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The introduction of fluorine atoms or trifluoromethyl groups in short cationic peptides enhances their antimicrobial activity

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Abstract—The effect of introducing fluorine atoms or trifluoromethyl groups in either the peptidic chain or the C-terminal end of cationic pentapeptides is reported. Three series of amide and ester peptides were synthesised and their antimicrobial properties evaluated. An enhanced activity was found in those derivatives whose structure contained fluorine, suggesting an increase in their hydrophobicity.

1. Introduction

The widespread use of antibiotics led to the development of numerous multidrug resistant strains, resulting in an urgent need to develop new effective antimicrobial agents capable of being established as therapies for bacterial infections.[1] These molecules should be as natural as possible with a wide range of action over several pathogens, easy to produce and not prone to inducing resistance. Native peptide molecules known as antimicrobial peptides (AMPs) completely fit this description.[2] This group of molecules, termed as 'natural antibiotics,' are endogenous peptides that are found in a wide range of eukaryotic organisms. They are mobilised shortly after microbial infection to neutralise a broad range of microbes and constitute a primitive immune defence mechanism.[3]

Antimicrobial peptides show great structural diversity, but some features common to them all are their relatively small size (usually less than 50 amino acid residues), their cationic nature due to multiple R and/or K residues and a substantial portion of hydrophobic amino acids (around 50%).[4]

The exact action mechanism is not totally established for all cationic peptides, although there is a consensus in considering that these peptides selectively disrupt cell membranes, possibly by transient pore formation or disruption of lipid packing.[5] The majority of these peptides are unstructured in solution; upon binding to bacterial membranes however, most of them will adopt a well-defined amphipathic structure[6] that plays an important role in the peptide-membrane interaction.[2–4,7] The preferential activity against bacteria over mammalian cells could be owing to the different nature and net charge of the anionic lipids in each case.[7]

Natural AMPs have excellent properties that could make them the best candidates to be used as therapeutic agents, for example, a decreased potential for resistance induction.[8] Despite this however, there are many other obstacles for their utilisation.[9] One main obstacle is their size as they are very large, which results in a high production cost. For this reason, the investigation has been centered on the production of smaller peptides with significant antibiotic activity, which led to the necessity of establishing the minimal structural requirements for it. Some approaches to the problem have been taken by using combinatorial libraries that allow for the identification of short active sequences.[10] Other investigations have been carried out by taking complex AMPs such as Lactoferrin[11] or truncated analogues of Indolicin,[12] or Tritrypticin[13] as starting material. These studies revealed that short fragments

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could maintain full antimicrobial activity, and that positively charged residues of R and the hydrophobic residues of W are always the repeating motif, which are important in electrostatic and hydrophobic interactions with phospholipids, respectively. A minimum of three from each residue, an amidated C-terminal end and a free N-terminal amino group, were necessary for the activity. Furthermore replacing R by K produced worse results, and the best natural hydrophobic residue was W, which was larger and bulkier than F or Y. On the other hand however, peptides containing four or more W residues displayed greater haemolytic activity.[14a]

Certain modifications have been made in order to improve antibacterial activity and new, short designed peptides have been obtained by employing unnatural amino acids in some cases.[15] Replacement of W residues with bulkier and more lipophilic unnatural aromatic amino acids and the esterification of the C-terminal end with lipophilic residues always resulted in an increase in antibacterial activity.[9,14b]

By taking into account the above-mentioned important contributions, our main interest was to modify the physico-chemical properties in short cationic peptides by introducing fluorine atoms or trifluoromethyl groups in both the peptidic chain and C-terminal end. It is known that substituting a hydrogen atom by fluorine in a molecule introduces minimal steric alterations owing to its relatively small size, a fact that can facilitate interactions of a fluorinated biomolecule with the receptor site.[16] Besides, the introduction of fluorine as a highly electronegative centre, which participates as a hydrogen acceptor and enhances lipophilicities, can usually alter the physico-chemical properties of the molecule significantly (such as solubility or the log P) in a predictable way. This could result in an improvement of antibacterial activity, an increase in stability, or in a reduction of toxicity to eukaryotic cells contributing to improved therapeutic efficiency. Also, substitution of hydrogen by a trifluoromethyl group could act in a similar way. In this case, the size is much larger (the volume of a trifluoromethyl group should be close to that of an isopropyl group),[17] and hence is not comparable at all. However, it is the enhanced volume what makes its contribution on hydrophobicity even higher than in the case of fluorine.

Strategies based on these special fluorine properties result in both the production and rational design of new pharmacological agents and drugs.[18]

2. Results and discussion

2.1. Peptides design

Although peptides with five residues were not been shown to be highly active,[9] we chose them for our analyses since their antimicrobial activity would probably be more sensitive to the structural modifications introduced.

Three series of peptides with an alternating sequence of hydrophobic and positively charged residues were synthesised. In the first one (Fig. 1) all the peptides had a net charge of +3, and R as polar amino acid. The second sequence (Fig. 2) was similar to the first, but K was introduced as a charged amino acid. The third one (Fig. 3) was built with the same residues as the first, but contained 3 polar and 2 hydrophobic residues, and is hence the net charge +4 instead of +3.

In order to determine the influence of fluorine on both the hydrophobicity and antimicrobial activity, two types of derivatives were synthesised. Modifications were introduced inside the chain of the first one, and amide peptides were obtained with the natural amino acids W or F, and with the unnatural 4-fluoro-L-phenylalanine (fF), or 4-trifluoromethyl-L-phenylalanine (cf_3F) as hydrophobic residues. In the second type, the modification was introduced at the C-terminal end, and acid peptides containing F as the hydrophobic residue were esterified with benzylic alcohol, 4-fluorobenzylic alcohol, 3-fluorobenzylic alcohol, 3,5-difluorobenzylic alcohol or 4-trifluoromethylbenzylic alcohol.

2.2. Effect of fluorine atom and trifluoromethyl groups on antibacterial activity

Antibacterial activity was assayed against Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria. Tables 1–3 show the results obtained in both cases.

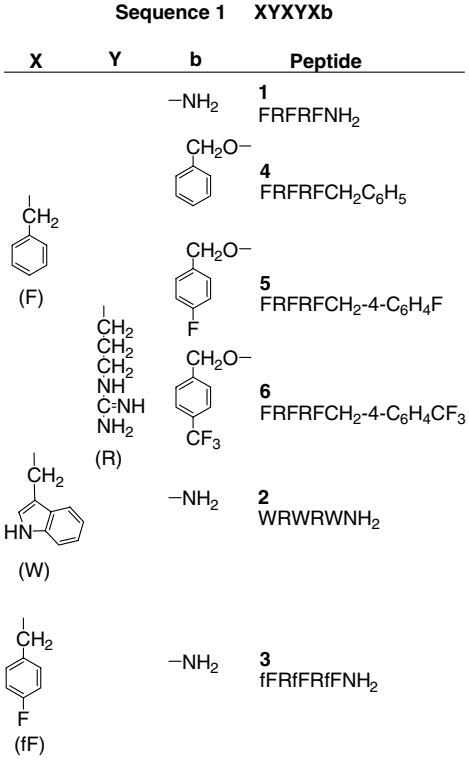

Figure 1. Sequence 1. X and Y represent the amino acids in the peptidic chain. The side chain for every residue is indicated in each case; b represents the terminal end, that could be an amide or an ester.

Sequence 2 $X_1YX_2YX_3b$

X_i	Y	b	Peptide
		$-NH_2$	**7** $FKFKFNH_2$
		$-OCH_2CH_3$	**13** $FKFKFCH_2CH_3$
		$-OCH_2CF_3$	**14** $FKFKFCH_2CF_3$
		CH_2O-	**15** $FKFKFCH_2C_6H_5$
CH_2 (F)		CH_2O- F	**16** $FKFKFCH_2$-4-C_6H_4F
		CH_2O- F	**17** $FKFKFCH_2$-3-C_6H_4F
	$CH_2CH_2CH_2CH_2NH_2$ (K)	CH_2O- F F	**18** $FKFKFCH_2$-3,5-C_6H_3FF
		CH_2O- CF_3	**19** FKFKFCH2-4-$C_6H_4CF_3$
CH_2 HN (W)		$-NH_2$	**8** $WKWKWNH_2$
CH_2 F (fF)		$-NH_2$	**9** $fFKfFKfFNH_2$
CH_2 CF_3 (cf_3F)		$-NH_2$	**12** $cf_3FKcf_3FKcf_3FNH_2$
X_1,X_3=F X_2=cf_3F		$-NH_2$	**10** $FKcf_3FKFNH_2$
X_2=F X_1,X_3=cf_3F		$-NH_2$	**11** $cf_3FKFKcf_3FNH_2$

Figure 2. Sequence 2. Letter code as in Figure 1.

Sequence 3 YXYXYb

Y	X	b	Peptide
		$-NH_2$	**20** $RFRFRNH_2$
	CH_2 (F)	CH_2O-	**23** $RFRFRCH_2C_6H_5$
		CH_2O- CF_3	**24** $RFRFRCH_2$-4-$C_6H_4CF_3$
$CH_2CH_2CH_2NHC{=}NHNH_2$ (R)	CH_2 HN (W)	$-NH_2$	**21** $RWRWRNH_2$
		$-NH_2$	**22** $RfRfRfRNH_2$
	CH_2 F (fF)	CH_2O-	**25** $RfRfRfRCH_2C_6H_5$
		CH_2O- F	**26** $RfRfRfRCH_2$-4-C_6H_4F

Figure 3. Sequence 3. Letter code as in Figure 1.

By comparing the antimicrobial activity of the amide peptides with the same hydrophobic residue and a differently charged amino acid or net charge, as expected, the best results were obtained for peptides in the first sequence, with two R as the charged amino acid. Results were worse for amide peptides in the second and third series, with 2 K or 3 R in the chain (compare entries **1**,**7**,**20**; **2**,**8**,**21** and **3**,**9**,**22**). This is because the peptides with a net charge of +4 in the third sequence are too polar, thus the penetration in the lipophilic membrane is more difficult. On the other hand, interaction with the phosphate groups of the membrane is more favourable with R than with K as a result of a better fitting with the guanidinium-type side chain.[9] The effect due to the charged amino acid nature, or to the amount of charge, was notably diminished in the more lipophilic ester derivatives, and similar values were found for any of the series (see entries **4**,**15**,**23**; **5**,**16** and **6**,**19**,**24**).

The introduction of fluorine atoms or trifluoromethyl groups in either the peptidic chain or the C-terminal end always improved the antimicrobial activity of the short cationic peptides tested by diminishing the minimal inhibitory concentration (MIC). Ester peptides were always more active than amide peptides, although the effect of fluorine substitution was more significant in the latter case for which the MIC value was reduced at least by half (compare entries **1**,**3**; **7**,**9** and **20**,**22**). Despite what was expected,[15] the fluorinated amide peptides were more active than those containing the larger and bulkier residue W with very few exceptions (entries **2**,**3**; **8**,**9** and **21**,**22**).

As the second and third series showed higher MIC values than the first one, it is likely that their biological activity could be more sensitive to the introduction of modifications. For this reason, more derivatives from these sequences were obtained in order to determine their influence. In this sense amide peptides containing K and 4-cf_3F, as bulkier hydrophobic residue, were synthesised, and a significant improvement was observed when this last amino acid was introduced in the three hydrophobic positions, especially in *S. aureus* (compare entries **7**, **10**–**12**).

Table 1. Peptides in sequence 1

Entry/peptide	Peptide sequence	t_R[a]	MIC[b]		M_W	
			E. coli	*S. aureus*	Calcd	Obsd[c] [MH^+]
1	$FRFRFNH_2$	15.8	37.5	50	770.4	771.4
2	$WRWRWNH_2$	17.2	18.75	18.75	887.5	888.7
3	$fFRfFRfFNH_2$	17.4	12.5	25	824.9	825.4
4	$FRFRFCH_2C_6H_5$	22.6	12.5	6.25	861.5	862.4
5	$FRFRFCH_2$–4-C_6H_4F	23.9	6.25	3.12	879.5	880.6
6	$FRFRFCH_2$–4-$C_6H_4CF_3$	26.0	4.68	2.34	929.4	930.5

[a] Retention time, determined by HPLC, in minutes.
[b] Minimal inhibitory concentration (MIC) in $\mu g\,mL^{-1}$.
[c] Determined by MALDI-TOF-MS.

Table 2. Peptides in sequence 2

Entry/peptide	Peptide sequence	t_R[a]	MIC[b]		M_W	
			E. coli	*S. aureus*	Calcd	Obsd[c] [MH^+]
7	$FKFKFNH_2$	15.0	50	75	714.4	715.7
8	$WKWKWNH_2$	16.5	37.5	50	831.5	832.8
9	$fFKfFKfFNH_2$	16.9	37.5	25	768.4	769.8
10	$FKcf_3FKFNH_2$	18.1	37.5	25	782.4	783.4
11	$cf_3FKFK\ cf_3FNH_2$	20.5	37.5	18.75	850.4	851.5
12	$cf_3FKcf_3FK\ cf_3FNH_2$	22.6	18.75	4.68	918.4	919.5
13	$FKFKFCH_2CH_3$	18.9	75	75	743.4	744.6
14	$FKFKFCH_2CF_3$	20.7	37.5	37.5	797.4	798.5
15	$FKFKFCH_2C_6H_5$	21.8	12.5	9.37	805.5	806.5
16	$FKFKFCH_2$–4-C_6H_4F	22.8	9.37	6.25	823.4	824.6
17	$FKFKFCH_2$–3-C_6H_4F	23.0	9.37	9.37	823.4	824.5
18	$FKFKFCH_2$–3,5-C_6H_3FF	23.6	9.37	4.68	841.4	851
19	$FKFKFCH_2$–4-$C_6H_4CF_3$	25.1	4.68	2.34	873.4	874.5

[a] Retention time, determined by HPLC, in minutes.
[b] Minimal inhibitory concentration (MIC) in $\mu g\,mL^{-1}$.
[c] Determined by MALDI-TOF-MS.

Table 3. Peptides in sequence 3

Entry/peptide	Peptide sequence	t_R[a]	MIC[b]		M_W	
			E. coli	*S. aureus*	Calcd	Obsd[c] [MH^+]
20	$RFRFRNH_2$	11.7	75	75	779.5	780.6
21	$RWRWRNH_2$	12.8	75	75	857.5	858.6
22	$RfFRfFRNH_2$	13.2	37.5	37.5	815.5	816.6
23	$RFRFRCH_2C_6H_5$	18.1	18.75	9.37	870.5	871.4
24	$RFRFRCH_2$–4-$C_6H_4CF_3$	21.1	6.25	6.25	938.4	939.5
25	$RfFRfFRCH_2C_6H_5$	19.0	9.37	6.25	906.5	907.5
26	$RfFRfFRCH_2$–4-C_6H_4F	19.2	6.25	4.68	924.5	925.5

[a] Retention time, determined by HPLC, in minutes.
[b] Minimal inhibitory concentration (MIC) in $\mu g\,mL^{-1}$.
[c] Determined by MALDI-TOF-MS.

Fluorinated benzylic esters were also found to be more active than benzylic ones (compare entries **4**–**6**; **15**–**19**; **23**–**24** and **25**–**26**). When the position or the number of fluorine atoms in the aromatic ring was changed, *E. coli* was not seen to be sensitive. Thus, in the second sequence, the same MIC values were observed for the ester peptides derived from 3-fluorobenzylic alcohol **17**, 4-fluorobenzylic alcohol **16** or 3,5-difluorobenzylic alcohol **18**. In contrast, *S. aureus* was more sensitive to the difluorinated benzylic derivative, as it was to what was substituted at position 4 (entries **16**–**18**) between the two monofluorinated esters.

Once again the trifluoromethylated benzylic derivatives were found to be the best drugs for both bacteria in all three series. In this sense, the effect of the CF_3 group is especially obvious when comparing the MIC values for the ethanol and trifluoroethanol ester derivatives in sequence 2 (entries **13** and **14**), although the results were

worse than in the hydrophobic benzylic esters case. Very low MIC values were found in all fluorinated esters despite the presence of F as a hydrophobic residue in the chain, for which worse results have always been described.[15]

2.3. Correlation hydrophobicity-antibacterial activity

Each sequence of peptides contains the same charged amino acid, while the hydrophobic residue or the C-terminal end is modified. As the size of the fluorine and hydrogen atoms is similar, and monofluorinated amino acids are almost isosteric to their hydrocarbon counterparts,[19] the differences derived from the substitution by fF in the case of amide peptides or fluorobenzylalcohol in the case of ester peptides could be due to an increase in the hydrophobic character of the molecule that allows for a better availability and membrane penetration.

To prove this hypothesis, the apparent octanol–water partition coefficient (D) at pH 13 was determined for some amide peptides in the first and second sequences using the shake flask method[20] (Table 4). It was not possible to determine an accurate value for amide peptides in the third sequence with three R residues since no extraction was practically produced. By contrast, extraction was almost complete in the case of ester peptides.

According to the apparent partition coefficient, hydrophobicity was clearly higher in peptides in the first and second sequences containing 4-fF than in those with F. Similar values were obtained for the analogues with W. However, as the apparent partition coefficient depends on the pH, and the nature of the charged amino acid and hence the ionisation percentages at pH 13 are therefore different, it was not possible to compare peptides of different sequences with these measures. The first sequence of peptides, where R was the charged amino acid, seemed to be more hydrophilic than the second one containing K. This is owing to the higher pK_a value of R which determines that an important fraction of ionised molecules must be present. Attempts to carry out the extraction at pH 14 were unsuccessful due to the slow degradation of the peptides under these conditions. In fact, these data were found to be contradictory with the retention times, determined by HPLC, for each peptide when these were analysed under the same standard conditions (Tables 1 and 2). Higher lipophilicity was observed in this case for peptides of the first sequence containing R (compare t_R in **1**,**7**; **2**,**8**; **3**,**9**; **4**,**15**; **5**,**16** and **6**,**19**). Retention time seems to be a better parameter to measure hydrophobicity under neutral physiological conditions. Indeed, a very good correlation between retention time and MIC is observed as is shown for *S. aureus* in Figure 4. The only exceptions are the ethyl and trifluoroethyl peptide esters **13** and **14** in the second sequence, but despite this, a good fitting is observed if amide peptides (black circles) and ester peptides (white circles) are considered independently.

Table 4. Apparent octanol–water partition coefficient at pH 13 (D)

Sequence/peptide	Peptide sequence	log D[a]
1/**1**	$FRFRFNH_2$	−0.24
1/**2**	$WRWRWNH_2$	0.21
1/**3**	$fFRfFRfFNH_2$	0.33
2/**7**	$FKFKFNH_2$	0.83
2/**8**	$WKWKWNH_2$	1.30
2/**9**	$fFKfFKfFNH_2$	1.26

[a] Details are described in Section 4.

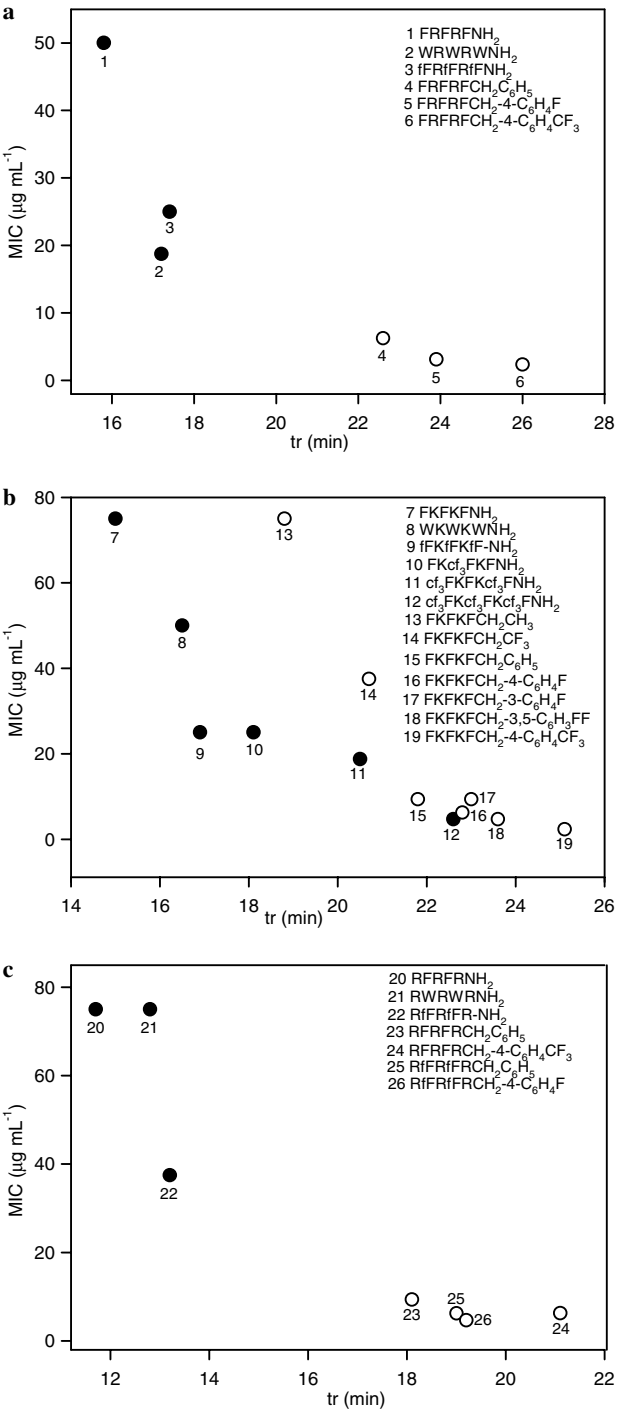

Figure 4. Representation of minimal inhibitory concentration (MIC) values in *Staphylococcus aureus* versus retention time (t_R), determined by HPLC. (a) Peptides in first sequence; (b) peptides in second sequence and (c) peptides in third sequence. The amide peptides are represented by black circles, and the ester peptides by white circles. Details of the determination of both measurements are described in Section 4.

It is worth mentioning that very similar values in the retention time were obtained for amide peptides containing W and for those containing fF, despite the higher biological activity displayed by the fluorine derivatives in almost all cases. This could indicate that the fluorine atom does not improve only the drug activity due to hydrophobicity modifications. Recent studies[21] on the folding of fluorinated helical peptides (based on trifluoroleucine and hexafluoroleucine) in membrane environments showed that fluorinated interfaces, which are simultaneously hydrophobic and lipophobic, are superior and more efficient at directing oligomer structures in phospholipid membranes than those based on aliphatic side chains alone, leading to the formation of stable fluorous aggregates. In the same way, an enhanced thermal and chemical stability has been described[16,22] in this kind of fluorinated peptides suggesting an increased stability of the resulting aggregated or protein assembly based on stronger interactions between fluoroalkyl groups than between their hydrocarbon analogues. The self-assembling trend of fluorinated peptides within the lipid environment could make pore formation easier in the bacterial membrane or drive to a destabilisation of the lipidic structure, which might account for the higher antimicrobial activity of our fluorinated peptides, especially those with the trifluoromethyl modification. Obviously further appropriate structural studies would be required to test this hypothesis.

Toxicity to eukaryotic cells is always a problem in compounds with a mechanism of action based on their interaction with membranes. When hydrophobicity is seen to exceed a certain level, selectivity between prokaryotic and eukaryotic membranes is lost, with a concomitant increase in cytotoxicity.[23] In order to discover the toxicity of these peptides to normal cells, they were all tested for their haemolytic activity against human erythrocytes. Lytic activity detected at a concentration of 250 μg/mL was lower than 5% in all cases.

3. Conclusions

To summarise, this study shows that the presence of fluorine atoms or trifluoromethyl groups in either the chain or the C-terminal end of short cationic peptides enhances their potential medical applications by improving antimicrobial activity (lower MIC values). Activity increased only in bacteria, and not in eukaryotic cells. All these facts together with the low molecular weight, simplicity and low modification cost, especially in the C-terminal end, make these drugs promising structures for their application as antibiotic agents.

4. Experimental

4.1. General

Amino acids protected with 9-fluorenyl-methoxycarbonyl group (Fmoc), Rink amide (aminomethyl)polyestyrene resin (1.1 mmol/g) and Wang resin (0.6–1 mmol/g) were purchased from Fluka. Side-chain protection scheme was *tert*-butoxycarbonyl for Lys (N_{ε}-Boc) and Trp (N_{in}-Boc), and 2,2,4,6,7-pentamethyldihydrobenzofuran-sulfonyl for Arg (N_{δ}-Pbf).

Dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole hydrate (HOBt) and other reagents for peptide synthesis were obtained from Aldrich as were the alcohols employed in the esterification of the acid peptides and the octanol for the apparent partition coefficient determination. Solvents for the analytical and semipreparative HPLC were acquired from Merck. Human red blood cells for the haemolytic assays and media for the antimicrobial assays were purchased from Sigma.

Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a Merck Hitachi Lachrom system. For analytical work, a LiChrospher 100 RP-18 (5 μm, 250 × 4 mm ID) column was used with a linear gradient from 10% to 90% of acetonitrile in water (containing 0.09% TFA) for 40 min, and with a flow rate of 1 mL/min. The wavelength for the detection was fixed at 214 nm. The semipreparative separation was performed with a LiChrospher 100 RP-18 (10 μm, 250 × 10 mm ID) column, using the appropriate mixture of acetonitrile and water in every case.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on a Reflex IV Bruker Daltonics instrument, using α-cyano-*p*-hydroxycinnamic acid as a matrix.

4.2. Solid-phase synthesis

All the pentapeptides were manually synthesised as amides using a Rink amide (aminomethyl)polyestirene resin (1.1 mmol/g), or as acids on a Wang resin (0.6–1 mmol/g). Coupling processes were carried out with 3 equiv of Fmoc amino acids, 3 equiv of HOBt and 3 equiv of DCC in DMF for at least 90 min, until completion was checked with the Kaiser's test. The Fmoc group in each cycle was removed by washing twice with 25% piperidine in DMF. After the synthesis, the resin was washed with methylene chloride and ethyl ether, and dried under vacuum.

For resin cleavage, the dried resin was suspended in a fresh mixture of trifluoroacetic acid, water, phenol and triisopropylsilane (88:5:5:2, 10 mL/g of resin) for 5–6 h. Then, the cleavage cocktail containing the peptide was filtered down into chilled diethyl ether (10 volumes in relation to the cleavage cocktail), and the precipitated peptide was stored at −80 °C overnight. The crude precipitated peptide was separated by centrifugation (3500 rpm × 15 min), washed twice with cold ethyl ether and air-dried. The peptide was directly purified by HPLC and characterised by mass spectrometry (MALDI-TOF) (see Tables 1–3).

4.3. Standard procedure for synthesis of ester peptides

The acid peptide (7.5 mg) was dissolved in the corresponding alcohol (ethanol, trifluoroethanol, benzylic

alcohol, 4-fluorobenzylic alcohol, 3-fluorobenzylic alcohol, 3,5-difluorobenzylic alcohol or 4-trifluoromethylbenzylic) (0.75 mL), and HCl gas was bubbled in the solution for an hour. The mixture was maintained stoppered at room temperature for 24 h and then it was poured into a mixture of water/ethyl ether (3:1.5 mL). After separation of phases, the organic layer was extracted with water (3× 1.5 mL) and all the water phases were joined and lyophilised to 2 mL. Finally, ester peptides were purified by HPLC using the appropriate conditions for each case, and characterised by MALDI-TOF-MS (see Tables 1–3).

4.4. Apparent octanol–water partition coefficient at pH 13 (*D*)

Octanol and an aqueous solution of NaOH (0.2 M, pH 13.02) were employed in the determination of the apparent partition coefficient (D = concentration of peptide in octanol/concentration of peptide in water at pH 13).

Both solvents were presaturated with the other by placing them in contact for at least 24 h. A solution of peptide in the NaOH aqueous solution was prepared (peptide concentration 0.5 μg/mL). Three series of extractions, each one in duplicated experiments, were prepared for every peptide. First 0.8 mL of the peptide solution and 0.8 mL of octanol (ratio v_{oct}/v_{water} 1) were added to a Teflon-capped glass tube, and the mixture was vigorously shaken for 10 min. After equilibration for a few minutes, the mixture was centrifuged (10 min at 2000 rpm) to achieve good separation. The final peptide concentration in the aqueous phase was determined twice by HPLC using an internal standard added after extraction (C_{fin}). The same internal standard was used to determine peptide concentration in the aqueous phase before extraction (C_{in}).

The second and the third extraction were performed in the same way as previously described, but using 0.4 mL of octanol (ratio v_{oct}/v_{water} 0.5) or 1.6 mL of octanol (ratio v_{oct}/v_{water} 2). The apparent partition coefficients were computed from the concentration ratios; these ratios were estimated by HPLC measurements in the aqueous phase by correcting unequal solvent volumes when they were used: $(C_{in} - C_{fin})\ V_{water}/C_{fin}V_{oct}$.

4.5. Antimicrobial activity

For antimicrobial testing, the microbroth dilution assay was used. *E. coli* (EC JM101) and *S. aureus* (BH1) were employed as test organisms. In order to determine the minimum inhibitory concentration (MIC), 2-fold serial dilutions of peptides starting with 200 and 150 μg/mL were prepared in 1% Bacto Peptone water broth. Bacteria were grown in 2% Bacto Peptone water until exponential growth, and then an inoculum of 2×10^6 cfu/mL of bacteria in 1% Bactopeptone water was added to each series and incubated for 20 h at 37 °C. The MIC value was determined as the lowest antimicrobial concentration at which there was no visible increase of the OD570. The peptides were tested in parallel using at least three independent dilutions. Gentamicin was used as an internal standard for the positive control (MIC 1.88 μg/mL for both bacteria). Results are shown in Tables 1–3.

4.6. Haemolytic activity

The haemolytic activity of the peptides was determined by using fresh human erythrocytes. The erythrocytes were washed three times (10 mM Tris, 150 mM NaCl, pH 7.4) prior to the assay. Then the cells were dissolved in buffer at a final cell concentration of 1.9×10^9 cells/mL. The cell suspension (100 μL) was added to a buffer solution containing peptide to provide a final volume of 750 μL. The peptide concentration was 250 μg/mL, and the final concentration of erythrocytes was 2.5×10^8 cells/mL. This suspension was incubated for 30 min at 37 °C in an Eppendorf thermomixer at 350 rpm. After cooling in ice water and centrifuging (5 min, 2000*g*, 4 °C), the optical density (OD_{540}) of the supernatant was determined. Zero haemolysis (blank) and 100% haemolysis (control) were determined using the supernatants after centrifuging 100 μL of the erythrocytes stock solution, which was incubated under the aforementioned conditions with buffer and 0.5% NH_4OH, respectively. Measurements were taken at least twice for each peptide, and the values determined differed by less than 5%.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.06.027.

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