CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200601583

A Combinatorial Approach to the Discovery of Biocidal Six-Residue Cyclic D,L-α-Peptides Against the Bacteria Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *E. coli* and the Biofouling Algae *Ulva linza* and *Navicula perminuta*

James T. Fletcher,[a, b] John A. Finlay,[c] Maureen E. Callow,[c] James A. Callow,*[c] and M. Reza Ghadiri*[a]

Abstract: A combinatorial approach has been used to rapidly identify cyclic D,L-α-peptide hexamer sequences that exert biocidal activity towards both methicillin-resistant *Staphylococcus aureus* (MRSA) and *E. coli* bacteria, as well as the marine algae *Ulva linza* and *Navicula perminuta*. Evaluation of the effects against marine algae was facilitated by the development of a reliable, automated assay for toxicity, which should be of general utility for biofouling investigations. While the selective toxicity of cyclic D,L-α-peptides towards bacteria has been proven to be highly sensitive to minor changes in amino acid composition, this study demonstrates that this phenomenon extends to eukaryotic species as well, despite their significant structural differences. In performing toxicity assays on both prokaryotic and eukaryotic organisms in parallel, we have discovered examples of six-residue cyclic D,L-α-peptide sequences with either broad-spectrum or highly selective biocidal activities. Sequence [KWFFFH] (underlined amino acid abbreviations represent D-amino acid residues) was found to display 100-fold selectivity towards *U. linza*, demonstrating that the approach described herein may help lead to the development of new biofouling tools which are not generally toxic to all organisms, but rather specifically target microbial agents of interest.

Keywords: algae · antibacterial agents · combinatorial chemistry · marine biofouling · peptides

Introduction

Self-assembling cyclic D,L-α-peptides are a versatile class of supramolecular structures with expanding utility in materials and biological settings.[1–5] They can be derived from appropriately designed six- and eight-residue cyclic D,L-α-peptides which partition into the membranes of targeted cells, thus destabilizing the membrane and causing rapid cell death (in minutes).[2–5] Among the attractive features of this class of antimicrobials are their facile synthesis and the ability to derive a large variety of bioactive peptides from the vast cyclic peptide sequence space. Therefore, cyclic D,L-α-peptides hold considerable promise as a class of supramolecular antimicrobial agents which can be designed or selected to exhibit broad-spectrum antimicrobial activities or can be tailored to act selectively on a desired range of microorganisms.[2–5] As a result of their abiotic structure, such molecules are both chemically and proteolytically stable and can be designed to operate in a variety of settings and environments. One such application in which targeted antimicrobial action is highly desirable is marine biofouling, which carries an annual financial burden in the billions of dollars.[6] Developing new antifouling technologies which do not release broad-spectrum biocides into the marine environment is an active area of research.[6] While the selective toxicity of cyclic D,L-α-peptides against prokaryotic organisms has been reported,[1] analogous activity against eukaryotic organisms

[a] Dr. J. T. Fletcher, Prof. M. R. Ghadiri
Departments of Chemistry and Molecular Biology
The Scripps Research Institute
10666 North Torrey Pines Road, La Jolla, CA 92037 (USA)
Fax: (+1) 858-784-2798
E-mail: ghadiri@scripps.edu

[b] Dr. J. T. Fletcher
Present address: Department of Chemistry
Creighton University, 2500 California Plaza
Omaha, NE 68178 (USA)

[c] Dr. J. A. Finlay, Dr. M. E. Callow, Prof. J. A. Callow
School of Biosciences, University of Birmingham
Birmingham B15 2TT (UK)
Fax: (+44) 121-414-5447
E-mail: j.a.callow@bham.ac.uk

has not yet been demonstrated. Hence, it was of interest to investigate whether cyclic-peptide activity might also be targeted towards eukaryotic organisms which participate in biofouling. With the long-term goal of creating next-generation antifouling coatings, such a discovery would serve both as a promising advance in the development of new antifouling tools and further exemplify the diverse and selective antimicrobial nature of cyclic D,L-α-peptides towards a wide range of target organisms.

We report herein, the discovery of six-residue cyclic D,L-α-peptides with diverse activity towards two prokaryotic bacteria (gram-positive MRSA and gram-negative *E. coli*) and two eukaryotic marine algae, *Ulva linza* and *Navicula perminuta*. *U. linza* is a macroalga (that is, a "seaweed") which colonizes a surface by the liberation of vast numbers of microscopic motile spores (5–7 μm in diameter).[7] The spores adhere permanently to a surface through the secretion of a powerful bioadhesive, later germinating to generate new attached plants. *N. perminuta* is a diatom, a single-celled alga, which attaches to a surface through secreted exopolymers.[6] In this case, cells can glide over the surface and divide, resulting in the formation of a biofilm. The algal species chosen represent two important groups of marine foulers, namely soft foulers in the case of *U. linza* and slime formers in the case of *N. perminuta*. Both have been used extensively as experimental models.[8,9] A targeted strategy to control fouling by these organisms involves engineering materials to inhibit initial surface colonization by exerting toxicity specifically towards the biofouling organisms as they initiate contact with the surface, but which remain generally nontoxic to the marine environment.

Due the fact that the antimicrobial activity of eight-residue D,L-α-peptides has been proven to be sensitive towards minor modifications of amino acid composition, coupled with the large potential bioactive sequence space of the cyclic D,L-α-peptide hexamer motif, target compounds in this study were identified by combinatorial screening.[3–5] Importantly, this required the use of reliable, high-throughput assays to evaluate activity against marine organisms in an analogous fashion to established methods for screening antibacterial activity. In addition to validation studies on the target compounds, two small analogue libraries were examined for the purpose of defining structure-activity relationships for selected cyclic peptide derivatives.

Results and Discussion

The cyclic D,L-α-peptide hexamer library utilized in this study (Figure 1) was inspired by analogous octamer sequences reported to show activity against gram-positive bacteria.[2–4] Two basic design principles were employed: the sequence needed to be 1) cationic in nature (proposed to attract it to the negatively charged bacterial cell wall) and 2) display a largely amphiphilic distribution of hydrophobic and hydrophilic residues (proposed to drive insertion into the cell membrane).[2–5,10–12] As a result of the universal

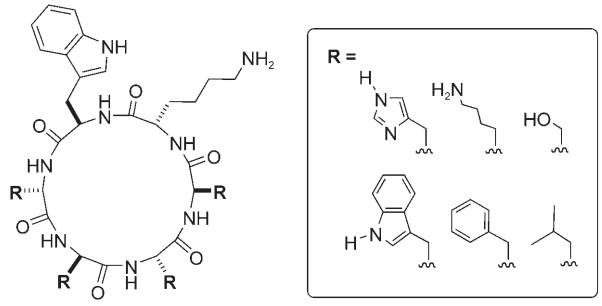

Figure 1. Design of the 1296-member cyclic D,L-α-peptide library.

usage of Lys (the location of resin immobilization for synthetic purposes), each library member was to be cationic (ranging from +1 to +5), while the distribution of hydrophobic and hydrophilic side chains was not statistically biased towards amphiphilicity. Because the cytoplasmic membrane compositions of the eukaryotes *U. linza* and *N. perminuta* are significantly different to those of either gram-positive bacteria (such as MRSA) or gram-negative bacteria (such as *E. coli*), it was unpredictable as to whether such a library designed for targeting bacteria would have any effect towards marine organisms.

Target compounds were identified by using two rounds of toxicity screening, in which activity towards preventing *U. linza* spore adhesion was the criterion for selection. Antibacterial screening was done in parallel to these experiments so that direct comparisons of peptide activity across disparate organisms could be made. Initial screenings of this cyclic peptide activity against *U. linza* were performed at a relatively high concentration (20 μM), resulting in a surprisingly large percentage of library members displaying activity. Of the 352 library members assayed, 88 members defined as "highly active" were selected, consolidated, and re-screened at lower concentrations (10, 5, and 2.5 μM). Activity was significantly diminished at the lower concentration screenings, in which of the 88 members screened, fifteen were active at 10 μM, eight at 5 μM, and only four at 2.5 μM.

Based on their activity against *U. linza*, MRSA, and *E. coli*, as well as the quality of their LC-MS/MS Sequence prediction,[13] nine members of the library were selected for validation studies. Each of these nine sequences were synthesized separately, purified, characterized by MS, and used as prepared in serial dilution assay plates so that their minimum thresholds of activity could be measured precisely. In addition to repeating assays against *U. linza*, MRSA, and *E. coli*, each derivative was tested for toxicity towards the diatom *N. perminuta* and for hemolytic activity.

At this point in the investigation, it was determined that the manual microscopic analysis employed for evaluating the survival of adhered spores of *U. linza* as a model for toxicity was not an ideal method for high-throughput analysis. Accordingly, a new automated assay was developed to measure toxicity directly. By correlating toxicity to the fluorescence intensity of chlorophyll from the eukaryote of interest, and normalizing this intensity with proper standards, a

quantitative value for each peptide corresponding to its LD_{66} (66% lethal dose) was readily obtained. From a data quality perspective, this is an advantage over the manual microscopic evaluation method in which wells were scored subjectively. From a high-throughput perspective, the new automated method is also much more efficient. Whereas in the manual method only 96 wells (one plate) could be scored in 40 minutes, the automated method allowed 96 wells to be analyzed in five minutes. This technique proved to be useful for evaluating toxicity towards both *U. linza* and *N. perminuta* and is proposed to be of general interest to screening the solution-phase toxicity of small molecules against a wide range of marine organisms.

The biological activities of the nine selected cyclic peptides are presented in Table 1. While these derivatives share many common structural features, they differ widely in their overall activity and selectivity towards this disparate range of organisms. Seven of the nine validated peptide sequences displayed reproducible toxicity towards *U. linza* in the automated assay, ranging in activity from 10 to 50 mM (considered to be largely in agreement with the values obtained in combinatorial screening). Two of the peptides (**8** and **9**) did not agree with the value predicted from the combinatorial screening. This disagreement could be attributed to the purity of the library samples, MS-based hit sequencing errors, or inaccuracies in the manual assessment of *U. linza* toxicity. Although hits were initially selected based on their activity against *U. linza*, surprising trends in antibacterial activities were observed. Cyclic peptides **1** and **2** displayed appreciable selectivity for gram-negative bacteria (*E. coli*) while peptides **3** and **4** showed an opposite gram-positive (MRSA) selectivity. Interestingly, while MRSA-selective cationic peptides **3** and **4** possess amphiphilic sequence structures, *E. coli*-selective enantiomeric peptides **1** and **2** have an unique mixed topology, suggesting that the histidine residue bound on either side by hydrophobic amino acids acts as a hydrophobic residue in its deprotonated state. Furthermore, in addition to the enantiomeric sequences **1** and **2**, peptides **5** and **6**, which are retroenatiomeric sequences, also display similar activity profiles, suggesting that side-chain properties are largely responsible for the observed biocidal behavior against both prokaryotic and eukaryotic organisms. Previous studies have reported similar observations for the antibacterial behavior of cyclic peptide octamers.[2–4] Based on the data obtained from the validation studies, two analogue libraries were constructed to examine the biocidal activity and species selectivity with respect to single amino acid substitutions (Figure 2).

Table 1. Activities of selected hits against bacteria, algae, and red blood cells.[a]

Peptide sequence[b]	MIC [μM][c]		LD_{66} [μM][d]		HD_{50}[e]
	MRSA	*E. coli*	*U. linza*	*N. perminuta*	[μM]
1 [K**W**F**H**W**K**]	30	15	10	20	>100
2 [K**W**F**H**W**K**]	>100	7.5	12.5	n.d.[f]	n.d.
3 [K**W**F**F**L**H**]	10	>100	10	>100	>100
4 [K**W**L**F**F**K**]	7.5	40	40	20	50
5 [K**W**W**F**W**K**]	30	100	10	40	>100
6 [K**W**F**W**W**K**]	100	>100	30	20	>100
7 [K**W**F**K**W**S**]	>100	>100	50	30	>100
8 [K**W**F**K**K**L**]	50	20	>100	30	>100
9 [K**W**F**L**W**H**]	100	>100	>100	40	>100

[a] See the Experimental Section for assay details. [b] Underlined amino acid abbreviations represent D-amino acid residues. [c] MIC = minimum inhibitory concentration. [d] LD_{66} = 66% lethal dose. [e] HD_{50} = 50% hemolytic dose (mouse erythrocytes). [f] n.d. = not detected.

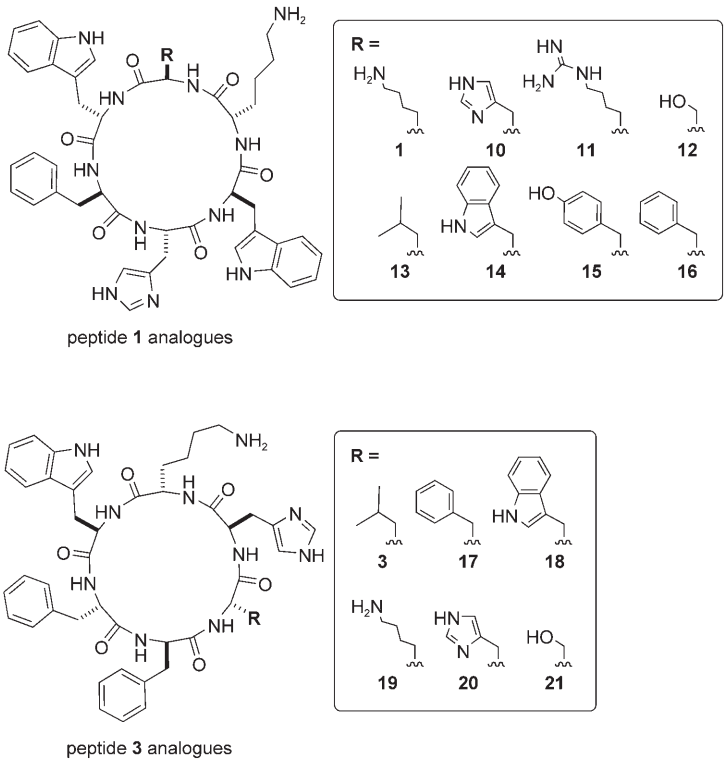

Figure 2. Analogue peptide libraries.

Peptide sequence **1** was chosen to derive the analogue family **1** of cyclic peptides (peptides **1**, **10**–**16**; Figure 2, Table 2) because of its appreciable broad-spectrum activity against the tested organisms. It is important to note that as the result of single amino acid substitutions, the overall cationic charge and amphiphilicity varies within this series of eight related peptide sequences. In comparing the activity profiles of analogue **1** peptides (Table 2), it seems that overall toxicity is strongly affected by the degree of amphiphilicity and not by the overall cationic charge. Sequences containing hydrophilic residues (Lys, Arg, His, or Ser) each display broad-spectrum activity towards the range of organisms assayed, while those sequences containing hydrophobic Trp, Tyr, or Phe substitutions were largely inactive in the range of concentrations tested. Peptide **13**, containing hydrophobic Leu, is the exception to this trend and displays >10-fold selectivity for MRSA.

Peptide **3**, which has the most selective activity profile (active only against MRSA and *U. linza*) was used to derive

Table 2. Activities of peptide **1** and **2** analogues against bacteria, algae, and red blood cells (mouse erythrocytes).[a]

Peptide sequence[b,c]	MIC [μM][d]		LD_{66} [μM][e]		HD_{50}
	MRSA	*E. Coli*	*U. linza*	*N. perminuta*	[μM][f]
analogue **1**					
1 [**K**WFHW**K**]	30	15	10	20	>100
10 [**H**WFHW**K**]	15	20	50	20	>100
11 [**R**WFHW**K**]	30	20	10	30	>100
12 [**S**WFHW**K**]	10	40	5	8	>100
13 [**L**WFHW**K**]	10	>100	>100	>100	>100
14 [**W**WFHW**K**]	>100	>100	>100	>100	>100
15 [**Y**WFHW**K**]	>100	>100	>100	>100	>100
16 [**F**WFHW**K**]	>100	>100	>100	>100	>100
analogue **3**					
3 [KWFF**L**H]	10	>100	10	>100	>100
17 [KWFF**F**H]	100	>100	1	100	>100
18 [KWFF**W**H]	50	>100	30	>100	>100
19 [KWFF**K**H]	15	10	10	8	>100
20 [KWFF**H**H]	7.5	50	8	10	90
21 [KWFF**S**H]	40	50	5	30	>100

[a] See Experimental Section for assay details. [b] Underlined amino acid abbreviations represent D-amino acid residues. [c] Bold amino acid abbreviations represent the variable residues. [d] MIC = minimum inhibitory concentration. [e] LD_{66} = 66% lethal dose. [f] HD_{50} = 50% hemolytic dose.

analogue **3** peptides (Figure 2). Single amino acid substitutions resulted in a series of six derivatives (peptides **3**, **17**–**21**) with varying degrees of the overall charge and amphiphilicity. Similar to the trend for analogue **1** sequences, hydrophilic Lys, His, or Ser amino acid substitutions (peptides **19**, **20**, and **21**) resulted in loss of species selectivity and gain of broad-spectrum activity (Table 2). These hydrophilic variants were consistently more active against *N. perminuta* than the parent peptide **3**. Most noteworthy, substituting Leu (Peptide **3**) with Phe (peptide **17**) resulted in the most potent and selective cyclic peptide derivative discovered in this study. We suggest that peptide **17** which has 100-fold selectivity towards *U. linza* might be a particularly attractive candidate for future development of antifouling agents.

Conclusion

A combinatorial approach has been used to rapidly identify cyclic D,L-α-peptide hexamer sequences which exert biocidal activity towards both MRSA and *E. coli* bacteria, as well as the marine algae *U. linza* and *N. perminuta*. Evaluation of the effects against marine algae was facilitated by the development of a reliable, automated assay for marine toxicity which should be of general utility for biofouling investigations. This study serves as another example of the remarkable scope and utility of the cyclic D,L-α-peptide architecture for the discovery of bioactive agents.

Because antimicrobial cyclic peptides are believed to exert biocidal behavior by partitioning into and destabilizing the external cytoplasmic membranes of targeted cells,[2–5] it is reasonable to expect that sequences with broad-spectrum toxicity could be identified for both eukaryotic and prokaryotic organisms. The cytoplasmic membranes of all living cells have a common structure based on the phospholipid bilayer and contain intercalated proteins that define their functional properties and are the main source of diversity across the major phyla of organisms. Although much is known about the molecular structure of the cytoplasmic membrane of prokaryotes (such as bacteria), there is little comparable molecular data for marine algae. However, considerable variations are anticipated due to the disparate evolutionary position of prokaryotes and eukaryotes (sterols for example are only found in eukaryotic cell membranes) and because their cytoplasmic membranes play different roles in their respective overall biology. A goal of this investigation was to discover biocidal molecules which are able to exploit these differences in order to target individual organisms selectively.

Due to the fact that self-assembling cyclic D,L-α-peptides often do not display intuitive structure-activity relationships, combinatorial screening followed by sequence optimization through parallel synthesis was shown to be an efficient approach towards discovering new sequences of interest. In this study, multiple cyclic D,L-α-peptide hexamers displaying broad-spectrum activity as well as a few with species-selective potencies coupled with low hemolytic profiles were identified. Analogous to previously reported antibacterial properties, toxicity towards the marine organisms in this study was shown to be highly sensitive towards single side-chain perturbations, enabling selective biocidal behavior to be identified from the screening process. A sequence displaying 100-fold selectivity towards *U. linza* was discovered, showing promise that the approach described herein may help lead to the development of new biofouling tools which are not generally toxic to all organisms, but rather engineered towards specific microbial agents of interest.

Experimental Section

Split-and-pool peptide library synthesis: Fmoc-Lys-OAll loaded trityl chloride macrobead resin was prepared as previously reported.[13] The peptides [KWXXXX], in which X = F, H, K ,L, S, or W were prepared by using a split-and-pool approach and Fmoc chemistry.[13–15] Each amino acid coupling step was performed by using four equivalents each of Fmoc amino acid, hydroxybenzotriazole (HOBt), and diisopropylcarbodiimide (DIC) in *N*-methylpyrrolidine (NMP) relative to resin loading and agitated with orbital shaking until all library members showed complete coupling by means of bromophenol blue dye monitoring (2–12 h).[16,17] Fmoc was deprotected by using piperidine/DMF 25:75 washings (2×20 min). After the sixth amino acid residue was coupled and Fmoc deprotected, the resin was washed with CH_2Cl_2 and the allyl protecting group was removed.[18,19] In two separate vessels, CH_2Cl_2 solutions of $Pd(PPh_3)_4$ (0.2 equivalents) and $PhSiH_3$ (10 equivalents) were degassed by using a nitrogen stream and then added to the resin and sealed under nitrogen. After agitating overnight, the resin was washed with CH_2Cl_2 (2×10 min), DMF (2×10 min), 1% dimethylthiocarbamic acid in DMF (2×10 min), 20% DIEA in DMF (2×10 min), and NMP (2×10 min). The linear deprotected peptide was then cyclized by treatment with a NMP solution containing one equivalent each of DIC and HOBt (4×1 h). The resin was washed with DMF (2×10 min), CH_2Cl_2 (2×10 min), and MeOH (2×10 min), and was then dried under vacuum overnight. Individual beads from the cyclic peptide library were separated into 96 well plates (one

bead per well) and cleaved from the resin by treatment with a cleavage cocktail (TFA/H_2O/TIS 95:2.5:2.5) for 4 h. After cleavage, the TFA mixture was evaporated under vacuum to remove all volatiles. An appropriate volume of DMSO was then added to each well to give approximate 2 mM concentrations of parent solutions (based on original loading of macrobead resin). These parent plates were then used to create assay plates by dilution into 0.5 M aq NaCl by using a Tecan Genesis solvent manipulation system.

LC-MS/MS sequencing: The amino acid sequences of library members of interest were identified by means of LC-MS/MS analysis as previously described.[13]

Peptide synthesis: Peptides synthesized for validation studies and parallel screening were prepared analogously to the library described above, except that 200 mesh trityl chloride resin was used, bromophenol blue monitoring was not employed, and amino acid couplings were performed by using standard HBTU/DIEA conditions. Peptides were purified by preparatory scale HPLC (MeCN/H_2O/TFA gradient) and characterized by tandem HPLC/MS analysis.

Preparation of library assay plates: In the initial round of assays, a Tecan Genesis system was used to dilute the 2 mM DMSO parent solutions into 0.5 M NaCl to give 96-well assay plates each containing 20 (for bacteria) or 40 μL (for marine organisms) solutions of 100 μM cyclic peptide (5% DMSO) in 0.5 M NaCl. For each different microbe to be tested, plates were prepared in triplicate. Upon addition of 80 (for bacteria) or 160 μL (for algae) microbial agent solution, a final assay concentration of 20 μM peptide (1% DMSO) was obtained. Of the original 1296-member library, 532 members were assayed, 88 active wells were selected, and their parent DMSO solutions were used to create "cherry picked" assay plates of 50, 25, and 12.5 μM concentrations of peptide in 0.5 M aq NaCl, which when diluted into microbe solution gave assay concentrations of 10, 5, and 2.5 μM peptide (containing 0.5, 0.25, and 0.125 DMSO, respectively).

Preparation of validation assay plates: A parent solution of each purified peptide obtained from direct synthesis was prepared by dissolution in DMSO and adjustment of the concentration to 2 mM by using the optical density (OD, 280 nm) and extinction coefficients of the Trp and Phe residues as applicable. These 2 mM DMSO solutions were used to prepare assay plates which resulted in final peptide concentrations ranging from 0–100 μM. Three copies of each serial dilution plate were prepared.

Anti-algal screening (manual adhesion assay for *U. linza*): Zoospores of *Ulva linza* were released from plants collected from Wembury Beach, Devon (UK) as previously described[7] An *U. linza* spore suspension in seawater of OD (660 nm) = 0.25 was prepared. Aliquots (160 μL) of this suspension was were added to the 96-well assay plates to give a final OD (660 nm) = 0.2, corresponding to approximately 2×10^6 spores ml^{-1}. After addition of spores, each assay plate was placed on an orbital plate shaker for 1 min at max speed to mix the contents, after which the plates were incubated in the dark at room temperature for 1 h. The plates were washed by hand shaking followed by refilling with 100 μL sea water and this process was repeated three times. Assessment of the number of spores attached to the well was made by viewing with an inverted microscope. Each well of each plate was assigned a score of 0, 1, or 2 depending on the quality and quantity of the attached spores. Poor-quality spores were ones that had damaged membranes and extruded cytoplasmic contents, whilst good-quality spores were ones that were rounded and appeared to be normal. A semiquantitative approach was used as automated counting could not discriminate between live and damaged spores. A score of 0 corresponded to 0 or very few poor-quality spores attached, a score of 1 corresponded to a mixture of poor and good-quality spores attached, and a score of 2 corresponded to a large number of good-quality spores attached (each as compared to the control wells). The scores for each of the three replicates were added, giving a total score of each individual well from zero to six. Those wells displaying a score of 0 or 1 were then used to create the "cherry-picked" assay plate tested at 10, 5, and 2.5 μM peptide concentrations.

Antibacterial screening: MRSA (ATCC #33591) and *E. coli* (ATCC #35218) bacteria were grown with agitation at 37 °C overnight and then were diluted into Muller–Hinton broth to give a concentration of 5×10^5 CFU ml^{-1}. Aliquots (80 μL) of these suspensions were added to 96-well assay plates containing 20 μL solutions of peptide in 0.5 M aq NaCl (the identical concentrations as described in the *U. linza* assays). After shaking in a 37 °C incubator for 24 h, the bacterial growth in each was determined by measuring OD (595 nm) by using a Tecan Genios platereader. Those wells displaying OD < 0.15 (transparent to the naked eye) were defined as suppressing bacterial growth. Identical methodology was used for both library and dilution plate screening.

Hemolytic activity: Heparinized murine blood was centrifuged at 1000 g for 10 min and the supernatant and the buffy coat removed. Erythrocytes were washed three times with 0.9% saline containing 10% FBS (v/v). Red blood cells were then treated with serial dilutions of the peptides in a 96-well plate at 37 °C for 30 min. Control samples included a saline solution and a 1% Triton X-100 as 0 and 100% hemolysis, respectively. Plates were centrifuged at 1000 g for 10 min and aliquots of the supernatant were diluted two times with saline solution and the absorbance was measured at 560 nm.

Automated toxicity assay for *U. linza* and *N. perminuta*: Zoospores were harvested from reproductive plants of *Ulva linza* as described for the manual assay. The spores were suspended in enriched seawater medium[20] and added to the wells of the plates to give an equivalent final OD (660 nm) of 0.2. The plates were agitated vigorously for 2 min on an orbital plate shaker to disperse the spores before being placed in the dark for a 2 h settlement period, followed by incubation in the light at 18 °C with a 16/8 hour light:dark cycle.

After 96 h, the sporeling biomass was measured by using a fluorescent plate reader (Tecan GENios Plus). By allowing healthy spores to germinate and grow into sporelings, the problem of discerning between healthy and moribund cells, which was inherent in the manual method of assessment was overcome. The mean data for each treatment were converted to a percentage of the control values for each plate. Wells in which growth was less than two thirds of the control (< 66%) were deemed to have contained inhibiting peptides.

A similar methodology was carried out for the diatom *Navicula perminuta*. Cells were grown to log-phase in conical flasks containing Guillard's F/2 medium[21] in an illuminated incubator as above. Cells adhering to the base of the flask were washed three times with fresh F/2 medium before being suspended and filtered for use. Cells were added to the dishes to produce an equivalent chlorophyll *a* concentration of 0.1 μg mL^{-1} (quantified by using the method of Shoaf and Lium)[22] and were agitated vigorously for two minutes on an orbital plate shaker. After a 2 h settlement period, the diatoms were incubated under the same conditions as the *U. linza* spores.

The growth of the diatoms was also evaluated by using the fluorescent plate reader. The data were converted to a percentage of the control values for each plate. Those wells with growth less than half of the control (< 50%) were scored as containing inhibiting peptides.

Acknowledgements

J.T.F. acknowledges the Skaggs Institute for Chemical Biology for a research fellowship. This research was supported by the Office of Naval Research (N00014–02–1–0520 to M.R.G. and N00014–02–1–0521 to J.A.C and M.E.C.).

[1] D. T. Bong, T. D. Clark, J. R. Granja, M. R. Ghadiri, *Angew. Chem.* **2001**, *113*, 1016–1041; *Angew. Chem. Int. Ed.* **2001**, *40*, 988–1011.
[2] S. Fernandez-Lopez, H. S. Kim, E. C. Choi, M. Delgado, J. R. Granja, A. Khasanov, K. Kraehenbuehl, G. Long, D. A. Weinberger, K. Wilcoxen, M. R. Ghadiri, *Nature* **2001**, *412*, 452–454.
[3] K. M. Wilcoxen, Ph.D. Thesis, **2002**, The Scripps Research Institute, La Jolla, California (USA).
[4] V. Dartois, J. Sanchez-Quesada, E. Cabezas, E. Chi, C. Dubbelde, D. Carrie, J. Granja, C. Gritzen, D. Weinberger, M. R. Ghadiri, T. R. Parr Jr., *Antimicrob. Agents Chemother.* **2005**, *49*, 3302–3310.

[5] S. W. Horne, C. M. Wiethoff; C. Cui, K. M. Wilcoxen, M. Amorin, M. R. Ghadiri, G. R. Nemerow, *Bioorg. Med. Chem.* **2005**, *13*, 5145–5153.
[6] M. E. Callow, J. A. Callow, *Biologist* **2002**, *49*, 1–5.
[7] M. E. Callow, J. A. Callow, J. D. Pickett-Heaps, R. Wetherbee, *J. Phycol.* **1997**, *33*, 938–947.
[8] A. Statz, J. Finlay, J. Dalsin, M. Callow, J. A. Callow, P. B. Messersmith, *Biofouling* **2006**, *22*, 391–399.
[9] J. A. Callow, M. E. Callow in *Biological Adhesives* (Eds.: A. M. Smith, J. A. Callow), Springer-Verlag, Berlin, Heidelberg, **2006**, pp. 63–78.
[10] H. G. Boman, *Annu. Rev. Immunol.* **1995**, *13*, 61–92.
[11] L. Andreu, L. Rivas, *Biopolymers* **1998**, *47*, 415–433.
[12] Z. Oren, Y. Shai, *Biopolymers* **1998**, *47*, 451–463.
[13] J. E. Redman, K. M. Wilcoxen, M. R. Ghadiri, *J. Comb. Chem.* **2003**, *5*, 33–40.
[14] K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski, R. J. Knapp, *Nature* **1991**, *354*, 82–84.
[15] A. Furka, F. Sebestyen, M. Asgedom, G. Dibo, *Int. J. Pept. Protein Res.* **1991**, *37*, 487–493.
[16] K. S. Lam, M. Lebl, V. Krchnak, *Chem. Rev.* **1997**, *97*, 411–448.
[17] R. Liu, J. Marik, K. S. Lam, *J. Am. Chem. Soc.* **2002**, *124*, 7678–7680.
[18] N. Theriet, J. Alsina, E. Girlat, F. Guibe, F. Albericio, *Tetrahedron Lett.* **1997**, *38*, 7275–7278.
[19] M. Dessolin, M.-G. Guillerez, N. Thieriet, F. Guibe, A. Loffet, *Tetrahedron Lett.* **1995**, *36*, 5741–5744.
[20] R. C. Starr, J. A. Zeikus, *Int. J. Pept. Protein Res.*, **1987**, *23*, S1–27.
[21] R. R. L. Guillard, J. H. Ryther, *Can. J. Microbiol.* **1962**, *8*, 229–239.
[22] T. W. Shoaf, B. S. Lium, *Limnol. Oceanogr.* **1976**, *21*, 926–928.

Received: November 7, 2006
Published online: February 16, 2007

