *S. aureus*, including MRSA [18]. In addition, it was approved in Europe in 2006 for the treatment of complicated skin and soft tissue infections. Daptomycin is marketed under the trade name Cubicin.

Daptomycin is given intravenously at a dose of 4–6 mg/kg once daily. It has linear pharmaco-kinetics with a half-life of 8–9 h and steady state is reached by day 3 [57]. The primary route of excretion is via kidneys with approximately 78% urinary recovery [58]. Despite high protein binding (up to 94%), protein-bound daptomycin is bioavailable and the binding is independent of the antibiotic concentration [59–61]. Daptomycin does not interact with the P450 cytochrome and, consequently, there are no known cytochrome P450-mediated drug–drug interactions between daptomycin and other drugs [57,58,62]. The volume of distribution is low (~0.1 l/kg) and corresponds to the concentration of the drug in plasma and interstitial fluid [58,62].

Although daptomycin exhibits strong activity against a variety of Gram-positive bacteria, including *Streptococcus pneumoniae*, the experimental and clinical data for mice and humans demonstrate that daptomycin lacks *in vivo* efficacy against Gram-positive bacterial lung infections [63,64]. In a Phase III clinical trial for the treatment of patients with community-acquired pneumonia (CAP) daptomycin (applied at a dose of 4 mg/kg) failed to achieve superiority over ceftriaxone, a drug of choice for the treatment of CAP. Reported efficacies for daptomycin and ceftriaxone are 79 and 87%, respectively [63,64]. The poor efficacy of daptomycin against CAP may be attributed to its interaction with pulmonary surfactant components [63]. Recent attempts to generate more potent daptomycin derivatives in the presence of bovine surfactant utilize combinatorial biosynthesis approach [65,66].

A54145 is a family of cyclic lipodepsipeptides produced by *Streptomyces fradiae* very similar to daptomycin, considering both structural similarity and the mode of action. Structure of A54145A **(5)**, a representative member of this family is presented in Figure 3 & Table 2 [47,48,67–69]. In general, these antibiotics exhibited potent *in vitro*, as well as *in vivo*, activity against *S.aureus* and *S. pyogenes*. However, acute mouse toxicities hampered their further development as a novel drug [67]. Interestingly, some of the A54145 antibiotics exhibited better activity in the presence of 1% bovine surfactant than daptomycin, providing, therefore, a lead structure for modifications to explore structure–activity relationship. Baltz *et al*. reported preparation of novel A5415 analogs by modifying A54145 NRPSs using segments of daptomycin NRPS genes [65,70–72]. Three mutants defective in *IptJ*, *IptK* or *IptL* genes encoding the enzymes involved in the biosynthesis of hydroxy-Asn^3 and methoxy-Asp^9 were generated. These deletions were combined with the deletion of the *IptI* gene involved in the biosynthesis of 3-methyl-Glu^{12} and with the plasmid containing combination of *Ipt* genes to produce a series of novel A5415/daptomycin analogs. For all of the compounds, removal of the methoxy group from Asp^9 and the hydroxy group from Asn^3 yielded compound A54145(Asn^3Asp^9), with comparable antibacterial activity to the parent A54145 or daptomycin, and without loss in activity in the presence of bovine surfactant [70]. The structure of A54145(Asn^3Asp^9), predicted from the genetic changes and bioinformatics studies was confirmed by chemical transformations, amino acid quantitation by enantiomer labeling, LC–MS/MS and 2D NMR techniques [72].

The development of resistance to daptomycin during the treatment has been well characterized for *S. aureus*, and to lesser extent for enterococci [73,74]. Multiple mechanisms involving changes in the net cell surface charge and/or altered cell membrane order (fluidity) leading to reduced affinity for daptomycin have been associated with

resistance to daptomycin [21,73–81]. In addition to bacterial membrane changes, thickening of the cell walls in *S.aureus* was also correlated with resistance to daptomycin [80,82]. However, recent studies demonstrated that cell wall thickness is not a universal marker for *S. aureus* reduced susceptibility to daptomycin [74]. Interestingly, the majority of these phenotypic perturbations have also been related to the reduced susceptibility to cationic antimicrobial peptides [74,78,81].

Several genes have been associated with the occurrence of daptomycin resistance in *S. aureus* and enterococci. These include:

- Genes encoding for the two- or three-component regulatory systems involved in the homeostasis of the cell envelope: *yvqF-vraSR* in *S. aureus* and *liaFSR* in enterococci [73,83];
- Genes encoding for enzymes involved in the metabolism of the cell membrane phospholipids: *mprF*, *cls2* and *pgsA* [75,83,84] in *S. aureus*, as well as *gdpD* and *cls2* in enterococci [73,85];
- Genes encoding for sensor histidine kinase that regulates cell wall metabolism and virulence in *S.aureus : walK* (previously *yycG*) [86];
- Genes encoding for β subunits of RNA polymerase in *S. aureus: rpoB/rpoC* [84,86,87].

The most notable genetic perturbations linked to daptomycin resistance in *S.aureus* are single point mutations in the *mprF* open reading frame [75,86,88,89]. MprF is a membrane protein that mediates both lysinylation of phosphatidyl-glycerol (PG) as well as its translocation to the outer leaflet of the membrane [90]. Point mutations in this protein appear to cause a gain-in-function, thus resulting in the accelerated translocation of lysyl-PG (LPG) and, therefore, leading to reduced net negative charge which may repel calcium–daptomycin complexes [76]. Also, increased expression of the *dltABCD* operon responsible for D-alanylation of teichoic acids in the cell wall contributes to the increase in the net positive surface charge [76]. Peleg *et al*. identified two mutations in the genes responsible for the production of anionic phospholipids, *pgsA* phosphatidyltransferase involved in biosynthesis of PG and *cls2* synthase involved in the biosynthesis of cardiolipin [84]. These mutations were correlated with changes in the phospholipid membrane composition and changes in the cell wall thickness in the *S. aureus* clinical isolates [84]. These mutations may occur in isolation or in concert, in particular mutations in *mprF* and *cls2*, and *in vivo* pressures from daptomycin exposure can result in a whole range of genetic mutations, including those involving virulence regulatory genes [84]. In laboratory studies, individual mutations in *mprF*, *rpoB*, *rpoC* and *walK* account for approximately twofold increase in MIC and only combinations of these mutations can give rise to a higher level of resistance while daptomycin-resistant clinical isolates had mutations in *mprF* [86,88,91] or *walK* [86].

Ramoplanins/enduracidins

Ramoplanin, originally named A-16686, was isolated by a research group from Gruppo LePetit (Biosearch Italia, Gerenzano, Italy) in 1984 as an antibiotic complex from the fermentation broth of *Actinoplanes* sp. (ATCC 33076; Figure 4; Table 3). The isolated antibiotic complex was a mixture of three structurally similar compounds **6–8**, among which compound **7** was the major component [92].

Early studies of these natural products included chemical degradation, MS and 2D NMR experiments, and revealed the structural complexity of this family of natural products [93]. The isolated compounds consist of a cyclic lipoglycodepsipeptides containing 17 amino acids (49-membered macrocycle), which include L- and D-amino acids along with other

nonproteinogenic amino acids, such as L-hydroxyphenylglycine (L-Hpg), D- and L-*a*Thr, L-*threo*-β-hydroxyasparagine (L-*t*HyAsn), D-ornithine (D-Orn) and L-3-chloro-4-hydroxyphenylglycine (L-Chp).

The macrocycle is linked via lactone between the L-Chp^{17} carboxyl group and the β-hydroxyl group present in L-*t*HyAsn^{2}. This residue is connected to Asn^{1}, which bears an unusual unsaturated acyl chain on its N-terminus. The stereochemistry of ramoplanin's lipid chain was initially assigned to be (2*Z*,4*Z*) [93], however, this was subsequently reexamined and corrected to (2*Z*,4*E*) [94,95]. Although the amino acid sequence is highly conserved within the ramoplanin family of natural products, they differ in the length and structure of their unsaturated acyl chain, (Figure 4; Table 3). The presence of L-Hpg^{11} residue that carries a disaccharide moiety, α-D-mannosyl-(1→2)-α–D-mannose, attached to its hydroxyl group further increases ramoplanin's structural complexity. Ramoplanins are structurally similar to ramoplanose **(9)**, a trimannosylated variant of ramoplanin also isolated from *Actinoplanes* sp. [96] and to enduracidins. Enduracidin A and B (**10 & 11**, respectively; Figure 5) were first isolated in 1968 from *Streptomyces fungicides* B5477 culture broth [97,98], differing only in the length of the lipidic tail. Enduracidins are 17-mer peptides possessing a macrocyclic core composed of 16-amino acids cyclized via a lactone bridge between Thr^{2} and L-Hpg^{17} residues (Figure 6; Table 4). Enduracidins display various unusual amino acids as part of their primary sequence, such as D- and L-endur-acididine (D-, L-End), L-citrulline (L-Cit), and L-3,5-dichloro-4-hydroxyphenylglycine (L-Dpg). Structural similarity between enduracidins and ramoplanins suggest a possible similarity in the mode of action and a common active pharmacophore [99]. However, it is worth noting that enduracidins do not have a sugar moiety.

Multidimensional NMR experiments in aqueous solution performed in 1996 by Kurtz and Guba, permitted the elucidation of the 3D structure of ramoplanin A2 **7**. The structure is characterized by two antiparallel β-sheets connected with six intramolecular hydrogen bonds and one reverse β-turn [94]. Almost identical structural characteristics were found previously in **9**, since 3D NMR revealed a double-stranded antiparallel β-sheet with seven intramolecular hydrogen bonds and two reverse turns [96]. Interestingly, Walker *et al.* demonstrated that ramoplanin A2 **(7)** in methanol is present as a mixture of monomer and dimer in slow exchange [100]. Under these conditions, **7** forms a C_2-symmetric dimer at the interface of residues 10–14, which is stabilized by four intermolecular hydrogen bonds. In 2009 McCafferty and colleagues reported the first x-ray structure of ramoplanin A2, demonstrating that the compound exists in the crystal as an amphiphatic dimer with C_2-symmetry. The mutual orientation of monomers permits the interaction of amino acid residues 9–15 and the formation of six intermolecular hydrogen bonds, resulting in a four-stranded antiparallel β-sheet [101]. The remarkable ability of compound **7** to form dimers in a hydrophobic solvent that mimics the environment at the bacterial cell surface and in the crystal, strongly supports previous findings about the way this antibiotic interacts with its bacterial target, Lipid II (*vide infra*). Not surprisingly, due to amino acid sequence similarities, the overall backbone structure of enduracidin and ramoplanin exhibit several common structural features [102]. Thus, enduracidins form two antiparallel β-sheets that involve residues 5–7 and 10–12 connected by a turn composed of the residues 8 and 9. Three hydrogen-bonding interactions between residues D-*a*Thr^{5} and D-Ser^{12}, D-Hpg^{7} and D-End^{10}, and D-Hpg^{7} and L-Cit^{9} take part in stabilizing this β-hairpin arrangement [102].

Ramoplanins A1–A3 **(6–8,** Figure 4) showed identical *in vitro* antibiotic activities and are active against variety of Gram-positive bacteria, including resistant strains such as vancomycin resistant enterococci (VRE) and MRSA at MIC levels <1 μg/ml [103–106]. They exhibit similar spectrum of antibacterial activities as vancomycin, however, they are four–eight times more potent [107]. Moreover, where vancomycin displays bacteriostatic

activity at its MICs, ramoplanin A2 exhibits bactericidal activity at concentrations close to its MICs for most Gram-positive bacteria, and at concentrations two times MIC against VRE [105,108]. On the other hand, ramoplanins **6–8** demonstrated no activity against Gram-negative bacteria [107,109]. Less than a decade after the isolation of ramoplanins A1–A3, three other ramoplanin analogs were isolated from the culture broth of the producing microorganism and named A1–A3. These new compounds present a monosaccharide unit linked to residue L-Hpg^{11}, instead of the α-1,2-dimannose moiety found on ramoplanins A1–A3 [110]. Remarkably, ramoplanin A2 exhibited better activity against certain bacterial strains than A2 [110]. Enduracidins **10–11** (Figure 5) demonstrated a similar spectrum of *in vitro* antimicrobial activityes to ramoplanins, whereas no antibacterial activity has been reported for **9** [111].

Due to the intrinsic structural complexity of ramoplanins and its great potential as novel antimicrobial agent, many efforts have been focused in the elucidation of its minimal bio-active pharmacophore. Ciabatti *et al*. reported the synthesis of tetrahydroramoplanin **(12)** by hydrogenation of ramoplanin A2, (Figure 6; Table 4) [202]. Also, the same group described the semisynthetic access to ramoplanin A2 aglycon **(13)** by treatment with trimethylsilyl iodide [203,204]. These modifications had no significant effect on the antibacterial activity of ramoplanin. Later, McCafferty *et al*. confirmed these results and suggested a key role of the disaccharide of ramoplanin in its hydrolytic (acidic media) stability and conformational rigidity. Assuming a fundamental role of the D-Orn^4 and D-Orn^{10} side chains in the interaction of ramoplanin with peptidoglycan biosynthetic intermediates, this group also reported the synthesis of several ramoplanin analogs via simple chemical modification of these basic residues (Figure 6; Table 4) [99]. Hence, guanidylation of ramoplanin using 1*H*-pyrazole-1-carboxamidine gave [Orn^4, Orn^{10}]-diguanidylated derivative **14** with comparable potency to the unmodified ramoplanin.

Reductive amination of the Orn side chains with isovaleryl aldehyde in the presence of NaC-NBH_3 afforded analog **15**, which preserves the original cationic character while increasing the steric bulk. This analog exhibit significantly reduced antimicrobial activity in comparison to ramoplanin, however, it maintained the ability to bind peptidoglycan biosynthetic intermediates. In contrast, a remarkable loss of antibiotic activity was reported for the acetylated analog of ramoplanin **16**, as well as for the analog in which the lactone linkage was hydrolyzed **17** [99,112,113]. Total solution-phase syntheses of **7**, **9** and ramoplanin A1–A3 aglycons, were accomplished by Boger and colleagues [95,112,114]. The retrosynthetic analysis was based on ramoplanin A2 3D structure in solution. Supported on this analysis, three linear peptide units (heptapeptide composed of residues 3–9, pentadepsipeptide composed of residues 1,2 and 15–17, and pentapeptide composed of residues 10–14) were synthesized and sequentially coupled. In order to maximize the efficacy of the final cyclization, the coupling sites were chosen to prevent potential racemization and to favor a β-sheet secondary structure that would place the reactive sites in close proximity. Following this approach, the 17-amino acid linear intermediate was efficiently cyclized to yield the ramoplanin aglycon macrocyclic core. Ramoplanin A1 and A3 aglycons were prepared by coupling of the lipid side chains to the synthetic ramoplanin macrocyclic core [115]. Boger's group also described the synthesis of ramoplanin A2 analogs in which the labile lactone bond was replaced by a more stable amide [116]. Therefore, the non-proteinogenic amino acid L-*t*HyAsn^2 was replaced by L-2,3-diaminopropionic acid (L-Dap) or L-2,4-diaminobutyric acid to yield the corresponding amide aglycon analogs [116]. While the L-Dap-analog maintained the same activity as ramoplanin A2, the L-2,4-diaminobutyric acid analog, containing an additional methylene group in the macrocycle, was devoid of antimicrobial activity. To explore the potential role and importance of each amino acid residue on the antibacterial activity of the L-Dap analog of ramoplanin, an alanine-scanning has been employed [117]. The Ala-scanning study

defined the amino acids and regions amenable to modifications aiming for a better understanding of the molecular interactions related to ramoplanin's mode of action. In addition, modifications of the lipidic part of ramoplanin A2 to improve its tolerability profile were reported as well [118]. Ozonolysis of the [Orn^4, Orn^{10}]-diFmoc protected ramoplanin followed by reductive amination of the resulting aldehyde with benzylamine provided an intermediate possessing a terminal *N*-Bn glycine. This amino acid was conveniently removed by Edman degradation and the product acylated with wide range of carboxylic acids. Recently, chemoenzymatic methods have been described for the efficient deacetylation of ramoplanin A2 with excellent yields and purities (84%) [119]. The modification of ramoplanin's lipid fragments proved to be a valid strategy in the search of analogs with enhanced tolerability [118]. In contrast, there are no reports on synthetic modification of enduracidins. However, biosynthesis of enduracidin A **(10)** analogs with altered halogenation patterns has been reported in 2010, and initial *in vitro* antibacterial evaluation showed that these analogs retained activity against *S. aureus* [120].

In 1990, Somner and Reynolds reported that ramoplanin A2 blocks bacterial cell wall biosynthesis at the step catalyzed by membrane-associated glycosyltrensferase MurG [121]. They suggested a mechanism of action in which **7** binds to MurG substrate Lipid I and, therefore, inhibit the synthesis of peptidoglycan's key building block Lipid II (Figure 7). Later, Brötz and coworkers revealed that ramoplanin A2 **7**, as well as some lantibiotics, interact with Lipid II. These results support an alternative mode of action for **7** [122]. Further studies by Walker *et al.* confirmed that ramoplanin A2 exhibits higher affinity for Lipid II than Lipid I, and proposed the transglycosylation step of the peptidoglycan assembly as its primary target. Inhibition kinetic experiments revealed that ramoplanin A2 binds Lipid II with a stoichiometry of 2:1 [123]. Besides, fibril formation of Lipid II analogs was observed upon titration with ramoplanin A2 **7** [99,124]. Walker's group also found that ramoplanin A2 inhibits MurG via direct interaction with the enzyme rather than by a mechanism involving binding to Lipid I [125]. In addition, since Lipid I and MurG are intracellular and, therefore, not accessible to ramoplanin [124,126], and considering ramoplanin's higher affinity toward Lipid II, it is not clear whether inhibition of MurG is of physiological relevance. Enduracidin has shown similar mode of action as ramoplanin A2, however, enduracidin produced an inhibition curve consistent with substrate binding [124].

Ramoplanin A2 is currently in Phase III clinical trials for the temporary suppression of VRE gastrointestinal carriage and for the treatment of *Clostridium difficile*-associated diarrhea. Although adverse gastrointestinal effects, including nausea, diarrhea, abdominal pain, dyspepsia and flatulence, have been observed, two Phase II studies have documented the tolerability and safety of oral ramoplanin [127,128]. Recently, the US FDA has granted ramoplanin A2 fast-track status for the prevention of VRE and treatment of *C. difficile* colitis [129].

Laboratory-generated *S. aureus* strain resistant to this antibiotic has been described recently [130]. Both the thickened cell wall and the decreased activity of autolytic enzymes in ramoplanin-resistant *S. aureus* probably contribute to the decreased susceptibility to ramoplanin. Although ramoplanin, vancomycin and nisin (polycyclic antibacterial peptide) exert their antibacterial activities via different mechanisms, a cross-resistance among these three antibiotics in *S. aureus* was observed [130].

Fusaricidins/LI-Fs

Fusaricidins or LI-Fs **(18–29)** are a new family of cyclic lipodepsipeptide antifungal antibiotics isolated from *Paenibacillus* sp. (Figure 8; Table 5) [29–31]. Common structural characteristics of these natural products are the peptide ring consisting of six amino acid

residues, three of which, L-Thr[1], D-*a*Thr[4], D-Ala[6] are conserved throughout the family, and a 15-guanidino-3-hydroxypentadecanoic acid attached via amide bond to the N-terminal Thr[1]. Fusaricidins/ LI-Fs are cyclized by a lactone bridge between *N*-terminal Thr[1] hydroxyl group and C-terminal D-Ala[6].

In 1987, Kurusu and Ohba reported isolation of LI-Fs **18–29** from *Paenibacillus polymyxa* L-1129 strain [29,32]. Although initial analysis of the natural products mixture isolated from the fermentation broth showed the presence of at least 10 compounds, five cyclic lipodepsipeptides were isolated and subsequently named LI-F03, F04, F05, F07 and F08. It was also found that each isolated product contains a mixture of two homologous components [29]. In 2000 Kuroda *et al.* reported sequences of all isolated LI-F natural products (Figure 8; Table 5) [32]. Isolated cyclic lipodepsipeptides demonstrated high *in vitro* activity against a variety of fungi and Gram-positive bacteria, whereas no activity was observed against Gram-negative bacteria [29]. Approximately a decade after the discovery of the LI-F family of anti-fungal antibiotics, Kajimura and Kaneda isolated a series of compounds named fusaricidins **(18–21)** from the culture broth of *P. polymyxa* KT-8 strain [30,31]. Fusaricidins A–D **(18–21;** Figure 8; Table 5) are analogous to the LI-F family of antimicrobial antibiotics. In fact, LI-F peptides were later determined to be mixtures containing previously structurally characterized and new fusaricidins [32,131]. Out of the four isolated fusaricidin antibiotics, fusaricidin A/LIF[04]a **(20)**, demonstrated the most promising *in vitro* antimicrobial activity against a variety of fungi, including the clinically important *Candida albicans* and *Cryptococcus neoformans* [32]. Fusaricidin A/LI-F04a **(20)** also exhibits potent *in vitro* activity against Gram-positive bacteria such as *S. aureus* (MIC values ranging from 0.78 to 3.12 μg/ml), and no activity against Gram-negative bacteria was observed [29,30]. Low acute toxicity in mice has been reported for intraperitoneally injected LI-F03, LI-F04, LI-F05, LI-F07, and LI-F08 (LD_{50} of 150–200 mg/kg) [46]. Precise mode of action of fusaricidins/LI-Fs is yet to be determined.

Two solid-phase synthetic approaches toward the most active fusaricidin A/LI-F04a **20** and related analogs have been reported recently, representing important initial steps toward full exploitation of this class of cyclic lipodepsipeptides antimicrobial potentials. The authors have reported complete Fmoc solid-phase synthesis of a fusaricidin A/LI-F04a analog **35** containing 12-guanidino dodecanoic acid instead of naturally occurring 15-guanidino-3-hydroxy-hexadecanoic acid (Figure 9; Table 6) [33,132]. The synthetic approach includes resin attachment of the first amino acid, D-Asp[5] via side chain, successful combination of four quasiorthogonal temporary protecting groups, stepwise Fmoc solid-phase synthesis of a linear precursor peptide, lipidic tail attachment followed by last amino acid coupling via ester bond and on-resin head-to-tail macrolactamization. This strategy allows the complete suppression of the undesired side reactions, and an efficient automated solid-phase synthesis of cyclic lipodepsipeptides [132].

Since the entire synthesis was accomplished on the solid support, this opened a possibility for structure activity relationship analysis of fusaricidin A/LI-F04a analogs. For this purpose a series of 18 fusaricidin A/LI-F04a analogs **30–47** was prepared and the effect of their structure modification on conformation, serum stability, antibacterial activity and human cell toxicity was investigated (Figure 9; Table 6). Similar to the natural products, these synthetic cyclic lipopeptides are active only against Gram-positive bacteria. Unfortunately, due to the differences in experimental conditions, it is difficult to compare directly antibacterial activities of the synthetic analogs **30–47** with the natural products **18–29**.

The positively charged guanidinium group at the terminus of the 12-carbon lipidic tail and the presence of hydrophobic amino acids in the depsipeptide sequence are crucial for antibacterial activity. Introduction of neutral and hydrophobic amino acids into position 3 of

the depsipeptide ring sequence allowed manipulation of the overall hydrophobicity and amphiphilicity of fusaricidin/LI-F analogs without loss of antibacterial potency (e.g., analogs **35** and **40**). However, these structural changes leading to an increase in the overall hydrophobicity and amphiphilicity of the depsipeptide resulted in an increase in nonselective cytotoxicity, as demonstrated by hemolytic activity assay (Figure 10).

On the other hand, substitution of an ester bond in depsipeptides by an amide bond gave more stable cyclic lipopeptide analogs **(43–46;** Figure 9; Table 6) with *in vitro* antibacterial activities comparable with the parent depsipeptides, yet greatly decreased human cell toxicity (Figure 11). Lower overall hydrophobicity/ amphiphilicity of amide analogs relative to their parent depsipeptides most likely contributed to the dissociation of antibacterial activity from human cell cytotoxicity. More stable and less cytotoxic amide analogs may have significant advantages over naturally occurring fusaricidin A/LI-F04a and its depsipeptide analogs as lead structures for the development of new antibacterial agents.

Total synthesis of fusaricidin A/LI-F04a **(48;** Figure 12; Table 7) was reported by Cochrane *et al.* [133,134]. In this approach, a linear peptide sequence was assembled on a solid-support using Fmoc-chemistry, followed by peptide cleavage, in solution macrolactonization, and final attachment of 15-guanidino-3-hydroxyhexadecanoic acid. Macrolactonization, a key step in this synthetic approach, was achieved by applying the Corey–Nicolaou, Boden–Keck or Yamaguchi lactonization procedures [134]. However, in all three cases analysis of the reaction, products revealed the mixtures of cyclic diastereoisomers, suggesting that the C-terminal D-Ala^6 underwent undesired epimerization during the macrolactonization reactions [134]. By total synthesis of both (*R*)- and (*S*)-15-guanidino-3-hydroxyhexadecanoic acid it has been determined that the absolute configuration of this fatty acid hydroxy group is (*R*) in the natural product fusaricidin A/LI-F04a **(20)** [133,134]. Nevertheless, it is not yet known whether this stereochemistry is conserved within the family. Synthetic fusaricidin A/LI-F04a **(48)** was found to exhibit similar antifungal activity against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* to that reported for the isolated natural product **(20;** Figure 12; Table 7). Inversion of fatty acid hydroxyl group stereochemistry, isomer **49**, or hydroxyl group removal, derivate **50,** resulted in a decrease of antifungal activity. On the other hand, epimerization of the C-terminal D-Ala^6, isomers **51** and **52**, led to a complete loss of antifungal activity. No antibacterial activity was reported for synthetic fusaricidin A/LI-F04a **(48)** and related derivatives **49–52**.

Two groups, Jensen *et al.* [135] and Park *et al.* [136], have recently reported identification and isolation of putative fusaricidin/LI-F synthetase gene, *fusA*, from *P. polymyxa*, opening the possibility for the development of biosynthetic approaches toward this family of naturally occurring cyclic lipodepsipeptides and their analogs.

Future perspective

Cyclic lipodepsipeptides belong to a large and diverse family of nonribosomally synthesized peptides that have a wide variety of important biological activities. However, occurrence of multidrug-resistant bacteria and approval of daptomycin in the USA, EU and Canada has paved the way for their exploitation as lead compounds for new antibiotic discovery. Daptomycin, ramoplanin and fusaricidin/LI-F families of cyclic lipodepsipeptides are the most illustrative examples of the antibacterial potentials that these compounds possess.

Due to their unique mode of actions, these peptides are active against Gram-positive bacteria that are resistant to many other antibiotics in current clinical use. Despite the promise that these cyclic lipodepsipeptides offer, the need for the discovery novel antibiotics continues. It

is inevitable that resistant strains of bacteria will emerge in response to widespread use of antibiotics. For example, clinical isolates of *S. aureus* and enterococci with a loss of daptomycin susceptibility have already being reported [19,20,137]. These examples of daptomycin-resistant pathogens additionally emphasize a continuous need for the discovery and development of new antibacterial drugs with novel modes of action.

Combinatorial biosynthesis and/or synthetic combinatorial chemistry can, in theory, be extended to fermentation-derived cyclic lipodepsipeptides to generate large libraries of novel compounds for antibacterial activity screening. Therefore, novel antibacterial agents based on a cyclic lipopeptide scaffold are expected to play a significant role in the discovery of novel and more potent antibiotics capable of reverting infections caused by multidrug-resistant bacteria.

Key Terms

ESKAPE pathogens	Multidrug-resistant *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species responsible for the majority of healthcare-associated infections
Depsipeptides	Microbial secondary metabolites that contain one or more ester bonds along with the amide bonds
Fusaricidins/LI-Fs	New family of cyclic lipodepsipeptide antifungal antibiotics isolated from *Paenibacillus* sp. The natural products and their synthetic derivatives are active against a variety of fungi and Gram-positive bacteria

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Patents

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Executive summary

Bacterial resistance & need for new antibacterial drugs

- Antibiotic resistance has become a global public health problem.
- The majority of life-threatening hospital-acquired infections are caused by the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* sp.).
- The lack of new antibiotics against multidrug-resistant pathogens emphasizes the need for discovery and development of new treatment options.

Cyclic lipodepsipeptides as promising source of novel antibacterial drugs

- Naturally occurring cyclic lipodepsipeptides, which contain one or more ester bonds along with the amide bonds, have emerged as an attractive source of promising lead structures for the development of new antibiotics.
- The cyclic lipodepsipeptide daptomycin (Cubicin [Cubist Pharmaceuticals Inc., MA, USA]) has already been approved for the treatment of infections caused by multidrug-resistant bacteria.
- Ramoplanin (Nanotherapeutics Inc. [FL, USA]) is in Phase III clinical trials for the treatment of *Clostridium difficile*-associated diarrhea.
- Due to unique mode of actions, daptomycin and ramoplanin are active against Gram-positive bacteria that are resistant to many other antibiotics in current clinical use.
- Fusaricidin/LI-F and their synthetic analogs possess neutral peptide sequence and a single positively charged guanidinium group located at the termini of the lipidic tail.
- Structural differences between fusaricidin/LI-F family of antibacterial agents and daptomycin or ramoplanin suggest that these antibacterial peptides exert their effects through different mode of action, and that they may be active against daptomycin- and ramoplanin-resistant Gram-positive strains.
- Combinatorial biosynthesis and synthetic combinatorial chemistry can be extended to fermentation-derived cyclic lipodepsipeptides to generate large libraries of novel compounds for antibacterial activity screening.

Conclusion

- Cautious use of existing antibiotics may slow further development of resistance; however, in order to provide effective treatment options for the future, innovative antibiotics are necessary, preferably with novel modes of action and/or belonging to novel classes of drugs.

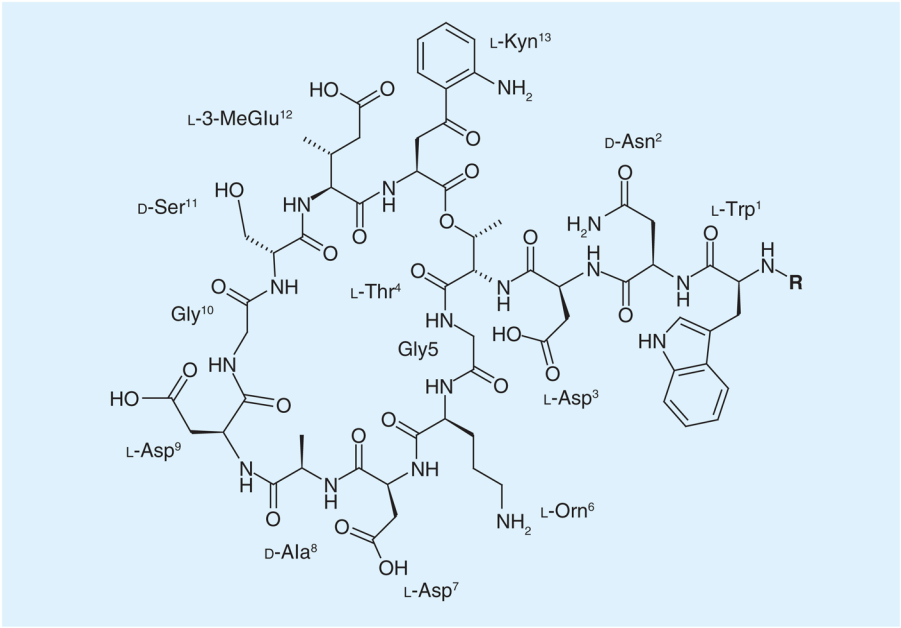

Figure 1. Isolated A21978C antibiotics (1–4)
See Table 1 for R groups.

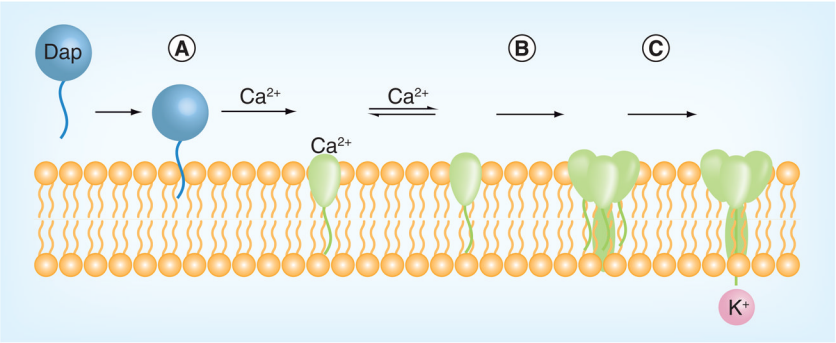

Figure 2. Proposed model for the bactericidal mechanism of action of daptomycin

(A) Insertion of daptomycin into bacterial cytoplasmic membrane in a calcium-dependent fashion, **(B)** oligomerization and disruption of the functional integrity of the cytoplasmic membrane and **(C)** release of intracellular ions and rapid cell death.

Dap: Daptomycin.

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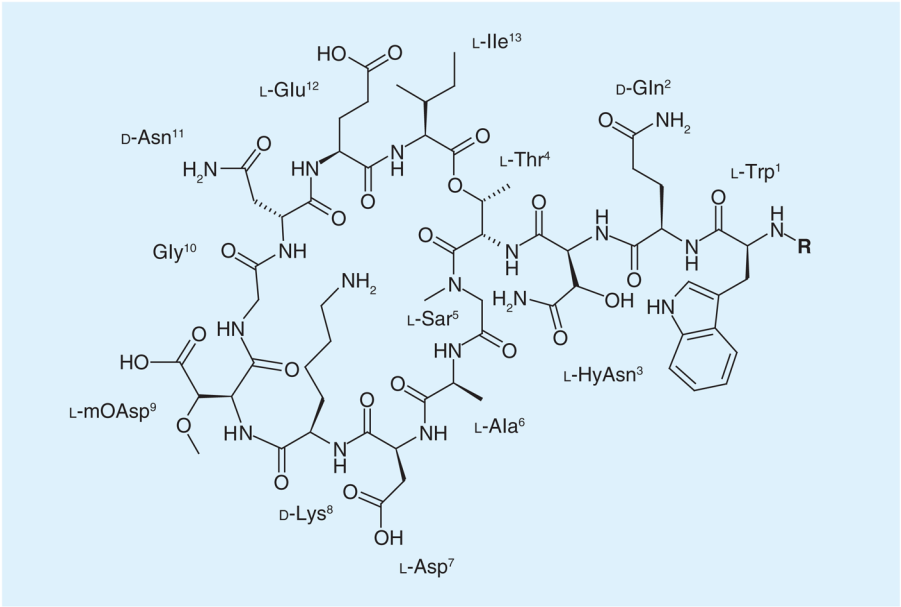

Figure 3. A54145A (5)
See Table 2 for R group.

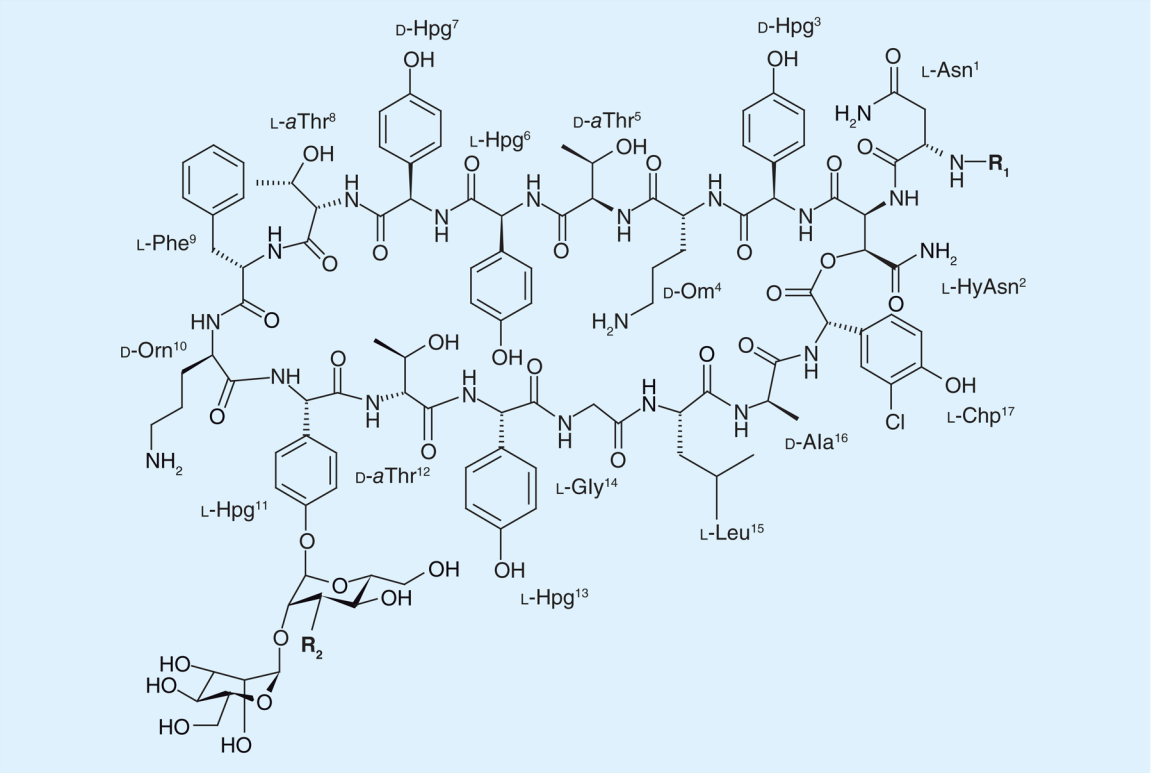

Figure 4. Ramoplanins A1–A3 (6–8) and ramoplanose (9)
See Table 3 for R groups.

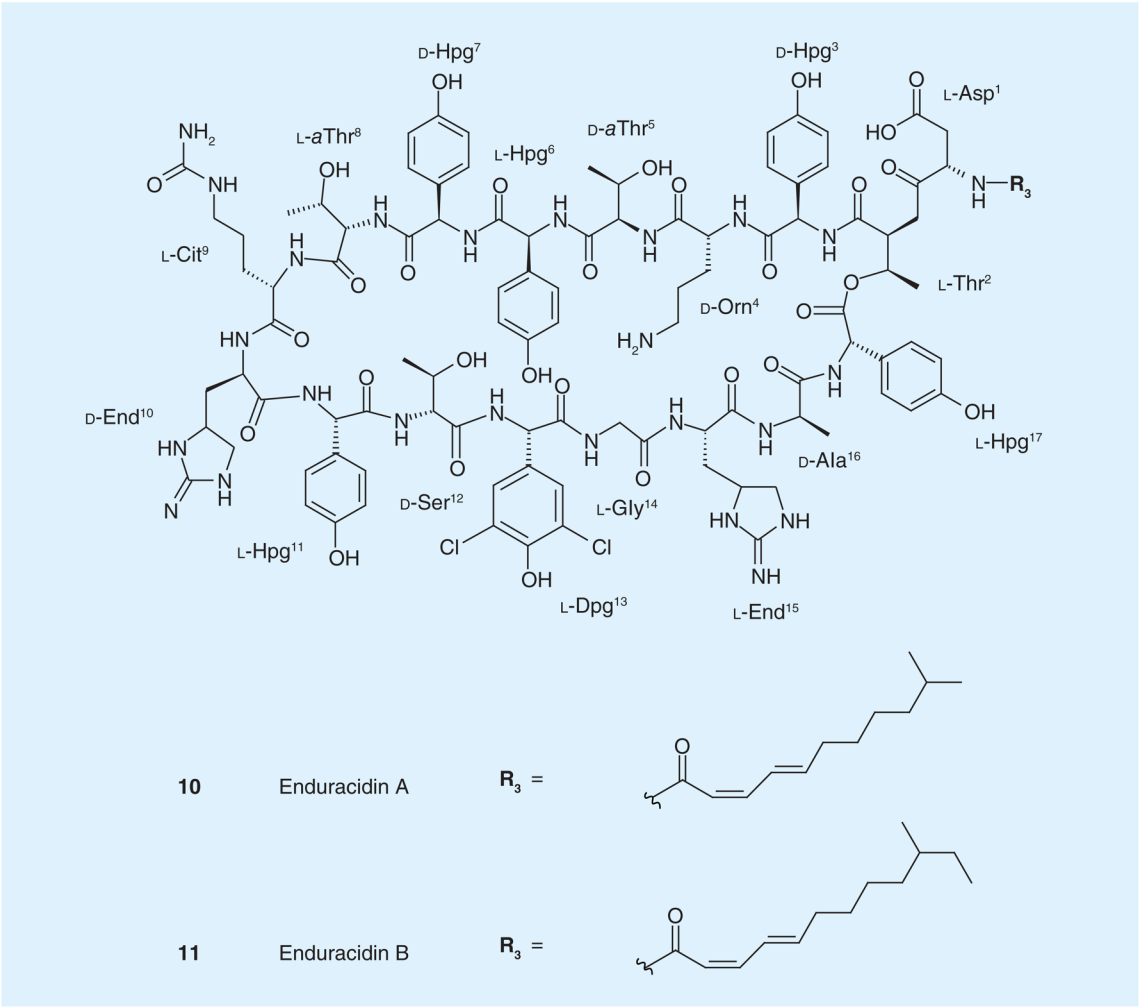

Figure 5.
Enduracidins A (10) and B (11).

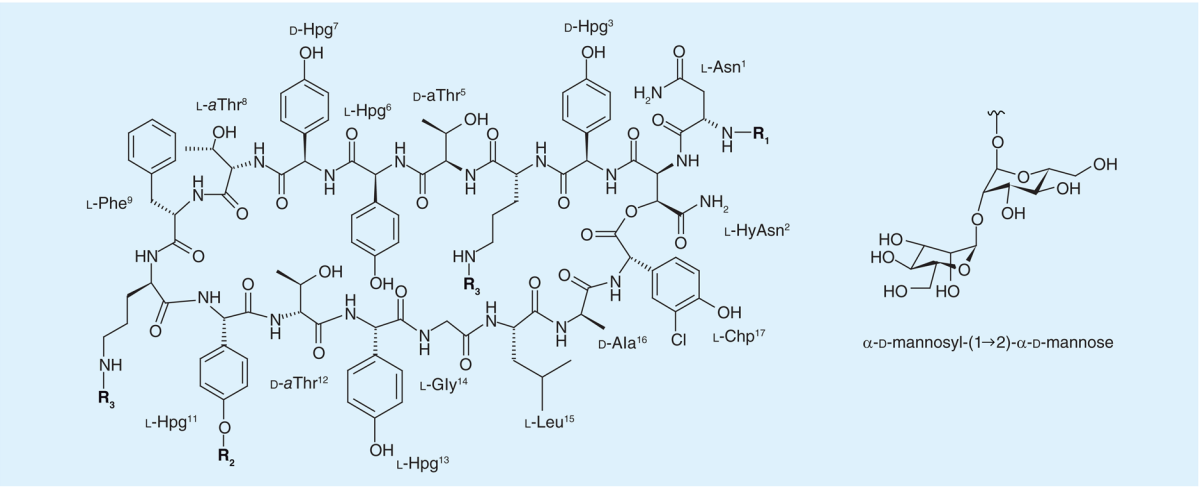

Figure 6. Ramoplanin A2 (7) and analogs (12–17)
See Table 4 for R groups.

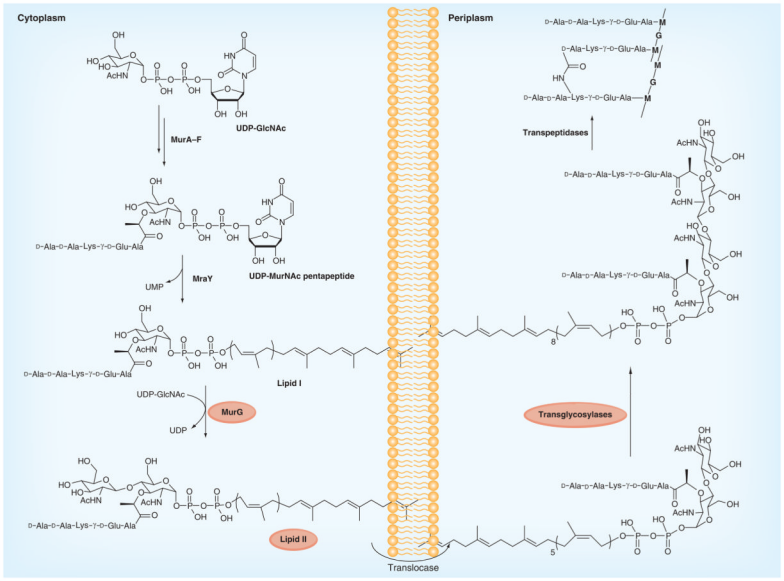

Figure 7.
Peptidoglycan biosynthesis and potential targets of ramoplanins (circled in red).

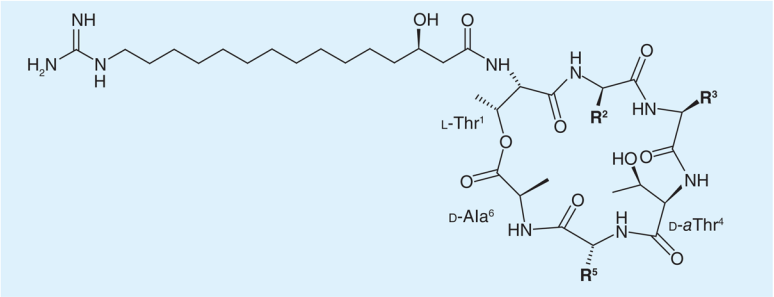

Figure 8. Fusaricidin/LI-F family of natural products
See Table 5 for amino acid sequences.

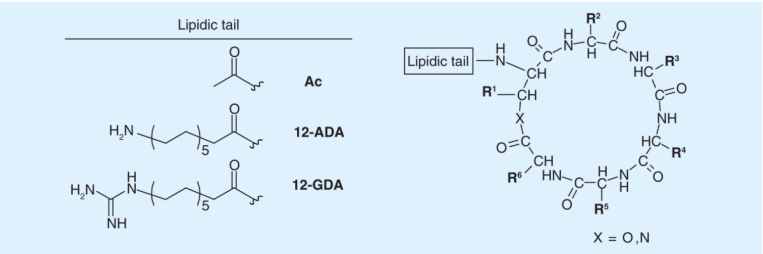

Figure 9. Synthetic analogs of fusaricidin A/LI-F04a
See Table 6 for lipidic tail and amino acid sequence.

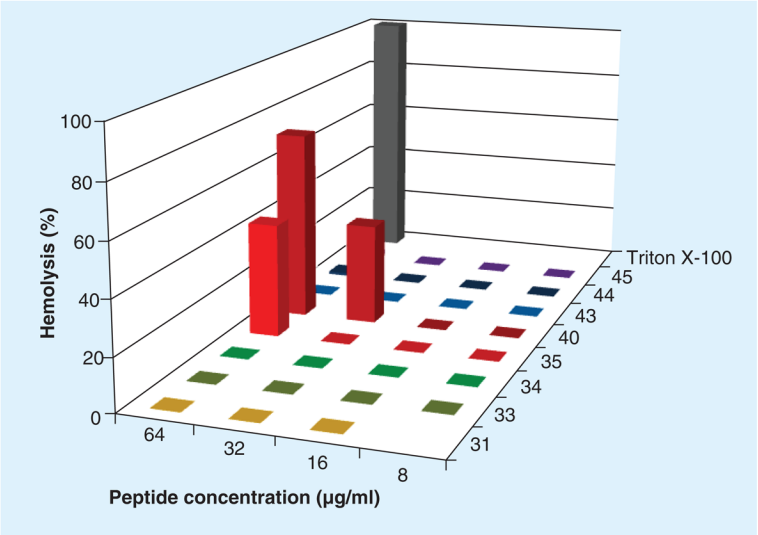

Figure 10. Hemolytic activities of selected depsipeptide analogs (31, 33–55, 40) and amide analogs (43–45)

0.5% of Triton X-100 was used as a reference for 100% hemolysis.

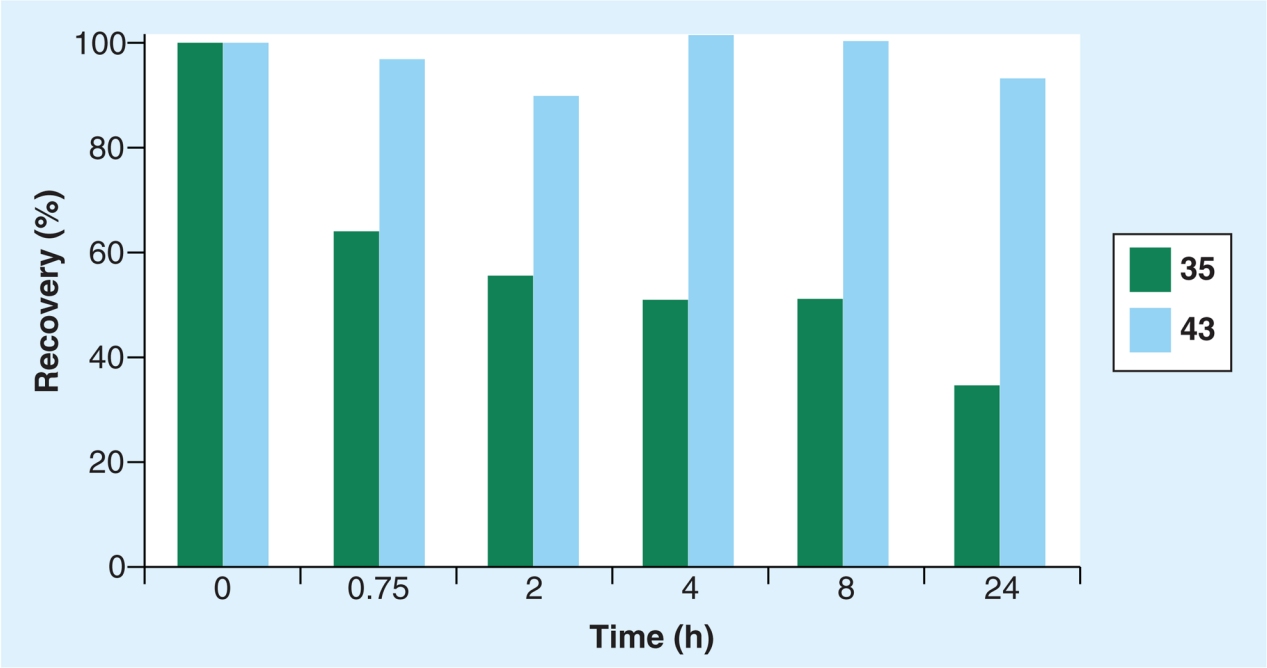

Figure 11. Stabilities of representative cyclic lipodepsipeptide (35) and its amide analog (43) in 50% human serum

Recovery of **35** and **43** upon incubation with human serum was determined by analytical HPLC and MALDI-TOF MS.

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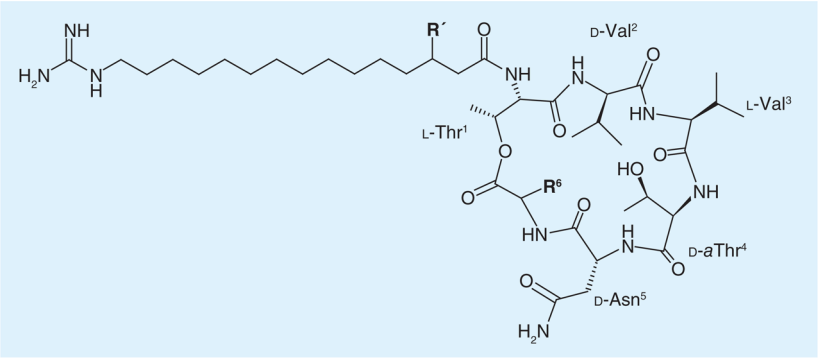
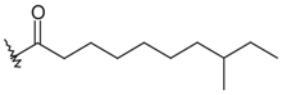
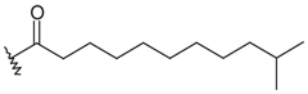
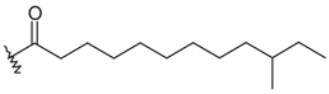
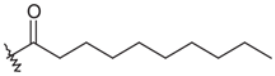


Figure 12. Synthetic fusaricidin A/LI-F04a (48), dehydroxy derivate (50) and related stereoisomers (49, 51 & 52)
See Table 7 for R groups.

Table 1

In vitro activities of isolated A21978C antibiotics 1–4 against *Staphylococcus aureus*.

Compound	R	MIC (μg/ml) *Staphylococcus aureus*
1 A21978C_1		1
2 A21978C_2		0.5
3 A21978C_3		0.13
4 Daptomycin		0.125–0.5

Data taken from [36].

Table 2

In vitro activity of A54145A (5) against *Staphylococcus aureus*.

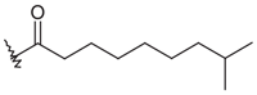
Compound	R	MIC (μg/ml) *Staphylococcus aureus*
5 A54145A		1

Table 3

In vitro antibacterial activities of ramoplanins A1–A3 (6–8) and ramoplanose (9).

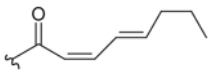
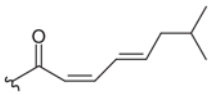
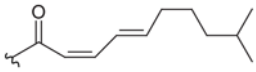
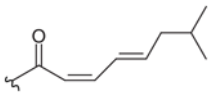
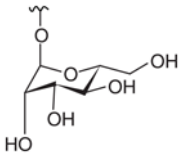
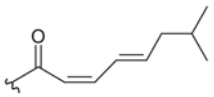
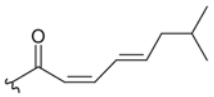
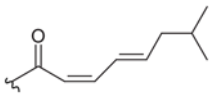
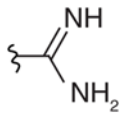
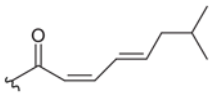
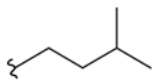
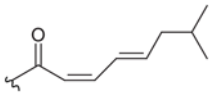
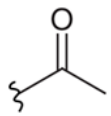
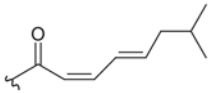
Compound	R_1	R_2	MIC (μg/ml)	
			***Staphylococcus pyogenes* L49**	***Staphylococcus pneumoniae* L44**
6 Ramoplanin A1		-OH	0.012	0.025
7 Ramoplanin A2		-OH	0.012	0.025
8 Ramoplanin A3		-OH	0.012	0.025
9 Ramoplanose			–	–

Table 4

In vitro antimicrobial activities of ramoplanin A2 (7) and analogs (12–17).

Compound	R_1	R_2	R_3	MIC (μg/ml)	
				Staphylococcus aureus†	*Bacillus subtilis*‡
7		α-D-mannosyl-(1→2)- α-D -mannose	H	0.5–1.56	0.03
12		α-D-mannosyl-(1→2)- α- D -mannose	H	2§	–
13		H	H	0.25–0.78	0.03
14		α-D-mannosyl-(1→2)- α -D -mannose		–	0.25
15		α-D-mannosyl-(1→2)- α-D-mannose		–	4
16		α-D-mannosyl-(1→2)- α-D-mannose		–	16

Compound	R_1	R_2	R_3	MIC (μg/ml)	
				Staphylococcus aureus†	*Bacillus subtilis*‡
17		α-D-mannosyl-(1→2)- α- D -mannose (lactone hydrolyzed)	H	>128	64

†Staphylococcus aureus *ATCC 25923*.

‡Bacillus subtilis *ATCC 8037*.

§Staphylococcus aureus *Tour*.

Table 5

R groups for fusaricidin/LI-F family of natural products.

Compound	R^2	R^3	R^5
18 Fusaricidin C/LI-F03a	D-Val	L-Tyr	D-Asn
19 Fusaricidin D/LI-F03a	D-Val	L-Tyr	D-Gln
20 Fusaricidin A/LI-F04a	D-Val	L-Val	D-Asn
21 Fusaricidin B/LI-F04a	D-Val	L-Val	D-Gln
22 LI-F05a	D-Val	L-Ile	D-Asn
23 LI-F05b	D-Val	L-Ile	D-Gln
24 LI-F06a	D-*a*Ile	L-Val	D-Asn
25 LI-F06b	D-*a*Ile	L-Val	D-Gln
26 LI-F07a	D-Val	L-Phe	D-Asn
27 LI-F07b	D-Val	L-Phe	D-Gln
28 LI-F08a	D-Ile	D-Ile	D-Asn
29 LI-F08b	D-Ile	D-Ile	D-Gln

Table 6

Synthetic analogs of fusaricidin A/LI-F04a and their *in vitro* activities against selected Gram-positive bacteria.

			MIC (μg/ml)			
Compound	**Lipidic tail**	**Sequence**	***S. aureus*†**	***S. aureus*‡**	***S. epidermidis*§**	***S. pyrogenes*¶**
30	Ac	L-Thr[1]-D-Val[2]-L-Val[3]-**D-Thr[4]**-D-Asn[5]-D-Ala[6]	ND	>128	ND	ND
31	Ac	L-Thr[1]-D-Val[2]-L-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	ND	>128	ND	ND
32	12-ADA	L-Thr[1]-D-Val[2]-L-Val[3]-**D-Thr[4]**-D-Asn[5]-D-Ala[6]	ND	>128	ND	ND
33	12-ADA	L-Thr[1]-D-Val[2]-L-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	ND	>128	ND	ND
34	12-GDA	L-Thr[1]-D-Val[2]-L-Val[3]-**D-Thr[4]**-D-Asn[5]-D-Ala[6]	16	16	16	32
35	12-GDA	L-Thr[1]-D-Val[2]-L-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	8	16	8	16
36	12-GDA	L-Thr[1]-D-Val[2]-L-Val[3]-**D-Ala[4]**-D-Asn[5]-D-Ala[6]	ND	64	32	64
37	12-GDA	L-Thr[1]-D-Val[2]-**L-Ala[3]**-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	16	16	8	16
38	12-GDA	L-Thr[1]-**D-Ala[2]**-L-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	ND	64	16	64
39	12-GDA	L-Thr[1]-D-Val[2]-L-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-**Gly[6]**	ND	64	64	64
40	12-GDA	L-Thr[1]-D-Val[2]-**L-Phe[3]**-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	8	16	8	8
41	12-GDA	L-Thr[1]-D-Val[2]-**L-Tyr[3]**-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	>128	ND	16	64
42	12-GDA	**L**-ε **Lys[1]**-D-Val[2]-L-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	ND	64	64	64
43	12-GDA	**L-Dap[1]**-D-Val[2]-L-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	8	16	16	16
44	12-GDA	**L-Dap[1]**-D-Val[2]-**L-Ala[3]**-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	ND	ND	64	64
45	12-GDA	**L-Dap[1]**-D-Val[2]-**L-Phe[3]**-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	8	ND	16	16
46	12-GDA	**L-Dap[1]**-D-Val[2]-**L-Tyr[3]**-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	ND	ND	64	64
47	12-GDA	L-Thr[1]-D-Val[2]-L-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	>128	ND	>128	>128

Differences among the sequences of naturally occurring fusaricidin A/LI-FO4a **20** *and synthetic analogs* **30–47** *are highlighted in bold.*

[†] Staphylococcus aureus *ATCC 29213.*

[‡] Staphylococcus aureus *Mu50 ATCC 700699.*

[§] Staphylococcus epidermidis *ATCC 27626.*

[¶] Staphylococcus pyrogenes *ATCC 19615.*

ND: Not determined.

Table 7

In vitro antifungal activities of synthetic fusaricidin A/LI-F04a (48), dehydroxy derivate (50) and related stereoisomers (49, 51 & 52).

Compound	R′	R[6]	MIC (μg/ml)		
			Candida albicans†	*Cryptococcus neoformans*‡	*Aspergillus fumigatus*§
48	OH	D-Ala	4.9	2.5	38.8
49	OH	D-Ala	19	9.7	19
50	H	D-Ala	38	19	19
51	OH	L-Ala	>77	>77	>77
52	OH	L-Ala	>77	>77	>77

†Candida albicans *ATCC 10231*.

‡Cryptococcus neoformans *ATCC 90112*.

§Aspergillus fumigatus *ATCC 204305*.