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Effects of Cyclic Lipodepsipeptide Structural Modulation on Stability, Antibacterial Activity, and Human Cell Toxicity

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Bacterial infections are becoming increasingly difficult to treat due to the development and spread of antibiotic resistance. Therefore, identifying novel antibacterial targets and new antibacterial agents capable of treating infections by drug-resistant bacteria is of vital importance. The structurally simple yet potent fusaricidin or LI-F class of natural products represents a particularly attractive source of candidates for the development of new antibacterial agents. We synthesized 18 fusaricidin/LI-F analogues and investigated the effects of structure modification on their conformation, serum stability, antibacterial activity, and toxicity toward human cells. Our findings show that substitution of an ester bond in depsipeptides with an amide bond may afford equally potent analogues with improved stability and greatly decreased cytotoxicity. The lower overall hydrophobicity/amphiphilicity of amide analogues in comparison with their parent depsipeptides, as indicated by HPLC retention times, may explain the dissociation of antibacterial activity and human cell cytotoxicity. These results indicate that amide analogues may have significant advantages over fusaricidin/LI-F natural products and their depsipeptide analogues as lead structures for the development of new antibacterial agents.

Introduction

Antibiotic resistance is increasing at a faster rate than the development of new antibiotics,[1–4] and the prevalence of multidrug-resistant (MDR) bacteria has become a global public health problem.[4] The majority of life-threatening infections worldwide are caused by the *ESKAPE* pathogens (***E**nterococcus faecium*, ***S**taphylococcus aureus*, ***K**lebsiella pneumoniae*, ***A**cinetobacter baumannii*, ***P**seudomonas aeruginosa*, and ***E**nterobacter* spp.).[5,6] This group of bacteria is encountered in more than 40% of hospital-acquired infections, and is resistant to the majority of commonly used antibiotics. Therefore, the identification of novel antibacterial targets and new antibacterial agents capable of treating infections by drug-resistant bacteria is crucial.

Naturally occurring cyclic lipodepsipeptides that contain one or more ester bonds along with the amide bonds have emerged as promising lead compounds for the discovery of new antibiotics.[7–10] The biosynthesis of these peptides proceeds non-ribosomally and is catalyzed by a complex of multifunctional enzymes termed non-ribosomal peptide synthases (NRPSs). NRPSs have a unique modular structure in which each module contains the requisite domains for the recognition and activation of a single amino acid, generating a wide structural and functional diversity of non-ribosomal peptides.[11] Within this class of natural products, the cyclic lipodepsipeptide daptomycin (Cubicin, Cubist Pharmaceuticals Inc.)[7,12,13] has already been approved for use in the USA, European Union, and Canada for the treatment of infections caused by MDR bacterial strains. Ramoplanin (Nanotherapeutics Inc.)[7,14–16] is another example of a cyclic lipodepsipeptide with the potential for reverting bacterial multidrug resistance. Ramoplanin is currently entering phase III clinical trials for the treatment of *Clostridium difficile*-associated diarrhea.

Despite progress in the development of new antibacterial agents, it is inevitable that resistant strains of bacteria will emerge in response to widespread use of a particular antibiotic and limit its usefulness. Structurally simple yet potent fusaricidins or the LI-F family of natural products (Figure 1) represent particularly attractive candidates for the development of new antibacterial agents capable of reverting infections caused by MDR bacteria.[7,17–20]

Fusaricidins/LI-Fs are cyclic lipodepsipeptide antifungal antibiotics isolated from *Paenibacillus* sp. Their common structural feature is the macrocyclic ring consisting of six amino acid residues, three of which, Thr^1, D-*allo*-Thr^4 (D-*a*Thr^4), and D-Ala^6, are conserved throughout the family, as well as the 15-guanidino-3-hydroxypentadecanoic acid tail attached to the N-terminal Thr^1 residue by an amide bond. Fusaricidins/LI-Fs are cyclized by a lactone bridge between the N-terminal Thr^1 hydroxy group and the C-terminal D-Ala^6. Among isolated fusaricidin/LI-F antibiotics, fusaricidin A or LI-F04a (Figure 1)[20] have shown the most promising antimicrobial activity against a variety of

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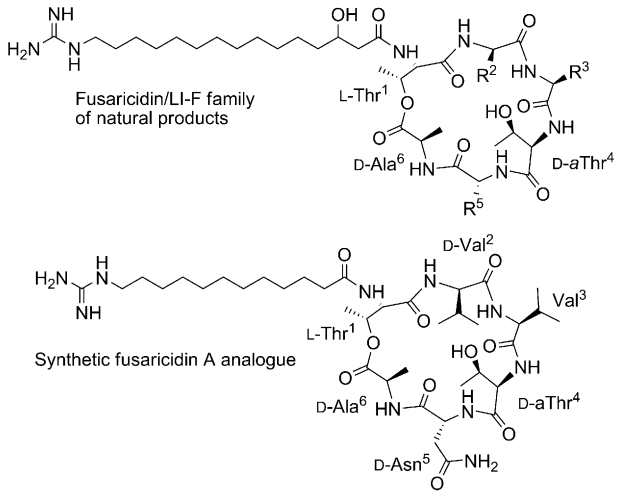


Compound	R^2	R^3	R^5
LI-F03a/Fusaricidin C	D-Val	Tyr	D-Asn
LI-F03b/Fusaricidin D	D-Val	Tyr	D-Gln
LI-F04a/Fusaricidin A	D-Val	Val	D-Asn
LI-F04b/Fusaricidin B	D-Val	Val	D-Gln
LI-F05a	D-Val	Ile	D-Asn
LI-F05b	D-Val	Ile	D-Gln
LI-F06a	D-*a*Ile	Val	D-Asn
LI-F06b	D-*a*Ile	Val	D-Gln
LI-F07a	D-Val	Phe	D-Asn
LI-F07b	D-Val	Phe	D-Gln
LI-F08a	D-Ile	D-Ile	D-Asn
LI-F08b	D-Ile	D-Ile	D-Gln
Synthetic fusaricidin A analogue[24]	D-Val	Val	D-Asn

Figure 1. Structures of the fusaricidin/LI-F family of compounds.

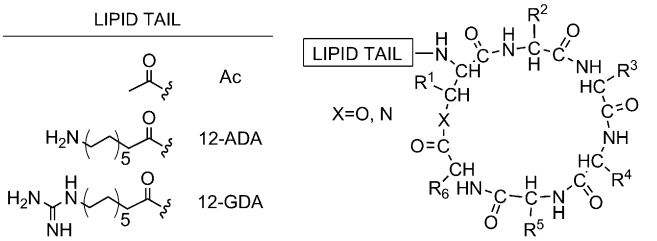
fungi, including the clinically important *Candida albicans* and *Cryptococcus neoformans*, and against Gram-positive bacteria such as *S. aureus* (MIC value range: 0.78–3.12 μg mL^{-1}). Fusaricidins/LI-Fs have not, however, shown activity against Gram-negative bacteria.[18,19] The mode of action of fusaricidins/LI-Fs is still unknown. The total synthesis of the fusaricidin A/LI-F04a natural product using a combination of solid- and solution-phase synthetic approaches was recently reported by Cochrane et al.[21] Two research groups, Jensen and colleagues[22] and Park and co-workers,[23] reported the identification and isolation of the putative fusaricidin/LI-F synthetase gene, *fusA*, from *Paenibacillus polymyxa*, opening the possibility for the development of biosynthetic approaches toward this family of naturally occurring cyclic lipodepsipeptides and their analogues.

We previously reported a complete Fmoc solid-phase synthesis of a fusaricidin A/LI-F04a analogue containing 12-guanidinododecanoic acid instead of the naturally occurring 15-guanidino-3-hydroxypentadecanoic acid (Figure 1).[24] Total synthesis of fusaricidin/LI-F antifungal antibiotics, and particularly unlimited access to their synthetic analogues, represent important initial steps toward full exploitation of their antimicrobial potential. Herein we describe our efforts to determine the structural aspects of fusaricidin/LI-F peptides that are required for antimicrobial activity, improved serum stability, and separation of antibacterial activity from toxicity toward human cells.

Results

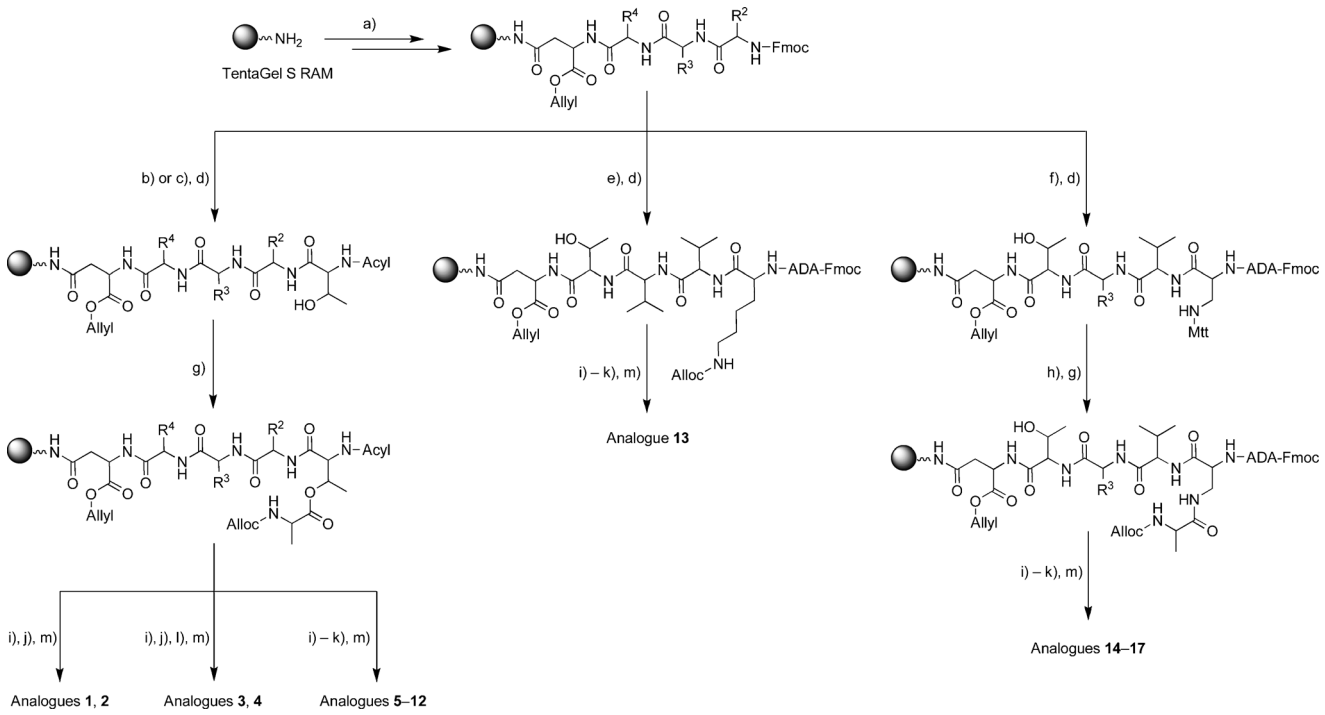
Solid-phase synthesis

The amino acid sequences and lipid tails of synthesized fusaricidin A/LI-F04a analogues **1**–**18** are shown in Figure 2. Analogues **1**–**12** are depsipeptides that contain an ester bond between Thr[1] and D-Ala[6] or Gly[6] residues. In analogue **13**, naturally occurring amino acid residues Thr[1] and D-Ala[6] are replaced with Lys, thereby substituting the ester moiety as well as two chiral centers, while keeping the same number of the atoms in the ring. The remaining four cyclic analogues **14**–**17** have an ester bond replaced with an amide bond by substituting Thr[1] for Dap[1]. Analogue **18** is a linear version of **6** and was prepared as a control. The synthesis of depsipeptide analogues **1**–**12** is shown in Scheme 1.[24] In the first step, the C-terminal amino acid Fmoc-D-Asp-OAllyl was attached to a PEG–PS-based amide resin (TentaGel S RAM) via the side chain by using a standard HBTU/HOBt/NMM synthetic protocol. Standard



Analogue	Lipid Tail	Sequence[a]	R_t [min][b]
1	Ac	Thr[1]-D-Val[2]-Val[3]-**D-Thr[4]**-D-Asn[5]-D-Ala[6]	11.89
2	Ac	Thr[1]-D-Val[2]-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	11.96
3	12-ADA	Thr[1]-D-Val[2]-Val[3]-**D-Thr[4]**-D-Asn[5]-D-Ala[6]	14.78
4	12-ADA	Thr[1]-D-Val[2]-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	14.87
5	12-GDA	Thr[1]-D-Val[2]-Val[3]-**D-Thr[4]**-D-Asn[5]-D-Ala[6]	15.76
6	12-GDA	Thr[1]-D-Val[2]-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	15.81
7	12-GDA	Thr[1]-D-Val[2]-Val[3]-**D-Ala[4]**-D-Asn[5]-D-Ala[6]	15.16
8	12-GDA	Thr[1]-D-Val[2]-**Ala[3]**-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	14.95
9	12-GDA	Thr[1]-**D-Ala[2]**-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	14.7
10	12-GDA	Thr[1]-D-Val[2]-Val[3]-D-*a*Thr[4]-D-Asn[5]-**Gly[6]**	15.48
11	12-GDA	Thr[1]-D-Val[2]-**Phe[3]**-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	16.82
12	12-GDA	Thr[1]-D-Val[2]-**Tyr[3]**-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	15.18
13	12-GDA	ε**Lys[1]**-D-Val[2]-Val[3]-D-*a*Thr[4]-D-Asn[5]	15.44
14	12-GDA	**Dap[1]**-D-Val[2]-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	15.12
15	12-GDA	**Dap[1]**-D-Val[2]-**Ala[3]**-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	14.17
16	12-GDA	**Dap[1]**-D-Val[2]-**Phe[3]**-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	16.27
17	12-GDA	**Dap[1]**-D-Val[2]-**Tyr[3]**-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	14.58
18	12-GDA	Thr[1]-D-Val[2]-Val[3]-D-*a*Thr[4]-D-Asn[5]-D-Ala[6]	15.01 Linear

Figure 2. Sequences of synthesized fusaricidin A/LI-F04a analogues. [a] Differences among the sequences of naturally occurring fusaricidin A/LI-F04a and synthetic analogues are highlighted in bold. [b] R_t: retention time obtained by analytical RP HPLC; method: 2% solvent B for 0.5 min followed by linear gradient 2→98% solvent B over 30 min, for which solvent A is 0.1% TFA in H_2O, and B is 0.08% TFA in CH_3CN.



Scheme 1. Synthesis of fusaricidin A/LI-F04a analogues **1**–**17**. *Reagents and conditions:* a) Fmoc-D-Asp-OAllyl and Fmoc-AA-OH, standard Fmoc SPPS deprotection and coupling protocols; b) Ac-Thr-OH, standard Fmoc SPPS deprotection and coupling protocols; c) Fmoc-Thr-OH, standard Fmoc SPPS deprotection and coupling protocols; d) Fmoc-ADA-OH, standard Fmoc SPPS deprotection and coupling protocols; e) Fmoc-Lys-OH, standard Fmoc SPPS deprotection and coupling protocols; f) Fmoc-Dap(Mtt)-OH, standard Fmoc SPPS deprotection and coupling protocols; g) Alloc-D-Ala-OH or Alloc-Gly-OH (4 equiv), DIC (4 equiv), DMAP (0.2 equiv), CH_2Cl_2, RT, 18 h; h) TFA/CH_2Cl_2 (1% *v/v*), RT, 30 min; i) $Pd(Ph_3P)_4$ (0.1 equiv), $HN(CH_3)_2{\cdot}BH_3$ (4 equiv), CH_2Cl_2, RT, 2×10 min; j) PyBOP/HOBt/DIEA (2:2:6 equiv), DMF, RT, 18 h; k) piperidine/DMF (20% *v/v*), RT, 25 min, *N*,*N*-bis(*tert*-butoxycarbonyl)thiourea (3 equiv), 2-chloro-1-methylpyridinium iodide (3 equiv), TEA (4 equiv), DMF, RT, 18 h; l) piperidine/DMF (20% *v/v*), RT, 25 min; m) TFA/TIA/H_2O (95:2.5:2.5 *v/v/v*), RT, 3 h.

Fmoc SPPS strategies were used throughout. The ester bond was formed between the Thr^1 side chain hydroxy group and Alloc-D-Ala^6-OH or Alloc-Gly^6-OH carboxyl groups using DIC/DMAP coupling conditions in CH_2Cl_2. To avoid potential epimerization during Alloc-D-Ala^6-OH coupling, a catalytic amount of DMAP (0.2 equiv) was used.[25,26] Under applied experimental conditions no epimerization was observed as indicated by analytical RP HPLC (data not shown). The lipid tail, Fmoc-aminododecanoic acid (Fmoc-ADA-OH), was incorporated into the linear peptide precursor prior to D-Ala^6/Gly^6 coupling via ester bond and on-resin cyclization in order to avoid an undesired O→N acyl shift known to occur under basic conditions required for Fmoc removal.[24,27,28]

After selective removal of Alloc and Allyl protecting groups by treatment with $Pd(Ph_3P)_4$ and non-basic borane dimethylamine complex as scavenger,[13] the linear peptide was cyclized through an amide bond between D-Ala^6 or Gly^6 and D-Asn^5 residues using PyBOP. The conversion of the lipid tail amino group into the desired guanidino function was achieved by removal of the Fmoc protecting group using a standard piperidine deprotection protocol and subsequent treatment of the peptidyl resin with *N*,*N*-bis(*tert*-butoxycarbonyl)thiourea followed by Mukaiyama's reagent, 2-chloro-1-methylpyridinium iodide.[14] Final deprotection and cleavage from the resins was carried out with a cleavage cocktail of TFA/TIA/H_2O (95:2.5:2.5 *v/v/v*).

Amide analogues **13**–**17** were prepared by replacing the Thr^1 residue with Lys (compound **13**) or Dap (**14**–**17**; Scheme 1). For this purpose, an identical Fmoc SPPS strategy was employed. In the case of analogue **13**, upon synthesis of the linear precursor, Allyl and Alloc protecting groups were removed, and the peptide was cyclized through the Lys^1 ε-amino group and the D-Asn^5 α-carboxyl group. In the case of analogues **14**–**17**, selective removal of the Mtt protecting group from Dap^1 with 2% TFA in CH_2Cl_2 allowed coupling of Alloc-D-Ala-OH through standard coupling protocols. The final synthetic steps were performed as described above. Linear analogue **18** was prepared on 2-chlorotrityl chloride resin (Cl-TrtCl)[29] using standard Fmoc chemistry, starting with the attachment of Fmoc-D-Ala-OH via the carboxylic group. Loading of the resin was determined to be 0.5 mmol g^{-1}.[30] The use of Cl-TrtCl resin in this case afforded a linear peptide with a C-terminal carboxylic group, identical to the hydrolysis product of **6**. All analogues were purified by preparative RP HPLC, and purity (≥ 95%) was confirmed with analytical RP HPLC and MALDI-TOF MS.

Conformational studies

The structural features of representative fusaricidin analogues **6** and **14** as well as the linear peptide **18** were monitored by circular dichroism (CD) spectroscopy (Figure 3). CD spectra were recorded in aqueous media as well as in less polar tri-

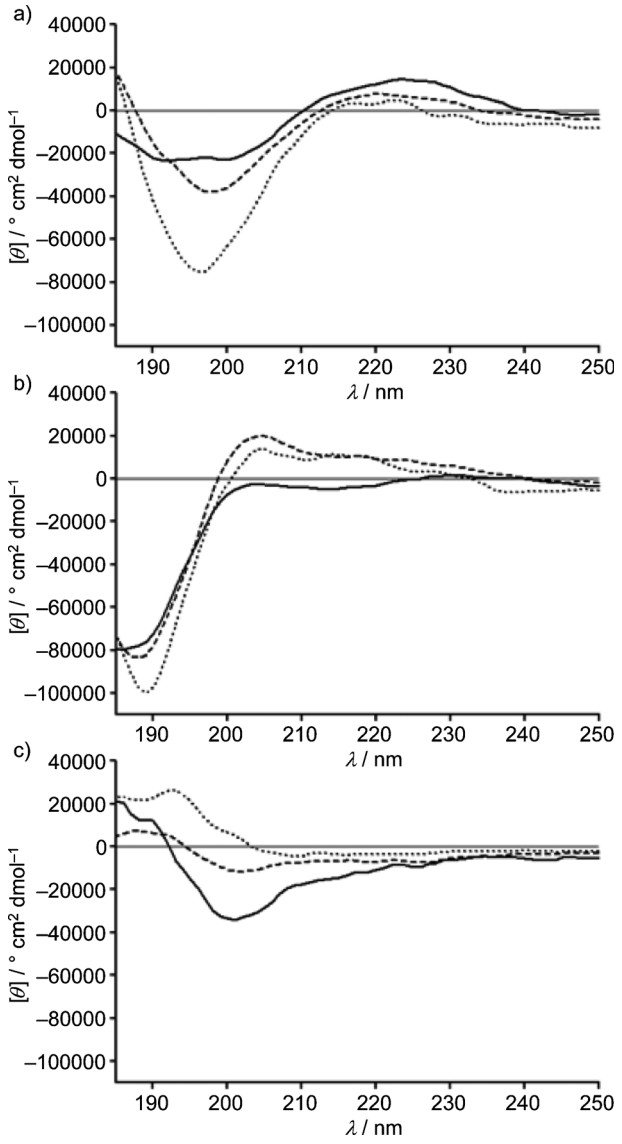


Figure 3. CD spectra of fusaricidin A/LI-F04 analogues a) **6**, b) **14**, and c) **18** in 0.5% AcOH (••••), 50% TFE (-----), or 100% TFE (——).

fluoroethanol (TFE), a membrane-mimicking solvent system.[31] Besides its membrane mimicking properties, TFE is also known to induce formation of stable conformations for peptides that are otherwise unstructured in aqueous solutions.[32] To increase the solubility of peptides in aqueous media and to inhibit potential peptide aggregation at the concentrations required for CD experiments, all analogues studied were dissolved in 0.5% aqueous AcOH.[33,34]

Because small cyclic peptides still have considerable mobility in their backbones,[35–38] it is reasonable to expect that the ester-to-amide substitution in our case would also lead to significant conformational changes. Therefore, we expected different CD spectra for depsipeptide **6** and amide analogue **14** in water and less polar TFE, due to differences in conformational flexibility and the ability of TFE to promote intramolecular hydrogen bonds and to stabilize a preferential conformation.[32] Importantly, D-amino acid residues predominate the sequences of depsipeptide **6**, amide **14**, and linear peptide **18**, causing inversion of their CD spectra (Figure 3). The CD spectrum of depsipeptide **6** in 0.5% AcOH exhibits a minimum at ~197 nm and a weak maximum ~220 nm, reminiscent of an inverted antiparallel β-sheet structure (Figure 3a). Water replacement with the less polar 50% TFE/H_2O mixture and 100% TFE resulted in a drastic change in the CD spectra of depsipeptide **6**. The spectrum in 100% TFE shows a double minimum at 192 and 201 nm and a maximum at 225 nm. In addition to these changes, a marked decrease in the intensity of the CD spectrum in TFE was also observed. All these spectral changes are characteristic of overall β-sheet/β-turn structures.[39–41] β-Sheet-/β-turn-containing small cyclic peptides are not unusual, and examples can be found in gramicidin S and its analogues.[40,42,43] CD spectra of amide analogue **14** differ markedly from those of parent depsipeptide **6** in both aqueous and TFE solutions, indicating significant differences in structural flexibility and conformations induced by ester-to-amide substitution. The CD spectrum of **14** in 0.5% AcOH is characterized by a maximum at 205 nm and minimum at 188 nm, whereas in TFE the maximum at 205 nm is completely lost, and the intensity of the spectral minimum at 188 nm is decreased and slightly shifted toward shorter wavelengths (Figure 3b).

As expected, due to the lack of structural constraints, linear peptide **18** shows different CD spectra in aqueous medium and TFE. In 0.5% AcOH, peptide **18** yields characteristics of an inverted unordered structure with a weak maximum at 195 nm, whereas in TFE the weak minimum at 200 nm indicates the presence of an inverted type I β-turn (Figure 3c).[39,41] Besides conformational changes, ester-to-amide substitution in cyclic peptides may also alter overall hydrophobicity and amphiphilicity, structural characteristics that are important for the biological activities of peptides.[45–49] Typically, the RP HPLC retention times (R_t) of peptides are used as indications of their overall hydrophobicity.[43,44] R_t values of synthesized fusaricidin A/LI-F04a analogues, listed in Figure 2, show that amide analogues are less hydrophobic than their parent depsipeptides, and this is expected due to the loss of a Thr^1 methyl group. The hydrophobicity of synthetic analogues was further altered by replacing the Val^3 residue with Ala, Tyr, and Phe, based on the amino acid sequences found in fusaricidin/LI-F natural products. The observed change in overall hydrophobicity in both depsipeptide and amide analogues, as expressed by R_t, is in the order: Ala < Tyr < Val < Phe.

To gain better insight into the conformational changes induced by ester-to-amide substitution in fusaricidin A/LI-F04a analogues, we conducted molecular dynamics (MD) simulations. Low-energy conformations were generated by conformational analysis in Molecular Operating Environment (MOE)[46] using a Monte Carlo search with the Generalized Born solvation model implemented in MOE.[47] The amphiphilic moment descriptor (μ), which is an established measure of balance between hydrophilic and lipophilic moieties, was computed in MOE for the lowest-energy conformations.[48]

As shown in Figure 4, the results of MD studies are in qualitative agreement with the experimental CD data, indicating

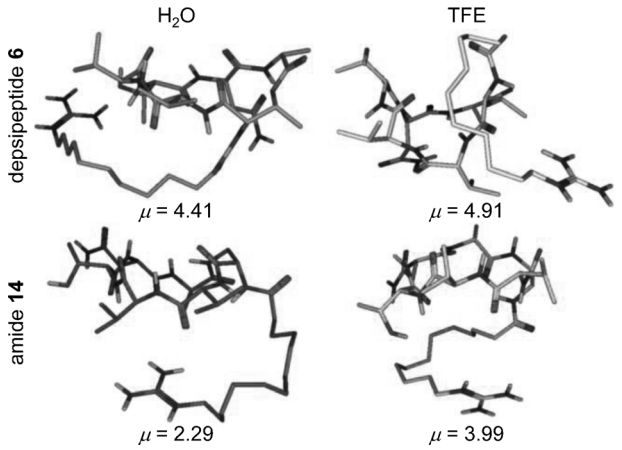


Figure 4. Representative structures of low-energy conformers of depsipeptide **6** and amide **14** in H_2O and TFE (as indicated) along with their calculated amphiphilic moments (μ; see Experimental Section for details).

that depsipeptide **6** can adopt different conformations in polar and nonpolar environments, whereas conformational differences for amide analogue **14** under the same conditions are less pronounced, suggesting a more rigid peptide backbone structure. Furthermore, calculated amphiphilic moments for depsipeptide **6** and amide **14** are markedly different, with the depsipeptide being more amphiphilic in both solvent systems (Figure 4).

Structure–activity studies

To identify structural determinants for antibacterial activity and cytotoxicity, a total of 18 fusaricidin A/LI-F04a analogues were prepared (Figure 2). Structural variations of the natural product include modification of the lipid tail, substitution of amino acid residues including an alanine scan (Ala-scan), ester-to-amide substitution, and a linear control peptide for comparison. The antibacterial in vitro activity of synthesized fusaricidin A/LI-F04a analogues **1**–**18**, expressed as the minimum inhibitory concentration (MIC, $\mu g\,mL^{-1}$), was determined for Gram-positive and Gram-negative bacteria using a standard microdilution broth method in 96-well plates (Table 1).[49,50] Similar to the natural products, fusaricidin/LI-F analogues are active against Gram-positive bacteria, including antibiotic-resistant strains, and do not show activity against Gram-negative bacteria (data not shown). To probe the role of the lipid tail in antibacterial activity, depsipeptides containing an acetyl group (**1** and **2**), 12-ADA (**3** and **4**), or 12-GDA (**5**–**12**) attached to Thr^1 were synthesized. No antibacterial activity was observed for the analogues containing an acetyl group or 12-ADA, even at the highest concentration tested. In contrast, depsipeptide analogue **6**, containing 12-GDA, showed potent antibacterial activity (MIC = 8 $\mu g\,mL^{-1}$) against Gram-positive bacteria (Table 1). Ala-scan and several other substitutions at key amino acid residues were performed to reveal the role of each individual amino acid in the antimicrobial activity of synthetic fusaricidin analogues. However, the requirements of our solid-phase synthetic approach[24] (Scheme 1) permitted the replacement of

Table 1. Antimicrobial activity of fusaricidin A/LI-F04a analogues.

Compd	MIC [$\mu g\,mL^{-1}$]				
	S. aureus ATCC 29213	*S. aureus* ATCC 33591	*S. aureus* ATCC 700699	*S. epidermidis* ATCC 27626	*S. pyogenes* ATCC 19615
1	ND[a]	>128	>128	ND	ND
2	ND	>128	>128	ND	ND
3	ND	>128	>128	ND	ND
4	ND	>128	>128	ND	ND
5	16	16	16	16	32
6	8	8	16	8	16
7	ND	64	64	32	64
8	16	16	16	8	16
9	ND	32	64	16	64
10	ND	64	64	64	64
11	8	8	16	8	8
12	>128	>128	ND	16	64
13	ND	64	64	64	64
14	8	16	16	16	16
15	ND	64	ND	64	>64
16	8	8	ND	16	16
17	ND	32	ND	64	64
18	>128	>128	ND	>128	>128

[a] ND: not determined.

four (D-Val^2, Val^3, D-*a*Thr^4, and D-Ala^6) out of six amino acids. The MIC values obtained (Table 1) show that substitution of Val^3 with Ala (analogue **8**) preserves the antibacterial activity of the parent depsipeptide **6**, whereas substitutions of D-*a*Thr^4 and D-Val^2 with D-Ala (analogues **7** and **9**, respectively) results in a significant decrease in antimicrobial activity. In contrast, replacement of either D-Ala^6 with Gly (analogue **10**) or both Thr^1 and D-Ala^6 with εLys^1 (analogue **13**) leads to a complete loss of antibacterial activity. No significant decrease in antimicrobial activity was observed for the case in which D-*a*Thr^4 was replaced by D-Thr^4: analogue **5**. Quite interestingly, despite significant changes in hydrophobicity, amphiphilicity, and conformation caused by ester-to-amide substitution, the antibacterial activities of amide analogues **14**–**17** parallel those of the parent depsipeptides **6**, **8**, **11**, and **12**. As mentioned earlier, fusaricidins/LI-F are a family of naturally occurring cyclic lipodepsipeptide antibiotics consisting of 12 compounds in total, with conserved Thr^1, D-*a*Thr^4, and D-Ala^6 residues.[7] Aliphatic nonpolar amino acid residues are present at position 2 (D-Val, D-Ile, and D-*a*Ile), and polar amino acids D-Asn and D-Gln are present at position 6 of the peptide sequence. Most diverse changes occur at position 3, where Tyr, Val, Ile, Phe, and D-Ile are found. Taking this into consideration, we synthesized fusaricidin/LI-F analogues possessing Phe (analogues **11** and **16**) and Tyr (analogues **12** and **17**) residues at position 3 in the sequence. Replacement of Val^3 with polar Tyr resulted in a decrease in antimicrobial activity, whereas substitution with nonpolar hydrophobic Phe did not affect antibacterial activity (Table 1). In addition, MIC data obtained for depsipeptides **6**, **8**, **11**, and **12** show that an increase in overall hydrophobicity, as indicated by HPLC retention times (Figure 2) does not correlate with their antibacterial activities. Analogues **6**, **8**, and **11** showed nearly identical antibacterial activities (within experimental error) for tested bacterial strains, whereas analogue **12** showed

lower activity. In contrast, similar comparison of amide analogues **14**–**16** showed different results. Amide analogues with hydrophobic residues at position 3 of the peptide sequence, such as Val and Phe (analogues **14** and **16**), exhibited greater antibacterial activity than analogues with more polar Ala and Tyr residues (analogues **15** and **17**), in this case indicating a correlation between the peptides' overall hydrophobicity and antibacterial activity. Despite a much higher degree of conformational flexibility and complete sequence homology with biologically active depsipeptide **6**, the linear control peptide **18** did not show any biological activities in the described assays.

Cytotoxicity

Hemolytic activity was determined against human erythrocytes (0.5% in PBS), as described in the Experimental Section below. PBS and 0.5% Triton X-100 were used as references for 0 and 100% hemolysis, respectively. Peptides inactive in the antimicrobial assays did not give detectable hemolytic activity. None of the active depsipeptides **6**, **8**, and **11** and their amide counterparts, peptides **14**–**16**, showed hemolytic activity at MICs. However, at higher concentrations, depsipeptides **6**, **8**, and **11** showed considerable hemolysis relative to reference Triton X-100. The degree of hemolysis appears to correlate with the increase in depsipeptide hydrophobicity. For example, at 64 $\mu g\,mL^{-1}$ (8×MIC) the least hydrophobic depsipeptide **8** with Ala^3 exhibited the lowest hemolytic activity (~30%) followed by analogue **6** with Val^3, causing ~45% hemolysis, whereas the most hydrophobic analogue **11** with Phe^3 showed the highest level of hemolysis at ~65%. Interestingly, none of the amide analogues **14**–**16** exhibited appreciable hemolytic activity at this concentration. At much higher concentrations, 128 and 256 $\mu g\,mL^{-1}$ (16× and 32×MIC), depsipeptide analogues **6** and **11** showed hemolysis similar to that of Triton X-100, whereas amide analogues **14** and **16** at 256 $\mu g\,mL^{-1}$ reached 15 and 50% hemolysis, respectively (Figure 5). Control peptide **18** did not exhibit appreciable hemolytic activity even at the highest tested concentration of 64 $\mu g\,mL^{-1}$.

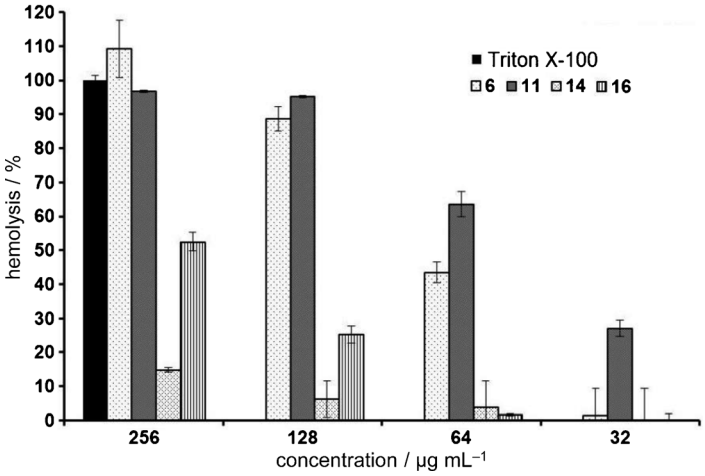


Figure 5. Hemolytic activity of fusaricidin A/LI-F04a analogues (see Supporting Information).

Notably, in this assay DMSO (up to 5% *v/v* final concentration) was added to increase the solubility of the tested fusaricidin A/LI-F04a analogues. Therefore, potential aggregation due to lower solubility of amide analogues at higher concentrations may contribute to the observed hemolytic activity.[51–53] Although DMSO is hemolytic,[54,55] our control experiments showed that under the conditions used, DMSO does not cause hemolysis.

To further assess the therapeutic potential of synthetic fusaricidin A/LI-F04a analogues, we tested the in vitro toxicity of analogues **1**, **4**, **6**, and **14** on the human liver embryonic cell line WRL 68 and the HepG2 cancer cell line. The known cytotoxic drug doxorubicin (Adriamycin)[56] was used as a positive control in these assays. Depsipeptide analogue **1**, lacking a lipid tail and inactive in the antimicrobial assays, did not show appreciable cytotoxicity toward WRL 68 or HepG2 cells within the tested concentration range. Interestingly, lipidated depsipeptide analogues **4** and **6**, regardless of their antibacterial or hemolytic activities, were found to be cytotoxic at higher concentrations toward both cell lines tested. Analogue **4** was highly cytotoxic at 150 $\mu g\,mL^{-1}$, whereas the more active depsipeptide analogue **6** showed a roughly similar trend in cytotoxicity to that of doxorubicin and was highly cytotoxic at 90 $\mu g\,mL^{-1}$. In comparison, the cytotoxicity of the less hydrophobic and amphiphilic amide analogue **14** was much lower at the same concentrations (data not shown).

Serum stability

To investigate the effect of ester-to-amide substitution on cyclic peptide proteolytic stability, the disappearance of the intact peptide incubated in 50% human serum for 24 h at 37 °C was followed by RP HPLC[57] (Figure 6). Depsipeptide **6** and amide **14** were used in this study as representative examples of each group of synthetic fusaricidin A/LI-F04a analogues. An approximate 35% change in the concentration of depsipeptide **6** was observed within 1 h incubation in 50% human serum. However, prolonged incubation of **6** did not result in complete depsipeptide degradation; after 24 h, ~35% of **6** was still detected in the serum. The stability of **6** in EMEM containing 10% FBS used for cytotoxicity assays was significantly higher. In this case a ~23% loss in concentration of **6** was observed after 24 h (see Supporting Information). Analysis of depsipeptide **6** degradation using RP HPLC and MALDI-TOF mass spectrometry revealed that the main degradation product is a result of ring opening via ester bond hydrolysis (depsipeptide **6**: $R_t = 15.8$ min, $[M+H]^+$ $m/z = 825.9709$; hydrolysis product: $R_t = 15.04$ min, $[M+H]^+$ $m/z = 843.9988$). Additional confirmation of this finding was obtained by identical MALDI-TOF m/z and RP HPLC R_t values for the degradation product of depsipeptide **6** and the control peptide **18** ($R_t = 15.05$ min, $[M+H]^+$ found $m/z = 844.1160$, calculated $m/z = 843.0228$). In contrast, no degradation was observed for amide analogue **14** under the same experimental conditions (Figure 6).

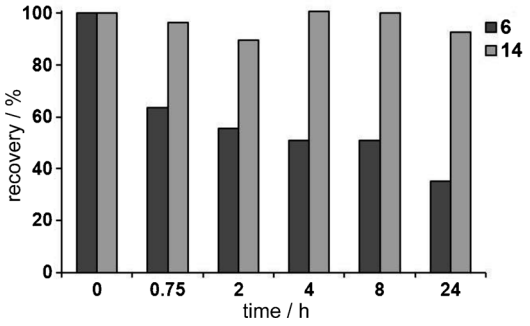


Figure 6. Stability of depsipeptide **6** and amide **14** in 50% human serum.

Discussion

Due to their structural diversity and high potency, naturally occurring cyclic depsipeptides have attracted a great deal of attention for the discovery of new antibiotics with superior activity against MDR bacteria.[7,58] However, full exploitation of the antimicrobial potential of this class of natural products strongly depends on synthetic access to their structures, in particular their analogues, and an understanding of the details important for their mode of action. It has been reported that the antibacterial and hemolytic activities of antimicrobial peptides depend on various structural components, such as charge, size, secondary structure, hydrophobicity, and amphipathicity.[42,59–61] Among naturally occurring antibacterial peptides, the fusaricidins/LI-F family of cyclic lipodepsipeptides represents an attractive series of lead compounds for the development of new antibiotics because of their unique structure in which peptide sequences are composed of six mostly hydrophobic amino acids and a positive charge located at the end of the lipid tail.[7] In addition, there is substantial evidence to show that substitution of the ester bond in depsipeptides with an amide bond may afford derivatives with similar activities and markedly increased serum stabilities.[62–64] With this in mind, we synthesized 18 fusaricidin A/LI-F04a analogues and investigated the effects of structural modification on their overall hydrophobicity/amphiphilicity, conformation, stability, and biological activity. All analogues were synthesized on a solid support using Fmoc methodology. However, solid-phase synthesis of depsipeptides **1**–**13** turned out to be particularly challenging. Our strategy for the solid-phase synthesis of cyclic lipodepsipeptides involved attachment of D-Asp^5 via side chain stepwise Fmoc synthesis of a linear peptide precursor and on-resin head-to-tail cyclization (Scheme 1). The key step in the synthesis of cyclic depsipeptides is ring closure. Considering the greater reactivity of the amino group, and therefore minimal possibility of side reactions, we chose macrolactamization for depsipeptide ring closure, as opposed to macrolactonization. Although this synthetic strategy appears to be a better choice for solid-phase depsipeptide synthesis, an undesired intramolecular O→N acyl shift may occur under basic conditions.[27,65] Reversible intramolecular O→N or N→O acyl shifts are well described side reactions in peptide chemistry.[27,65] Typically, peptides containing Ser or Thr residues undergo N→O acyl shift under acidic conditions, whereas the exposure of corresponding depsipeptides to basic conditions leads to the opposite O→N acyl shift. Incorporation of the lipid tail or acetyl group into the linear peptide precursor prior to D-Ala^6 coupling via ester bond and on-resin cyclization completely suppressed the O→N acyl shift, resulting in the desired cyclic lipodepsipeptide (Scheme 1). In addition, we found that the efficacy of ester bond formation strongly depends on the nature of the solid support and solvent. Optimal results were obtained with PEG-based resins, a DIC/DMAP coupling method, and CH_2Cl_2.[24] The superiority of PEG–PS over PS-based resins in ester bond formation could be explained by a better solvation of peptide–PEG–PS resins,[66,67] rapid DIC activation of the carboxylic group,[68] and significant suppression of *N*-acylurea by-product formation[69] in a nonpolar solvent such as CH_2Cl_2. On the other hand, synthesis of amide analogues **13**–**17** using the same approach was straightforward (Scheme 1). The desired cyclic lipopeptides were obtained in satisfactory yields.

Several studies have highlighted the importance of amide-to-ester substitution on peptide conformation.[35,70–72] In general, replacement of a hydrogen-donating NH group with an oxygen atom in depsipeptides results in removal of the backbone hydrogen bond, thus affecting the conformation of peptides. As long as the side chains are unaltered, intramolecular hydrogen bonds affect the equilibrium distribution of peptide conformers. The effect of ester-to-amide substitution on the conformation of fusaricidin A/LI-F04a analogues was assessed by CD spectroscopy (Figure 3). The dramatic differences observed in CD spectra of depsipeptide **6** in aqueous medium and less polar TFE, and the absence of such spectral changes for amide analogue **14**, indicate a higher degree of flexibility of the ester bond as opposed to the amide bond. These differences also suggest the greater ability of synthesized depsipeptide analogues to alternate conformations in different cellular environments. However, complete interpretation of both depsipeptide **6** and amide **14** CD spectra in aqueous medium and TFE is rather difficult, as the spectra obtained cannot be attributed to a single conformation. MD calculations were performed to further explore the conformational differences caused by ester-to-amide substitution in fusaricidin A/LI-F04a analogues and to assess the possibility of forming more organized structures under conditions that mimic the membrane environment. The results are shown in Figure 4. Our experimental CD data fully support the MD simulations, indicating higher conformational flexibility of depsipeptide analogues. The MD calculations showed no significant conformational differences between depsipeptide **6** and amide analogue **14** in water, whereas the differences become markedly more pronounced in a less polar environment such as TFE.

Besides conformational changes, ester-to-amide substitution altered the biochemical properties of the fusaricidin analogues. Assessments of the cytotoxicity and stability of synthesized fusaricidin A/LI-F-04a analogues in human serum are important secondary screening assays, mainly because these assays eliminate cytotoxic analogues and those with short half-lives.[73] Our experimental data suggest that the low serum stability of depsipeptide **6** can be attributed mainly to the hydrolysis of its ester bond. This finding is in accordance with published data

showing that cyclic depsipeptides can be degraded through competition between protease and esterase activities. However, the depsipeptides were more easily subjected to hydrolysis by esterase-type enzymes.[74–76] The cyclic structure, the presence of D-amino acids, and the greater stability of an amide bond altogether contribute to the enhanced proteolytic stability of the amide analogues.[77–84] Quite importantly, ester-to-amide substitution did not affect the antibacterial potency of these peptides. A positive charge positioned at the end of the lipid tail, hydrophobicity, and amphiphilicity appeared to be crucial for the antibacterial activities of fusaricidin A/LI-F04a analogues as well as separation of their antibacterial activity and toxicity toward human cells. Antibacterial assays showed that only analogues with a guanidylated lipid tail are effective against Gram-positive bacteria, indicating the importance of the lipid tail and positively charged guanidino functionality for their antimicrobial activity (Table 1). Based on the role of the lipid tail in other lipopeptide antibiotics[85–88] and our experimental data showing that nonlipidated analogue **2** is inactive against tested bacterial strains, we can assume that the lipid moiety helps target fusaricidin analogues to the bacterial membrane. The antibacterial activity of peptides with a guanidylated lipid moiety could be explained by the strong ability of the guanidinium group to bind the anionic phosphate of the bacterial phospholipid membrane through a combination of hydrogen bonding and charge–charge interactions. In general, a high pK_a value, diffused charge density, and the geometry of the guanidinium group which allows better alignment of hydrogen bonds, all contribute to better interactions with the phosphate anion relative to the ammonium group.[89]

The results of the antibacterial assays of the Ala-scan analogues revealed that residues D-Val^2, D-*a*Thr^4, and D-Ala^6 are important for antibacterial activity. Replacement of any of these amino acids with corresponding Ala, analogues **7**, **9**, and **10**, resulted in complete loss of depsipeptide antibacterial activity (Table 1). On the other hand, position 3 in the depsipeptide sequence is more tolerable to changes. Depsipeptide analogues containing neutral Ala^3 (**8**) and bulky hydrophobic Val^3 (**6**) or Phe^3 (**11**) exhibited nearly identical antibacterial activities, whereas analogue **12** with polar Tyr^3 was inactive. Equally potent in vitro antibacterial activity was observed for their amide counterparts, analogues **14** and **16**, possessing hydrophobic Val^3 and Phe^3, respectively. However, differences in antibacterial activity were observed between depsipeptide **8** and its amide counterpart **15**, both containing Ala at position 3. Depsipeptide **8** exhibited antibacterial activity similar to the most potent analogues, whereas amide **15** showed significant decrease in activity (Table 1). Because the relative overall hydrophobicity ranking order of Ala $<$ Val $<$ Phe is the same for both amide and depsipeptide analogues, the greater change in amphiphilicity upon substitution of lipophilic Val^3 with neutral Ala^3 in structurally constrained amide **15** may explain the loss of its antibacterial activity.

As mentioned earlier, comparisons of the overall hydrophobicity of depsipeptides **6**, **8**, **11**, and **12** (Figure 2) show no correlation with their antibacterial activities. In contrast, an increase in depsipeptide hydrophobicity can be associated with the enhanced hemolytic activities (Figure 5). More hydrophobic depsipeptides **6** and **11** were also more hemolytic. Similarly, depsipeptides **6**, **8**, **11**, and **12** exhibited higher hemolytic activities than their less hydrophobic amide counterparts **14**–**17**. At concentrations 8 × MIC (64 μg mL^{-1}) none of the amide analogues **14**–**17** were found to be hemolytic. The fact that linear peptide **18** is not hemolytic (Supporting Information) suggests that hydrolysis of the ester bond which leads to opening of the depsipeptide ring is not a cause of hemolysis.

Liver toxicity is one of the most critical issues in drug development, often leading to the delay or failure of drug candidates.[90–92] Therefore, peptides that show no in vitro liver cell toxicity may prove to be better candidates for further preclinical or clinical studies. HepG2 and WRL 68 cell lines were used to assess the potential toxicity of fusaricidin A/LI-F04a analogues. In both cell lines, depsipeptide **6** exhibited much higher in vitro cytotoxicity than amide counterpart **14**. However, the absence of cytotoxicity toward human cells observed for amide analogue **14** cannot be explained solely by lower hydrophobicity. Conformational differences among these fusaricidin/LI-F analogues, as illustrated by the MD calculations and the differences in CD spectra between analogues **6** and **14** (Figures 3 and 4), should be taken into consideration as well. Whereas hemolytic depsipeptide **6** has the ability to form more ordered structures in a membrane-mimicking environment, non-hemolytic amide analogue **14** fails to undergo any significant conformational change, regardless of the solvent system. Conformational changes and hence changes in amphipathicity have been ascribed to the low hemolytic activity of some synthetic and naturally occurring cyclic cationic antimicrobial peptides.[42,59,61,93] Typically, for these cyclic peptides, the presence of cationic amino acids in the sequence, a defined secondary structure (β-sheet and hairpin loop), and amphiphilic character are common structural characteristics that determine their biological activities.[59,93] In the case of fusaricidin A/LI-F04a and its analogues, a positive charge is positioned at the terminus of the 12-carbon-atom lipid tail, and peptide sequences are composed mostly of hydrophobic amino acids (Figure 2). Nevertheless, experimental CD data and molecular modeling studies showed that the peptide ring in both depsipeptide **6** and amide **14** analogues can adopt different amphiphilic conformations depending on the environment, with depsipeptides being more amphiphilic. Relatively lower amphiphilicity of the amide analogues may contribute to the dissociation of antibacterial activity from human cell cytotoxicity.

Conclusions

Structure–activity relationship studies of fusaricidin A/LIF-04a depsipeptide analogues reported herein reveal key structural requirements for antibacterial activity and decreasing cytotoxicity. The positively charged guanidinium group at the end of the 12-carbon-atom lipid tail and the presence of hydrophobic amino acids in the depsipeptide sequence are crucial for antibacterial activity. Ala-scan results and comparison of the fusaricidin/LI-F natural product sequences suggest that position 3 in the depsipeptide sequence is more tolerable to amino acid

change. By introduction of neutral and hydrophobic amino acids into this position, we were able to manipulate the depsipeptide's overall hydrophobicity and amphiphilicity without loss of antibacterial potency. However, structural changes leading to an increase in the depsipeptide's overall hydrophobicity and amphiphilicity resulted in an increase in cytotoxicity. On the other hand, substitution of an ester bond in depsipeptides by an amide bond gave more stable cyclic lipopeptide analogues with preserved in vitro antibacterial activities, yet greatly improved serum stabilities and minimized human cell toxicity. Lower overall hydrophobicity/amphiphilicity of amide analogues in relative to their parent depsipeptides may explain the dissociation of antibacterial activity from human cell cytotoxicity. More stable and less cytotoxic amide analogues may have significant advantages over naturally occurring fusaricidin A/LI-F04a and its depsipeptide analogues as lead structures for the development of new antibacterial agents. In addition, amide analogues are synthetically more accessible than the parent depsipeptides, allowing for further structural optimization using a combinatorial chemistry approach. Synthesis of a focused combinatorial library based on fusaricidin A/LI-F04a amide analogues and elucidation of the mode of action of both the depsipeptide and amide analogues are currently underway.

Experimental Section

Chemicals and instrumentation

TentaGel S RAM resin was obtained from Advanced ChemTech (Louisville, KY, USA). 2-Chlorotrityl chloride was obtained from Novabiochem (Gibbstown, NJ, USA). Fmoc-protected amino acids and coupling reagents (HOBt, HBTU, PyBOP) were purchased from Chem-Impex (Wood Dale, IL, USA) or Novabiochem. DIC was purchased from Acros Organics (Thermo Fisher Scientific, Waltham, MA, USA). DMAP was purchased from Sigma–Aldrich (St. Louis, MO, USA). All solvents were purchased from Fisher Scientific (Atlanta, GA, USA) or Sigma–Aldrich, and were analytical reagent grade or better. Linear peptidyl-resin precursors were synthesized on a PS3 automated peptide synthesizer (Protein Technologies Inc., Tucson, AZ, USA). Mass spectrometry was performed on MALDI-TOF Voyager-DE STR (Applied Biosystems, Foster City, CA, USA) in reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix and positive mode. Analytical RP HPLC analyses and peptide purifications were performed on 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA) liquid chromatography systems equipped with a UV/Vis detector. For analytical RP HPLC analysis, a C_{18} monomeric column (Grace Vydac, 250×4.6 mm, 5 μm, 120 Å), 1 mL min^{-1} flow rate, and elution method with a linear gradient of 2→98% B over 30 min, where A is 0.1% TFA in H_2O, and B is 0.08% TFA in CH_3CN was used. For peptide purification, a preparative C_{18} monomeric column (Grace Vydac, 250×22 mm, 10 μm, 120 Å) was used. Elution method was identical to the analytical method except for the flow rate, which was 19 mL min^{-1}. CD spectra were recorded on a JASCO 810 spectropolarimeter (Easton, MD, USA) using a quartz cell of 0.1 mm optical path length. Spectra were measured over a wavelength range of 180–250 nm with an instrument scanning speed 200 nm min^{-1} and a response time of 1 s. The concentrations of peptides were 0.1–0.2 mM. Cytotoxicity assays were analyzed on a Synergy H4 microplate reader (BioTek, Winooski, VT, USA). Microbial strains and human cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Dehydrated culture media and agar, and polystyrene plates used for antimicrobial assays were purchased from BD (Franklin Lakes, NJ, USA). Control antibiotics were purchased from Sigma–Aldrich. Antimicrobial activity assays were carried out in standard sterile 96-well plates, and MIC values were determined by measuring turbidity at 600 nm using a Stat Fax 2100 Microplate reader (Awareness Technology Inc., Palm City, FL, USA). Human red blood cells (hRBCs) were purchased from Innovative Research (Novi, MI, USA). Human serum was purchased from Sigma–Aldrich.

General procedure for peptide synthesis and purification

Linear peptidyl-resin precursors for cyclic lipopeptides **1**–**17** were synthesized on amide TentaGel S RAM resin (substitution 0.26 mmol g^{-1}, 0.25 mmol scale) using an automated peptide synthesizer. The solid-phase synthesis of cyclic peptides **1**–**17** was started by attaching C-terminal Fmoc-D-Asp-OAllyl via side chain to the resin using HBTU/HOBt/NMM protocol. The same coupling protocol was used throughout, including coupling of the lipid tail (Fmoc-ADA-OH, 1.5 equiv). In the case of depsipeptide analogues **1**–**12**, Alloc-D-Ala-OH (4 equiv) or Alloc-Gly (4 equiv) was coupled manually via ester bond to the hydroxy group of Fmoc-Thr using DIC (4 equiv) and DMAP (1 equiv) coupling reagents in CH_2Cl_2. Amide analogues **13**–**17** were prepared by coupling Fmoc-Dap-(Mtt) instead of Fmoc-Thr-OH using the same coupling protocol as above. Selective removal of Allyl and Alloc protecting groups was performed by treatment of peptidyl-resin precursors with borane dimethylamine complex (4 equiv), followed by addition of $Pd(PPh_3)_4$ (0.1 equiv) in CH_2Cl_2 under argon.[94] Mtt was selectively removed under mild acidic conditions (1% TFA in CH_2Cl_2, 30 min). Solid-phase cyclization of linear precursors was carried out in a manual reaction vessel overnight using PyBOP/HOBt/DIEA (2:2:6 equiv) in DMF. The conversion of the lipid tail amino group into the desired guanidino group was achieved by removal of the Fmoc protecting group using standard piperidine deprotection protocol and treatment of the peptidyl-resin with *N*,*N*-bis(*tert*-butoxycarbonyl)thiourea (3 equiv) followed by Mukaiyama's reagent 2-chloro-1-methylpyridinium iodide/TEA (3:4 equiv) in DMF.[95]

Control peptide **18** was synthesized on 2-chlorotrityl chloride resin (substitution 1.3 mmol g^{-1}). The synthesis started by attaching Fmoc-D-Ala-OH (4 equiv) to the resin using an equimolar amount of DIEA in CH_2Cl_2 followed by resin end-capping with MeOH,[64] and chain elongation using standard Fmoc chemistry. Quantitative Fmoc substitution of the resin (0.5 mmol g^{-1}) was determined by Fmoc cleavage and absorption measurement at 304 nm.[30] In all cases, the reaction progress was monitored by RP HPLC, MALDI-TOF MS, and where applicable, ninhydrin colorimetric test.[96]

Peptides were removed from the resin using TFA/TIA/H_2O (95:2.5:2.5 *v*/*v*/*v*) for 3 h. The crude peptides were precipitated with cold methyl *tert*-butyl ether, and purified using preparative RP HPLC. HPLC fractions were analyzed for purity, combined, and lyophilized to give a white powder. The final purity of synthesized peptides was confirmed by analytical RP HPLC, and was ≥95% in all cases.

Concentrations of peptides in all experiments were determined using RP HPLC and calibration curve based on analogue **6**. The peptide content of **6** was determined by quantitative amino acid analysis to be 62.32%.

Circular dichroism

All CD spectra were recorded on JASCO 810 spectropolarimeter at 25 °C using a 0.1 cm path length cell. The spectra were acquired in the range 180–250 nm, 1 nm bandwidth, four accumulations and 200 nm min^{-1} scanning speed. All spectra were obtained using 0.1–0.2 mM concentrations in 0.5% or 1% AcOH, 25–100% TFE/H_2O (*v*/*v*) solution. Spectra at 0.5 and 1% AcOH were virtually identical for each peptide. Each experiment was repeated at least once and at various concentrations. No concentration-dependent CD spectral changes were observed.

Molecular modeling

First low-energy conformations were generated by conformational analysis in Molecular Operating Environment (MOE)[46] using a Monte Carlo search with the Generalized Born solvation model implemented in MOE.[47] The dielectric constant was set to 26.14 or 80 to simulate the search in TFE or water, respectively. The search was conducted with the MMFF94x force field using default parameters. The lowest-energy conformers found for each compound in the Monte Carlo search were the starting points of 40 ns MD simulation using the MacroModel 9.9 module from Maestro software.[97] In brief, the initial structures were equilibrated by 2 ps. The "Bonds to Hydrogens" option from the Shake procedure was selected in order to use 2 fs as time step. The simulation temperature was set at 300.0 K as default. The optimized potential for liquid simulation (OPLS)-2005 force field[98] was used to calculate the potential energy. Conformations were sampled every 20 ps and optimized using the same force field. The dielectric constant was set to 26.14 to simulate the search in TFE. The GB/SA solvation model implemented in MacroModel[99] was used for water simulation.

The amphiphilic moment descriptor, μ, which is an established measure of the balance between hydrophilic and lipophilic molecular properties,[26] was computed in MOE for the lowest-energy conformations obtained in the dynamics within 5 kcal mol^{-1} of the lowest minimum.

Antibacterial activity

A total of six Gram-positive and two Gram-negative bacterial strains were used, including MDR bacterial strains: *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* (MRSA) ATCC 33591, *Staphylococcus aureus* Mu50 (VRSA) ATCC 700699, *Staphylococcus epidermidis* (MRSE) ATCC 27626, *Streptococcus pyogenes* ATCC 19615, *Escherichia coli* K-12 ATCC 29181, and *Klebsiella pneumoniae* K6 ATCC 700603. Quantification of the antibacterial activity of synthesized analogues **1**–**18** was performed in sterile 96-well flat-bottomed polystyrene plates by the standard microdilution broth method.[49,50] Tests were performed using Müller–Hinton broth (MHB) without dilution. Controls on each plate were media without bacteria, bacterial inoculum without antimicrobials added, and bacterial inoculum containing methicillin or vancomycin. Assay setup: Stocks of microorganisms maintained at −80 °C in 30% glycerol were thawed and grown in media recommended by ATCC protocols for each particular microorganism. The following day, an aliquot of the bacterial suspension (100 μL) was transferred into 10 mL fresh media and incubated for 4–5 h, until OD_{600} of the suspension was 0.3–0.4. Bacterial suspension (100 μL) was then transferred into sterile tubes (Eppendorf) and centrifuged for 5 min at 1300 *g*. The supernatant was discarded, and cell pellet resuspended in MHB. Upon measuring OD_{600} of the suspension, appropriate dilution was made so that the final OD of the suspension was ~0.001 (based on calculation). Concentrations of analogues **1**–**18**, as well as control antibiotics, were in the range 1–128 μg mL^{-1}. All samples were loaded in duplicate, and the average OD value was taken for calculating MIC. Each assay was repeated twice. Stock solutions of synthesized analogues **1**–**18**, as well as control antibiotics were prepared in 5–10% DMSO/H_2O (*v*/*v*) solvent mixture, depending on the analogue solubility. After dilutions with MHB, final concentration of DMSO in wells was 0.5–1%. Plates were loaded with 90 μL bacterial suspension (with initial OD_{600} of 0.001) of the tested microorganism, and 10 μL aliquots of twofold serial dilutions of the analogues **1**–**18** or control antibiotics. Plates were then incubated at 37 °C overnight with gentle shaking. In the case of *S. pyogenes*, plates were incubated under 5% CO_2 atmosphere. Inhibition of bacterial growth was determined by measuring OD_{600}; a decrease in OD_{600} indicates inhibition of bacterial growth.

Hemolytic activity

Human red blood cells (hRBCs) were diluted with PBS to 1%. Depending on solubility, analogues **1**–**18** were dissolved in 5–10% DMSO/H_2O (*v*/*v*) solvent mixture to concentrations of 16–512 μg mL^{-1}. Into each well of the clear, flat-bottom 96-well plate, 50 μL of the hRBCs were placed followed by addition of 50 μL analogue solution to final peptide concentrations of 8–256 μg mL^{-1}. Assays were performed in triplicate, and each experiment was repeated twice. To determine the potential effect of DMSO on hemolytic activity, controls containing 5–10% DMSO in H_2O (*v*/*v*) were added to the assay setup. As a positive control, 50 μL Triton X-100 in H_2O was used at a final concentration of 0.5% (*v*/*v*). As a negative control, 50 μL H_2O and PBS was used. Plates were incubated for 1 h at 37 °C. To each well 100 μL of PBS was added, and the plates were centrifuged for 10 min at 1000 *g*. Supernatants (150 μL) were transferred into a new plate, and absorbance at 405 nm was measured. Within the tested concentration range, the effect of DMSO on hemolysis was minimal, and was subtracted to obtain solely hemolytic activity of the peptides. The degree of hemolysis was expressed in percent relative to total hemolysis effected by Triton-X.

Cytotoxicity

Cytotoxicity of analogues **1**–**18** was determined using the MTT colorimetric assay. Assays were set up in flat-bottom polystyrene 96-well plates with 10000 cells per well grown in EMEM containing 10% FBS, 5% penicillin/streptomycin, and 5% L-glutamine (*v*/*v*). After an overnight incubation at 37 °C under a humidified atmosphere with 5% CO_2, media were removed, and fresh media containing analogues **1**–**18** in a concentration range of 1–250 μM were added. Plates were again incubated at 37 °C under a humidified atmosphere with 5% CO_2. As a control, doxorubicin was used in the same concentration range. After incubation for 24 or 48 h, media were removed, and 100 μL MTT dissolved in serum-free medium (1 mg mL^{-1}) was added to each well. Plates were again incubated for 3 h under the same conditions. Media containing MTT were removed, and 100 μL DMSO was added to each well. Plates were shaken for 5 min before reading absorbance at 540 nm.

Stability assays

The stabilities of selected fusaricidin A/LI-F04a analogues in 50% human serum, as well as in EMEM (used in cell toxicity assays) were determined. For stability in 50% human serum, peptides **6**

and **14** (0.5 mg each) were dissolved in 200 μL DMSO, to which 800 μL H_2O and 1 mL human serum were added. The solution was incubated at 37 °C. After 0 min, 45 min, 2, 4, 8, and 24 h, three samples (3×100 μL) of each peptide were taken and precipitated by the addition of 20 μL 15% aqueous TCA. Samples were quickly vortexed and then centrifuged at 9300 *g* for 10 min. The supernatant was analyzed by analytical RP HPLC and MALDI-TOF MS. For the stability of depsipeptide **6** in EMEM containing 10% FBS, 0.25 mg of the peptide was dissolved in 10 μL DMSO, followed by the addition of 990 μL media and incubation at 37 °C. Peptide samples were treated and analyzed in the same way as described above.

Abbreviations

AcOH, acetic acid; Alloc, allyloxycarbonyl; 12-ADA, 12-aminododecanoic acid; CD, circular dichroism; Dap, diaminopropionic acid; DIC, diisopropylcarbodiimide; DIEA, diisopropylethyl amine; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EMEM, Eagle's minimal essential medium; FBS, fetal bovine serum; Fmoc, fluorenylmethyloxycarbonyl; 12-GDA, 12-guanidinododecanoic acid; HOBt, *N*-hydroxybenzotriazole; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; hRBCs, human red blood cells; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MHB, Müller–Hinton broth; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *S. aureus*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Mtt, 4-methyltrityl chloride; NMM, *N*-methylmorpholine; PBS, phosphate-buffered saline; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; RP HPLC, reversed-phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; TIA, thioanisole; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TFE, trifluoroethanol; VRSA, vancomycin-resistant *S. aureus*.

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