Consequences of N-Acylation on Structure and Membrane Binding Properties of Dermaseptin Derivative K₄-S₄-(1–13)*

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Acyl conjugation to antimicrobial peptides is known to enhance antimicrobial properties. Here, we investigated the consequences of aminolauryl (NC₁₂) conjugation to the dermaseptin derivative K₄-S₄-(1–13) (P) on binding properties to bilayer models mimicking bacterial plasma membrane, which is often cited as the ultimate site of action. Isothermal titration calorimetry revealed that acylation was responsible for enhancing the binding affinity of NC₁₂-P compared with P (Kₛₒₐₜ = 13 × 10⁵ and 1.5 × 10⁵ M⁻¹, respectively). Surface plasmon resonance measurements confirmed the isothermal titration calorimetry results (Kₛₒₐₜ = 12.6 × 10⁵ and 1.53 × 10⁵ M⁻¹, respectively) and further indicated that enhanced adhesion affinity (Kᵢₖₛₚₒₐₜ = 3 × 10⁵ and 1 × 10⁵ M⁻¹, respectively) was coupled to enhanced tendency to insert within the bilayer (Kᵢₖₛᵣᵢₒₐₜ = 4.5 and 1.5, respectively). To gain insight into the molecular basis for these observations, we investigated the three-dimensional structures in the presence of dodecylphosphocholine using NMR. The ensemble of NMR-calculated structures (backbone root mean square deviation <0.6 Å) showed that the acyl moiety was responsible for a significant molecular reorganization, possibly affecting the electrostatic potential distribution in NC₁₂-P relative to that of P. The combined data present compelling evidence in support of the hypothesis that N-acylation affects antimicrobial properties by modifying the secondary structure of the peptide in a manner that facilitates contact with the membrane and consequently increases its disruption.

Peptide-based cytolitic compounds represent a promising class of novel antimicrobial agents (1–5). Antimicrobial peptides (AMPs) are believed to kill target cells by destabilizing the structure of cell membranes (5–7), although the fine details of this mechanism have yet to be fully understood. Various potential targets other than the cytoplasmic membrane have also been proposed; AMPs were shown to activate microbicidal activities of leukocytes (8–10) or suppress the production of inflammatory cytokines, thereby providing protection against the cascade of events that leads to endotoxic shock (11–13). Other potential uses of these multifunctional peptides include food preservation (14, 15), imaging probes for detecting infection loci (16), and linings for medical/surgical devices (17, 18). Clearly, the externally localized site of action and receptor-independent mechanism of AMPs may significantly reduce or prevent development of drug-resistance. However, since this mechanism is also largely responsible for unselective activity over a wide range of cell types, only topical therapeutic applications have been considered so far (19, 20). Thus, a major challenge toward the safe use of AMPs is to improve our understanding of the mechanism of action and consequently conceive AMPs with an increased therapeutic index. Numerous strategies have been adopted toward this goal, including synthetic combinatorial libraries (21), extracting sequence patterns after comparing to natural counterparts (22), or designing AMPs based on secondary structure requirements (23–25). Various attempts were made to modify natural peptides or assemble chimeric peptides from segments of different natural peptides (10, 17, 26). Last, naturally occurring AMPs characterized by a lipophilic acyl chain at the N terminus were discovered in various microorganisms (27). Moreover, acyl conjugation to certain AMPs proved a useful technique for improving antimicrobial characteristics (28–32).

Dermaseptins are known to exert cytolitic activity against a variety of pathogens (33–36). Bacteria were shown to develop resistance to commercial antibiotics but not to the L- or D-isomers of the dermaseptin derivatives (37). Various acylated derivatives were investigated for their effect on malaria-infected red blood cells, taking advantage of their ability to translocate spontaneously across the plasma membrane of mammalian cells (38). Although certain acylated dermaseptin derivatives displayed increased specific antiparasitic efficiency combined with reduced hemolysis of infected or normal erythrocytes (30), acylation, in general, was shown to enhance hemolysis, antiprotozoan activity, and antibacterial activity against Gram-positive but not Gram-negative bacteria (39). However, the corresponding aminoacyl analogs behaved essentially the same as the nonaminated acyl counterparts but displayed enhanced activity against all microorganisms tested, including Gram-negative bacteria (39). A general correlation between increased potency and increased secondary structure was reported for various AMPs (32, 40, 41). Other studies reported that acylation enhanced antiprotozoan activity (29), antifungal activity (28), and activity against bacteria (21, 32). Thus, whereas a variety of studies report that antimicrobial activity of AMPs can be enhanced through conjugation to an acyl moiety, the underlying molecular basis is far from being fully understood. To explore this issue, the 13-mer dermaseptin S₄ derivative, K₄-S₄-(1–13), was conjugated to either aminolauryl or lauryl moieties and probed in
terms of binding and structural properties compared with the acyl-free peptide.

EXPERIMENTAL PROCEDURES

Peptides—The peptides were synthesized by the solid phase method applying the Fmoc (9-fluorenylmethoxycarbonyl) active ester chemistry on an Applied Biosystems model 433A peptide synthesizer. 4-Methylbenzhydrylamine resin (Novabiochem, Darmstadt, Germany) was used to obtain amidated peptides. The acylated analogs were prepared by linking the amino terminus of the peptides to lauric or oleic acid, as described (39). The crude peptide was purified to \( \geq 95\% \) chromatographic homogeneity by reverse-phase high performance liquid chromatography (HPLC) (LC-MS Alliance-ZQ Waters). The purified peptides were subjected to mass spectrometry analysis to confirm their composition. Peptides were stocked as lyophilized powder at \(-20\, ^\circ C\). Prior to testing, fresh solutions were prepared in water, briefly vortexed, sonicated, centrifuged, and then diluted with the appropriate medium. Buffers were prepared with distilled water (mQ; MilliQ Corp.). All other reagents were analytical grade.

Liposome Preparation—Small unilamellar vesicles (SUVs) composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPC/POPG, 3:1 molar ratio) were prepared in PBS by the sonication method as per the manufacturer’s (Avanti Polar) instructions using a G112SP1 bath sonicator (Labconco Corp.). Liposomes were suspended in PBS buffer, pH 7.4, and vortexed, sonicated, centrifuged, and then diluted with the appropriate medium. SUVs in PBS buffer, pH 7.4, at 25 \( ^\circ C \) were prepared in PBS by the sonication method as per the manufacturer’s (Avanti Polar) instructions using a G112SP1 bath sonicator (Labconco Corp.). Liposomes were suspended in PBS buffer, pH 7.4, and measurements were performed at 25 \( ^\circ C \).

Isothermal Titration Calorimetry (ITC)—Experiments were performed using a calorimeter (Microcal, Northampton, MA) calibrated electronically. Heats of dilution were determined in control experiments by injecting either peptide solution or lipid suspension into the calorimeter (Microcal, Northampton, MA) calibrated at 4.725 ppm. TOSCY (44), DQF COSY (45), and NOESY (46) were collected with six mixing times ranging from 50 to 300 ms in order to gain maximal NOE buildup with a minimal contribution from spin diffusion. The buildup curves were examined for all of the amide protons and the tryptophan aromatic protons, and structural data were subsequently acquired with a mixing time of 150 ms using 160 transients for each \( t_\alpha \). A list of NOE restraints used is available from the authors. Spectra were processed and analyzed with the XWINNMR and Viewer software packages (Bruker Analytische Messtechnik GmbH) and SPARKY (provided by T. D. Goddard and D. G. Kneller, SPARKY 3; University of California, San Francisco) on a Silicon Graphics Indigo2 R10000 work station. Zero filling in the 1 dimension and data apodization with a shifted squared sine bell window function in both dimensions were applied prior to Fourier transformation. The base line was further corrected in the 2 dimension with a quadratic polynomial function.

Resonance assignment was done according to the sequential assignment methodology developed by Wüthrich (48) based on the TOCSY and NOESY spectra measured under identical experimental conditions. The volumes of the NOE peaks were calculated by SPARKY, categorized according to volume and designated as strong, medium, weak, and very weak, and assigned distance values of up to 3.0, 4.0, 5.0, and 6.0 \( Å \), respectively. The three-dimensional structures of the peptides were generated using XPLOR (version 3.856) (49) as described (40). Molmol (50) was used for visual analysis and presentation. Low energy structures chosen for further analysis had no NOE violations, deviations from ideal bond lengths of less than 0.05 \( Å \), and bond angle deviations from ideality of less than 5\(^\circ\). PROCHECK (51) was used to analyze the secondary structures of the calculated conformations.

Hydrophobic properties were mapped onto the space-filling model of the molecule according to a consensus scale (52).

Electrostatic free energies were derived from finite difference solutions of the Poisson-Boltzmann equation using the DelPhi program (53). The AMBER force field (54) was employed, and a full Coulombic calculation was performed. The ionic strength of the solution was 78 \( mM \). The internal peptide dielectric value was 5.0, and when the solution dielectric was varied between 5.0 and 40 to reflect the lipid environment, there was no significant change in the shapes of the calculated electrostatic potential distribution, although their values differed. The positive and negative 5 \( kT/e \) isopotential surfaces were presented using CHIMERA (55).

RESULTS AND DISCUSSION

To understand the interaction of acylated peptides with phospholipid membranes, we investigated the effects of conjugating aminolauryl (NC12) or lauryl (C12) to the dermaseptin S4 derivative K4S4-(1–13) (P) on properties of binding to bilayer models mimicking bacterial plasma membranes (the sequence is shown in Table 1). The identity of the synthetic products was confirmed by mass spectrometry post purification. Peptide purities were \( \geq 95\% \) as determined by analytical HPLC and subsequently by NMR (data not shown).

ITC—The ability of the peptides to bind to liposomes was compared by titrating a solution of each peptide (12 \( \mu M \)) with a suspension of SUVs. Dynamic light scattering measurements indicated that the liposome size was 25 \( \pm 5\, \mu M \) and remained unchanged in the presence of

| Table 1: Primary structure of peptides investigated |
|-----------------------------------------------|
| Peptide | Designation | Sequence |
| K4S4-(1–13) | P | ALWKTLLKKVLKANH2 |
| Lauryl-K4S4-(1–13) | C12-P | C12-ALWKTLLKKVLKANH2 |
| Aminolauryl-K4S4-(1–13) | NC12-P | NC12-ALWKTLLKKVLKANH2 |
the highest peptide concentration used in the titration experiment (data not shown), Fig. 1A depicts the interaction of a representative peptide with the anionic liposomes. Fig. 1B shows the enthalpy changes for each injection shown in Fig. 1A, integrated and fit using a single binding site algorithm. Table 2 summarizes the affinity constants obtained for the dermaseptin derivatives. The acyl-free peptide displayed a binding constant of $1.5 \times 10^5$ M$^{-1}$, but the acylated peptides displayed unambiguously higher affinities, with $K$ values of $13.0 \times 10^6$ and $3.1 \times 10^8$ M$^{-1}$, respectively, for NC$_{12}$-P and C$_{12}$-P. The derivative C$_{12}$-P displayed an affinity that was surprisingly 4-fold lower than its acylated analog and (unlike NC$_{12}$-P) was found to be aggregated at the peptide concentrations, the ITC apparatus was unable to yield reliable results due to technical limitations. Therefore, the $K$ value obtained for C$_{12}$-P must be considered as indicative only.

SPR—To further investigate the lipophilic properties of the peptides, the same SUVs used for ITC measurements were adsorbed onto a sensor chip (L1) to form a supported lipid bilayer that chemically and physically resembles the surface of a cell membrane. Peptide solutions were passed over the immobilized lipid surface, and the changes in the resonance signal during the binding process (composed of association and dissociation phases) were detected, reflecting the accompanying changes in the mass during the reaction. The membrane-binding affinities of the dermaseptin derivatives were analyzed with a two-stage model. A two-stage reaction describes the binding process involving a conformational change that gradually leads to a more stable complex. This model portrays the interaction of antimicrobial peptides with lipid bilayers as a two-stage process comprising surface adhesion and membrane insertion. The insertion stage, which takes place after the initial adhesion of the peptide to the membrane surface, is consistent with a conformational change that occurs in the peptide-membrane complex and leads to increased stability of the complex. The binding profile obtained for NC$_{12}$-P is portrayed in Fig. 2A. The binding levels increased as a function of peptide concentration, which is indicative of a specific interaction between the peptide and the phospholipid membrane. Table 2 summarizes the kinetic rate constants and the derived affinity constants obtained for the interaction of P and NC$_{12}$-P as determined using integrated equations of a two-step model. The individual affinity constants of each step of the interaction were calculated as the ratio of the respective association and dissociation rate constants. The association affinity constant of the first step of the interaction, $K_{\text{adhesion}}$, indicates the affinity of the peptide for adhering to the membrane surface, whereas $K_{\text{insertion}}$ is a unitless constant that reflects the tendency of the peptide to insert into the membrane (or efficacy of bilayer penetration by the peptide) and is referred to as the insertion affinity (42). The apparent affinity constant for the global interaction, $K_{\text{app}}$, is a product of the individual affinity constants: $K_{\text{app}} = K_{\text{adhesion}} \times K_{\text{insertion}}$.

As shown in Table 2, P associated with a $k_{a1}$ value of $1.18 \times 10^3$ M$^{-1}$ s$^{-1}$ and dissociated with a $k_{d1}$ value of $1.16 \times 10^{-2}$ s$^{-1}$, giving the adhesion affinity $K_{\text{adhesion}}$ of $1.02 \times 10^5$ M$^{-3}$. After adhering to the membrane, P inserted into the bilayer with a $k_{a2}$ value of $3.42 \times 10^{-3}$ M$^{-1}$ s$^{-1}$ and "dissociated" (from the inserted form into the superficially adhering form) with a $k_{d2}$ value of $2.28 \times 10^{-3}$ s$^{-1}$, giving the insertion affinity $K_{\text{insertion}}$ of 1.50. These individual affinity constants resulted in

![FIGURE 1. Binding properties based on ITC. A, a representative liposome titration assay obtained for NC$_{12}$-P (12 μM) titrated with 5 μL of 38 mM POPC/POPG (3:1) SUVs in PBS buffer, pH 7.4. B, the enthalpy changes for each titration data point shown in the upper pane after being integrated and fitted with a one-binding site algorithm ($\chi^2 < 100$).](image-url)

**TABLE 2**

| Thermodynamic parameters* | P             | NC$_{12}$-P | C$_{12}$-P |
|----------------------------|---------------|-------------|------------|
| $\Delta H$ (kcal/mol)      | $-23.8$ (0.3) | $-17.8$ (0.2) | $-33.9$ (0.6) |
| $\Delta S$ (cal/mol/K)     | $-55.2$ (0.4) | $-30.5$ (0.4) | $-87.4$ (0.6) |
| $\Delta G$ (kcal/mol)      | $-7.3$ (0.2)  | $-8.6$ (0.1)  | $-7.8$ (0.3)  |
| $K$ (M$^{-1}$) $\times 10^6$ | 1.5 (0.1)     | 13.0 (0.2)    | 3.1 (0.3)     |

| Binding parameters*         | P             | NC$_{12}$-P | C$_{12}$-P |
|---------------------------|---------------|-------------|------------|
| $k_{a1}$ (s$^{-1}$)        | $1.18 \times 10^3$ | $1.17 \times 10^3$ | $6.80 \times 10^5$ |
| $k_{d1}$ (s$^{-1}$)        | $1.16 \times 10^{-2}$ | $4.21 \times 10^{-3}$ | $3.44 \times 10^{-4}$ |
| $K_{\text{adhesion}}$ (M$^{-1}$) $\times 10^5$ | 1.02 (0.1) | 2.13 (0.3) | 4.53 (0.03) |
| $K_{\text{insertion}}$     | 1.50 (0.01)   | 4.53 (0.03)   | 1.56 (0.02)   |
| $K_{\text{app}}$ (M$^{-1}$) $\times 10^5$ | 1.53 (0.1) | 12.6 (0.1) | 3.11 (0.2) |

| MIC** (μM)                 | S. aureus     | E. coli     |
|----------------------------|---------------|-------------|
|                            | 9.0 (3)       | 4.5 (1.5)   |
|                            | 0.75          | 1.5         |
|                            | >50           |             |

* ITC data were obtained from two independent experiments. Values represent the mean and S.D. (in parentheses).

* SPR kinetic rate constants and binding affinity constants for the peptide's interaction with the lipid bilayer, determined by numerical integration using a two-step reaction model as detailed (42). Binding affinity constant values represent the mean and standard deviation (in brackets) of two independent experiments. Kinetic rate constants are from one representative experiment. $\chi^2$ values for the fitted models were less than 100.

** MIC is defined as the lowest peptide dose that induced 100% growth inhibition of S. aureus and E. coli, determined after 24 h of culture in Luria-Bertani medium.
an apparent binding affinity of 1.53 × 10^5 M⁻¹. Comparison with NC12-P shows that the association rate is 4-fold faster than P, but since the dissociation rates were the same, the resulting adhesion affinity was higher by more than 3-fold (K_{adhesion} = 2.78 × 10^5 M⁻¹). NC12-P not only adhered to the membrane with a higher affinity than P but also displayed a higher tendency to insert within the membrane (K_{insertion} = 1.50 and 4.53, respectively, for P and NC12-P). The affinity constant (K_{app}) obtained from SPR is in fair agreement with the binding constant (K) obtained from ITC, thereby validating the experimental system. Table 2 also shows the antimicrobial activity of the peptides for comparison. A correlation can be seen between both SPR/ITC affinity constants and the antimicrobial activities of P and NC12-P, as discussed below.

**Binding Properties of C12-P**—The effect of acyl deamination on the binding properties was investigated using both ITC and SPR methodologies. Using the same experimental conditions, titrating C12-P yielded a higher binding affinity than P, with K = 3.1 × 10^5 M⁻¹ (Table 2). Surprisingly, C12-P displayed about 4-fold lower affinity than its aminated analog. A very similar outcome resulted from the SPR methodology (Table 2). Comparing the SPR binding profiles of the three peptides at a single concentration of 12 µM (Fig. 2B) shows how the peptides differ in their binding levels as well as by their kinetics of interaction (the rate of response signal change for association and dissociation phases). C12-P binding proceeded with slower kinetics, but its binding capacity was significantly higher. Moreover, C12-P continued to accumulate on the membrane surface during the entire injection phase without reaching an equilibrium level, even after increased injection times (not shown). This behavior is most likely related to the degree of self-assembly of the peptides in solution and complex modes of interpeptide interaction on the membrane surface. As described above, C12-P undergoes self-assembly in solution at the peptide concentrations used. Since at monomeric concentrations both ITC and SPR methods were unable to yield reliable results (exceeding minimal detection limits), the binding parameters obtained for C12-P must be considered as indicative only.

Collectively, the binding data show that alterations in the physicochemical properties of the peptide have enhanced its interaction affinities as seen by both ITC and SPR. Although there is a recent increase in the number of reports using either one of these methodologies as model systems to investigate the mechanism(s) of action of AMPs, the relevance of their data remains to be convincingly established. Moreover, to the best of our knowledge, the present study compares both methodologies for the first time. Use of SPR not only enabled us to validate the ITC data but also provided additional information pertaining to fine details beyond the mere binding affinity, namely the respective contributions of the adhesion and insertion affinities to binding (42, 58). The ITC data showed that the binding affinity of the acyl-free peptide was of an order of magnitude comparable with that of other antimicrobial peptides (24, 59, 60), whereas acylation was responsible for increasing the binding affinity. SPR data further suggested that beyond the increased adhesion affinity, structural alterations caused by acylation also increased the propensity of the peptide to insert into the bilayer. In general, we find that antimicrobial activity correlates very nicely with K_{insertion} rather than with K_{adhesion}. Since certain peptides may adhere strongly (i.e. high K_{adhesion}), but, being unable to insert into the membrane (low K_{insertion}), they do not permeate it or cause its disruption (42).

**NOE Measurements, Resonance Assignment, and Experimental Distance Restraints**—The NOESY spectra of P (Protein Data Bank code 2DD6) and NC12-P (Protein Data Bank code 2DCX) were taken in DPC, which forms micelles that were more amenable to NMR work than liposomes usually formed by POPC/POPG mixtures. SDS samples showed a large degree of line broadening and were incompatible with
structure determination by NMR (data not shown). The spectra in DPC showed numerous interactions for P and NC12-P (Figs. 3 and 4, left and right, respectively). All of the amino acid peaks and hydrogens were accounted for. The amino lipid moiety was partially unresolved, but the resolved and overlapped peaks in the one-dimensional spectrum of NC12-P all gave integration values that correlated with the molecular structure in this portion of the molecule. The coupling constants could not be resolved due to broad peak widths. The amide region of the spectrum was spread over almost 1 ppm in both cases. The entire peptide for P, together with the additional five proximate methylenes and the two methylenes adjacent to the amine of the lipid amino acid for NC12-P, was resolved in two dimensions and assigned (Table 3). The up-field deviations of the α proton chemical shifts from random values, characteristic of a local α-helical structure (56), were calculated relative to random-coil values in a 2,2,2-trifluoroethanol-d$_6$/water solution to match the dielectric constant of the solution (57), and values were qualitatively compared with each other (data not shown). Looking at deviations of more than −0.2 ppm from random coil, α proton values of P show evidence of α-helical structure in residues 2, 4–8, and 10–11. The helicity of NC12-P is evident in residues 4, 6 and 7, and 10–12.

A list of NOE interactions and statistics is given in Table 4. Overlapping NOE peaks in the aliphatic region were not counted or used in the structure calculation. P shows a few peaks, in particular long range peaks indicative of the peptide helicity. All of the $i,i+1$ HN-HN connectivities were clearly seen, except for Leu$^5$-Leu$^7$ and Val$^{10}$-Leu$^{13}$, which were too close to the diagonal. Additional do$_{(N(i+1),i)}$ and do$_{(N(i+3),i)}$ cross-peaks, typical of α-helical structures, were observed in the region of Ala$^1$–Lys$^{12}$ in P, NC12-P showed fewer NOE interactions than P, in particular interactions indicative of a clear helical structure. Sparse long range peaks were seen in the region of Trp$^3$–Lys$^{1}$, with a break in the region of Leu$^2$–Lys$^9$. This break in helical values was also seen in the NH values.

NMR-calculated Secondary and Tertiary Structures and Electrostatic Potential Distribution—Molecular modeling of the three-dimensional structures of P and NC12-P was done using a starting ensemble of 50 structures for each. Of these, 66 and 58%, respectively, had no NOE violations, had bond lengths that were correct to within 0.05 Å, had angles and dihedral violations within 5° of ideal values, and had residues that fell in the core and favored regions of the Ramachandran criteria (except at the termini) according to PROCHECK. The Ramachandran plot of P showed that residues 2 and 4–13 were all in α-helix regions of the plot; the residues in all of the structures were in the core and the rest in allowed conformational space of NC12-P less helical, but it is less structured as well.

The secondary structures of these subsets were characterized using PROCHECK. P structures showed over 95% of residues 5–13 in a helical secondary conformation or series of hydrogen-bonded turns, residues 3

### Table 3

Proton chemical shifts (ppm) of P (top) and NC12-P (bottom) in DPC

| Residues | HN   | Har  | Hβ   | Others                  |
|----------|------|------|------|-------------------------|
| P        |      |      |      |                         |
| Ala$^{1}$| 7.29 | 7.01 | 4.14 | 1.76                    |
| Leu$^{2}$| 6.73 | 3.59 | 1.49 | 1.28                    |
| Trp$^{3}$| 8.44 | 4.25 | 3.37 | 3.22                    |
| Lys$^{4}$| 7.54 | 3.78 | 1.92 |                         |
| Thr$^{5}$| 7.68 | 3.81 | 4.30 |                         |
| Leu$^{6}$| 8.20 | 4.01 | 1.84 | 1.59                    |
| Leu$^{7}$| 8.15 | 4.00 | 1.80 | 1.59                    |
| Lys$^{8}$| 7.89 | 3.90 | 1.90 |                         |
| Lys$^{9}$| 7.67 | 4.07 | 2.07 | 1.91                    |
| Val$^{10}$| 7.97 | 3.75 | 2.26 |                         |
| Leu$^{11}$| 7.99 | 4.12 | 1.83 | 1.56                    |
| Lys$^{12}$| 7.64 | 4.14 | 1.90 |                         |
| Ala$^{13}$| 7.75 | 4.16 | 1.48 |                         |

| NC12-P   |      |      |      |                         |
| Lipid    | 8.64 | 4.14 | 1.50 |                         |
| Ala$^{1}$| 8.56 | 4.23 | 1.67 | 1.48                    |
| Leu$^{2}$| 7.70 | 4.40 | 3.48 | 3.34                    |
| Trp$^{3}$| 7.88 | 3.76 | 1.79 | 1.69                    |
| Lys$^{4}$| 7.86 | 3.91 | 4.25 |                         |
| Thr$^{5}$| 7.96 | 4.08 | 1.86 | 1.72                    |
| Leu$^{6}$| 8.31 | 3.99 | 1.73 |                         |
| Lys$^{7}$| 7.72 | 4.11 | 2.10 | 1.97                    |
| Lys$^{8}$| 7.68 | 4.18 | 1.94 |                         |
| Val$^{10}$| 8.03 | 3.83 | 2.30 |                         |
| Leu$^{11}$| 8.04 | 4.16 | 1.95 |                         |
| Lys$^{12}$| 7.89 | 3.93 | 1.95 |                         |
| Ala$^{13}$| 7.80 | 4.21 | 1.51 |                         |

### Table 4

NOE connectivity statistics and root mean square deviation (RMSD) values for P and NC12-P

| Connectivity | P        | NC12-P   |
|--------------|----------|----------|
| Total        | 250      | 241      |
| $i,i+1$      | 83       | 109      |
| $i,i+2$      | 78       | 44       |
| $i,i+3$      | 34       | 19       |
| $i,i+4$      | 37       | 39       |
| $i,i+5$      | 18       | 30       |

| RMSD          |          |          |
|---------------|----------|----------|
| Structures that upheld Ramachandran criteria | 33/50    | 29/50    |
| Backbone      | 0.27     | 1.38     |
| Heavy         | 0.88     | 2.17     |
| 15 low energy structures (residues 2–12)      | 0.12     | 0.58     |
| Backbone      | 0.40     | 1.25     |
The end of the ligand and the peptide, which is not supported (or refuted) by direct experimental data. The root mean square deviation for the backbone of residues 2–12 of these 15 structures was 0.12 and 0.40 Å for the backbones of P and NC12-P, and 0.58 and 1.25 Å for the heavy atoms (Table 4).

Structural differences between P and NC12-P are evident in the N terminus, for example, where the tryptophan residue is directed toward the top of the peptide axis in P, and the first four residues show no helical or hydrogen-bonded turn conformation, whereas in NC12-P the tryptophan extends toward the side of the peptide axis and all of the N terminus residues adopt a helical or hydrogen-bonded turn conformation. In addition, the side chains of NC12-P are not as fully extended as in P.

Fig. 5B shows the hydrophobic distribution of the peptide residues, where yellow represents hydrophilic residues and green represents hydrophobic residues. P shows a clear delineation between the hydrophobic and hydrophilic regions of the peptide along the axis of the peptide, whereas NC12-P has a contiguous hydrophilic patch that is not as defined as in P.

The full Coulomb electrostatic potential distribution was calculated for P and NC12-P, and the isopotential surfaces of the +5 kT/e (blue) and −5 kT/e (red) are shown in Fig. 5C. The electrostatic potentials presented correspond to the longer range affinities that we are addressing in this work. The electrostatic potential distribution reflects the amphipathic nature of P with two delineated lobes as in the case of the hydrophobic/hydrophilic profile above. Both the positive and negative lobes are separated along the axis of the peptide. The positive potential extends much further beyond the Van der Waals radii of the molecule than the negative one, making it likely to participate in long range affinity toward negatively charged membranes. The positive electrostatic potential of NC12-P differs from that of P; the positive potential engulfs the negative distribution, and there is no bilobed separation. The positive distribution of NC12-P also extends further from the backbone than the negative one but to a lesser degree than in P.

Overall, the NMR data indicate that the added aminoacyl moiety stabilizes the N-terminal residues, probably due to a steric effect in which Trp5 extends toward the side of the molecule and stabilizes the first residues in a hydrogen-bonded turn conformation. The Trp5 side chain in P is extended toward the top of the molecule due to steric constraints. This results in a pronounced separation of the hydrophobic and charged (lysine) residues, as is evident in Fig. 5B, and a marked difference in the distribution of the positive electrostatic potential (Fig. 5C).

Raising the degree of helicity of antimicrobial peptides was proposed to correlate increased activity (24, 40), although the manner in which changes in helicity participate in the mechanism of action is yet unclear. Specifically, we have hitherto not been able to differentiate between (i) increased activity due to long range affinity of the peptide to the membrane, presumably caused by a larger degree of amphipathic separation extending the reach of the electrostatic binding potential, and (ii) increased activity due to the ability of the peptide to intercalate into the membrane. Lockwood et al. (31) recently showed that acylation of SC4 dodecapeptide increases its bactericidal potency. In their work, they show an ensemble of NMR-derived structures of the acylated peptide in DPC micelles showing the helical conformation associated with antimicrobial peptides with the common amphipathic separation of hydrophobic and hydrophilic residues. They state that their data show increased stability of the acylated SC4 in these micelles. In this respect, we show here that the increase in activity that follows acylation does not correlate with increased helicity based on the direct comparison of structures.
Although the cytoplasmic membrane of target cells (including that of bacteria) is widely believed to be the ultimate target of many AMPs, various intracellular potential targets have been proposed (26, 61, 62) to account for the ability of certain AMPs to spontaneously translocate across the plasma membrane of various cell types at nontoxic concentrations (63, 64). Whether representing a final or intermediate step in the mechanism of action, it is nevertheless clear that the interaction of AMPs with the plasma membrane plays an important role in their biological activity. At our present state of understanding the mode(s) of action of AMPs, activity is typically affected either by peptide properties (e.g. three-dimensional structure, amphipathic characteristics, and organizational state in solution (40, 65, 66)) or by the membrane properties (e.g. charge distribution, fluidity, and the presence of a cell wall). In this respect, *Staphylococcus aureus* and *Escherichia coli* are characterized by different cell walls, suggesting that the antibacterial behavior of AMPs (especially acylated AMPs) is very likely to be dictated by the nature of these external barriers. We speculate that enhanced activity of C<sub>12</sub>-P on *S. aureus*, but not on *E. coli* (Table 2), indicates that the peptidoglycan-based cell wall is permeable to this peptide but not the lipopolysaccharide-based, tightly packed and highly hydrophilic external membrane of *E. coli*. By introducing an amino group on the acyl moiety, hydrophobicity was reduced, aggregation was inhibited, and access of the peptide to the plasma membrane became possible (39). This hypothesis is supported by experiments comparing peptide activity after introducing defects into the external membrane using EDTA, which artificially increased permeability to aggregated C<sub>12</sub>-P (39).

In conclusion, the data presented indicate that conjugation of NC<sub>12</sub> has increased the hydrophobicity and changed the structure of P. The resulting increased antimicrobial activity is therefore proposed to be due to an increased efficacy of the interaction of the peptide with the plasma membrane, leading either to its permeation or disruption.

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