Reversible Antifouling Effect of the Cyclotide Cycloviolacin O2 against Barnacles†

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Cycloviolacin O2, a plant peptide of the cyclotide family, is shown to have potent effects against fouling barnacles (*Balanus improvisus*), with complete inhibition of settlement at a concentration of 0.25 μM. The effect of cycloviolacin O2 against barnacles is reversible and nontoxic in the bioassay employed in these studies. Cycloviolacin O2 was isolated from the terrestrial plant *Viola odorata* by strong cation exchange and reversed-phase HPLC and identified by mass spectrometry following aminoethylation and enzymatic cleavage.

Fouling marine organisms such as barnacles, blue mussels, and hydroids cause severe technical and economical problems for aquaculture and shipping. Tri-*n*-butyl tin oxide (TBTO), copper oxide, and herbicides in marine coatings have been used extensively to solve this problem. But these agents, besides being toxic to their target organisms, exhibit unwanted effects against several nonfouling marine organisms. For example, in oysters, effects such as reproduction failure and decrease in adult growth,[1] and in gastropods, effects such as the development of imposex, as in the dog whelk,[2] have been attributed to TBTO. Because of these unwanted side effects, the use of TBTO will be stopped by future bans. Indeed, the International Marine Organisation (IMO) states in a resolution that a complete prohibition on the presence of organotin compounds that act as biocides in antifouling systems on ships will be ensured by January 2008.[3] Hence, finding new nontoxic alternatives that exert a specific action on target organisms and that are also biodegradable is urgent.

One promising approach to finding such antifouling agents is to explore natural compounds occurring in the marine environment,[4] especially those produced by sessile fouling-free marine organisms.[5,6] By describing the potent antifouling effect against barnacles (*Balanus improvisus* Darwin) of the plant peptide cycloviolacin O2, we show that this search for biologically active natural antifouling products can be beneficially expanded to include products isolated from terrestrial sources as well.

This peptide, cycloviolacin O2, isolated from the sweet violet, *Viola odorata* Linn. (Violaceae), is a member of the family of macrocyclic plant peptides named cyclotides.[7] This family delineates the so-called cyclic cystine knot motif.[7−9] That is, the characterizing amide backbone of members of the family is circular, and thus all members lack both N- and C-terminals, and they contain six cysteine residues involved in three disulfide bridges in a cystine knotted arrangement. These structural features, in combination with their size, ranging from 28 to 37 amino acid residues, render these peptides a unique structure that is very compact and stable.

Approximately 50 cyclotides have been described. Most of them have been isolated from the Violaceae and Rubiaceae plant families, in which they are expressed as complex mixtures in a number of plants.[10−13] The first cyclotide was discovered as the active substance in a uterocontracting native medicine used to facilitate childbirth.[14,15] Since then, a wide variety of other effects have been attributed to this peptide family, including antimicrobial,[16] HIV-inhibitory,[17−20] trypsin inhibitory,[16,21] anticancer,[22,23] and insecticidal[24] effects. Their function within the plant, however, has yet to be fully elucidated, but probably they are involved in the plant's host-defense system.[11,24] This ability for carrying such an array of activities, combined with their unique structure, has also made the cyclotides highly interesting as scaffolds for molecular engineering.[25]

In this study we show that one of the cyclotides, cycloviolacin O2, which was first described by Craik and co-workers,[7] by having a potent, nontoxic, and reversible effect against fouling barnacles, has promise as a marine antifouling substance.

Experimental Section

General Experimental Procedures. HPLC (cation exchange and reverse-phase) was done on an Äkta Basic system (Amersham Biosciences, Uppsala, Sweden). MS was done using a nanospray-ion trap MS [Protana's NanoES source (MDS Protana A/S, Odense, Denmark) mounted on a LCQ (Thermo Finnigan, San Jose, CA)] in the positive ion mode. Samples were sprayed using a solution (60% MeOH, 1% HOAc in water); the capillary temperature was set at 150 °C and the spray voltage, at 0.5 kV. For MS/MS sequencing, the relative CID was typically set at 25%. Unless otherwise stated, average masses were used.

Isolation of Cycloviolacin O2. Dried and ground plant material (*Viola odorata* Linn., obtained from Galke, Gittelde, Germany) was defatted with CH_2Cl_2 before the main extraction was carried out using 50% aqueous EtOH. This extract was concentrated in vacuo (i.e., all EtOH was removed); then the acidified (final concentration 2% HOAc) extract was filtered through polyamide to remove tannins before being partitioned between H_2O and *n*-BuOH. Up to this point, the isolation procedure was carried out according to the procedures outlined in Claeson et al.[26] and Broussalis et al.[12] The BuOH fraction was then evaporated and redissolved (in 25% MeCN, 0.1% TFA in water) before being pumped through a Vydac sulfonic acid polymeric strong cation exchange column (400VHP575, 5 μm, 7.5 mm i.d. × 50 mm) at a flow rate of 1 mL/min, until the binding sites of the gel were saturated, that is, until peptides could be detected (by RP-HPLC) in the eluting solvent. Bound

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substances were then eluted with a salt gradient ranging from 0 to 1 M NaCl. The peak elution in the salt gradient was collected and analyzed using RP-HPLC and MS. The major peptide was then isolated by means of RP-HPLC, in a manner analogous to that described in Göransson et al. and Broussalis et al.[12,13]

Acetylation of Lysines. Dry peptide (~3 nmol) was dissolved in 20 μL of 50 mM NH_4HCO_3. To this, 50 μL of acetic anhydride in MeOH (1:3) was added. After 1 h incubation at room temperature, the mixture was lyophilized to dryness.

Aminoethylation and Tryptic Cleavage. This procedure was essentially carried out according to published methods[10] as follows. Peptide (~3 nmol) was reduced with dithioerythritol (DTE, 390 nmol) in 0.25 M Tris-HCl containing 1 mM EDTA and 6 M guanidine-HCl (pH 8.5; 24 °C; 1 h). Reduced peptide was then alkylated by the addition of 25 equiv of bromoethylamine versus DTE (9.75 μmol) dissolved in 10 μL of the Tris-HCl buffer. The reaction was then incubated overnight (37 °C), after which it was terminated by injection on RP-HPLC. The derivatized peptide was collected manually and lyophilized, and then cleaved overnight with modified trypsin (Promega Co., WI) (0.2 μg of trypsin in 50 μL of 50 mM NH_4HCO_3, pH 7.8, 37 °C).

Larval Bioassay. The brood stock of adult barnacles (*Balanus improvisus* Darwin) were allowed to settle on Plexiglas panels on a raft in the sea outside the Tjärnö Marine Biological Laboratory (58°53′ N, 11°8′ E). After being cleaned of epiphytes, the brood stock was brought to the laboratory and immediately placed in trays with running seawater (salinity 32 ± 1%). *Balanus improvisus*, when regularly fed with nauplii of *Artemia salina*, will spawn throughout the year. For larval rearing, we used the method of Berntsson and coworkers.[27] The experiment for evaluating the effect on settlement and mortality was conducted using polystyrene Petri dishes (ø 48 mm) to which was added 10 mL of the peptide dissolved to different concentrations in filtered seawater (0.2 μm, 32 ± 1%). Competent cyprids (20 ± 2 individuals) were added to each dish in four replicates, with dishes containing filtered seawater serving as controls. Dishes were maintained for 3−4 days at room temperature, after which they were examined under a stereomicroscope for attached and metamorphosed individuals, and also for dead cyprids.

Reversibility Test. For each Petri dish incubated at the highest tested concentration (2.5 μM), half the cyprids were moved to a Petri dish containing 10 mL of fresh seawater. These Petri dishes, as well as the original ones incubated at this concentration, were examined (as described above) after 5 days of incubation.

Statistical Method. Results from a 1-factor analysis of variance (ANOVA) are reported as means ± standard error.

Results and Discussion

After isolating the pure peptide (cycloviolacin O2) from the cyclotide-containing material obtained from the plant *Viola odorata*, the antifouling activity of the peptide against barnacles was measured. The peptide's identity was established by MS of the native peptide and by MS/MS of fragments obtained after derivatization and enzymatic digestion.

Extraction of plant material and isolation of cycloviolacin O2 was carried out as previously described,[12,13,26] with the exception of the use of strong cation exchange chromatography (SCX), which effectively captured positively charged cyclotides, as shown in Figure 1. SCX was done at pH 2 to ensure that peptides were positively charged: cycloviolacin O2 is predicted to have a net charge of +3 at this pH. To further promote charge−charge interactions between the peptide and the SCX gel, organic solvent (MeCN) was included in the mobile phase, improving thereby the retention of the peptides and also preventing unwanted hydrophobic substances in the extract from interfering with the separation. Such methods of promoting charge−charge

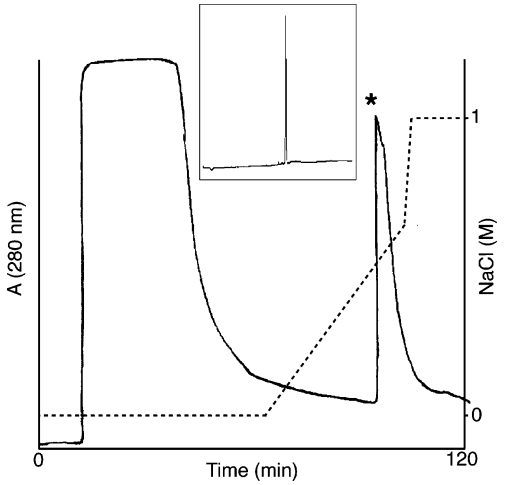

Figure 1. Isolation by ion exchange chromatography. Having a basic net charge, cyclotide molecules were effectively captured by strong cation exchange chromatography (SCX). After elution of noncharged substances, the bound peptides (marked *) were eluted in a NaCl gradient (0−1 M). Cycloviolacin O2 was then isolated from this mixture of cyclotides by RP-HPLC. The inset shows the purity of the tested compound as judged by RP-HPLC.

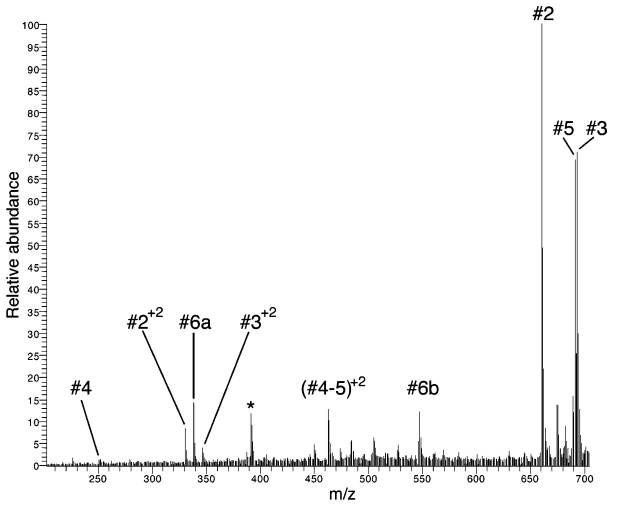

Figure 2. MS of intercysteine loops after tryptic digestion. Ions (*m*/*z*) and their corresponding loop number are shown. Loop 1 appears absent in this particular experiment: this loop contains the only acidic residue within its sequence, GESC, and therefore the abundance of this ion was very low. *Also present in the blank cleavage buffer.

interactions between the peptide and the SCX gel proved to efficiently complement the final purification done by RP-HPLC, because completely different features of the cyclotides are exploited (i.e., charge versus hydrophobicity). Using RP-HPLC and MS analyses, the peak eluting in the salt gradient was collected and found to contain a mixture of peptides. The major peptide, cycloviolacin O2, was then isolated by means of preparative RP-HPLC, analogously with previous isolations of cyclotides.[12,13,26]

Using nanospray MS, the molecular weight of the isolated peptide was determined to 3140.4, which is consistent with the molecular weight of the earlier reported cycloviolacin O2[7] (calculated MW: 3140.8). As there are several cyclotides reported with this particular molecular weight in common, the peptide was then subjected to the recently described intercysteine loop sequencing protocol to unambiguously establish its identity.[10] In short, this protocol involves conversion of cysteines into their aminoethylated derivatives (acting as enzymatic cleavage sites) that, following tryptic digestion, yield fragments corre-

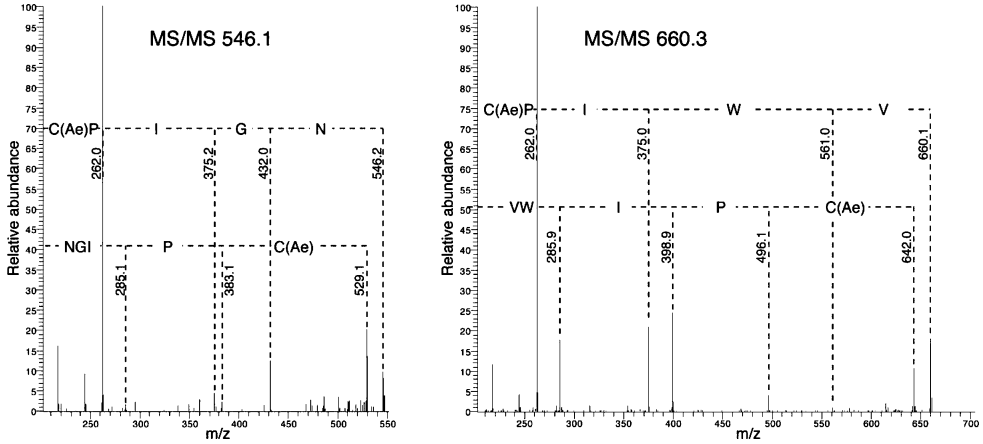

Figure 3. MS/MS sequencing. The MS/MS spectra of the ions *m/z* 546.1 and 660.3 show full sequence coverage of loop 6b and loop 2 through their C- and N-terminal fragmentation series. Equivalent spectra were obtained for all intercysteine loops.

Figure 4. Complete sequence of cycloviolacin O2. The identified intercysteine loops are underlined, showing the complete sequence coverage obtained by MS analyses of the derivatized peptide. Cysteines and loops are numbered according to the praxis outlined in ref 7 (note that the starting point of this macrocyclic peptide is arbitrary).

sponding to the intercysteine loops of the peptide. These shorter and linear fragments are then ideal targets for MS/MS sequencing.

For cycloviolacin O2 the analysis, however, is complicated by the occurrence of native cleavage sites. To limit this problem, the two lysines in the sequence of cycloviolacin O2 were protected from cleavage by acetylation, allowing then the sequence KSKVC to be identified as one fragment. Figure 2 shows the MS analysis of the obtained fragments, which all had masses consistent with the calculated masses for digested cycloviolacin O2. Each fragment was then subjected to sequencing by MS/MS, as exemplified in Figure 3. Table 1 (provided as Supporting Information) shows the assigned C- and N-terminal fragmentation series for all enzymatic cleavage products. Following these experiments, the complete sequence was established, as shown in Figure 4.

The effect of cycloviolacin O2 on the mortality of *Balanus improvisus* and its dose-dependent inhibition of settlement for that species is shown in Figure 5. At the lowest tested concentration (0.0025 μM), the degree of settlement was the same as in the control (saltwater only), with 68 $\pm$ 3% and 67 $\pm$ 3% settlement, respectively. At 0.025 μM cycloviolacin O2, only 21 $\pm$ 0.5% of the cyprids settled, and at 0.25 μM, none settled (i.e., complete inhibition).

As shown in Figure 5, no increase in mortality could be detected compared to the control, even at the highest tested concentration (2.5 μM). Cyprids incubated at this concentration were then moved into Petri dishes containing fresh seawater (without cycloviolacin O2), where they then regained their normal settlement behavior. Figure 6 shows that 27% of the transferred cyprids had settled after 5 days. No increase in mortality was observed.

Having now described the isolation and identification of the plant cyclotide cycloviolacin O2 and its potent effects against fouling barnacles, we conclude that the antifouling of cycloviolacin O2 is reversible and, in this particular assay, nontoxic, because cyprids exposed to the peptide regained their normal settlement behavior after being transferred to fresh seawater. This result, which distinguishes cycloviolacin O2 from other antifouling agents used today, may well represent a substantial step toward a solution to the major problems caused by the acute toxicity and side effects of other agents, and for this reason and others, a major step toward solving the severe problem of marine biofouling has been made.

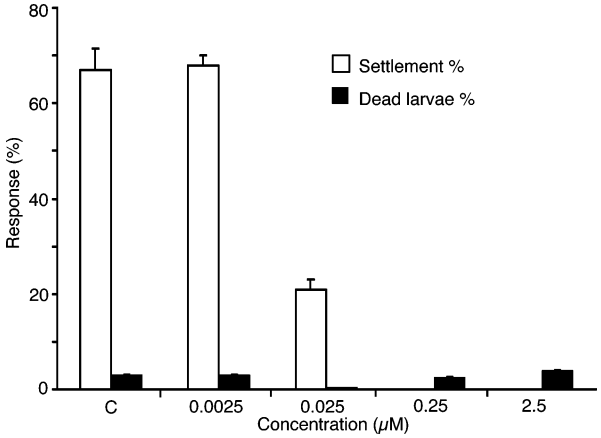

Figure 5. Antifouling effect of cycloviolacin O2. The settlement of barnacles was inhibited in a dose-dependent manner. At 0.25 μM cycloviolacin O2, settlement was completely inhibited. Not one of the tested concentrations (given as means $\pm$ SE; $n = 4$) showed an increase in mortality compared to the control (C, seawater only).

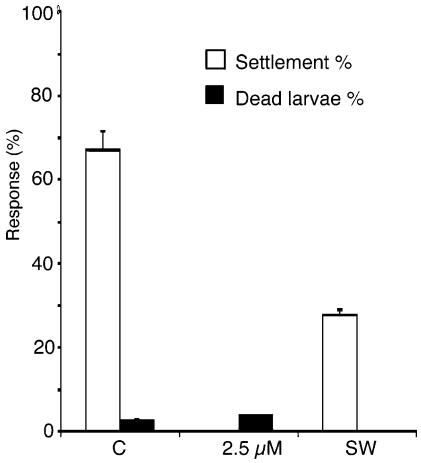

Figure 6. Reversibility test. Cyprids exposed to a high concentration (2.5 μM cycloviolacin O2) regained their innate settlement behavior when transferred to fresh seawater (SW). Compared to the control (C), a lower percentage of transferred cyprids settled. However, after long incubation times (in this case 3+5 days), reduced probability of settlement is normal, perhaps because of exhaustion. Mortality (given as means $\pm$ SE; $n = 4$) did not increase.

The many cyclotides known to occur in the family of Violaceae,[7,10] and their high degree of sequence homology made molecular weight determination alone an insufficient tool for unambiguous identification of the isolated peptide. Hence, for intercysteine loop sequencing of cyclotides, we used that method,[10] which comprises introduction of charges and of cleavage sites on the cysteines, followed by enzymatic digestion to produce peptide fragments suitable for sequencing by MS. The number of cleavage sites was limited by acetylation of lysine residues, which allowed loop 5 (KSKVC) to be sequenced as one entity instead of being cleaved into three smaller fragments. The full sequence was determined to be cyclo-(VWIPCISSAIGCSCKSKVCYRN-GIPCGESC), and therefore the peptide was unambiguously identified as the previously reported cycloviolacin O2.[7]

The inhibitory effect of cycloviolacin O2 against fouling barnacles was well within the same range as that for TBTO: the inhibition by cycloviolacin O2 was complete at a concentration of 0.25 μM. This degree of inhibition might be compared to an EC_{50} value of 0.15 μM (0.09 μg mL^{-1}) for TBTO in the same assay.[28] We stress though that the EC_{50} value for TBTO represents the concentration at which 50% of the cyprids are dead,[28] whereas the effect of cycloviolacin O2 in this assay is nontoxic and reversible, giving cycloviolacin O2 a distinct advantage over TBTO and other commercially used marine antifouling agents.

To our knowledge, this is the first report of an evaluation of a natural product from a terrestrial source against fouling barnacles. The studied substance represents, curiously, a class and size of molecules—peptides—that generally are considered nonattractive due to their instability. On the contrary the cyclotides are recognized for their stability: they resist enzymes and conditions as harsh as boiling water.[14,15] Thus they are regarded as an interesting scaffold for drug design and development,[25,29] which has triggered the development of methods for cyclotide synthesis.[25,30,31] The opportunity to utilize this knowledge for building structure–activity relationships pertaining to antifouling activity is intriguing; the structure may then be optimized for potency and to develop a suitable formulation (e.g., one might covalently bind the cyclotide directly to a surface or into an antifouling paint).

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Supporting Information Available: A table with the assigned C- and N-terminal fragmentation series for all products following aminoethylation and tryptic cleavage of cycloviolacin O2 is provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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