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Lipopeptides as anti-infectives: a practical perspective

Review Article

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Abstract: Lipopeptide antibiotics represent an old class of antibiotics that were discovered over 50 years ago, which includes the old polymyxins but also new entries, such as the recently approved daptomycin. They generally consist of a hydrophilic cyclic peptide portion attached to a fatty acid chain which facilitates insertion into the lipid bilayer of bacterial membranes. This review presents an overview of this class of antibiotics, focusing on their therapeutic applications and putting particular emphasis on chemical modifications introduced to improve their activity.

Keywords: *Lipopeptides • Antimicrobial peptides • Antibiotics • Semi-synthetic analogues • Daptomycin • Polymyxin • Echinocandin • Lipid membranes • LPS*

1. Introduction

Exploring the possibility of developing new classes of anti-infective compounds has emerged prominently in the last two decades or so, spurred by the looming threat of microbial resistance. With our arsenal of antibiotic molecules getting old and less efficient, only few truly original replacements have become available. Due to a number of different reasons - including daunting R&D costs for putting a new molecule on a highly competitive market and the inherent difficulty of identifying innovative antibiotic targets [1,2] - such a shortage in recruitment has translated into an accelerated occurrence of outbreaks (especially in nosocomial settings) of infective agents that no longer respond to first-line antibiotics.

Decreasing effectiveness of therapeutic intervention extends across all types of microbial ailments, whether caused by bacteria (both Gram-positive and -negative), viruses, fungi and parasites, and to every major class of antibiotic, and does not spare developed countries. New molecules, new medical practises and new diagnostic tools are very much needed to solve this alarming problem.

Amongst the candidate new antibiotics, lipopeptides are now on stage, thanks to a handful of compounds with different therapeutic indications that have reached the market in recent years and more examples in the biotech pipeline (Table 1). While most natural antimicrobial lipopeptides are secondary metabolites produced by soil bacteria *via* non-ribosomal pathways, the most promising compounds pertaining to this group are almost semi-synthetic in nature, being often derived from fermentation products. A common feature is an acyl chain conjugated to a linear or cyclic peptide sequence; the peptide portion can contain either cationic or anionic residues, and may contain non-proteinaceous amino acids or other unusual components [3,4].

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Compound name (commercial name)	Therapeutic area	Method of manufacture	Lead compound and producing organism	Company	Phase
Daptomycin (Cubicin)	Complicated infections of skin and skin structure/*S. aureus* bacteremia and right-sided endocarditis	Fermentation	Daptomycin and *Streptomyces roseosporus* NRRL11379	Cubist	Market
CB-182,804	Gram-negative infections	Semi-synthetic	--------	Cubist	IND[1]
WAP-8294A_2	MRSA infections	Fermentation	WAP-8294A_2 and *Lysobacter* sp.	aRigen	Phase I
NAB739 & NAB7061	MDR Gram-negative bacterial infections	Semi-synthetic	Polymyxin B/colistin and *Bacillus polymyxa*	Northern Antibiotics	Preclinical
MX-2401	Serious Gram-positive bacterial infections	Semi-synthetic	Amphomycin and *Streptomyces canus* ATCC 12237	Migenix	Preclinical
Lipohexapeptides HB1275 & HB1345	Acne, rosacea, MRSA and coutaneous mycoses	Synthetic	HB1275 & HB1345 None	Helix Biomedix	Preclinical
Telavancin	Complicated skin and skin structure infections (cSSSI) caused by Gram-positive bacteria	Semi-synthetic	Vancomyncin and *Amycolatopsis orientalis*	Theravance	NDA[2]
Caspofungin (Cancidas)	Antifungal	Semi-synthetic	Echinocandins and *Glarea lozoyensis*	Merck and Co.	Market
Micafungin (Mycamine)	Antifungal	Semi-synthetic	Echinocandins and *Coleophoma empetri*	Astellas Pharmaceuticals	Market
Anidulafungin (Eraxis)	Antifungal	Semi-synthetic	Echinocandins and *Aspergillus nidulans*	Pfizer Pharmaceuticals	Market

Table 1. Selected natural product-derived lipopeptide antibiotics currently in preclinical and clinical development.

1: Investigational New Drug Application Process
2: New Drug Application Process

In this review, we will discuss the main chemical features, the process of lead optimization to develop a candidate, the mechanism of action and the therapeutic applications for selected lipopeptides, including daptomycin, polymyxin derivatives, derivatives of amphomycin, echinocandins, anti-infective lipohexapeptides and telavancin. As we will highlight in the following sections, lipopeptides make a promising class of antibiotic compounds whose potential for therapeutic application has been explored only to a limited extent, and can contribute significantly to replacing molecules whose effectiveness is vanishing because of resistance.

Polymyxins –cationic cyclic peptides– are probably the best known naturally-occurring lipopeptides. Polymyxin B and polymyxin E (also known as colistin), both produced by the bacterium *Bacillus polymyxa*, have been known for decades and are the best studied examples, but novel compounds belonging to this class, such as polymyxin M (mattacin) isolated from *Paenibacillus kobensis*, continue to be discovered [5]. In general, polymyxins have a remarkable affinity for the lipopolysaccharide (LPS) component of the outer cell membrane (OM) of Gram-negative bacteria, and not surprisingly their clinical use is limited to these microbial species. Pressed by the rising tide of bacterial resistance and the lack of viable alternatives, the use of polymyxin B and colistin for the treatment of severe infections caused by Gram-negative bacteria has re-emerged, a last resort when every other option has failed [6,7].

While polymyxins and derivatives are undergoing a resurgence, daptomycin is the new star in the lipopeptide constellation, and has grasped headlines for some time recently. First identified by Eli Lilly and Company in the early 1980s, this anionic cyclic lipopeptide was approved in 2003 by the FDA for the treatment of infections caused by Gram-positive bacteria, under the market name Cubicin.

Another interesting example of lipopeptides with clear therapeutic value are echinocandins. These large natural and semi-synthetic lipopeptides were first identified in 1974 as antifungal compounds, being

particularly active against several species of *Candida* and, to a minor extent, *Aspergillus* [8].

At this point some definitions are useful. Some authors [3] consider lipopeptides as a peculiar class of antimicrobial peptides (AMPs). These –isolated by the hundreds from virtually every class of organism, from bacteria to fungi, plants and animals– are a key component of innate immunity, a complex mix of cellular and molecular factors that act non-specifically to defend the host from infection by microbial pathogens. AMPs and lipopeptides do in reality share some basic features, such as the ability to fold into an amphipathic conformations when in a hydrophobic environment. This, in turn, is a prerequisite for exerting their antimicrobial action, thought to proceed in both cases mainly through binding to the target cell membrane, which leads to its destabilization, eventually causing an increase in permeability and leakage of the cellular contents. This broad disruption of the lipidic component of bacterial membranes hampers the development of microbial resistance, making lipopeptides and AMPs attractive as antibiotic drugs. In addition, given a naturally-occurring sequence, a number of synthetic analogues can be developed that retain or improve upon a peptide's original biological properties. This intriguing research avenue is being actively explored currently for both lipopeptides and AMPs. In contrast with lipopeptides, which are themselves a structurally diverse group, AMPs are generally gene-encoded and synthesized on ribosomes, cationic in nature, and endowed with a limited variability of non-conventional amino acids. Furthermore, it is increasingly recognized that a number of mammalian AMPs, including the defensins and cathelicidins, not only have direct microbicidal activity, but also play an important immunomodulatory and immunostimulatory role, somehow functioning at the interface between innate and adaptive immunity. Stemming from the recognition of this broader, multifunctional role for some AMPs, the term 'host defence peptides' has been coined [9].

Owing to the differences noted above, we do prefer to keep lipopeptides distinguished from AMPs, and direct the interested reader to the abundant literature available on the latter group [10-14]. The use of lipopeptides in the development of self-adjuvanting vaccines [15], or as tools for plant disease biocontrol [16], falls out of the scopes of this paper and will not be covered here.

2. Daptomycin and derivatives

Daptomycin represents the first member of the novel class of cyclic lipopeptide antibiotics, discovered in the early 1980s and approved for clinical use by US FDA in 2003. Daptomycin belongs to the complex A21978C, produced through the action of nonribosomal peptide synthetases (NRPSs) in *Streptomyces roseosporus* [17]. In particular, three major components are present in this complex, having a common peptide nucleus and different lipid chains [18-20].

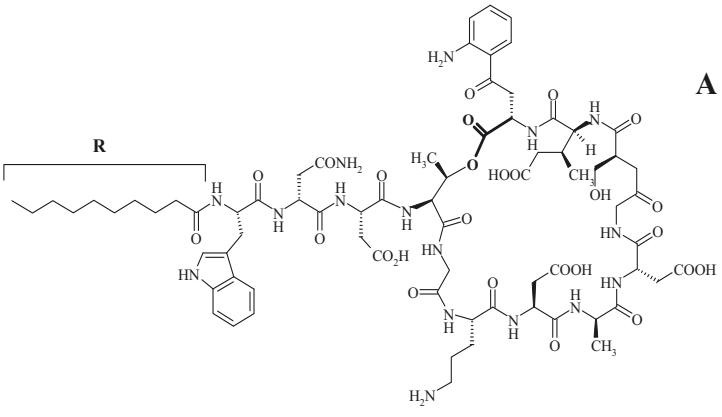
The peptide structures of these three components were determined by incubation with *Actinoplanes utahensis* NRRL 12052, a bacterial strain producing a deacylase enzyme, which is capable of selectively removing the fatty acyl groups from the cyclic peptides. The common peptide nucleus isolated is a depsipeptide and contains 13 amino acids, with 10 residues forming a cyclic frame linked by an ester bond between the terminal kynurenine (Kyn13) and the hydroxyl group of threonine (Thr4) [17,21-23]. Non-proteinogenic and D-amino acids are included in the structure, in particular ornithine (Orn), D-Ala, D-Ser, L-*threo*-3-methylglutamic acid (3mGlu), D-Asn and L-Kyn [3-(2-aminobenzoyl)-L-alanine], to give the final peptide sequence ^{1}W^{D}NDTGOD^{D}ADG^{D}SmEKyn [24,25]. The branched chain of the fatty acyl group attached to the *N*-terminus of Trp1 in the lipo-complex was found to be *anteiso*-undecanoyl, *iso*-dodecanoyl and *anteiso*-tridecanoyl for A21978C$_{1\text{-}3}$, respectively [22].

In order to improve the known antibacterial activity of this complex, a semi-synthetic approach was followed. After a deacylation step, a reacylation step was carried out with small changes in the acyl side-chain length [21]. Activity and toxicity of reacylated A21978C derivatives was strongly affected by structural changes in the acyl group [21,26,27]. Furthermore, different daptomycin analogues were also prepared by a chemoenzymatic approach, which was used to substitute some non-proteinogenic amino acids, such as *threo*-3-methylglutamic acid (3mGlu), with glutamic acid resulting in a significantly decreased antibacterial activity [28].

In vitro studies against *Staphylococcus aureus* and *Streptococcus pyogenes* indicated that activity increased with acyl chain length, and *in vivo* studies, in particular LD$_{50}$ determination, indicated an increased toxicity for a chain length longer than C$_{10}$ [21]. Consequently, more detailed antibacterial and toxicological studies were carried out on the decanoyl, undecanoyl, *d*ecanoylphenylalanyl and *d*odecanoyl-p-aminophenacetyl derivatives.

Among these, the decanoyl derivative, originally designated as LY146032, was later renamed daptomycin (Figure 1A) and recognized as a potentially useful analogue, having the best therapeutic index in mice. Subsequently, the production of daptomycin was carried out by fermentation, replacing the semi-synthetic approach used in the exploratory work, by adding decanoic acid to the growth medium during fermentation of *S. roseosporus*. However, this method of production presented several technical challenges [19,29]. Indeed, due to toxicity of decanoic acid to *S. roseosporus*, which caused complete lysis of cultures if allowed to accumulate, three fundamental parameters had to be defined and fixed before proceeding, such as: cultivation temperature (30°C), feeding rates, and a suitable formulation for feeding. A mixture of decanoic acid and methyl oleate in a ratio of 1:1 proved to the best option, the resulting fermentation process yielding daptomycin as the major component produced, thus facilitating the subsequent purification step [30]. To date, the highest yield for daptomycin production is still provided by cultures of *S. roseosporus* (about 150 mg/L), but many improvements have been made also in *S. lividans* (TK23 and TK64 strains) daptomycin production, including the site-specific insertion of a 128 kbp region of cloned *S. roseosporus* DNA containing the daptomycin gene cluster into the *φC31 attB* site [31].

Due to its net negative charge at neutral pH, daptomycin has a high solubility in an aqueous environment. However, the presence of the lipid tail and



	R	R^1	R^2	R^3	
WAP-8294A$_1$	$(CH_2)_4CH_3$	CH_2	CH_3	CH_3	
WAP-8294A$_2$	$(CH_2)_3CH(CH_3)_2$	CH_2	CH_3	CH_3	*Lysobacter* sp mixture components
WAP-8294A$_4$	$(CH_2)_4CH(CH_3)_2$	CH_2	CH_3	CH_3	
WAP-8294Ax$_8$	$(CH_2)_3CH(CH_3)_2$	CH_2	CH_3	H	
WAP-8294A$_{x9}$	$(CH_2)_3CH(CH_3)_2$	CH_2	H	CH_3	
WAP-8294A$_{x13}$	$(CH_2)_3CH(CH_3)_2$	$(CH_2)_2$	CH_3	CH_3	

Figure 1. General structures for daptomycin (A) and WAP-8294A complex (B), two lipodepsipeptides. The acyl group (R) in daptomycin is a decanoyl fatty acyl chain. WAP-8294A$_2$, whose substituents R are reported in the accompanying table and are indicated by an arrow, is one of the components isolated from the mixture complex produced through fermentation by *Lysobacter* sp. The site of macrolactamization of both compounds is pointed out in bold.

some hydrophobic amino acids promotes an overall amphipathic behaviour. Due to this amphipathic nature, daptomycin forms aggregates, in particular micelles, in aqueous solution, as evidenced by surface tension measurement at pH 10.0, which indicated a critical micellar concentration (CMC) around 2 mM [32].

Currently, the mechanism of action accepted for daptomycin involves the binding of the lipopeptide to the cytoplasmic bacterial membrane in a Ca^{2+}-dependent manner and the oligomerization of daptomycin in the membrane. The anionic character of daptomycin is masked by its complexation with divalent cations such as Ca^{2+}, enabling the lipopeptide to perturb bacterial membranes in a detergent-like manner, analogously to what has been suggested for cationic antimicrobial peptides [33-35].

A more recent proposal of a modified mechanism of action [36,37] suggests that daptomycin oligomerizes in the presence of Ca^{2+} to form micelles and subsequently dissociates in proximity to the membrane, allowing daptomycin insertion into the membrane. A further oligomerization step then occurs in the membrane. The ability of daptomycin to perturb membranes by inducing a positive curvature strain on the lipids, in a Ca^{2+} dependent manner, was demonstrated by means of DSC (differential scanning calorimetry) and solid state ^{31}P NMR studies, carried out in the presence of lipid bilayers and Ca^{2+} [38]. Daptomycin depolarises the cell membrane causing disparate effects on cell physiology that lead to rapid cell death and a reduced occurrence of resistance, due to the difficulty for target cells to re-design the susceptible membrane features [39]. As a result of this interaction, which is proposed to ultimately lead to cell death, a loss of potassium occurs which in turn perturbs the synthesis of macromolecules [37,40].

Recent results of transcriptional profiling studies on the action of daptomycin on *S. aureus* indicate that inhibition of peptidoglycan biosynthesis, either directly or indirectly, together with membrane depolarization, takes part in the mode of action of daptomycin [41]. Daptomycin possess other unique features, such as its ability to be bactericidal without being bacteriolytic [42] (thus reducing over-stimulation of the immune response by the release of bacterial elements and a prolonged postantibiotic effect) and an efficacy maintained in both the static and growth phases of bacteria [43].

Daptomycin was widely studied by means of NMR spectroscopy in order to elucidate the basis of its mechanism of action, but various hurdles were encountered preventing full characterization by NMR, among which the most prohibitive was the difficulty of obtaining an uniformly isotopically labelled sample (^{13}C, ^{15}N and/or ^{2}H). To date, there are no studies reporting large scale production of labelled daptomycin, even though recent advances have demonstrated production of daptomycin and analogues by modifying the nonribosomal peptide synthetase (NRPS) in the biosynthetic pathway of daptomycin using different strains of *S. roseosporus* [44] or *Streptomyces coelicolor* [28,45]. Another difficult aspect in undertaking NMR structural studies was carrying out characterization of daptomycin under conditions in which the lipopeptide is most active, *i.e.* in the presence of high concentrations of Ca^{2+} [46-48]. With the Ca^{2+}/daptomycin molar ratio at 1:1 under these conditions, oligomerization occurs, resulting in 14 to 16 monomers forming micelles [35,49,50]. The arrangement into micelles is not accompanied by a change in lipopeptide conformation, as originally proposed [36]. Indeed, making a comparison with addition of magnesium instead of calcium to a daptomycin solution, the NOESY spectra closely resembled the NOESY spectra obtained for apo-daptomycin [51]. Also, in the presence of 1 eq of Ca^{2+}, two new structures quite similar to the apo-form are present [35], suggesting that the interaction of daptomycin with Ca^{2+} is weak and non-perturbing. Inspection of the structure of apo-daptomycin shows that the four acidic residues, Asp3, Asp7, Asp9 and 3mGlu12, are not close enough spatially to one another to render a Ca^{2+}-specific binding site, in the absence of a major conformational change. This suggests an electrostatic interaction between daptomycin and Ca^{2+} that aids aggregation [49]. Finally, to determine whether the interaction of daptomycin with lipids is accompanied by a change in structure, NMR TOCSY and NOESY experiments were carried out in the presence of Ca^{2+} and DHPC (dihexanoylphosphatidylcholine) micelles. Results suggested that daptomycin undergoes only a minor conformational rearrangement upon binding to DHPC in the presence of Ca^{2+} [51].

Daptomycin is marketed under the name Cubicin by Cubist Pharmaceuticals (www.cubist.com). Once-daily daptomycin is now approved for the treatment of complicated infections of skin and skin structure in North America, Europe and several other countries, and for *S. aureus* bacteremia and right-sided endocarditis in the USA alone [52].

The lack of suitable chemical approaches for the total synthesis of daptomycin analogues makes combinatorial biosynthesis an important alternative to generate and scale up derivatives of daptomycin for clinical evaluation [44]. As mentioned above, daptomycin and related cyclic lipopeptides are synthesized by NRPSs; these large enzymes usually comprise several subunits, with each subunit functionally organized into a series of modules. Each module catalyses the incorporation of one specific amino acid in a way in which the substrate binding

specificity of the series of modules reflects the order of amino acid residues in the final product (colinearity rule). The peptide is synthesized as a linear molecule that is subsequently cyclized by a terminally located thioesterase (Te) domain in the NRPS. In particular, the cyclic, branched, 13-membered lipopeptide backbone of daptomycin is produced by three giant NRPS multi-enzymes, DptA, DptBC and DptD, encoded by the *dptA*, *dptBC* and *dptD* genes, respectively [53].

In one study, module exchange in NRPS model systems has proven to be useful to generate novel daptomycin derivatives. λ-Red-mediated recombination was used to exchange single or multiple modules in the DptBC subunit of the NRPS to modify the daptomycin cyclic peptide core. The combination of several strategies including module exchange, NRPS subunit exchange, inactivation of the tailoring enzyme glutamic acid 3-methyltransferase, and natural variations of the lipid tail was used to generate a library of novel lipopeptides, some of which were as active as daptomycin against Gram-positive bacteria, and to elucidate structure-activity relationships [44]. In another study, the effect of module exchange at nucleotide sequences encoding inter-peptide linkers was explored in dptD, a gene encoding a di-modular NRPS subunit that incorporates 3-methylglutamic acid (3mGlu12) and kynurenine (Kyn13) into daptomycin. Two advantages of using module exchange, as opposed to subunit exchange, are that it conserves the natural inter-peptide docking sites required for efficient communication between DptBC and DptD, and it provides a mechanism to expand the number of substitutions at position 13 that play key roles in determining antibacterial potency [53].

Cubist has an additional lipopeptide program aimed at the development of novel antibiotic candidates with efficacy in treating infections caused by multi-drug resistant Gram-negative pathogens. These compounds have been generated by several approaches including chemical, chemoenzymatic and combinatorial biosynthetic approaches. One of them (named CB-182,804) showed *in vivo* efficacy against *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* and is expected to undergo a Phase 1 single-ascending-dose study in the next few months.

3. WAP-8294A$_2$ (WAP)

WAP-8294A$_2$ (WAP) is a water-soluble lipodepsipeptide membrane active agent isolated from the fermentation broth of the Gram-negative bacterium *Lysobacter* sp. WAP exhibits activity against methicillin-resistant *S. aureus* (MRSA) infections as well as various strains of *Propionibacterium acnes* including clinical isolates and multi-drug resistant strains [54].

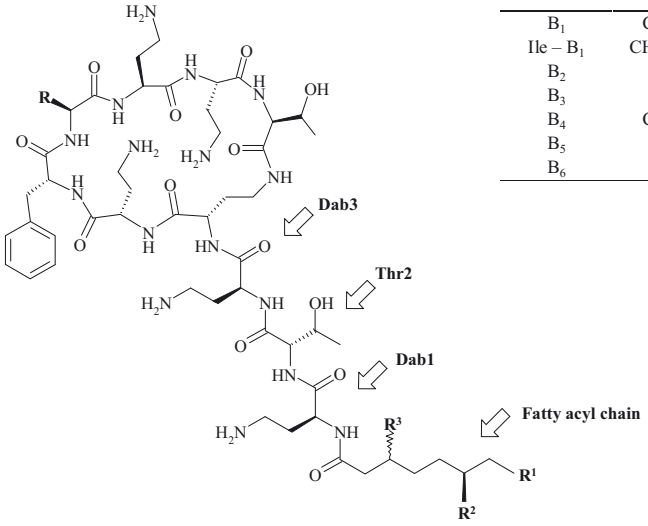
Among the 19 components observed by HPLC-UV analysis [54] and present in the fermentation complex, the major component WAP-8294A$_2$ demonstrated a relevant *in vitro* biological activity [55,56]. The other relatively abundant components, A$_1$, A$_2$, A$_4$, A$_{x8}$, A$_{x9}$ and A$_{x13}$, were also isolated and the general formula is shown in Figure 1B. The structures of the major and few minor components have been elucidated by chemical degradation and 2D-NMR [55], resulting in a common depsipeptide core containing several non-proteinogenic and D-amino acids. These include D-Trp, D-Asn, D-Orn, D-NMePhe, D-*threo*-β-OH-Asn and L-NMe-Val. The three components A$_1$, A$_2$, and A$_4$ were also isolated by high-speed counter-current chromatography (HSCCC) in a single run, providing a more efficient separation method than the previous conventional chromatographic procedure, thus helping reduce the costs of production [57].

WAP was demonstrated to have a 3 to 7-fold higher antimicrobial activity than vancomycin with the addition of human serum and no cross-resistance with other antibiotics. WAP-8294A$_2$ is currently under clinical development by aRigen (www.arigen.jp) and Janus Pharmaceutical (www.januspharma.com) for systemic use against MRSA and, formulated as a cream, for the treatment of acne.

4. Polymyxins and derivatives

Polymyxins are a family of closely related cyclic lipopeptide antibiotics originally isolated in the late 1940s from the soil bacterium *Bacillus polymyxa* [58]. They are membrane active molecules with high affinity for the lipid A component of the LPS, and are characterized by poor tissue penetration, a molecular weight of approximately 1100, and activity directed predominantly against Gram-negative aerobes. Only two of them, namely polymyxins B (PMB) and E (colistin), have been used in clinical practice so far.

PMB and colistin are very similar in their structure, containing a common group of amino acids mainly made of two residues of L-α,γ-diaminobutyric acid (Dab) and one of L-threonine, as elucidated by several authors [59-62]. Colistin differs from PMB by one amino acid, D-leucine instead of D-phenylalanine in the cyclic heptapeptide. Although the sequence difference is minimal, it results in a significant difference in potency between PMB and colistin against strains of *P. aeruginosa*, with PMB being more potent [59]. A side acyl chain is linked to the α-amino group of the terminal diaminobutyric acid



Polymyxin	R	R^1	R^2	R^3
B_1	$CH_2CH(CH_3)_2$	CH_3	CH_3	H
Ile – B_1	$CH(CH_3)CH_2CH_3$	CH_3	CH_3	H
B_2		CH_3	CH_3	H
B_3		CH_3	H	H
B_4	$CH_2CH(CH_3)_2$	H	H	H
B_5		CH_2CH_3	H	H
B_6		CH_3	CH_3	OH

Figure 2. General structure for polymyxin B. Polymyxin B is a complex constituted of six membres, differing in fatty acyl chain and in amino acid identity at position 7 of the cyclic portion (R substituents are reported in table). The arrows point out the sites modified to obtain NAB7061 and NAB739 polymyxin B derivatives. Both derivatives lack the Dab1 residue and bear an uncharged amino acid at position 3 instead of the charged Dab3. The NAB739 linear peptide portion is constituted of Thr-D-Ser and that of NAB7061 is constituted of Thr-Abu, reducing the overall number of charges from 5 to 3.

residue L-Dab both in polymyxin B and E. In particular, polymyxin B is in reality a complex mixture of closely related polypeptides differing from each other in the fatty acid moiety and by the presence or absence of additional amino acids (Figure 2) [62].

Even though several toxicity issues limited the use of polymyxins in the past, recent studies reported a better safety profile. For example, a revisited toxicity assessment along with the emergence of multi-drug-resistant Gram-negative bacteria, prompted the reintroduction of colistin and its derivatives in clinical practice [63]. Among the numerous efforts made to reduce the toxicity of polymyxins, the synthesis of colistin methanesulfonate (CMS), a pro-drug of colistin where the free amino groups are blocked by sulfomethylation, resulted in a reduced *in vitro* activity along with a dramatically decreased toxicity and avoidance of some local painful, undesirable side effects. CMS is currently used in aerosol systemic therapy, while colistin sulphate is available commercially for topical and oral use [64].

Several optimization programs were also carried out on PMB, focused on reducing its toxicity while preserving both the anti-endotoxin and antibacterial activities. In particular, removal of the fatty acid chain by proteolytic cleavage with ficin or related enzymes resulted in deacylated nonapeptides [65], with less acute toxicity than their parent polymyxins along with a reduced antibacterial activity [66-68]. Despite lacking bactericidal activity themselves, these polymyxin nonapeptides (PMBN) can damage the outermost cell wall structure of Gram-negative bacteria, greatly increasing its permeability to hydrophobic antibiotics, which may facilitate combinatorial use with other antibiotics to treat specific infections [69-75].

Northern Antibiotics (www.northernantibiotics.com) started an optimization program based on the structure-function relationships and antibacterial properties of novel synthetic PMB derivatives, modified both in the *N*-terminal fatty acyl chain and in the linear peptide portion. The main feature of the two lead PMB derivatives, NAB739 and NAB7061, consists in possessing no more than three positive charges under physiological conditions. Since nephrotoxicity of polymyxins seems to be related to the highly cationic nature of the molecules, a reduced number of charges is expected to result in a better safety profile. Nephrotoxicity is probably due to the interaction of polymyxins with megalin, a giant endocytic receptor abundant in the apical membrane of proximal tubules [76]. In fact, NAB739 and NAB7061 bind to the isolated brush-border membrane of rat kidney at an affinity which is only 1/7-1/5 of that for PMB, indicating a lower potential for nephrotoxicity [77]. Figure 2 depicts modifications introduced into PMB in order to create NAB739 and NAB7061, by reducing both the number of charges and changing the identity of the fatty acyl chain. In particular, the linear peptide portion of NAB7061 consists of Thr-Abu (amino butyric acid) instead of Dab-Thr-Dab in PMB. Analogously, the peptide linear portion

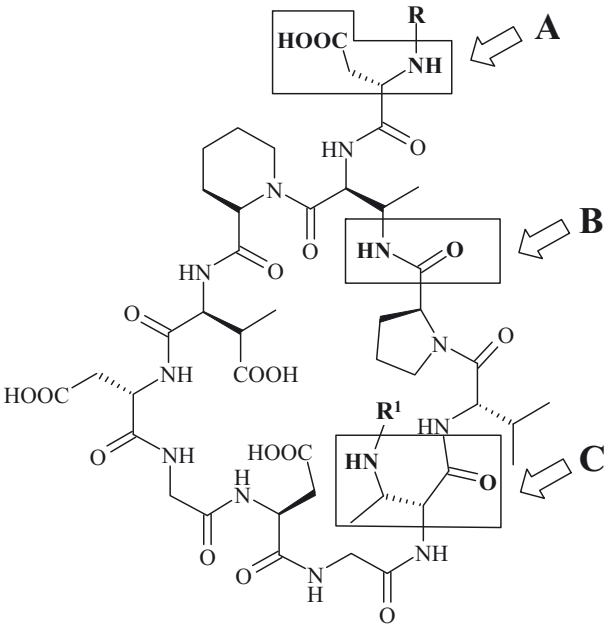


Figure 3. The general structure of the macrolactam amphomycin (the site of macrolactamization is indicated in box B) and the sites of modification identified in the optimization Migenix's program (see main text). The original fatty acid, D3-anteiso-trideceneoic acid originally linked to Asp1, was removed and by a structure-function study introducing several substituents (R) in Asp1 (box A), a first derivative more active than amphomycin was identified. In a second acylation step, Dab9 (box C) was modified by introducing several substituents to the b-NH_2 of Lthreo-a,b-diaminobutyric acid (R'). MX-2401 (see main text) is an amphomycin derivative modified both in Asp1 and in Dab9.

in NAB739 is made of Thr-D-Ser instead of the original Dab-Thr-Dab common to all components of the PMB mixture. Both NAB739 and NAB7061 carry octanoyl residues as the fatty acyl chain instead of a mixture of methyloctanoyl and methylheptanoyl residues as in the PMB complex, whereas the cyclic portion of both derivatives is identical to that of PMB.

Although the direct antibacterial activity of NAB7061 was very weak, it showed a strong synergism with hydrophobic antibiotics, *i.e.* rifampin, indicating that it is able to damage the bacterial OM permeability barrier in the same way as PMBN, the best-characterized OM permeability-increasing agent [68]. The direct bactericidal activity of NAB739 is more potent, but with no evidence of synergism with hydrophobic antibiotics. The different MIC values and synergism profiles of these two compounds suggest a different mechanism of action. Probably, the activity on the OM is not merely due to electrostatic interactions, but rather depends on a proper conformation of the derivatives. This is supported by work with the enantiomeric mirror analogue of PMBN (D-PMBN), which bound LPS similarly to L-PMBN but was unable to sensitize the bacteria to hydrophobic antibiotics [78].

At the moment, the two NAB lead compounds are undergoing late preclinical development. The results from both *in vitro* and *in vivo* studies indicate the desired efficacy and safety profiles.

5. Amphomycin and derivatives

Migenix (www.migenix.com) is developing a novel semi-synthetic injectable compound, named MX-2401, based on the lipopeptide amphomycin (A-1437 E, Figure 3), for the treatment of serious Gram-positive bacterial infections.

Amphomycin (Amp) is a cyclic 11-membered peptide isolated from the fermentation broth of *Streptomyces canus*, that is a complex of lipopeptide antibiotics having a common cyclic peptide core and bearing different lipid chains. Heinemann and Bodanzky reported the isolation from hydrolysates of three fatty groups linked to Amp: Δ3-*anteiso*tridecenoic acid (10-methyl-3-dodecenoic acid), the lactone of 4-hydroxy-*anteiso*-tridecanoic acid (4-hydroxy-10-methyl-dodecanoic acid), and 4-hydroxy-*iso*decanoic acid (4-hydroxy-10-methyl-hendecanoic acid) [79,80]. From acid hydrolysate of Amp, L-aspartic acid, L-glycine, L-*threo*-β-methylaspartic acid, L-proline, L-valine, D-pipecolic acid, L-*threo*-α,β-diaminobutyric acid, and D-*erythro*-α,β-diaminobutyric acid were also isolated and identified [81].

Two easily accessible sites for modifications on Amp, *i.e.* the amino acids Dab9 (diaminobutyric acid) and Asp1, have been exploited in order to evaluate the structure-activity relationship between the features of

the peptide's tail and its antibacterial activity. Indicated in Figure 3 are two sites modified to investigate the effect on activity under Migenix's optimization program. By introducing lipophilic tails condensed with i) succinimidyl esters to give amides and ii) isocyanate to give ureas, a set of compounds was generated and tested on methicilline-sensitive *S. aureus* (MSSA) [82]. The results indicated that a linear lipid chain of 15-16 carbon atoms in length introduced in Asp1 confers better activity when compared to bulky substituents in the same position. Furthermore, the effect of aromatic lipophilic substituents was investigated: short aromatic substituents produced inactive derivatives, whereas the antimicrobial activity was restored when the aliphatic chain was reintroduced. Analogues containing various heterocyclic groups flanked by aliphatic chains were, in general, active [83].

After the identification of a linear acyl chain containing 15 atom carbons linked to Asp1 as the group conferring an increased *in vitro* activity, a second position at Dab9 was used for further modifications. A set of compounds generated by introduction of several substituents in Dab-9 were thus prepared and screened *in vitro* against MSSA, keeping constant the C_{15} lipophilic acyl chain linked to Asp1 [83,84]. From this library, MX-2401 emerged, whose *in vitro* profile is characterized by a strong antimicrobial activity against clinically-relevant bacteria including *Streptococcus pneumoniae* and *S. aureus* as well as drug-resistant bacteria such as vancomycin-resistant enterococci (VRE), MRSA, methicillin-resistant *Staphylococcus epidermidis* (MRSE), macrolide-resistant *S. pneumoniae*, and penicillin-resistant *S. pneumoniae* (PRSP) in addition to multidrug resistant *S. aureus*.

Amp-derived lipopeptides share with daptomycin a Ca^{2+}-dependent mode of action on bacterial targets, but they bind to the undecaprenyl lipid carrier which in turn prevents the action of MraY (an enzyme that synthesizes lipid I) [52]. MX-2401 showed efficacy in several animal models of infection, including thigh and lung models, and is currently in the preclinical phase. Its bactericidal activity has been correlated to its calcium-dependent depolarization effect. However, the change in membrane potential by MX-2401 was reported to be relatively small compared to that caused by daptomycin, suggesting a different mechanism of action [85].

6. Echinocandins

The echinocandins comprise a new class of lipopeptide antifungal agents. All molecules in clinical use or development are amphiphilic cyclic hexapeptides with an *N*-linked acyl lipid side-chain and a molecular weight of about 1200 Da [8]. Echinocandins were isolated for the first time from a strain of *Aspergillus ruglosus* and *A. nidulans* and are characterized by their strong antifungal and antiyeast activities [86,87].

Three echinocandin structures were isolated. While the echinocandin B (ECB) structure was determined by chemical degradation studies in combination with X-ray crystallographic analysis [87,88], the structures of echinocandins C and D were elucidated by their conversion into a common intermediate derived from the B form [89]. They are all acylated at the *N*-terminus with a linoleic acid molecule. Furthermore, the total synthesis of echinocandins C and D was carried out by Yasufumi and colleagues in 1986, *via* the stereocontrolled synthesis of all constituent amino acids such as (2S, 3S, 4S)-3-hydroxy-4-methylproline, (2S, 3R)-3-hydroxyhomotyrosine and γ,δ−dihydroxyornithine [90,91].

The structural features common to these lipopeptides include a cyclic peptide and an *N*-terminal fatty acid group, which usually is either branched or unbranched with a chain length of 14 to 18 carbon atoms. Full biological activity seems to require both the cyclic structure [92] and an acyl side chain, emphasizing the importance of the acyl group for the mode of action [93,94].

In order to isolate new and more active antifungal derivatives, deacylation of ECB has been accomplished using an *Actinoplanes utahensis* culture, providing the corresponding inactive deacylated cyclic peptide [95]. With the reintroduction of the *N*-acyl group, the biological activity was restored, as long as certain structural requirements were met. Indeed, while small groups such as acetyl, benzoyl or cyclohexanoyl were ineffective in restoring activity, increase in the chain length from C_{12} up to C_{17}-C_{18}, was proportional with an increase in activity [95,96].

The echinocandin drugs on the market consist of three agents (Figure 4): caspofungin (Cancidas; Merck and Co., Whitehouse Station, New Jersey, USA), micafungin (Mycamine; Astellas Pharmaceuticals, Grand Island, New York, USA) and anidulafungin (Eraxis; Pfizer Pharmaceuticals, New York, USA). Echinocandins act by blocking the synthesis of 1-3-β-D-glucan, a critical component of the fungal cell wall, through noncompetitive inhibition of the enzyme 1-3-β-D-glucan synthase. Fungicidal activity is caused by osmotic disruption after loss of cell wall integrity [97]. The echinocandin antifungal spectrum is restricted with few exceptions to *Candida* spp. and *Aspergillus* spp. and these drugs are available only as intravenous injections. These agents have been studied in multiple

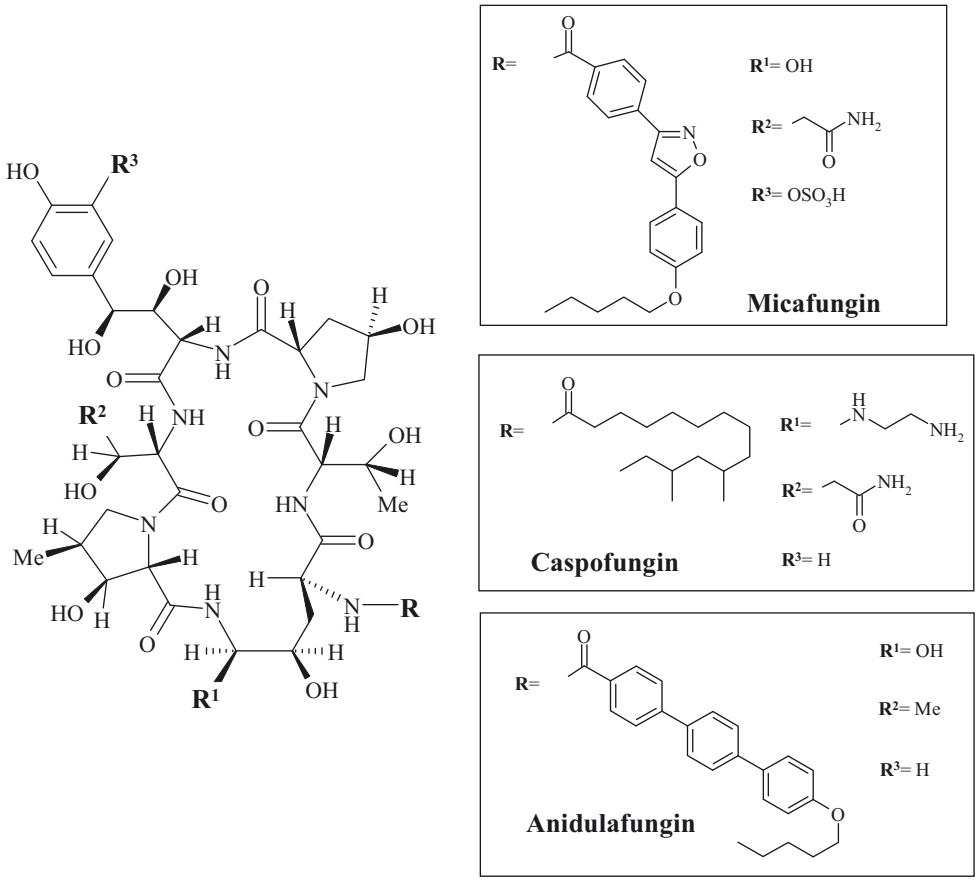


Figure 4. The general structure of echinocandin B. The semi-synthetic derivatives micafungin, caspofungin and anidulafungin and relative characteristic substituents are reported.

and varied clinical settings ranging from invasive, oral and esophageal candidiasis to the treatment of invasive aspergillosis.

7. HB1345

Helix Biomedix (www.helixbiomedix.com) has an extensive proprietary library of peptides derived from the innate defense system including a novel class of short bioactive peptides named lipohexapeptides. These molecules showed an array of activities ranging from antimicrobial, immune modulation, wound healing and angiogenesis. In particular, HB1345 is a synthetic lipohexapeptide anti-infective product with potent and broad spectrum antimicrobial activity against skin pathogens (including *Propionibacterium acnes*). It is bactericidal, non-cross resistant, exhibits low to no resistance emergence, active in serum and lipid mixtures and it is under development for the treatment of acne (http://www.helixbiomedix.com/antiinfective.html).

The company included this class of molecules in both its clinical programs for the development of therapeutics capable of treating skin conditions such as acne, rosacea, MRSA and cutaneous mycoses, and in non-clinical products such as skin care cosmetics [98,99].

8. Semi-synthetic lipoglycopeptides

In the early 1990s, Eli Lilly initiated a large-scale program aimed at discovering semi-synthetic derivatives of vancomycin that may be unaffected by conventional resistance mechanisms. In the wake of this quest, three new glycopeptide antibiotics—dalbavancin, telavancin and oritavancin—have emerged, which are now either in an advanced stage of development or waiting for FDA approval. These antibiotics were generated by the addition of a hydrophobic moiety to the disaccharide functionality of vancomycin, with oritavancin and telavancin being modified at the vancosamine nitrogen, and dalbavancin at the glucosamine nitrogen. They

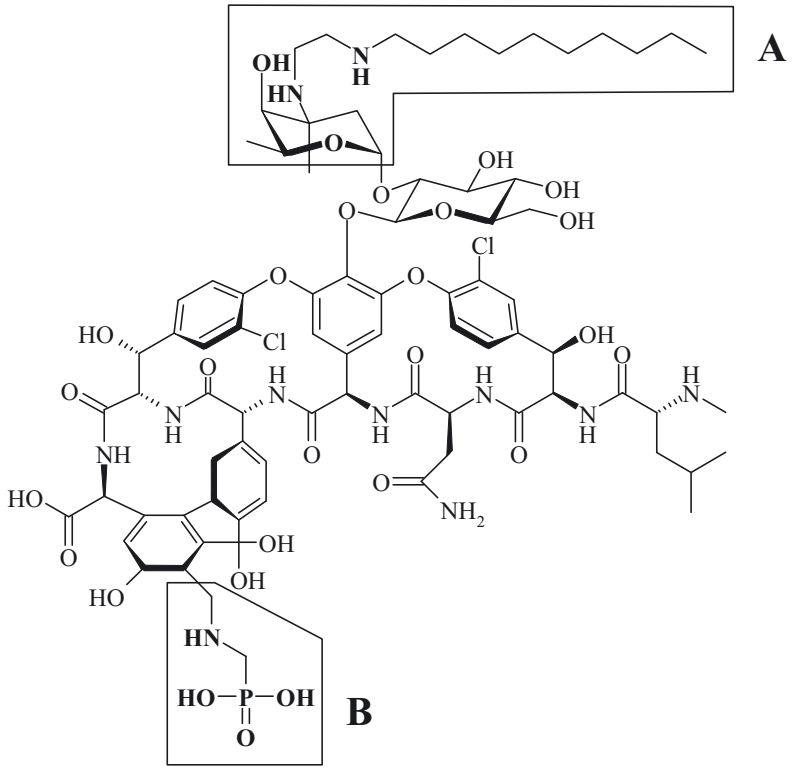


Figure 5. Telavancin structure. The introduction of a decylaminoethyl chain (box A) on the vancosamine sugar and a hydrophilic phosphonomethylaminomethyl group (box B) on the resorcinol-like 4′ position of amino acid 7 improve the ADME (adsorption, distribution, metabolism, excretion) profile of telavancine with respect to vancomycin.

show multiple modes of action and new pharmacological properties [52].

In fact, lipidation of glycopeptides antibiotics with hydrophobic tails may result in an improved activity due to the combined action of a cell wall synthesis inhibitor and a membrane active compound. Unfortunately, this hydrophobic group imparts unfavourable absorption, distribution, metabolism and excretion (ADME) profiles, resulting in a long elimination half-life and high tissue accumulation along with poor excretion and distribution to liver and kidney tissue [100,101]. In terms of activity, the lipid tail confers membrane perturbing properties in bacteria at higher concentrations [102].

On the other hand, the addition of a hydrophilic group to a lipidated vancomycin derivative was demonstrated to improve the *in vitro* activity of this class of compounds. In particular, telavancin contains a phosphonomethylaminomethyl group on the resorcinol-like 4′ position of amino acid, as shown in Figure 5. This approach demonstrated that the addition of a negatively charged auxiliary hydrophilic group to the decylaminoethyl side chain on the vancosamine sugar might result in increased urinary excretion, a shorter half-life, and lower cellular accumulation compared with vancomycin [103].

Telavancin was developed by Theravance (www.theravance.com). This vancomycin derivative is characterized by the presence of a vancomycin pharmacophore that allows binding to the bacterial D-Ala–D-Ala motif. Binding causes inhibition of peptidoglycan biosynthesis, in particular peptidoglycan polymerization (transglycosylation) and subsequent cross-linking (transpeptidation) steps. Recently, it was demonstrated that telavancin's antibacterial activity derives from at least two mechanisms of action. The second mechanism of action of telavancin is related to perturbation of the electrochemical potential and permeability of the bacterial cell membrane, causing membrane permeabilization similarly to the mode of action of antimicrobial peptides [40,102,104,105]. Dissipation of the cell membrane potential is concentration-dependent, rapid (15 minutes), and can be detected starting with telavancin at its 10-fold MIC values [102]. This activity appeared to require interaction with peptidoglycan intermediates, suggesting a molecular basis for bacterial membrane selectivity.

The emerging multifunctional mechanism of action of telavancin confers advantageous antibacterial properties

such as a rapid, concentration-dependent bactericidal activity against extracellular as well as intraphagocytic forms of *S. aureus*, including MRSA, VISA and VRSA strains, along with a reduced potential for development of resistance observed for telavancin both *in vitro* and *in vivo* [106]. The long half-life (7 to 9 hours) and postantibiotic effect (4 to 6 hours) allow for once-daily intravenous administration. The company submitted a New Drug Application (NDA) for the treatment of complicated skin and skin structure infections (cSSSI) caused by Gram-positive bacteria. In addition, two large Phase III studies in patients with healthcare associated pneumonia were recently completed.

9. Conclusions

Daptomycin's approval in the USA in 2003 paved the way for renewed exploration and eventual exploitation of the therapeutic potential of lipopeptides . Due to their mechanism of action, these molecules have several advantages over other agents. These include a rapid and concentration-dependent bactericidal effect which should promote not only clinical cure but also eradication of pathogenic organisms, minimal induction of resistance, and activity against isolates that display various resistance phenotypes. The main limitation of these agents, on the problematic side, is related to solubility issues, their potential toxicity due to a rather unspecific mechanism of action (when used as membrane active antibacterial agents), or to the risk of intracellular and tissue accumulation.

Despite the promise that lipopeptide agents such as daptomycin offer, the need for novel antibiotics continues. To be more specific, the class of lipopeptide antibiotics should be further investigated to determine whether other molecules can be identified with activity against Gram-negative organisms, where we currently have very limited therapeutic options available. This approach has been followed by Cubist Pharmaceuticals by drawing on its suite of biosynthesis technologies constructed upon the success of Daptomycin. The preclinical studies conducted by Cubist for CB-182,804 were promising, and the company announced the submission of an Investigational New Drug Application with the US FDA on December 2008. Additional research is required to investigate the relationships between structural diversity, safety and efficacy in different conditions (*i.e*, the presence of lung surfactant). The coupling of chemical modifications with combinatorial biosynthesis can, in theory, be extended to other fermentation-derived lipopeptides to generate large libraries of compounds for screening and testing. Thus, novel synthetic antibacterial agents based on a lipopeptide scaffold are expected to be an important option to treat different types of drug-resistant pathogens in the near future.

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