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Bulky Aromatic Amino Acids Increase the Antibacterial Activity of 15-Residue Bovine Lactoferricin Derivatives

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Abstract: A model peptide, FKCRRWQWRMKKLGA, residues 17–31 of bovine lactoferricin, has been subjected to structure–antibacterial activity relationship studies. The two Trp residues are very important for antibacterial activity, and analogue studies have demonstrated the significance of the size, shape and aromatic character of the side chains. In the current study we have replaced Trp residues in the model peptide with bulky aromatic amino acids to elucidate further the importance of size and shape. The counterproductive Cys residue in position 3 was also replaced by these aromatic amino acids. The largest aromatic amino acids employed resulted in the most active peptides. The peptides containing these hydrophobic residues were generally more active against *Staphylococcus aureus* than against *Escherichia coli*, indicating that the bacterial specificity as well as the antibacterial efficiency can be altered by employing large hydrophobic aromatic amino acid residues. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antibacterial peptide; unnatural aromatic amino acid; bovine lactoferricin; tryptophan.

INTRODUCTION

Bovine lactoferricin is a 25-residue antibacterial peptide isolated from gastric cleavage of bovine lactoferrin [1]. We have used the membrane active peptide FKCRRWQWRMKKLGA (LFB), residues 17–31 of the mature bovine lactoferrin protein, as a model peptide for structure–antibacterial activity relationship studies of highly cationic peptides [2]. An Ala-scan of LFB has revealed that the Trp residues in positions 6 and 8 are essential for antibacterial activity [3]. The importance of Trp for the antibacterial activity is further substantiated by the presence of both residues in the antibacterial core of bovine lactoferricin, which has been determined by Tomita *et al.* [1] to be RRWQWR. The amidated derivative of this hexapeptide adopts a well-defined amphipathic structure on interaction with artificial membranes, and the two Trp residues are important in the interaction with sodium dodecyl sulphate (SDS) micelles [4].

It is known that membrane proteins have an increased content of Trp compared to soluble proteins [5]. In membrane proteins, Trp residues play a special role by functioning as an anchor, and by playing an important role in the folding of the proteins [5,6]. Trp residues are also proposed to act as needles for pulling transmembrane helices across membranes [5,7], as has been demonstrated by the ability of *N*-acetyl-tryptophan-octylamide and *N*-acetyl-tryptophan-octylester to traverse phosphatidylcholine and phosphatidylglycerol bilayers [7]. In a study of vesicle formation by *N*-alkyl and 3-alkyl indoles, Abel *et al.* [8] showed that the indole moiety is capable of serving as a head-group in the vesicles, and it is suggested that Trp may function as an organizing

Abbreviations: ATCC, American Type Culture Collection; Ath, β-(anthracen-9-yl)alanine; Bal, β-(benzothien-3-yl)alanine; Bip, β-(4,4′-biphenyl)alanine; Dip, β-diphenylalanine; LFB, lactoferricin B residues 17–31; MIC, minimal inhibitory concentration; 1-Nal, β-(naphth-1-yl)alanine; PAC-PEG-PS, 4-hydroxy methylphenoxy acetic acid–polytehylene glycol–polystyrene resin; Tbt, β-(2,5,7-tri-*tert*-butyl-indol-3-yl)alanine.

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element in both membrane-bound peptide and protein structures.

The importance of Trp in short derivatives of lactoferricin indicates that the anchoring effect might be of importance for the antibacterial activity [3]. The Trp residues are also important for antibacterial activity in the cathelicidin-derived tritrpticin (tritrypticin) [9,10], which has the minimal functional domains RRFPWWW and WWWPFRR [9]. The membrane affinity of tritrpticin has been demonstrated for the truncated sequence FPWWWPFL, which adopts an amphipathic two-turn structure when bound to micelles, with the Trp residues embedded in the SDS micelles [11]. Recently, the physical basis for the interfacial preference of Trp in bilayers has been explained by the aromaticity of the amino acid, rather than its ability to participate in hydrogen bonding or the amphipathicity of the indole side chain [12]. We have shown that the antibacterial activity of LFB derivatives is not dependent on the presence of Trp residues per se [13], but rather that the size of the aromatic residues is more important than the amphipathicity or hydrogen bonding ability [13]. Furthermore, we demonstrated that the shape of the aromatic side chain is important for the antibacterial activity. These findings have led us to investigate more closely how the biological activity of LFB peptides can be affected by employing different aromatic amino acids. To address this question, a series of peptides where the two Trp residues in positions 6 and 8 were replaced by large aromatic amino acids were prepared and tested for antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. To further elucidate the effects of different aromatic amino acids on the antibacterial activity of LFB peptides, the counterproductive Cys residue normally present in position 3 was also replaced [3]. These replacements allowed us to explore the structure–antibacterial activity relationships of LFB derivatives containing up to four aromatic amino acids. Structures and abbreviations of the aromatic amino acids used in this work are shown in Figure 1.

Materials and methods

Peptide synthesis

Peptides were synthesized on a Millipore 9050 Plus PepSyntheziser (Milligen, Milford, MA, USA) using Fmoc chemistry with DMF as solvent. The *C*-terminal Ala residue was preattached to a PAC-PEG-PS resin, which ensured a free *C*-terminal carboxylic acid after final acid treatment (see below). Coupling reactions with Fmoc amino acids activated as Pfp-esters were catalyzed by HOBt (1.3 equivalents). Unactivated Fmoc amino acids were activated *in situ* using HATU or HBTU, and coupling reactions were base-catalyzed with DIPEA (2.4 equivalents). A fourfold excess of Fmoc amino acid was employed during every coupling step. For unnatural amino acid residues the coupling reactions were performed

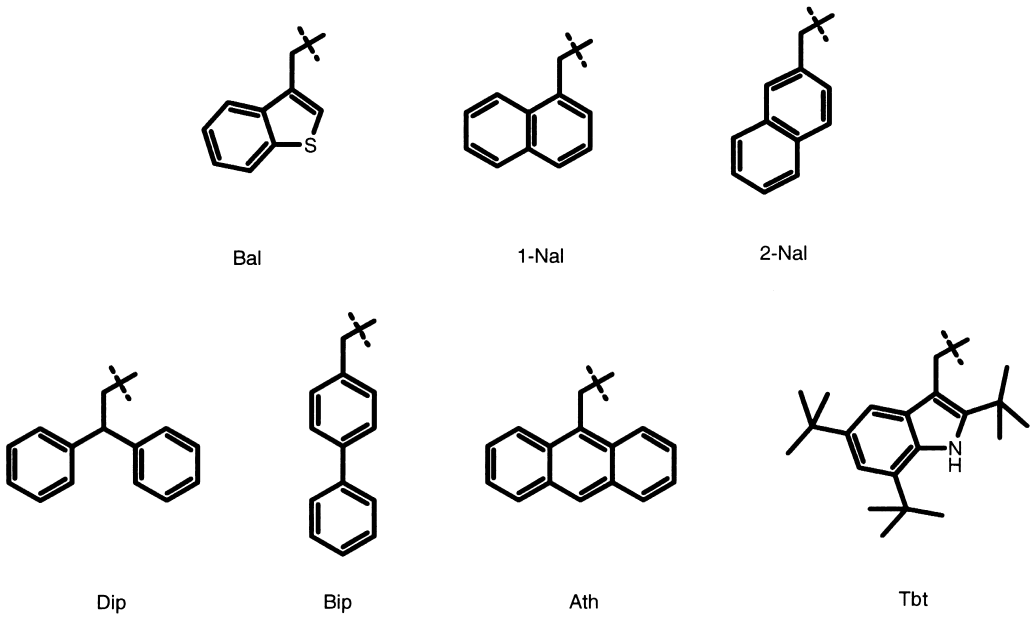

Figure 1 Structure of side chains and abbreviations for the corresponding aromatic amino acid residues employed.

using a twofold excess of Fmoc amino acid, and prolonged coupling time. All amino acid residues with reactive side chains, except Cys, were protected with acid-labile protecting groups and deprotected during cleavage of the peptide from the solid support upon treatment with Reagent K [14] (82.5% TFA, 5% thioanisole, 2.5% ethanedithiol and 5% water) for no more than 3 h. Cys was irreversibly protected in all peptides with an Acm group. Deprotection and cleavage reactions were performed in the dark. After cleavage, the solid support was removed by filtration, and the filtrate was concentrated under reduced pressure. The crude peptides were precipitated from diethyl ether, washed several times with diethyl ether and dried under reduced pressure. The peptides were purified using preparative reverse-phase high performance liquid chromatography (RP-HPLC) on a C_{18} column (Delta-Pak™C18, 100 Å, 15 μm, 25 × 100 mm, Waters Corp., Milford, MA, USA) with a mixture of water and acetonitrile (both containing 0.1% TFA) as mobile phase and UV-detection at 254 nm. For LFB Dip6,8 UV-detection at 214 nm was used. The homogeneity of the purified peptides was analyzed on an analytical C_{18} HPLC column (Delta-Pak™C18, 100 Å, 5 μm, 3.9 × 150 mm; Waters Corp., Milford, MA, USA). The purity of all peptides was found to be above 95%. The integrity of the peptides was checked by positive ion electrospray ionization mass spectrometry on a VG Quattro quadrupole mass spectrometer (VG Instruments Inc., Altringham, UK). Peptides were lyophilized before biological testing.

Chemicals

All natural Fmoc amino acids, Fmoc-L-Ala-PEG-PS resin, HOBt, HATU, HBTU, DMF, piperidine, DIPEA and TFA were purchased from PerSeptive Biosystems (Hertford, UK). Phenol, ethanedithiol and *tert*-butanol were purchased from Fluka (Buchs, Switzerland). Thioanisole was purchased from Sigma (St. Louis, MO, USA). Fmoc-β-(benzothien-3-yl)alanine and Fmoc-β-(naphth-1-yl)alanine were purchased from Bachem (Bubendorf, Switzerland). Fmoc-β-diphenylalanine and Fmoc-β-(4,4′-biphenyl)alanine were purchased from Synthetech (Albany, USA). Fmoc-β-(anthracen-9-yl)alanine was purchased from Peninsula laboratories (San Carlos, USA). β-(2,5,7-tri-*tert*-butyl-indol-3-yl)alanine (Tbt) was prepared by treating Trp with an excess of *tert*-butanol in TFA at room temperature as described by Löw *et al.* [15]. The Fmoc-protecting group was introduced by a standard procedure using Fmoc-OSu [16]. Fmoc-Tbt-OH was isolated as a white solid line ($R_f = 0.16$, 5% MeOH-CH_2Cl_2): m.p. 117–123°C (dec); $[\alpha]_D^{23} + 6.2$ (c 0.50, methanol); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.73 (s, 1H), 7.99 (d, 2H, $J = 7.7$ Hz), 7.80 (d, 1H, $J = 8.8$ Hz), 7.69 (d, 1H, $J = 7.7$ Hz), 7.64 (d, 1H, $J = 7.3$ Hz), 7.48 (s, 1H), 7.40 (q, 2H, $J = 7.7$, 7.3 Hz), 7.31 (t, 1H, $J = 7.3$ Hz), 7.23 (t, 1H, $J = 7.7$ Hz), 7.00 (s, 1H), 4.25–4.13 (m, 4H), 3.45–3.37 (m, 1H), 3.14 (q, 1H, $J = 8.4$, 6.2 Hz), 1.47 (s, 9H), 1.45 (s, 9H), 1.31 (s, 9H); ^{13}C-NMR (100 MHz, DMSO-d_6): 174.3, 156.6, 144.4, 144.3, 142.9, 141.2, 141.0, 132.1, 130.8, 130.3, 128.2, 128.1, 127.6, 125.9, 120.6, 115.8, 113.1, 106.2, 66.3, 56.7, 47.1, 34.9, 34.8, 33.8, 32.5, 31.3, 30.8, 27.5; HRMS calculated for $C_{38}H_{46}N_2O_4$ 594.3458, found 594.3481.

Antibacterial activity

The bacterial strains, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, were grown in 2% Bacto Peptone water (Difco 1807-17-4) until exponential growth. A standard microdilution technique with an inoculum of 2×10^6 colony-forming units per ml was used. The minimal inhibitory concentration (MIC) of the peptides was determined in 2% Bacto Peptone water after incubation overnight at 37°C. The concentration range used for the modified LFB peptides was between 300 and 1.0 μg/mL. All peptides were tested in duplicate. For the Ath and the Tbt peptides, the MIC values are given as median values from three to six measurements.

Calculations

Calculations of molecular volumes for the side chains of aromatic amino acids were performed using MacroModel Version 6.5 [17] (Table 2). To exclude conformational variations, the amino acid moieties were replaced by hydrogen atoms, and thus calculations performed for methyl-substituted aryls. The lengths of the side chains were measured as the longest distance between the methyl group carbon atom (β-carbon atom in amino acids) and the most distant carbon atom. The width was measured as the longest distance between two carbon atoms in the side chain. For width measurements, the β-carbon atom was excluded.

RESULTS

The antibacterial activity of the LFB derivatives is compiled in Table 1. The introduction of large aromatic amino acids as replacements for Trp gave in

Table 1 Antibacterial Activity of LFB Peptides Containing Large Unnatural Aromatic Amino Acids in Positions 6 and 8

Peptide name[a]	Molecular mass[b]		MIC[c] *E. coli*		MIC[c] *S. aureus*	
LFB	2064.8	(2064.5)	50	(24)	100	(48)
LFB Dip6	2102.6	(2101.8)	7.5	(3.6)	35	(17)
LFB Dip8	2102.6	(2102.0)	7.5	(4.8)	35	(17)
LFB Dip6,8	2139.7	(2138.8)	7.5	(3.5)	15	(7.0)
LFB Bip6	2102.6	(2102.0)	25	(12)	15	(7.1)
LFB Bip8	2102.6	(2102.0)	15	(7.1)	15	(7.1)
LFB Bip6,8	2139.7	(2138.8)	12.5	(5.8)	3	(1.4)
LFB Ath6	2126.7	(2125.6)	12.5	(5.9)	35	(16)
LFB Ath8	2126.7	(2125.8)	10	(4.7)	15	(7.1)
LFB Ath6,8	2187.8	(2187.2)	10	(4.6)	7.5	(3.4)
LFB Tbt6	2233.9	(2232.6)	12.5	(5.6)	10	(4.5)
LFB Tbt8	2233.9	(2232.9)	12.5	(5.6)	5	(2.2)
LFB Tbt6,8	2402.2	(2401.6)	20	(8.3)	7.5	(3.1)

[a] Sequence: FKCRRW^{6}QW^{8}RMKKLGA.
[b] Molecular mass calculated and (observed) including Acm-protected cysteine.
[c] Minimal inhibitory concentration in μg/ml and (μM).

Table 2 Structural Properties of Aromatic Amino Acids in this Study

Amino acid	Volume (Å^3)	Area (Å^2)	Length[a] (Å)	Width[b] (Å)
Phe	100.0	274.6	4.341	2.429
Trp	129.4	318.9	5.415	4.659
Bal	135.0	324.4	5.350	4.796
1-Nal	142.1	333.8	5.185	4.976
2-Nal	142.6	340.0	6.476	4.973
Bip	172.3	391.8	8.695	2.429
Dip	172.4	387.9	4.343	7.119
Ath	185.4	391.3	5.141	7.274
Tbt	325.1	580.5	7.600	8.924

[a] Measured as the maximum distance between the C-β and a carbon atom in the side chain.
[b] Measured as the maximum distance between two carbon atoms in the side chain.

all cases an increase in the antibacterial activity. Molecular mechanics calculations of volume and shape of the residues are compiled in Table 2. There is a clear correlation between the antibacterial activity of the peptides and the molecular volumes of the residues employed as replacements for Trp. The larger residues impart higher antibacterial activity of the LFB derivatives. The molecular calculations also revealed that aromatic residues that are long or broad are preferred for the construction of highly active LFB derivatives.

The difference in shape also seems to influence the bacterial selectivity. In the case of *E. coli*, the most active peptides were obtained when Trp was replaced by Ath, Dip or Tbt. Peptides containing Bip were less active against *E. coli* than peptides containing the isomeric amino acid Dip. Of the peptides where one Trp was replaced, the most active peptides against *S. aureus* were LFB Tbt6 and LFB Tbt8. The most active peptide in this study was LFB Bip6,8, which showed a MIC value of 3 μg/ml against *S. aureus*. The peptides containing Bip were more active against *S. aureus* than the peptides containing Dip. The Ath peptides showed antibacterial activity that was inbetween that of the peptides containing Bip or Dip. Of the Ath peptides, LFB Ath6,8 was the most active derivative, showing a slightly higher antibacterial activity against *S. aureus* than *E. coli*.

For the replacement of Cys in position 3, aromatic amino acids from previous studies were also included. Results for the Cys replacements are compiled in Table 3.

Table 3 Antibacterial Activity of LFB Derivatives with Aromatic Residues in Position 3

Peptide name[a]	Molecular mass[b]		MIC[c] *E. coli*		MIC[c] *S. aureus*	
LFB	2064.8	(2064.5)	50	(24)	100	(48)
LFB Tbt3	2245.9	(2244.8)	20	(8.9)	5	(2.2)
LFB Bip3	2114.6	(2113.6)	12.5	(5.9)	5	(2.4)
LFB Dip3	2114.6	(2114.0)	7.5	(3.5)	5	(2.4)
LFB Ath3	2138.6	(2138.2)	12.5	(5.8)	7.5	(3.5)
LFB 1-Nal3	2088.6	(2087.8)	20	(9.6)	10	(4.8)
LFB Bal3	2094.6	(2093.5)	20	(9.5)	20	(9.5)
LFB Phe3	2038.5	(2037.6)	15	(7.4)	35	(17)

[a] Sequence: FKC[3]RRWQWRMKKLGA.
[b] Molecular mass calculated and (observed) including Acm-protected cysteine.
[c] Minimal inhibitory concentration in μg/ml and (μM).

The replacement of Cys with an aromatic amino acid gave in all cases more active peptides compared to the unmodified 15-residue peptide. For *E. coli*, there was no correlation between size of the aromatic residue in position 3 and the antibacterial activity. However, the peptides containing the largest aromatic residues in position 3 showed lower MIC values against *S. aureus* than the peptides containing smaller residues.

DISCUSSION

In this study we have investigated the effects of replacing Trp in the antibacterial peptide LFB by larger aromatic amino acids. We have also performed replacements of Cys 3 by natural and unnatural aromatic amino acids in order to elucidate the effects of introducing a fourth aromatic residue into the peptide. The peptides reported in this paper were all more active against the test strains than LFB itself (Tables 1 and 3). The structures of all the aromatic residues employed are shown in Figure 1. The MIC values for the LFB derivatives ranged from 35 to 3 μg/ml against *S. aureus* and from 25 to 7.5 μg/ml against *E. coli*. The increased antibacterial activity of the modified LFB peptides shows that Trp can be favourably replaced by any larger unnatural aromatic amino acid. Despite the relatively small differences in activity between the peptides reported, correlations between the chemical properties of the residues introduced as replacements for Trp and Cys and the antibacterial activity of the LFB derivatives were found.

The calculated molecular volumes of the side chains of the residues shown in Figure 1 are compiled in Table 2. When the biological activity of the LFB derivatives containing unnatural aromatic amino acids is compared with the side chain volume of these residues, it is evident that the size of the side chain is important for the antibacterial activity.

The incorporation of Bip, Dip or Ath as replacements for Trp in LFB gave peptide derivatives with enhanced antibacterial activity. The peptides in the present study are more active than the 1-Nal and 2-Nal LFB derivatives reported previously by us [13]. Bip, Dip and Ath are about 1.3–1.4 times the volume of Trp, whereas the Nal residues are more or less the same size as Trp. The Tbt residue, which gave peptides highly active against both bacterial strains, was found to be 2.5 times the volume of Trp. When the LFB derivatives where Trp residues were replaced by Ala reported by Strøm *et al.* [3] are also considered, the natural and unnatural aromatic amino acids employed as Trp replacements can be ranked by their ability to impart antibacterial activity of LFB derivatives. Clearly, Ala is the poorest replacement for Trp, while Phe is somewhat better. The Trp-sized benzothienylalanine and the two isomeric naphthylalanine residues constitute the middle of the scale, while the residues reported here, which are the largest, also gave the most active peptides. The size of the residues thus seems to be the major determinant for the antibacterial activity of the LFB peptides in which they are incorporated. The impact of side chain size on antibacterial activity is more pronounced against *S. aureus* than for *E. coli*, indicating that the bacterial selectivity can be altered by choosing from the residues given in Figure 1.

Although they have similar molecular volumes, the shapes of Ath, Bip and Dip are quite different. From the geometry optimizations when calculating the molecular volumes of the side chains, the

lengths and widths of the side chains as methyl-substituted aryls were measured (Table 2). From these measurements it can be seen that Bip is elongated, while Dip and Ath are short and broad. The rigid aromatic system of the anthracene moiety gives the Ath amino acid a planar side chain, whereas the two other residues have a more three-dimensional shape. Despite the fact that Ath is larger than Bip and Dip, the incorporation of this residue into LFB did not give peptides of higher activity compared to the Bip and Dip peptides. These results confirm that the shape of the side chain is an additional determinant of peptide activity. Ath does not have the length that seems to be an important feature of Bip, nor does it have the flexibility and three-dimensionality of Dip.

The two isomeric amino acids Bip and Dip gave peptides of different antibacterial selectivity when incorporated as replacements for Trp. The Dip peptides were clearly most active against *E. coli* and the Bip peptides most active against *S. aureus*. Since these residues are of essentially identical size, the topology of the side chain seemed to determine the biological activity of the peptide derivatives in which they are incorporated. Assuming that the biphenyl system adopts a conformation with a 45° angle between the planes of the two aromatic rings, the shape of the side chain of this residue can be described as a flattened barrel. In the diphenyl side chain, the two aromatic rings are more flexible, making this residue broad. The shape of Bip gives this residue a possibility to function as a needle, and hence we expect it to be more effective than Trp in pulling the rest of the peptide into the membrane, causing membrane disruption. The wedge shape of Dip makes it more efficient than Trp in causing a lateral disruption of the membrane.

The amino acid Tbt combines the structural features of Bip and Dip. Tbt is both broad and long, with the *tert*-butyl groups pointing out from the indole nucleus. This makes it capable of giving both a deep and a lateral disruption if inserted into bacterial cell membranes. Tbt is by far the largest residue in this study, and the Tbt peptides prepared were the most active peptides against *S. aureus* in this study. Comparing the effect of the small (Phe), the medium sized (Trp, Bal, 1-Nal and 2-Nal) and that of the large aromatic amino acids (Ath, Bip and Dip) with that of the extremely large Tbt, it was obvious that bulky residues were preferred. However, it seemed that there was an upper limit to the amount of bulk that could be introduced into the LFB derivatives. In the case of *S. aureus*, there was an increase in antibacterial activity when both Trp residues were replaced with Ath, Bip or Dip, compared to the derivatives containing only one unnatural aromatic residue, whereas for *E. coli* this increase was only seen when Bip was employed. When both Trp residues were replaced with Tbt, a slight decrease in the antibacterial activity against both *E. coli* and *S. aureus* was observed. Measurements of haemolytic activity of the four peptides that contained Tbt (for assay, see Strøm *et al.* [3]) revealed that LFB Tbt6 and LFB Tbt8 were non-haemolytic in the concentration range tested, i.e. $EC_{50} > 1000$ μg/ml. However, LFB Tbt3 and LFB Tbt6,8 were slightly haemolytic with EC_{50} values of 500 μg/ml and 230 μg/ml, respectively. Thus, the introduction of too much bulk rendered the peptides less selective against bacterial cell membranes. For magainins it has been shown that the hydrophobicity of the peptides is closely related to their haemolytic activity [18]. For indolicidin, the replacement of all five Trp residues with Phe results in a loss of haemolytic activity, whereas the antibacterial activity is retained [19].

Also the replacement of the counterproductive Cys residue in position 3 with an aromatic amino acid gave more active peptides (Table 3). The replacement of Cys with an aromatic amino acid introduced an extra hydrophobic residue, making the peptides overall more hydrophobic. As discussed by Strøm *et al.* [3], LFB may have more than the necessary amount of cationic residues, and thus the peptide may be deficient with respect to hydrophobic residues. Both for the replacements of Trp and Cys the introduction of hydrophobic residues increased the antibacterial activity, indicating that LFB is in fact deficient in hydrophobic residues. In the case of *E. coli*, the effect of introducing a fourth aromatic amino acid was not especially dependent on the nature of the residue. This was seen when comparing the activities of LFB Phe3 and LFB Tbt3, which showed the same MIC values. Against *S. aureus* the increase in antibacterial activity upon introduction of a fourth aromatic residue correlated well with the size and hydrophobicity of the residue. Thus, Tbt, Bip and Dip gave the most active peptides, whereas the introduction of Phe gave the least active peptide. Hence, the hydrophobicity of LFB peptides is an important structural feature for these peptides to be active against bacteria. The importance of having large aromatic residues, or an increased number of these hydrophobic residues, in LFB peptides seemed to be more pronounced against *S. aureus* than against *E. coli*. This was

observed both for peptides where the Cys in position 3 was replaced with an aromatic amino acid residue, and for the peptides where a Trp residue was replaced.

From the antibacterial activities of the Trp replacement peptides, it can be seen that the replacement of Trp 8 in several cases gave more active peptides than the replacement of Trp 6. This was also the case for the LFB derivatives containing Bal, 1-Nal and 2-Nal, previously reported by us [13]. This difference suggests that the residues have different orientations or are positioned in different environments in the active conformation of the peptide. In a recent study of nisin Z [20], the difference in depth of Trp residues in bilayers was suggested to be caused mainly by their relative position in the peptide. Generally, cationic antibacterial peptides often have random secondary structures in solution, but have the propensity to fold into amphipathic secondary structures upon interaction with bacterial cell membranes. The propensity of LFB to form an α-helical secondary structure in the presence of SDS micelles or in TFE is low [3]. This suggests that the active form of LFB is either a β-strand or that the peptide has some other form of amphipathic secondary structure. In solution the cyclic 25-residue bovine lactoferricin peptide, FKCRRWQWRMKKLGAPSITCVRRAF (LfcinB) was found by Hwang *et al.* [21] to adopt an antiparallel β-sheet-like conformation. This is different from the X-ray structure of the lactoferrin protein [22], where part of the fragment constituting LfcinB has an α-helical conformation. Hwang *et al.* [21] suggest that the high degree of hydrophobic interaction may be the principal driving factor for the folding of the LfcinB peptide. We expect that the replacement of Trp or Cys by a more hydrophobic aromatic residue does not enhance the propensity of LFB to form α-helices, and that the folding into an amphipathic structure dominated by hydrophobic interactions could be more pronounced. If the peptides adopt an amphipathic conformation upon interaction with bacterial cell membranes, it is possible that the distance between the charged Arg residues and the hydrophobic aromatic residues determines the membrane anchoring possibilities of these residues. This may be an explanation to the difference observed for the two Trp positions in the LFB peptides. In a comparison of linear cationic antimicrobial peptides that form α-helix versus β-sheet structures it was found that activity is not dependent on a particular secondary structure [23,24]. However, the ease by which the peptides adopted an amphipathic conformation upon interaction with bacterial cell membranes is more important. The elucidation of the active conformation of LFB derivatives is obviously an important issue that needs further research, and it would no doubt be essential in the determination of the peptide mechanism of action.

In conclusion, introducing unnatural aromatic amino acids that have larger volumes than Trp can increase the antibacterial activity of lactoferricin derivatives. The bacterial selectivity can be altered to a certain degree by the introduction of aromatic residues with different structural features. The observation that LFB peptides containing one Tbt instead of a Trp residue are highly active against bacteria but do not possess haemolytic activity is noteworthy. This study shows that engineering of LFB can possibly provide an antibacterial peptide with properties desired for clinical usage.

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