Tunichrome *Sp*-1: New Pentapeptide Tunichrome from the Hemocytes of *Styela plicata*

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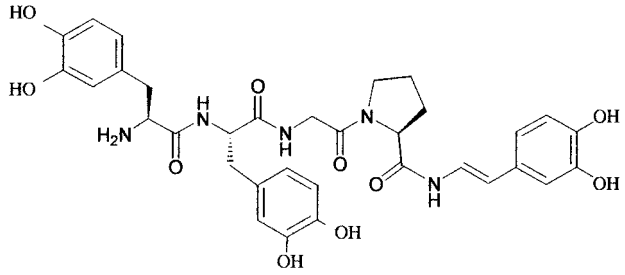
A modified pentapeptide has been isolated from the hemocytes of the ascidian *Styela plicata*. The structure of the peptide was determined by Edman sequence analysis, mass spectrometry, and NMR spectroscopy with the stereochemistry assigned by acid hydrolysis followed by both (a) GC−MS of the volatile amino acid derivatives on a chiral column and (b) ultrasensitive detection of fluorescent diasteromeric derivatives of the component amino acids after reversed-phase HPLC. The peptide L-DOPA-L-DOPA-Gly-L-Pro-dcΔDOPA (where DOPA = 3,4-dihydroxyphenylalanine and dcΔDOPA = decarboxy-(*E*)-α,*β*-dehydro-3,4-dihydroxyphenylalanine) we designate as tunichrome *Sp*-1.

The tunichromes are small modified peptides isolated from the hemocytes of ascidians. While investigations have sought to find a connection between these compounds and the phenomenon of vanadium and, to a lesser extent, iron accumulation and storage in ascidian blood cells,[1,2] their biological function remains equivocal.[3] The tunichromes described to date have been tripeptides containing one or more DOPA (3,4-dihydroxyphenylalanine) or TOPA (3,4,5-trihydroxyphenylalanine) residues or their unsaturated derivatives and have been isolated from ascidian species of two vanadium-accumulating genera, *Ascidia*[4] and *Phallusia*,[5] and one iron-accumulating genus, *Molgula*.[4] Tunichromes have subsequently been detected in numerous other ascidian species.[6] The halocyamines, modified tetrapeptides with an oxidatively decarboxylated 6-bromotryptophan-derived C-terminal amino acid residue, were described previously and were found to have broad spectrum antimicrobial activity.[7] Other peptides sharing structural features with the tunichromes and halocyamines have been isolated from sponges.[8−11]

Recently we undertook a survey of the constituents of the hemocytes of the ascidian *Styela plicata* (Lesueur, Styelidae) in an effort to identify molecules with antimicrobial or metal binding potential and identified a modified octapeptide called plicatamide.[12] Like the tunichromes, plicatamide contains an oxidatively decarboxylated C-terminal DOPA residue. This paper describes the structural elucidation of a modified pentapeptide from *S. plicata* hemocytes which we designate as a tunichrome called tunichrome *Sp*-1 (**1**).



Tunichrome *Sp*-1 corresponds to the major chromatographic peak when acetic acid extracts of *S. plicata* hemocytes are subjected to RP-HPLC. The peptide was first identified as a putative tunichrome by its unusual UV spectrum with maxima at 227, 280, and 311 nm and by its redox activity after acetic acid-urea polyacrylamide gel electrophoresis (AU-PAGE). As previously described for plicatamide, the spectral features reflect conjugation at the C-terminal residue, while the redox activity is derived from the oxidatively sensitive catechol and pyrogallol moieties.[12] Edman degradation analysis with appropriate standards for DOPA[13] indicated an *N*-terminal sequence of DOPA-DOPA-Gly-Pro. ESIMS measurements on an ion trap instrument indicated a $[M + H]^+$ ion of 664.1. The discrepancy in molecular mass between the sequence and the observed molecular ion indicated the likely presence of a C-terminal decarboxy dehydroDOPA (dcΔDOPA) residue. As described for plicatamide, the dcΔDOPA double bond was confirmed as being trans-disubstituted by the characteristic resonances and coupling constants of the α and *β* vinyl protons at *δ* 7.05 (1H, dd, $J = 15$, 10 Hz ΔDOPA-αCH) and *δ* 6.04 (1H, d, $J = 15$ Hz, ΔDOPA-*β*CH).[12] This is also in accordance with previously described C-terminal trans-disubstitutions for the tunichromes,[4] celenamides,[9−11] and clionamide,[8] but in contrast with the cis-disubstituted halocyamines.[7]

We gave high priority to the assignment of the absolute configurations of the amino acid residues in tunichrome *Sp*-1. The origin of this class of compounds is unknown. They either represent the products of secondary metabolism or are post-translationally cleaved from ribosomally derived polypeptide precursors with hydroxylation occurring either prior to or after cleavage.[3] The presence of D-amino acids in plicatamide and tunichrome *Sp*-1 would suggest that they are not ribosomally synthesized, although the possibility of post-translational epimerization as observed in some *Conus* peptides[14] could not be excluded. As described for plicatamide, we were sample-limited by having isolated only 800 *μ*g of pure compound. We adopted the highly sensitive method of fluorescence detection of diasteromeric amino acids after reaction with *o*-phthaldialdehyde and *N*-acetyl-L-cysteine to form an isoindole.[15] Elution of the derivatized amino acids from a reversed-phase C_{18} HPLC column was monitored at 450 nm after excitation at 340 nm. Diastereomers are efficiently separated and identified using this method. Likewise we confirmed an all-L-stereochemistry for plicatamide. Unfor-

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tunately this method is not applicable to the secondary amino acid proline. For this amino acid we resorted to GC−MS analysis of its volatile derivative on Chirasil-Val as has been reported previously[16] and also confirmed an L-configuration.

We designate the molecule as a tunichrome for the following reasons: (a) the peptide is isolated from tunicate hemocytes, (b) the peptide has an oxidatively decarboxylated C-terminus derived from a hydroxylated aromatic amino acid residue and (c) two or more hydroxylated aromatic amino acid residues in its sequence, and finally the peptide is (d) linear and (e) low molecular weight, i.e., under six residues in length.

Ascidians are remarkable marine invertebrates. They have the ability to sequester vanadium from seawater and store the metal in the trivalent state in their blood cells. They have a tough outer coat derived from cellulose, which is extremely unusual in the animal kingdom. They are invertebrate chordates, thus occupying an evolutionarily significant niche. The biological function of the tunichrome class of compounds in ascidian blood cells remains unknown, although metal sequestration, extracellular sclerotization, and antimicrobial activity have all been proposed as potential functions.[3,7] In view of the latter property demonstrated by the tunichrome-like halocyamines, we tested tunichrome *Sp*-1 against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* but found no significant bactericidal action. Since the peptides are present in extremely high concentrations in ascidian hemocytes, the elucidation of their function is likely to be critical in understanding some of their unique biological processes. Interspecies variation in tunichrome structure may provide some insight into the biological roles. For example we have observed a correlation between the presence of TOPA in vanadium-accumulating ascidians and DOPA in iron-accumulators. The significance of this and other structural variations is the subject of ongoing research.

Experimental Section

General Experimental Procedures. Sequencing by Edman degradation was performed on a Porton Instruments microsequenator (Tarzana, CA). Mass spectra were recorded on a Bruker Daltonics Esquire ion trap mass spectrometer. NMR data were acquired on a Varian 300 MHz inverse probe NMR. Stereochemistry was determined using the OPA/NAC reaction and by GC−MS after derivatization to form the *N*-(trifluoroacetyl)methyl ester moieties.

Animal Material. One hundred sixty five individual *S. plicata* were collected by hand from Mission Bay, California, from a submerged crab trap in approximately 3 m of water. The tunics of the organisms were gently cleaned with a coarse toothbrush before the animals were stored overnight in filtered, running seawater.

Characterization of Tunichrome *Sp*-1. Specimens were bled by incising individuals between the siphons and draining the blood (ca.700 mL total) through 70 μm nylon mesh into 50 mL centrifuge tubes on ice and subsequently centrifuged for 20 min at 800*g* and 4 °C. The plasma was decanted, and the blood cells forming the pellet were extracted with 5% acetic acid containing 8 M urea and 0.1 M EDTA. Acetic acid urea−EDTA extracts were subjected to solid phase extraction on Waters Sep-Pak Vac 6 cm^3 (1 g) C_{18} cartridges and eluted with 60% acetonitrile (0.085% TFA)/H_2O (0.1% TFA) after a 20 mL H_2O (0.1% TFA) wash. Eluant was lyophilized and resuspended in H_2O (0.1% TFA) for preparative scale reversed-phase HPLC on a Phenomenex 250 × 10 mm C_{18} column with a Vydac high-performance guard column using acetonitrile (0.085% TFA)/H_2O (0.1% TFA) gradients. Acid urea polyacrylamide gel electrophoresis (AU-PAGE) was performed on the acetic acid urea−EDTA extracts of blood cells followed by parallel staining with Coomassie for polypeptide and nitroblue tetrazolium (NBT) for redox active amino acids. Tunichrome *Sp*-1 was initially identified by its positive response to NBT stain. Approximately 800 μg of white powder was isolated from the peak described as *Sp*-1: UV λ_{max} 227, 280, 311 nm. ^{1}H, COSY, and TOCSY NMR spectra were recorded in DMSO in a Shigemi tube and confirmed the structure of tunichrome *Sp*-1: ^{1}H NMR (DMSO, 300 MHz, res. solv. δ 2.49 ppm); δ (ppm) 1.92 (2H, m, Pro-γCH_2); 2.08 (2H, m, Pro-βCH_2); 2.63 (1H, m, DOPA(1)-βCH); 2.64 (1H, m, DOPA(2)-βCH); 2.91 (1H, m, DOPA(2)-βCH); 3.01 (1H, m, DOPA(1)-βCH); 3.53 (2H, ddd, Pro-δCH_2); 3.82 (1H, m, br, DOPA(1)-αCH); 3.94 (1H, d, Gly-αCH); 4.02 (1H, d, Gly-αCH); 4.31 (1H, dd, Pro-αCH); 4.57 (1H, dd, DOPA(2)-αCH); 6.04 (1H, d, J = 14.7 Hz, DOPA(5)-βCH); 6.53−6.70 (9H, m, DOPA(1,2,5)ArH); 7.05 (1H, dd, J = 9.9 Hz, J = 14.7 Hz DOPA(5)-αCH); 7.89 (2H, br, DOPA(1)NH_2); 8.21 (1H, br, GlyNH); 8.69 (1H, d, J = 11.1 Hz, DOPA(2)NH); 8.79−8.87 (6H, br, DOPA(1,2,5)OH); 9.96 (1H, d, J = 9.9 Hz, DOPA-(5)NH) (DOPA residues numbered according to their distance from the N-terminus); HRMALDI−FTMS m/z $[M + Na]^+$ 686.2428, calcd for $C_{33}H_{37}N_5O_{10}Na$, 686.2432 (Δ 0.4 mmu).

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Supporting Information Available: A RP-HPLC profile of tunichrome *Sp*-1 during purification from *S. plicata* blood cell extracts and ^{1}H, COSY, and TOCSY NMR spectra are included as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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