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New Antimicrobial Hexapeptides: Synthesis, Antimicrobial Activities, Cytotoxicity, and Mechanistic Studies

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Synthetic antimicrobial peptides have recently emerged as promising candidates against drug-resistant pathogens. We identified a novel hexapeptide, Orn-D-Trp-D-Phe-Ile-D-Phe-His(1-Bzl)-NH_2, which exhibits broad-spectrum antifungal and antibacterial activity. A lead optimization was undertaken by conducting a full amino acid scan with various proteinogenic and non-proteinogenic amino acids depending on the hydrophobic or positive-charge character of residues at various positions along the sequence. The hexapeptide was also cyclized to study the correlation between the linear and cyclic structures and their respective antimicrobial activities. The synthesized peptides were found to be active against the fungus *Candida albicans* and Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* and methicillin-resistant *Staphylococcus epidermidis*, as well as the Gram-negative bacterium *Escherichia coli*; MIC values for the most potent structures were in the range of 1–5 $\mu g\,mL^{-1}$ (IC_{50} values in the range of 0.02–2 $\mu g\,mL^{-1}$). Most of the synthesized peptides showed no cytotoxic effects in an MTT assay up to the highest test concentration of 200 $\mu g\,mL^{-1}$. A tryptophan fluorescence quenching study was performed in the presence of negatively charged and zwitterionic model membranes, mimicking bacterial and mammalian membranes, respectively. The results of the fluorescence study demonstrate that the tested peptides are selective toward bacterial over mammalian cells; this is associated with a preferential interaction between the peptides and the negatively charged phospholipids of bacterial cells.

Introduction

The widespread and irrational use of antibiotics has led to the emergence of resistant strains of pathogenic microorganisms. Therefore, the search for novel antimicrobial agents has gained importance over the past several years. Nature has provided a solution to this problem in the form of antimicrobial peptides (AMPs) that are not only lethal to a broad spectrum of pathogens but also have a unique low tendency to cause development of resistance. A variety of AMPs and proteins have been isolated from virtually all kingdoms and phyla including plants, microbes, insects, larger animals, and humans.[1–4] Hundreds of such AMPs have been listed in the antimicrobial peptide database (APD).[5] These naturally occurring peptides are an integral part of the host defense system and provide an immediate response to invading microorganisms; they display a broad spectrum of bactericidal and fungicidal actions.[6] Consequently, AMPs are specifically toxic toward pathogens and are able to differentiate between host and microbial cells. This specific recognition may be governed by differences in cell membrane composition and hence charge, membrane asymmetry, transmembrane potential, and specific receptor binding.[7] Such selective action is also demonstrated by preferential interaction with the anionic lipopolysaccharide (LPS) of the bacterial cell membrane rather than with the net neutral mammalian cell membrane, which is composed of zwitterionic phospholipids and cholesterol.[8–10] The therapeutic potential of AMPs is attributed mainly to their membrane lytic properties. This includes initial binding of the peptide monomer to the microbial membrane followed by peptide aggregation and insertion, which generally results in the formation of pores or disruption of the cell membrane. The peptides have demonstrated their ability to rapidly kill a broad spectrum of microorganisms including multi-drug-resistant bacteria, fungi, and viruses. Development of resistance by sensitive microbial strains against these AMPs is less probable, because AMPs exert their action by forming multimeric pores in the cell membranes, leading to cell lysis,[11] or interaction with the RNA or DNA after penetration into the cell.[12] Models proposed to explain the mechanism of microbial cytoplasmic membrane disruption by AMPs include the 'barrel-stave' model, the 'toroid pore' or 'wormhole' model, and the 'carpet' model.[7,13–15] In general, the initial association of AMPs with the microbial membrane occurs

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through electrostatic interactions between the cationic peptide and the anionic LPS or other negatively charged components of the membrane bilayer, leading to membrane perturbation. Most of these peptides are cationic in nature with a significant number of hydrophobic residues, and their antimicrobial potency has been correlated with positive charge, although not always linearly.[16]

Although naturally occurring AMPs appear to be promising candidates against resistant pathogens owing to their specificity and infrequent problems of resistance development, their clinical utility is limited by problems of enzymatic degradation, bioavailability, and cost. Thus, synthetic congeners that possess similar structural features but which contain unnatural amino acids may provide the solution. Short cationic AMPs have reached the realm of classical drug molecules in terms of molecular size and complexity. They are attracting considerable attention and are the focus of more research in their development as future antibiotics to treat multi-drug-resistant infections.[17–19] Thus, our aim is to identify potential AMPs and to manipulate their structure to create synthetic analogues to successfully treat fungal and bacterial infections.

Results and Discussion

We came across an earlier study in which the hexapeptide His-D-Trp-D-Phe-Phe-D-Phe-Lys-NH_2 (**1**), structurally similar to growth hormone releasing hexapeptide (GHRP-6, His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2),[20] was found to display antifungal activity against *Candida albicans* (IC_{50} = 28.6 μM) and *Cryptococcous neoformans* (MIC = 6.8 μM). Further optimization of **1** was done by using the combinatorial approach in which D-amino acids at positions 2, 3, and 5, which were found to be essential for activity, were retained. This led to the identification of peptide **2**, Arg-D-Trp-D-Phe-Ile-D-Phe-His-NH_2, which is more potent against *C. albicans* (IC_{50} = 6.8 μM) but displays similar potency against *C. neoformans* (MIC = 6.8 μM). To examine the effect of increased peptide chain length on biological activity, peptide **2** was chosen as a motif upon which to develop a nonapeptide library. This approach led to the peptide Arg-D-Trp-D-Phe-Ile-D-Phe-His-Lys-Arg-Lys-NH_2, which is more potent than **2** against both *C. albicans* (IC_{50} = 3.3 μM) and *C. neoformans* (MIC = 2.4 μM).[21]

In our preliminary study, we set out to optimize hexapeptide **2** in terms of structure and biological activity, without increasing the peptide backbone length, by replacing its key constituent amino acids with selected unnatural amino acids. We concentrated our efforts on a systematic amino acid replacement strategy with peptide **2**. Keeping in mind the observations made earlier,[20] the residues at positions 2–5 in **2** were kept intact in all designed peptides. Modulation of histidine residue(s), either by substitution on the imidazole ring or by replacement with other amino acids, could profoundly influence a peptide's biological activities.[22] Moreover, it has been reported that the replacement of basic amino acids, such as arginine and lysine, with histidine increases the antimicrobial activity of peptides, particularly under acidic conditions.[23] Therefore, we first investigated the role of His 6 and synthesized peptides in which it is replaced by D-His, 1-Bzl-His, and 2-pyridylalanine (2-Pal) (Table 1). In one particular case (peptide **3**), in addition to replacement of His 6 with 1-Bzl-His, the role of Arg 1 was also investigated by its replacement with the less basic non-proteinogenic ornithine (Orn) residue.

Table 1. In vitro antifungal and antibacterial activities of representative hexapeptides **3**–**6**.

Peptide	Sequence[a]	*C. neoformans*		MRSA		MSSA	
		MIC[b]	IC_{50}[c]	MIC[b]	IC_{50}[c]	MIC[b]	IC_{50}[c]
3	O-*W*-*F*-I-*F*-H(1-Bzl)-NH_2	5.0	1.5	10.0	3.5	10.0	3.5
4	R-*W*-*F*-I-*F*-*H*-NH_2	10.0	6.0	NT	>20	NT	>20
5	R-*W*-*F*-I-*F*-H(1-Bzl)-NH_2	5.0	3.5	10.0	6.5	10.0	6.0
6	R-*W*-*F*-I-*F*-Pal-NH_2	5.0	3.5	5.0	3.5	10.0	3.5

[a] O = ornithine; amino acids in italics represent the D-isomers. [b] MIC [μg mL^{-1}] (minimum inhibitory concentration): the lowest test concentration that completely inhibits microorganism growth; NT: not tested. [c] IC_{50} [μg mL^{-1}]: the concentration that affords 50% inhibition of bacterial growth; standards used: *C. neoformans* (amphotericin B: IC_{50} = 0.34 μg mL^{-1}), MRSA (vancomycin: IC_{50} = 0.14 μg mL^{-1}), MSSA (ciprofloxacin: IC_{50} = 0.05 μg mL^{-1}).

Peptides **3**–**6** displayed promising antifungal activity against *C. neoformans* (Table 1), but they were not active against *Aspergillus fumigatus* and showed moderate inhibition (IC_{50} = 20 μg mL^{-1}) against *C. albicans* (data not shown). Peptide **3** was the most promising antifungal compound of the series, displaying pronounced antifungal activity with an MIC value of 5.0 μg mL^{-1} (IC_{50} = 1.5 μg mL^{-1}) against *C. neoformans* (Table 1), whereas peptides **4**–**6** displayed MIC values in the range between 5.0 and 10.0 μg mL^{-1}. Peptides **3**, **5**, and **6** displayed promising antibacterial activities against methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). Peptide **3** was the most potent and exhibited antibacterial activity with MIC values of 10 μg mL^{-1} (IC_{50} = 3.5 μg mL^{-1}) against MRSA and MSSA. The in vitro cytotoxicities of peptides **3**–**6** were tested against four human cancer cell lines and two noncancerous mammalian kidney cell lines up to a concentration of 23.8 μg mL^{-1} using a neutral red assay procedure, as described earlier.[24,25] None of the peptides showed any cytotoxic effects up to the highest test concentration. The antimicrobial activities discussed above led to the identification of the hexapeptide Orn-D-Trp-D-Phe-Ile-D-Phe-His(1-Bzl)-NH_2 (**3**) as a lead compound, which provides an interesting pharmacophore for the design of more potent AMPs. Interestingly, upon careful examination the chemical structure of peptide **3**, the clear-cut demarcation between the positively charged termini (residues X_1 and X_6) and the inner hydrophobic core sequence (residues X_2–X_5) is readily apparent (Figure 1). This segregation of positively charged and hydrophobic features correlates directly with the amphiphilic structure of AMPs, and this has been

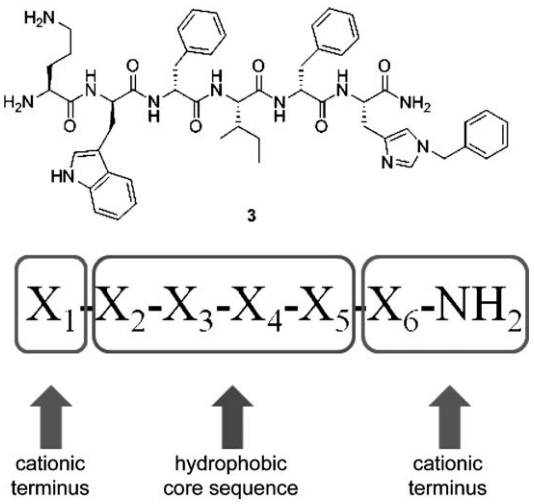


Figure 1. Lead hexapeptide **3** with cationic termini and inner hydrophobic amino acids.

widely reported to be essential for the antimicrobial activity of this peptide class. Encouraged by these observations, we decided to undertake lead optimization in the form of a full amino acid scan with various proteinogenic and non-proteinogenic (natural/unnatural) amino acids on lead hexapeptide **3**. Our aim was to increase antimicrobial activity and to identify residues that increase activity at various sequence positions in the lead compound. In particular, the positively charged terminal amino acids were replaced with similar cationic residues at positions 1 and 6 of lead peptide **3**. The selection of replacement residues was made on the assumption that they partially or fully retain the cationic character of amino acids already present. Similarly, the amino acids at positions 2–5 of lead peptide **3** were replaced with residues in a manner that conserves the hydrophobic character at this position.

We also synthesized a specific peptide in which the lead hexapeptide **3** is constrained by cyclization. This was undertaken to correlate the linear and cyclic structures of the peptides with their respective antimicrobial activities. The synthesized peptides were evaluated for antimicrobial activity against two Gram-positive bacteria, three Gram-negative bacteria, and one fungal strain. To further assess their therapeutic potential, cytotoxicity studies were performed with the MTT test on mouse fibroblasts. To investigate the relative extent of peptide burial in the model membranes, we performed a fluorescence quenching experiment in small unilamellar vesicles (SUVs) using the water-soluble neutral fluorescence quencher acrylamide.

Modifications at positions 1 and 6

The Orn 1 and His(1-Bzl) 6 residues at the respective N- and C-termini of peptide **3** were selectively replaced with cationic amino acids based on the assumption that they partially or fully retain the cationic character at positions X_1 and X_6. The substitute cationic residues chosen were Lys, Arg, His, D-Lys, D-Arg, D-His, 2,4-diaminobutyric acid (Dab), 4-amino-L-phenylalanine [Phe(4-NH_2)], His(1-Bzl), and Orn; this resulted in peptides **7**–**15** (substituted at X_1) and **146**–**154** (substituted at X_6) (Table 2). These peptides were synthesized using the Boc strategy for solid-phase peptide synthesis (SPPS).

Modifications at positions 2–5

Keeping the hydrophobic character intact, the D-Trp 2, D-Phe 3, Ile 4 and D-Phe 5 groups of peptide **3** were systematically replaced with various proteinogenic and non-proteinogenic amino acids, including aminobutyric acid (Abu), 4,4′-biphenylalanine (Bip), 4-fluoro-L-phenylalanine [Phe(4-F)], 4-methyl-L-phenylalanine [Phe(4-Me)], cyclohexylalanine (Cha), phenylglycine (Phg), 3-benzothienylalanine (Bal), 4-*tert*-butyl-L-phenylalanine [Phe(4-*t*Bu)], 1-napthylalanine (Nal), 4-trifluoromethyl-L-phenylalanine [Phe(4-CF_3)], 3-(9-anthryl)-L-alanine [Ala(9-anth)], dibutylglycine (Dbg), 3,3-diphenylalanine (Dip), norleucine (Nle), pentafluoro-L-phenylalanine (Pfp), L/D-Trp, L/D-Phe, L/D-Leu, L/D-Ile, L/D-Val, L/D-Tyr, L/D-Cys, L/D-Met, L/D-Ala, and L/D-Pro. These replacements resulted in peptides **16**–**47** (substituted at X_2), **48**–**80** (substituted at X_3), **81**–**112** (substituted at X_4) and **113**–**145** (substituted at X_5) (Table 2), which were synthesized using the Fmoc strategy for SPPS.

Cyclization

To investigate the antimicrobial activities of linear versus cyclic structures, we also synthesized an N-to-C-terminus-cyclized version of peptide **3**. The linear hexapeptide, which was synthesized by Fmoc SPPS, was cyclized in the presence of PyBop, HOAt, and DIEA in DMF to afford the cyclic peptide **155** (Scheme 1).

Antimicrobial activity

The synthesized peptides were evaluated for antibacterial and antifungal activities against three Gram-negative bacteria [*Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 9027), and *Klebsiella pneumoniae* (ATCC 700603)], two Gram-positive bacteria [MRSA (DSM 50128509) and methicillin-resistant *Staphylococcus epidermidis* (MRSE; DSM 50160384)], and one fungal strain [*C. albicans* (ATCC 10231)], which were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and cultured using procedures described earlier.[26–28]

The antimicrobial activities of peptides **7**–**155** are listed in Table 2. Among peptides **7**–**15**, which represent replacements at position X_1 of peptide **3**, peptides **10** (X_1 = D-Lys) and **11** (X_1 = Lys) are the most potent against *C. albicans* (MIC = 20 $\mu g\,mL^{-1}$; IC_{50} = 9.9–11.7 $\mu g\,mL^{-1}$). These observations clearly indicate that the presence of Lys, either as the L or D isomer, increases the potency of X_1-position-substituted peptides toward the fungus to the greatest extent. They also reflect that the presence of an amino group (as a positively charged species) on the side chain at position X_1, consistent with peptide **3** (X_1 = Orn), is essential for activity against *C. albicans*. Regarding Gram-positive bacterial strains, analogue **8** displayed potent activity against MRSA, with an excellent MIC value of 2 $\mu g\,mL^{-1}$ (IC_{50} = 0.75 $\mu g\,mL^{-1}$). Analogues **7** and **11** (X_1 = D-

Table 2. Antifungal and antimicrobial activities of hexapeptides **7**–**155**.

Peptide	Sequence[a]	MIC (IC_{50}) [$\mu g\,mL^{-1}$][b]					
		C. albicans	MRSA	MRSE	*E. coli*	*K. pneumoniae*	*P. aeruginosa*
7	K-*W*-*F*-I-*F*-H(1-Bzl)-NH_2	20 (11.7)	10 (4.8)	5 (3.7)	50 (36.1)	>200	100 (52.1)
8	R-*W*-*F*-I-*F*-H(1-Bzl)-NH_2	50 (18.6)	2 (0.75)	10 (6.7)	20 (13.3)	>200	100 (66.9)
9	H-*W*-*F*-I-*F*-H(1-Bzl)-NH_2	100 (20.9)	>200	>200	>200	>200	200
10	K-*W*-*F*-I-*F*-H(1-Bzl)-NH_2	20 (9.9)	100 (17.2)	20 (8.0)	100 (43.1)	>200	200
11	*R*-*W*-*F*-I-*F*-H(1-Bzl)-NH_2	100 (27.4)	5 (2.6)	5 (1.9)	100 (56.8)	>200	200
12	*H*-*W*-*F*-I-*F*-H(1-Bzl)-NH_2	100 (37.2)	>200	>200	>200	>200	>200
13	Dab-*W*-*F*-I-*F*-H(1-Bzl)-NH_2	100 (22.8)	>200	50 (14.3)	>200	>200	>200
14	F(4-NH_2)-*W*-*F*-I-*F*-H(1-Bzl)-NH_2	100 (47.3)	>200	>200	>200	>200	200
15	H(1-Bzl)-*W*-*F*-I-*F*-H(1-Bzl)-NH_2	50 (18.0)	>200	>200	200	200	100 (51.8)
16	O-Abu-*F*-I-*F*-H(1-Bzl)-NH_2	200	>200	>200	>200	>200	200
17	O-Bip-*F*-I-*F*-H(1-Bzl)-NH_2	50 (36.7)	50 (21.4)	50 (16.5)	200	>200	>200
18	O-F(4-F)-*F*-I-*F*-H(1-Bzl)-NH_2	5 (4.0)	100 (3.4)	50 (22.0)	200	>200	>200
19	O-F(4-Me)Abu-*F*-I-*F*-H(1-Bzl)-NH_2	2 (1.5)	100 (26.9)	100 (32.6)	100 (32.4)	>200	>200
20	O-Cha-*F*-I-*F*-H(1-Bzl)-NH_2	5 (2.9)	10 (7.3)	5 (3.6)	200	>200	>200
21	O-Phg-*F*-I-*F*-H(1-Bzl)-NH_2	20 (11.2)	>200	50 (28.4)	>200	>200	>200
22	O-Bal-*F*-I-*F*-H(1-Bzl)-NH_2	10 (9.4)	>200	200	>200	>200	>200
23	O-F(4-*t*Bu)-*F*-I-*F*-H(1-Bzl)-NH_2	50 (27.1)	50 (19.0)	50 (22.1)	200	>200	>200
24	O-Nal-*F*-I-*F*-H(1-Bzl)-NH_2	2 (1.1)	10 (3.7)	10 (3.8)	>200	>200	>200
25	O-F(4-CF_3)-*F*-I-*F*-H(1-Bzl)-NH_2	10 (7.2)	50 (16.2)	50 (33.6)	>200	>200	>200
26	O-A(9-anth)-*F*-I-*F*-H(1-Bzl)-NH_2	200	>200	>200	>200	>200	>200
27	O-Dbg-*F*-I-*F*-H(1-Bzl)-NH_2	200	>200	>200	200	>200	>200
28	O-W-*F*-I-*F*-H(1-Bzl)-NH_2	10 (7.9)	5 (1.1)	10 (6.1)	100 (23.6)	>200	>200
29	O-*F*-*F*-I-*F*-H(1-Bzl)-NH_2	50 (35.0)	50 (38.0)	20 (12.4)	10 (13.5)	100 (37.6)	200
30	O-F-*F*-I-*F*-H(1-Bzl)-NH_2	10 (14.9)	>200	200	>200	>200	>200
31	O-L-*F*-I-*F*-H(1-Bzl)-NH_2	100 (39.0)	>200	>200	>200	>200	200
32	O-L-*F*-I-*F*-H(1-Bzl)-NH_2	200	>200	>200	>200	>200	>200
33	O-*I*-*F*-I-*F*-H(1-Bzl)-NH_2	100 (59.1)	>200	>200	>200	>200	>200
34	O-I-*F*-I-*F*-H(1-Bzl)-NH_2	50 (20.7)	50 (23.1)	50 (11.6)	200	>200	>200
35	O-*V*-*F*-I-*F*-H(1-Bzl)-NH_2	100 (43.0)	>200	>200	>200	>200	>200
36	O-V-*F*-I-*F*-H(1-Bzl)-NH_2	100 (27.0)	100 (31.5)	>200	100 (36.0)	>200	200
37	O-*Y*-*F*-I-*F*-H(1-Bzl)-NH_2	100 (64.3)	100 (42.1)	>200	>200	>200	200
38	O-Y-*F*-I-*F*-H(1-Bzl)-NH_2	5 (4.5)	50 (8.6)	5 (2.0)	2 (1.4)	100 (56.1)	>200
39	O-*C*-*F*-I-*F*-H(1-Bzl)-NH_2	50 (40.3)	>200	>200	>200	>200	>200
40	O-C-*F*-I-*F*-H(1-Bzl)-NH_2	100 (64.1)	>200	>200	>200	>200	100 (72.3)
41	O-*M*-*F*-I-*F*-H(1-Bzl)-NH_2	10 (6.1)	>200	>200	>200	>200	>200
42	O-M-*F*-I-*F*-H(1-Bzl)-NH_2	100 (57.3)	200	200	200	>200	200
43	O-*A*-*F*-I-*F*-H(1-Bzl)-NH_2	200	>200	>200	>200	>200	>200
44	O-A-*F*-I-*F*-H(1-Bzl)-NH_2	100 (72.2)	>200	100 (36.5)	>200	>200	>200
45	O-P-*F*-I-*F*-H(1-Bzl)-NH_2	200	100 (49.7)	>200	200	>200	>200
46	O-*P*-*F*-I-*F*-H(1-Bzl)-NH_2	50 (23.1)	>200	200	>200	>200	>200
47	O-Dip-*F*-I-*F*-H(1-Bzl)-NH_2	200	200	>200	>200	>200	100 (65.8)
48	O-*W*-Abu-I-*F*-H(1-Bzl)-NH_2	100 (78.7)	>200	>200	>200	200	200
49	O-*W*-Bip-I-*F*-H(1-Bzl)-NH_2	100 (60.3)	5 (3.9)	5 (2.7)	>200	200	>200
50	O-*W*-F(4-F)-I-*F*-H(1-Bzl)-NH_2	20 (13.0)	2 (0.25)	5 (2.4)	50 (28.7)	>200	200
51	O-*W*-F(4-Me)-I-*F*-H(1-Bzl)-NH_2	10 (6.6)	5 (1.8)	1 (0.2)	50 (16.2)	200	200
52	O-*W*-Cha-I-*F*-H(1-Bzl)-NH_2	2 (1.0)	2 (0.11)	2 (0.86)	100 (18.9)	>200	200
53	O-*W*-Nle-I-*F*-H(1-Bzl)-NH_2	50 (14.8)	10 (6.8)	5 (1.6)	>200	200	200
54	O-*W*-Phg-I-*F*-H(1-Bzl)-NH_2	100 (28.1)	20 (8.8)	20 (6.9)	20 (5.9)	100 (79.9)	200
55	O-*W*-Bal-I-*F*-H(1-Bzl)-NH_2	50 (17.7)	2 (1.8)	1 (1.0)	100 (52.6)	200	>200
56	O-*W*-F(4-*t*Bu)-I-*F*-H(1-Bzl)-NH_2	10 (7.2)	2 (1.1)	1 (0.56)	100 (79.0)	>200	200
57	O-*W*-Nal-I-*F*-H(1-Bzl)-NH_2	50 (27.7)	2 (1.7)	1 (0.02)	200	200	200
58	O-*W*-F(4-CF_3)-I-*F*-H(1-Bzl)-NH_2	10 (4.0)	2 (0.94)	1 (0.81)	50 (21.5)	200	200
59	O-*W*-A(9-anth)-I-*F*-H(1-Bzl)-NH_2	NT	NT	NT	NT	NT	NT
60	O-*W*-Dbg-I-*F*-H(1-Bzl)-NH_2	100 (63.9)	200	200	100 (32.5)	>200	>200
61	O-*W*-F-I-*F*-H(1-Bzl)-NH_2	20 (13.5)	100 (22.7)	10 (3.8)	>200	>200	200
62	O-*W*-*W*-I-*F*-H(1-Bzl)-NH_2	20 (11.2)	10 (9.0)	1 (0.18)	200	100 (58.8)	200
63	O-*W*-W-I-*F*-H(1-Bzl)-NH_2	10 (5.1)	50 (18.3)	2 (1.1)	>200	>200	200
64	O-*W*-L-I-*F*-H(1-Bzl)-NH_2	10 (8.1)	100 (61.7)	10 (6.3)	200	>200	200
65	O-*W*-L-I-*F*-H(1-Bzl)-NH_2	50 (12.2)	100 (21.1)	20 (11.5)	>200	>200	>200
66	O-*W*-*I*-I-*F*-H(1-Bzl)-NH_2	20 (9.6)	50 (17.1)	10 (8.6)	>200	>200	200
67	O-*W*-I-I-*F*-H(1-Bzl)-NH_2	50 (20.9)	200	100 (76.8)	>200	>200	200
68	O-*W*-*V*-I-*F*-H(1-Bzl)-NH_2	100 (31.1)	20 (4.0)	100 (36.4)	>200	>200	200
69	O-*W*-V-I-*F*-H(1-Bzl)-NH_2	50 (7.9)	>200	100 (54.1)	>200	>200	200
70	O-*W*-*Y*-I-*F*-H(1-Bzl)-NH_2	100 (53.8)	>200	100 (26.4)	>200	>200	>200
71	O-*W*-Y-I-*F*-H(1-Bzl)-NH_2	50 (22.6)	200	100 (46.9)	>200	>200	200

Table 2. (Continued)

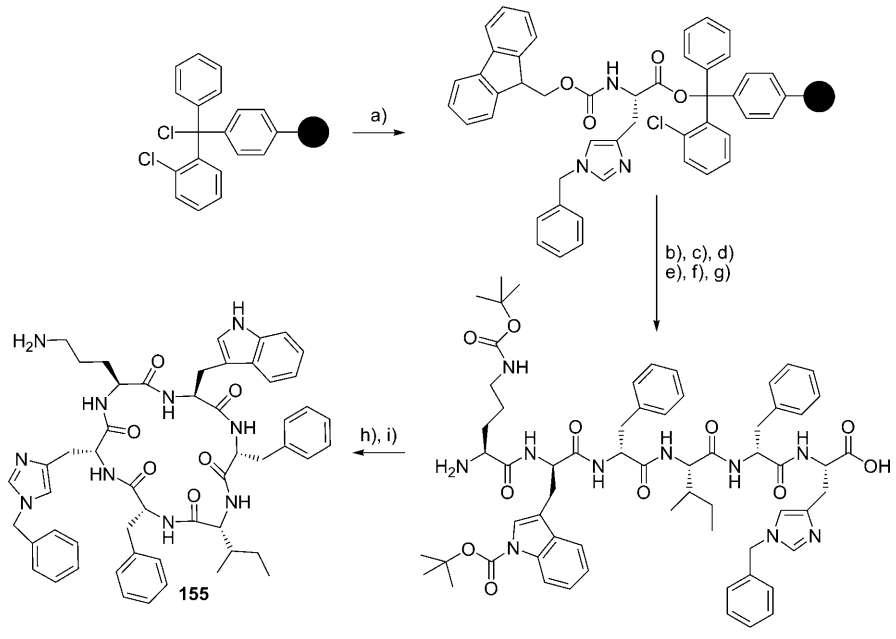
Peptide	Sequence[a]	MIC (IC_{50}) [$\mu g\,mL^{-1}$][b]					
		C. albicans	MRSA	MRSE	*E. coli*	*K. pneumoniae*	*P. aeruginosa*
72	O-*W*-*C*-I-*F*-H(1-Bzl)-NH_2	100 (28.4)	10 (5.6)	2 (1.4)	100 (40.6)	> 200	200
73	O-*W*-C-I-*F*-H(1-Bzl)-NH_2	200	5 (2.1)	10 (4.8)	50 (20.9)	100 (62.5)	200
74	O-*W*-*M*-I-*F*-H(1-Bzl)-NH_2	100 (39.2)	200	20 (11.1)	> 200	> 200	200
75	O-*W*-M-I-*F*-H(1-Bzl)-NH_2	100 (66.9)	100 (26.6)	100 (33.9)	> 200	> 200	> 200
76	O-*W*-*A*-I-*F*-H(1-Bzl)-NH_2	200	> 200	200	> 200	> 200	200
77	O-*W*-A-I-*F*-H(1-Bzl)-NH_2	100 (38.5)	200	200	> 200	> 200	200
78	O-*W*-P-I-*F*-H(1-Bzl)-NH_2	100 (51.1)	> 200	100 (17.7)	> 200	> 200	200
79	O-*W*-*P*-I-*F*-H(1-Bzl)-NH_2	200	> 200	> 200	> 200	> 200	> 200
80	O-*W*-Dip-I-*F*-H(1-Bzl)-NH_2	10 (6.4)	2 (1.2)	1 (0.58)	100 (35.6)	> 200	200
81	O-*W*-*F*-Abu-*F*-H(1-Bzl)-NH_2	200	> 200	50 (12.8)	200	> 200	> 200
82	O-*W*-*F*-Bip-*F*-H(1-Bzl)-NH_2	200	2 (1.4)	2 (1.2)	100 (52.5)	> 200	200
83	O-*W*-*F*-F(4-F)-*F*-H(1-Bzl)-NH_2	100 (63.4)	2 (0.95)	1 (0.78)	10 (8.2)	100 (65.6)	> 200
84	O-*W*-*F*-F(4-Me)-*F*-H(1-Bzl)-NH_2	20 (15.3)	1 (0.42)	1 (0.07)	200	200	200
85	O-*W*-*F*-Cha-*F*-H(1-Bzl)-NH_2	20 (11.3)	> 200	> 200	50 (34.0)	> 200	> 200
86	O-*W*-*F*-Nle-*F*-H(1-Bzl)-NH_2	20 (7.0)	> 200	> 200	> 200	> 200	> 200
87	O-*W*-*F*-Phg-*F*-H(1-Bzl)-NH_2	100 (26.8)	20 (9.4)	1 (1.2)	100 (36.2)	> 200	100 (46.6)
88	O-*W*-*F*-Bal-*F*-H(1-Bzl)-NH_2	200	1 (0.58)	1 (0.23)	100 (60.6)	> 200	> 200
89	O-*W*-*F*-F(4-*t*Bu)-*F*-H(1-Bzl)-NH_2	100 (48.7)	20 (12.8)	1 (0.63)	10 (6.5)	> 200	100 (38.7)
90	O-*W*-*F*-Nal-*F*-H(1-Bzl)-NH_2	100 (38.9)	> 200	> 200	> 200	> 200	> 200
91	O-*W*-*F*-Pfp-*F*-H(1-Bzl)-NH_2	50 (23.8)	1 (0.63)	20 (19.5)	> 200	> 200	200
92	O-*W*-*F*-F(4-CF_3)-*F*-H(1-Bzl)-NH_2	100 (49.7)	200	5 (2.5)	50 (32.0)	> 200	200
93	O-*W*-*F*-A(9-anth)-*F*-H(1-Bzl)-NH_2	50 (17.7)	200	100 (38.9)	> 200	> 200	200
94	O-*W*-*F*-Dbg-*F*-H(1-Bzl)-NH_2	100 (31.5)	100 (32.6)	100 (42.0)	> 200	> 200	> 200
95	O-*W*-*F*-*F*-*F*-H(1-Bzl)-NH_2	20 (4.4)	5 (2.1)	5 (4.0)	100 (68.63)	100 (75.6)	200
96	O-*W*-*F*-*F*-*F*-H(1-Bzl)-NH_2	200	50 (18.4)	20 (10.3)	> 200	> 200	> 200
97	O-*W*-*F*-*W*-*F*-H(1-Bzl)-NH_2	100 (63.6)	20 (10.5)	20 (16.3)	> 200	> 200	> 200
98	O-*W*-*F*-W-*F*-H(1-Bzl)-NH_2	> 200	10 (3.6)	20 (9.5)	200	> 200	200
99	O-*W*-*F*-L-*F*-H(1-Bzl)-NH_2	200	100 (37.9)	200	> 200	> 200	> 200
100	O-*W*-*F*-L-*F*-H(1-Bzl)-NH_2	50 (29.3)	200	200	200	> 200	200
101	O-*W*-*F*-*I*-*F*-H(1-Bzl)-NH_2	100 (31.7)	> 200	> 200	> 200	> 200	> 200
102	O-*W*-*F*-*V*-*F*-H(1-Bzl)-NH_2	50 (12.5)	100 (26.9)	50 (20.3)	> 200	> 200	> 200
103	O-*W*-*F*-V-*F*-H(1-Bzl)-NH_2	100 (54.1)	50 (7.8)	50 (25.8)	50 (22.2)	200	100 (58.5)
104	O-*W*-*F*-*Y*-*F*-H(1-Bzl)-NH_2	50 (26.2)	50 (20.4)	100 (56.8)	> 200	> 200	> 200
105	O-*W*-*F*-*C*-*F*-H(1-Bzl)-NH_2	200	> 200	> 200	> 200	> 200	> 200
106	O-*W*-*F*-C-*F*-H(1-Bzl)-NH_2	> 200	> 200	> 200	> 200	> 200	> 200
107	O-*W*-*F*-*M*-*F*-H(1-Bzl)-NH_2	NT	NT	NT	NT	NT	NT
108	O-*W*-*F*-*A*-*F*-H(1-Bzl)-NH_2	200	> 200	> 200	> 200	> 200	> 200
109	O-*W*-*F*-A-*F*-H(1-Bzl)-NH_2	200	200	> 200	> 200	> 200	200
110	O-*W*-*F*-*P*-*F*-H(1-Bzl)-NH_2	200	> 200	100 (57.4)	200	> 200	> 200
111	O-*W*-*F*-*P*-*F*-H(1-Bzl)-NH_2	200	> 200	> 200	200	> 200	200
112	O-*W*-*F*-Dip-*F*-H(1-Bzl)-NH_2	50 (22.6)	50 (12.4)	1 (0.70)	> 200	> 200	> 200
113	O-*W*-*F*-I-Abu-H(1-Bzl)-NH_2	200	100 (20.1)	> 200	20 (6.5)	> 200	> 200
114	O-*W*-*F*-I-Bip-H(1-Bzl)-NH_2	200	200	200	> 200	> 200	> 200
115	O-*W*-*F*-I-F(4-F)-H(1-Bzl)-NH_2	50 (30.2)	200	200	> 200	200	> 200
116	O-*W*-*F*-I-F(4-Me)-H(1-Bzl)-NH_2	100 (44.7)	> 200	20 (8.0)	200	> 200	> 200
117	O-*W*-*F*-I-Cha-H(1-Bzl)-NH_2	50 (16.1)	50 (14.6)	200	100 (34.2)	> 200	> 200
118	O-*W*-*F*-I-Nle-H(1-Bzl)-NH_2	> 200	200	50 (19.0)	> 200	> 200	> 200
119	O-*W*-*F*-I-Phg-H(1-Bzl)-NH_2	200	> 200	100 (29.7)	> 200	> 200	200
120	O-*W*-*F*-I-Bal-H(1-Bzl)-NH_2	200	> 200	100 (43.1)	> 200	> 200	> 200
121	O-*W*-*F*-I-F(4-*t*Bu)-H(1-Bzl)-NH_2	20 (5.8)	> 200	100 (27.4)	50 (27.1)	> 200	> 200
122	O-*W*-*F*-I-Nal-H(1-Bzl)-NH_2	200	200	200	> 200	100 (56.0)	> 200
123	O-*W*-*F*-I-F(4-CF_3)-H(1-Bzl)-NH_2	100 (65.9)	> 200	100 (58.8)	> 200	> 200	> 200
124	O-*W*-*F*-I-A(9-anth)-H(1-Bzl)-NH_2	200	> 200	> 200	> 200	> 200	> 200
125	O-*W*-*F*-I-Dbg-H(1-Bzl)-NH_2	200	200	200	> 200	200	> 200
126	O-*W*-*F*-I-F-H(1-Bzl)-NH_2	50 (20.5)	200	> 200	> 200	> 200	> 200
127	O-*W*-*F*-I-I-H(1-Bzl)-NH_2	50 (26.4)	200	> 200	> 200	> 200	> 200
128	O-*W*-*F*-I-W-H(1-Bzl)-NH_2	100 (41.5)	200	200	> 200	> 200	> 200
129	O-*W*-*F*-I-L-H(1-Bzl)-NH_2	> 200	> 200	> 200	> 200	> 200	> 200
130	O-*W*-*F*-I-L-H(1-Bzl)-NH_2	100 (34.0)	> 200	> 200	100 (42.7)	> 200	> 200
131	O-*W*-*F*-I-*I*-H(1-Bzl)-NH_2	200	200	200	> 200	100 (37.5)	> 200
132	O-*W*-*F*-I-*V*-H(1-Bzl)-NH_2	100 (50.8)	200	200	> 200	100 (32.2)	> 200
133	O-*W*-*F*-I-V-H(1-Bzl)-NH_2	200	200	> 200	20 (8.6)	> 200	> 200
134	O-*W*-*F*-I-*Y*-H(1-Bzl)-NH_2	100 (27.6)	> 200	200	> 200	> 200	200
135	O-*W*-*F*-I-Y-H(1-Bzl)-NH_2	200	> 200	> 200	> 200	> 200	> 200
136	O-*W*-*F*-I-*C*-H(1-Bzl)-NH_2	100 (39.4)	200	100 (56.5)	100 (40.1)	> 200	> 200

Table 2. (Continued)

Peptide	Sequence[a]	MIC (IC_{50}) [$\mu g\,mL^{-1}$][b]					
		C. albicans	MRSA	MRSE	*E. coli*	*K. pneumoniae*	*P. aeruginosa*
137	O-*W*-*F*-I-C-H(1-Bzl)-NH_2	200	200	>200	>200	>200	>200
138	O-*W*-*F*-I-*M*-H(1-Bzl)-NH_2	>200	>200	>200	>200	>200	>200
139	O-*W*-*F*-I-M-H(1-Bzl)-NH_2	200	200	>200	>200	>200	200
140	O-*W*-*F*-I-*A*-H(1-Bzl)-NH_2	100 (32.6)	>200	>200	>200	>200	>200
141	O-*W*-*F*-I-A-H(1-Bzl)-NH_2	50 (19.2)	>200	>200	>200	>200	>200
142	O-*W*-*F*-I-P-H(1-Bzl)-NH_2	100 (48.2)	50 (17.9)	20 (14.7)	>200	>200	>200
143	O-*W*-*F*-I-*P*-H(1-Bzl)-NH_2	100 (30.3)	>200	20 (10.5)	>200	>200	>200
144	O-*W*-*F*-I-*W*-H(1-Bzl)-NH_2	20 (11.3)	>200	20 (14.7)	100 (59.9)	>200	>200
145	O-*W*-*F*-I-Dip-H(1-Bzl)-NH_2	50 (17.3)	100 (35.5)	20 (9.5)	>200	>200	200
146	O-*W*-*F*-I-*F*-K-NH_2	50 (15.1)	200	20 (11.0)	200	>200	>200
147	O-*W*-*F*-I-*F*-R-NH_2	100 (28.6)	5 (1.6)	1 (0.12)	50 (16.5)	>200	>200
148	O-*W*-*F*-I-*F*-H-NH_2	20 (12.7)	200	10 (4.7)	50 (13.2)	>200	>200
149	O-*W*-*F*-I-*F*-*K*-NH_2	50 (11.8)	>200	20 (9.5)	5 (1.5)	50 (25.1)	>200
150	O-*W*-*F*-I-*F*-*R*-NH_2	100 (39.4)	50 (20.0)	10 (8.4)	20 (5.6)	200	>200
151	O-*W*-*F*-I-*F*-*H*-NH_2	50 (18.6)	20 (11.9)	10 (6.2)	20 (8.2)	200	>200
152	O-*W*-*F*-I-*F*-Dab-NH_2	10 (2.6)	50 (15.1)	10 (3.9)	20 (10.4)	>200	200
153	O-*W*-*F*-I-*F*-O-NH_2	20 (10.8)	20 (6.7)	5 (1.2)	50 (12.3)	200	>200
154	O-*W*-*F*-I-*F*-F(4-NH_2)-NH_2	50 (15.3)	10 (4.1)	5 (3.3)	20 (6.4)	>200	>200
155	cyclo-[O-*W*-*F*-I-*F*-H(1-Bzl)]	>200	>200	200	>200	>200	200

[a] O = ornithine; amino acids in italics represent the D-isomers. [b] Standards used: *C. albicans* (amphotericin B: $IC_{50} = 0.34\ \mu g\,mL^{-1}$), MRSA (vancomycin: $IC_{50} = 0.14\ \mu g\,mL^{-1}$), MRSE (vancomycin: $IC_{50} = 0.14\ \mu g\,mL^{-1}$), *E. coli* (streptomycin: $IC_{50} = 0.73\ \mu g\,mL^{-1}$), *K. pneumoniae* (neomycin: $IC_{50} = 0.6\ \mu g\,mL^{-1}$), *P. aeruginosa* (ciprofloxacin: $IC_{50} = 1.18\ \mu g\,mL^{-1}$).

Arg) were also promising, with MIC values of 10 and 5 $\mu g\,mL^{-1}$, respectively. On the other hand, analogue **11** was active against MRSE, with an excellent MIC value of 5 $\mu g\,mL^{-1}$ ($IC_{50} = 1.9\ \mu g\,mL^{-1}$). As in the case of MRSA, it appears that D-Arg at X_1 is also best suited among the tested peptides for activity against MRSE. In the case of Gram-negative bacterial strains, analogue **8** was found to be most potent against *E. coli*, with an MIC value of 20 $\mu g\,mL^{-1}$ ($IC_{50} = 13.3\ \mu g\,mL^{-1}$). However, none of the peptides was active against *K. pneumoniae*, whereas peptides **7**, **8**, and **15** were moderately active against *P. aeruginosa*. Therefore, in general, out of all the peptides in which Orn 1 of hexapeptide **3** is replaced, peptides **7** and **8** (X_1 = Lys and Arg, respectively) were active against four tested strains, exhibiting a broad spectrum of antimicrobial activities.



Scheme 1. Reagents and conditions: a) Fmoc-His(Bzl)-OH, DIEA, CH_2Cl_2, 2 h; b) Fmoc-D-Phe-OH, TBTU, DIEA, DMF, 3 h, then 20% piperidine in DMF, 15 min; c) Fmoc-Ile-OH, TBTU, DIEA, DMF, 3 h, then 20% piperidine in DMF, 15 min; d) Fmoc-D-Phe-OH, TBTU, DIEA, DMF, 3 h, then 20% piperidine in DMF, 15 min; e) Fmoc-D-Trp(Boc)-OH, TBTU, DIEA, DMF, 3 h, then 20% piperidine in DMF, 15 min; f) Fmoc-Orn(Boc)-OH, TBTU, DIEA, DMF, 3 h, then 20% piperidine in DMF, 15 min; g) 1% TFA in CH_2Cl_2, 10 min, then pyridine, 5 min; h) PyBop, HOAt, DMF, 24 h, room temperature; i) 20% TFA in CH_2Cl_2, 30 min, room temperature.

Among peptides **16**–**47**, which are substituted at position X_2 of the lead peptide **3**, analogues **24** (X_2 = Nal), **19** [X_2 = Phe(4-Me)] and **20** (X_2 = Cha) were observed to be the most potent against *C. albicans*, with MIC values of 2, 2, and 5 $\mu g\,mL^{-1}$ ($IC_{50} = 1.1$, 1.5, and 2.9 $\mu g\,mL^{-1}$), respectively. Interestingly, most of the peptides that are active against *C. albicans* have unnatural amino acids as the substituent at position X_2. In the case of MRSA, analogue **28** exhibited highly potent activity (MIC = 5 $\mu g\,mL^{-1}$; $IC_{50} = 1.1\ \mu g\,mL^{-1}$). Peptide **38** was the most potent analogue against MRSE, with an MIC value of 5 $\mu g\,mL^{-1}$ ($IC_{50} = 2.0\ \mu g\,mL^{-1}$). This observation clearly indicates that the presence of hydrophobic amino acids with aromatic rings on the side chain at position X_2 increases the poten-

cy against the tested Gram-positive bacterial stains. Of all the tested analogues, **38** was the most active, with an MIC value of 2 $\mu g\,mL^{-1}$ ($IC_{50}=1.4\ \mu g\,mL^{-1}$) against *E. coli*. Most of the peptides were inactive against *K. pneumoniae* and *P. aeruginosa*, with the exception of analogues **29** ($X_2=$ D-Phe), **38**, **40**, and **47**, which displayed modest activities. Broadly, out of all peptides evaluated with substitutions at position X_2, **38** and **28** (substituted with Tyr and Trp, respectively) were found to be active against five and four tested microbial strains exhibiting a broad spectrum of activities.

In the case of peptides **48**–**80**, in which D-Phe 3 of peptide **3** is replaced, analogue **52** ($X_3=$Cha) is the most potent against *C. albicans*, with an MIC value of 2 $\mu g\,mL^{-1}$ ($IC_{50}=1.0\ \mu g\,mL^{-1}$). In fact, **52** was the most active analogue against *C. albicans* among all the tested peptides. Analogues **58** [$X_3=$Phe(4-CF_3)], **63** ($X_3=$Trp), and **80** ($X_3=$Dip) also exhibited promising activities against *C. albicans*. The presence of a bulky ring substitution at position X_3 seems to be critical for potency against the fungal strain. In the same manner, peptides **52**, **50** [$X_3=$Phe(4-F)], and **58** exhibited potent activity, with MIC values of 2 $\mu g\,mL^{-1}$ ($IC_{50}=0.11$, 0.25 and 0.94 $\mu g\,mL^{-1}$, respectively) against MRSA. Analogue **57**, on the other hand, displayed potent activity against MRSE ($MIC=1\ \mu g\,mL^{-1}$; $IC_{50}=0.02\ \mu g\,mL^{-1}$). Other peptide analogues such as **51** [$X_3=$Phe(4-Me)], **52**, **56**, **58**, **62** ($X_3=$ D-Trp), and **80** also displayed high activities against MRSE. In general, most of the peptides exhibited good activities against both the Gram-positive bacterial strains. Comparatively fewer peptides were active against *E. coli*, with analogue **54** ($X_3=$Phg) displaying the greatest potency, with an MIC value of 20 $\mu g\,mL^{-1}$ ($IC_{50}=5.9\ \mu g\,mL^{-1}$). In parallel with the observation made earlier, most peptides were found inactive against *K. pneumoniae* and *P. aeruginosa*. To conclude, peptide **54**, containing a Phg residue, was found to be active against five tested microbial strains exhibiting a broad spectrum of activities, whereas analogues **51**, **52**, **58**, **73** ($X_3=$Cys), and **80** displayed promising activity against four tested microbial strains.

For peptides **81**–**112**, in which substitutions at position X_4 of peptide **3** were carried out, analogues **95** ($X_4=$ D-Phe) and **86** ($X_4=$Nle) displayed moderate activity against *C. albicans*, with MIC values of 20 $\mu g\,mL^{-1}$ ($IC_{50}=4.4$ and 7.0 $\mu g\,mL^{-1}$, respectively). In the case of MRSA, analogues **84**, **88** ($X_4=$Bal), **91** ($X_4=$Pfp), and **83** [$X_4=$Phe(4-F)] were highly potent, with excellent MIC values of 1, 1, 1, and 2 $\mu g\,mL^{-1}$ ($IC_{50}=0.42$, 0.58, 0.63, and 0.95 $\mu g\,mL^{-1}$), respectively. Analogue **84** was highly potent against MRSE, with an excellent MIC value of 1 $\mu g\,mL^{-1}$ ($IC_{50}=0.07\ \mu g\,mL^{-1}$). Some other analogues such as **83** [$X_4=$Phe(4-F)], **88**, **89** [$X_4=$Phe(4-*t*Bu)], and **112** ($X_4=$Dip) also displayed high activities, with MIC values of 1 $\mu g\,mL^{-1}$ ($IC_{50}=0.78$, 0.23, 0.63, and 0.70 $\mu g\,mL^{-1}$, respectively) against MRSE. Interestingly, most of the active peptides against MRSA and MRSE contained an unnatural amino acid with a bulky residue as the side chain at position X_4. In the case of Gram-negative bacterial strains, analogue **89** was most active against *E. coli*, with an MIC value of 10 $\mu g\,mL^{-1}$ ($IC_{50}=6.5\ \mu g\,mL^{-1}$). However, consistent with earlier results, these peptides were inactive against *K. pneumoniae* and *P. aeruginosa*, with the exception of analogues **83**, **87**, **89**, **95**, and **103**, which displayed modest activities. Broadly speaking, analogue **95** displayed activity against five tested microbial strains, thereby exhibiting a broad spectrum of activities, whereas analogues **83**, **89**, and **103** displayed activity against four tested microbial strains.

The antimicrobial data for peptides **113**–**145**, with substitutions at position X_5 of the lead peptide **3**, show that **121** [$X_5=$Phe(4-*t*Bu)] is the most potent against *C. albicans* ($MIC=20\ \mu g\,mL^{-1}$; $IC_{50}=5.8\ \mu g\,mL^{-1}$). In the case of MRSA, peptides **117**, **113** ($X_5=$Abu), and **142** ($X_5=$Pro) exhibited moderate activity, with MIC values of 50, 100, and 50 $\mu g\,mL^{-1}$ ($IC_{50}=14.6$, 26.1 and 17.9 $\mu g\,mL^{-1}$), respectively. On the other hand, analogues **116** [$X_5=$Phe(4-Me)], **142**, **143** ($X_5=$ D-Pro), **144**, and **145** were active against MRSE, with MIC values of 20 $\mu g\,mL^{-1}$ ($IC_{50}=8.0$, 14.7, 10.5, 14.7, and 9.5 $\mu g\,mL^{-1}$, respectively). In general, the range of activities for peptides **113**–**145** is smaller than that of the preceding series of peptides toward Gram-positive bacterial strains. Analogue **133** ($X_5=$Val) was the most active peptide, with an MIC value of 20 $\mu g\,mL^{-1}$ ($IC_{50}=8.6\ \mu g\,mL^{-1}$) against *E. coli*. In parallel with the observation made earlier, most of the peptides were inactive against *K. pneumoniae* and *P. aeruginosa*. In summary, analogues **117**, **121**, and **145**, respectively containing Cha, Phe(4-*t*Bu), and Dip residues, were active against three tested microbial strains, exhibiting a broad spectrum of activities.

For peptides **146**–**154**, in which His(1-Bzl) 6 in peptide **3** is replaced, peptide analogue **152** ($X_6=$Dab) was the most potent against *C. albicans*, with an MIC value of 10 $\mu g\,mL^{-1}$ ($IC_{50}=2.6\ \mu g\,mL^{-1}$). Analogue **147** ($X_6=$Arg) exhibited the highest potency, with an MIC value of 5 $\mu g\,mL^{-1}$ ($IC_{50}=1.6\ \mu g\,mL^{-1}$) against MRSA. Similarly, for MRSE, peptide **147** exhibited the most promising activity, with an excellent MIC value of 1 $\mu g\,mL^{-1}$ ($IC_{50}=0.12\ \mu g\,mL^{-1}$). Some other analogues such as **153** and **154** also displayed high activities, with MIC values of 5 $\mu g\,mL^{-1}$ ($IC_{50}=1.2$ and 3.3 $\mu g\,mL^{-1}$, respectively). In the case of *E. coli*, analogue **149** ($X_6=$ D-Lys) was the most active, with an MIC value of 5 $\mu g\,mL^{-1}$ ($IC_{50}=1.5\ \mu g\,mL^{-1}$). Except **149** ($MIC=50\ \mu g\,mL^{-1}$), no compound was found to be active against Gram-negative bacterial strains of *K. pneumoniae* and *P. aeruginosa*. Generally speaking, analogues **149**, **151**, **152**, **153**, and **154**, containing D-Lys, D-His, Dab, Orn, and Phe(4-NH_2) residues, respectively, at position X_6, were active against four tested microbial strains, exhibiting a broad spectrum of activities.

The cyclic peptide **155** was inactive against all the tested strains ($MIC\geq200\ \mu g\,mL^{-1}$). This observation clearly indicates that linearity is essential for activity against microbial strains in this particular series of peptides. The constraint induced by cyclization possibly limits the flexibility of the peptide to a large extent, thereby preventing the cationic termini from interacting with the anionic phospholipids of the microbial cell membrane for disruption.

Cytotoxicity experiments

All the synthesized peptides were screened for cytotoxicity using an MTT assay with mouse fibroblasts using a procedure

described earlier.[27] Most of the tested linear synthetic hexapeptides showed no cytotoxic effects up to the highest test concentration used (200 μg mL^{-1}). Peptides **29** and **55**, however, displayed some cytotoxic behavior. Peptide **29** exhibited high cytotoxicity, with an observed IC_{50} value of 10 μg mL^{-1}, whereas **55** was 50% toxic at 100 μg mL^{-1}.

Tryptophan fluorescence quenching studies

The selectivity of active peptides toward bacterial cells may be related to differences in their interaction with the outer membrane monolayers of bacterial versus mammalian cells. Such differences can be elucidated by the relative extent of peptide burial in model membranes; to measure this, we performed a tryptophan fluorescence quenching study with both negatively charged and zwitterionic model membranes, which mimic bacterial and mammalian membranes, respectively. For this purpose, we selected the twelve hexapeptides **11**, **15**, **70**, **75**, **87**, **113**, **118**, **134**, **146**, **147**, **149**, and **152** that exhibited modest to promising activity against the microorganism strains tested. All the selected peptides contain D- or L-tryptophan in their sequence, along with phenylalanine and tyrosine in some of the hexapeptides.

The Stern–Volmer plots of acrylamide-mediated quenching of tryptophan in the absence and presence of lipid vesicles are shown in Figure 2. Tryptophan fluorescence in the peptides decreased in a concentration-dependent manner through the addition of acrylamide to the peptide solution, both in the absence and presence of liposomes. However, relative to the measurements in the absence of liposomes, the slopes were decreased in the presence of small unilamellar vesicles (SUVs) of egg yolk L-α-phosphatidylcholine (EYPC)/egg yolk L-α-phosphatidyl-D,L-glycerol (EYPG) (7:3 *w*/*w*) or SUVs of EYPC/cholesterol (10:1 *w*/*w*), suggesting that the indole moiety of tryptophan is buried in the bilayers, where it is inaccessible for quenching by acrylamide. In the presence of EYPC/EYPG (7:3 *w*/*w*) SUVs, which mimic the negatively charged bacterial membrane, all of the peptides exhibited similar extents of quenching, suggesting that their indole moieties are buried effectively in the negatively charged phospholipid membranes. Likewise, the order for the relative extent of tryptophan fluorescence quenching in the presence of EYPC/cholesterol (10:1 *w*/*w*) SUVs, which mimic the mammalian membrane, also exhibited similar extents of quenching. In particular, the slope for the tested peptides in the presence of EYPC/cholesterol (10:1 *w*/*w*) SUVs was much higher than in the presence of EYPC/EYPG (7:3 *w*/*w*) SUVs, suggesting that the peptides have a preferential interaction with negatively charged phospholipids. This clearly indicates that the peptides effectively embed into the negatively charged membrane but not into the zwitterionic membrane, suggesting that the selectivity of these peptides toward bacterial cells is associated with preferential interaction with negatively charged phospholipids. Thus, it can be safely stated that the observations made from the tryptophan fluorescence quenching studies with the selected most active hexapeptides are in line with the differences between bacterial (negatively charged phospholipids) and mammalian (zwitterionic phospholipids and cholesterol) cell membrane composition.

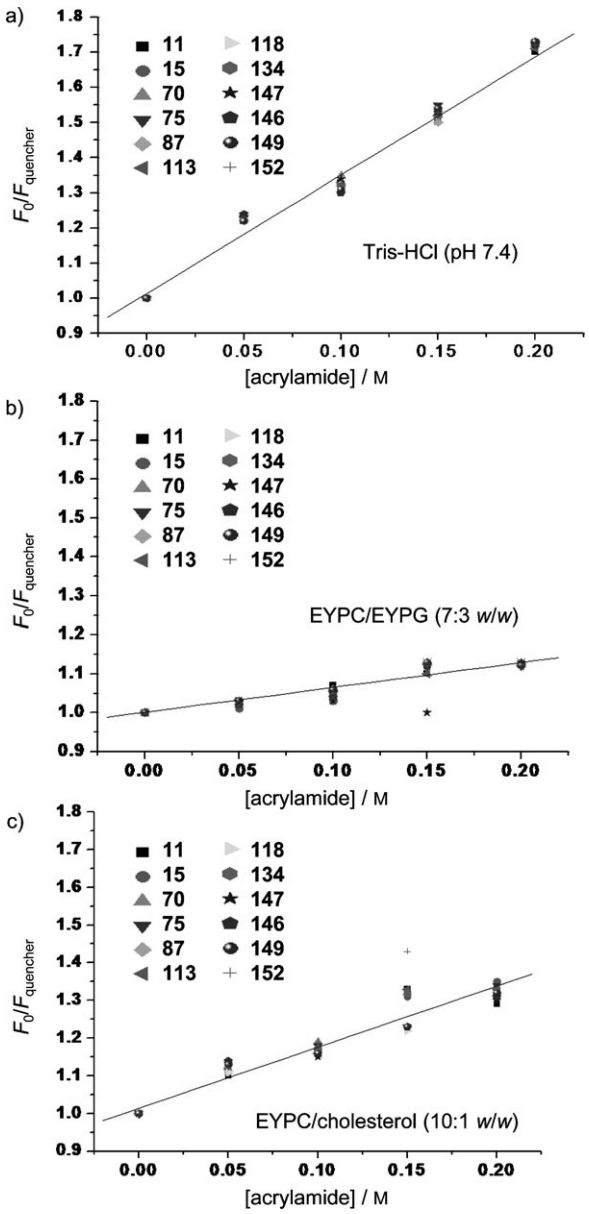


Figure 2. Stern–Volmer plots for the quenching of Trp fluorescence in the peptides by the aqueous quenching agent, acrylamide.

Conclusions

Solid-phase peptide synthesis (SPPS) protocols were used with peptide **3** as a starting point in the design and synthesis of small synthetic peptides containing natural and unnatural amino acids, which were successfully used to identify leads against various microbial pathogens. The synthesized peptides displayed a broad spectrum of activities, and were particularly more active against the Gram-positive bacterial strains. These peptides also exhibited potent activities against the fungus *C. albicans*. Of all the peptides tested against *C. albicans* and MRSA, Orn-D-Trp-Cha-Ile-D-Phe-His(1-Bzl)-NH_2 (**52**) was the

most active. Peptide **52** also displayed an excellent IC_{50} value against MRSE and moderate activity against *E. coli*. Notably, **52** exhibited better activities than peptide **3** against all the tested fungal, Gram-positive, and Gram-negative bacterial strains. Thus, peptide **52** has been identified as a new lead that displays broad-spectrum activities against a panel of pathogenic microorganisms. On the other hand, Orn-D-Trp-D-Phe-Ile-D-Phe-D-Lys-NH_2 (**149**) was most active against both *E. coli* and *K. pneumoniae*, whereas Orn-D-Trp-Nal-Ile-D-Phe-His(1-Bzl)-NH_2 (**57**) and Orn-D-Trp-D-Phe-Phe(4-*t*Bu)-D-Phe-His(1-Bzl)-NH_2 (**89**) exhibited the best potency against MRSE and *P. aeruginosa*, respectively. It seems that an N-to-C-terminus cyclization restricts the flexibility of the peptide and leads to blockage of terminal functional groups, resulting in the loss of antimicrobial activity in peptide **155**. The fluorescence studies not only indicate that the active hexapeptides interact preferentially with the negatively charged bacterial membrane over the neutral mammalian membrane, but also that these hexapeptides exhibit their activity in a well-proven manner. Therefore, it can be safely stated that the synthesized active peptides might employ any of the traditional mechanistic models (barrel-stave, carpet, or toroid pore) in disruption of the cell membrane that ultimately results in killing of the microbial cell.

Experimental Section

Materials

Amino acids, coupling reagents, *N*,*N*-diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were purchased from either Chem-Impex International or NovaBiochem (Merck Ltd.). Egg yolk L-α-phosphatidylcholine (EYPC), egg yolk L-α-phosphatidyl-D,L-glycerol (EYPG), acrylamide, and cholesterol were supplied by Sigma Chemical Co. The buffers were prepared in double glass-distilled water. All solvents used for synthesis were of analytical grade and were used without further purification unless otherwise stated. All other reagents were of analytical grade. We used both Fmoc and Boc methods of peptide synthesis to optimize various sets of conditions for the series of hexapeptides reported herein.

General procedure for Boc SPPS

MBHA·2 HCl resin was used as the solid support, and was swelled in CH_2Cl_2 and neutralized with 10% DIEA in CH_2Cl_2. Free resin was coupled with the Boc-protected first amino acid using *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) as coupling agent in the presence of DIEA as activating base in DMF for 90–120 min. The completion of coupling reactions was routinely monitored by Kaiser's test. The Boc group was then removed using 20% TFA for 20 min in CH_2Cl_2. The peptide was neutralized using 10% DIEA in CH_2Cl_2 to afford the resin-coupled amino acid. This cycle of coupling, deprotection, and neutralization was repeated with subsequent Boc-protected amino acids to afford the resin-coupled hexapeptides. The resin and orthogonal protecting groups were cleaved using 30% HBr solution in acetic acid, in the presence of pentamethylbenzene and thioanisole as scavengers in TFA for 1 h at ambient temperature to yield the linear hexapeptides. After filtration of the resin and subsequent TFA evaporation, crude peptides were precipitated and washed with Et_2O before being dried under vacuum.

General procedure for Fmoc SPPS

TentaGel S Ram resin was used as the solid support. The Fmoc group in TentaGel S Ram resin was first removed using 20% piperidine in DMF for 15 min to yield free resin. The free resin was coupled with the first Fmoc-protected amino acid in the presence of TBTU or benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBop) as coupling agent (depending on the amino acid to be coupled) and DIEA as activating reagent in DMF for 3 h. The Fmoc group was removed using 20% piperidine in DMF for 15 min to afford the resin-coupled amino acid. This cycle of coupling and deprotection was repeated with subsequent Fmoc-protected amino acids to afford the resin-coupled hexapeptides. Finally, the resin and orthogonal protecting groups were cleaved using TFA in the presence of 3% triisopropylsilane as scavenger and 2% H_2O for 2.5 h. After filtration of the resin and subsequent TFA evaporation, crude peptides were precipitated and washed with *tert*-butylmethyl ether before being dried under vacuum.

Synthesis of cyclic peptide 155

Peptide **155** was synthesized as shown in Scheme 1. 2-Chlorotrityl chloride polystyrene resin, which was used as the solid support, and Fmoc-His(1-Bzl)-OH were coupled by adding DIEA and dry CH_2Cl_2, and shaking the reaction mixture for 2 h at ambient temperature. The optical density of the Fmoc group was measured by UV/Vis spectroscopy of the cleaved Fmoc group. Depending on the loading of amino acid on the resin, the resin-coupled His(1-Bzl) was coupled with Fmoc-D-Phe-OH using TBTU and DIEA for 3 h to afford the Fmoc-protected resin-coupled dipeptide. The Fmoc group was cleaved by using 20% piperidine in DMF for 15 min. This cycle of coupling and deprotection was repeated with Fmoc-Ile-OH, Fmoc-D-Phe-OH, Fmoc-D-Trp(Boc)-OH, and Fmoc-Orn(Boc)-OH to afford the resin-coupled orthogonally protected hexapeptide. Treatment with three cycles of 1% TFA in CH_2Cl_2 cleaved the protected hexapeptide from the resin. The filtrate was treated with pyridine to neutralize the TFA, and the solvent was removed under vacuum. The resulting residue was dissolved in 1,4-dioxane and lyophilized to yield the linear hexapeptide Orn(Boc)-D-Trp(Boc)-D-Phe-Ile-D-Phe-His(1-Bzl). This linear hexapeptide was allowed to cyclize in the presence of PyBop, 1-hydroxy-7-azabenzotriazole (HOAt), and DIEA in DMF as solvent for 24 h at ambient temperature. Finally, the Boc groups were removed using 20% TFA in CH_2Cl_2 for 30 min to yield the required cyclic hexapeptide **155**.

Peptide purification and characterization

The initial mass spectra were recorded with a Finnigan Mat LCQ spectrometer (APCI) and a Kratos III MALDI mass spectrometer. Preparative HPLC was done using a Merck Hitachi HPLC system equipped with a Nucleosil 100 C_{18}, 250×40 mm column. All final peptides were checked for homogeneity on a Shimadzu SPD-M20A HPLC system using a Supelcosil™ LC-8 (5 μm, 250×4.6 mm i.d.) column by employing a solvent system of CH_3CN/H_2O (0.1% TFA) with a 14 min gradient of 0→50% CH_3CN over 12 min, 50→60% CH_3CN over 1 min, and 60→100% CH_3CN over 1 min. Final confirmation of peptide purity was done on an Agilent 1100 series LCMS with an Applied Biosystems API100 (MS) using a Polymer Labs PLRP-S 100A (3 μm, 50×2.1 mm) column by employing a solvent system of CH_3CN/H_2O (0.1% TFA) with a 15 min gradient of 5→95% CH_3CN over 10 min and 100% CH_3CN over 5 min. In general, the percentage purity obtained for the synthesized compounds

was found to be >90%. However, peptides **24**, **26**, and **93** showed a purity of 86, 76, and 86%, respectively, mainly because of the presence of inseparable racemic sequence isomers.

Preparation of small unilamellar vesicles (SUVs)

SUVs were prepared by a standard procedure with required amounts of either EYPC/EYPG (7:3 *w/w*) or EYPC/cholesterol (10:1 *w/w*) for tryptophan fluorescence. Dry lipids were dissolved in $CHCl_3$ in a small glass vessel. Solvents were removed by rotary evaporation to form a thin film on the wall of a glass vessel. The dried thin film was resuspended in Tris-HCl buffer by vortex mixing. The lipid dispersions were then sonicated in an ice/H_2O mixture for 10–20 min with an ultrasonicator until the solution became transparent.

Quenching of Trp emission by acrylamide

The fluorescence quenching was measured using a PerkinElmer spectrofluorimeter (LS 50B), and the experiment was performed in small unilamellar vesicles (SUVs) using the water-soluble neutral fluorescence quencher acrylamide, which has been widely used for the same purpose.[29] To minimize absorbance by acrylamide, excitation of Trp was conducted at $\lambda = 295$ nm instead of 280 nm.[30,31] Aliquots of the 4.0 M solution of this water-soluble quencher were added to the peptide in the absence or presence of liposomes at a peptide/lipid molar ratio of 1:200. The acrylamide concentration in the cuvette was 0–0.20 M. The effect of acrylamide on the fluorescence of each peptide was analyzed with the Stern–Volmer equation:

$$F_0/F = 1 + K_{SV}[Q]$$

in which F_0 and F represent the fluorescence intensities in the absence and the presence of acrylamide, respectively, K_{SV} is the Stern–Volmer quenching constant, and [Q] is the concentration of acrylamide.

Abbreviations

AMPs = antimicrobial peptides, APD = antimicrobial peptide database, Boc = *tert*-butyloxycarbonyl, DIEA = *N*,*N*-diisopropylethylamine, EYPC = egg yolk L-α-phosphatidylcholine, EYPG = egg yolk L-α-phosphatidyl-D,L-glycerol, Fmoc = 9-fluorenylmethyloxycarbonyl, HPLC = high-pressure liquid chromatography, IC_{50} = inhibitory concentration that affords 50% inhibition of microbial growth, LPS = lipopolysaccharide, MBHA = 4-methylbenzhydrylamine, MIC = minimum inhibitory concentration, MRSA = methicillin-resistant *Staphylococcus aureus*, MRSE = methicillin-resistant *Staphylococcus epidermidis*, MSSA = methicillin-sensitive *Staphylococcus aureus*, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide, PyBop = benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate, SUVs = small unilamellar vesicles, TBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate, TFA = trifluoroacetic acid, YPD = yeast extract/peptone/dextrose.

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Keywords: antimicrobial peptides · cytotoxicity · fluorescence · peptides · solid-phase synthesis

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