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Synthesis and antimicrobial activity of dermaseptin S1 analogues ☆

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ABSTRACT

Dermaseptins are peptides found in the skin secretions of Phyllomedusinae frogs. These peptides exert lytic action on some microorganisms without substantial haemolysis. In an attempt to understand the antimicrobial activity of these peptides we designed several dermaseptin S1 (ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ) (DS1) analogues. All peptides were tested on the growth of prokaryotic (Gram-positive and Gram-negative bacteria) and eukaryotic (the yeast *Candida albicans* and the protozoon *Leishmania major*) organisms. Our data showed a dose-dependent killing effect by most DS1 derivatives. Maximal antibacterial activity was displayed by a 16-mer peptide that was more active than native DS1.

1. Introduction

Infectious diseases are the second leading cause of death worldwide. Accordingly, there is an increasing need to identify new antibacterial agents with a particular emphasis on multi-drug-resistant bacteria and newly emerging pathogens. Evolution of bacteria towards resistance to antimicrobial drugs, albeit an unavoidable aspect of the general evolution of bacteria, is a major public health concern as it is extremely difficult to overcome.[1] New hopes to delay the emergence and subsequent dissemination of resistant microorganisms or resistant genes come from the discovery of natural products that may act as efficient leads in the development of novel therapeutic agents.[2,3] Prominent amongst these natural products are the members of the cationic host defence peptide family, which are widely distributed in nature as a component of the immediate non-specific defence against infections. This defence peptide system exists in species of all kingdoms from insects to plants to mammals and non-mammalian vertebrates and its effectiveness has been assessed by demonstrating direct antimicrobial activity against bacteria, fungi, eukaryotic parasites and/or viruses.[4] The advantages of these peptides in clinical application include their potential for broad-spectrum activity, their rapid bactericidal activity and low propensity for development of resistance. However, there are possible disadvantages such as cost, limited stability and unknown toxicology.[5]

We focused on dermaseptins, a set of peptides expressed by different cells found in the skin of frogs belonging to the Phyllomedusinae subfamily.[6–8] They consist of a characteristic polypeptide chain of 28–34 amino acids with 3–6 lysine residues and a highly conserved tryptophan residue in the third position from the N-terminus. These peptides that differ in net charge and hydrophobicity exert a selective lytic action on some bacteria, protozoa, yeast and filamentous fungi at micromolar concentrations.[8] Unlike polylysine peptides, dermaseptins show little or no detectable haemolytic activity.[8,9] Using phospholipid liposomes or live cells the antimicrobial action of these peptides was shown to be mediated by selective interaction of the basic and amphipathic α-helical moiety with plasma membrane phospholipids, leading to permeabilization and lysis.[8,10,11] Recently, it has been demonstrated that dermaseptins possess a potent spermicidal activity against human sperm.[12] In addition, some synthetic peptides derived from dermaseptin S4 showed activity against *Neisseria gonorrhoeae*[13] indicating a potential role as microbicidal compounds. There has been renewed interest in the antimicrobial activity of dermaseptins in response to the isolation and characterization of DS 01, from the skin secretions of the Brazilian species *Phyllomedusa oreades*, a novel dermaseptin characterized by antibacterial and anti-protozoal activity.[9,14]

In this structure–activity relationship study, we chose dermaseptin S1 (DS1) because this peptide is characterized by the lowest haemolytic activity.

Abbreviations: DMF, N,N-dimethylformamide; DS1, dermaseptin S1; FSC, fetal calf serum; HATU, [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; MIC, minimal inhibitory concentration; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid.

☆ Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977. Amino acid symbols denote L-configuration unless indicated otherwise.

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As a useful guide we also made use of two enlightening papers on dermaseptin S4 and its analogues.[15,16] In contrast to other S dermaseptin peptides, the 28-residue dermaseptin S4 is highly toxic to erythrocytes but, as noted by Feder et al.[15] and Rydlo et al.[16], reducing hydrophobicity or by increasing the net positive charge improved peptide bioactivity. Shorter derivatives still displayed high antibacterial activity in vitro and in vivo along with a more acceptable toxicity profile against human erythrocytes, albeit still rather high.[17]

In an attempt to explain why dermaseptins have markedly different biological activities despite a similar tendency to form amphipathic helices, we synthesized a series of analogues of DS1. In particular, we investigated the importance of the C-terminal portion of DS1 on the growth of several microorganisms. Amongst possible single residue changes we focused on position 3 (Trp^3) to assess the importance of the dermaseptin conserved indole moiety on bioactivity.[18] Here we report the design, synthesis and biological evaluation of 15 DS1 analogues.

2. Results and discussion

The main goal of our study was to identify a shorter bioactive analogue of DS1 and then attempt to improve its biological profile with respect to that of the parent peptide. In order to fulfil this goal we used the following criteria: (i) progressive C-terminal shortening (intact N-terminus is essential for antimicrobial activity[7,8,19]), (ii) perform single residue modifications on the shortest active analogue, with particular emphasis on the third residue.[7,8]

In order to characterize the antimicrobial properties of DS1 and its analogues, *Staphylococcus aureus* and *Escherichia coli* were selected as representative Gram-positive and Gram-negative bacteria. The data obtained in the primary antimicrobial screening are reported in Table 1. As mentioned above, in a first round of synthesis the C-terminal portion of DS1 was progressively shortened and amidated. Both chemical modifications are fully tolerated, as shown by the fact that for peptides **1–3** the MIC concentration is in the range from 2.7 to 35.9 μM. A small but consistent increase in antimicrobial activity was observed for compound DS1(1–29)-NH_2, whilst deletion of residues beyond position 15 at the C-terminus produces a marked decrease in biological activity. DS1(1–14)-NH_2 is about fivefold less active than the reference peptide on both Gram-positive and Gram-negative bacteria. DS1(1–15)-NH_2 is the minimum fragment showing antimicrobial properties comparable to the parent peptide (DS1) both in terms of MIC and LD_{50}.

Having selected peptide DS1(1–15)-NH_2 as a template for further structure-activity investigations, we focused on the importance of the highly conserved tryptophan in position 3[8,18] on antimicrobial activity. Replacement of the indole with a naphthyl moiety generated compounds **7** and **8** that showed an antimicrobial potency comparable to that of the reference DS1(1–15)-NH_2. The presence of amino acids with a simple phenyl side chain (compounds **9** and **10**) in position 3 reduces antibacterial activity possibly indicating that the distance between the side chain and the peptide backbone seems to be particularly important in modulating activity against *E. coli*. Similar results were obtained replacing Trp^3 with amino acids characterized by different chemical entities, 1H-imidazole (compound **11**), *p*-hydroxyphenyl (compound **12**) or isobutyl (compound **13**) as side chain. Collectively, these data confirm the crucial role of position 3 for membrane bacterial interaction and indicate as a critical feature the presence of an indole nucleus. A key factor is probably the steric encumbrance of the side chain, since Trp is believed to be a very good anchor point in the membrane.[20,21]

In an attempt to improve the potency of DS1(1–15)-NH_2 we added a Lys residue to the N-terminus. Recent findings indicated that acylation of dermaseptin S4 fragments with long chain fatty acids, with or without an amino function in their structures, is well tolerated in terms of antimicrobial activity and is instrumental in modulating the potency and spectrum of activity in different assay conditions.[16,22] Compound **14** showed a small increase in MIC against *S. aureus* and maintained the same activity as DS1(1–15)-NH_2 against *E. coli*. Interestingly, the LD_{50} against *E. coli* decreased about twofold with respect to that of DS1(1–15)-NH_2 indicating faster bactericidal kinetics. The presence of an additional Lys residue in the N-terminal peptide increases the positive charge with respect to DS1(1–15)-NH_2 and this may promote an interaction with the cell wall of Gram-negative bacteria leading to a more effective cell permeabilization. Combining in the same template two modifications potentially able to increase antibacterial properties, that is, the replacement in position 3 with 1Nal and the addition to the N-terminal amino function of a Lys residue, yielded a

Table 1
Amino acid sequence, antibacterial activity and lysis of human red blood cells of DS1 and analogues

N	Compound	Amino acid sequence	MIC[a] (μM)		LD_{50}[b] (μM)		HD_{50}[c] (μM)
			S. aureus	*E. coli*	*S. aureus*	*E. coli*	
	DS1	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	12	12	5	3.1	>100
1	DS1(1–29)-NH_2	ALWKTMLKKLGTMALHAGKAALGAAADTI-NH_2	2.7	2.7	<1.5	2.2	>100
2	DS1(1–19)-NH_2	ALWKTMLKKLGTMALHAG-NH_2	35.9	3.6	10.7	<1.8	>100
3	DS1(1–15)-NH_2	ALWKTMLKKLGTMAL-NH_2	11.5	2.9	2.7	1.4	>100
4	DS1(1–14)-NH_2	ALWKTMLKKLGTMA-NH_2	>48.8	48.8	28.3	20.5	>100
5	DS1(1–13)-NH_2	ALWKTMLKKLGTM-NH_2	>50.6	50.6	50.6	22.7	>100
6	DS1(1–12)-NH_2	ALWKTMLKKLGT-NH_2	>54	>54	54	54	>100
7	[1Nal^3]DS1(1–15)-NH_2	AL-1Nal-KTMLKKLGTMAL-NH_2	11.5	2.8	2.4	0.9	75
8	[2Nal^3]DS1(1–15)-NH_2	AL-2Nal-KTMLKKLGTMAL-NH_2	11.5	2.8	2.5	0.9	>100
9	[hPhe^3]DS1(1–15)-NH_2	AL-hPhe-KTMLKKLGTMAL-NH_2	46.8	11.7	9.3	3.0	>100
10	[Phe^3]DS1(1–15)-NH_2	AL-Phe-KTMLKKLGTMAL-NH_2	>47.1	23.5	16.7	4.5	>100
11	[His^3]DS1(1–15)-NH_2	AL-His-KTMLKKLGTMAL-NH_2	>44.9	>44.9	>44.9	>44.9	>100
12	[Tyr^3]DS1(1–15)-NH_2	AL-Tyr-KTMLKKLGTMAL-NH_2	>46.7	>46.7	>46.7	>46.7	>100
13	[Leu^3]DS1(1–15)-NH_2	AL-Leu-KTMLKKLGTMAL-NH_2	>47.9	23.9	12.0	6.0	>100
14	[Lys^0]DS1(1–15)-NH_2	Lys-ALWKTMLKKLGTMAL-NH_2	20.8	2.6	2.7	<0.8	>100
15	[$Lys^0$1Nal^3]DS1(115)-NH_2	Lys-AL-1Nal-KTMLKKLGTMAL-NH_2	5.1	1.2	0.8	<0.8	80

Underline denote amino acids substitution performed on DS1(1–15)-NH_2.
[a] MIC, minimal inhibitory concentration.
[b] LD_{50}, dose responsible of the 50% lethality of bacteria after 3 h incubation.
[c] HD_{50}, peptide concentration causing 50% lysis of human red blood cells.

very interesting analogue: compound **15**. This compound acts as a highly potent antimicrobial peptide against *S. aureus* and *E. coli* with LD_{50} values in the sub-micromolar range. The values obtained indicated an activity similar to that reported by Brand et al.[9] and was more potent than that reported by Rotem et al.[23] with other dermaseptin derivatives. This result indicated that different chemical modifications can be combined in the same molecule with a final synergistic effect on both MIC and LD_{50} values.

Cytotoxic activity of the synthesized peptides against human RBC indicated 50% haemolysis (Table 1) at a peptide concentration in excess of 75 μM, in most cases >100 μM, confirming that DS1 is weakly haemolytic, possibly because the N-terminus is only moderately hydrophobic.[15] It is worthy of note that only with the compounds **7**, **8** and **15** at a concentration of 100 μM did haemolysis in excess of 30% occur. With the other analogues, at the same concentration, lytic activity was between 4% and 16%. These values are substantially lower than that reported for other dermaseptins.[9,24–26] DS1, DS1(1–15)-NH_2 and analogues **7–15** were also tested against different microorganisms, found in opportunistic and chronic diseases and multi-drug-resistant (Table 2). In these assays, the most active compounds (**7**, **8**, **14** and **15**) are characterized by the presence of the indole or naphthyl nucleus in position 3, by a further N-terminal Lys residue or, as in analogue **15**, by the presence of both chemical modifications in the template DS1(1–15)-NH_2. However, the activity of these peptides on multi-drug-resistant bacteria was lower, and in particular the susceptibility of *Burkholderia cepacia* and *Stenotrophomonas maltophilia* (two Gram-negative non-fermenting strains) is very low. The antibacterial behaviour of antimicrobial peptides is very likely to be dictated by the nature of the external barriers of the microorganisms examined.[6] Virulent strains of *Pseudomonas aeruginosa* may produce alginic acid, a highly anionic capsular exopolysaccharide, that interferes with antimicrobial activity of peptides in vitro.[27] Along with capsular shielding, this microorganism exploits other mechanisms of resistance through inherent modifications of outer membrane structures. Marr et al.[5] and Yeaman and Yount[27] reported that Burkholderia (ex *Pseudomonas* sp.) was one of the few naturally peptide-resistant organisms. In the multi-drug-resistant *S. aureus* strain the lack of peptide activity could be due to the polysaccharidic capsule coating the peptidoglycan-based bacterial cell wall, that sequesters the compound and neutralizes it prior to reaching its target or to some constitutive alterations in cytoplasmic membrane structure or function.[27] However, it is possible that dermaseptins are involved in a process supporting other bacterial targets, such as RNA synthesis, as reported by Patrzykat et al.[28]

The yeast *Candida albicans* was more susceptible to compounds **9** and **10**, characterized by the presence of a phenyl ring in position 3 suggesting that this position may be useful for improving selectivity against a selected pathogen. Moreover, in a preliminary assay, the first C-terminal truncated DS1 analogues were assayed against *Leishmania major*. The results reported in Table 3 are obtained after 24 h incubation of the protozoon with different peptides. Similar inhibitory and killing activities were observed after 3 h of treatment. We also observed dose-dependent activity against *L. major* promastigotes (data not shown). Standard deviations of LC_{50} were ⩽10% of the values reported. These results demonstrate that in this assay shortening of the C-terminal peptide sequence seems to have a detrimental effect against the leishmanicidal activity and the sequence 1–29 appears to be the minimal active fragment. Compounds DS1(1–29)-NH_2 had a LC_{50} similar or marginally increased to that of DS1 and dermaseptin S4 as reported by Gaidukov et al.[24] on *L. major*, and also increased compared to temporins A and B reported by Mangoni et al.[29] on *L. donovani*. Moreover, leishmanicidal activity was observed at concentrations significantly lower than RBC cytotoxic concentrations indicating more selectivity compared to the dermaseptin analogues tested by Gaidukov et al.[24]

Table 3
Activity of DS1 and C-terminal truncated analogues on *Leishmania major*

Compound	MIC[a]		LC_{50}[b]	
	μg/mL	μM	μg/mL	μM
DS1	50	12	9.9	2.4
DS1(1–29)-NH_2	25	6.1	9.6	2.3
DS1(1–19)-NH_2	50	17.9	22	7.9
DS1(1–15)-NH_2	50	22.8	19	8.7
DS1(1–14)-NH_2	100	48.8	50	24.4
DS1(1–13)-NH_2	100	50.6	50	25.3
DS1(1–12)-NH_2	100	54.2	50	27.1

[a] MIC, minimal concentration inhibiting the growth of promastigotes after 24 h incubation.
[b] LC_{50}, concentration which caused a 50% reduction of survival or viability of promastigotes after 24 h incubation.

The antimicrobial activity observed by some of the synthetic compounds tested are similar to those obtained by other authors with defensins[30] and with peptides purified from *Phyllomedusa distincta*.[31] However, at least 15 amino acids (or 14 with changes in the initial part of the molecule) are required to display potent activity against bacteria and *Candida*.

The anti-protozoal activity was superior with native dermaseptin (34 aa) or derivatives with a long chain (29 aa), without a parallel haemolytic activity. Similarly, Brand et al.[9] demonstrated an anti-protozoal activity against *Trypanosoma cruzi* by DS01 (29-residue peptide) and by two synthetic derivatives, without a toxicity against mouse erythrocytes and white blood cells. The observed activity seems superior to that reported by Mangoni et al.[29] for temporins (from *Rana temporaria*).

Leishmaniasis is a significant health problem in many regions of the world, especially as it has high prevalence in HIV positive patients.[32] As there is currently a limited number of chemotherapeu-

Table 2
MIC evaluation of DS1, DS1(1-15)-NH_2 (compound **3**) and analogues against several Gram-positive and Gram-negative bacteria and the yeast *Candida albicans*

Microorganism	MIC[a] (μM) of different peptides										
	DS 1	3	7	8	9	10	11	12	13	14	15
S. aureus ATCC 6538 P	6	5.7	2.8	2.8	5.8	23	>45	>45	24	2.6	1.3
S. aureus multi-drug-resistant	NT[b]	>47	22.5	>25	>46	>46	>45	>45	>48	>42	21
B. subtilis ATCC 9466	NT[b]	5.7	11.2	5.6	23	23	>45	>45	24	21	5.2
P. aeruginosa ATCC 15442	>24	>47	5.6	5.6	>46	>46	>45	>45	>48	5.2	2.6
P. aeruginosa multi-drug-resistant	NT[b]	>47	11.2	11.2	46	>46	>45	>45	>48	21	10.5
B. cepacia 6/06	>24	23.7	25	25	23	>46	>45	>45	>48	42	42
Stenotrophomonas maltophilia FC	>24	>45	22.5	25	23	>46	>45	>45	>48	21	21
C. albicans	>24	23.7	11.2	11.2	5.8	5.8	>45	>45	12	10.4	10.5

[a] MIC, minimal inhibitory concentration.
[b] NT, not tested.

tic agents for use in this patient population (as reviewed by Alberola et al.[33]) dermaseptin analogues represent a novel approach worthy of further investigation.

3. Conclusion

Investigating the structure–activity features of DS1, we identified that (i) the minimum bioactive sequence is the DS1(1–15)-NH_2; (ii) the Trp in position 3 is important for biological activity and can be substituted with non-natural amino acids bearing a naphthyl nucleus on the side chain; (iii) a further Lys residue in the N-terminal improves the antimicrobial activity against *E. coli* and (iv) modifications in position 3 and the N-terminus can be combined in the DS1(1–15)-NH_2 template to generate novel antimicrobial peptides. Some of these novel small synthetic peptides (belonging to the family of dermaseptins) revealed a selective activity against some microorganisms indicating a potential use as new selective antimicrobials.

4. Experimental

4.1. General information

Amino acids, protected amino acids, and chemicals were purchased from Bachem, Novabiochem, Fluka or Chem-Impex International. All other reagents were from Sigma Chemical Co. (Poole, UK) or E. Merck (Darmstadt, Germany) and were of the highest purity available. Peptides were stored as a lyophilized powder at −20 °C. Solutions were prepared in PBS, vortexed, and used as stock solutions in experiments.

4.2. Peptide synthesis

All the peptides were synthesized by solid-phase methods using Fmoc/^{t}Bu chemistry[34] with a SYRO XP synthesizer (MultiSyntech, Witten Germany). Rink resin (0.65 mM/g) and Wang resin preloaded with Fmoc-Gln(Trt) (0.45 mM/g) (Fluka, Buchs SG, Switzerland) were used as a support for the synthesis of peptides as amide or free acid, respectively. The resin (0.2 g in all synthesis) was treated with piperidine (20%) in DMF and the Fmoc amino acid derivatives (fourfold excess) were coupled to the growing peptide chain using HATU[35] (fourfold excess). Piperidine (20%) in DMF was used to remove the Fmoc group at all steps.

After deprotection of the last Fmoc group, the peptide resin was washed with methanol and dried in vacuo to yield the protected peptide-Resin. Protected peptides were cleaved from the resin by treatment with Reagent B[36] TFA/phenol-triisopropysilane/H_2O (88:5:2:5 v/v) 5 mL/0.2 g of resin at room temperature for 2 h. After filtration of the resin, the solvent was concentrated in vacuo and the residue triturated with ether. The crude peptides were purified by preparative reverse phase HPLC to yield a white powder after lyophilization.

4.3. Peptide purification and analytical determinations

Crude peptides were purified by preparative reversed-phase HPLC using a Waters Delta Prep 4000 system with a Phenomenex Jupiter C_{18} column (250 × 30 mm, 300 A, 15 μm spherical particle size column). The column was perfused at a flow rate of 25 mL/min with solvent A (10%, V/V, acetonitrile in 0.1% aqueous TFA), and a linear gradient from 0% to 60% of solvent B (60%, V/V, acetonitrile in 0.1% aqueous TFA) over 25 min was adopted for peptide elution. Analytical HPLC analyses were performed on a Beckman 125 liquid chromatograph equipped with a Beckman 168 diode array detector. Analytical purity and retention time (t_R) of the peptides were determined using two different HPLC conditions. Retention time I was obtained using a Alltech C_{18} column (4.6 × 150 mm 5 μm particle size) with the above solvent system (solvents A and B) programmed at a flow rate of 1 mL/min using a linear gradient from 5% to 50% B over 25 min. Retention time II was obtained using a TSK gel super ODS C_{18} column (4.6 × 150 mm, 5 μm particle size) with solvent A: 35 mM NaH_2PO_4 (pH 2.1) and solvent B: 59 mmol NaH_2PO_4 (pH 2.1)/acetonitrile (60:40 v/v). The column was perfused at a flow rate of 1 mL/min with a linear gradient from 0% to 30% B over 25 min. All analogues showed >95% purity when monitored at 220 nm. Molecular weights of compounds were determined using a mass spectrometer ESI Micromass ZMD-2000; values are expressed as MH^+. The analytical properties of DS1 analogues are reported in Table 4.

4.4. Antibacterial activity

In the first series of experiments two bacterial strains (*S. aureus* Cowan 1 NCTC 8530 and *E. coli* HB101), one Gram-positive and one Gram-negative, were used to investigate antimicrobial activity. Microorganisms were grown in static culture at 37 °C in Mueller Hinton Broth (Oxoid) and diluted to 5×10^5 CFU/mL. The MIC was determined using the microdilution serial twofold assay (from 100 μg/mL of each preparation) and performed in sterilized 96-well plates (Nunc) in a final volume of 200 μL[16,37]; control wells without peptides were included. The MIC was considered the lowest peptide concentration that showed no increase in turbidity after overnight incubation at 37 °C. To evaluate bactericidal kinetics, another series was prepared and incubated for 3 h; afterwards, the cultures were subjected to serial 10-fold dilutions in PBS and bacterial counts were determined after 24 h incubation at 37 °C in Mueller Hinton Agar (Oxoid) plates. Peptide dose causing 50% lethality (LD_{50}) was assessed.[15] More active compounds were assessed for the MIC against several other bacteria, three Gram-positive (*S. aureus* ATCC 6538P, *S. aureus* multi-drug-resistant and *Bacillus subtilis* ATCC 9466) and four Gram-negative (*P. aeruginosa* ATCC 15442, *P. aeruginosa* multi-drug-resistant, *B. cepacia* 6/06 and *Stenotrophomonas maltophilia* FC). All results represent the mean values obtained from at least two independent experiments performed in duplicate.

4.5. Antifungal assay

One yeast species, *C. albicans* (wild strain), was assayed. The in vitro assay (in triplicate) was performed using the broth microdilu-

Table 4
Abbreviated names and analytical properties of DS1 and analogues

No.	Abbreviated names	t_R[a]		MH^+[b]	
		I	II	Calculated	Found
	DS1	12.31	26.22	3456.6	3456.2
1	DS1(1–29)-NH_2	12.66	26.93	2953.6	2953.0
2	DS1(1–19)-NH_2	11.86	23.90	2098.2	2098.2
3	DS1(1–15)-NH_2	13.06	25.33	1705.2	1705.1
4	DS1(1–14)-NH_2	11.05	23.24	1592.2	1592.3
5	DS1(1–13)-NH_2	11.07	22.97	1520.3	1520.6
6	DS1(1–12)-NH_2	10.30	21.51	1389.3	1389.6
7	[1Nal^3]DS1(1–15)-NH_2	10.02	20.60	1715.3	1715.8
8	[2Nal^3]DS1(1–15)-NH_2	9.86	19.42	1715.3	1715.0
9	[hPhe^3]DS1(1–15)-NH_2	9.78	20.15	1680.2	1680.7
10	[Phe^3]DS1(1–15)-NH_2	9.54	19.73	1666.2	1666.7
11	[His^3]DS1(1–15)-NH_2	9.51	17.49	1655.1	1655.8
12	[Tyr^3]DS1(1–15)-NH_2	9.43	18.32	1682.2	1682.3
13	[Leu^3]DS1(1–15)-NH_2	9.67	19.44	1632.2	1632.6
14	[Lys^0]DS1(1–15)-NH_2	9.67	19.41	1832.4	1832.8
15	[Lys^{0}1Nal^3]DS1(1–15)-NH_2	9.58	16.65	1844.2	1844.8

[a] t_R is the retention time determined by analytical HPLC.
[b] The mass ion (MH^+) was obtained by electro spray mass spectrometry.

tion method according to the recommendations of the NCCLS[38] and Pfaller et al.[39] The MIC was considered the lowest peptide concentration that showed no increase in turbidity after overnight incubation at 37 °C.

4.6. Haemolytic activity

Investigation of haemolytic activity was essentially as reported.[9,31] The compounds were serially diluted in Eppendorf test tubes and incubated under agitation with 100 μL containing 10^8 human red blood cells (RBC), obtained from human washed blood, at 37 °C for 30 min. After a 900g centrifugation for 10 min, the absorbance of the supernatant (as an index of haemoglobin leakage) was measured spectrophotometrically (Microplate Reader 450, Bio-Rad) at 550 nm. Maximum haemolysis was determined by adding distilled water. The peptide concentration (mean) causing 50% lysis (HC_{50}) was obtained from at least two independent experiments performed in triplicate.

4.7. Leishmanicidal activity

Leishmania major (strain MHOM/IL/67/JERICHO-II) promastigotes were grown at 25 °C in 'complete medium'[37,40], comprising medium 199 (Invitrogen, California, USA) supplemented with 20% heat-inactivated FCS (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), 5 μg/mL hemin (in 50% triethanolamine) and 1 μg/mL 6-biotin (in 95% ethanol) *L. major* were resuspended in fresh medium to a final concentration of 5×10^5 viable promastigotes/mL and treated with serial dilutions of the different peptides. Protozoal viability was assessed after 3 and 24 h incubation at 25 °C under agitation by counting in a haemocytometer (Thoma chamber) after vital staining with trypan blue (dye exclusion method). The experiments were performed in triplicate and the results reported as mean of the MIC values. The peptide concentration which caused a 50% reduction in survival or viability (LC_{50}) after 24 h incubation in comparison to that in an identical culture without the compound was estimated. This value was determined by nonlinear regression analysis using GraphPad Prism 3 software. Standard deviation of LC_{50} was $\leqslant$10% of the values reported.

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