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Sequence Requirements and an Optimization Strategy for Short Antimicrobial Peptides

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Summary

Short antimicrobial host-defense peptides represent a possible alternative as lead structures to fight antibiotic resistant bacterial infections. Bac2A is a 12-mer linear variant of the naturally occurring bovine host defense peptide, bactenecin, and demonstrates moderate, broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria as well as against the yeast *Candida albicans*. With the assistance of a method involving peptide synthesis on a cellulose support, the primary sequence requirements for antimicrobial activity against the human pathogen *Pseudomonas aeruginosa* of 277 Bac2A variants were investigated by using a luciferase-based assay. Sequence scrambling of Bac2A led to activities ranging from superior or equivalent to Bac2A to inactive, indicating that good activity was not solely dependent on the composition of amino acids or the overall charge or hydrophobicity, but rather required particular linear sequence patterns. A QSAR computational analysis was applied to analyze the data resulting in a model that supported this sequence pattern hypothesis. The activity of selected peptides was confirmed by conventional minimal inhibitory concentration (MIC) analyses with a panel of human pathogen bacteria and fungi. Circular-dichroism (CD) spectroscopy with selected peptides in liposomes and membrane depolarization assays were consistent with a relationship between structure and activity. An additional optimization process was performed involving systematic amino acid substitutions of one of the optimal scrambled peptide variants, resulting in superior active peptide variants. This process provides a cost and time effective enrichment of new candidates for drug development, increasing the chances of finding pharmacologically relevant peptides.

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Introduction

Antibiotic therapy is under severe pressure due to increased antibiotic resistance. According to the 2004 report of the National Nosocomial Infection Surveillance (NNIS) [1], the rate of resistance to methicillin of coagulase-negative *Staphylococci* (CNS) reached 89.1%, while that of *Staphylococcus aureus* (well known as MRSA) increased to 59%. Similar trends were seen for other pathogenic bacteria against third-generation cephalosporins such as ceftriaxone, cefotaxime, and ceftazidime, for example, *Klebsiella pneumoniae* for which resistance increased nearly 2-fold to reach 20.6% and *Pseudomonas aeruginosa*, which had become 31.9% resistant.

Cationic antimicrobial peptides provide potential templates for a new generation of antimicrobials. They kill microorganisms rapidly and directly, demonstrate synergy with conventional antibiotics, and in some cases, activate host innate immunity. Moreover, some seem to counteract harmful inflammatory/septic responses induced by bacterial endotoxin [2], which is extremely important since rapid killing of bacteria and subsequent liberation of bacterial components such as LPS or peptidoglycan can induce potentially fatal immune dysregulation [3, 4].

The bovine cationic peptide bactenecin (also called dodecapeptide) is one of the smallest naturally occurring antimicrobial peptides. It was discovered in bovine neutrophils by Romeo et al. in 1988 [5]. Bactenecin (RLCRIVVIRVCR) is stabilized by an internal disulfide bridge and is moderately active against Gram-negative bacteria and against certain Gram-positive bacteria (e.g., *S. pyogenes*, *C. xerosis*). A linear variant, Bac2A (RLARIVVIRVAR-NH_2), in which the cysteine residues were replaced with alanines, showed similar activity against Gram-negative bacteria but improved activity against Gram-positive bacteria [6]. The features of Bac2A, namely small size, linearity, and activity against both Gram-positive and Gram-negative bacteria, make this peptide an ideal lead structure to develop new antimicrobial drugs.

There are hundreds of cationic peptides from nature with a wide variety of sequences, structures, and spectra of activity that vary substantially in potency [7]. This diversity is matched by a number of complex and contentious models that attempt to describe and explain their modes of action, although central to their mode of action is their ability to interact with membranes [8]. Previously, detailed studies of primary sequence requirements were

Table 1. Substitution Analysis of a Single Position of Three Different Peptides

Original Sequence	Activity Relative to the Parent Peptide with Different Substituted Amino Acids[a]																			
	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
RLARIVVIRV**A**R[a]	6.3	**2.5**	13.6	19.4	5.5	**4.3**	**3.8**	**3.1**	**2.7**	**4.4**	5.1	5.4	9.4	5.5	**2.3**	5.8	5.3	5.7	5.4	**3.2**
RL**W**RIVVIRV**A**R	1.0	3.9	12.9	12.4	3.5	1.3	1.0	2.5	**0.2**	2.0	1.6	0.8	1.2	1.2	**0.7**	1.4	2.1	2.1	2.5	3.1
RL**R**RIVVIRV**A**R	1.0	0.9	3.2	2.7	**0.5**	1.0	**0.6**	1.0	**0.6**	**0.7**	0.8	1.0	1.7	0.9	**0.6**	1.1	1.3	0.9	**0.6**	**0.5**

The amino acids, given in the one letter code, are substituted against the underlined and bold position in the peptides. This assay was performed with peptides synthesized on cellulose and a luminescent indicator strain (see Experimental Procedures). The first peptide is called Bac2A, and the other two peptides are variants of Bac2A in which the third position (bold) is substituted by a Trp or Phe, respectively. The numbers in the table are all relative EC_{50} values. All observed EC_{50} values are divided by the EC_{50} value of the parental peptide and, for comparison, normalized by using the MIC whereby the MIC of the first peptide is 6.3-fold higher than for the next two peptides. Consequently, values higher than 6.3 for the first peptide or 1 for the next two peptides correspond to weaker activity (relative to the parent peptide), and values lower than 6.3 for the first peptide or 1 for the next two peptides correspond to stronger activities. Bold numbers represent peptides with antimicrobial activities against *Pseudomonas aeruginosa* strain H1001 at least 30% stronger than the parent peptide.

[a] Data taken from Hilpert, 2005 [9]. Normalized according to the MICs of the parent peptides for *P. aeruginosa*. The MIC of Bac2A against wild-type *Pseudomonas aeruginosa* was determined to be 50 μg/ml, whereas both variants of Bac2A showed an MIC of 8 μg/ml [9].

restricted by the high cost and the time consumption to synthesize larger quantities of peptides on resin. Peptide arrays on cellulose provide an effective and relatively inexpensive method for surveying large numbers of peptide variants for particular activity determinants. Recently, we reported the application of this technique [9] to investigate the killing of *Pseudomonas aeruginosa* strain H1001 [10]. Strain H1001 has a luciferase gene cassette incorporated and can be used to monitor viability of these bacteria. The peptides synthesized on a cellulose support can be cleaved from the support by ammonia gas; inserted and serially diluted into a microtiter plate. Finally, the peptides are incubated with the indicator bacterium at 37°C, and luminescence is detected in a time-dependent manner. In this paper, we have applied this newly developed method to increase understanding of the primary sequence requirements for short antimicrobial peptides (12 amino acids) in respect to killing the pathogenic Gram-negative bacterium *P. aeruginosa*.

Results and Discussion

Single Position Substitution Analyses

Previous studies utilized a complete substitution analysis of the peptide Bac2A (RL**A**RIVVIRV**A**R-NH_2) to demonstrate, for example, that Ala residues at positions 3 and 11 can be substituted by many other amino acids [9]; with Arg, Phe, and Trp, in particular, being favored at position 3. For example, exchanging Ala with Arg or Trp at position 3 led to two Bac2A variants (R3 = RL**R**RIVVIRVAR-NH_2 and W3 = RL**W**RIVVIRVAR-NH_2) showing superior minimal inhibitory concentrations (MICs) of 8 μg/ml against wild-type *P. aeruginosa* strain H103, compared to 50 μg/ml for the parent peptide Bac2A [9]. To investigate whether different occupancy at position 3 favored particular substitutions at position 11, single-position substitution analyses at position 11 for the peptides Bac2A, R3, and W3 were analyzed for their ability to kill *P. aeruginosa* strain H1001 (Table 1). Only substitutions with the positively charged amino acids Arg and Lys were able to universally improve activity relative to the respective parent peptides, Bac2A, R3, or W3. For Bac2A, substitutions of Ala with Arg, Cys, Gly, His, Lys, Ile, Leu, and Tyr all improved activity, while most other substitutions, with the exception of the acidic residues Asp and Glu, were well tolerated. In case of peptide W3, where position 3 is filled with the hydrophobic amino acid Trp, the activity decreased if position 11 was occupied by another hydrophobic amino acid such as Phe> Tyr>Trp/Ile>Thr and Val. Only substitutions with Lys and Arg led to improved activity, while Asn and His were well tolerated. In contrast, peptide R3, with a positively charged hydrophilic amino acid substituted at position 3, was much better able to accommodate substitutions at position 11, including hydrophobic residues like Phe/Tyr>Trp>Leu, as well as hydrophilic positively charged ones such as Arg, His, or Lys, resulting in improved activity, while only Asp and Glu were detrimental. It is quite clear from these results that changes in any single position of the peptide may impact on the preferred residues at other positions in the peptide. Each single substituted peptide variant may lead to different preferred substitutions at any other position, which was chosen for optimization. Each of these variants could, in principle, be used for a further optimization process, but this would result in a very complex optimization strategy.

Luminescence-Based Killing Assay with Scrambled Bac2A Peptides

To investigate the flexibility of amino acid arrangements for creating active peptides, we decided to use a very simple approach by scrambling the sequence of an active peptide. Consequently 49 peptide variants of Bac2A were created by a nonbiased random computer-driven scrambling of sequences (Table 2). Each of these variants were composed of the same amino acids and had the same length, net charge, and amount of hydrophilic and hydrophobic amino acids and thus were ideally suited for testing the importance of the primary amino acid sequence for activity. For example, it has been demonstrated that scrambling of a sequence destroys sequence-specific interactions, as observed, e.g., with antibodies recognizing linear epitopes. For this type of sequence-specific interaction, scrambled peptides are normally used as a negative control to prove the sequence specificity. Conversely if amino acid composition was the sole determinant of killing activity, all scrambled peptides should be active.

Assessment of killing of *P. aeruginosa* by the scrambled peptides indicated that they fell into six different activity classes (Table 2), whereby six were substantially more active than Bac2A (activity class 1), four slightly

Table 2. Effect of Scrambled Variants of Bac2A on Antibacterial Activity against *P. aeruginosa* Strain H1001 as Assessed by the Rate of Decrease of Luminescence

Peptide	Sequence	Activity Class[a]	Peptide	Sequence	Activity Class[a]
Bac006	VRIRARRVILVA	1	Bac007	RRLVAIVAVRRI	4
Bac010	AAVRRRVRLVII	1	Bac011	AVRVRRRAILVI	4
Bac014	RAVAVIIRLRRV	1	Bac021	ARIARRVRILVV	4
Bac020	RRAAVVLIVIRR	1	Bac024	RAIIRRVLVRVA	4
Bac034	VRLRIRVAVIRA	1	Bac032	ARRARIRILVVV	4
Bac043	RVLIVIRARRVA	1	Bac036	RARRVRVLIAIV	4
Bac08	VLIRIRRVARAV	2	Bac037	RAIRVRRIVLAV	4
Bac016	RARIVRVRVILA	2	Bac041	RLRVAIVAIVRR	4
Bac018	RRVAIVVIARLR	2	Bac048	VVRALRRIIARV	4
Bac029	IILAVRAVRRVR	2.5	Bac023	RRRAIVRVVAIL	4.5
Bac2A	**RLARIVVIRVAR**	**3**	Bac030	IVVRRRRAALVI	4.5
Bac002	RRIARVIVAVLR	3	Bac003	ARRLIVRVRVIA	5
Bac004	IRARIAVRRVVL	3	Bac009	IIRAALRRVRVV	5
Bac005	IVRVAVALRRIR	3	Bac017	VILARRRVRIAV	5
Bac012	IAARRLIRVVRV	3	Bac019	ILVARVIRRRVA	5
Bac013	VARIVVRLIRAR	3	Bac028	VIVRLAARRVRI	5
Bac015	AVRAIRVLRVIR	3	Bac031	LAIVRRARVRIV	5
Bac025	ARAILIRVVRRV	3	Bac033	IRVRRLVAAVIR	5
Bac026	IARRIVAVRLRV	3	Bac035	RVLRVVRAAIRI	5
Bac042	ILVIVRRRARAV	3	Bac038	VVIRAAIRRVRL	5
Bac045	VIALRIAVRRVR	3	Bac039	RIVLRRAAVIRV	5
Bac046	RRRVIVAVLARI	3	Bac040	VLARVVARRIRI	5
Bac047	RVLIAARVIRRV	3	Bac027	RVLIARVVRAIR	5.5
Bac049	VIALVRARVRRI	3	Bac022	ILRRVRVRAVAI	6
Bac050	RRVIAIAVRRLV	3	Bac044	VIRRRRILAAVV	6

[a] The table is ordered by the activity classes as follows: class 1, peptides showing stronger killing than Bac2A at a 4- to 8-fold higher dilution; class 2, peptides showing stronger killing compared to that of Bac2A at a 4-fold higher dilution; class 3, peptides similar to Bac2A; class 4, peptides similar to Bac2A at the highest concentration but demonstrating weaker killing at a 2-fold dilution; class 5, peptides giving very weak or no killing at a 2-fold dilution; class 6, peptides with weak killing activity at the highest peptide concentration.

more active (activity class 2), 14 showed similar activity (activity class 3), 11 showed slightly lower activity (activity class 4), 12 lower activity (activity class 5), and two were completely inactive (activity class 6). Examination of the sequences within a given activity class demonstrated that peptides that varied substantially from the primary amino acid-specific sequence of Bac2A were able to kill *P. aeruginosa*. These data therefore clearly showed that there were no sequence-specific interactions and thus favored a scenario whereby, in addition to the appropriate amino acid composition, there is one or more additional features that determined activity. A similar observation with an α-helical antimicrobial peptide was made in that a scrambled variant of a wild-type peptide showed a similar or stronger activity against different bacterial strains [11].

To confirm these data and the observed activity classes, three of the most active class 1 peptides and two of the least active class 6 peptides were chosen to be synthesized on resin. The minimal inhibitory concentrations (MIC) were determined for the important medical bacterial pathogens *P. aeruginosa*, *E. coli*, *Salmonella typhimurium*, *S. aureus*, *S. epidermidis*, and *E. faecaelis* and the yeast *Candida albicans*. The measured MIC values (Table 3) confirmed the classification of the peptides. All three activity class 1 peptides showed lower MICs than Bac2A against *Pseudomonas aeruginosa*, with the best being about 7-fold more active. The two activity class 6 peptides showed an MIC that was at least 5-fold higher than Bac2A. The MICs for *P. aeruginosa* grown under high and low magnesium conditions demonstrated the same 4-fold difference in MIC for both Bac2A and the activity class 1 peptides (data not shown), indicating that increased activity reflected more than just decreased antagonism by divalent cations.

The data indicate that the tactic of scrambling a peptide sequence is potentially useful as an optimization approach for short linear antimicrobial peptides. This easy and rapid approach opens up novel possibilities to enrich the pool of excellent candidate peptides that can then be further optimized or screened for other desired qualities, like bioavailability, nontoxicity, stability, and solubility.

QSAR Computational Analysis

Sequence alignments of the scrambled Bac2A peptides failed to demonstrate any correlation between peptide sequence and activity. This result supports the hypothesis that there is no sequence-specific interaction involved in killing activity against bacteria. To identify any general structural or molecular properties that might correlate with activity, a QSAR computational analysis with the program MOE (Chemical Computing Group, Inc.) was performed. For this analysis, peptides were separated into two major pools: active (similar or better than Bac2A) and less active peptide variants (worse than Bac2A). Given the lack of three-dimensional structures and the similar composition of the peptides (same scrambled sequence), descriptors were developed that related to sequence ordering and fragment-based hydrophobicity. A classification model (Figure 1) was developed on the basis of these descriptors [12] and demonstrated that the active peptides could be classified on the basis of the distance between the middle two Arg residues and possession of a certain hydrophobicity (>2) in a span of five consecutive residues

Table 3. Determination of the Minimal Inhibitory Concentrations in Mueller-Hinton Media for Five Different Bacteria and the Yeast *Candida albicans*

			Minimal Inhibitory Concentrations (MIC) (μg/ml)					
Sequence[a]	Name	Rel. EC_{50}[b]	*P. aeruginosa*	*E. coli*	*Salmonella*	*S. aureus*	*S. epidermidis*	*C. albicans*
RLARIVVIRVAR	Bac2A		50	17	34	17	4	9
RAVAVIIRLRRV	Bac014		23	11	11	23	6	6
RRAAVVLIVIRR	Bac020		11	6	23	11	1.5	11
ILRRVRVRAVAI	Bac022		250	136	250	>250	136	250
VIRRRRILAAVV	Bac044		>250	136	136	250	136	136
VRLRIRVAVIRA	Bac034	1	25	3	12	25	3	12
VRFRIRVAVIRA	F3	0.89	6	1.6	6	12	1.6	6
VRWRIRVAVIRA	W3	0.66	6	1.6	6	12	1.6	12
VRLWIRVAVIRA	W4	1.89	>50	6	>50	50	3	50
VRLRIRVAVRRA	R10	0.81	12	6	6	12	1.6	6
VRLRIRVAVIRK	K12	0.48	6	3	6	6	1.6	3
VQLRIRVRVIRK	opt1		6	3	6	12	1.6	6
VRLRIRVRVIRK	opt2		3	1.6	6	6	0.8	3
KQFRIRVRVIRK	opt3		6	3	6	12	1.6	6
KRFRIRVRVIRK	opt4		6	1.6	3	6	0.8	12
KRWRIRVRVIRK	opt5		3	1.6	3	3	0.8	6

The MICs are given as the most frequently observed value except for Bac2A, which is an average value of all measurements obtained with this peptide (frequently used as a control). The values are rounded to achieve full numbers.
[a] All peptides had an amidated C terminus.
[b] Proxy EC_{50} from Figure 4 for the single substitution peptides only.

somewhere in the molecule (a so-called hydrophobic patch) and, in some cases, the specific presence of a Val or Leu residue at the beginning of the hydrophobic patch. These descriptors implicate global hydrophobicity as opposed to position-specific hydrophobicity, indicating that according to this model, the position of the hydrophobic patch is not relevant to activity.

For the ten peptides that had better activity than Bac2A, a similar model-building exercise yielded a model with an accuracy of 95% that indicated that the best activity was favored by a hydrophobic patch and a predicted water-accessible surface area of all atoms with a partial charge <0.2 (data not shown); however, the number of active peptides was insufficient for creation of a robust model.

Structure/Activity Relationships for Selected Peptides

The hydrophobic patch found by the QSAR analysis is consistent with the requirement of certain necessary structural features for activity of the scrambled peptides. It has been shown that the activity of many peptides, including bactenecin and a linear version thereof, is driven by membrane interaction, which involves the induction of a defined peptide structure [8, 13]. Thus it seemed likely that one purpose of the hydrophobic patch referred to above would be in conjunction with membrane interaction. (A recent discussion of the interactions of antimicrobial peptides with model phospholipids membranes was presented by Papo and Shai [14].) To determine if the activity of the scrambled peptide variants reflected differences in membrane interaction, CD spectrometry was performed in buffer and in anionic liposomes. In Tris buffer (Figure 2A), all the peptides demonstrated a typical random structure. In liposomes (Figure 2B), the activity class 1 peptides demonstrated spectra typical of β structures, whereas both activity class 6 peptides displayed more unstructured spectral characteristics at all liposome concentrations used. This is consistent with the suggestion that those

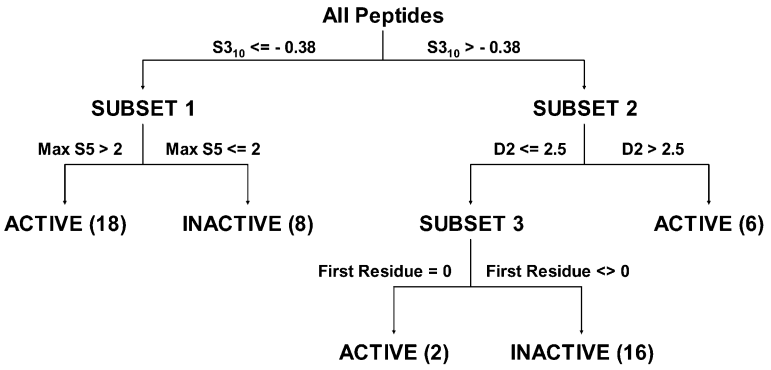

Figure 1. QSAR Modeling

The peptide sequences were analyzed by assessing which of several descriptors were effective for grouping, into separate classes, the active and inactive/less-active peptides. Using the binary classification algorithm of the model-building program MOE (Chemical Computing Group, Inc.), a set of differentiating criteria were determined as outlined in the figure. In particular, it was apparent that the active peptides were included in three main subsets: subset 1 was characterized by all peptides with a nonhydrophobic tail ($S3_{10} \leq -0.38$) and with an internal hydrophobic "patch" characterized by a maximum hydrophobicity $S3_{10}$ over five residues (Max S5) of >2. Hydrophobicity ($S3_{10}$) was determined according to the Eisenberg hydrophobicity scale [21]. This division correctly predicted the activity of 16/18 peptides. Peptides with a more hydrophobic C-terminal tail were further subdivided in subset 2 by the presence of a small internal hydrophobic patch between the second and third arginine residues (D2 > 2.5), which yielded a correct prediction for four of six active peptides. The two active peptides among the remaining 18 peptides were manually classified in subset 3 based on their "FirstResidue" parameter being = 0 (see Experimental Procedures). Overall, 21/25 active peptides and 22/25 inactive peptides were correctly classified with this model, leading to a classification accuracy of 74% for both active and inactive peptides.

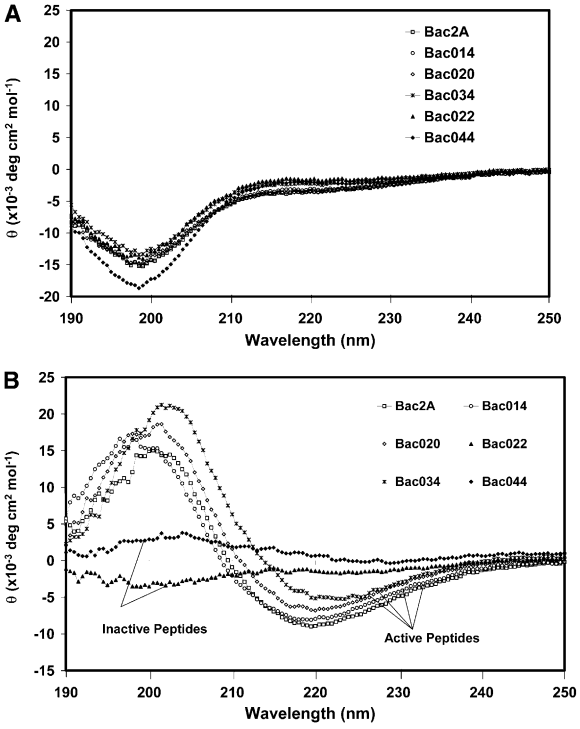

Figure 2. Structural Characterization of Different Scrambled Peptides by Circular-Dichroism Spectroscopy

(A and B) Spectra in 10 mM Tris buffer (pH 7.4) (A), or spectra in liposomes (POPG/POPC 1:1) (B).

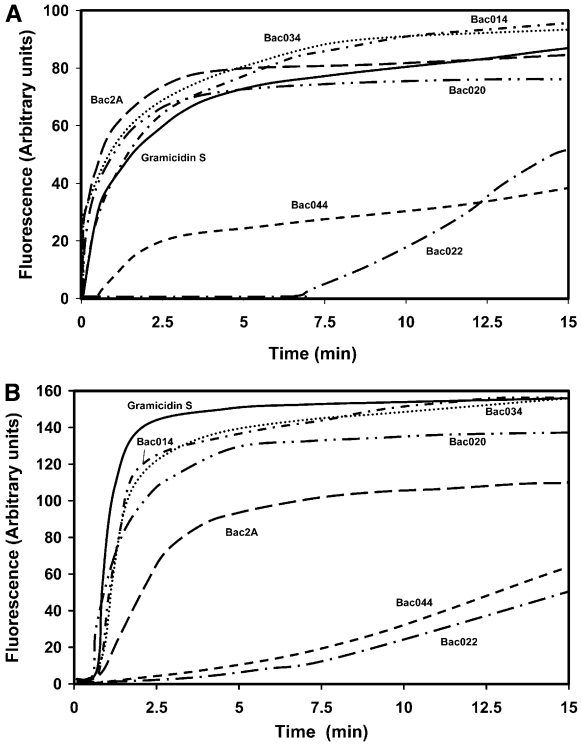

Figure 3. Membrane Depolarization Assay

Gramicidin S (solid line), Bac2A (long dash), Bac014 (dash dot), Bac020 (long dash dot dot), Bac022 (long dash dot), Bac034 (round dot), and Bac044 (dash) were used to determine the mechanism of action of peptides as assessed by measuring the ability to depolarize *S. aureus* cells (A) or *E. coli* strain DC2 (B) with the membrane-potential-sensitive dye $diSC_35$. Either 20 μg/ml of peptides or, as a positive control, 29 μg/ml of gramicidin S were added. The fluorescence values were measured every 5 s. Smoothed lines were used to describe the data points.

peptides with low antimicrobial activity are less able to interact with membranes.

Since liposomes are simplified models of biological membranes, an additional assay was performed with living bacteria and biologically intact membranes. Determination of the ability of peptides to depolarize the cytoplasmic membrane, which occurs with most antimicrobial peptides at high enough concentrations, thus permits the direct assessment of peptide interaction with bacterial membranes, and this can be monitored by the dequenching of the fluorescent probe $diSC_35$ [15]. The peptide Bac2A and all activity class 1 peptides were able to rapidly depolarize the membrane of *S. aureus*, with similar rates to the positive control gramicidin S (Figure 3A). In contrast, less active class 6 peptides showed a much reduced ability to depolarize the membrane. Similar results were achieved with Gram-negative bacterium *E. coli* strain DC2 (Figure 3B). Thus, these data are consistent with the proposal that peptide activity is influenced by the ability to interact with the bacterial membranes by adopting certain structural features that are embedded in the peptide sequence.

Using Scrambled Peptides for Additional Optimization Process

The screening of the scrambled Bac2A peptides represented a new approach to optimization. To gain even more strongly active peptides, a second optimization approach was used for one of the best scrambled peptides (Bac034) by using a substitution analysis synthesized on cellulose. The killing activity of all these peptides was determined with *P. aeruginosa* strain H1001 as described above (Figure 4) (N.B., Cys was omitted in this series due to concerns about its tendency to form covalent dimers). The core amino acid residues Ile-5, Arg-6, and Val-7 and also Arg-11 proved to be very important for killing activity. Substitutions of the original amino acid residues in peptide Bac034 with the structure disrupting amino acids Pro and Gly at positions 4, 5, 6, 7, and 9 led to a strong decrease in activity. These results tend to indicate that there are important structural features at these positions in the center of the molecule that help to determine antimicrobial activity. Also, certain amino acid positions demonstrated substantial flexibility in substitutions including the C-terminal residue Ala-12 and Ala-8 that could be optimized with 5 and 7 favored substitutions, respectively.

Five peptides were chosen to validate the substitution analysis, demonstrating that the proxy EC_{50} used to assess relative efficiency in decreasing luminescence represented a reasonable method of assessing improved killing activity in peptides. The peptide K12 in particular had a very good broad spectrum activity profile. Using the information from the Bac034 analysis, we chose selected substitutions to be combined to design new peptides (Table 3). Opt5 in particular had excellent broad spectrum activity. The results demonstrated that the creation of improved antimicrobial peptides could be

Original Amino Acids		Substituted Amino Acids																		
		A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
1	V	0.99	2.01	2.27	1.10	1.07	0.93	0.99	1.03	1.01	1.00	1.25	1.25	1.04	1.14	1.23	1.24	1.00	1.30	1.23
2	R	1.59	5.83	5.64	1.19	1.62	1.23	1.55	0.96	1.30	1.95	1.47	1.93	1.22	1.00	1.22	1.35	1.32	1.57	1.22
3	L	1.29	2.32	3.26	0.89	1.73	1.78	1.03	1.14	1.00	1.08	1.88	2.11	2.22	1.02	1.77	1.50	1.12	0.66	1.08
4	R	2.26	I*	I	2.52	3.23	1.44	2.36	0.91	2.12	2.01	1.67	I	1.57	1.00	1.80	1.79	2.10	1.89	2.52
5	I	3.02	I	I	0.91	I	I	I	I	1.22	1.27	I	I	I	4.32	I	7.80	0.96	0.88	1.48
6	R	9.35	6.49	I	I	5.92	2.64	11.0	0.95	9.33	10.2	1.89	I	3.37	1.00	3.33	3.32	5.55	6.17	5.19
7	V	2.17	I	I	1.18	4.76	4.22	0.86	4.30	1.05	1.61	I	I	4.63	1.80	4.43	2.88	1.00	1.22	1.95
8	A	1.00	2.18	2.86	0.99	0.99	0.64	1.02	0.60	1.11	1.05	0.86	2.42	0.84	0.61	0.84	1.13	1.12	1.24	0.84
9	V	1.57	3.71	4.32	1.11	2.59	2.22	1.03	1.42	1.28	0.84	1.99	3.43	2.56	1.12	1.68	1.40	1.00	0.98	1.02
10	I	0.94	1.73	1.35	1.05	0.88	0.95	1.00	0.93	0.99	1.01	1.24	1.60	0.90	0.81	1.83	1.07	0.98	1.17	0.95
11	R	1.89	I	I	2.52	1.99	1.24	2.72	0.92	2.69	3.10	2.28	1.52	2.87	1.00	2.45	2.59	3.59	4.74	2.35
12	A	1.00	2.16	1.53	1.04	0.96	0.80	1.53	0.48	1.33	1.12	0.77	1.03	1.66	0.79	0.91	0.69	0.95	1.14	1.01

* I = inactive.

Figure 4. Complete Substitution Analysis for Bac034

The first two columns give the position (indicated as the row number) and the one-letter code sequence of the original peptide Bac034. The second row gives the amino acids substituted at each amino acid position. For example, the peptide in the upper left-hand corner (column 3, row 3) is ARLRIRVAVIRA. The results presented within each box represent the relative EC_{50} value, determined by treatment of the *lux* reporter strain H1001 with peptide for 4 hr. Results are color coded as black, superior activity to the parent peptide Ba034; dark gray with white lettering, equivalent activity to the parent peptide Bac034; light gray with black lettering, inferior activity to the parent peptide Bac034; white, very little activity.

successfully achieved by starting from a single template (Bac2A), by first scrambling the sequence (Bac020, Bac034, and others), and then by using a substitution matrix to improve the activity by single or multiple amino acid substitutions (K12, opt 5, and others). This new process, combined with the dramatic reduction in both cost of the peptides and assay time, may help to improve the speed of development of a broad range of new peptide-based antimicrobial drugs against antibiotic resistance bacteria.

Significance

Short antimicrobial peptides are promising candidates for overcoming the critical and accelerating problem of bacterial resistance to currently utilized antibiotics. Despite the fact that the first antimicrobial peptide is close to clinical approval, little is known about the primary sequence requirements of short nonhelical peptides. To provide a road map for development of improved second generation therapeutics, we have investigated in this study these sequence requirements by using scrambled peptides, QSAR modeling, and substitution analyses. The resultant study adds significantly to the field, demonstrating that the complexity of primary amino acid sequence requirements is much higher than previously anticipated and, for the first time in an unbiased fashion, has revealed intriguing structure activity relationships. Our newly described time- and cost-efficient optimization strategies will have a strong impact on the development of new drugs through the rapid enrichment of lead candidates.

Experimental Procedures

Strains

For the screening assay, a mini-*Tn5-lux fliC::luxCDABE* mutant strain H1001 of *P. aeruginosa* PAO1 was utilized. This strain contained a transcriptional fusion resulting in constitutive expression of luciferase [10]. The bacterial strains used for the antimicrobial activity assay included *E. coli* UB1005 (F−, nalA37, metB1), a wild-type *Salmonella enterica* ssp. Typhimurium (*S. typhimurium*), wild-type *Pseudomonas aeruginosa* (*P. aeruginosa*) H103, *Enterococcus faecalis* (*E. faecalis*) ATCC29212, *Staphylococcus aureus* (*S. aureus*) ATCC25923, and a clinical isolate of *Staphylococcus epidermidis* (*S. epidermidis*) obtained from Dr. D. Speert (Department of Pediatrics, UBC). Antifungal activity was tested with a lab isolate of *Candida albicans* (*C. albicans*) obtained from Dr. B. Dill (Department of Microbiology and Immunology, UBC).

Peptide Synthesis

Peptide syntheses on cellulose were performed with a pipetting robot (Intavis, Köln, Germany) and Whatman 50 cellulose membranes (Whatman, Maidstone, United Kingdom) by using two glycine residues as a linker, as described previously [16, 17]. For further characterization, free peptides were synthesized by Fmoc chemistry in our labs (Humboldt-Universität, Berlin) or purchased from Thermohybaid (Thermo-Electron-Corporation; Ulm, Germany). The peptide concentration was estimated from the weighed sample. Advantages and disadvantages for the peptide spot synthesis were discussed previously [9].

Minimal Inhibitory Concentration Determination

The minimal inhibitory concentration (MIC) of the peptides was measured by a modified broth microdilution method [6] in Mueller-Hinton (MH) medium. After incubation at 37°C for 12–15 hr, the MIC was taken as the concentration at which no growth was observed.

Screening Assay for the Peptides from Cellulose Support

The peptides were cleaved from the dried membrane in an ammonia atmosphere overnight, resulting in free peptides with an amidated C terminus. The liberated peptides were amidated and contained two glycines at the C terminus due to the linker between the cellulose membrane and the peptide sequence. The peptide spots were punched out and transferred in a 96-well microtiter plate. The active wild-type peptide and an unrelated peptide (GATPEDLNQKLS) were used as positive and negative controls, respectively. An overnight culture of *P. aeruginosa* strain H1001 was diluted 1:500 with 100mM Tris buffer (pH 7.3), 20mM glucose and was added to the wells (100 μl/well) containing the peptide spots. After 30 min incubation time to resolve the peptides from the cellulose support, serial dilutions were carried out from the membrane spots. The luminescence produced by the FMN-dependent luciferase system was detected in a time-dependent manner with a Tecan Spectra Fluor plus (Tecan U.S., Inc., Durham, NC).

Circular-Dichroism Spectroscopy

Circular-dichroism (CD) spectra were recorded with a Jasco J-810 spectropolarimeter (Tokyo, Japan). Spectra were measured

between 190 and 250 nm at a scan speed of 10 nm/min and a total of ten scans per sample, with a quartz cell with 1 mm path length at room temperature. The data were collected and processed with Jasco software. The peptide concentration used was 70 μM. Spectra were recorded in 10 mM Tris buffer (pH 7.3) and in unilamellar liposomes made by extrusion of a 1:1 mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG). In all cases, the spectrum in the absence of peptide was subtracted from that in the presence of peptide.

Membrane Depolarization

To assess bacterial membrane depolarization, a membrane-potential-sensitive dye, $diSC_3 5$, was used [15] as described previously [18, 19]. This cationic dye concentrates in the cytoplasmic membrane under the influence of the membrane potential (which is oriented internal negative) resulting in a self-quenching of fluorescence. Upon disruption of the membrane potential, the dye dissociates into the buffer, leading to an increase in fluorescence [13]. Depolarization was monitored over a period of 900 s with 20 μg/ml peptide or 29 μg/ml gramicidin S in a Luminescence Spectrometer LS50B (Perkin Elmer, Woodbridge, Canada) at room temperature. The depolarization of the cytoplasmic membrane was determined with the Gram-positive bacterium *S. aureus* ATCC 25923 and the Gram-negative bacterium *E. coli* DC2 mutant with increased outer membrane permeability [20] to permit the dye to reach the cytoplasmic membrane.

Computational Analyses

The peptide sequences were analyzed with the program MOE (Chemical Computing Group, Inc.). Given the qualitative nature of the data, a classification model [12] was derived in an attempt to discern the difference between active (activity classes 1, 2, and 3) and inactive (activity classes 4, 5, 6) peptides. The three-dimensional structures of these peptides were not known, thus descriptors based on exact spatial relationships could not be used. Moreover, due to the nature of the scrambled sequences, many of the traditional two-dimensional descriptors did not discriminate between the peptides (e.g., net charge, logP, etc.). Therefore, a number of descriptive indices related specifically to sequence ordering and hydrophobicity were derived including: (1) distance between the first and second (D1), second and third (D2), and third and fourth (D3) arginines measured as the number of residues separating them; (2) the maximum and average distance between any two consecutive arginines (Dmax, Davg); (3) water accessible surface area of all hydrophobic ($|q_i| < 0.2$) atoms (ASA_H); (4) hydrophobicity using the Eisenberg scale [21] measured over consecutive strings (S) of three, four, or five residues of the peptide (S3x, S4x, S5x) and maximum and minimum values of the hydrophobicity scale (e.g., S4max, S4min, etc.); (5) the maximal stretch of hydrophobic residues (count H) or the presence of a string of six hydrophobic amino acids including alanine (6HA); and (6) the presence of a string of four amino acids, preferably beginning with isoleucine (first residue: 1 = YES, 0 = string begins with V or L instead of I, 2 = NO).

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