Rapid identification of compounds with enhanced antimicrobial activity by using conformationally defined combinatorial libraries

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We have combined the strength of our synthetic combinatorial library approach for the rapid identification of highly active compounds with prior knowledge of the relationship between the antimicrobial activities of individual peptides with specific induced conformations in order to identify new peptides with enhanced activity relative to a starting known antimicrobial sequence. In the current study, conformationally defined combinatorial libraries were generated based on an 18-mer antimicrobial peptide known to be induced into an α-helical conformation in a lipidic environment. Not only were novel sequences readily identified with 10-fold increases in activity, but detailed information about the structure–activity relationships of the peptides studied was also obtained during the deconvolution process. By using circular dichroism spectroscopy it was found that the individual 18-mer peptides could be induced into α-helical conformations on interaction with the cell lipid layer and/or sialic acids, which could result in bacterial cell lysis due to perturbation of the lipid packing of the cell wall.

INTRODUCTION

The screening of large 'collections' of known individual compounds, as well as the diversity present in natural sources such as soil samples, marine waters, insects and tropical plants (reviewed in [1,2]) for which the modes of action are initially unknown, represents the primary current means for the identification of new antimicrobial agents. The continuing improvement in the understanding of the mechanisms of bacterial resistance enables inhibition targets and pathways to be explored [3]. This in turn permits the synthetic design of modified versions of existing antimicrobial compounds. Over the past 5–10 years, peptide and steroidal antimicrobial compounds isolated from various mammals such as frogs [4,5], insects [6] and sharks [7] have emerged as potential therapeutic agents. Although peptides are not traditionally considered as effective systemic therapeutic agents owing to their lack of oral bioavailability and rapid enzymic degradation, it appears that insects and mammals use peptides to counter bacterial infections as one of a number of primary host defence systems [8]. Because they have different modes of action from classical antibiotics (i.e. peptides generally exert their activity by altering the permeability properties of bacterial membranes [9]), peptides can be expected to have value as alternative agents in the fight against new resistant microbial strains. The characterization of such new antimicrobial peptides and the design of analogues with improved activities has allowed a better understanding of the structure–activity relationships of these peptides (reviewed in [9]). For instance, earlier studies directed towards understanding the structure–activity relationships of a number of antimicrobial peptides isolated from natural sources [10,11] indicated that amphipathic structures, combined with the presence of positively charged residues, were required for their antimicrobial activities. These studies have led to the design of a large number of novel cationic peptides with potent antimicrobial activities, all of which were found to be capable of adopting an amphipathic α-helical structure in the presence of lipid/cell membranes [12–15].

To overcome the limitations inherent in the traditional structure–activity relationship design of the new antimicrobial agents described above (i.e. the lengthy and laborious procedures and the large number of analogues that must be screened), synthetic combinatorial libraries (SCLs) have recently been shown to be a powerful means for the rapid development of new classes of antimicrobial compounds [16–21]. For instance, a range of L-amino acid hexapeptides [16,17,19,21] and tetrapeptides composed of L-, D- and unnatural amino acids [18] have been identified that have potent antimicrobial activity against Gram-positive (e.g. *Staphylococcus aureus*, *Streptococcus sanguis*) and Gram-negative (e.g. *Escherichia coli*, *Pseudomonas aeruginosa*) bacteria, and/or fungi (e.g. *Candida albicans*). More recently, peptidomimetic SCLs have been used to discover novel anti-staphylococcal compounds [20]. Although, in most instances, active sequences have been identified from the screening of SCLs, it should be noted that none of the library approach guarantees the identification of the most active compound(s) present in the library in each assay system. This mostly depends on the specificity of the assay systems (i.e. the relative effect of replacing each residue on the activity of interest). Negative synergistic effects between sequences present in a given mixture of thousands of related sequences may also prevent the identification of a number of active sequences (e.g. simultaneous presence of agonists and antagonists, or peptide–peptide interactions that prevent the occurrence of peptide–receptor interactions).

In the current study we have combined prior knowledge of the importance of induced secondary-structure capability in known antimicrobial peptides with the strength of the SCL approach to

Abbreviations used: ACN, acetonitrile; CFU, colony-forming unit; EPC, egg phosphatidylcholine; HD_{50}, concn. needed to lyse 50 % of red blood cells; $[LK]^{3.6}$, Ac-LKLLKKLLKKLKKLLKKL-NH_2; MIC, minimum inhibitory concentration; PS, phosphatidylserine; RBC, red blood cell; RP-HPLC, reversed-phase HLPC; SCLs, synthetic combinatorial libraries; SUVs, small unilamellar vesicles; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

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rapidly identify active compounds from a combinatorial library made up of thousands of closely related sequences. Conformationally defined SCLs have been prepared on the basis of a known antimicrobial sequence (Ac-LKLLKKLLKKLKKL-LKKL-NH_2, referred as to $[LK]^{3,6}$ [22]). This starting 18-mer peptide was found in earlier studies to adopt an amphipathic α-helical conformation in lipidic environments. The generation of SCLs within a known biologically active sequence can be expected not only to accelerate greatly the development of individual analogues of equal or greater activity when compared with the original sequence, but also to allow detailed structure–activity relationship information to be obtained. As illustrated in the present work, the use of SCLs also enables detailed structure–activity relationship studies to be accomplished concurrently with the selection process involved in the identification of highly active sequences.

MATERIALS AND METHODS

SCLs and individual peptide synthesis

The SCLs and individual peptides were prepared by simultaneous multiple peptide synthesis using *t*-butoxycarbonyl chemistry as described elsewhere [23]. The mixture positions were obtained by using a mixture of 19 L-amino acids (cysteine was omitted) based on a predefined chemical ratio [24] at each coupling step. Final cleavage and deprotection steps were performed with a 'low–high' hydrogen fluoride procedure [25,26] and a 120-vessel cleavage apparatus. Individual peptides were purified by preparative reversed-phase HPLC (RP-HPLC) on a DeltaPrep 3000 RP-HPLC combined with a Foxy fraction collector (Millipore, Waters Division, San Francisco, CA, U.S.A.). The crude peptides were solubilized in water containing 5 % (v/v) acetonitrile (ACN) and purified on a Waters Delta Pak C_{18}-100 Å column (25 mm × 100 mm). Solvent A consisted of 0.05 % trifluoroacetic acid (TFA) in water, and solvent B consisted of 0.05 % TFA in ACN. The peptides were purified with a gradient starting at 5 % B, increasing to 35 % B in 5 min, and then increasing to 50 % B in 30 min. The elution was monitored at 230 nm. Analytical RP-HPLC and laser desorption time-of-flight mass spectroscopy (Kratos Kompact MALDITOF mass spectrometer; Kratos, Ramsey, NJ, U.S.A.) were used to determine the purity and identity of the individual peptides. The determined mass values varied by ± 1 Da from the theoretical molecular mass.

CD measurements

All measurements were carried out on a Jasco J-720 CD spectropolarimeter (Eaton, MD, U.S.A.), in conjunction with a Neslab RTE 110 water bath and temperature controller (Dublin, CA, U.S.A.). Ellipticity is reported as mean residue ellipticity [θ] (degrees·cm^2·$dmol^{-1}$). Peptide concentrations were determined by UV spectrophotometry at 276 nm in buffer, taking $\epsilon = 1420\ M^{-1}\cdot cm^{-1}$ for tyrosine [27] and $\epsilon = 5570\ M^{-1}\cdot cm^{-1}$ for tryptophan [28]. For liposome binding studies and sialic and colominic acid titrations, stock solutions were separately prepared with: (1) peptide in buffer (5 mM Mops/NaOH, pH 7.0), (2) 5.6 mM egg phosphatidylcholine (EPC) and phosphatidylserine (PS) (8 % PS, mol/mol), (3) sialic acid (1 mg/ml) and (4) colominic acid [poly(2,8-*N*-acetyl neuraminic acid); 1 mg/ml]. The samples studied were then prepared by mixing the appropriate solutions at a range of defined ratios. Samples were tested for peptide-induced liposome aggregation by using a Hewlett Packard 8452A diode-array UV spectrophotometer (Palo Alto, CA, U.S.A.).

Antimicrobial and haemolytic assays

Microdilution assays were performed against *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC in 96-well tissue-culture plates as described elsewhere [12]. In brief, the exponential phase from an overnight culture grown at 37 °C in Mueller Hinton broth (Becton Dickinson Microbiology Sysytems, Lincoln Park, NJ, U.S.A.) at a final concentration of $(1–5) \times 10^5$ colony-forming units (CFUs) per ml was used in all assays. Bacterial suspension in 2 × broth was added to the mixtures at concentrations varying from 1000 to 1 μg/ml derived from serial 2-fold dilutions. The plates were then incubated for 21 h at 37 °C. The relative percentage growth of the bacteria found for each mixture was determined by the attenuance at 620 nm (A_{620}) with a Titertek Multiskan Plus apparatus. The IC_{50} values were then calculated with sigmoidal-curve-fitting software (Graphpad; ISI Software, San Diego, CA, U.S.A.). The minimum inhibitory concentration (MIC) is defined as the lowest concentration of mixture at which no change in A_{620} occurs between time 0 and 21 h. The IC_{50} values allow a more accurate relative activity to be determined when comparing mixtures within a given SCL [29].

Antifungal microdilution assays were performed in a similar manner against *Candida albicans* ATCC 10231 at 30 °C for 48 h in yeast medium [21].

The haemolytic activities of the peptides and/or peptide mixtures were determined on human red blood cells (RBCs). The blood was collected in heparin, maintained at 4 °C and used on either the same day or the following day. The cells were washed three times with PBS (35 mM phosphate buffer/0.15 M NaCl, pH 7.0) and resuspended in PBS. The haemolytic activities were determined as described [30] by using 96-well tissue culture plates. In brief, 100 μl of a 0.5 % RBC solution was added to an equal volume of each peptide or mixture in PBS. The plates were incubated for 1 h at 37 °C and the A_{414} value of the supernatant was measured. The concentration of peptide or mixture necessary to lyse 50 % of the RBCs (HD_{50}) was then determined by a sigmoidal-curve-fitting method (Graphpad).

RESULTS

Design of conformationally defined SCLs

Amphipathic α-helical conformations are known to play a key role in a number of the biological activities of peptides [31]. Alteration of the chemical nature of either side of an amphipathic α-helix was therefore expected to affect such activity. Two SCLs were initially prepared by selectively replacing either individual leucine or lysine residues, respectively, of a non-*N*-acetylated form of $[LK]^{3,6}$ with mixtures of amino acids to generate a combinatorial array. A tyrosine was also incorporated at the N-terminus to allow accurate determination of the concentration of the peptides and peptide mixtures for CD spectroscopic studies (the original sequence was YKLLKKLLKKLKKLLKKL-NH_2). The first SCL was prepared by maintaining or individually replacing Leu-4 with one of 18 L-amino acids (cysteine was omitted; this individually defined position is represented by the letter 'O'), and replacing the leucines at positions 7, 11 and 14 with a close to equimolar mixture of the same 19 amino acids (these mixture positions are represented by the letter 'X'). If one assumes that the peptide adopts an ideal amphipathic α-helix [22], then these four leucine residues are located in the middle of the hydrophobic face, i.e. at the maximum distance from the hydrophobic/hydrophilic interface. This library can be represented by the general formula $YKLO^4KKX^7LKKX^{11}$-

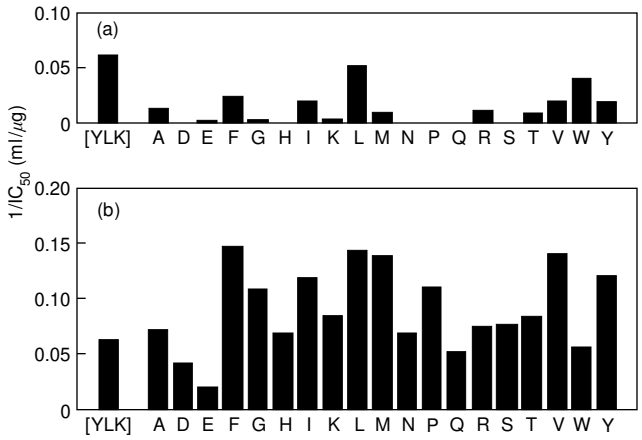

Figure 1 Antimicrobial activity against *S. aureus* of a conformationally defined library constructed on (a) the hydrophobic face of the helix and (b) the hydrophilic face of the helix

The IC_{50} was determined for each peptide mixture of (**a**) YKLO^{4}KKX^{7}LKKX^{11}K KX^{14}LKKL-NH_2 and (**b**) YKLLKO^{6}LLX^{9}KLKX^{13}LLX^{16}KL-NH_2) against (1–5) $\times 10^5$ CFU/ml. Each bar in each panel represents the inverse IC_{50} value for each peptide mixture, with the *x*-axis representing the defined amino acid ('O' position). The first bar in each graph represents the inverse IC_{50} value obtained for the original sequence [YLK]. Each bar represents the average of three assay determinations. The relative rank order of the activity between the mixtures within an SCL was consistent in three assays, although up to a 15% variation in activity was found from one assay to another (i.e. $1/IC_{50}$ varied by ± 0.05 ml/μg).

KKX^{14}LKKL-NH_2 and is composed of 19 separate peptide mixtures, with each mixture made up of 6859 (19^3) individual peptides. The second SCL was similarly prepared with the defined position replacing Lys-6, and the mixture positions replacing Lys-9, Lys-13 and Lys-16 (represented as YKLL-KO^{6}LLX^{9}KLKX^{13}LLX^{16}KL-NH_2). Interestingly, the 19 separate mixtures making up the SCL in which the hydrophilic side was varied (i.e. by replacing the lysine residues) appeared α-helical in buffer, whereas the peptide mixtures in which the hydrophobic face was varied were in a random conformation under the same conditions (results not shown). All peptide mixtures adopted α-helical conformations in the presence of 80% trifluoroethanol (TFE).

Screening of SCLs

Each peptide mixture making up the two libraries was assayed for antimicrobial activity against *S. aureus*. Higher activities relative to the original sequence were found when the hydrophilic face of the helix was varied (i.e. by replacing the lysine residues), whereas the peptide mixtures derived from substituting the leucine residues showed little or no activity (Figure 1). These results may be explained by the lack of spatial stretches of hydrophobic residues on the hydrophobic side of the helix, which, in our earlier studies with single-omission analogues, appeared to be necessary for activity to occur [12]. Because it had high overall activity, the library constructed on the hydrophilic face of the helix was selected for a detailed study to determine if individual analogues having potent antimicrobial activity could be identified.

Two different approaches for the deconvolution process have been described for the identification of active individual peptides from mixtures after the screening of an SCL. The first involves an iterative process of selection and further synthesis in which, by successive steps of synthesis, screening and selection of the most active mixtures, the amino acids at each position are sequentially defined [16]. The second approach is termed a positional scanning process, which allows the direct determination of key residues at each library position [32]. A positional scanning SCL is composed of a number of single-position SCLs, each representing the same diversity of compounds, but differing from one to another by the

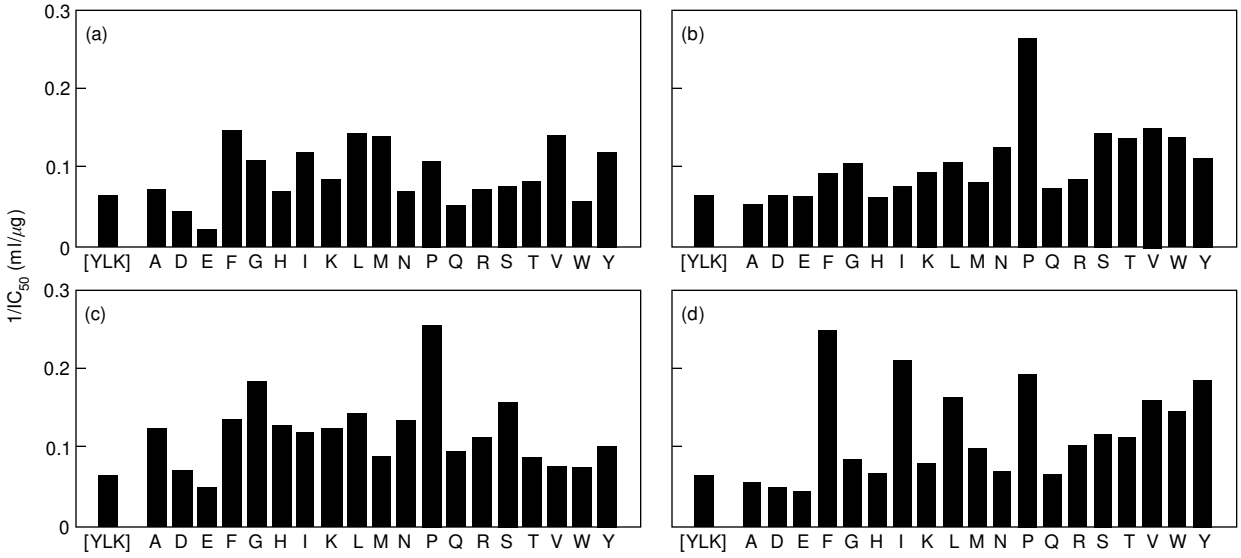

Figure 2 Antimicrobial activity against *S. aureus* of a conformationally defined positional scanning SCL constructed on the hydrophilic face of the helix

Each graph shows the inverse IC_{50} values obtained for each of the four separate positional SCLs: (**a**), YKLLKO^{6}LLX^{9}KLKX^{13}LLX^{16}KL-NH_2; (**b**), YKLLKX^{6}LLO^{9}KLKX^{13}LLX^{16}KL-NH_2; (**c**), YKLLKX^{6}LLX^{9}KLKO^{13}LLX^{16}KL-NH_2; (**d**), YKLLKX^{6}LLX^{9}KLKX^{13}LLO^{16}KL-NH_2. The bars are as defined in Figure 1.

Table 1 Activity of individual peptides derived from a PS-SCL

Name	Sequence	MIC (μg/ml) against:			HD_{50} (μg/ml)
		S. aureus	*Ps. aeruginosa*	*C. albicans*	
$[L^6P^9G^{13}F^{16}]$	YKLLKLLLPKLKGLLFKL-NH_2	2–3	6–12	120–185	24
$[L^6P^9P^{13}F^{16}]$	YKLLKLLLPKLKPLLFKL-NH_2	3–5	7–14	130–175	55
$[L^6P^9G^{13}I^{16}]$	YKLLKLLLPKLKGLLIKL-NH_2	4–6	14–28	130–206	28
$[L^6P^9P^{13}I^{16}]$	YKLLKLLLPKLKPLLIKL-NH_2	4–6	8–16	120–174	69
$[L^6P^9G^{13}P^{16}]$	YKLLKLLLPKLKGLLPKL-NH_2	11–14	5–30	70–100	> 125
$[L^6P^9P^{13}P^{16}]$	YKLLKLLLPKLKPLLPKL-NH_2	45–55	30–60	> 170	> 125
[YLK]	YKLLKKLLKKLKKLLKKL-NH_2	30–55	10–20	50–78	6.1
$[W^6L^9A^{13}E^{16}]$	YKLLKWLLLKLKALLEKL-NH_2	60–120	45–90	> 170	6.3

location of the defined position. The advantage of positional scanning SCLs is that each of the separate positional libraries addresses only a single position in the peptide. Thus, when used in concert, the data derived from each positional SCL yields information about the most important amino acids or building blocks at every position. This information can then be used to prepare individual sequences. Thus no specific deconvolution steps are required but only the confirmation of the screening results by the synthesis of individual compounds. By its nature, the positional scanning process permits the identification of active individual sequences in a more rapid manner than the iterative process, with only the significant specificity of the residues at a given position being necessary in the selection process. It should be noted that 'hits' (i.e. single-position defined mixtures) generally have substantial signal-to-noise differences from other mixtures, which permits the direct identification of active individual sequences.

A second library built around the hydrophilic face of the α-helix was prepared in a positional scanning format, which involved four separate series of mixtures (i.e. having the defined position at position 6, 9, 13 or 16). As shown in Figure 2, although relatively redundant, the presence of a hydrophobic residue at position 6 resulted in higher anti-staphylococcal activity relative to the original sequence (i.e. peptide mixtures defined by F, L, V and Y have IC_{50} values ranging from 6 to 8 μg/ml, compared with 14 μg/ml for the original sequence). Greater specificity was found for the three other positions, with proline defining the most active peptide mixtures (IC_{50} = 3.9, 4.0 or 4.9 μg/ml for proline at positions 9, 13 or 16 respectively). It should be noted that although the differences in activity between the most active mixtures in a single positional SCL and the next most active mixtures may not appear dramatically significant (1.5–2-fold decreases in IC_{50}), the relative activity between the peptide mixtures within a separate positional SCL was reproducible between different assays. This reproducibility permitted a definite selection of amino acids for the deconvolution process. The selection process for the amino acids chosen at each position enables the generation of a series of individual peptides. Two main criteria are used: high anti-staphylococcal activity and as much difference in chemical character for those amino acids chosen for a given position. Thus leucine was selected at position 6 as a representative hydrophobic residue, proline at position 9, proline and glycine at position 13, and phenylalanine, isoleucine and proline at position 16. Six individual peptides, which represent all possible combinations of these selected amino acids ($1 \times 1 \times 2 \times 3 = 6$), were then synthesized and characterized by mass spectral analyses and RP-HPLC (sequences shown in Table 1).

Antimicrobial activity of the individual peptides identified from the SCLs

The six individual peptides were assayed against *S. aureus* in a manner similar to that used for the SCLs (Table 1). As expected, all except $[L^6P^9P^{13}P^{16}]$ showed improved activity relative to the original sequence, [YLK]. Four of these peptides showed MICs ranging from 2 to 6 μg/ml, representing a 10-fold increase in activity relative to the original sequence. It should be noted that even in those cases in which the range in activity between mixtures is relatively small, the single amino acids defining the most active mixture at each position (i.e. L, P, P and F at positions 6, 9, 13 and 16 respectively), when combined, led to one of these four most active individual peptides. Thus one could have selected just these single amino acids from the screening results and obtained a single peptide, $[L^6P^9P^{13}F^{16}]$, with enhanced activity relative to the original sequence, [YLK]. The peptides exhibited similar high activity against the Gram-negative bacteria *E. coli* and *Ps. aeruginosa*, but lower activities against *C. albicans* (Table 1). The six peptides all had lower haemolytic activity than the original sequence (Table 1).

Owing to its helix-breaking propensity [33–35], the presence of proline residues in the active peptide mixtures was surprising. To understand this observation further, an analogous peptide, designed in an unrelated study to have a high α-helical propensity, was assayed in a similar manner. This peptide was found to adopt a tetrameric α-helical conformation in buffer (results not shown). Although this negative control peptide only differed from the proline-containing peptides by the residues at positions 6, 9, 13 and 16 (i.e. having W, L, A and E respectively), it was found to have significantly weaker activity than the original sequence or the proline-containing peptides identified through the screening of the SCLs. Because W, L, A and E residues at positions 6, 9, 13 and 16 respectively were not found to represent peptide mixtures having the highest activity in the respective single positional SCLs, these results further support the potential of connecting residues derived from the screening of a library in a positional scanning format. Furthermore, this result demonstrates the potential of positional scanning SCLs for the rapid identification of active peptide analogues from pools of thousands of sequences.

Conformational studies

To further our understanding of the relationships between the induction into an α-helical conformation and the antimicrobial activity of these peptides, structural studies were performed by means of CD spectroscopy under various conditions. The struc-

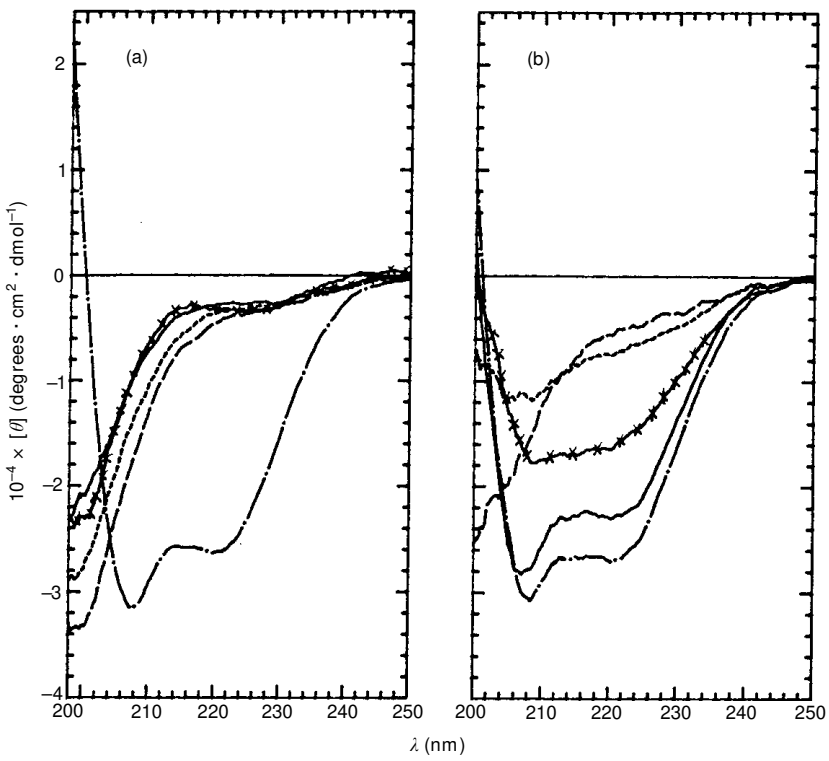

Figure 3 CD spectra of individual peptides (a) in Mops buffer or (b) in the presence of colominic acid

The CD spectra were recorded at a peptide concentration of 45 μM in 5 mM Mops buffer (**a**), and in the presence of colominic acid at a ratio $R_{co} = 4$ (**b**). The mean residue ellipticities, $[\theta]$, are shown for [$L^6P^9G^{13}F^{16}$] (−×−×−), [$L^6P^9P^{13}F^{16}$] (·-·-), [$L^6P^9P^{13}P^{16}$] (−−−), [$W^6L^9A^{13}E^{16}$] (—·—) and [YLK] (—).

tural behaviour of two of the most active individual peptides against *S. aureus* ([$L^6P^9G^{13}F^{16}$] and [$L^6P^9P^{13}F^{16}$]), the least active anti-staphylococcal peptide derived from the deconvolution process ([$L^6P^9P^{13}P^{16}$]), the original sequence, [YLK], and the highly helical, negative control peptide described above ([$W^6L^9A^{13}E^{16}$]) were investigated further.

In Mops buffer, the CD spectra of the three peptides derived from the library screening ([$L^6P^9G^{13}F^{16}$], [$L^6P^9P^{13}F^{16}$] and [$L^6P^9P^{13}P^{16}$]) exhibited a negative band at approximately 198 nm, which is characteristic of a random coil conformation (Figure 3a). The CD spectra of [$W^6L^9A^{13}E^{16}$] showed strong negative bands at 208 and 222 nm, characteristic of an α-helical conformation, which results from tetrameric self-association (results not shown). In the presence of TFE, a solvent known to induce helicity in polypeptides [36], the CD spectra of all of the peptides studied, except [$L^6P^9P^{13}P^{16}$], showed indications of α-helical content. These results indicate that the helical character found either in buffer or in organic solvents such as TFE cannot be directly correlated with the biological activity of these peptides. This is in agreement with earlier studies involving the haemolytic and antimicrobial activity of melittin and analogues with different induced conformations in such environments [37].

The structural behaviour of these peptides was further evaluated in the presence of two main components that make up biological membranes: naturally occurring polymers of *N*-acetylneuraminic acid (referred to as polysialic acids), which are present on the surface of cell membranes, and negatively charged small unilamellar vesicles (SUVs) composed of EPC/PS (92:8 molar ratio) to mimic the lipid bilayer of the cell membranes. Two distinct structural behaviours were observed when the peptides were in the presence of colominic acid (Figure 3b). A clear conformational change was found for [$L^6P^9G^{13}F^{16}$], [$L^6P^9P^{13}F^{16}$] and [YLK] on binding to colominic acid. Thus the three peptides were induced into an α-helical conformation with an observed maximum at $R_{co} = 4$ (R_{co} = [colominic acid]/[peptide], w/w). In contrast, the two other peptides studied, [$L^6P^9P^{13}P^{16}$] and [$W^6L^9A^{13}E^{16}$], remained random and α-helical, respectively, on binding to colominic acid (Figure 3b). It is noteworthy that no conformational change was observed for any of the peptides in the presence of the monomeric sialic acid (results not shown).

The effects on peptide conformation on binding to SUVs were then examined in the presence of different phospholipid/peptide ratios (molar ratio R_{lp} = [EPC/PS]/[peptide]). Thus, on increasing R_{lp}, a gradual increase in α-helical content was observed for [$L^6P^9G^{13}F^{16}$], [$L^6P^9P^{13}F^{16}$] and [YLK]. In contrast, [$L^6P^9P^{13}P^{16}$] remained random in the presence of liposomes at the highest R_{lp} tested ($R_{lp} = 30$). No conformational change was observed for [$W^6L^9A^{13}E^{16}$], which remained fully α-helical in the presence of liposomes. To simplify the comparison between the extent of conformational changes that are induced by the SUVs, changes in peptide ellipticity at 222 nm, normalized to the initial ellipticity values in buffer, are shown in Figure 4(a). To ensure that the various conformational changes observed were not due to differences in binding affinity to liposomes, UV spectroscopy studies were also performed at different R_{lp} values. Thus vari-

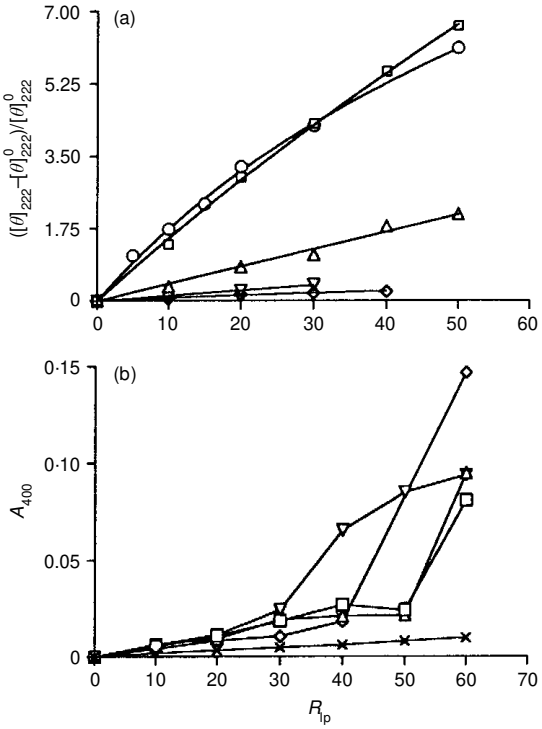

Figure 4 Binding-affinity curves

(**a**) The CD spectra were recorded at a peptide concentration of 45 μM in 5 mM Mops buffer in the presence of EPC/PS SUVs. The $[\theta]_{222}$ values were calculated for R_{lp} varying from 0 to 60. The $[\theta]_{222}$ values, which were normalized to the $[\theta]_{222}$ at $R_{lp} = 0$ ($[\theta]^0_{222}$), are shown for [$L^6P^9G^{13}F^{16}$] (□), [$L^6P^9P^{13}F^{16}$] (△), [$L^6P^9P^{13}P^{16}$] (▽), [$W^6L^9A^{13}E^{16}$] (◇), and [YLK] (○). (**b**) The UV attenuance was recorded at 400 nm and is plotted as a function of R_{lp} for [$L^6P^9G^{13}F^{16}$] (□), [$L^6P^9P^{13}F^{16}$] (△), [$L^6P^9P^{13}P^{16}$] (▽), [$W^6L^9A^{13}E^{16}$] (◇), and [YLK] (○). The values for SUVs without peptides are also shown (×). It should be noted that the attenuance values for [$L^6P^9G^{13}F^{16}$] and [YLK] overlap.

ations in liposome size due to peptide–lipid interactions were monitored by the resulting attenuance at 400 nm. The A_{400} values were found to increase for all the peptides studied on addition of EPC/PS SUVs (Figure 4b). Interestingly, a dramatic increase in attenuance, indicative of vesicle aggregation, was observed in the same range of R_{lp} (40–50) for all the peptides. These results suggest that the five peptides are able to bind to the SUVs in a similar manner. Because the initial driving force for peptide interactions with phospholipid membranes is believed to be mainly electrostatic interactions, the similar binding affinity found is probably due to the fact that these peptides have the same extent of positively charged residues (except for [YLK]).

DISCUSSION

Induced conformation-based diversity has been shown here to be a useful means for the rapid development of analogues with enhanced activity relative to known biologically active peptides. Furthermore, this approach allows structure–activity relationship studies to be performed not only on the final active peptides, but also on related less active sequences prepared during the selection process. Interestingly, the antimicrobial peptides identified from the screening of the library all contain at least one proline residue within their sequence. On the basis of prior work in this and other laboratories about the importance of the amphipathic α-helical conformation on the antimicrobial activity of peptides [10–12], the insertion of a clear helix-breaker residue such as proline was not expected to be a sequence modification of choice. In the SCL approach, all possible substitution analogues for the positions of interest are included in a sequence, and are simultaneously presented to a particular target (e.g. a micro-organism). One can consider this approach as a 'natural' selection process. However, the initial selection of the combinatorial library positions in a peptide sequence of interest, when based on prior structure–activity relationship knowledge, is expected to direct such selection processes.

The synthetic peptides identified in the current studies have activities similar to peptides isolated from natural sources, such as the magainins, cecropins and defensins (reviewed in [1]). These newly identified peptides were also found to undergo conformational changes in the presence of liposomes or polysialic acids. It is noteworthy that the least active of the peptides studied, [$W^6L^9A^{13}E^{16}$], was, in contrast to the other peptides, α-helical in mild buffer with no conformational change detected in the presences of liposomes or polysialic acids. These conformational studies lead us to the conclusion that induction into α-helical conformations on interaction with the lipid layer and/or sialic acids present in bacterial cell membranes promotes the lysis of the cells. One can thus envisage that the structural constraint induced by this peptide–lipid association results in the perturbation of the lipid packing. This in turn results either in leakage of the cell contents or in an initial penetration of the peptides past the membrane through hydrophobic interactions between the leucine face of the helix and the phospholipid chains. Both of these potential mechanisms result in the death of the cells.

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