Title
Novel Post-Translationally Modified Peptide Antibiotics from Solitary Tunicates

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Novel, Post-Translationally Modified Peptide Antibiotics From Solitary Tunicates
Project # R/MP-93A

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Aims of the proposal.

Although the pharmaceutical industry has "mined" the world of soil bacteria, streptomycetes and fungi to find novel antimicrobial molecules, the emergence of resistant superbugs necessitates identifying new antibiotic sources. Recent research into the innate immune system has focused attention on antimicrobial peptides – molecules that equip animals to resist infection without assistance from antibodies and other accoutrements of adaptive immunity. In recent years, potent antimicrobial peptides have been identified in the blood cells (hemocytes) of horseshoe crabs, shrimp, mussels and tunicates ("sea squirts"). Our studies centered on three tunicates (*Styela clava, Styela plicata* and *Ciona intestinalis*) that are abundant in Southern California's shallow waters.

In our initial studies (Taylor, S.W., et al, J Biol Chem. 275:38417-38426, 2000), we observed that some tunicate antimicrobial peptides contained unusual post-translational modifications more commonly found in the classical "secondary metabolite" antibiotics produced by microbes. However, unlike most conventional antibiotics produced by prokaryotes, the endogenous antimicrobial peptides of animals are gene-encoded- a feature that offers exciting "down-the-road" possibilities for their recombinant production or transgenic expression in agricultural and aquacultural crops.

Research Findings:

An important end-result of this project was the characterization of plicatamide, a small peptide that we isolated from the blood cells of *Styela plicata*. Plicatamide is composed of only eight amino acids, making it one of the smallest antimicrobial peptides found to date. It showed excellent activity against methicillin-resistant Staphylococcus aureus ("MRSA"), and appeared to kill this important human pathogen in an unusual manner that we described in Reference 4. In addition to studying the native plicatamide peptide, we produced over 60 synthetic analogs of it to perform structure:activity studies and to see if we could further reduce its size or broaden its antimicrobial spectrum. We have also examined, but not yet published, experiments describing the ability of plicatamide to bind metals and identifying the residues that do so.

References describe studies related to the clavanin family. These peptides are found in the blood cells of *Styela clava*, a solitary tunicate. We had previously described four alpha-helical peptides that we named clavanins A, B, C and D. These peptides contained 23 amino acid residues, were histidine-rich, and were C-terminally amidated. In Reference 1 we described an an additional member of this family that we named clavaspirin. Unlike clavanins A-D, clavaspirin

had potent cytotoxic and hemolytic activity. Reference 3 used immunohistochemistry to identify the blood cells that contained clavanins.

In working with tunicate blood cells, it did not take long to become impressed by their diversity and intrinsic beauty. As tunicate hematology has not been widely practiced during the past half century, we undertook a structural analysis of the blood cells of Styela clava. Our findings are described in Reference 4, which also describes a novel histochemical method that allowed us to identify the cells that contained styelins.

Publications:

1) Lee I.H., Zhao, C, Nguyen, T., Menzel, L., Sherman, M.A., Waring, A.J., and R.I. Lehrer. 2001. Clavaspirin, an antibacterial and hemolytic peptide from *Styela clava*, J. Pept. Res. 58: 445-56.

2) Lee, I-H., Lee, Y.S., Kim, C.H., Kim, C.R., Hong, T., Menzel, L., Boo, L.M., Pohl, J., Sherman, M.A., Waring, A., Lehrer, R.I. 2001. Dicynthaurin: an antimicrobial peptide from hemocytes of the solitary tunicate, *Halocynthia aurantium*. Bichim. Biophys. Acta. 1527: 141-148.

3) Menzel, L.P., Lee, I.H., Sjostrand, B., Lehrer, R.I. 2002. Localization of clavanins in *Styela clava* hemocytes. Dev. Comp. Immunol. 26: 505-515.

4) Tincu, J.A., Menzel L.P., Azimov, R., Sands, J., Hong, T., Waring, A.J., Taylor, S.W., Lehrer, R.I. 2003. Plicatamide, an antimicrobial octapeptide from *Styela plicata* hemocytes. J Biol Chem. 278:13546-13553.

5) Lehrer, R.I., Tincu, J.A., Taylor, S.W., Menzel, L.P. and A.J. Waring. 2003. Natural peptide antibiotics from tunicates: Structures, functions and potential uses. Integrative and Comparative Biology 43. 313–322.

Novel, Post-Translationally Modified Peptide Antibiotics from Solitary Tunicates
Project # R/MP-93B

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Aims of the proposal:

Although the pharmaceutical industry has "mined" the world of soil bacteria, streptomycetes and fungi to find novel antimicrobial molecules, the emergence of resistant superbugs necessitates identifying new antibiotic sources. Recent research into the innate immune system has focused attention on antimicrobial peptides – molecules that equip animals to resist infection without assistance from antibodies and other accoutrements of adaptive immunity. In recent years, potent antimicrobial peptides have been identified in the blood cells (hemocytes) of horseshoe crabs, shrimp, mussels and tunicates ("sea squirts"). Our studies centered on three tunicates (*Styela clava, Styela plicata* and *Ciona intestinalis*) that are abundant in Southern California's shallow waters.

In our initial studies (Taylor, S.W., et al, J Biol Chem. 275:38417-38426, 2000), we observed that some tunicate antimicrobial peptides contained unusual post-translational modifications more commonly found in the classical "secondary metabolite" antibiotics produced by microbes. However, unlike most conventional antibiotics produced by prokaryotes, the endogenous antimicrobial peptides of animals are gene-encoded- a feature that offers exciting "down-the-road" possibilities for their recombinant production or transgenic expression in agricultural and aquacultural crops.

Research Findings:

Plicatamide is a modified octapeptide from the ascidian *Styela plicata* having the structure Phe-Phe-His-Leu-His-Phe-His-decarboxy□DOPA (where decarboxy□DOPA = decarboxy-(*E*)- □,□-dehydro-3,4-dihydroxyphenylalanine) (Tincu, Craig and Taylor Biochem Biophys Res Commun. 2000 Apr 13;270(2):421-4). During the course of the project we characterized this peptide's antimicrobial activity (Reference 1); identified a structurally related peptide (Reference 2) as well as caveats in the mass spectrometric characterization of this class of compounds (Reference 3). We also developed a new chemoenzymatic synthesis route to probe structure-activity relationships in plicatamide's antimicrobial activity (Reference 4).

We hypothesized that plicatamide was the product of post-translational cleavage from a polypeptide precursor as observed in many other antimicrobial peptides (References 5 and 6). Attempts to develop degenerate primers for use in polymerase chain reactions and library screens based upon the amino acid sequence of plicatamide were undertaken. The goal of the investigation was to address the biosynthetic origins of plicatamide utilizing the techniques of degenerate PCR, RNA ligase mediated rapid

amplification of cDNA ends, and oligonucleotide screening of a cDNA library. Ultimately the eight residue sequence was insufficient for the identification of a polypeptide precursor and its existence remains unproven.

In efforts to gain insight to what blood cell types contain plicatamide in *S. plicata* we attempted immunolocalize the peptide. Native plicatamide and the synthethic derivative PL-101 (FFHLHFHY[$CONH_2$]) were coupled to keyhole limpet hemocyanin and injected in rabbits to produce -plicatamide and -PL-101 antibodies. -Plicatamide antisera *did not* recognize plicatamide using western and dot blot analysis. -PL-101 antisera *did* recognize PL-101 using western and dot blot analysis but *did not* recognize plicatamide using the same techniques. Additional synthetic derivatives of plicatamide were tested for reactivity with -PL-101 antisera which partially determined the binding epitope of the -PL-101 antibody which unfortunately has no utility for the immunolocalization of native plicatamide.

Two antimicrobial peptides from the ascidian *C. intestinalis* were isolated and partially characterized. These peptides, designated cionarin H and cionarin I, have many characteristics in common with larger molecular weight polypeptides and proteins isolated from ascidian blood cells including ferreascidin, the Ascidia and Mogula blood cell polypeptides and morulin Pm. These characteristics include resistance to Edman degradation sequencing and protease cleavage, possibly resulting from unknown post-translational modifications. Unlike these other peptides cionarins do not appear to contain DOPA or TOPA residues. While the cionarins eluded total characterization in the current study, preliminary results employing modern techniques of tandem mass spectrometry appear promising in elucidating the structures of this elusive class of biomolecules.

Publications:

1) Tincu JA, Menzel LP, Azimov R, et al. Plicatamide, an antimicrobial octapeptide from Styela plicata Hemocytes. J BIOL CHEM 278 (15): 13546-13553 APR 11 2003.
2) Tincu JA, Taylor SW Tunichrome Sp-1: New pentapeptide tunichrome from the hemocytes of Styela plicata. J NAT PROD 65 (3): 377-378 MAR 2002.
3) Taylor SW, Kassel DB, Tincu JA, et al. Fragmentation of tunichrome Sp-1 is dominated by an unusual gas-phase intramolecular rearrangement. J MASS SPECTROM 38 (10): 1105-1109 OCT 2003.
4) Taylor SW. Chemoenzymatic synthesis of peptidyl 3,4-dihydroxyphenylalanine for structure-activity relationships in marine invertebrate polypeptides. ANAL BIOCHEM. 302(1): 70-4. MAR. 2002
5) Tincu JA, Taylor, SW. Antimicrobial peptides from marine invertebrates. ANTIMICROBIAL AGENTS CHEMOTHERAPY 48 (10):3645-3654 OCT 2004.
6) Lehrer RI, Tincu JA, Taylor SW, et al. Natural peptide antibiotics from tunicates: Structures, functions and potential uses. INTEGR COMP BIOL 43 (2): 313-322 APR 2003.