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Styelin D, an Extensively Modified Antimicrobial Peptide from Ascidian Hemocytes*

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Steven W. Taylor‡§, A. Grey Craig¶, Wolfgang H. Fischer¶, Minkyu Park¶, and Robert I. Lehrer‖

From the ‡Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California San Diego, La Jolla, California 92093-0204, the ¶Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, California 92037, and the ‖School of Medicine, UCLA, Los Angeles, California 90095

We isolated styelin D, a 32-residue, C-terminally amidated antimicrobial peptide, from the blood cells (hemocytes) of the solitary ascidian, *Styela clava*. Styelin D had remarkably extensive post-translational modifications, containing two novel amino acids, dihydroxyarginine and dihydroxylysine, and two distinctly unusual ones, 6-bromotryptophan and 3,4-dihydroxyphenylalanine. In addition, the peptide exhibited microheterogeneity because of differential mono- and dihydroxylation of several lysine residues. The primary sequence of one variant was: GW*LR**K**AAK**SVGK**FY*Y*K**HK*Y*Y*IK*AAWQIGKHAL-*NH*$_2$, where W* is 6-bromotryptophan, R** is dihydroxyarginine, Y* is 3,4-dihydroxyphenylalanine, K* is 5-hydroxylysine, and K** is dihydroxylysine. Styelin D exhibited activity against Gram-negative and Gram-positive bacteria, and this activity was retained in 200 mM NaCl. The role of the extensive modifications may be to preserve activity at low pH and/or high salinity because, under these conditions, the native peptide was considerably more active against the Gram-positive bacterial strains than its unmodified synthetic analogue. The peptide was also hemolytic and quite cytotoxic to eukaryotic cells. These broad ranging activities, combined with its relative abundance in ascidian hemocytes, suggest that styelin D plays a significant role in the innate immune mechanisms of *S. clava*.

As primitive chordates, ascidians are excellent subjects for research into the evolution of vertebrate innate immunity. Although ascidians lack the immunoglobins and T cell receptors found in higher vertebrates, their hemocytes can recognize and destroy foreign materials such as antigens or non-self-tissues that penetrate their tunic or contact their internal tissues (1). In certain ascidian species, vacuolated cells (including the berry-shaped morula cells) contain hemagglutinin (2, 3) and proteases (4, 5). The morula cells of *Halocynthia roretzi* contain halocyamines (tetrapeptides containing L-3,4-dihydroxyphenylalanine (DOPA)[1] and a 6-bromoindole) that are active against Gram-positive bacteria, fungi, marine bacteria, and some fish RNA viruses (6, 7). Phenoloxidase, perhaps acting in concert with DOPA-derived metabolites and proteins (8–10), may also be a critical player in cellular defenses.

Two families of antimicrobial polypeptides, clavanins and styelins, were identified from hemocytes of the stolidobranch ascidian, *Styela clava.* The former are histidine-rich, α-helical polypeptides with 23 amino acid residues and C-terminal amidation (11). The latter are phenylalanine-rich peptides with 32 amino acid residues (12). Whereas styelins demonstrated a broad pH optimum in killing bacteria, the clavanins showed optimal antibacterial activity at pH 5.5. The initial 20 amino acid residues of styelins A and B were sequenced and indicated an unusual structure consisting of several unidentified amino acid residues and hydroxylysine residues that are common only in collagen-like polypeptides. Although they were not specifically identified, the absorbancy of styelins at 280 nm suggested that methylated or otherwise modified tyrosine residues were present.

Amino acid sequences corresponding to both the clavanins and styelins were identified using partial sequences and a cDNA library prepared from hemopoietic pharyngeal tissue (13, 14). Although cDNA for styelins A and B were not detected, a closely related cDNA sequence was identified and designated as styelin C. Two additional sequences, styelins D and E, were identified by these cloning studies. Styelins D and E closely resembled each other, differing by only two residues in the sequence of the mature peptide, but differed significantly from styelins A-C (see Table I). In contrast to styelins A and B, styelins C–E had not been previously isolated as peptides. Overall, these studies established that styelins are highly basic polypeptides whose prepropeptides have a signal sequence and a polyanionic C-terminal extension whose charge counter balances the cationic residues in the mature peptide domain. Similarities were found in the gene sequences with cecropins, antimicrobial polypeptides found in many insects, and the pig intestine (15).

During our search for DOPA and 3,4,5-trihydroxyphenylalanine (TOPA)-containing proteins from ascidian blood cells (16–18), we isolated and characterized the mature styelin D peptide. The peptide exhibits *in vitro* cytotoxic and antimicrobial activity and possesses extensive post-translational amino acid modifications.

The abundant evidence that ascidian hemocytes exert cytotoxic and antimicrobial activity (19–23) has generated interest from natural product chemists to identify the molecules responsible for these actions. Because most previous researchers have

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§ To whom correspondence should be addressed: Center for Marine Biotechnology and Biomedicine, Scripps Inst. of Oceanography, University of California San Diego, La Jolla, CA 92093-0204. Fax: 858-534-1305; E-mail: swtaylor@ucsd.edu.

[1] The abbreviations used are: DOPA, 3,4-dihydroxyphenylalanine; AU-PAGE, acetic acid urea-polyacrylamide gel electrophoresis; MS/MS, tandem mass spectrometry; PTH, phenylthiohydantoin; TOPA, 3,4,5-trihydroxyphenylalanine; HPLC, high performance liquid chromatography; RP, reverse phase; GC, gas chromatography.

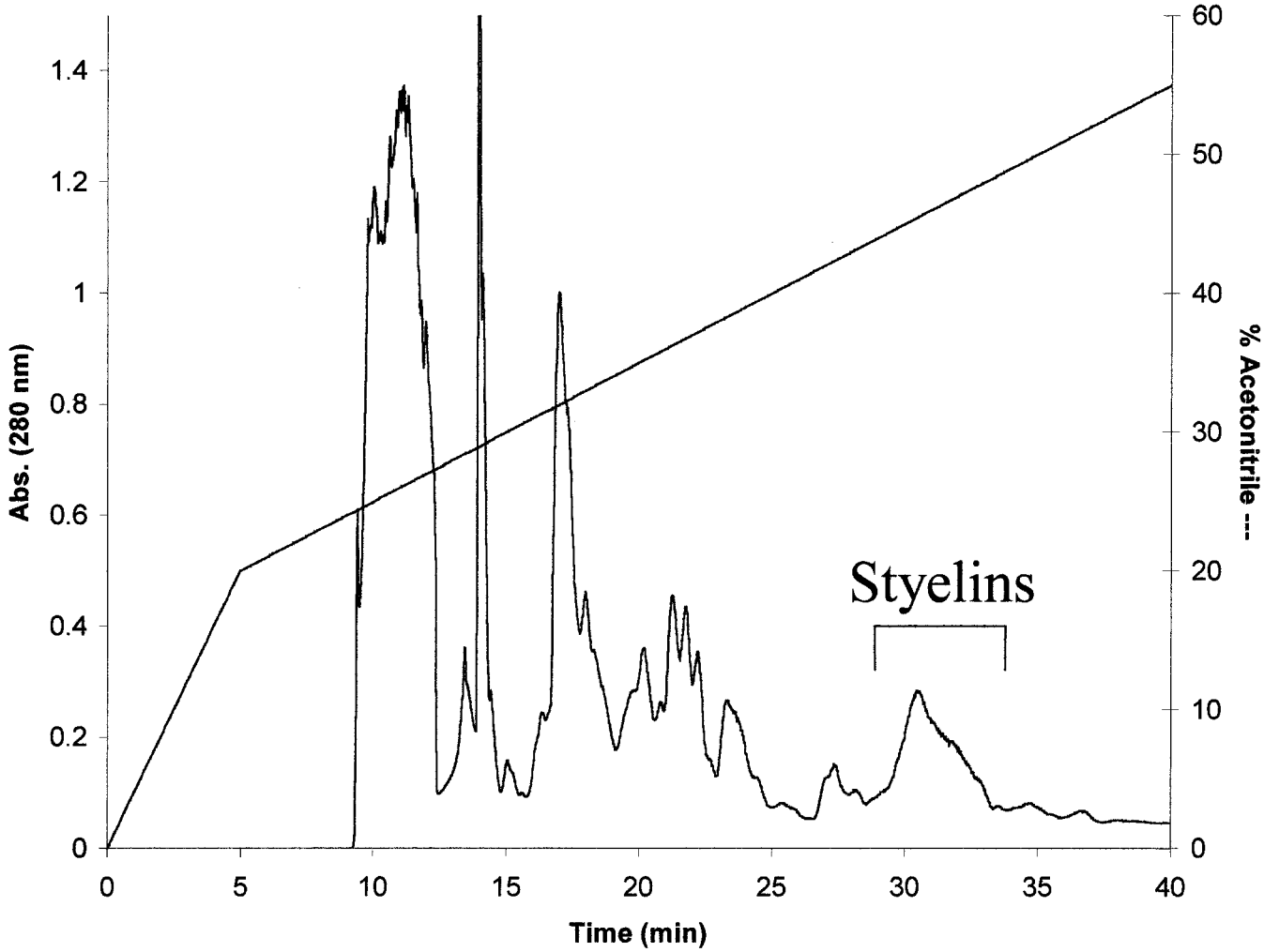

FIG. 1. **RP-HPLC of *S. clava* blood cell acetic acid-urea-EDTA extract.**

concentrated on extracting cytotoxic secondary metabolites from intact tunicates (see Ref. 24 and references therein), less attention has been devoted to detailed characterizations of active polypeptides known to be in aqueous extracts of ascidian hemocytes (25, 26).

EXPERIMENTAL PROCEDURES

Purification—S. clava specimens were collected from docks in local marinas in San Diego. After severing the base of the animals' stalks, blood was filtered through 70-μm mesh into iced 50-ml centrifuge tubes and spun at 800 $\times$ *g* for 20 min in a refrigerated centrifuge at 4 °C. The plasma was decanted and the pellet extracted in ~1 ml 5% acetic acid solution containing 8 M urea and 0.1 M EDTA (saturated). Extracts were subjected to acetic acid urea-polyacrylamide gel electrophoresis (AU-PAGE) followed by parallel Coomassie staining for polypeptide and nitroblue tetrazolium staining for redox active amino acids such as DOPA and TOPA (16, 17). Purification involved one or two HPLC runs. The crude extract was directly loaded onto a Phenomenex 250 $\times$ 10 mm Jupiter C-18 column and eluted with water (0.1% trifluoroacetic acid)/acetonitrile (0.085% trifluoroacetic acid) gradients. Redox active polypeptides (later identified as mature styelins D and E) eluted around 30 min using a linear gradient of 0–20% over 5 min followed by 20–60% mobile phase over 40 min (see Fig. 1). Fractions absorbing at 280 nm were collected, freeze dried as a white powder, and resuspended in water (0.001% trifluoroacetic acid) and then stored at -80 °C until further analysis. Further purification of the polypeptides was carried out by a second HPLC step on an analytical Phenomenex 250 $\times$ 4.6 mm Jupiter C-18 column at a flow rate of 1 ml/min column using linear gradients (see Fig. 2). Purity was monitored by AU-PAGE (see Fig. 2, *inset*). Synthetic (unmodified) styelin D (C-terminal amide) was produced by Research Genetics Inc. (Huntsville, AL) and also purified to homogeneity by RP-HPLC on C-18 using acetonitrile/water/trifluoroacetic acid gradients.

Charaterization and Peptide Mapping—Fractions containing a single band on AU-PAGE were directly subjected to mass spectrometry on a Bruker Daltonics Esquire ion trap mass spectrometer to determine molecular masses. Sequencing of pure fractions by Edman degradation was performed on a Procise 494 PE ABI protein sequencer. In addition to the standard 20 amino acids, we used DL- and DL-allo-5 hydroxylysine (Fluka), L-DOPA (Sigma), DL-5-bromotryptophan (Aldrich), and DL-6-bromotryptophan (Biosynth AG, Staad, Switzerland) as standards. A modified gradient system was employed which differentiates between the PTH-derivatives of the 5- and 6-bromotryptophan isomers.[2]

Styelin D (1.75 mg) obtained after the first HPLC step was proteolytically digested with a high concentration of chymotrypsin (~1:1 substrate to enzyme) for 1.25 h at ambient temperature in 0.1 M Tris-ascorbate buffer, pH 7.5, in a final volume of 275 μl. The reaction was terminated with 10 μl of 20% trifluoroacetic acid, and the mixture was directly loaded onto a semi-preparative C-18 column and eluted with a 0–40% gradient of mobile phase over 60 min (see Fig. 3). The peptide fragments were identified based on Edman sequence analysis and MS and tandem mass spectrometry (MS/MS) analysis.

GC-MS Characterization of Volatile Derivatives of the Amino Acids—The styelin D (1.0 mg) obtained after the first HPLC step was hydrolyzed using the fast acid hydrolysis method (5 M HCl with 8% trifluoroacetic acid and 8% phenol *in vacuo* at 155 °C for 45 min) of Tsugita and co-workers (27). The solvent was subsequently evaporated using a stream of dry, high purity nitrogen at 60 °C. A subsequent wash with ethanol and re-evaporation yielded clean hydrolysate. The volatile *N*-trifluoroacetyl, *O*-methyl derivatives of the amino acids were prepared as described previously (16) and subjected to GC-MS on a Hewlett Packard 5988 instrument using a Chirasil-Val column (28). A synthetic

[2] W. H. Fischer, unpublished results.

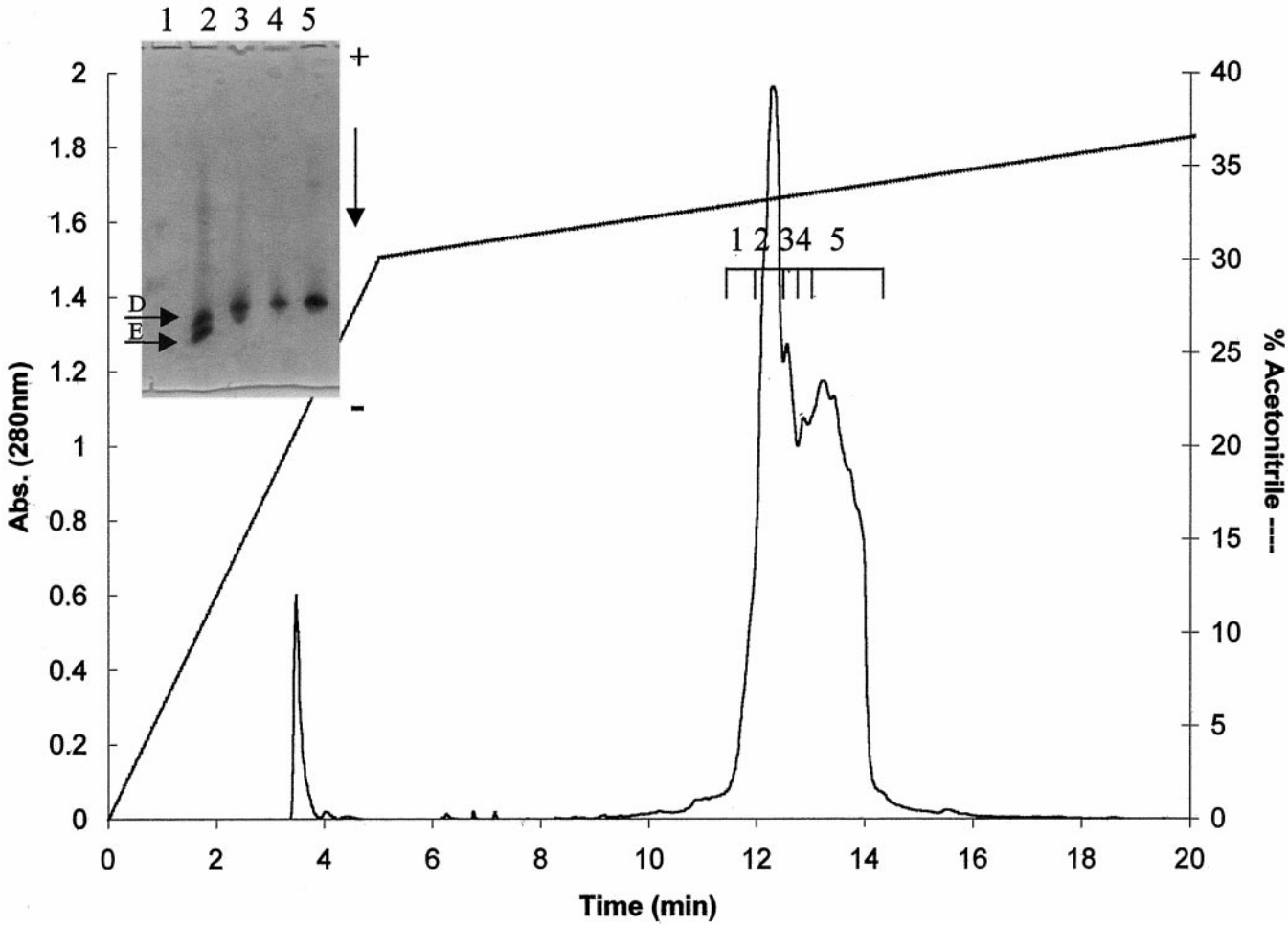

FIG. 2. **RP-HPLC chromatogram of the styelin variants.** *Inset*, AU-PAGE of the fractions.

TABLE I
The styelins

Mature styelins A and B from N-terminal sequence analysis (12)	
Styelin A	GXFGK*AFXSVSNFAK*K*HK*TA??????
Styelin B	GXFGPAFHSVSNFAK*K*HK*TA??????
Styelin precursor sequences deduced from cDNA only (14)	
Styelin C	GWFGKAFRSVSNFYKKHKTYIHA-GLSAATLLG
Styelin E	GWLRKAAKSVGKFYYKHKYYIKAAWKIGRHALG
Styelin D	GWLRKAAKSVGKFYYKHKYYIKAAWQIGKHALG
Mature styelin D (this work)	

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
GW*LR**K**AAK**S VGK**F Y*Y*K**H K*Y*Y*I K*A A W Q I G K H A L-NH_2

collagen amino acid standard mixture (Sigma) was similarly prepared for the sake of comparison.

Circular Dichroism Spectra—CD spectra were recorded on a AVIV 61DS spectropolarimeter with a 0.5-nm bandwidth, 0.5-nm step size, and a 4.0-s time constant over a wavelength range of 300–205 nm to assess the conformation of the peptide backbone in the presence an absence of trifluoroethanol. The instrument was calibrated with a standard solution of 10-camphorsulfonic acid.

Biological Activity—Antimicrobial testing was performed by two-stage radial diffusion assays (29). Underlay gels contained 1% agarose, 10 mM sodium phosphate buffer, 0.3 mg/ml trypticase soy broth powder, and either 100 or 200 mM NaCl. The sample wells were 3.2 mm in diameter and received 8-μl aliquots of the peptide, serially diluted in acidified water containing 0.01% albumin. Overlay gels were poured after 3 h, and zone sizes were read to the nearest 0.1 mm (1 unit) after an overnight incubation. In radial diffusion assays, the x intercept of a least mean squares regression line ($\log_{10}$ concentration *versus* zone diameter) corresponds to the minimal inhibitory concentration.

Hemolytic activity was tested by incubating a 2.5% (v/v) suspension of washed human or sheep red blood cells for 30 min with serial 2-fold dilutions of the peptides (maximum concentration, 80 μg/ml). The tubes were centrifuged, and hemoglobin release to the supernatant was measured spectrophotometrically at 460 nm. Peptide free and 100% lysis controls (0.1% Triton X-100) were included, and the percentage of lysis was calculated conventionally.

Cytotoxicity was measured in 96 well culture plates by a tetrazolium technique, using Cell Proliferation Kit 1 according to the manufacturer's (Roche Molecular Biochemicals) instructions. Briefly, wells were seeded with 5×10^3 cells in 0.1 ml of RPMI medium with 10% fetal calf serum, 50 μg/ml gentamicin, and 2 mM L-glutamine. After 5 h, peptide was added, and the plates were incubated in 5% CO_2 and room air for 20 h at 37 °C. Then, MTT (3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyltetrazolium bromide) reagent was added, and the incubation was continued for an additional 4 h before the solubilizer was added so that the optical density could be read.

Bacterial membrane permeabilization was measured spectrophotometrically, using *Escherichia coli* ML-35p as the target. This organism, which is lactose-permease deficient, contains a periplasmic β-lactamase and a cryptic cytoplasmic β-galactosidase. Nitrocefin (20 μM) was used in place of PADAC (7-(thienyl-2-acetamido)-3(2-(4-*N*,*N*-dimethyl amino phenylazo)-pyridinium-methyl)-3-cephen-4-carboxylic acid which is no longer available) to detect outer membrane permeabilization, and *o*-

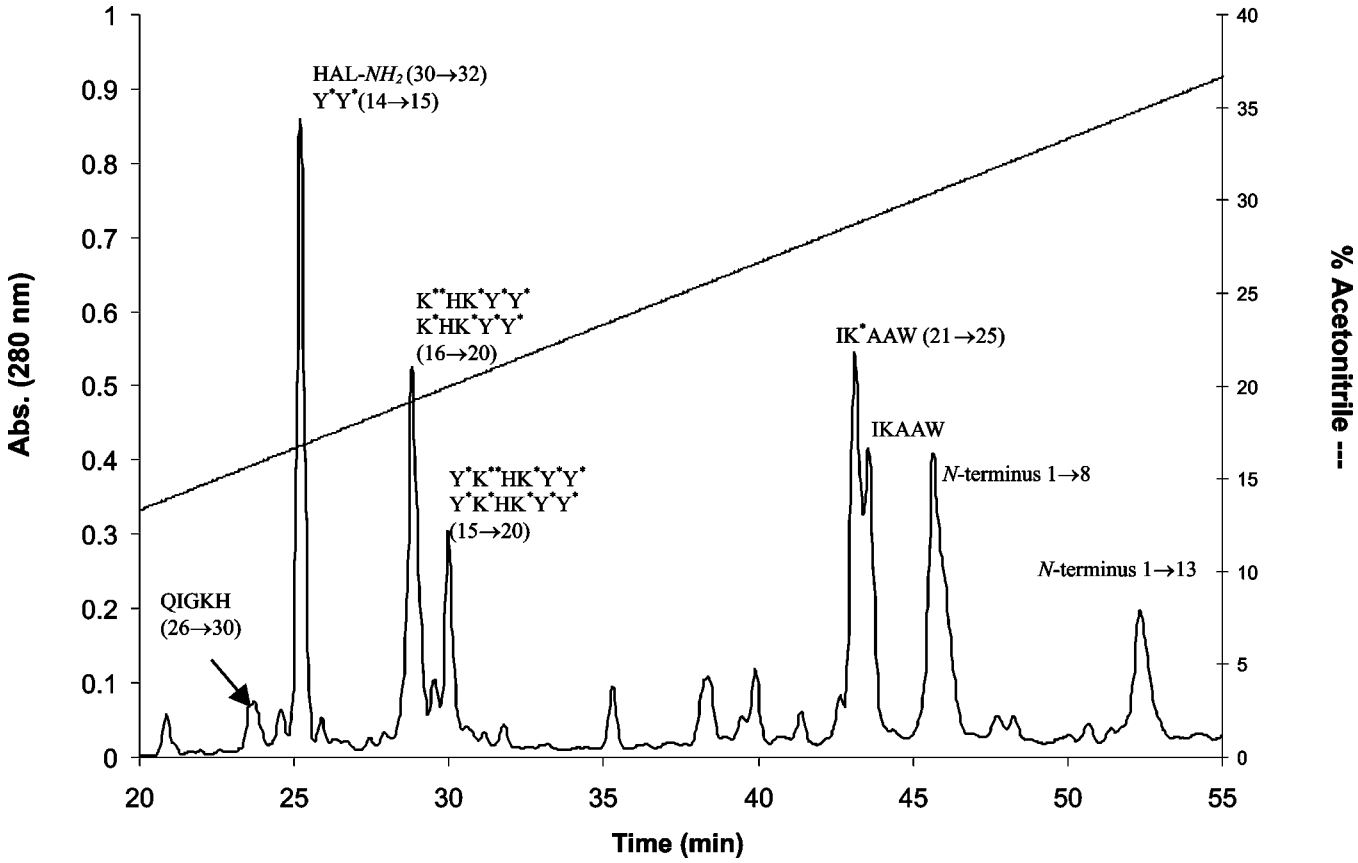

FIG. 3. **RP-HPLC of the styelin chymotrypsin digestion mixture.**

nitrophenyl β-D-galactopyranoside was used to detect inner membrane permeabilization, as described previously (30, 31).

RESULTS

Purification and Characterization of the Intact Styelins—Approximately 8.7 mg of styelin D (with styelin E as a minor contaminant) was obtained from 108 medium sized individuals collected from Mission Bay, San Diego in November, 1999. Typical RP-HPLC profiles for the styelins are shown in Figs. 1 and 2. The styelins were observed as two closely migrating bands that were stained with Coomassie and with nitroblue tetrazolium. In the *inset* of Fig. 2, styelin D runs above styelin E, because of its lower positive charge, resulting from the substitution of an uncharged Gln-26 for the charged Lys/5-hydroxylysine (Table I). Interestingly, styelin E (the lower band) was the major variant isolated during preliminary studies on East Coast *S. clava* specimens collected from Stone Harbor, New Jersey in summer, 1997.[3]

N-terminal Edman sequencing of fraction 4 (Fig. 2) covered the first 29 residues: GW*LX(K*/Z)AA(K*/Z)SVG(K*/Z)FY*Y*(K*/Z) HK*Y*Y*I(K/K*)AAWQIGK, where *X* and *Z* were not any of the common amino acids and the PTH-derivatives of residues in parentheses co-eluted in their respective cycles. Based on the cDNA sequence observed by Lee and co-workers (Table I), this fraction was proposed to closely correspond to that of styelin D. However, extensive post-translational modification and microheterogeneity appeared to be present in the peptide. The PTH-derivative in the second cycle was found to elute at an identical position to 6-bromotryptophan and not 5-bromotryptophan. PTH-DOPA was detected as the sole amino acid in cycles 14, 15, 19, and 20 by comparison with an authentic standard. The presence of PTH-hydroxylysine and nonstandard peaks was indicated in cycles 5, 8, 12, 14, and 15 (cycle 18 appeared to contain PTH-hydroxylysine alone). A trace of PTH-arginine was observed in the cycle corresponding to residue 4; however, the yield was far less than expected compared with the other residues (glycine, bromotryptophan, leucine), suggesting that it was not the major amino acid in this position. Electrospray ionization mass spectra indicated molecular masses of 4081, 4096, 4112, 4127, and 4144 (±1) Da (calculated from the +5 and +6 ions, data not shown). Based on the MS and the chemical sequence analysis, we proposed that there was microheterogeneity of the peptide because of differential hydroxylation of styelin D.

Peptide Mapping and Characterization of the Post-translational Modifications—HPLC purified styelin D was digested for preparative peptide mapping studies using chymotrypsin at high enzyme to substrate ratios. Two N-terminal peptide fragments were identified by the presence of doublets of equal intensity separated by 2 *m/z* for the molecular ion peaks in the electrospray ionization mass spectra consistent with the presence of bromine. Edman sequence analysis showed that the late eluting peak in Fig. 3 corresponded to the N-terminal peptide (1 → 13) GW*LX(Z/K*)AA(Z/K*)SVG(Z/K*)F. The electrospray ionization mass spectrum showed four variants, each 16 Da different in molecular mass. As we increased our enzyme to substrate ratio, the N-terminal octapeptide (1 → 8) GW*LX(Z/K*)AA(Z/K*) having two variants differing by 16 Da increased in relative concentration. We immediately noted that the molecular masses observed for the variants of peptide (1 → 13) were 32, 48, 64, and 80 mass units higher than expected for GW*LRK*AAK*SVGK*F (where K* is 5-hydroxylysine). Similarly, the variants of peptide (1 → 8) were 48 and 64 mass units higher than expected for fully hydroxylated GW*LRK*AAK*.

Clearly, hydroxylation of lysine to 5-hydroxylysine could not account for the masses observed (*i.e.* two to five extra sites of

[3] S. W. Taylor, unpublished results.

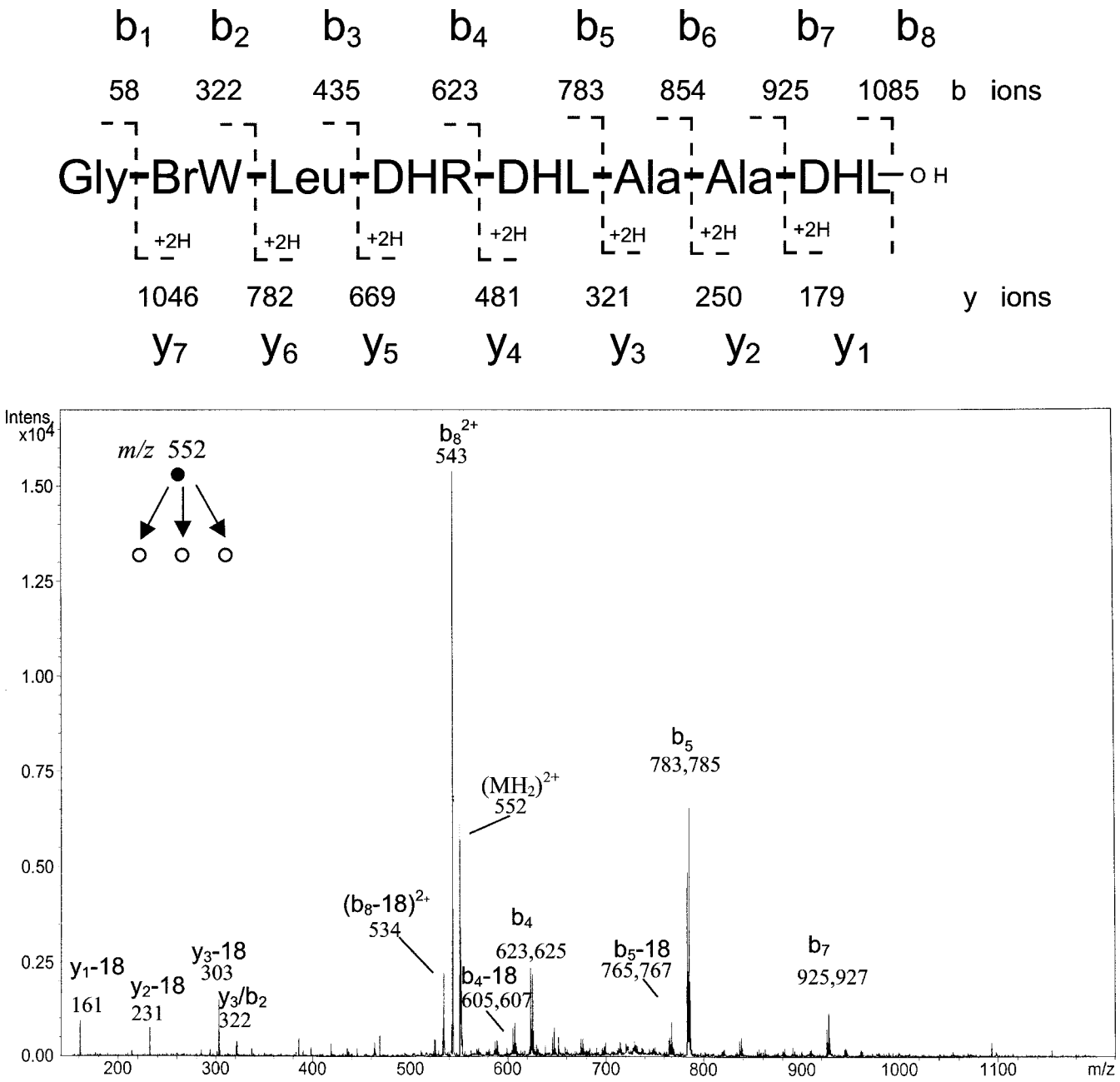

FIG. 4. **Sequence and mass spectrum obtained from the MS/MS of the doubly protonated $[M+2H]^{2+}$ fully hydroxylated variant of the N-terminal octapeptide (1 → 8).** A *dashed line* represents peptide bond cleavage (except at the terminus) resulting in N-terminal b ions or C-terminal y ions. *BrW*, 6-bromotryptophan; *DHR*, dihydroxyarginine; *DHL*, dihydroxylysine.

hydroxylation in the respective peptides; see cDNA sequence data in Table I). We characterized both variants of peptide (1 → 8) by MS/MS. The fragmentation pattern observed for the $[M+2H]^{2+}$ ion of the more hydroxylated variant (molecular mass, 1102, 1104) shown in Fig. 4 was consistent with the sites of extra hydroxylation occurring on residues 4, 5, and 8. The b_4 (*m/z*, 623, 625) ion indicates that 32 Da of extra mass is associated with the residue in the fourth position, whereas the b_5 (*m/z*, 783, 785)/b_7 (*m/z*, 925, 927) and b_8^{2+} (*m/z*, 543, 544) ions are consistent with dihydroxylation of Lys-5 and Lys-8, respectively. Edman sequence analysis of peptide (1 → 8) contained unidentified PTH-derivative peaks only in cycles 4, 5, and 8, ruling out further modifications to the other amino acid residues. MS/MS of the lesser hydroxylated variant of peptide 1 → 8 (molecular mass, 1086, 1088) was indicative of an isobaric mixture, also with dihydroxylation of Arg-4, but with differential hydroxylation of 5-hydroxylysine at positions 5 and 8, respectively (data not shown). This indicated that differential hydroxylation of 5-hydroxylysine to dihydroxylysine is a source of microheterogeneity.

Similarly, MS and MS/MS of other proteolytic fragments revealed a variant with an additional 16 Da localized on 5-hydroxylysine-16 (data not shown). We obtained additional evidence for the presence of dihydroxylysine by GC-MS analysis of volatile derivatives of acid hydrolysates of the styelins (Figs. 5 and 6). Dihydroxylysine elutes after 5-hydroxylysine (which has an identical elution time and MS fragmentation pattern to one of the 5-hydroxylysine diastereoisomers in an authentic synthetic standard) on Chirasil-Val (Fig. 5). In the absence of a suitable standard for dihydroxylysine, the amino acid qualitatively appears to be present in the polypeptides at similar levels to 5-hydroxylysine with the Lys content being much less.

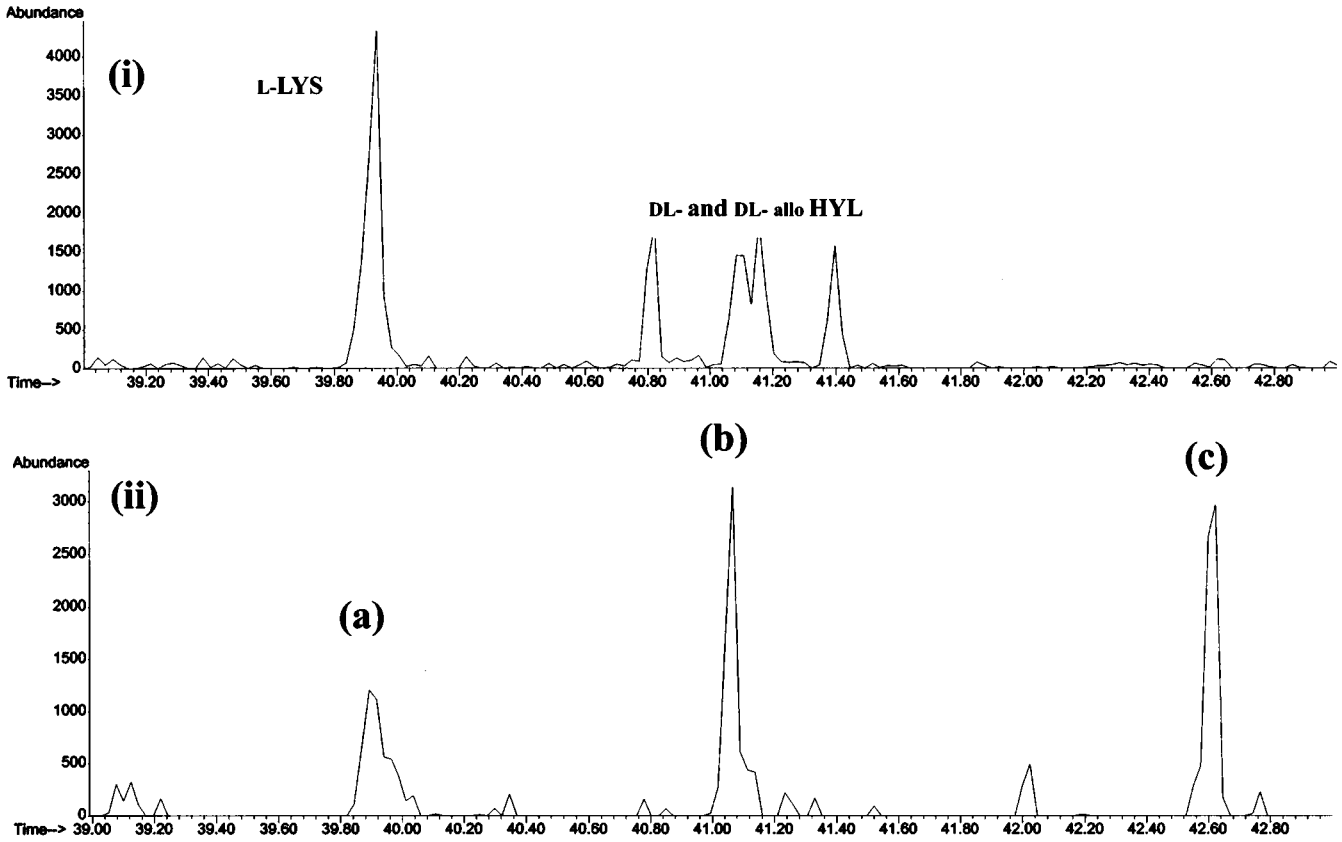

FIG. 5. **GC-MS chromatogram of lysine and hydroxylysine diasteromers separated on Chirasil Val: *(i)*, Sigma amino acid standard for collagen hydrolysates; *(ii)*, styelin hydrolysate.**

The fragmentation pattern of the *N*-trifluoroacetic acid, *O*-Me derivative is fully consistent with trifluoroactelyation of the two amines and two side chain hydroxyls of dihydroxylysine (Fig. 6). Neither Arg nor dihydroxyarginine were observed because of their lability during derivatization (28). Finally, the last feature of the microheterogeneity was differential hydroxylation of Lys to 5-hydroxylysine at residue 22. The absence of a brominated I(K/K*)AAW (21 → 25) fragment in the digest indicated that only the N-terminal tryptophan was modified as has been previously observed in a 33-residue bromosleeper peptide from *Conus radiatus* (32). Table II compares the observed and calculated molecular masses of differentially hydroxylated styelin D variants.

Secondary Structure—The far UV circular dichroism spectrum shows that the styelin D has an α-helical structure in solution in the presence of trifluoroethanol (Fig. 7). The helix shown should convey considerable amphiphilicity to the molecule as observed for the antimicrobial melittin, magainins and cecropin peptides (33). The mean hydrophobic moment is likely to be substantially increased by these post-translational modifications, because bromotryptophan is more hydrophobic than tryptophan, and hydroxy- and dihydroxy-lysine are more hydrophilic than lysine. It is this amphiphilicity that has been associated with the efficacy of this group of polypeptides as antibiotics, giving the molecules the ability to interact with and disrupt bacterial membranes (33).

Biological Activity—Native and synthetic styelin D showed activity against methicillin-resistant and susceptible strains of *S. aureus* (Fig. 8). However, whereas the activity of the synthetic peptide diminished considerably if the acidity or salinity of the assay medium increased, the native peptide was unaffected by these changes. Both native and synthetic styelin D were equally active against *Pseudomonas aeruginosa* (Table III), and neither pH nor salinity affected their behavior significantly.

We noted that styelin D acted sequentially on the outer and inner membranes on *E. coli* to make the former permeable to nitrocefin (a β-lactamase substrate) and the latter permeable to *o*-nitrophenyl β-D-galactopyranoside (a β-galactosidase substrate) (Fig. 9). Similar effects have been demonstrated with many other antimicrobial peptides, including clavanins, the other family of antimicrobial peptides found in *S. clava* hemocytes.

Styelin D was also cytotoxic to HCT-116 cells (IC_{50}, 10.1 μg/ml) and to human ME-180 cervical epithelial cells (EC_{50}, 50 μg/ml). In addition, it was potently hemolytic toward human (EC_{50}, 10 μg/ml) and sheep (EC_{50}, 40 μg/ml) erythrocytes (Fig. 10). This impressive combination of antimicrobial and cytotoxic activity suggests that styelin D could play multiple roles in the innate immune responses of *S. clava.*

DISCUSSION

Although there have been extensive studies on the immune systems of marine invertebrates, not until recently have some of the actual effector molecules been characterized. The most extensive work in this area involves tachyplesins, a family of molecules identified in hemocytes of the horseshoe crab, *Tachypleus tridentatus* (*Limulus polyphemus* is its more familiar counterpart). These cationic peptides are composed of 17–18 amino acid residues and have a C-terminal arginine α-amide. They adopt a fairly rigid conformation, constrained by two disulfide bridges in a hairpin β-sheet-like structure. The tachyplesins are active against both Gram-negative and Gram-positive bacteria as well as the pathogenic fungus *Candida albicans* (see Ref. 34 and references therein). Schnapp and coworkers (35) partially characterized a proline-rich 6.5 kDa

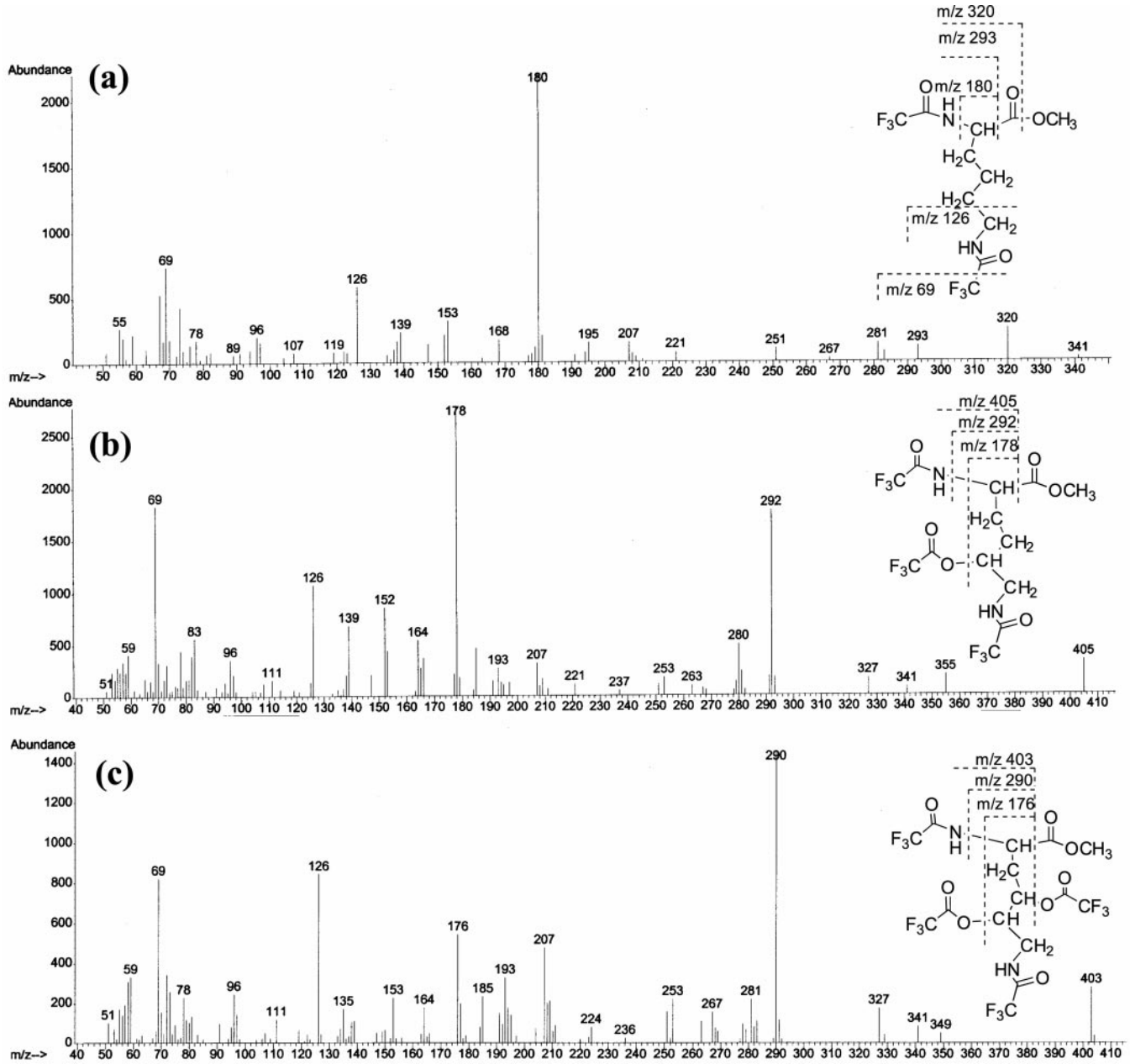

FIG. 6. **Electron impact fragmentation pattern of the peaks in Fig. 5.** *(a)*, lysine derivative. *(b)*, 5-hydroxylysine derivative. The spectrum is consistent with those observed in the four diastereoisomers in the standard and with the literature (53). *(c)*, dihydroxylysine derivative. Although the 4,5-dihydroxylysine derivative is depicted, the correct isomer remains to be determined. Fragment ions were assigned according to Leimer *et al.* (54).

TABLE II

Comparison of molecular masses observed for styelin D (the mean value calculated from $[M + 5H]^{5+}$ ion and $[M + 6H]^{6+}$ ion peaks) with masses calculated from the cDNA sequence adjusted for post-translational modifications

Modifications to styelin D cDNA sequence	Observed mass unresolved (±1 Da)	Average mass calculated
C-terminal amidation, 1R**, 4K**, 2K*, 4Y*, 1W*	4144	4145.46
C-terminal amidation, 1R**, 3K**, 3K*, 4Y*, 1W*	4127	4129.46
C-terminal amidation, 1R**, 2K**, 4K*, 4Y*, 1W*	4112	4113.46
C-terminal amidation, 1R**, 1K**, 5K*, 4Y*, 1W*	4096	4097.46
C-terminal amidation, 1R**, 0K**, 6K*, 4Y*, 1W*	4081	4081.46

peptide with broad-spectrum antibacterial activity from the hemocytes of another crusteacean, the shore crab *Carcinus maenas*. Very recently, the same laboratory described a 11.5-kDa cysteine-rich cationic antibacterial protein from *C. maenas* that was active only against Gram-positive bacteria (36). Other antimicrobial peptides have been more recently isolated from the hemocytes of aquacultured marine invertebrates, including shrimp and mussels. Destoumieux *et al.* (37) recently described penaeidins-shrimp (*Penaeus vannamei*) peptides that are active against fungi and especially Gram-positive bacteria. The pennaedins contain 50–62 residues, including an N-terminal domain that is rich in proline residues and a C-terminal domain that contains three intramolecular disulfide bridges. One penaeidin has a post-translationally modified N-terminal glu-

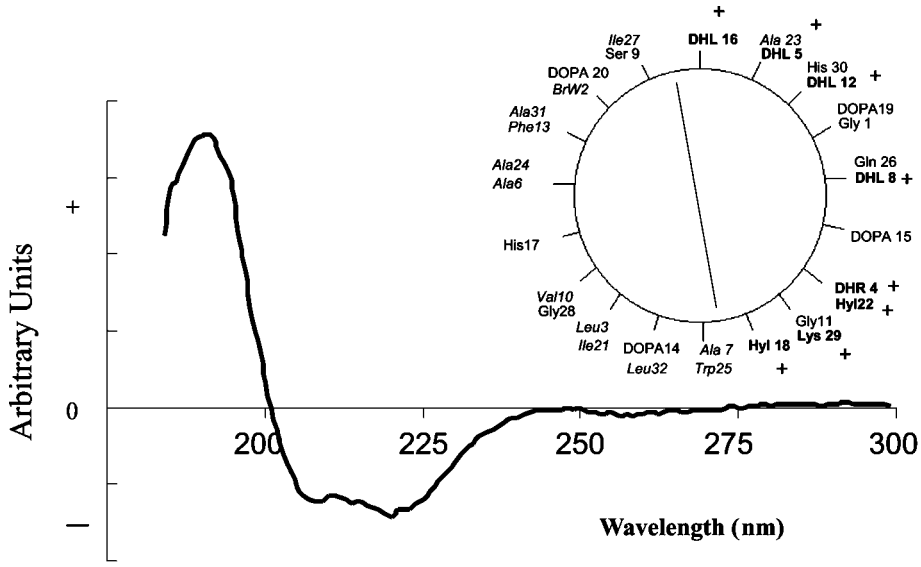

FIG. 7. **Far UV circular dichroism spectrum for styelin D in aqueous solution containing 50% trifluoroethanol indicating a propensity to form an α-helical conformation.** *Inset*, helical wheel diagram for styelin D. Amino acid side chains that are charged around pH 8.0 (seawater) are in *bold type*, and aliphatic amino acids are in *italic type*. The hydrophobic face is to the *left* of the line, and the hydrophilic face (with eight positively charged side chains) is to the *right*. The incremental angle is 100°. *Hyl*, 5-hydroxylysine. The other abbreviations are as in the legend to Fig. 4.

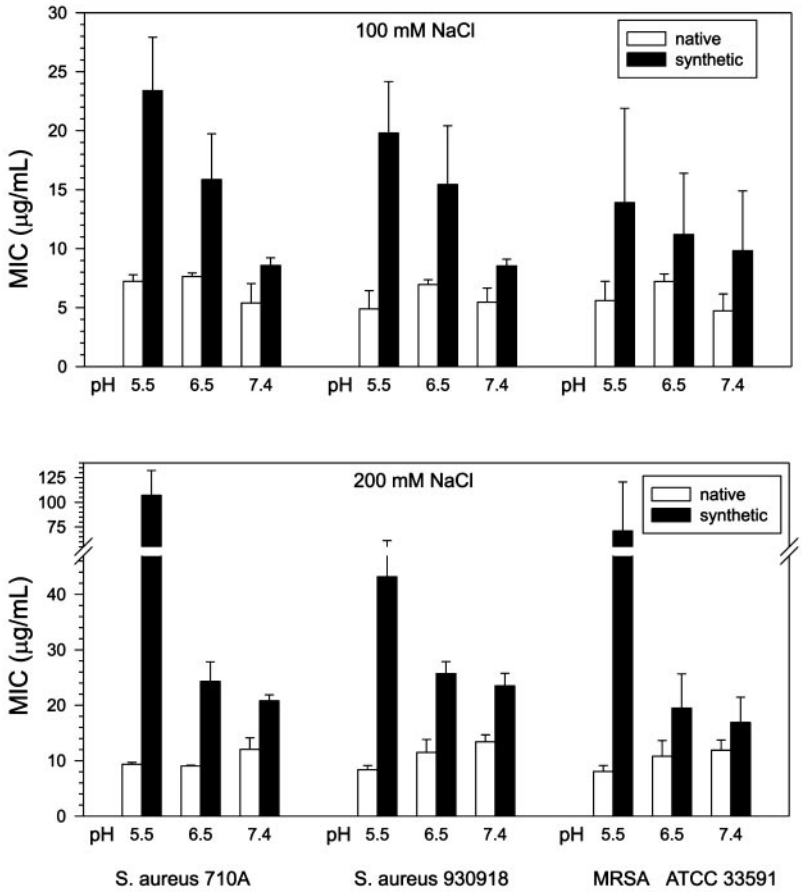

FIG. 8. **Effect of salinity and pH on activity of styelin D against staphylococci.** The susceptibility of three different isolates of *S. aureus*, one of which was a methicillin-resistant (*MRSA*) strain, were tested at pH 5.5, 6.5, or 7.4 in underlay gels that contained either 100 or 200 mM NaCl. Mean ± S.E. values of the minimal inhibitory concentration are shown ($n = 3$). Note that the native, extensively modified styelin outperformed its unmodified synthetic counterpart when the acidity or salinity increased.

TABLE III
Activity of native and synthetic styelin D against P. aeruginosa MR3007

The data show minimal inhibitory concentration values (μg/ml), from radial diffusion assays performed with underlay gels that contained 100 or 200 mM NaCl at pH 5.5, 6.5, or 7.4.

Styelin D	100 mM NaCl			200 mM NaCl		
	pH 5.5	pH 6.5	pH 7.4	pH 5.5	pH 6.5	pH 7.4
Native	2.1	5.7	4.9	2.8	4.2	8.1
Synthetic	1.1	5.9	2.4	1.5	5.4	4.8

tamate which has been converted into a pyroglutamate residue. Several small, cationic cysteine-rich antimicrobial peptides have been isolated from mussels including mytilin (34 residues), myticin (40 residues) as well as peptides belonging to the arthropod defensin family (38). An antifungal peptide called mytimycin was also partially characterized. Little information

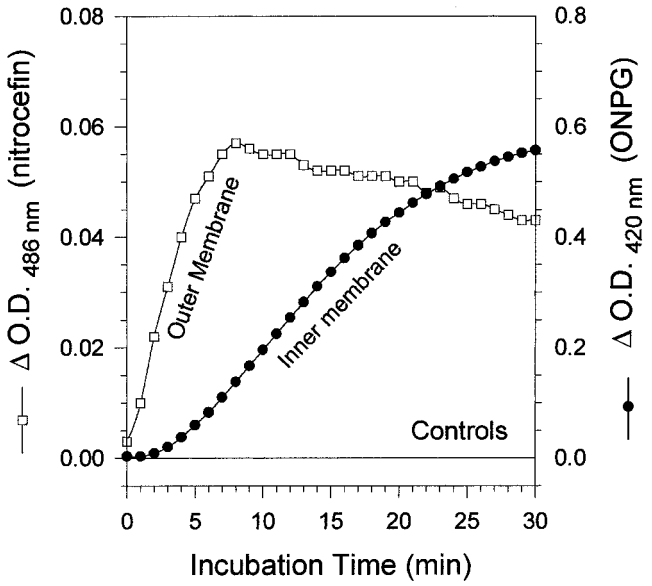

FIG. 9. **Membrane permeabilization.** Stationary phase *E. coli* ML-35p (2.5×10^6 colony forming units) was suspended in a pH 6.5 medium that contained 100 mM NaCl, 10 mM sodium phosphate buffer, 0.3 mg/ml trypticase soy broth powder, and either 2.5 mM *o*-nitrophenyl β-D-galactopyranoside or 10 μM nitrocefin. The final volume was 100 μl. Immediately after the addition of 25 μg/ml of native styelin, the loss of inner and outer membrane integrity was monitored at 420 and 486 nm, respectively.

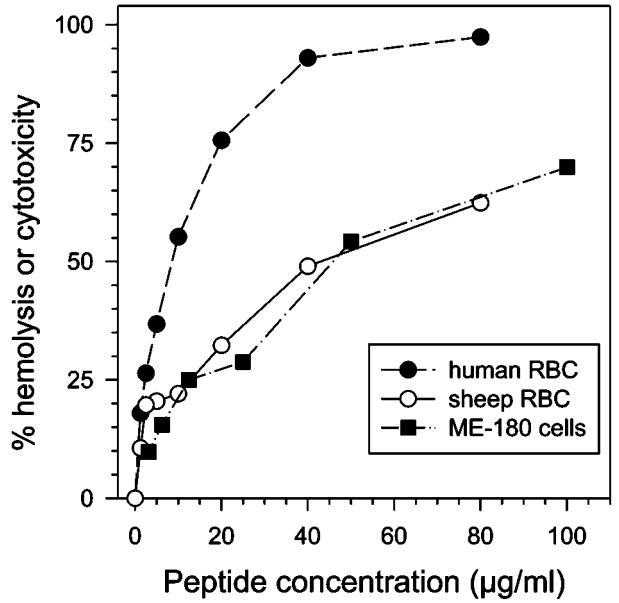

FIG. 10. **Hemolytic and cytotoxic activity of styelin D.** Both human and sheep erythrocytes were lysed by native styelin D, with human cells showing considerably greater susceptibility. The cytotoxicity of the peptide for human ME-180 cells, a line of well differentiated cervical cancer epithelial cells, resembled its ability to lyse the sheep erythrocytes.

is available on gene-encoded antimicrobial peptides from other marine invertebrates.

The styelins and clavanins were discovered during a recent search for antimicrobial peptides in ascidian hemocytes. Prior to this, there had been numerous reports of *in vitro* antimicrobial and cytotoxic activity by ascidian hemocytes but relatively little information about the effector molecules. Proliferating lymphocyte-like hemocytes accumulate around incompatible transplants in Styelid ascidians, apparently mediating graft rejection through cytotoxic destruction of allogenic tissue. Cytotoxic activity has also been demonstrated by hemocytes from other styelid ascidians (21), *H. roretzi* (39) and *Ciona intestinalis* (20, 25, 26). Styelin D is cytotoxic, a property implicating the polypeptide as a mediator of allogenic cytotoxicity in *S. clava* (19). Its propensity to form an extremely amphipathic α-helix may reflect a mechanism like the cecropins and/or an ability to disrupt cellular membranes. Learning exactly how *S. clava* cells protect themselves from the cytotoxic properties of their own styelin D provides an interesting area for future research.

The cDNA sequences for styelins D and E have been reported (14), but the corresponding peptide molecules have not been previously identified. Zhao *et al.* (14) found significant sequence homology for the cDNA sequences of the styelins to the four cecropins. The precursor sequences of styelins D and E have the same overall homology as styelin C (Table I) but with different alignment. Additional residues Leu-3; Lys-8 and Ala-31 match the cecropin sequences, whereas residues 28–30 (Gly-Lys-His) are not homologous with the Ala-Ala-Thr sequence observed in styelin C and the cecropins.

This is the first example of dihydroxylysine and dihydroxyarginine in the primary sequences of gene-encoded polypeptides. Isomers of both amino acids have only recently been reported for the first time in secondary metabolites. 3,4-Dihydroxyarginine has been found in a cyclic tripeptide from a Palauan sponge (40) and 3,5-dihydroxylysine in an antibiotic dipeptide from *Streptomyces antibioticus sp. antibioticus* Tü (41, 42). 4-Hydroxyarginine has been previously reported as a post-translationally modified amino acid in a DOPA-containing protein from the mussel *Mytilus edulis* (43). We are currently determining the isomers of the dihydroxylysine and dihydroxyarginine residues in a high resolution nanoprobe NMR study on the fully hydroxylated N-terminal octapeptide.[4] This approach is necessary because of the low recoveries of the peptide and amino acids from proteolytic digests and total acid hydrolysates, respectively. Once the position of the hydroxyl substituents on dihydroxylysine and dihydroxyarginine side chains are identified, synthesis of all possible diastereoisomers should allow determination of their stereochemistry and absolute configurations by a comparison of the natural and synthetic amino acids.

6-Bromotryptophan was first reported as a post-translational modification in gene-encoded peptides from cone snail venom (32, 44) and in morulin Pm from the ascidian *Phallusia mammillata* (16). Earlier, a partially characterized antimicrobial peptide from the most primitive of vertebrates, the hagfish *Myxine glutinosa* (45), was found to contain bromotryptophan, although the isomer was not identified.

As DOPA-containing polypeptides, the styelins belong to a group of molecules from ascidian hemocytes that include ferreascidin (46), the Ascidia and Molgula blood cells polypeptides (17), and morulin Pm (16). Each of these molecules has only been partially characterized because of protease resistance, premature termination of Edman sequencing, and extensive microheterogeneity because of uncharacterized post-translational modifications. Primarily because of their high content of DOPA and TOPA, various roles have been suggested for these molecules, including participation in metal accumulation, adhesion, and sclerotization of the tunic. The cytotoxic activity of these polypeptides has generally not been investigated, although morulin Pm tested negative for antimicrobial activity by a conventional disc diffusion assay. It is noteworthy that when we performed a similar assay with styelin D, we found the peptide adhered firmly to the paper discs at neutral pH and

[4] S. W. Taylor and G. E. Martin, unpublished results

did not diffuse into the agar. In a marine environment, a tendency to bind cellulose might allow styelins to impregnate the cellulose-like tunic without being leached away.

It is significant that our studies show that, in the case of *S. aureus* and methicillin-resistant *S. aureus*, activity of the native styelin D was considerably higher than the synthetic peptide under acidic and/or high salt conditions. Because the native and synthetic styelin D peptides differed only with respect to the presence or absence of the described post-translational modifications, these modifications clearly enhanced the performance of the peptide. This suggests that a role for these extensive modifications may be in preserving activity against certain bacteria under conditions of low pH that occur within the phagocytic vacuoles (phagosomes) of invertebrate hemocytes (47) and vertebrate leukocytes (48). Notably, the clavanins were most active at lower pH yet lacked the extensive modification found in the styelins (49). Although there have been extensive measurements of the intracellular pH of vanadium-accumulating ascidians because of the extraordinary low values proposed (see Ref. 50 and references therein) fewer measurements have been made on iron-accumulating stolidobranch ascidians such as *S. clava*. Near neutral intracellular pH values for the stolidobranch ascidians *Pyura stolonifera* and *Boltenia ovifera* have been earlier reported (51, 52). Our priorities for future research on *S. clava* hemocytes include measuring intracellular and intravacuolar pH and examining the trafficking of styelins (*e.g.* their synthesis, storage sites, extracellular secretion, and intracellular translocation to phagosomes). Further studies will seek to correlate the structure of the styelins and other DOPA/TOPA polypeptides with biological function.

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