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High-throughput generation of small antibacterial peptides with improved activity

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Cationic antimicrobial peptides are able to kill a broad variety of Gram-negative and Gram positive bacteria and thus are good candidates for a new generation of antibiotics to treat multidrug-resistant bacteria. Here we describe a high-throughput method to screen large numbers of peptides for improved antimicrobial activity. The method relies on peptide synthesis on a cellulose support and a *Pseudomonas aeruginosa* strain that constitutively expresses bacterial luciferase. A complete substitution library of 12-amino-acid peptides based on a linearized variant (RLARIVVIRVAR-NH_2) of the bovine peptide bactenecin was screened and used to determine which substitutions at each position of the peptide chain improved activity. By combining the most favorable substitutions, we designed optimized 12-mer peptides showing broad spectrum activities with minimal inhibitory concentrations (MIC) as low as 0.5 μg/ml against *Escherichia coli*. Similarly, we generated an 8-mer substituted peptide that showed broad spectrum activity, with an MIC of 2 μg/ml, against *E. coli* and *Staphylococcus aureus*.

The treatment of bacterial infections with antibiotics is one of the mainstays of human medicine. Unfortunately, the effectiveness of antibiotics has become limited owing to an increase in bacterial antibiotic resistance in the face of a dearth of discovery of new classes of antibiotics. For example, nosocomial bacterial infections that are resistant to therapy cost more than $2 billion and account for more than 80,000 direct and indirect deaths a year in North America alone.

A major limitation in antibiotic development has been the difficulty of finding new structures with the same characteristics as conventional antibiotics, namely low toxicity for the host and a broad action against bacterial pathogens. Recent novel antibiotic classes, including the oxazolidinones (linezolid), the streptogramins (quinupristin/dalfopristin) and the glycolipids (daptomycin) have all been limited in their spectrum of activity to Gram-positive pathogens. It is thus a challenge for scientists to design antibiotics with novel structures and/or modes of action.

Cationic antimicrobial peptides represent good templates for a new generation of antimicrobials. They often kill both Gram-negative and Gram-positive microorganisms rapidly and directly, do not easily select mutants, work against clinically common drug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus*, can show a synergistic effect with conventional antibiotics and can often activate host innate immunity without displaying immunogenicity[1]. Moreover, some also seem to counteract some of the more harmful aspects of inflammation (e.g., sepsis, endotoxemia)[2].

Cationic antimicrobial peptides comprising sequences of natural L-amino acids were discovered in the hemolymph of insects in the late 1970s. Today, more than 600 cationic peptides have been described in bacteria, fungi, insects, tunicates, amphibians, crustaceans, birds, fish and mammals including humans (http://www.bbcm.units.it/~tossi/pag1.htm). They can be described through their physical chemical characteristics with a size ranging from 12 to 50 amino acids, a net positive charge >2 due to excess arginine and lysine residues and $\sim$50% hydrophobic amino acids[1]. The multitude of cationic peptide sources, structures and spectra of activity is matched by a number of complex and controversial models attempting to describe and explain the modes of action of these peptides[3]. Most antimicrobial peptides bind to the lipopolysaccharide (LPS) of Gram-negative bacteria or to the lipoteichoic acid of Gram-positive bacteria, and subsequently associate with, and either permeabilize or cross, the cytoplasmic membrane and act on internal targets. The precise mechanisms of how they kill the target cells are not fully understood.

The cationic peptide bactenecin (also called bovine dodecapeptide; RLCRIVVIRVCR) is one of the smallest natural occurring antimicrobial peptides. It was discovered in bovine neutrophils in 1988 (ref. 4). This peptide is modestly active against both Gram-negative (*E. coli*, *P. aeruginosa*) and certain Gram-positive bacteria (*Streptococcus pyogenes*, *Corynebacterium xerosis*). The natural peptide is stabilized by an internal disulfide bridge, but a linear variant, Bac2A (RLARIVVIRVAR-NH_2), shows a similar activity against Gram-negative bacteria and higher activity against Gram-positive bacteria[5]. These features, namely small size, linearity and activity against both Gram-positive and Gram-negative bacteria, make this peptide a good candidate for a lead structure to develop a new class of antimicrobial drugs. However, the development of antimicrobial peptides has been hampered by the lack of a generally useful low-cost method for randomly varying and screening large numbers of peptides.

The method of synthesizing peptides on cellulose sheets was developed in 1992 (ref. 6). Large numbers of peptides can be synthesized on a cellulose sheet (20 × 30 cm) in a parallel manner,

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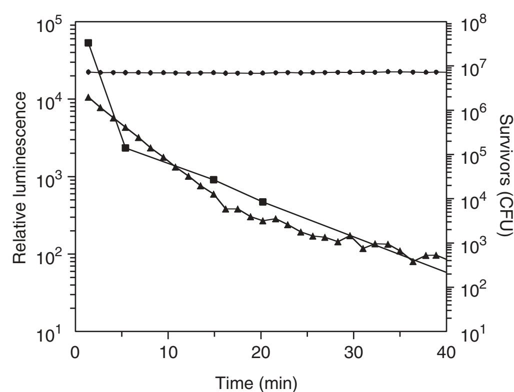

Figure 1 Comparison of a 'classical' killing curve and a *lux* killing assay of *Pseudomonas aeruginosa* Bac2A. Bac2A was used at threefold the MIC for both methods and the triangles demonstrate the decrease in luminescence whereas the squares assess surviving bacterial colony counts. The circles demonstrate the killing assay without peptide. Both assays were carried out in 0.1 M Tris-buffer, pH 7.3 (containing 20 mM glucose in the case of the *lux* assay) with a starting concentration of between 2×10^6 and 10^7 colony forming units (CFU) per ml.

and each spot is positionally addressable. The peptides are created from the C to the N terminus by the F-moc solid-phase peptide synthesis strategy. This allows use of the natural occurring L- and corresponding D-amino acids, unnatural amino acids and other organic compounds. The technique can be carried out manually or with the help of pipetting robots. For solid-phase assays, up to 8,000 different peptides can be synthesized on one cellulose sheet, and for solution-phase assays (where larger amounts, that is, larger spots, are required), up to 1,000 (refs. 7,8). Here, we have adapted this method to create a large number of variants through systematic modifications of a peptide sequence and used a luciferase-based screen to investigate their ability to kill *P. aeruginosa*. We demonstrate the utility of this approach in the design of small (eight amino acid) peptides with excellent antimicrobial activity.

Peptide synthesis on cellulose is an effective and inexpensive way to investigate many different peptide variants for one particular activity. However, one problem of this technique is the small amount of peptides synthesized on the membrane, about 280 nmol per cm^2 (ref. 9). The average peptide spot size used for the experiments presented below was about 0.3 cm^2, resulting in a maximum of ~100 μg of peptide. Therefore, an assay had to be developed that was sensitive enough to show relative activity in low volumes without dilution and rapid enough for screening. *P. aeruginosa* PAO1 strain H1001, isolated in our laboratory, contains a luciferase gene cassette, *luxABCDE*, incorporated into the bacterial chromosome in a gene (*fliC* involved in flagellar biosynthesis) that is constitutively expressed[10]. It will thus produce light as long as ATP is provided by the bacterium. A decrease in the amount of light produced thus signals a decrease in ATP levels, which could occur if the bacteria is killed by an antimicrobial peptide (for example, by the consumption of ATP without new ATP generation and/or the leakage of ATP from cells). This action can be monitored by detecting luminescence in a microtiter plate luminescence reader over time. We optimized the volume and amount of cells per well for this assay. After screening, we were able to monitor the killing action with the parent peptide Bac2A (**Fig. 1**) and other peptides, even at concentrations below the MIC (data not shown). The actual killing of *P. aeruginosa* could be confirmed by measuring residual colony counts (**Fig. 1**); an irrelevant peptide, GAT PEDLNQKLS-NH_2, served as a negative control (data not shown).

To analyze the positional importance of the specific amino acids in Bac2A, we substituted, one by one, all of the 19 conventional amino acids for each Bac2A amino acid, creating a total of 228 unique peptides. Antimicrobial activity was assessed using the *Pseudomonas lux*-based assay (**Fig. 2**). The original Bac2A peptide was present in each series of analyses (*e.g.*, **Fig. 2**, row 3, column 1, where A was retained in position 3). Conversely, the irrelevant peptide, GATPEDLNQKLS-NH_2, always served as a negative control and lacked the ability to cause a decrease in luminescence. The results, presented as the proxy IC_{50} values, revealed information about the positional specificity of particular amino acids and demonstrated many substitutions that improved the activity of Bac2A (that is, preferred substitutions).

The data are presented as **Figure 2**, which has been shaded to demonstrate preferred substitutions (darker grays in the insert boxes), and are summarized in **Table 1**. Looking at the effects of specific substitutions, it was clear that some amino acids, particularly C > W > R > H, K, were often preferred to the parent residue. In contrast, some residues never led to an improvement in activity, namely A, D, E, P and V. Substitutions were rarely conservative and could not be predicted by obvious substitutions, such as that of one hydrophobic

Original amino acid		Substituted amino acid																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
1	R	0.15	0.27	0.34	0.41	0.18	0.15	0.24	0.17	0.14	0.16	0.35	0.34	0.17	0.33	0.13	0.29	0.25	0.21	0.06	0.20
2	L	0.17	0.08	0.33	0.21	0.13	0.06	0.10	0.12	0.06	0.13	0.18	0.18	0.15	0.16	0.05	0.10	0.17	0.12	0.06	0.09
3	A	0.13	0.09	0.18	0.16	0.04	0.12	0.09	0.07	0.05	0.09	0.14	0.12	0.14	0.09	0.03	0.17	0.11	0.15	0.04	0.11
4	R	0.31	0.35	0.49	0.41	0.45	0.46	0.35	0.59	0.11	0.75	0.39	0.28	0.25	0.26	0.13	0.30	0.31	0.27	0.25	0.29
5	I	0.31	0.05	0.42	0.30	0.29	0.26	0.23	0.13	0.10	0.21	0.23	0.26	0.33	0.26	0.08	0.22	0.20	0.17	0.06	0.20
6	V	0.25	0.06	0.44	0.47	0.09	0.43	0.20	0.20	0.12	0.20	0.26	0.30	0.75	0.25	0.15	0.29	0.23	0.13	0.07	0.13
7	V	0.37	0.06	0.25	0.20	0.17	0.17	0.09	0.05	0.03	0.11	0.20	0.10	0.60	0.05	0.05	0.26	0.09	0.13	0.19	0.11
8	I	0.48	0.06	0.75	0.75	0.14	0.50	0.23	0.13	0.15	0.18	0.38	0.40	0.31	0.31	0.16	0.42	0.52	0.13	0.16	0.16
9	R	0.39	0.09	0.75	0.75	0.38	0.23	0.41	0.48	0.18	0.41	0.27	no fit	0.41	0.40	0.13	0.41	0.31	0.49	0.22	0.19
10	V	0.61	0.06	0.75	0.75	0.21	0.39	0.21	0.11	0.14	0.16	0.22	0.29	0.22	0.41	0.18	0.41	0.33	0.13	0.08	0.13
11	A	0.13	0.04	0.21	0.23	0.12	0.08	0.08	0.06	0.06	0.08	0.09	0.12	0.18	0.13	0.05	0.10	0.10	0.10	0.13	0.07
12	R	0.38	0.75	0.75	0.75	0.75	0.33	0.20	0.27	0.19	0.47	0.47	0.25	0.42	0.32	0.13	0.40	0.29	0.37	0.75	0.54

Figure 2 Complete substitution analysis for Bac2A. The first two columns give the position (indicated as the row number) and the one-letter code sequence of the original peptide Bac2A. The second and third rows give the column number and the amino acids, respectively, substituted at each amino acid position. Thus for example the peptide in the upper left hand corner (column 1, row 1) is ALRARIVVIRVAR, and the next peptide in row 1 is CLRARIVVIRVAR; the peptide column 1, row 2 is RARARIVVIRVAR; the parent peptide Bac2A appears in column 1, row 3, RLARIVVIRVAR; in column 2, row 4 is RRACIVVIRVAR. The results presented within each box represent the proxy IC_{50} value, determined by treatment of the *P. lux* reporter strain H1001 with peptide for 4 h. Results are color coded as black for superior activity to the parent peptide Bac2A; dark gray with white lettering is equivalent activity to the parent peptide Bac001; light gray with black lettering is inferior activity to the parent peptide Bac2A; white is very little activity.

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Table 1 Favored amino acid substitutions in Bac2A

Position	Parent amino acid	Most favored	Not favored
1	R	W	E
2	L	C, G, H, K, R, S, W, Y	
3	A	C, F, H, I, K, L, Q, R, W	
4	R		D, E, F, G, I, L
5	I	C, R, W	D
6	V	C, F, W	D, E, G, P
7	V	C, H, I, K, N, Q, R, T	P
8	I	C	I, D, E, G, N, S, T
9	R	C	I, D, E, H, I, L, N, P, Q, S, V
10	V	C, W	I, D, E, G, Q, S
11	A	C, G, H, I, K, L, M, R, S, Y	
12	R		C, D, E, F, L, M, P, S, W, Y

residue for another. Some amino acid positions were particularly rich candidates for substitution, namely positions 2, 3, 7 and 11 and these positions also had the fewest unfavorable substitutions. Five positions did not readily accept substitutions, namely positions 4, 8, 9, 10 and 12, and for these the parent (Bac2A) amino acid was often the best residue. There was some asymmetry in the preferred substitutions in that the C terminus was generally less tolerant of substitutions, which may reflect asymmetric evolution (that is, optimization) of the original bactenecin gene. Overall, 46 peptides demonstrated improved activity compared with Bac2A, whereas a further 52 neutral substitutions (having similar activity to Bac2A) were identified.

A specific example of the findings at a given amino acid position included the first residue, arginine (position 1), which could be substituted by 6 out of 19 amino acids without losing activity compared to Bac2A. The introduction of hydrophobic amino acids, with the exception of tryptophan, did not increase activity. A completely different pattern was observed for the arginine residues at positions 9 and 12. These were very sensitive to all exchanges, and even substitution by lysine led to a modest decrease in activity.

The valine residues at positions 6 and 7 showed similarities and differences in the effects of substitutions. The strong decrease in activity by substituting proline for valine at both positions indicated that this region may have structural constraints. Substitution at position 6 by arginine and lysine resulted in peptides with similar activity; in contrast the same substitutions at position 7 led to stronger activity. The opposite behavior was detected for tryptophan and phenylalanine, showing a stronger activity when substituted into position 6 and similar or weaker activity in position 7.

The fact that nearly 50% of the 229 peptide variants screened were improved or equivalent to the parent peptide, and only 5 showed no activity at all, indicates that the mechanism of action of the peptides cannot be easily disturbed by a single substitution. This might be one reason why these peptides have retained activity despite substantial variation during evolution.

Bovine bactenecin contains a disulfide bridge, as evidenced by the lack of free sulfhydral groups in the native molecule[4]. The original peptide Bac2A was created by substituting alanine for the two cysteine residues, although a dual serine substitution provided a peptide that was almost as active[5]. These two positions were remarkably flexible, and alanine was by no means the preferred residue at this position; nine and ten other substitutions at Ala-3 and Ala-11, respectively, were preferred to alanine, including cysteine, which is found in the native peptide. One possible explanation would be the tendency of the cysteines to dimerize, resulting in a larger, more charged peptide.

To confirm the data in **Figure 2**, we synthesized and assayed 15 peptides by conventional MIC susceptibility tests. Three of these proved to be insoluble. The remaining peptides demonstrated similar trends to those observed using the *lux* assay, when screened against a range of microbes, including three Gram-negative bacteria, three Gram-positive bacteria and a fungal pathogen (representing the most important nosocomial pathogens in Western society; **Table 2**). The parent peptide Bac2A was confirmed to have rather modest broad spectrum activity, with preferential activity against *Staphylococcus epidermidis*[5]. As predicted by the *lux* assay (**Fig. 2**), peptide P7 was clearly much worse than the parent, being totally inactive. Peptide G1 was similar to the parent peptide, whereas peptides R2, R3, W3, W10 and R11 were much better. Overall there was a good correlation between the IC_{50} as measured by the luminescence assay and the measured MICs for *P. aeruginosa* (R value of 0.895, $P < 0.01$ by ANOVA), although peptide K7, for example, demonstrated a low IC_{50} but only a mediocre MIC.

To determine whether the information obtained from the above substitution analysis was specific for peptides with single substitutions, or whether it could be combined and extrapolated to peptides with multiple substitutions, we made variants with two, three, five and six substitutions based on the data of **Figure 2** using preferred and/or equivalent substitutions at various sites. The peptide with two substitutions (Sub2) had a similar activity to one of its parent peptides R2 (**Table 2**). On the other hand, peptides with three and five 'favorable' substitutions had excellent broad spectrum activities. Conversely, the peptide Sub6 with six substitutions showed only a slightly lower MIC than Bac2A, indicating that in some cases individual substitutions are substitutions might be redundant or counterproductive.

We further extrapolated these data to the design of even shorter peptides. One of the major drawbacks of peptide antibiotics is that they are more expensive than conventional antibiotics. To minimize cost it is advisable to minimize the size of the peptide, although smaller peptides tend to have a narrow spectrum of activity and be salt sensitive. We started with the 8-mer peptide RIVVIRVA representing amino acids 4–11 of Bac2A. This parent peptide was quite insoluble. The substitution of three to four individual residues with preferred or equivalent amino acid substitutions, as determined from the single substitution analysis (**Fig. 2**), led to one peptide, Bac8d, with slightly weaker activity than Bac2A; two peptides, Bac8a and Bac8b, with similar activity to this parent peptide; and one, Bac8c, with clearly improved activities, especially against Gram-positive bacteria and *E. coli*.

The development of most pharmaceutical drugs involves, in the first phase, the semi-random synthesis and screening of large numbers of compounds. Such procedures have not been available to individuals who are interested in designing improved antimicrobial peptides. This paper demonstrates a procedure and a screening assay that permit the synthesis of large numbers of peptide variants, allowing the effects of large numbers of substitutions to be studied simultaneously. We demonstrated the utility of this method by performing a complete substitution analysis on a 12-mer peptide. We observed, as have others, that the introduction of positively charged amino acids or tryptophan at particular positions can lead to a more active peptide. More importantly, this study showed that some positions are more affected by substitutions, indicating targeted locations for substitution. It is worth noting that activity against the yeast *Candida albicans* tended to track with general antibacterial activity, for the tested peptides, with the exceptions of R3 and Sub2 (**Table 2**).

Table 2 Minimal inhibitory concentration (MIC) determinations

Sequence	Name	Proxy IC_{50}[a]	MIC (μg/ml)[b]						
			P. aerug.	*E. coli*	*S. typhim.*	*S. aureus*	*S. epi.*	*E. faecalis*	*C. albicans*
RLARIVVIRVAR-NH_2	Bac2A	0.13	50	17	34	17	4	17	9
GLARIVVIRVAR-NH_2	G1	0.15	125	16	63	63	16	31	125
R**G**ARIVVIRVAR-NH_2	G2	0.06	31	8	31	31	16	31	16
R**R**ARIVVIRVAR-NH_2	R2	0.05	8	4	8	16	2	16	8
RL**R**RIVVIRVAR-NH_2	R3	0.03	8	4	8	8	2	8	16
RL**W**RIVVIRVAR-NH_2	W3	0.04	8	2	8	2	0.5	2	8
RLAR**R**VVIRVAR-NH_2	R5	0.08	63	16	31	63	16	125	125
RLARIV**P**IRVAR-NH_2	P7	0.60	>250	250	>250	>250	>250	>250	>250
RLARIV**K**IRVAR-NH_2	K7	0.03	31	16	63	125	31	125	31
RLARIVVIR**W**AR-NH_2	W10	0.08	16	4	8	8	2	4	8
RLARIVVIRV**R**R-NH_2	R11	0.05	16	8	8	8	2	8	8
RLARIVVIRVA**G**-NH_2	G12	0.33	63	16	63	63	31	31	63
RL**R**RIVVIRV**R**R-NH_2	Sub2		8	2	8	8	2	4	31
R**RW**RIVVIRV**R**R-NH_2	Sub3		2	0.5	4	2	0.2	4	4
R**RWK**IVVIR**WR**R-NH_2	Sub5		2	4	8	2	0.5	2	4
R**WWK**I**W**VIR**WW**R-NH_2	Sub6		31	4	16	8	1	8	31
RIVVIRVA-NH_2	Bac8		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
KI**W**VIR**WR**-NH_2	Bac8a		16	8	31	16	4	16	16
RI**W**VIR**WR**-NH_2	Bac8b		16	4	16	4	4	8	16
RI**W**VIW**R**R-NH_2	Bac8c		8	2	8	2	2	2	8
R**RW**VI**WR**R-NH_2	Bac8d		250	16	31	16	8	32	63

The positions labeled in bold represent the amino acid exchanges with respect to Bac2A.
[a]Proxy IC_{50} from **Figure 2** for the single substitution peptides only. [b]The MICs are given as the most frequently observed value, except for Bac2A, which is an average value of all measurements done with this peptide (frequently used as a control). n.s., not soluble.

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The substitution analysis provided a set of guidelines for improving antibiotic activity, which we extrapolated with some success to multiple substitutions, leading to peptides with excellent activities, as assessed by conventional MIC assays, against some of the more recalcitrant microorganisms in our society. Furthermore, the knowledge gained from the substitution analysis allowed the design of shorter peptides containing only eight amino acids. We designed and synthesized four 8-mer peptides, one of which showed two- to eightfold higher MIC values compared with the parent 12-mer peptide Bac2A.

In summary, synthesis on cellulose support provides an inexpensive and fast screening method for thousands of single peptides using simple equipment. We estimate that with the assistance of two pipetting robots, 100,000 single peptides could be produced and tested using this assay in one year. A strength of the *lux* method is that it can be easily adapted to the screening of any microbial pathogen of interest.

In contrast, although solid-phase synthesis on resin—an alternative method of peptide synthesis—is automated, creating a library of several thousand members this way is costly and involves substantial manpower for purification. Typically, combinatorial libraries are synthesized and used for many different screens. However, the larger scale of such syntheses and the resultant costs of chemicals and equipment and supplies for synthesis, purification and screening are a major limitation. Nevertheless, useful information can be gained for smaller peptides using a mixed peptide deconvolution strategy. For example, such a strategy was applied to obtain 6-mer peptides that were unfortunately specific for *S. aureus*[11]. For larger peptides, cost issues can often limit library design to rather simple strategies such as an alanine scan, which yields useful but limited information.

Another possible method for designing and screening peptide libraries is phage display libraries. It is a powerful technique for finding desired peptides in a large library but is limited in that it involves multiple simultaneous changes (which may oppose each other) and because the variant peptide is presented within the scaffold of a phage protein. Therefore, it is difficult to address questions about structure/function relationships or mode of action systematically by varying each position of the molecule, because only those peptides with the strongest activity will be enriched during screening.

The limitations of the method presented here are that (i) for liquid-phase assays, peptides can be used only once and new peptides must be synthesized to confirm the data (although multiple spots for any given peptide can be synthesized to provide repetition); (ii) the peptides are not purified and may contain incorrect sequences; (iii) the peptide sequence has two additional glycine residues that are required as a linker to the cellulose support (which we predict would not substantially affect activity or solubility); (iv) the amounts of synthesized peptides can vary according to the sequence at any given position; (v) in solution-phase assays, the peptide concentration is probably relatively low and the linker amino acids may influence peptide solubility; and (vi) in solid-phase assays, the cellulose support can influence the interactions. In addition, the data obtained must be verified by synthesis of selected peptides on resin. Although the actual amounts of peptide recovered from the cellulose support vary and the peptides synthesized are not pure (that is, they may contain varying amounts of contaminants, assessed as less than 30% in previous studies), for screening assays it is not cost effective to estimate the amount and purity of peptide recovered from each spot. However, the high degree of correlation of activity between proxy MICs and measured MICs of the soluble peptides (**Table 2**) indicates that issues of yield and purity do not substantially distort results, and repeated synthesis of the same peptides led to very similar proxy MICs (*e.g.*, 0.13 ± 0.04 for 50 replicate assays with Bac2A). Therefore, the potential disadvantages described above are not insurmountable, and peptide

synthesis on cellulose supports should provide a valuable method for making improved cationic antimicrobial peptides. Such tools will permit the development of a broader range of molecules with which to conduct *in vivo* tests of efficacy, bioavailability and toxicity.

METHODS

Peptide synthesis. Peptide syntheses on cellulose were performed using a pipetting robot (Abimed) and Whatman 50 cellulose membranes (Whatman), as described previously[12]. Briefly, the cellulose membranes were incubated in an activated glycine[13] solution to achieve a base for peptide synthesis. Peptide synthesis at discrete spots addressed by the robot was performed by an F-moc strategy using an F-moc-protected N terminus and a C-terminal Opfp-activating group, with double coupling at each cycle to ensure higher coupling efficiency at each amino acid position. The cleavage of the side-chain protecting group was carried out with 90% trifluoroacetic acid, 3% tri-isobutylsilane, 2% water, 1% phenol in dichloromethane for 30 min following a 120-min incubation with 50% trifluoroacetic acid, 3% tri-isobutylsilane, 2% water and 1% phenol in dichloromethane. The peptides were cleaved from the membrane by incubating the membrane overnight in an ammonia gas atmosphere. We estimate that the variation in amount of peptide obtained was between 10% and 20% based on the variation in assay results for replicates of peptide spots.

For further characterization, peptides were synthesized on by solid-phase methods according to standard F-moc machine protocols using a multiple peptide synthesizer (Abimed). The peptides were purified by high-performance liquid chromatography up to at least 75% purity and characterized by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. The peptide concentration was estimated from the weighed sample.

Minimal inhibitory concentration (MIC) determination. The MIC of the peptides were measured using a modified broth microdilution method[14] in Mueller Hinton medium, whereby the assay was performed in sterile 96-well, round-bottom polypropylene microtiter plates (Costar) with an inoculum of 2–7×10^5 bacteria or fungi per ml. The plates were incubated at 37 °C for 12–15 h and the MIC was taken as the concentration at which no growth was observed.

Screening assay for the cleaved 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 free peptides contained two glycines at the C terminus, in addition to being amidated owing to the linker between the cellulose membrane and the peptide sequence. The peptide spots were punched out and transferred to a 96-well microtiter plate. Serial dilutions were carried out from the membrane spots. In the first row, four wells contained controls, including two Bac2A and two with an unrelated peptide (GATPEDLNQKLS). The other eight wells were used for the peptide variants. An overnight culture of *P. aeruginosa* strain H1001 was diluted 1:500 using 100 mM Tris buffer pH 7.3, 20 mM glucose and was added to the wells (100 µl/well) containing the peptide spots. In all other wells 50 µl was added. The microtiter plate was incubated for 30 min at 37 °C to release the peptides from the membrane. A dilution series was carried out and the plate was incubated at 37 °C. The luminescence produced by the ATP-dependent luciferase system was detected in a time dependent manner using a Tecan Spectra Fluor plus (Tecan US). Over the entire data set, the IC_{50} values for the parent peptide, Bac2A, were very stable giving mean residual luminescence values ± s.d. of 0.13 ± 0.04 for 50 replicate assays with only two replicates having to be discarded since they gave IC_{50} values greater than 2 s.d. from the mean. The loss of luminescence coincided with peptide-induced lethality as confirmed by plating the contents of wells from the *lux* assays containing peptide Bac2A compared to the negative control peptide. Thus plating the contents of wells untreated with peptide or treated with a Bac2A concentration equal to one half the MIC resulted in no colonies (3.7×10^6 and 1.6×10^5, respectively) whereas treatment with three- or tenfold the MIC lead to complete killing.

Strains. For the screening assay a mini-*Tn5-lux* mutant in *P. aeruginosa* PAO1 strain H103 was used. The strain is called H1001 and contains a *fliC::luxCDABE* 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 *P. aeruginosa* H103, *Enterococcus faecalis* (*E. faecalis*) ATCC29212, *S. aureus* ATCC25923 and a clinical isolate of *S. epidermidis* obtained from D. Speert (Department of Medicine, University of British Columbia). Antifungal activity was tested using a lab isolate of *Candida albicans* (*C. albicans*) obtained from B. Dill (Department of Microbiology and Immunology, University of British Columbia).

Calculation of peptide activity. Luminescence values were measured for each peptide at a range of dilutions. Controls (two replicates of the parent peptide, one or two replicates of the unrelated peptide) were present on every plate. Peptide activity values (proxy IC_{50} values) were calculated by the following procedure. The maximum luminescence value across the entire data set was calculated (L_{max}). Each data point was subtracted from the L_{max} value and the resultant decrease in luminescence plotted against the log of the apparent concentration. Linear regression lines were calculated for each data set and as long as the r^2 (correlation coefficient) value was >0.9, then the concentration at half maximal killing obtained (as half of L_{max} minus the lowest subtracted luminescence values for the parent peptide on the respective plate). This IC_{50} value was chosen to account for plate-to-plate variations across the data set. Occasionally the plot of subtracted luminescence versus log concentration clearly demonstrated saturation of the curve at high or low concentrations. In these instances, these values were removed from the calculation of the linear curve and the interpolation procedure was repeated as long as the resulting r^2 was >0.9. For data sets containing insufficient (<4) data points for curve fitting, an extrapolated value (that is, below that of the lowest concentration giving activity or above that of the highest active concentration) was assigned.

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COMPETING INTERESTS STATEMENT
The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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