The Vertebrate Peptide Antibiotics Dermaseptins Have Overlapping Structural Features but Target Specific Microorganisms*

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The physiological significance of the occurrence of sequence similar antimicrobial peptides in frog skin, as the bombinins in Bombina, the magainins in Xenopus, and the dermaseptins in Phyllomedusa, is a major unanswered question. Dermaseptins s1, s2, s3, s4, and s5, a family of cationic (lysine-rich), amphipathic antifungal peptides of 28-34 residues were thus synthesized, purified to homogeneity, and evaluated for their growth-inhibition activity *in vitro* against various pathogenic microorganisms. Although all five of these peptides shared a similar spectrum of lytic activity against the filamentous fungi that are responsible for opportunistic lethal infections that follow the immunodeficiency syndrome or the use of immunosuppressive agents, they exhibited marked differences in their potencies to arrest the growth of Gram-positive and Gram-negative pathogenic bacteria and yeasts. Likewise, whereas dermaseptin s1 and s5 were devoid of hemolytic activity, dermaseptin s4 caused lysis of erythrocytes at micromolar concentrations. The dermaseptins exhibited dramatic synergy of action upon combination, resulting in some cases in a 100-fold increase in antibiotic activity of the mixture over the activity of the peptides separately. Shortening the peptide chain of dermaseptin s3 to dermaseptin s3-(1-16)-NH2 did not affect the antimicrobial potency of the peptide. Further reduction of the chain length yielded peptide derivatives gradually showing reduced activity. Surprisingly, however, analogs of dermaseptin s3 as shorter as 10-12 residues in length remained fully active against Enterococcus faecalis, Cryptococcus neoformans, and against Aeromonas caviae, the causal agent of red-leg disease in amphibians. Overall, these results suggest that, despite 40% sequence similarities, the dermaseptins have distinct spectra of antimicrobial activity and may act in concert to circumvent host invasion by providing frogs with a better shielding against a broad array of microorganisms. They also demonstrate the potential usefulness of short analogs of these peptides as potential candidates for biorational design of germicides.

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these peptides adopted random conformation in dilute aqueous solutions, but could be induced to form helical amphipathic structure in the presence of apolar solvents (13). These peptides differ in their chain lengths, charge ratio, and hydrophobicity, thus allowing the effect of these parameters on peptide activity and selectivity to be investigated.

Toward this goal, we have synthesized the five dermaseptins as well as a series of analogs and evaluated their ability, either alone or in combination, to inhibit the growth of various pathogenic agents. The results reported herein suggest that the simultaneous presence of the five dermaseptins provides frogs with a better shielding against a wider range of infecting microbes. They also demonstrate the potential usefulness of these peptides as candidates for bioregional fungicides or models for the synthesis of such fungicides.

MATERIALS AND METHODS

Solid Phase Peptide Synthesis—Peptides were prepared by stepwise solid phase synthesis using Fmoc polypeptide-active ester chemistry on a Milligen 9050 pepsin synthesizer. All Fmoc-amino acids were from Milligen. 4-hydroxy-2,3-dehydro-4-oxo-benzotriazine (Dhbt) esters were from Milligen/Bioresearch. Side chain protection were tert-butyl for Ser and His, t-butylxycarbonyl for Lys, and oxygen tert-butyl for Glu and Asp. Cleavage of peptidyl-resin and side chain deprotection were carried out at a concentration of 5 mg of peptidyl-resin in 1 ml of a mixture composed of trifluoroacetic acid, para-cresol, thioanisole, and ethyl methyl sulfide for 2 h at room temperature. After filtering to remove the resin and ether, the crude peptides were purified by a combination of Sephadex gel filtration, ion exchange chromatography, and preparative HPLC. Homogeneity of the synthetic peptides was assessed by analytical HPLC, amino acid analysis, solid phase sequence analysis, and mass spectrometry as described elsewhere (12).

Biological Assays—Antimicrobial assays were performed in sterilized 96-well plates (Nunc F96 microtiter plates, Denmark) in a final volume of 100 ml as follows. 50 ml of suspension containing 10^2 spores or microconidia/ml in Sabouraud glucose broth or 10^6 bacteria/ml in Luria-Bertani (LB) culture medium were incubated in the presence of serial 2-fold dilutions of the synthetic dermaseptins in 50 ml of water, or in the presence of 50 ml of 0.4% formaldehyde in water as a negative control, or in the presence of 50 ml of water as a positive control. The synthetic peptides were weighed in a microbalance and solubilized in water at the desired primary dilution. Inhibition of growth was determined by measuring optical density at 492 nm with a Titertek Multiskan MCC after an incubation time of 24 h at 30 °C for bacteria. Each minimal inhibitory concentration (MIC) was determined from two independent experiments performed in duplicate.

Hemolytic activity of the synthetic peptides was assayed with heparinized fresh human blood rinsed three times with phosphate-buffered saline by centrifugation for 15 min at 900 \text{x} g. Red blood cells (10^6/ml) were then incubated under agitation at 37 °C in distilled water for 100% hemolysis, in phosphate-buffered saline (50 mM sodium phosphate, 150 mM NaCl, pH 7) for control, or in phosphate-buffered saline containing various concentrations of the peptide (up to 0.250 mg/ml) in a final volume of 0.2 ml. Release of hemoglobin was monitored after centrifugation at 900 \text{x} g by measuring the absorbance of 100 ml of supernatant at 541 nm after 1 and 24 h of incubation. Part of the supernatant was dispatched for HPLC analysis as described below.

Reverberality of growth inhibition was assessed as follows. Peptides (0.2 mg/ml) were incubated in 0.5 ml suspension containing 1 x 10^5 cells/ml of Candida albicans (IP886-65), A. castae (IP67-16T), or Staphylococcus aureus (IP76-25) in Sabouraud glucose broth at 30 °C for yeast and in LB medium at 37 °C for bacteria. After various incubation times (10 min, 1 h, and 24 h) aliquots of the suspensions (100 ml) were drawn and centrifuged at 900 \text{x} g. To verify the reverberability of the inhibition, the pellet was washed three times in 1 ml of water by successive centrifugations and reincubated for 24 h at 30 °C (37 °C for bacteria) in fresh culture medium.

Stability of the Peptides to Enzymatic Degradation—Stability was assessed for dermaseptins s3, s4-1 (16)-NH₂, and s4-2 (10)-NH₂ as follows. Peptides (0.2 mg/ml) were incubated in 0.5 ml of suspension containing 10^6 cells/ml of C. albicans (IP886-65), A. castae (IP67-16T), or S. aureus (IP76-25) in Sabouraud glucose broth at 30 °C for yeast and in LB medium at 37 °C for bacteria. After various incubation times (10 min, 1 h, and 24 h) aliquots of the suspensions (100 ml) were drawn and centrifuged at 900 \text{x} g. The supernatant (40 ml) was analyzed by HPLC using a reversed-phase analytical column (Nucleosyl C18, 5 μm, 250 x 4.6 mm). The column was equilibrated with water containing 0.1% trifluoroacetic acid and eluted for 60 min at a flow rate of 0.5 ml/min with a linear gradient 0-60% of acetonitrile containing 0.07% trifluoroacetic acid. This procedure was also applied to verify peptide stability after 1 h of incubation with red blood cells.

RESULTS

Spectrum of Antimicrobial Activity of the Dermaseptins—The synthetic replicates of dermaseptins s1, s2, s3, s4, and s5 were investigated for their ability to affect the viability of various prokaryotic and eukaryotic cells in culture media. Their ability to induce cytolysis or to inhibit cell proliferation is reported in terms of MIC, defined as the dose at which 100% inhibition of growth was observed after 24 h of incubation at 30 °C (37 °C for bacteria). As shown in Table II, each dermaseptin revealed to be endowed with a large spectrum of antimicrobial activity, including Gram-positive and Gram-negative actinomycetales bacteria, cocci, and rods, as well as yeasts and filamentous fungi. Interestingly, the dose-response profiles obtained showed sharp curves in which 0–100% inhibition was generated within a 2-3-fold peptide dilution (Fig. 1). However, a close inspection of the tabulated values reveals complex patterns of antimicrobial potencies. Indeed, despite extensive structural similarities between the dermaseptin family members, they exhibited marked differences in their efficacy to inhibit microbial proliferation. These differences are most pronounced with bacteria. For instance, whereas dermaseptins s1, s2, s3, and s4 showed high potency against the causal agent of “red-leg” disease in amphibians, A. castae (MIC = 0.5–1 μM), or against E. coli (MIC = 1–4 μM), dermaseptin s5 exhibited low potency.
TABLE II

Growth inhibition activity in vitro of the dermaseptins (DS) against bacteria, yeasts, and filamentous fungi

| Organism                      | Minimal inhibitory concentrationa (µM) |
|-------------------------------|----------------------------------------|
|                               | DSs1 | DSs2 | DSs3 | DSs4 | DSs5 |
| Aeromonas caviae (IP67-16T)   | 0.5  | 1    | 1    | 0.5  | 35   |
| Escherichia coli (IP76-24)    | 1    | 2.5  | 2.5  | 4    | 35   |
| Enterococcus faecalis (IP103214) | 5    | 10   | 10   | 20   | 40   |
| Staphylococcus aureus (IP75-25) | 5    | 20   | 10   | 10   | 2    |
| Nocardia brasiliensis (IP16-80) | 35   | 20   | 5    | 10   | 40   |
| Saccaromyces cerevisiae (IP118079) | 5    | 5    | 5    | 20   | 5    |
| Candida albicans (IP984-65)   | 10   | 5    | 10   | 20   | 10   |
| C. albicans (IP886-65)        | 10   | 10   | 10   | 20   | 10   |
| Cryptococcus neoformans (IP960-67) | 1    | 1    | 1    | 2    | 1    |
| C. neoformans (IP962-67)      | 0.5  | 1    | 1    | 1    | 1    |
| Microsporum canis (IP1194)    | 15   | 15   | 15   | 15   | 15   |
| Trichophyton rubrum (IP2043-92) | 35   | 35   | 40   | 40   | 20   |
| T. mentagrophytes (IP77-71)   | 20   | 20   | 20   | 20   | 20   |
| Arthroderma simii (IP1063-74) | 30   | 30   | 30   | 30   | 30   |
| A. simii (IP1063-74 mutant)   | 30   | 20   | 20   | 20   | 30   |
| A. simii J43 (IP1065-65)      | 15   | 10   | 10   | 30   | 30   |
| Aspergillus niger (IP218-53)  | 30   | 20   | 10   | 20   | 20   |
| A. niger (IP1025-70)          | 30   | 20   | 20   | 20   | >70  |

Hemolysisb

| Human erythrocytes          | >70  | 70  | 80  | 1   | >90  |

*a The MIC is defined as the dose at which 100% inhibition of growth was observed after 24 h of incubation in culture media (36 h for M. canis, T. rubrum, and T. mentagrophytes).

*b 100% hemolysis after 1 h of incubation.

Fig. 1. Dose-dependent inhibition curves obtained for each dermaseptin (upper case) after 24 h of incubation with A. fumigatus (left) or N. brasiliensis (right). The lower diagrams show the respective curves obtained after incubation of various concentrations of dermaseptin s3, either alone (O) or in the presence (X) of 0.25 µM of each of the other dermaseptins.

against these bacteria (MIC = 35 µM). Conversely, dermaseptin s5 turned out to be the most efficient peptide in inhibiting the proliferation of the Gram-positive cocci S. aureus (MIC = 2 µM) compared with dermaseptins s1, s2, s3, and s4 (MIC = 5, 20, 10, and 10 µM, respectively). On the other hand, the order of potency against Nocardia brasiliensis, a Gram-positive filamentous branching bacteria, was dermaseptins s3 > s4 > s2 > s1 > s5. Although to a lesser extent, these differences in potency were also found against the yeasts Cryptococcus neoformans, C. albicans, and Saccharomyces cerevisiae (Table II). In sharp contrast, all the five peptides were almost as efficient in inhibiting the growth of pathogenic fungi at concentrations ranging from 10 to 40 µM (Table II). The only noticeable exception being dermaseptin s5 which showed reduced potency against the fil-
Table III
Growth inhibition activity in vitro of dermaseptin s3 (DSs3) in the absence and in the presence of a mixture of DSs1, DSs2, DSs4 and DSs6 (0.25 µM each).

| Organism                        | Minimal inhibitory concentration* DSS3 | DSS3 + DSs1, DSs2, DSs4, DSs6 |
|---------------------------------|----------------------------------------|-----------------------------|
| Aeromonas caviae (IP67-16T)     | 1                                     | <0.1                        |
| Staphylococcus aureus (IP76-25) | 10                                    | <0.1                        |
| Nocardia brasiliensis (IP76-80) | 5                                     | 1                           |
| Saccharomyces cerevisiae (IP118079) | 5                                  | 0.6                          |
| Candida albicans (IP884-65)     | 10                                    | 3                           |
| C. albicans (IP886-65)          | 10                                    | 3                           |
| Cryptococcus neoformans (IP962-67) | 1                                  | <0.1                        |
| C. neoformans (IP960-67)        |                                       | <0.1                        |
| Aspergillus niger (IP218-53)    | 10                                    | 4                           |
| Aspergillus fumigatus (IP1025-70) | 20                               | 3                           |

* The MIC is defined as the dose at which 100% inhibition of growth was observed after 24 h of incubation in culture media.

Amentous mold Aspergillus fumigatus. Another noticeable difference is evidenced by the ability of the dermaseptins to interact with human red blood cells. As reported in the lower part of Table II, 1 h treatment with dermaseptins s1 or s5 did not permeate the erythrocytes up to the highest concentration assayed (70 and 90 µM, respectively). Hemolysis of erythrocytes however occurred after treatment with dermaseptins s2 or s3 at 70 and 80 µM, respectively. Under the same conditions, treatment with dermaseptin s4 resulted in 100% hemolysis at 1 µM (50% hemolysis at 0.5 µM).

**The Dermaseptins Exhibited Synergy of Action upon Combination**—To investigate a possible synergetic relation between the dermaseptins, each peptide was tested in serial 2-fold dilutions against various microorganisms in the presence of a constant equimolar mixture composed with the remaining four peptides. As an example, Fig. 1 shows typical dose-dependent curves of growth inhibition obtained for A. fumigatus or N. brasiliensis after 24 h of treatment by the individual dermaseptins. In the absence of the other family members, the peptides exhibited MIC values ranging between 20 and >70 µM for A. fumigatus, and between 5 and 40 µM for N. brasiliensis. However, in the presence of a mixture composed of 0.25 µM of each of the dermaseptins s1, s2, s4, and s5, the MIC of dermaseptin s3 dropped from 20 to 3 µM and from 5 to 1 µM, respectively (Fig. 1). Note that, individually, none of the five dermaseptins is active at 1 µM against either one of the two microorganisms. This potency enhancement was observed for dermaseptin s3 (Table III) as well as for the other dermaseptins (data not shown) with various microorganisms. As shown in Table III, synergy of action upon combination resulted in some cases in a 100-fold increase in antibiotic activity of the mixture over the activity of the peptides separately.

**Short Dermaseptin Analogos Are Highly Potent against Several Microorganisms**—To evaluate the structural features responsible for the antimicrobial activity of the dermaseptins, COOH-terminally truncated fragments of dermaseptin s3 were assayed against various microorganisms. The data collected are summarized in Table IV. Neutralization of the negative charge at the COOH terminus of dermaseptin s3 by conversion of the carboxylate to a carboxamide yields a peptide derivative exhibiting enhanced antimicrobial potency (2-10-fold) over a broad array of pathogenic microorganisms. Stepwise elimination the COOH-terminal 14 residues of dermaseptin s3 to give dermaseptins s3-(1-20)-NH₂ and s3-(1-16)-NH₂ did not significantly alter the peptide potency. For instance, the 16-residue peptide retained many of the features of the parent compound and conserved a large spectrum of activity. Only in a few cases, such as with S. aureus and N. brasiliensis, these shorter analogs showed reduced potency compared with the parent molecule. Interestingly, although highly potent against pathogenic microorganisms, s3-(1-16)-NH₂ was devoid of hemolytic activity even at higher concentrations (150 µM) compared with dermaseptin s3 (Table IV). Further stepwise shortening of the chain length down to dermaseptin s3-(1-10)-NH₂ yielded peptide derivatives exhibiting a slow gradual loss in potency compared to dermaseptin s3, namely, against S. aureus or N. brasiliensis as well as against the filamentous fungi. Surprisingly, however, these shorter analogs still displayed high potency against bacteria, such as Aeromonas caviae, Escherichia coli, Enterococcus faecalis, and the yeasts. In few cases, s3-(1-10)-NH₂ was practically as efficient as the native peptide, yet this shorter s3 version was devoid of hemolytic activity.

**Stability of the Dermaseptins**—To evaluate the susceptibility of peptides to enzymatic degradation, aliquots from the suspensions assayed with dermaseptins s3, s3-(1-16)-NH₂ and s3-(1-10)-NH₂ were analyzed by HPLC at various incubation times against S. albitscins, A. caviae, S. aureus, and human erythrocytes. Whereas dermaseptin s3 and s3-(1-16)-NH₂ displayed potent inhibitory activity against S. aureus, their concentrations in the suspensions gradually decreased in time and vanished after 24 h of incubation (Fig. 2). While devoid of antimicrobial activity against S. aureus, dermaseptin s3-(1-10)-NH₂ was conserved almost intact in the suspensions after 24 h of incubation time. On the other hand, although these three peptides were active against C. albicans and A. caviae, their concentrations were stable throughout the experiment as at least 90% of the initial peptide could be recovered after 24 h of incubation (data not shown). While the disappearance of the active peptides may result from differential susceptibility to intracellular proteolytic enzymes released consequently of their cytolytic activity, these results suggest that there may not be necessarily a direct correlation between peptide stability and activity. In addition, they clearly rule out the possibility that the loss of activity is due to enzymatic degradation. Therefore, the observation that dermaseptin s3-(1-10)-NH₂ is devoid of antimicrobial activity against S. aureus may mostly reflect the lack of the molecular elements that are responsible for membrane recognition and activity. The fact that dermaseptin s3, a hemolytic peptide, is partially degraded after 1 h of incubation with human erythrocytes while the nonhemolytic peptides dermaseptins s3-(1-16)-NH₂ and s3-(1-10)-NH₂ are stable is in accordance with this scheme (data not shown).

**Dermaseptins Cause Irreversible Growth Inhibition**—To gain insight into the mechanism of action of the dermaseptins, suspensions of C. albicans, A. caviae, and S. aureus were treated each with 0.2 mg/ml of dermaseptins s3, s3-(1-16)-NH₂ or s3-(1-10)-NH₂. After various incubation times, the microorganisms were harvested by centrifugation, thoroughly washed and reincubated in fresh medium for 24 h. After 10 min of treatment with any one of the three peptides, washed C. albicans and A. caviae did not proliferate. Whereas washed S. aureus did not proliferate after treatment previously with dermaseptins s3 or with s3-(1-16)-NH₂, it did proliferate when treated with dermaseptin s3-(1-10)-NH₂. These results remained unchanged after 1 or 24 h of treatment. This correlates nicely with the results reported in Table IV and demonstrates that the effects of the dermaseptins are instantaneous and irreversible.

**DISCUSSION**

In the attempt to understand possible reasons for the occurrence of many structurally related dermaseptins in frog skin, dermaseptins s1, s2, s3, s4, and s5 were prepared by stepwise solid phase synthesis, purified to homogeneity, and investi-
Antimicrobial Activity of Dermaseptin Peptides

Growth inhibition activity in vitro of dermaseptin s3 and truncated analogs against bacteria, yeasts, and fungi

| Organism                        | s3 | s3-(1-30)a | s3-(1-20)a | s3-(1-16)a | s3-(1-15)a | s3-(1-12)a | s3-(1-10)a |
|--------------------------------|----|------------|------------|------------|------------|------------|------------|
| Aeromonas caviae (IP67-16T)    | 1  | 0.5        | 2          | 2          | 3          | 6          | 7          |
| Escherichia coli (IP76-24)     | 2.5| 0.5        | 2          | 2          | 3          | 5          | 14         |
| Enterococcus faecalis (IP103)  | 10 | 2          | 2          | 3          | 6          | 7          |
| Staphylococcus aureus (IP76-25)| 10 | 1          | 8          | 60         | >150       | >150       | >150       |
| Nocardia brasiliensis (IP16-80)| 5  | 2.5        | 30         | 70         | >150       | >150       | >150       |
| Saccaromyces cerevisiae (IP118079)| 5 | 2.5        | 4          | 4          | 10         | 20         | 40         |
| Candida albicans (IP884-65)    | 10 | 4          | 4          | 8          | 20         | 40         | 150        |
| Cryptococcus neoformans (IP960-67)| 1 | 0.5        | 1          | 2          | 3          | 6          | 25         |
| Arthrodema simili (IP1063-74)  | 30 | 10         | 15         | 8          | 30         | 70         | >150       |
| Aspergillus niger (IP218-53)   | 10 | 10         | 15         | 15         | 40         | 90         | >150       |
| Aspergillus fumigatus (IP1025-70)| 20| 10         | 15         | 8          | 20         | 90         | >150       |

Hemolysis

| Human erythrocytes | s3 | s3-(1-30)a | s3-(1-20)a | s3-(1-16)a | s3-(1-15)a | s3-(1-12)a | s3-(1-10)a |
|--------------------|----|------------|------------|------------|------------|------------|------------|
|                    | 80 | ND         | ND         | >150       | ND         | ND         | >150       |

The MIC is defined in the legend of Table II, a = amide.

100% hemolysis after 1 h of incubation.

Not determined.

gated for their ability to affect the viability of various prokaryotic and eukaryotic cells in vitro. Overall, the data presented show for each peptide broad, but not identical, spectra of antimicrobial activity. At micromolar concentrations, each dermaseptin was highly efficient in killing selectively certain microbes (Table II). Moreover, their activity was considerably amplified upon combination. Hence the biological significance of the presence of five closely related dermaseptins in frog skin may thus be understood in terms of advantage for survival under hostile environmental conditions. To defend itself against pathogenic microbes, the naked skin of frog would produce a fixed repertoire of small-sized and structurally related antimicrobial peptides that are promptly synthesized at a low metabolic cost, easily stored in large amounts, and readily available to provide the frog with a maximum coverage over a wide range of possible invading microorganisms.

Concerning the mode of action of the dermaseptins, previous studies performed with dermaseptin s1 have suggested that basicity and amphiphility of the NH₂-terminal domain are responsible for binding and aggregation of the peptide at the plasma membrane, inducing consequently cell lysis (21–23).

Although the precise mechanism of action of the four new dermaseptins is yet to be demonstrated, it is likely that they act in a manner similar to that established for dermaseptin s1 (21), i.e. (i) complementarity between the charge distributions of the peptide and the polar head groups of phospholipids initiates association of the peptide with the membrane surface via electrostatic interactions; (ii) upon interacting with the apolar interface, the peptide adopts α-helical structure corresponding to its hydrophobic period that provides a low energy conformation in which the hydrophobic residues are maximally dehydrated and the polar residues are maximally hydrated; (iii) the helical peptide penetrates deeply into the membrane contacting fatty acyl chains and disrupting the fluidity of the membrane. Concordant with this view are the following observations: (a) multiple sequences alignment of the five dermaseptins yielded 40% amino acid positional identity (Table I); (b) the dose response curves (Fig. 1), obtained with all dermaseptins, evolved from 0 to 100% inhibition generally within the range of 2–3-fold peptide dilutions, suggesting that peptide aggregation occurs at a...
threshold concentration (21); and (c) the effect induced by treatment with each dermaseptin was rapid (<10 min) and irreversible.

Since the dermaseptins differ only in their net charge, charge distribution, hydrophobicity of the nonpolar face, and average angle subtended by the polar face (Table I), the effect of these parameters on lipid affinity, and thus on peptide potency, can be investigated (29). As shown in Table II however, some dermaseptins exhibited a similar potency against several microorganisms despite the fact that they greatly differ in their above cited structural parameters. On the other hand, despite extensive homology in their structural profiles, some dermaseptins exhibited up to 70-fold differences in potency to inhibit the growth of various microorganisms (Table II). In a similar vein, one property of the dermaseptins that should be explained is their differential ability to target specific microorganisms. Indeed, the dermaseptins displayed a heterogeneous profile of potencies over the different microorganisms tested (Table II). Perhaps, the most remarkable divergence between them is their preferential affinity for pathogenic microbes versus mammalian cells, as most dermaseptins are barely or not toxic for human erythrocytes while dermaseptin s4 is highly hemolytic. A possible explanation for this may lie in the fact that s4 has, by far, the highest hydrophobic moment. When compared with the remaining dermaseptins, the summed hydrophobic indexes of its side chains is +28.9 compared with +6.3, +13.0, +10.5, and +9.7, respectively, for dermaseptins s1, s2, s3, and s5 (Table I). Also, the highest difference in hydrophobicity is localized at the NH₂ terminus, a domain being of crucial importance for effective interactions with membranes (23). Therefore one is tempted to hypothesize that highly hydrophobic NH₂ terminus within amphipathic cationic peptides would confer hemolytic activity. This hypothesis is experimentally supported by the use of the 27-residue antimicrobial nonhemolytic peptide, dermaseptin bicolor (DS b1): DVLKKQGIVALVAGKAALGA-VADTISQ-NH₂ (28). Addition of hydrophilic residues to its NH₂ terminus resulted in a considerable enhancement of its antimicrobial potency but did not affect its hemolytic properties (data not shown). Further addition of hydrophobic residues yielded peptide derivatives increasingly hemolytic with increasing hydrophobicity i.e. 100% hemolysis was observed at 15 μg/ml for LMWKK-DS b1, at 60 μg/ml for WKK-DS b1; no hemolysis was observed up to 1000 μg/ml for KK-DS b1 or for native DS b1. In that regard, it is interesting to note that a weak hemolytic activity was observed for dermaseptin s2 but not for s1, dermaseptin s2 being slightly more hydrophobic due to two substitutions i.e. K/F and D/N at positions 4 and 27, respectively. Accordingly, the hemolytic capacity of dermaseptin s3 (Table IV) may have been abolished by decreasing its hydrophobicity, the summed hydrophobic indexes of s3-(1-16)-NH₂ and s3-(1-10)-NH₂ being +0.2 and +3.2, respectively, compared with +10.5 for the native peptide.

Thus, whereas slight structural variations, e.g. the net charge, the charge distribution around the polar face, and bulkiness of the nonpolar face seem important, although not fully understood, factors for fine tuning the lipid targeting of these peptides, it is most probable that lipid affinity may vary considerably depending on the composition of the fatty acyl chains, the content of cholesterol, and the hydrophobic thickness of the lipid bilayers. Therefore, in contrast to the currently growing opinion that often generalize the bioactive properties of cationic-amphiphilic peptides, this study showed that this view may be oversimplified since subtle structural differences in the dermaseptin family members lead to marked differences in both the spectrum of activity and in potency.

In a preliminary study that concerned dermaseptin s1 (23), we showed that the mere deletion of 2 residues from its NH₂ terminus lowered its lytic potency against C. albicans and C. neoformans. On the other hand, dermaseptin s1-(1-18) displayed a comparable potency with that of the native peptide, and dermaseptin s1-(16-34) was devoid of antimicrobial activity (23). In addition, amidation of the carboxyl at the COOH-terminal end of dermaseptin s1-(1-18) improved its potency. This suggested that the α-helical NH₂-terminal domain of dermaseptin s1 contains the essential features responsible for its activity. To probe further these properties we evaluated the minimal structural requirements for potent antimicrobial activity using dermaseptin s3. This peptide represents a convenient tool to investigate the structure-function relationships in terms of how the chain length and charge content correlate with lytic potency since, among the five dermaseptins, dermaseptin s3 presents the most regular structure, with lysine residues punctuating every fourth amino acid position along the peptide chain. In agreement with previous observations, we find in this study that amidation of the carboxyl at the COOH-terminal end of the native peptide improves its potency (Table IV). However, analysis of the results obtained with COOH-terminally truncated analogs revealed complex patterns of behavior, making it impossible to draw general predictive rules. For the sake of clarity the results obtained from these experiments were divided into two groups (Fig. 3): a group of microorganisms for which stepwise deletions from 30 to 10 residues gradually led to loss of potency (Fig. 3, upper diagram), and a group for which potency was hardly affected by these modifications. In the first group, deletion of the 10 COOH-terminal residues, which did not vary the net positive charge of the peptide, resulted in a general increase in MIC except for E. faecalis and C. albicans for whom the MIC remained un-
Antimicrobial Activity of Dermaseptin Peptides

31641

changed. Further reductions in chain length down to derma-
septin s3-(1–16)-NH₂ which, conserve the net positive charge
of the parent peptide, resulted in a further increase in MIC for
S. aureus, N. brasiliensis, and C. albicans, while no change in
MIC was observed for Aspergillus niger. Interestingly, the
chain reduction resulted in a more efficient inhibition of Arth-
roderma simii and A. fumigatus. Reduction of the chain length
down to derma-
septin
roderma simii
and, although to a lesser extent, for
C. albicans, A. simii, A. fumigatus, and A. niger which still
displayed a good sensitivity. Finally, all members of this group
were insensitive to the 10-mer version probably due to the loss
of the molecular elements responsible for its activity against
these microorganisms. As illustrated in the lower diagram of
Fig. 5, all of the mentioned above modifications hardly affected
the members of the second group (A. caviae, E. coli, E. faecalis,
C. neoformans, and S. cerevisiae). The MIC observed for all
peptides remained practically unchanged, analogs of derma-
septin s3 as shorter as 10–12 residues in length being highly
active against these microorganisms. These results demon-
strate the potential usefulness of these short analogs as candi-
dates for biorational-targeted antibiotics or as a model for the
design of such antibiotics. Finally, in the search of a universal
minimal pharmacophore, this study revealed the feasibility
of such a task only when it is targeted against individual
microorganisms.

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REFERENCES
1. Bevins, C. L., and Zasloff, M. (1990) Annu. Rev. Biochem. 59, 395–414
2. Lehrer, R. I., Ganz, T., and Selsted, M. E. (1991) Cell 64, 229–239
3. Nicolas, P., Mor, A., and Delfour, A. (1992) Medicine/Sciences 8, 425–431
4. Lazarus, L. H., and Attila, M. (1993) Prog. Neurobiol. 41, 473–507
5. Boman, H. G., and Hultmark, D. (1987) Annu. Rev. Microbiol. 41, 103–126
6. Hoffmann, J. A., Dimarco, J. L., and Bulet, P. (1992) Medecine/Sciences 8, 462–469
7. Gibson, B. W., Tang, D., Mandrell, R., Kelly, M., and Spindel, E. R. (1991) J. Biol. Chem. 266, 23103–23111
8. Simmaco, M., Barra, D., Chiarrini, F., Noviello, L., Melchiori, P., Kreil, G., and Richter, K. (1991) Eur. J. Biochem. 199, 217–222
9. Giovanni, M. G., Poulter, L., Gibson, B. W., and Williams, D. H. (1987) Biochem. J. 245, 115–120
10. Zasloff, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5449–5453
11. Terry, A. S., Poulter, L., Williams, D. H., Ntukis, J. C., Giovannini, M. G., Moore, C. H., and Gibson, B. W. (1988) J. Biol. Chem. 263, 5745–5751
12. Mor, A., Nguyen, V. H., Delfour, A., Migliore, D., and Nicolas, P. (1991) Biochemistry 30, 8824–8830
13. Mor, A., and Nicolas, P. (1994) Eur. J. Biochem. 219, 145–154
14. Zasloff, M., Martin, B., and Chen, H. C. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 910–913
15. Williams, R. W., Starman, R., Taylor, K. M., Gable, K., Beeler, T., Zasloff, M., and Covell, D. (1990) Biochemistry 29, 4490–4496
16. Cruccu, A. R., Barker, L. D., Zasloff, M., Chen, H. C., and Colaneri, O. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 3752–3756
17. Rana, F. R., Macias, K. A., Sultany, C. M., Modzrakowski, M. C., and Blazyk, J. (1991) Biochemistry 30, 6556–6566
18. Chung, A. L., Leer, D. J., and DeGrado, F. W. (1992) Biochemistry 31, 6606–6616
19. Blondelle, S. E., and Houghten, B. A. (1992) Biochemistry 31, 12988–12994
20. McLean, L. R., Hagaman, K. A., Owen, T. J., and Krstenanski, J. L. (1991) Biochemistry 30, 31–37
21. Pouy, Y., Rasaport, D., Mor, A., Nicolas, P., and Shai, Y. (1992) Biochemistry 31, 12416–12423
22. Hernandez, C., Mor, A., Daguer, F., Nicolas, P., Hernandez, A., Benedetti, L., and Dunia, I. (1992) Eur. J. Cell Biol. 59, 414–424
23. Mor, A., and Nicolas, P. (1994) J. Biol. Chem. 269, 1934–1939
24. Kotelchuck, E., Sola, M., Simian, H. I., and Berger, A. (1964) The Proteins: Composition, Structure and Function (Nehrath, H., ed) Vol. 2, Chapter 10, pp. 406–581, Academic Press, New York
25. Onellette, A. J., Greco, R. M., James, M., Frederick, D., Naftilan, J., and Pallen, J. T. (1989) J. Cell Biol. 108, 887–895
26. Dodici, R. F. (1985) Trends Biochem. Sci. 10, 227–233
27. Mor, A., Nguyen, V. H., and Nicolas, P. (1991) J. Med. Chem. 34, 216–220
28. Mor, A., Aniche, M., and Nicolas, P. (1994) Biochemistry 33, 6642–6650
29. Segrest, J. P., De Loof, H., Dohman, J. G., Broutinie, C. G., and Anantharamaiah, G. M. (1990) Proteins 8, 103–117
30. Huggins, D. G., and Sharp, P. M. (1989) Comput. Appl. Biochem. 5, 151–153
31. Garnier, J., Osguthorpe, D., and Robson, B. (1978) J. Mol. Biol. 120, 97
32. Kise, J., and Dodici, R. F. (1982) J. Mol. Biol. 157, 105–132