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Improved antimicrobial activity of h-lysozyme (107–115) by rational Ala substitution

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The most challenging target in the design of new antimicrobial agents is the development of antibiotic resistance. Antimicrobial peptides are good candidates as lead compounds for the development of novel anti-infective drugs. Here we propose the sequential substitution of each Ala residue present in a lead peptide with known antimicrobial activity by specific amino acids, rationally chosen, that could enhance the activity of the resultant peptide. Taking the fragment 107–115 of the human lysozyme as lead, two-round screening by sequentially replacing both Ala residues (108 and 111) by distinct amino acids resulted in a novel peptide with 4- and 20-fold increased antimicrobial activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213, respectively. These results reinforce the strategy proposed, which, in combination with simple and easy screening tools, will contribute to the rapid development of new therapeutic peptides required by the market. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: alanine scanning; antimicrobial activity; peptides; lysozyme

Introduction

Antimicrobial peptides (AMPs) are produced by almost all living organisms, at low metabolic cost, as a component of their innate non-specific immune system [1–4]. AMPs are much appreciated as lead compounds for the discovery of human therapeutics to tackle the development of antibiotic resistance. Furthermore, the interest of pharmaceutical industries in peptides has been rekindled. Today, more than 50 peptides are in the world pharmaceutical market, and more than a hundred are in several clinical phases. As drugs, some peptides show unique characteristics (high biological activity, high specificity and low toxicity), thereby making them particularly attractive therapeutic agents [5,6]. Moreover, small peptides can be prepared industrially at a relatively low cost, thus meeting the needs of the medical, agrochemical and food industries.

Several robust tools can be used to develop new AMPs; these include soluble synthetic combinatorial libraries made up of natural and/or unnatural amino acids in conjunction with iterative selection processes, as described by Blondelle *et al*. [7,8]. These methods allow the systematic assessment of thousands to millions of peptides by means of simple microdilution assays. Furthermore, single substitution of amino acids of a lead peptide with known antimicrobial activity can dramatically affect its secondary structure and its biological activity, thus resulting in an improved AMP [9].

The *alanine-scanning* technique is the sequential substitution of each amino acid of a lead peptide by an Ala residue in order to determine the amino acids that are crucial for biological activity [10]. Ala is chosen because it is considered the most neutral amino acid. Therefore, with an α-substitution, the side chain of Ala is the least voluminous of all the amino acids. Furthermore, it is not highly hydrophobic, has no charge and does not fit in any pocket. These features result neutral for a structure–activity relationship [11]. The *alanine-scanning* method has been widely used for defining the structure–activity relationship of many potential therapeutic molecules as well as for designing analogs with improved activity and/or selectivity [12–17]. Here, we propose an alternative of the *alanine scanning* that involves the sequential substitution of each Ala residue present in a lead peptide with known biological activity by specific amino acids, rationally chosen, that could contribute to the biological activity of the resultant novel peptide. We postulate that this method may improve the development of new compounds with enhanced biological activity from a lead peptide.

As a model to test the validity of the new strategy, a fragment of the human lysozyme (hLz), with known antibacterial activity against both Gram-positive and Gram-negative microorganisms, was chosen.

In addition to the enzymatic (muramidase) antimicrobial activity of lysozyme, its digestion brings about several catalytically independent peptides with broad antimicrobial activity. Many authors have described the activity and mechanism of action of these peptides against several bacterial strains. AMPs released by clostripain digestion of hen egg white lysozyme (cLz) were analyzed by Pellegrini *et al*. [18] and Ibrahim *et al*. [19]. Moreover,

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107 115
RAWVAWRNR

Figure 1. Sequence of 107–115 hLz.

AMPs released by peptic–tryptic digestion of cLz were studied by Mine *et al*. [20], providing evidence of the potential release of AMPs from cLz in the digestive system to prevent bacterial infections. The attributes consensus of these peptides are the presence of positively charged and hydrophobic amino acids and an amphiphilic helical conformation, which interact directly with the microbial membrane to generate pores that lead to microbial death. The lethal step in most cationic AMPs is the disruption of the cytoplasma membrane; this process is accomplished in two steps: membrane binding, predominantly governed by electrostatic interactions, and membrane insertion/permeation, which is directly related to the hydrophobicity of the peptide and its capacity to partition into the plasma membrane [21]. Weinberg *et al*. [22], Hancock and Lehrer [23], Jenssen *et al*. [24] and Papagianni [25] reviewed the research on cationic AMPs.

Thus, the fragment 107–115 (Figure 1) of hLz was chosen. This peptide belongs to the *C*-terminal part of the helix-loop-helix domain located at the upper lip of the active site cleft and shows almost full identity with cLz [19].

The aim of this study was to find an analog of peptide 107–115 hLz with enhanced antibacterial activity by sequentially replacing the two Ala residues of the sequence (108 and 111) with specific amino acids that could contribute to the biological activity of the novel peptide.

Peptide 107–115 hLz and 13 analogs, punctually substituted in position 108, were analyzed for antibacterial activity against *Escherichia coli* ATCC 25 922 and *Staphylococcus aureus* ATCC 29 213 in a first round of screening. An analog with improved antibacterial activity was selected for the second round in order to find a substitution for position 111 that enhances its antimicrobial activity.

Materials and Methods

Materials

Rink-amide resin, Fmoc amino acids, coupling reagents and solvents were from Applied Biosystems (Foster City, CA, USA). Solvents for peptide purification were of HPLC grade and were purchased from Tedia Company Inc. (Fairfield, CA, USA). Müeller–Hinton (MH) broth and agar were from Britania (Buenos Aires, Argentina).

Microorganisms

Bacterial strains *E. coli* ATCC 25 922 and *S. aureus* ATCC 29 213 were from the American Type Culture Collection (Manassas, VA, USA).

Peptide Synthesis

Peptides were synthesized in an ABI 433A Synthesizer (Applied Biosystems) using N^{α}-Fmoc protection following the FastMoc 0.10-mmol protocol. Peptides were deprotected and cleaved from the resin using TFA/H_2O/TIS (95 : 2.5 : 2.5) solution for 3 h at room temperature.

Peptide Purification

Peptides were purified by RP-HPLC on an Ultrasphere ODS column (Beckman Instruments, Palo Alto, CA, USA) using a linear gradient 10–55% acetonitrile in water containing 0.05% TFA.

Peptide Identification

Peptides were identified by ESI-MS in a LCQ-Duo (ion trap) mass spectrometer (Thermo Fisher, San José, CA, USA). Samples were introduced from a Surveyor pump (Thermo Fisher) in a 40 µl/min solvent flow. Peptide analysis was performed by full Scan 200–2000 amu.

Antibacterial Assays

Both bacterial strains were grown at 37 °C in MH broth until logarithmic phase was reached (1×10^8 CFU/ml). A final bacterial concentration of $1–5 \times 10^5$ CFU/ml was used in all assays.

The inhibition growth assay was performed in test tubes. Briefly, bacterial suspension in 2× concentrated MH broth was added to the peptide solutions, at concentrations that varied from 2.5 to 0.1 mg/ml in serial two-fold dilutions, and these were then incubated at 37 °C for 21 h. Bacterial growth was determined by measuring the optical density at 620 nm in an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Positive growth control was performed by replacing the peptide solution with water and was considered as the 100% growth. Negative growth control was performed in the absence of bacterial suspension.

The microdilution assay was performed in 96-well flat-bottomed micro plates for those peptides with the highest inhibitory activity in order to determine the minimum inhibitory concentration (MIC). MICs were determined as the lowest concentration of peptide that inhibits bacterial growth when incubated in MH broth at 37 °C for 21 h. Peptide concentrations varied from 1.25 mg/ml to 1 µg/ml in serial two-fold dilutions. Bacterial growth was determined by measuring the optical density at 620 nm. All assays were performed in triplicate in two independent experiments.

Instability Index Determination

The instability index (II) was determined by the ProtParam tool of the Expasy server (http://www.expasy.org/tools/protparam.html). This index provides an estimation of the stability of a protein or a peptide in a test tube [26,27]. It was revealed by a statistical analysis of unstable and stable proteins that the occurrence of certain dipeptides was significantly different in the unstable proteins compared with those in the stable ones. Based on the impact of these dipeptides on the protein stability (unstable proteins over the stable ones), a weight value of instability is assigned to each of the dipeptides. For a given protein, the summation of these weight values normalized to the length of its sequence helps to distinguish between potential unstable and stable proteins. This tool was used with the aim to simplify the screening; the peptides with significant II, over 40, were discarded for further analysis.

Hemolytic Assays

The hemolytic activity of peptides was determined on human red blood cells (RBCs). The blood was collected in heparin and RBCs were washed three times with phoshate-buffered saline (PBS) (35 mM phosphate buffer, 0.15 M NaCl, pH 7.0) and resuspended

in PBS. Briefly, 500 µl of a 0.5% RBC suspension was added to an equal volume of each peptide solution at concentrations of 15, 50 and 125 µg/ml. The mixtures were incubated for 1 h at 37 °C and centrifuged at 2800 rpm for 5 min. The supernatant $A_{414\ nm}$ value was then measured. PBS and 0.1% Triton X-100 were used as negative and positive hemolysis controls, respectively.

The hemolysis percentage was calculated as follows:

$$[(A_{peptide} - A_{PBS})/(A_{Triton} - A_{PBS})] \times 100 \quad (1)$$

Results and Discussion

Ala 108

Design

Ala was substituted by Arg, Asn, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val. These amino acids were chosen on the basis of their potential capacity to enhance the antimicrobial activity of the peptide. All positively charged and hydrophobic amino acids were included because of their contribution to the amphiphilic features of the resultant peptides; features that have been proposed to be responsible for peptide interaction with microbial membranes [25]. On the basis of results reported by Blondelle [7] from combinatorial libraries, where it was noteworthy that none of the peptide mixtures with antimicrobial activity had aspartic, β-aspartic, glutamic or γ-glutamic acid in their sequences, we excluded negatively charged amino acids in order to prevent positive-charge neutralization. Cys was excluded in order to prevent dimer formation. Similar amino acids were represented by one member of the group. The selection criteria used were based on the antimicrobial activity and the predicted stability of each peptide.

Peptide Identification

Table 1 shows the comparison of the theoretical and experimental molecular weights of the peptides synthesized. Theoretical molecular weights were calculated with the ProtParam tool of the expasy server (http://www.expasy.org/tools/protparam.html).

Table 1. Theoretical and experimental molecular weight (MW) of peptide 107–115 hLz (A) and its analogs

Sequence	Theoretical MW	Experimental MW
RAWVAWRNR (A)	1214.4	1214.4
RRWVAWRNR (R)	1299.5	1299.5
RNWVAWRNR (N)	1257.4	1257.4
RHWVAWRNR (H)	1280.4	1280.5
RIWVAWRNR (I)	1256.4	1256.5
RKWVAWRNR (K)	1271.4	1271.5
RMWVAWRNR (M)	1274.5	1274.3
RFWVAWRNR (F)	1290.5	1290.6
RPWVAWRNR (P)	1240.4	1240.6
RSWVAWRNR (S)	1230.4	1230.5
RTWVAWRNR (T)	1244.4	1244.4
RWWVAWRNR (W)	1329.5	1329.3
RYWVAWRNR (Y)	1306.5	1306.5
RVWVAWRNR (V)	1242.4	1242.6
RKWVKWRNR (K′)	1328.5	1328.2
RKWVWWRNR (W′)	1386.6	1386.6
RKWVYWRNR (Y′)	1363.5	1362.8

Screening

Figure 2 shows the results of the first inhibition growth screening for *E. coli* ATCC 25 922 and *S. aureus* ATCC 29 213, performed with 0.1 mg/ml peptide solutions. This concentration allowed us to discriminate between peptides with high and low inhibitory activity.

From this screening, we selected peptides R, K, W and Y and determined their MICs on the basis of their high inhibitory power. Results are compared in Table 2, which also shows the II as well as the peptide stability classification.

The results of the first screening showed that A 108 substitution by either a positively charged or an aromatic amino acid would improve the antibacterial activity of the resultant peptides, as judged by the significant decrease in their MIC values. This trend

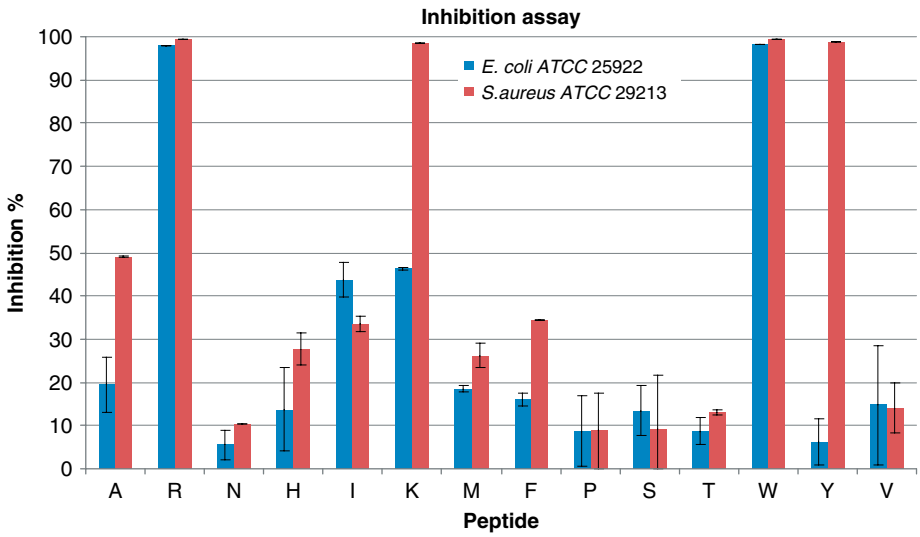

Figure 2. Inhibition assay at 0.1 mg/ml of peptide 107–115 hLz and its 13 analogs A 108 substituted, for *E. coli* ATCC 25 922 and *S. aureus* ATCC 29 213. Each group of bars is segregated by the A 108 substitution. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

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Figure 3. Sequence of peptide K.

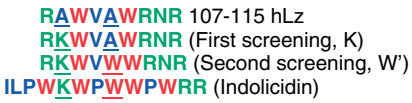

Figure 4. Sequence homology between 107–115 hLz, K and W′ analogs, and indolicidin. The Protein Colourer tool from the expasy server was used (http://www.ebi.ac.uk/cgi-bin/proteincol/ProteinColourer.pl). Color code: Blue, aliphatic residues and proline; Red, aromatic residues; and Green, polar and charged residues.

Table 2. MIC values, instability index (II) and peptide classification of peptide 107–115 hLz (A) and selected analogs

Sequence 108 substitution	MIC (μM), *E. coli* ATCC 25 922	MIC (μM), *S. aureus* ATCC 29 213	II	Peptide classification[a]
R**A**WVAWRNR	412	206	13.17	Stable
R**R**WVAWRNR	96	96	140.46	Unstable
R**K**WVAWRNR	98	49	13.17	Stable
R**W**WVAWRNR	47	94	76.81	Unstable
R**Y**WVAWRNR	191	24	−6.73	Stable

[a] Peptide classification: II below 40 predicts stable peptides and II over 40 predicts unstable peptides [27].

was observed for both microorganisms analyzed (Gram-positive and Gram-negative) and in both cases MIC values were reduced between two- and eight-fold. As expected, in agreement with results reported previously [18,19], Gram-positive bacteria were more sensitive to AMPs than Gram-negative bacteria. This observation is attributed to the fact that AMPs interact directly with the plasma membrane. In Gram-negative bacteria, most peptides must cross the outer membrane to reach the plasma membrane, thus making these microorganisms less susceptible to AMPs [20,21].

Primary structural analysis of 107–115 hLz and analogs R, K, W and Y predicted that two of them, R and W, may result in unstable peptides (Table 2). Consequently, we substituted A 108 for K, thus K became the lead peptide for the second screening (Figure 3).

Ala 111

Design

The amino acids used for the Ala 111 substitution were chosen taking into account the results of the first screening and the predicted stability of the resulting peptides. Thus, only R, K, W and Y were chosen as candidates for the second screening. The resultant peptides were named R′, K′, W′ and Y′, with K being the reference peptide (K 108–A 111).

Screening

First of all, the IIs of the selected peptide (K) and analogs were determined, and results are shown in Table 3.

Peptide R′ was discarded for the second screening because of its predicted instability. Peptides K′, W′ and Y′ were synthesized and their MICs determined and compared with those of the lead peptide (A) and the first screening analog (K). MIC values are compared in Table 4.

Table 3. II of peptide K and its potential analogs

Sequence 111 substitution	II	Peptide classification
R**A**WVAWRNR		
A (107–115 hLz)	13.17	Stable
RKWV**A**WRNR		
K	13.17	Stable
RKWV**R**WRNR		
R′	76.81	Unstable
RKWV**K**WRNR		
K′	9.97	Stable
RKWV**W**WRNR		
W′	13.17	Stable
RKWV**Y**WRNR		
Y′	−6.73	Stable

Table 4. Second screening: MIC values of peptide 107–115 hLz and selected analogs

Peptide (A 111 substitution)	*E. coli* ATCC 25922		*S. aureus* ATCC 29213	
	μg/ml	μM	μg/ml	μM
A (107–115 hLz)	500	412	250	206
K	125	98	62.5	49
K′	125	94	31.25	24
W′	125	90	15.6	11
Y′	125	92	31.25	23

Table 5. Hemolysis percentage of peptide 107–115 hLz, K and W′ analogs at 15, 50 and 125 μg/ml against human RBCs, respectively

Peptide	% Hemolysis (μg/ml)		
	15	50	125
107–115 hLz	1.4	2.0	4.0
K	2.1	3.2	3.0
W′	2.6	5.4	30.7

Given the four-fold decrease in MIC against *S. aureus* ATCC 29213 resulting from the A 111 substitution, peptide W′ was chosen for further experiments. None of the A 111 substitutions improved the antibacterial activity of K against *E. coli* ATCC 25922.

Hemolysis Assays

These analyses were carried out for peptide 107–115 hLz and the resultant analogs from the two screenings (K and W′). Results are compared in Table 5.

Hemolytic activity of the selected analog (W′) was significant at ten-fold its anti-staphylococcal MIC. This result encourages further characterization of W′ potentiality as an active pharmaceutical ingredient against staphylococcal strains.

Primary structure analysis of the lead peptide (107–115 hLz) and its two analogs derived from the first (K) and second (W′) screening

indicates that an additional positive charge at the *N*-terminus and an aromatic residue at the center of the molecule will contribute to enhance the antibacterial activity of the resultant peptide.

The new analog (W′) displays an additional positive charge near the *N*-terminus (108) and an extra Trp residue at the center of the molecule (111), suggesting that this profile improves the interaction of the novel peptide with the bacterial membrane, thus enhancing its anti-staphylococcal activity. Our results are consistent with those of Pellegrini *et al*. [18], who studied particular amino acid constellations as prerequisites for bactericidal activity in peptides derived from cLz enzymatic digestion. Their focus was pointed on the *C*-terminal side of fragment 98–112, which displays antimicrobial action (fragment 106–112). They found that replacement of Asn 106 or Ala 107 by Arg, at the *N*-terminus of the peptide strongly increased its bactericidal activity. They also observed that replacement of Trp 108 or Trp 111 by Tyr significantly reduced or abolished the antimicrobial activity, respectively. These findings highlight the relevance of Trp at the center of the molecule for the antimicrobial activity of the peptide. Furthermore, these results are in agreement with those of Ibrahim *et al*. [19], who analyzed the participation of both Trp residues (108 and 111, 109 and 112, in cLz and hLz, respectively) in the membrane interaction. They demonstrated that active peptides derived from cLz and hLz interact with liposomal membranes, prepared from phospholipids of *E. coli,* and that the Trp residues were located at the membrane interface. The additional Trp residue of the new analog (W′) at the center of the molecule may contribute to the localization of the peptide at the bacterial membrane interface, thus stabilizing the interaction that leads to pore formation.

Moreover, we found that both substitutions introduced in the lead peptide increased its sequence homology with indolicidin, a natural AMP isolated from the cytoplasmic granules of bovine neutrophils. Indolicidin is a 13-residue peptide with the highest content of Trp among naturally occurring peptides [28]. The sequence homology with indolicidin and the Trp content of our novel peptide, W′, increased from 33 to 56% and 22 to 33%, respectively. Staubitz *et al*. [29] reported the relevance of positions 5 and 12 in the antimicrobial activity of indolicidin by single Ala replacement. (Ala^5)- indolicidin and (Ala^{12})-indolicidin were the analogs with the lowest activity. Interestingly, these positions were shared between W′ and indolicidin, position 5 (Lys) corresponding to the substitution found in the first screening.

Figure 4 shows the sequence homology comparison between 107–115 hLz, selected analogs from the two screenings, and indolicidin.

Concluding Remarks

This alternative *alanine scan* has been shown to be a useful tool to find a novel peptide with enhanced antimicrobial activity from the lead peptide 107–115 hLz. With a simple two-round screening, an analog with 4- and 20-fold increased antimicrobial activity against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213, respectively, was found. This strategy could be extended to other Ala-containing peptides and may provide an additional tool for the discovery of compounds with enhanced biological activity from lead peptides.

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References

1 Hancock REW, Diamond G. The role of cationic antimicrobial peptides in innate host defenses. *Trends Microbiol.* 2000; **8**: 402–410.
2 Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol.* 2003; **3**: 710–720.
3 Rivas-Santiago B, Serrano CJ, Enciso-Moreno JA. Susceptibility to infectious diseases based on antimicrobial peptide production. *Infect. Immun.* 2009; **77**: 4690–4695.
4 Menéndez A, Ferreira RBR, Brett FB. Defensins keep the peace too. *Nat. Immunol.* 2010; **11**: 49–50.
5 Bruckdorfer T, Marder O, Albericio F. From production of peptides in milligram amounts for research to multi-tons quantities for drugs of the future. *Curr. Pharm. Biotechnol.* 2004; **5**: 29–43.
6 Zompra AZ, Galanis AS, Werbitzky O, Albericio F. Manufacturing peptides as active pharmaceutical ingredients (API). *Future Med. Chem.* 2009; **1**: 361–377.
7 Blondelle SE, Takahashi E, Weber PA, Houghten RA. Identification of antimicrobial peptides by using combinatorial libraries made up of unnatural amino acids. *Antimicrob. Agents Chemother.* 1994; **38**: 2280–2286.
8 Blondelle SE, Pérez-Payá E, Houghten RA. Synthetic combinatorial libraries: novel discovery strategy for identification of antimicrobial agents. *Antimicrob. Agents Chemother.* 1996; **40**: 1067–1071.
9 Pérez-Payá E, Houghten RA, Blondelle SE. Determination of the secondary structure of selected melitin analogues with different hemolytic activities. *Biochem. J.* 1994; **299**: 587–591.
10 Cunningham BC, Wells JA. High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science* 1989; **244**: 1081–1085.
11 Beck-Sickinger AG, Wieland HA, Wittneben H, Willim K-D, Rudolf K, Jung G. Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur. J. Biochem.* 1994; **225**: 947–958.
12 Galoppini C, Patacchini R, Meini S, Vigano S, Tancredi M, Quartara L, Triolo A, Maggi CA, Rovero P. A structure-activity study on the bradykinin B1 antagonist desArg10-HOE 140: the alanine scan. *Lett. Pept. Sci.* 1999; **6**: 123–127.
13 Nicole P, Lins L, Rouyer-Fessard C, Drouot C, Fulcrand P, Thomas A, Couvineau A, Martínez J, Brasseur R, Laburthe M. Identification of key residues for interaction of vasoactive intestinal peptide with human VPAC1 and VPAC2 receptors and development of a highly selective VPAC1 receptor agonist. Alanine scanning and molecular modeling of the peptide. *J. Biol. Chem.* 2000; **275**: 24003–24012.
14 Quartara L, Ricci R, Meini S, Patacchini R, Giolitti A, Amadesi S, Rizzi C, Rizzi A, Varani K, Borea PA, Maggi CA, Regoli D. Ala scan analogues of HOE 140. Synthesis and biological activities. *Eur. J. Med. Chem.* 2000; **35**: 1001–1010.
15 Muñoz A, López-García B, Pérez-Payá E, Marcos JF. Antimicrobial properties of derivatives of the cationic tryptophan-rich hexapeptide PAF26. *Biochem. Biophys. Res. Commun.* 2007; **354**: 172–177.
16 Nam J, Shin D, Rew Y, Boger DL. Alanine scan of [L-Dap2]ramoplanin A2 aglycon: assessment of the importance of each residue. *J. Am. Chem. Soc.* 2007; **129**: 8747–8755.
17 Haversen L, Kondori N, Baltzer L, Hanson LA, Dolphin GT, Duner K, Mattsby-Baltzer I. Structure-microbicidal activity relationship of synthetic fragments derived from the antibacterial alpha-helix of human lactoferrin. *Antimicrob. Agents Chemother.* 2010; **54**: 418–425.
18 Pellegrini A, Thomas U, Bramaz N, Klauser S, Hunziker P, von Fellenberg R. Identification and isolation of a bactericidal domain in chicken egg white lysozyme. *J. Appl. Microbiol.* 1997; **82**: 372–378.
19 Ibrahim HR, Thomas U, Pellegrini A. A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. *J. Biol. Chem.* 2001; **276**: 43767–43774.

20 Mine Y, Ma F, Lauriau S. Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. *J. Agric. Food Chem.* 2004; **52**: 1088–1094.
21 Papo N, Shai Y. Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes? *Peptides* 2003; **24**: 1693–1703.
22 Weinberg A, Krisanaprakornkit S, Dale BA. Epithelial antimicrobial peptides: review and significance for oral applications. *Crit. Rev. Oral Biol. Med.* 1998; **9**: 399–414.
23 Hancock REW, Lehrer R. Cationic peptides: a new source of antibiotics. *Trends Biotechnol.* 1998; **16**: 82–88.
24 Jenssen H, Hamill P, Hancock REW. Peptide antimicrobial agents. *Clin. Microbiol. Rev.* 2006; **19**: 491–511.
25 Papagianni M. Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. *Biotechnol. Adv.* 2003; **21**: 465–499.
26 Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A. Protein identification and analysis tools on the ExPASy server. In *The Proteomics Protocols Handbook*, Walker JM (eds). Humana Press: Totowa, New Jersey, 2005; 571–607.
27 Guruprasad K, Reddy BVB, Pandit MW. Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence. *Protein Eng.* 1990; **4**: 155–161.
28 Ryge TS, Doisy X, Ifrah D, Olsen JE, Hansen PR. New indolicidin analogues with potent antimicrobial activity. *J. Pept. Res.* 2004; **64**: 171–185.
29 Staubitz P, Peschel A, Nieuwenhuizen WF, Otto M, Gotz F, Jung G, Jack RW. Structure-function relationships in the tryptophan-rich, antimicrobial peptide indolicidin. *J. Pept. Sci.* 2001; **7**: 552–564.

