Antimicrobial lipopeptides: *in vivo* and *in vitro* synthesis

A. Etchegaray¹ and M. T. Machini²*

¹Faculty of Chemistry, Pontifical Catholic University of Campinas, Rod. D. Pedro I Km 136, Pq. das Universidades, Campinas, SP, 13086-900, Brazil
²Department of Biochemistry, Institute of Chemistry, University of São Paulo, Av. Prof. Lineu Prestes, 748, B8 Sup., sala 0854, São Paulo, SP, 05508-000, Brazil

*Also cited as Miranda, MTM.

The non-ribosomally assembled lipopeptides display a number of biological activities, including antibiotic, antitumor, immune-modulating and immune-suppressive activities. This review starts with general comments on antimicrobial peptides (AMPs), a large family of compounds that includes non-ribosomally assembled lipopeptides. Then, it brings together information on *in vivo* synthesis (biosynthesis) of such hybrid biomolecules using as models the surfactins, potent anionic biosurfactants produced by *Bacillus subtilis* that have potential environmental and medical applications, but limited productivity due to low yields and the high costs associated with microbial growth and surfactant recovery. It follows a brief statement on how important is to overcome those limitations as well as a discussion about *in vitro* synthesis (chemical synthesis) of this type of compounds. Hence, the methods currently used for making peptides are outlined and given examples of lipopeptides and analogues that have been chemically synthesized by the solid-phase method using the Fmoc-based chemistry. It becomes clear that most of these compounds have only been successfully produced in small scale and that a challenge is set up to upscale productivity of non-ribosomally assembled lipopeptides with low costs, high yields and, hopefully, using environmentally friendly methods, techniques and conditions. In this context, modifications of the protocols used for non-ribosomal synthesis and microwave-assisted chemical synthesis seem to be promising alternatives.

**Keywords** antimicrobial peptides; non-ribosomal peptides; lipopeptides; solid-phase peptide synthesis

### 1. Antimicrobial peptides

The continually evolving resistant microbes are challenges for medical care to overcome with novel antimicrobials. Therefore, the research on discovery, properties, production and mechanisms of action of bactericidal and/or fungicidal compounds has become more urgent than ever. To better appraise such urgency, one should look throughout time how many lives have been saved since the discovery of penicillin [1].

Among the natural antibiotics known so far [2,3] is the family of antimicrobial peptides (AMPs), which is composed of several subfamilies [4-6]. Based on how they are biosynthesized, these immune-defense molecules can also be classified in ribosomally and non-ribosomally assembled peptides [3].

The first class has a large number of members. Hundreds of them have been extensively studied worldwide [7], including those termed bacteriocins [5]. However, probably mostly due to their high salt-sensitivity and susceptibility to proteolysis, only a few have been approved for use in therapeutics or in food industry and are now in the market [8].

As to the non-ribosomally assembled peptides, many of them have already been commercialized and used, such as vancomycins (glycopeptides [9]), tyrocidines (cyclic peptides; [10]), gramicidins (cationic cyclic peptides [11]) and polymyxins (cyclic lipopeptide [8]). The last decade has seen a growing interest in anionic or cationic AMPs covalently attached to a fatty acid moiety (lipopeptides), also members of this class, as is the case of polymyxins [12].

### 2. Lipopeptides

Lipopeptides are linear or cyclic peptides acylated by a lipid, usually a fatty acid side chain (examples are shown in Figure 1). It has been described that these compounds are produced only in bacteria and fungi of various habitats during cultivation on carbon and nitrogen sources [13,14].

The literature is rich in descriptions of lipopeptide biological actions, such as antibacterial [15], antifungal [16,17], improved bacterial motility [18], humoral and/or cell-mediated immunity [19,20], surfactant [21,22] and many others [18]. It has been proposed that their natural functions are in reality strictly related to the biology of the producing microorganism [23,18]. Table 1 lists some lipopeptides, their roles and producing microorganisms.

Due to their antimicrobial, toxic and surfactant properties, lipopeptides are scientifically and economically valued molecules. A good example is daptomycin, which, even though it is somewhat toxic to human cells, has been approved by the Food and Drug Administration (FDA) for therapeutic application in systemic and life-threatening infections caused by Gram-positive bacteria [24]. Presently, caspofungin, micafungin, and anidulafungin are other examples of lipopeptides used as commercial antibiotics [25]. Needless to say that, as a consequence, these compounds must be produced in large amounts using *in vivo* and *in vitro* synthetic methods (biosynthesis and chemical synthesis, respectively).
Table 1: Some lipopeptides, their biological roles and their producing microorganisms

<table>
<thead>
<tr>
<th>Lipopeptide</th>
<th>Function</th>
<th>Producing microorganism (reference)</th>
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<tbody>
<tr>
<td>Surfactin</td>
<td>Surfactant activity</td>
<td><em>Bacillus subtilis</em> [21]</td>
</tr>
<tr>
<td>Fengycins</td>
<td>Antifungal activity</td>
<td><em>Bacillus subtilis</em> [16]</td>
</tr>
<tr>
<td>Viscosin</td>
<td>Increase of bacterial motility</td>
<td><em>Pseudomonas fluorescens</em> [18]</td>
</tr>
<tr>
<td>Lipopeptide present in MCF-7 cells</td>
<td>Cytotoxicity, cell-cycle arrest and apoptosis in MCF-7 cell</td>
<td><em>Bacillus natto</em> [26]</td>
</tr>
<tr>
<td>Iturins</td>
<td>Antifungal activity</td>
<td><em>Bacillus subtilis</em> [29]</td>
</tr>
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</table>

3. Biosynthesis of lipopeptides

Lipopeptides can be produced by the non-ribosomal biosynthetic pathway, an assembly line that parallels protein synthesis but it is ribosomal-independent and elaborated by a complex mechanism involving multifunctional synthetases.

Figure 1 shows the structure of two lipopeptides elaborated by the non-ribosomal pathway, an antibiotic of the polymyxin family and a biosurfactant of the surfactin family [27,21]. The uniqueness of these the surfactins is the macrocyclization that is accomplished, in the case of surfactin, produced by *Bacillus subtilis*, due to the substrate beta-hydroxy fatty acid, which is linked to the N-terminal amino acid by an amide bond (involving the carboxyl moiety of the fatty acid) and to the C-terminal amino acid by an ester bond (involving the beta-hydroxyl side chain of the fatty acid). A database of non-ribosomal peptides and, specifically, non-ribosomally assembled lipopeptides can be consulted at the Norine database (http://bioinfo.lifl.fr/norine/ [28]).

In the biosynthesis of surfactins, in order to produce generate their unusual peptide structure, a combination of enzyme-catalyzed steps is carried out by three modular non-ribosomal peptide synthetases (NRPSs), the surfactin synthetases A, B and C [30,31] presented in Figure 2. The enzymatic steps involve aminoacyl-adenylation, thioesterification, condensation, epimerization and thioesterase activities, depending on the specific enzyme module.

Fig. 1 Primary structures of polymyxins, where Dba is 1-diaminobutyric acid, (A) and of surfactins, showing the beta-hydroxylated fatty acid that links the carboxyl and aminoterminals of the heptapeptide (B).

In the biosynthesis of surfactins, in order to produce generate their unusual peptide structure, a combination of enzyme-catalyzed steps is carried out by three modular non-ribosomal peptide synthetases (NRPSs), the surfactin synthetases A, B and C [30,31] presented in Figure 2. The enzymatic steps involve aminoacyl-adenylation, thioesterification, condensation, epimerization and thioesterase activities, depending on the specific enzyme module.

Fig. 2 Enzymatic assembly line involved on the biosynthesis of surfactins (adapted from the work of Kraas et al. [31]). The NRPSs are involved in the steps leading to surfactin synthesis. On every enzyme module there are specific domains enrolled on adenylation of each amino acid as denoted for Glu, Leu, Val and Asp; a domain involved in condensation (C); a peptidyl carrier protein domain (PCP); an epimerization domain (Ep) and a thioesterase domain (Te).
Figure 3 shows the catalytic steps of surfactin synthesis in detail. The biosynthesis of surfactin, like in many examples of non-ribosomal peptide biosynthesis, is carried out by the thiotemplate mechanism, a step-by-step enzyme catalyzed reaction that involves attachment of the alpha-carboxyl of each amino acid to an SH group within the enzyme.

The referred SH group originates from a 4'-phosphopantetheine (PAN) cofactor, which is attached to the peptidyl carrier protein domain (PCP domain). The name PCP is coined to this domain in analogy to a similar domain involved in fatty acid biosynthesis, the acyl carrier protein domain (ACP). Therefore, the steps involved in non-ribosomal biosynthesis are aminoacyl-adenylation at the expense of ATP; generation of a reactive intermediate in the form of thioester; condensation; and tailoring steps, such as epimerization, heterocyclization and others [32].

The first reaction catalyzed by peptide synthetases is important because it involves substrate selection and activation of the amino acid carboxyl as mixed anhydride with AMP (eq. 1) [33].

$$AA + ATP \rightarrow AA-AMP + PPi$$ (1)
The active form (Holo-form) of a peptide synthetase has a 4’-phosphopantetheine cofactor attached to the PCP domain. Owing to this functionalization, an SH group allows the formation of a reactive intermediate of the substrate amino acids, activated at the carboxyl end. The following equation (eq. 2) shows the formation of an enzyme-substrate intermediate, as thioester, catalyzed by the PCP domain.

\[
\text{AA-AMP + Enz-SH} \rightarrow \text{Enz-S-AA} \quad (2)
\]

Before the first peptide bond is formed, as in the example of other biosynthetic clusters involved in the synthesis of lipopeptides, the N-terminal amino acid is acylated by a fatty acid. As stated above, in surfactins, the fatty acid incorporated into the N-terminal amino acid is a beta-hydroxyl-fatty acid, which can vary from 13 to 15 carbons in length [21]. The catalytic domain involved in the aminoaclylation reaction is a special type of condensation domain, which is normally found in other synthetases that receive an aliphatic fatty acid or aryl compounds. This is also found at the N-terminus of several subunits of bacterial non-ribosomal peptide synthetases in order to transfer a growing peptide chain from one synthetase to the next collinear subunit. Such enzyme architecture can be seen in the synthetases presented in Figure 3. In our previous work, we showed that condensation domains located at N-terminus of peptide synthetases present higher sequence similarity. The alignment of C-domains also allowed us to group separately the condensation domains catalyzing acylation of aromatic derivatives and those that promote acylation of aliphatic fatty acids [34].

Thus, the formation of peptide bonds and N-terminal acylation reactions are catalyzed by condensation domains, where the catalytic mechanism involves conserved histidines, which will carry out the abstraction of protons from the middle module which is normally found in other synthetases that receive an aliphatic fatty acid or aryl compounds. This is also found at the N-terminus of several subunits of bacterial non-ribosomal peptide synthetases in order to transfer a growing peptide chain from one synthetase to the next collinear subunit. Such enzyme architecture can be seen in the synthetases presented in Figure 3. In our previous work, we showed that condensation domains located at N-terminus of peptide synthetases present higher sequence similarity. The alignment of C-domains also allowed us to group separately the condensation domains catalyzing acylation of aromatic derivatives and those that promote acylation of aliphatic fatty acids [34].

The corresponding acylation of the N-terminal amino acid (glutamic acid) is similarly performed. However, the fatty acid is activated as thioester by a different enzyme that activates the fatty acid as coenzyme A intermediate, demonstrating that the condensation domain in fact acts on a preliminarily activated fatty acid thioester [35].

In surfactin biosynthesis, there are two tailoring conversions of L-leucine into D-Leucine. Both are catalyzed by an epimerase domain and take place at the C-terminal modules of the srfAA and SrfAB synthetases. According to the work of Luo et al. [36], epimerization occurs after amino acid esterification. These authors have studied the conversion of L to D-phenylalanine in the biosynthesis of gramicidin, another cyclic peptide produce by the Bacillus genus, having showed that the ratio of D-isomer in the thioester form over its adenylate counterpart is approximately 3,000 [36]. Therefore, in Figure 3 the aminoaoyl-adenylates were presented as L-amino acids rather than in the D-form; conversely, the corresponding thioesters were shown as D-amino acids.

The last biosynthetic step is the hydrolysis of the peptide from the last PCP domain, a reaction that is catalyzed by the thioesterase (Te) domain of srfAC (Figure 3). This step is a concerted reaction that also promotes cyclization of the peptide by a transesterification reaction in which the hydroxyl of the fatty acid makes an ester linkage with the carboxyl end of the C-terminal amino acid of surfactin, thus leading to a cyclic peptide as shown in Figure 1 and Figure 3C. There are many other lipopeptides, such as daptomycin, produced by a similar pathway.

An interesting feature of the thiotemplate mechanism is the possibility of using the enzymatic machinery for combinatorial biosynthesis, given that substrate selection is not restricted to the primary substrates. The partially relaxed selection of substrates by the adenylation and thioesterification domains has been explored by some authors, allowing the biosynthesis of novel peptides using combinatorial biosynthesis, a strategy that can be used by feeding novel substrates or precursors in the growth medium [37]. Another approach that has been explored is based on the engineering of synthetases by swaping modules from one synthetase into another, a strategy that leads to a novel peptide structure and, therefore, an “unnatural product” that is yet produced as natural product [38].

However, the bottle neck in non-ribosomal production of AMPs, including lipopeptides, is the cost of production considering the low yields at the end of the growth of the producing organism. To overcome this problem, several studies have been carried out using cheaper or recycled substrate specifically for production of surfactins and other lipopeptide families [39]. Besides, chemical synthesis may provide higher yields and, undoubtedly, allow insertion of novel amino acids, cyclizations and modifications in the structures to create analogues with modified biological properties, which can be more appropriate for specific purposes such as specificity and capacity to avoid microbial resistance.

### 4. Chemical synthesis of lipopeptides

It is well known that chemical peptide synthesis is based on amide bond formation between amino acid or peptide derivatives as shown in the following reaction, in which R-COOH represents an amino acid or a peptide derivative, X represents a good leaving group and \( \text{H}_2\text{N-HR} \) represents an amino acid or a peptide derivative:
Peptide chain assembly can be achieved by the classical or the solid-phase method using one of the two strategies specially developed for such purpose: tert-butyloxycarbonyl (Boc) and fluorenylmethyloxycarbonyl (Fmoc) (Figure 4). As shown, these methods and synthetic strategies share as the common features the needs for (i) orthogonal protection and for $\alpha$-carboxyl-group activation during peptide chain building as well as (ii) full deprotection when the assembly is complete. Their main differences are the blocker of the $\alpha$-aminogroup of the amino acids that act as the acyl donors in peptide bond formation (temporary: Boc or Fmoc) and protecting groups of their reactive side-chains (semi-permanent: benzyl or tert-butyl group derivative, respectively) [40,41].

In classical or traditional peptide synthesis, the starting materials (fully or partially protected amino acids), coupling reagents, bases, acids, byproducts and products are fully soluble in the reaction media (Figure 4A; [42,43]). In such anhydrous organic conditions, it is imperative to monitor formation of the products by a colorimetric or chromatographic method as well as to separate them from the reagents, solvent and byproducts using extraction, precipitation and/or liquid chromatography. As the whole synthetic process comprise many steps of $\alpha$-aminogroup deprotection, $\alpha$-carboxylgroup deprotection and amino acid coupling, classical peptide synthesis is time-consuming and not always an easy task. Full deprotection gives the crude desired peptide to be purified and chemically characterized prior to use.

Solid-phase peptide synthesis (SPPS) has been conceived to make peptide chain assembly fast and simple. In fact, as the amino acids, partially or fully protected, are incorporated on a resin that is insoluble in the reaction media, the soluble reagents, bases, acids and byproducts are removed by simple filtration followed by alternate washings of the aminoacyl- and growing peptidyl-resin with solvents able to swell or shrink them (Figure 4B; [44,45]). As a result, SPPS has become the method of choice to synthesize trouble-free peptides manually [46] or automatically, separately or in parallel [47], conventionally (at room temperature as first proposed by Merrifield; [48]) or at elevated temperature, using conventional heating [49-51], or with the assistance of microwaves [52-54]. Detachment from the resin and simultaneous full deprotection give the crude peptide, which is usually contaminated with byproducts generated from undesired reactions that also take place during the steps of amino acid deprotection and coupling.

Despite the advances and general applicability of both methods, it is of interest to improve them even more because there are still peptides extremely difficult to be obtained by the classical or solid-phase methods. Moreover, many research groups around the world have attempted to chemically synthesize modified proteins [55], not found in natural sources and impractical to produce by the recombinant DNA methodology. For such purposes, peptide derivatives, rather than amino acid derivatives, are used as starting materials.

**Fig. 4** Chemical synthesis of a tripeptide containing three different amino acid residues using the classical (A) or solid-phase method (B). Aa$_1$, Aa$_2$ and AA$_3$ represent the amino acids, R represents the resin employed, R1, R2 and R3 are the amino acid side-chains; circles and ovals represent the protecting groups for the reactive amino acid side-chains and the triangle is the Boc or Fmoc group.
The methods of classical chemical synthesis and, mostly, SPPS have been extremely useful to study biologically active peptides [56], including those with antibiotic activity [57]. Indeed, the last two decades have witnessed an overflow of reports on the properties, structure and mode of actions of antimicrobial peptides (AMPs) that would not exist without the possibility of using their synthetics forms (generally, isolation from natural sources provides little amounts of purified bioactive peptides). In addition, structure-activity relationship of natural AMPs has been widely explored simply because the chemical synthesis methodologies have made possible to prepare their truncated, modified and/or cyclic analogues [58-60]. Table 2 illustrates this point by listing some synthetic AMPs and analogues that have been obtained and studied, expanding the current knowledge on such remarkable biomolecules.

There are several excellent reviews about lipopeptides focusing on their biosynthesis, properties, biological roles, structures and potential to be employed in the industry [37,61-64,18,65], but not on their chemical synthesis. In 2001 and in commemoration of 100 years of peptide synthesis, Pegoraro and Moroder [66] comprehensively reviewed this subject. With an impressive collection of information, these authors showed that chemical synthesis has been successfully used for peptide assembly during the preparation of N-myristoyl peptides, S-prenylated peptides, S-palmitoylated peptides, doubly lipidated peptides, nonnatural mono- and multiple-lipidated peptides [66]. Besides giving chemical reactions and detailed protocols for the preparation of modified amino acids (building blocks), the authors reminded that modification of synthetic lipopeptides with groups that can be traced by proper analytical techniques is essential to make them suitable for biological studies [67]. Therefore such additional step(s) must be considered when designing the synthetic strategy to be employed.

From 1994 to date, dozens of lipopeptides and analogues with variable biological functions have been chemically synthesized, being most of the synthesis performed on resin by the Fmoc strategy with the aim to demonstrate the efficiency of the strategy studied or to obtain the desired lipopeptide to be used in exploratory works. In fact, for instance, Casey and Buss reported optimized synthetic procedures specially developed to furnish simple lipoderivatives [68]. Okeye et al. proposed a procedure to prepare prenylated peptides, which is based on chemoselective transformations of peptide containing Se-phenylselenocystein [69]. DeOgny et al. described the rapid and convenient synthesis of tripalmitoylglycerol analogs of the N-terminal sequences of several bacterial lipopeptides, which also present immunogenic activity [70]. Pagadoy et al. synthesized the racemic mixture and the R isoform of surfactin, lipopeptide still regarded as the most powerful biosurfactant [71]. Into et al. reported the synthesis of a dipalmitoylated lipopeptide derived from paralogous lipoproteins of Mycoplasma pneumonia with immunostimulatory activity [72]. JolcK et al. [73] developed a novel approach for the synthesis of PEGylated lipopeptides based on SPPS and the use of the Cu(I) catalyzed azide/alkene Huisgen 1,3-dipolar cycloaddition (CuAAC “Click” conjugation) previously reported by Meldal and coworkers [74] and Sharpless and coworkers [75]. Schieck et al. prepared the myristoylated-peptide Myr-HBVpreS/2-78, which is a Hepatitis B virus entry inhibitor, by using solid-phase synthesis at 50 °C [76]. Bionda et al. have just described the suitability of guanilation of resin-bound peptidyl amines for the preparation of fusaricidin/LI-F class of cyclic lipopeptides, which present broad antimicrobial activity [77]. Vilà et al. also have just reported different synthetic strategies for obtaining cyclic lipopeptides with potential to act against plant pathogens [78].

In these examples, peptide assembly was performed in small scale on different resins, such as MBHA (Boc strategy), Fmoc-RINK-MBHA, TentaGel R RAM, Rapp Polymere or NovaSyn TentaGel (Fmoc strategy). The lipid moiety was successfully used for peptide assembly during the preparation of N-myristoyl peptides, S-prenylated peptides, S-palmitoylated peptides, doubly lipidated peptides, nonnatural mono- and multiple-lipidated peptides [66]. Besides the Cu(I) catalyzed azide/alkene Huisgen 1,3-dipolar cycloaddition (CuAAC “Click” conjugation) previously reported by Meldal and coworkers [74] and Sharpless and coworkers [75]. Schieck et al. prepared the myristoylated-peptide Myr-HBVpreS/2-78, which is a Hepatitis B virus entry inhibitor, by using solid-phase synthesis at 50 °C [76]. Bionda et al. have just described the suitability of guanilation of resin-bound peptidyl amines for the preparation of fusaricidin/LI-F class of cyclic lipopeptides, which present broad antimicrobial activity [77]. Vilà et al. also have just reported different synthetic strategies for obtaining cyclic lipopeptides with potential to act against plant pathogens [78].

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Table 2 A few examples of synthetic analogues of antimicrobial peptides (AMPs) with improved features

<table>
<thead>
<tr>
<th>Original AMP</th>
<th>Analogue (reference)</th>
</tr>
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<tbody>
<tr>
<td>Bovine α-hemoglobin fragment 33-61 (Hb33-61)</td>
<td>amidated bovine α-hemoglobin fragment 40-61 (Hb40-61a) [58]</td>
</tr>
<tr>
<td>Tachypleusin (TP)</td>
<td>[Gly6]ecTP* [79]</td>
</tr>
<tr>
<td>Crotamine</td>
<td>Nucleolar targeting peptides (NrTPs) [80]</td>
</tr>
<tr>
<td>Pyrrococerin (pyrr)</td>
<td>Pip-pyr-MeArg** dimer [81]</td>
</tr>
<tr>
<td>Mellitin</td>
<td>Cyclic mellitin [82]</td>
</tr>
</tbody>
</table>

* cc is cyclic cystine; **MeArg is N-methyl-arginine and Pip is 4-amino-piperidine-4-carboxylic acid

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References


