In Vitro Discriminative Antipseudomonal Properties Resulting from Acyl Substitution of N-Terminal Sequence of Dermaseptin S4 Derivatives

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SUMMARY

Truncation and acylation were combined to investigate the broad-spectrum bactericidal and hemolytic peptide S4(1–15). Substitution of up to seven residues with dodecanoic acid (C_{12}) gradually led to specific antipseudomonal activity: out of 40 bacterial strains tested in vitro, C_{12}-S4(8–15) displayed similar minimal inhibitory concentrations (MICs) as S4(1–15) against *Pseudomonas aeruginosa* sp. (identical MIC_{90}) but was practically inactive against most other bacteria or erythrocytes. Surface plasmon resonance and isothermal titration calorimetry experiments revealed the binding properties of S4(1–15) to be consistent with its nonselective activities, while discriminative activities of C_{12}-S4(8–15) correlated with high binding affinity to a membrane containing pseudomonal lipopolysaccharides and with lower affinities to membranes containing nonpseudomonal lipopolysaccharides or cholesterol. Various mechanistic studies failed to detect significant differences in secondary structure, bactericidal kinetics, or ability to perturb the cytoplasmic membrane, pointing to a similar mode of action.

INTRODUCTION

Antimicrobial peptides (AMPs) represent a ubiquitous component of the innate immune system, whose function includes control of invading pathogens [1–4]. Over the past 20 years, AMPs have been shown to be effective killers of viruses [5–9], bacteria, fungi [10–12], protozoa [12–16], and cancer cells [17–19]. Inspection of over 900 known AMP sequences reveals no consensus motif in terms of primary or secondary structure, besides amphipathic organization which seems to accentuate their overall positive charge and hydrophobicity [4, 20]. These physical properties represent essential elements in nonspecific interactions with multiple targets [21], although various fine details of the mode(s) of action are yet to be fully understood.

AMPs' main target is often cited to be the plasma membrane, though recent studies suggest intracellular targets, at least for some peptides [22–24]. The molecular basis for peptide specificity is presumably linked to differences in membrane composition between target and nontarget cells such as charge density, membrane fluidity [25, 26], and transmembrane potential [27]. Whichever mechanism is used by an AMP, its interaction with surface components (cell wall or plasma membrane) is likely to play a major role in antimicrobial or cytolytic actions. Accordingly, AMPs were proposed to induce their effect(s) via disruption of the cell membrane [28–30] and/or cytoplasmic translocation followed by interaction with various anionic elements [22]. Such nonspecific mechanisms are likely to inhibit the innate talents of bacteria to develop resistance [31, 32] and, because of this, AMPs present an obvious advantage over conventional antibiotics.

Due to their simple structure and broad-spectrum activity, AMPs represent exquisite candidates for various antimicrobial applications [33, 34]. However, although various topical applications are considered [31, 34, 35], they notoriously lack adequate specificity, while their relative toxicity toward red blood cells limits their potential systemic use. Another significant and prohibitive factor is their relatively high cost, at least as long as they are produced by chemical synthesis. Thus, new strategies are needed to reduce toxicity and cost.

Dermaseptins are a large family of 24–34 residue long linear AMPs [13, 15, 36–41] whose cytolytic properties are triggered after interaction of N-terminal residues with the plasma membrane [42, 43]. N-terminal acylation of dermaseptin S4 derivatives was shown to increase antimicrobial activity but hemolytic activity was found to increase as well, especially when using long-chain hydrophobic acyls [13, 36, 44]. Similar results were obtained with other AMPs [13, 36, 45]. To circumvent the risk of excessive hydrophobicity of acyl-conjugated peptides, we attempted in this study to limit hydrophobicity increase by exchanging the hydrophobic N-terminal amino acid residues by a fatty acid of moderate length (dodecanoic acid). This strategy was inspired by results obtained in previous investigations: truncation of three to four residues from the N terminus of the active sequence of various AMPs significantly hampered antimicrobial activity [41, 46, 47]; C-terminal truncation resulted in active dermaseptin derivatives, of which the 15-mer S4(1–15) was the shortest derivative having the highest growth inhibition activity against *Escherichia coli* [41]. We therefore tested the hypothesis that the combination of truncation and acylation strategies when applied to S4(1–15) would avoid aggregation in solution and would thereby enable the assessment of the role of hydrophobicity in selectivity. *Pseudomonas aeruginosa* was targeted for its medical relevance. *P. aeruginosa* is a clinically common pathogen due to its natural resistance to many antimicrobial agents and plays a major role in lung infections, namely in cystic fibrosis [48].

RESULTS

Characterization of the Reference Peptide and Its Derivatives

Initially, antibacterial activity was routinely assessed in terms of minimal inhibitory concentration (MIC) against two Gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and two Gram-negative bacteria (*E. coli* and *P. aeruginosa*), and cytotoxicity toward human red blood cells (RBCs) was assessed in terms of minimal concentration that induced 50% hemolysis (LC_{50}). For comparison purposes, Table 1 also lists the properties of two antimicrobial peptides that were assessed in human clinical trials, the magainin derivative (MSI-78) and the protegrin derivative (IB-367), as well as conventional antibiotics (polymixin, rifampin, and piperacillin), which were all assayed under identical conditions.

Results summarized in Table 1 show that the 15-mer reference peptide is endowed with large-spectrum antibacterial activity (MIC ranging from 3 to 9 μM) as well as significant hemolytic activity (LC_{50} 18 μM). Whereas truncation of the N terminus invariably decreased all activities, dodecanoylation led to heterogeneous consequences: dodecanoyl substitutions initially resulted in nonselective antibacterial activities that culminated with C_{12}-S4(5–15). Beyond the 11-mer derivative, a gradual reversal was observed where potencies of the shorter acylated derivatives were reduced except against *P. aeruginosa*, whose sensitivity was maintained up to the acylated 8-mer derivative. The potentiating limits of dodecanoylation were approached with the 7-mer derivative C_{12}-S4(9–15), which was virtually inactive in all assays. To assess the specific contribution of the acyl moiety, C_{12} was replaced by C_{14} in S4(8–15) and S4(9–15). The results demonstrate that increasing only hydrophobicity reverted to nonselective and hemolytic activities. These results are consistent with the hypothesis that specificity toward *P. aeruginosa* might emerge when an AMP attains a specific set of structural properties (charge/hydrophobicity?).

S4(1–15) versus C_{12}-S4(8–15)

To delimit mechanistic differences, the peptides were compared in terms of spectrum of activity, kinetics, secondary structure, and binding properties, as detailed below.

Antipseudomonal Activity

Shown in Table 2 are the MIC values obtained in 13 additional strains of *P. aeruginosa*. The MIC values for 50% and 90% of the strains tested (MIC_{50} and MIC_{90}, respectively) were 3 and 25 μM for the parent peptide, while C_{12}-S4(8–15) displayed MIC_{50} and MIC_{90} values of 12.5 and 25 μM, respectively. This confirmed potency of both peptides over bacterial species considered extremely difficult to treat [48, 49]. Discriminative properties were examined using 23 additional bacterial strains including various Gram-positive and Gram-negative bacteria (American Type Culture Collection [ATCC] as well as clinical isolates). As shown in Table 2, all strains tested were more resistant to C_{12}-S4(8–15).

Bactericidal Kinetics

Bactericidal properties were compared at equal concentrations representing 1, 2, and 4 multiples of the MIC value against a representative strain of *P. aeruginosa* (ATCC 9027). Figure 1A shows the dose-dependent effect, demonstrating that both peptides have rapid bactericidal kinetics, namely, both peptides managed to reduce the colony-forming unit (CFU) count by >6 log units within 15 min of incubation at 4 multiples of the MIC value.

Hemolytic Properties

For comparison purposes, hemolytic properties were initially assessed in terms of LC_{50}, as listed in Table 2 and in the literature [13–15, 36, 39, 40]. Although these data indicated that C_{12}-S4(8–15) was less hemolytic than S4(1–15), the peptide activities were further compared following the recommendations of antibacterial peptide protocols [50]. Figure 1B compares hemolytic activity at 60, 120, and 180 μM (concentrations are equal, respectively, to 10, 20, and 30 multiples of the MIC value against the reference strain); hemolytic activity was drastically altered. For example, at ten MICs, C_{12}-S4(8–15) displayed 0.03% hemolysis versus 12.45% for S4(1–15).

Interaction with the Cytoplasmic Membrane

The peptides' ability to affect bacterial cytoplasmic membrane function was assessed using a membrane-potential-sensitive fluorescent probe, $diSC_3$-5. Testing *P. aeruginosa* requires permeabilization of its outer membrane with EDTA (1 mM). EDTA did not interfere with $diSC_3$-5 fluorescence or influence the peptides' MIC (up to 2 mM EDTA). Cell viability was monitored by sampling bacteria at various time intervals during the $diSC_3$-5 assay and plating for CFU count. As shown in Figure 2, both peptides caused rapid (Figure 2A) and dose-dependent (Figure 2B) depolarization of the cytoplasmic membrane. Maximal fluorescence did not change after up to 30 min of monitoring (data not shown). Both peptides displayed faster bactericidal kinetics (negative cultures were obtained within 5 min) in the presence of EDTA, probably reflecting facilitated access of the peptides to the plasma membrane due to EDTA-mediated destabilization of the outer membrane.

Circular Dichroism

A global indication for structural differences was obtained using circular dichroism (CD) measurements in PBS in the presence of POPC:POPG (3:1) liposomes. In PBS alone, all peptides had an unordered structure (Figure 3). In the

Table 1. List of Investigated Compounds and Their Properties

Sequence	Designation	H[b]	Q[c]	LC_{50}[d] (μM)	MIC (μM)[e] *Bc*	*Sa*	*Ec*	*Pa*
$ALWKTLLKKVLKAAA_{amide}$	S4(1–15)[a]	48	5	18	3	9 ± 3	3	6
C_{12}-$ALWKTLLKKVLKAAA_{amide}$	C_{12}-S4(1–15)	75	4	<3	12.5	4.5 ± 1.5	37 ± 13	12.5
$LWKTLLKKVLKAAA_{amide}$	S4(2–15)	47	5	36	4.5 ± 1.5	18 ± 7	4.5 ± 1.5	12.5
C_{12}-$LWKTLLKKVLKAAA_{amide}$	C_{12}-S4(2–15)	69	4	<3	18 ± 7	9 ± 3	25	12.5
$WKTLLKKVLKAAA_{amide}$	S4(3–15)	44	5	>100	>50	>50	37 ± 13	25
C_{12}-$WKTLLKKVLKAAA_{amide}$	C_{12}-S4(3–15)	68	4	<3	18 ± 7	9 ± 3	18 ± 7	12.5
$KTLLKKVLKAAA_{amide}$	S4(4–15)	34	5	>100	>50	>50	>50	>50
C_{12}-$KTLLKKVLKAAA_{amide}$	C_{12}-S4(4–15)	67	4	4	9 ± 3	9 ± 3	9 ± 3	9 ± 3
$TLLKKVLKAAA_{amide}$	S4(5–15)	38	4	>100	>50	>50	>50	>50
C_{12}-$TLLKKVLKAAA_{amide}$	C_{12}-S4(5–15)	72	3	3	4.5 ± 1.5	3	6	6
$LLKKVLKAAA_{amide}$	S4(6–15)	31	4	>100	>50	>50	>50	>50
C_{12}-$LLKKVLKAAA_{amide}$	C_{12}-S4(6–15)	64	3	32	9 ± 3	12.5	12.5	6
$LKKVLKAAA_{amide}$	S4(7–15)	29	4	>100	>50	>50	>50	>50
C_{12}-$LKKVLKAAA_{amide}$	C_{12}-S4(7–15)	61	3	100	12.5	18 ± 7	12.5	6
$KKVLKAAA_{amide}$	S4(8–15)	22	4	>100	>50	>50	>50	>50
C_{12}-$KKVLKAAA_{amide}$	C_{12}-S4(8–15)	55	3	>100	25	25	50	6
$KVLKAAA_{amide}$	S4(9–15)	25	3	>100	>50	>50	>50	>50
C_{12}-$KVLKAAA_{amide}$	C_{12}-S4(9–15)	60	2	>100	>50	>50	>50	50
C_{14}-$KKVLKAAA_{amide}$	C_{14}-S4(8–15)	60	3	22	6	9 ± 3	6	6
C_{14}-$KVLKAAA_{amide}$	C_{14}-S4(9–15)	64	2	27	25	18 ± 7	12.5	25
Reference Antibacterial Compounds								
	MSI-78	46	10	45	nd	9 ± 3	1.5	1.5
	IB-367	45	4	7	nd	3	4.5 ± 1.5	19 ± 6
	Polymixin B	nd	nd	nd	nd	>50	2	0.75
	Rifampin	nd	nd	nd	nd	<0.1	7.5	12.5
	Piperacillin	nd	nd	nd	nd	50	5.5	12.5

Bc, *Bacillus cereus*; Sa, *Staphylococcus aureus*; Ec, *Escherichia coli*; Pa, *Pseudomonas aeruginosa*; nd, not determined.
[a] The reference peptide.
[b] Hydrophobicity, defined as the percent acetonitrile eluent on a C_{18} HPLC column.
[c] Charge at physiological pH.
[d] Lowest peptide concentration that induced 50% hemolysis (1% RBC) after 3 hr incubation in PBS at 37°C.
[e] Lowest peptide concentration that fully inhibited bacterial growth after 24 hr incubation at 37°C. Values represent the mean ± standard deviation obtained from at least two independent experiments performed in duplicate. Lack of standard deviation reflects consistency. Decimal values were rounded up to the nearest half-unit for simplicity.

presence of liposomes, S4(1–15) displayed a typical elipticity profile of an α helix as characterized by double minima at 208 and 222 nm (Figure 3A). Whereas the truncated derivative S4(8–15), which also did not exhibit a detectable activity, displayed unordered structure in both media (Figure 3B), its acylated counterpart C_{12}-S4(8–15) displayed a reduced yet unambiguous helical profile (Figure 3C). Consistent with the bioassays, the shortest derivative tested, C_{12}-S4(9–15), which had weak antipseudomonal activity, displayed less helicity (Figure 3D) than C_{12}-S4(8–15).

Binding Properties

Binding affinities were determined by both surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) using three liposomal preparations whose compositions mimic bacterial or erythrocyte membranes. Binding constants are summarized in Table 3. Binding properties to liposomes whose compositions mimic the zwitterionic membrane of erythrocytes (POPC:cholesterol) (9:1) were drastically different between the peptides. Compared with S4(1–15), the acylated derivative exhibited 60- and 52-fold lower adhesion and insertion

Table 2. Growth Inhibition of a Large Panel of Bacterial Strains

Bacteria	Strain	S4(1–15) MIC (μM)[a]	C_{12}-S4(8–15) MIC (μM)[a]
Pseudomonas aeruginosa	C.I. 12848[b]	3	25
	C.I. 8537	3	25
	C.I. 12777	3	25
	C.I. 13216	6	25
	C.I. 11662	25	25
	C.I. 8634	3	25
	C.I. 11668	50	12.5
	C.I. 11128	25	12.5
	C.I. 8732	25	12.5
	ATCC 9027	6	6
	C.I. 13720	1.5	3
	C.I. 12360	0.8	1.5
	C.I. 11496	0.4	1.5
	C.I. 12459	0.8	1.5
Escherichia coli	C.I. 14213	1.5	>50[c]
	C.I. 16328	1.5	>50[c]
	C.I. 16350	1.5	>50[c]
	C.I. 16348	3	>50[c]
	C.I. 16229	1.5	>50
	C.I. 14182	3	>50
	C.I. 14384	3	>50
	C.I. 16233	1.5	>50
	C.I. 14517	1.5	>50
	C.I. 16377	3	>50
	ATCC 35218	3	50
Vibrio cholera	Environmental isolate	12.5	>50
Yersinia kristensenii	ATCC 33639	6	>50
Salmonella choleraesuis	ATCC 7308	6	50
Acinetobacter baumannii	ATCC 19606	6	>50
Acinetobacter lwoffii	ATCC 15309	6	>50
Enterococcus faecalis	ATCC 29212	25	>50
Bacillus cereus	ATCC 11778	2	25
Staphylococcus aureus	C.I. 15877	25	>50
	C.I. 17314	12.5	>50
	C.I. 15852	25	>50
	C.I. 15916	12.5	>50
	C.I. 20745	25	>50
	C.I. 15886	6	>50
	ATCC 25923	9	25
	C.I. 15903	6	25

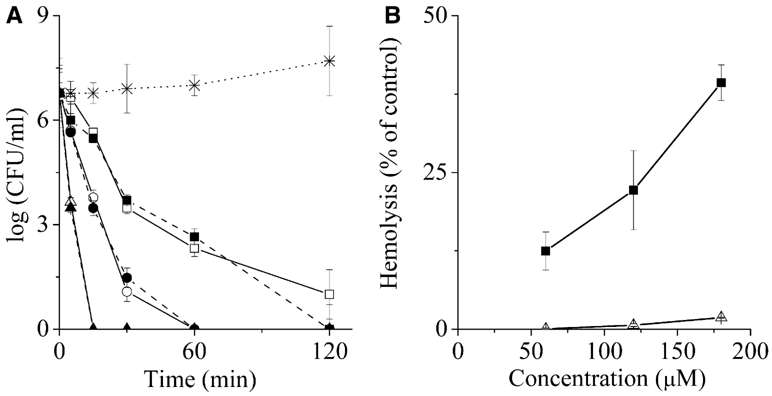

Figure 1. Bactericidal Kinetics and Hemolytic Activity

(A) *P. aeruginosa* were exposed to S4(1–15) or C_{12}-S4(8–15) at different concentrations, sampled after 5, 15, 30, 60, and 120 min, subjected to serial 10-fold dilutions, and plated on LA agar dishes for CFU count after overnight incubation. Black, S4(1–15); white, C_{12}-S4(8–15); squares, circles, and triangles represent 1, 2, and 4 times the MIC value, respectively; stars, normal growth in the absence of peptide. Plotted values represent the mean ± standard deviation obtained from at least two independent experiments performed in duplicate.

(B) Peptide hemolytic properties were determined against washed human erythrocytes (10% hematocrit) exposed to PBS containing S4(1–15) or C_{12}-S4(8–15) at 60, 120, and 180 μM (concentrations equal to 10, 20, and 30 multiples of the MIC value, respectively). Hemolytic activity was determined after 1 hr incubation at 37°C by measuring absorbance (405 nm) of the supernatants and comparison with RBCs exposed to PBS containing 0.2% Triton X-100 (for 100% hemolysis) or to PBS alone (for baseline value). S4(1–15), black squares; C_{12}-S4(8–15), white triangles. Statistical data were obtained from two independent experiments performed in duplicate.

affinities, respectively, yielding an overall affinity constant ($K_{apparent}$) that was reduced by three orders of magnitude. This outcome was consistent with—and may explain—the observed hemolytic properties.

To assess the possible implication of bacterial outer membrane lipopolysaccharides (LPS) in peptides' abilities to discriminate between *E. coli* and *P. aeruginosa* sp., SPR experiments were conducted using negatively charged liposomes that incorporated LPS from either *E. coli* or *P. aeruginosa* sp. (Figure 4). Although the two-stage model may not accurately account for all events in this somewhat more complex interaction, the SPR data strongly suggested that C_{12}-S4(8–15) is endowed with higher (27-fold) binding affinity to *P. aeruginosa* LPS (K_{app} 180 × 10^4 M^{-1}) than to *E. coli* LPS (K_{app} 6.6 × 10^4 M^{-1}), unlike the reference peptide, which displayed 16-fold higher affinity to *E. coli* LPS. ITC experiments using liposomal suspensions of the same compositions as in SPR yielded nearly identical affinity constants (Table 3).

Overall, SPR and ITC data were both consistent with the biological activities reported in Tables 1 and 2 and support a role for binding affinities to target cell membranes in determining the selective activities observed for C_{12}-S4(8–15).

Peptide Organization in Solution

The light-scattering properties of various derivatives were investigated in PBS at the relevant (active) concentration range (data not shown). Whereas C_{12}-S4(1–15) aggregated at low micromolar concentrations, no evidence for self-assembly could be detected for the substituted versions, including C_{12}-S4(8–15).

DISCUSSION

In accordance with the hypothesis that activities of AMPs proceed by nonspecific mechanisms, we recently verified that the physicochemical properties of the dermaseptin derivative S4(1–13) can be exploited to promote significant discrimination between Gram-positive and Gram-negative bacteria [44] by manipulating the hydrophobicity of its N-terminal sequence. By extending the study, we show here that physicochemical properties can be exploited to reduce production costs and to promote specific antibacterial activity, although it is not clear how these lipidated peptides would behave in complex in vivo environments.

To our knowledge, this is the first time it has been shown that fatty acids can replace peptide sequences. Other studies have shown the consequences of fatty acid addition (conjugation) to AMPs [36, 44, 51, 52]. An interesting comparison can be made with regard to studies showing that acetylated hexapeptides derived from an 18-mer AMP maintained antimicrobial activity [51]. However, their nonselective activity, which was achieved at the cost of reduced potency [53], was proposed to involve peptide interaction with an intracellular target [54]. Furthermore, various optimization studies of either naturally occurring or de novo designed AMPs indicate that the optimal molecular length is represented by a 15-mer peptide [36, 41, 55–59]. In this respect, this study establishes that peptide length can be reduced to seven (or fewer) residues by replacing N-terminal amino acids with a single fatty acid while preserving the bactericidal mode of action. Such

[a] Lowest peptide concentration that inhibited bacterial growth by 100% after 24 hr incubation at 37°C. Values represent the mean ± standard deviation obtained from at least two independent experiments performed in duplicate. Lack of standard deviation reflects consistency.
[b] Clinical isolates.
[c] Strain whose MIC was verified to be >500 μM.

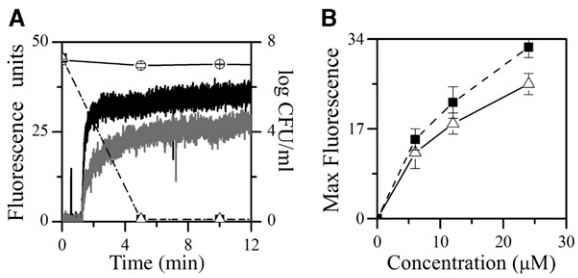

Figure 2. Cytoplasmic Membrane Depolarization and Viability of EDTA-Treated Bacteria

(A) Bacteria (*P. aeruginosa* ATCC 9027) in mid-logarithmic phase were permeabilized with EDTA (1 mM), and then diSC3-5 was added (1 μM) and quenching was allowed to occur at room temperature for 60 min. KCl (100 mM) was added to equilibrate the cytoplasmic and external K^+ concentrations. Peptides were added to cell suspensions at a concentration equal to 4 multiples of the MIC value and changes in fluorescence were recorded (excitation and emission wavelengths were 622 nm and 670 nm, respectively). At 0, 5, and 10 min intervals, aliquots were plated and incubated overnight at 37°C to assess cell viability. Shown are representative fluorescence records of S4(1–15) (black) and C_{12}-S4(8–15) (gray). Bacterial viability is shown in the absence of peptide (circles) and in the presence of S4(1–15) (squares) or C_{12}-S4(8–15) (triangles).

(B) The dose dependence for peptide concentrations equal to 1, 2, and 4 multiples of the MIC value is shown. Plotted values represent the maximum fluorescence recorded after 30 min exposure to peptides.

short lipopeptides present several advantages: besides potency aspects and being more resistant to proteolysis [36], short acylated peptides represent a considerable economic gain both because of the number of residues and because amino acids are considerably more expensive than fatty acids.

Acylation of the truncated peptide promoted selective activity. Compared with its parent peptide, C_{12}-S4(8–15) displayed reduced antibacterial activity except against pseudomonal species. We investigated the possibility that the derivatives use distinct modes of action:

(1) The peptides displayed similar helical structures. Moreover, CD data showed a correlation between active peptides and α-helical structure that was reduced or absent in inactive derivatives. Helical structure is known to stabilize amphipathic organization, which is critical for activity of many AMPs [46, 60–62] and of dermaseptins in particular [36, 39, 63]. In the present case, stabilization is conceivably mediated by interaction of the acyl chain with the hydrophobic face of the helix [64].
(2) SPR and ITC data indicated that both derivatives had lipophilic properties. Incidentally, S4(1–15) was found to bind better to *E. coli* LPS and was more active on most *E. coli* strains (average MICs are 2.1 versus 10.8, respectively, for *E. coli* and *P. aeruginosa*).
(3) Both peptides displayed rapid bactericidal kinetics, and bacterial death coincided with rapid disruption of the membrane potential.

Hence, our attempts to detect mechanistic differences strongly suggest that the peptides essentially use a similar mechanism of action. The only major difference detected concerned selectivity, which correlated with loss of two positive charges and a slight increase in molecular hydrophobicity. This resulted in reduced potency over most bacteria tested but not pseudomonal strains, apparently due to increased binding affinity mediated by the LPS component of the outer membrane. As the differences between these LPS molecules are undetermined, we are unable to further address this issue at this time. Of course, tight binding is not the only criterion for more potent biological function, especially when it comes to a complex multilevel binding event such as in the present case, where in order to kill bacteria the AMP is most likely to sequentially undergo binding events with the outer and

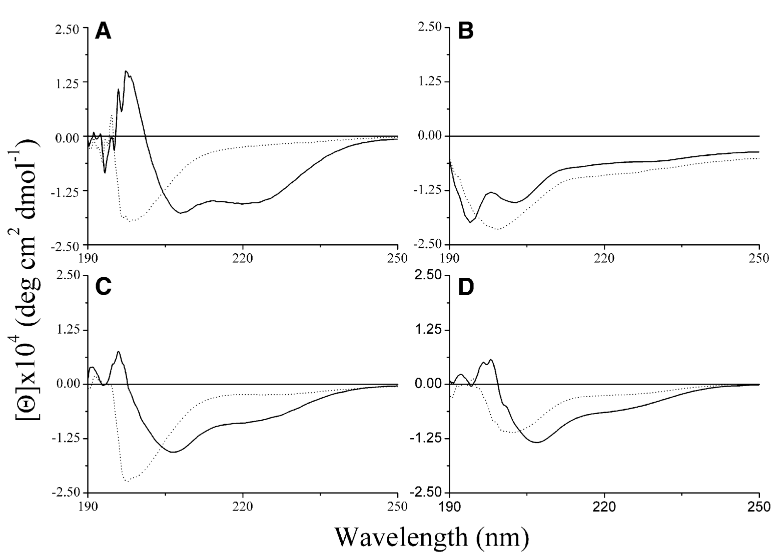

Figure 3. Effect of Truncation and Acylation on the Peptide's Secondary Structure

Circular dichroism spectra were measured for peptide samples (100 μM) that were dissolved in PBS alone (dashed line) or PBS containing POPC:POPG (3:1) (solid line). Data represent average values from three separate recordings.

(A) S4(1–15).

(B) S4(8–15).

(C) C_{12}-S4(8–15).

(D) C_{12}-S4(9–15).

Table 3. Peptide Binding Properties to Model Phospholipid Membranes Using SPR and ITC Technologies

	POPC:Cholesterol (9:1)		POPC:POPG (3:1) + *Pa* LPS		POPC:POPG (3:1) + *Ec* LPS	
	S4(1–15)	C_{12}-S4(8–15)	S4(1–15)	C_{12}-S4(8–15)	S4(1–15)	C_{12}-S4(8–15)
$K_{adhesion}$ $(M^{-1}) \times 10^{4a}$	79 ± 0.4	1.3 ± 0.8	1.5 ± 0.4	90 ± 0.6	5.6 ± 0.2	7.7 ± 0.1
$K_{insertion}{}^{a}$	236 ± 3	4.54 ± 0.02	2.25 ± 0.05	2.0 ± 0.04	10 ± 0.02	0.85 ± 0.03
$K_{apparent}$ $(M^{-1}) \times 10^{4a}$	1.9 ± 0.4 × 10^4	5.9 ± 0.7	3.4 ± 0.2	180 ± 0.3	56 ± 0.4	6.6 ± 0.3
K $(M^{-1}) \times 10^{4b}$	1.5 ± 0.1 × 10^4	6.0 ± 0.7	3.2 ± 0.4	140 ± 0.2	58 ± 0.8	6.6 ± 0.3

Chi^2 (reflecting the best fit) in both methods ranged between 2.5% and 10%.
Values represent the mean ± standard deviation obtained from two independent experiments.
[a] SPR affinity constants to each membrane were determined using integrated equations of a two-step model as detailed [38].
[b] ITC affinity constants to each membrane were determined by integrating enthalpy changes and fitting as described [64].

inner membranes plus eventual cytoplasmic components. In this context, high affinity between the AMP and LPS may be responsible for efficient recruitment of peptide molecules. The resulting LPS neutralizing effect of the initial wave will allow access of additional peptide molecules to inner bacterial targets and induce bacterial death. This concept is widely supported in the literature [29, 65–67]. Its weakness is indeed the unavailability of data on the detailed characteristics of peptide-LPS interaction. Regardless, the novelty of our data is the suggestion that a specific combination of hydrophobicity, charge, and secondary structure, such as those found in the lipopeptide C_{12}-S4(8–15), is involved in selective antibacterial activity due to its selective binding to pseudomonal LPS, albeit activity on Gram-positive bacteria and similar activity on certain pseudomonal strains seemingly argue against the proposed LPS-mediated mechanism of action. However, as can be seen in Table 2, C_{12}-S4(8–15) displayed a rather mild potency against only three out of ten bacteria tested, while in most cases (70%) the peptide was inactive with MIC > 50 μM. This residual activity is likely to occur via a different mechanism, as AMPs are known to target simultaneously multiple sites of action [21]. Similarly, as LPS varies between strains (including within the same species), this variation is very likely to explain the observed potency variations among pseudomonal strains. The fact remains that compared with its parent peptide, C_{12}-S4(8–15) displayed reduced antibacterial activity except against pseudomonal species.

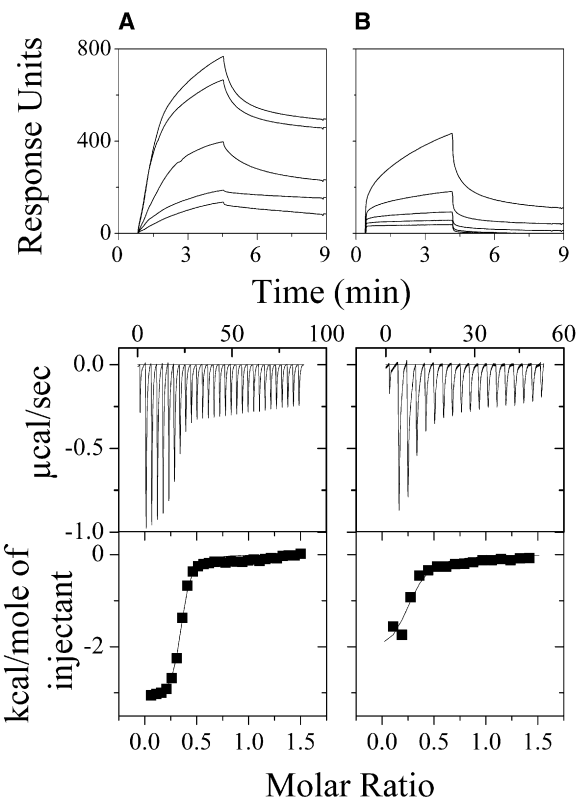

Figure 4. Peptide-Binding Properties to Model Membranes
Representative experiments from which were derived the binding parameters listed in Table 3 are shown in (A) and (B) for S4(1–15) and C_{12}-S4(8–15), respectively, using SPR (upper panel) and ITC (lower panels). SPR: shown are binding curves (association/dissociation rates) of five peptide doses (3, 6, 12, 25, and 50 μM) to a bilayer composed of POPC:cholesterol (9:1) in PBS (pH 7.4), using the L1 chip. The curves with greater intensity correspond to higher peptide concentration. Each sensorgram represents the mean of two different experiments. ITC: the middle panel is a representative isotherm obtained for titration of a 12 μM solution with 10 μl of 30 mM POPC:cholesterol (9:1) in PBS (pH 7.4). The lower panel shows the enthalpy changes for each titration data point after being integrated and fitted with a one-binding-site algorithm.

Acylation of the truncated peptide promoted reduced hemolysis. Acylation of antimicrobial peptides usually correlates with higher hydrophobic and hemolytic properties [13, 36, 45, 68]. Therefore, a surprising aspect of this study relates to the fact that C_{12}-S4(8–15) actually displayed reduced hemolysis. The dramatic strong binding of S4(1–15) to POPC:cholesterol might be of significance and therefore deserves full investigation in the future. However, there are plenty of data in the literature regarding binding of dermaseptins to erythrocytes and their correlation with hemolysis [14, 15, 38–41, 69]. Thus, based on the CD and SPR/ITC data, we propose that the N-terminal sequence which must be involved in secondary-structure induction (perhaps consequently) also induces tighter binding to the cholesterol component of the membrane (compare secondary structure and hemolysis of S4[1–15] and S4[8–15]). The fact that despite acylation, which increased its hydrophobicity, C_{12}-S4(8–15) displayed less structure, less hemolysis, and less cholesterol binding affinity, supports this hypothesis.

SIGNIFICANCE

Antimicrobial peptides present clear advantages over conventional antibiotics due to their simple structure, broad-spectrum rapid lytic activity, and ability to escape resistance. However, they notoriously lack adequate specificity and their toxicity toward red blood cells limits their potential systemic uses. The present study provides in vitro evidence in support of the concept that despite their nonspecific mechanism of action, AMPs' physicochemical properties can be exploited to promote discrimination. The data also suggest that further investigations along the presented lines of research have the potential to unravel new, safe, and economical derivatives of known antimicrobial peptides.

EXPERIMENTAL PROCEDURES

Peptides

The peptides were synthesized by the solid-phase method applying Fmoc (9-fluorenylmethyloxycarbonyl) active ester chemistry on an Applied Biosystems model 433A peptide synthesizer (Foster City, CA, USA) [70]. 4-methylbenzhydrylamine resin (Novabiochem, Darmstadt, Germany) was used to obtain amidated peptides. The acylated analogs were prepared by covalent linking of the peptide amino terminus to lauric acid as described [36]. The crude peptides were purified to $\geq$95% chromatographic homogeneity by reverse-phase high-performance liquid chromatography (HPLC) (Alliance-Waters, Milford, MA, USA). Purification and refolding of IB-367, which contains cysteine residues, were performed basically according to the procedure described by Harwig et al. [71] and repurified by HPLC as described above; the β-sheet content was confirmed by circular dichroism. The purified peptides were subjected to amino-acid analysis [72] and electrospray mass spectrometry (Micromass ZQ, Waters) to confirm their composition and stored as a lyophilized powder at -20°C. Prior to being tested, fresh solutions were prepared in water (10 mM acetate buffer for IB-367), briefly vortexed, sonicated, centrifuged, and then diluted in the appropriate medium. Buffers were prepared with bidistilled water. Polymixin B, rifampin, and piperacillin were obtained from Sigma (Jerusalem, Israel). All other reagents were analytical grade.

Bioassays

Growth Inhibition Assay

To assess peptide effect on bacterial proliferation, we used the microdilution susceptibility test [73] as modified [39] to determine the minimal inhibitory concentration (MIC), defined as the lowest peptide concentration that produced 100% inhibition of growth in overnight cultures. Briefly, bacterial suspension was grown overnight in Luria Broth medium (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl [pH 7.4]) and diluted to approximately 5×10^5 bacteria/ml. The cell populations were estimated by optical density measurements at 620 nm referred to a calibration curve. One hundred microliters from each dilution were added to 100 μl of culture medium containing no peptide (control) or various peptide concentrations (serial 2-fold dilutions) in 96-well plates (Nunc, Rochester, NY, USA). Inhibition of proliferation was determined by optical-density measurements after overnight incubation at 37°C. Antibacterial activity was routinely tested against two Gram-positive bacteria (*Bacillus cereus* ATCC 11778 and *Staphylococcus aureus* ATCC 25923) and two Gram-negative bacteria (*Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 9027). Further anti-pseudomonal and specificity tests were performed against 13 clinical isolates of *P. aeruginosa*, 10 clinical isolates of *E. coli*, and 7 clinical isolates of *S. aureus*, *Salmonella choleraesuis* (ATCC 7308), *Yersinia kristensenii* (ATCC 33639), *Acinetobacter baumannii* (ATCC 19606), *Acinetobacter lwoffii* (ATCC 15309), *Enterococcus faecalis* (ATCC 29212), and an environmental isolate of *Vibrio cholera*. The sources for pseudomonal clinical isolates listed in Table 2 are urine (ATCC 12848, 13720, 12777, 13216), abscess (ATCC 11668, 8732, 12360), sputum (ATCC 11496, 11128), catheter (ATCC 11662, 8634), peritoneal fluid (ATCC 8537), and bronchial wash (ATCC 12459).

Kinetic Studies

Bactericidal kinetics were assessed as described [37]. Briefly, bacterial suspensions of *P. aeruginosa* were added to culture medium containing 0, 1, 2, or 4 multiples of the MIC value. Bacteria were sampled after 5, 15, 30, 60, and 120 min exposure to the peptides, subjected to serial 10-fold dilutions, and plated on Luria Broth agar (LA) dishes for CFU count after overnight incubation.

Hemolysis

The peptide membranolytic potential as presented in Table 1 was measured against human red blood cells (RBCs) (1% hematocrit) after 3 hr incubation in PBS at 37°C to determine LC_{50} as described [36]. Alternatively (as specified in the Results and Figure 2), a 10% hematocrit was used and hemolysis was determined after 1 hr incubation as described [50].

Cytoplasmic Membrane Permeability Assay

The assay was performed using *P. aeruginosa* as described [74]. The lipophilic membrane-potential-sensitive cyanine dye $diSC_3$-5 concentrates within cells and self-quenches its own fluorescence. If the tested compound dissipates the membrane potential, $diSC_3$-5 will be released into the medium, causing a fluorescence increase. Bacteria (*P. aeruginosa* ATCC 9027) in mid-logarithmic phase were suspended in 5 mM HEPES (pH 7.4) to yield 0.05 optical density at 620 nm. Bacterial outer membrane was first permeabilized with EDTA to allow dye uptake, then $diSC_3$-5 was added (1 μM), and quenching was allowed to occur at room temperature for 60 min. KCl (100 mM) was then added to equilibrate the cytoplasmic and external K^+ concentrations. Peptides (1, 2, and 4 multiples of the MIC value) were added to 3 ml bacterial suspensions, and changes in fluorescence were continuously recorded (excitation and emission wavelengths at 622 nm and 670 nm, respectively). At the specified intervals, aliquots were plated on an LA plate and incubated overnight at 37°C to assess cell viability.

Liposomes

LPS-containing liposomes were prepared as described [75]. Briefly, a stock solution of LPS (30 mg/ml) in petroleum ether:chloroform: phenol mixture (8:5:2) is mixed with dried phospholipids (1:1, w/w), vacuumed overnight, suspended in PBS, heated to 60°C, vortexed, sonicated, and used as stock solution. Large unilamellar vesicles composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine/ 1-palmitoyl-2-oleoyl-sn-glycero-3 phosphoglycerol (POPC:POPG, 3:1 molar ratio) or (POPC:cholesterol, 9:1 molar ratio) were prepared in PBS by the extrusion method as per the manufacturer (Avanti Polar, Alabaster, AL, USA) instructions using a LiposoFast-Basic extrusion apparatus (Avestin, Ottawa, ON, Canada) to give a translucent solution (30 mM) of vesicles with a mean diameter of 100 nm as verified by dynamic light scattering using a BI-200SM research goniometer system (Brookhaven Instruments, Holtsville, NY, USA).

Surface Plasmon Resonance

Peptide binding to phospholipid membranes was determined using the optical biosensor system BIAcore 2000 (Biacore Life Sciences, Uppsala, Sweden). The experiments, analysis of binding kinetics, and determination of resulting affinity constants were performed as described [38]. Experiments were performed in PBS at 30°C to enable comparison with other relevant studies [42, 63].

Isothermal Titration Calorimetry

Experiments were performed using a VP-ITC microcalorimeter (Microcal Origin, Northampton, MA, USA) calibrated electronically. Heats of dilution were determined in control experiments by injecting either peptide solution or lipid suspension into buffer. The heats of dilution were subtracted from the heats determined in the corresponding peptide-lipid binding experiments. Each peptide (12 μM) was titrated with 10 μl injections of 30 mM (based on phospholipids) large unilamellar vesicles in PBS at 30°C. Enthalpy changes for each injection were integrated and fitted using the one-binding-site algorithm (Microcal Origin, version 5.0).

Circular Dichroism

CD spectra were recorded on a model J-810 spectropolarimeter (Jasco, Tokyo, Japan) connected to a Jasco spectra manager, using a QS Hellma quartz cell of 1 mm path length at 25°C between 190 and 250 nm at a scanning speed of 50 nm/min. The CD spectrum was scanned for peptide samples (100 μM) that were dissolved in sodium phosphate buffer in the presence or absence of liposomes (2 mM POPC:POPG [3:1]). Minor contributions of circular differential scattering were eliminated by subtracting the CD spectrum of buffer and liposomes without peptide. CD data represent average values from three separate recordings with 1200 scans per sample.

Peptide Self-Assembly

Aggregation properties were investigated by static light-scattering measurements as detailed [24]. Peptides at an initial concentration of 50 μM were successively diluted in 2 ml PBS at room temperature and light scattering was recorded. The static light-scattering signal is proportional to the number of aggregated molecules and the size of the aggregate. Therefore the slope is indicative of the aggregation tendency of the peptides, where a slope value above unity indicates the presence of a micellar form.

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