Comparative Biochemistry and Physiology, Part B 151 (2008) 110–117

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

The crosslinking and antimicrobial properties of tunichrome

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ARTICLE INFO

Article history:
Received 28 March 2008
Received in revised form 2 June 2008
Accepted 7 June 2008
Available online 12 June 2008

Keywords:
Antimicrobial activity
Crosslinking
Dehydrodopa
Natural product
Quinone methide
Tunicates
Tunichrome
Tunic formation

ABSTRACT

Tunichromes are small peptides containing one or more dehydrodopa derived units that have been identified in the blood cells of at least eleven species of tunicates. Incubation of tunichromes isolated from *Ascidia nigra* hemocytes (or model dopa-containing compounds) under oxidative conditions with either lysozyme, cytochrome *c* or ovalbumin resulted in a time-dependent polymerization of these test proteins to dimers, trimers, tetramers and potentially to other oligomers. These results indicate that the oxidation products of tunichromes possess inherent crosslinking properties. Hence it is possible that tunichromes participate in tunic production by forming adducts and crosslinks with structural proteins and/or carbohydrate polymers, similar to the well-understood process of insect cuticle hardening. Since such crosslinking potentials could also be beneficial for defense reactions against invading microorganisms, antibacterial activity of tunichromes was tested using both a radial diffusion assay and the Microtox® test. Tunichromes exhibited antimicrobial activity against gram-negative bacteria *Escherichia coli* and *Photobacterium phosphorium*. However, they did not show any antimicrobial activity against the gram-positive bacteria *Staphylococcus aureus* at the concentrations tested. We propose that the crosslinking and antimicrobial functions are both based on the reactivity of dehydrodopa units present in the tunichromes, and their subsequent ability to form highly reactive quinone methides.

1. Introduction

Tunicates (also called ascidians or simply sea squirts; subphylum Tunicata, Class Ascidiacea) are sessile marine filter-feeding invertebrates that exhibit features characteristic of the vertebrates, including gill slits, larval notocord and larval dorsal nerve cord, yet are considered to be a highly divergent offshoot from the other members of the Chordata (Zeng and Swalla, 2005). Several species of tunicates contain various, low molecular weight, dopa- (=3,4 dihydroxyphenylalanine-) or topa- (=3,4,5-trihydroxyphenylalanine-) containing peptides (or modified peptides) in their blood cells or other body tissues including styelins from *Styela clava* (Taylor et al., 2000), plicatamide from *Styela plicata* (Tincu et al., 2000), halocyamines from *Halocynthia roretzi* (Azumi et al., 1990a), lamellarins from *Didemnum chartaceum* (Lindquist et al., 1988), ferreascidin from *Pyura stolonifera* (Dorsett et al., 1987) and tunichromes from eleven species of tunicates (Macara et al., 1979; Bruening et al., 1985, 1986; Oltz et al., 1988; Bayer et al., 1992; Parry et al., 1992; Tincu and Taylor, 2002).

Tunichromes were the first members of this group to be isolated. They are small, modified peptides that have been extracted and characterized from blood cell lysates of the phlebobranch *Ascidia nigra* (An-1, An-2, An-3; Macara et al., 1979; Bruening et al., 1985, 1986; Oltz et al., 1988), the stolidobranch *Molgula manhattensis* (Mm-1, Mm-2; Oltz et al., 1988), and the phlebobranch *Phallusia mammillata* (Pm-1, Pm-2, Pm-3; Bayer et al., 1992). The structures of these peptidal derivatives are presented in Fig. 1. More recently, a modified pentapeptide tunichrome (Sp-1) has been isolated from the hemocytes of the stolidobranch ascidian *S. plicata* (Tincu and Taylor, 2002). Common to all tunichromes is the presence of dehydrodopamine units (Table 1 and Fig. 1). Many other peptides from tunicates, though not part of the tunichrome family of compounds, also possess dehydrodopamine units (Table 1), including the octopeptide plicatamide from *S. plicata* (Tincu et al., 2000), and the polycyclic lamellarins from *D. chartaceum* (Lindquist et al., 1988) (Table 1), as does the tetrapeptide halocyamines isolated from *H. roretzi* blood cells (Azumi et al., 1990a). In addition, two groups of peptides isolated from the sponge *Cliona celata,* the bromotryptophan containing dipeptide clionamide (Andersen and Stonard, 1979) and the tri- and tetrapeptide celenamides (Stonard and Andersen, 1980a,b) all contain dehydrodopamine units.

The biochemistry of dehydrodopa units has been extensively investigated in relation to cuticle formation in insects and byssal thread production in mussels (Sugumaran, 1998, 2002; Waite, 1990;

Abbreviations: dehydro NADA, 1,2-dehydro *N*-acetyldopamine; dopa, 3,4-dihydroxyphenylalanine; EDTA, ethylene diamine tetraacetate; MEC, minimal effective concentration; NADA, *N*-acetyldopamine; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; TFA, trifluoroacetic acid; TLC, thin layer chromatography; topa, 3,4,5-trihydroxyphenylalanine.

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doi:10.1016/j.cbpb.2008.06.004

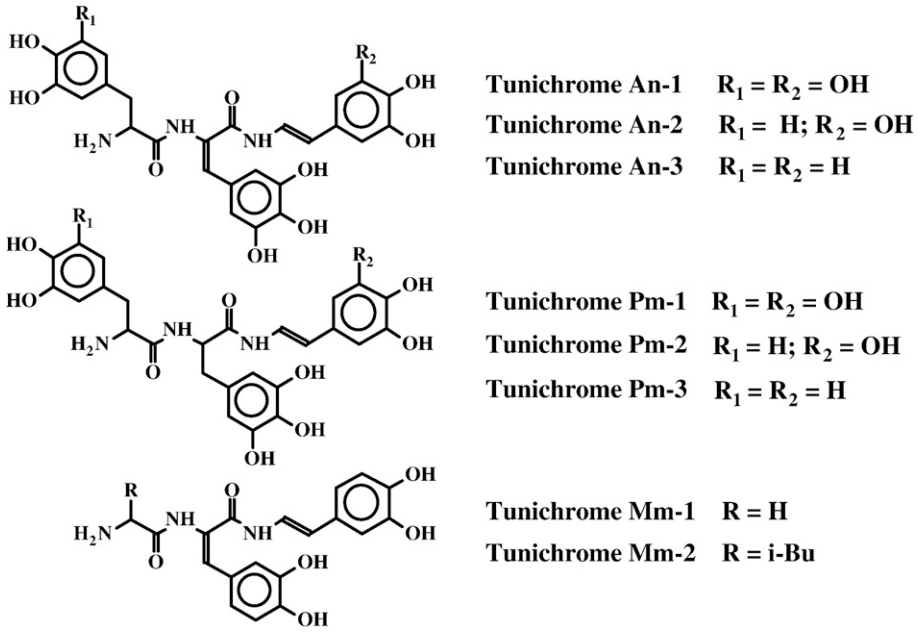

Fig. 1. Structures of some simple tunichromes.

Rzepecki and Waite, 1991; Sugumaran and Ricketts, 1995; Burzio and Waite, 2001). In general, dopa units are initially converted into the corresponding quinones by the action of phenoloxidases. The quinones generated are further transformed either enzymatically or nonenzymatically to dehydrodopa units. Subsequent nonenzymatic oxidation of the dehydrodopa units directly generates highly reactive quinone methide imine amide (Sugumaran, 2000) that can form crosslinks and adducts necessary for hardening the substratum (Sugumaran, 1998, 2002; Waite, 1990).

In light of theses findings, it is highly likely that the multiple dehydrodopamine units of tunichromes would also possess the ability to form crosslinks and therefore aid tunic formation. It has been known that the lysis of the blood cells of *P. mammillata* leads to the production of a greenish black fluid, called Henze solution, that subsequently yields a dark, sometimes fibrous precipitate (Nette et al., 2000; Ciancio et al., 2004). Furthermore, Robinson et al. (1986) found that tunic of 46-h-old *Ascidia callosa* larvae reared from dechorionated neurulae was either markedly reduced in thickness or absent altogether. The epidermis was fragile and cuticular fins failed to develop. Dechorionated neurulae treated with tunichrome showed an enhancement in tunic formation and rudimentary fin development (Robinson et al., 1986), suggesting that tunichrome plays a role in tunic formation. A number of investigators have subsequently proposed that tunichrome is involved in the hardening of the outer cuticle of the tunic, the tunic itself, or the crosslinking of tunic fibers (Robinson et al., 1986, 1996; Waite, 1990; Taylor et al., 1995, 1997a,b).

Table 1
Examples of low molecular weight dehydrodopamine-containing peptide derivatives isolated from tunicates

Compound	Structure
Tunichrome An-1	topa-dehydrotopa-dehydrotopamine
Tunichrome An-2	dopa-dehydrotopa-dehydrotopamine
Tunichrome An-3	dopa-dehydrotopa-dehydrodopamine
Tunichrome Pm-1	topa-topa-dehydrotopamine
Tunichrome Pm-2	dopa-topa-dehydrotopamine
Tunichrome Pm-3	topa-topa-dehydrodopamine
Tunichrome Mm-1	gly-dehydrodopa-dehydrodopamine
Tunichrome Mm-2	leu-dehydrodopa-dehydrodopamine
Tunichrome Sp-1	dopa-dopa-gly-pro-dehydrodopamine
Plicatamide	phe-phe-his-leu-his-phe-his-dehydrodopamine
Lamellarins (A-H)	polycyclic compounds having dehydrodopamines

Some of the tunichrome-related compounds that have been isolated from tunicate blood cells and possess dehydrodopa units exhibit antimicrobial properties, including styelins, clavanins, halocyamine and plicatamide (Azumi et al., 1990a,b; Lee et al., 1997a,b; Taylor et al., 2000; Tincu et al., 2003; Lehrer, 2003; Lehrer et al., 2001, 2003; Tincu and Taylor, 2004). Whether tunichrome itself exhibits *in vitro* antimicrobial properties, or whether any of these dopa-containing peptides possess *in vivo* antimicrobial activity, has yet to be determined. Since the crosslinking potential of dehydrodopa compounds could be beneficial in defense reactions against microorganisms, we therefore hypothesize that tunichromes would exhibit both crosslinking and antimicrobial activity. To assess such possible functions, we examined the crosslinking properties of tunichromes (and some of its analogs) by measuring the degree of polymerization of three test proteins (lysozyme, cytochrome *c* and ovalbumin). We also studied the antibacterial properties of tunichromes (and its analogs) with Microtox® and two-stage radial diffusion assays.

2. Materials and methods

2.1. Materials

A. nigra (Savigny) were purchased from Biomarine Technology (Key West, FL, USA). Waters Sep-Pak Vac C18 cartridges were procurred from Millipore (Billerica, MA, USA). Mushroom tyrosinase (E.C. 1.14.18.1.), trifluoracetic acid (TFA), urea, EDTA, sodium phosphate, lysozyme, cytochrome *c*, $FeCl_3$, SDS, glycerol, Tris, β-mercaptoethanol, bromophenol blue, Coomassie brilliant blue R, glycine-glycine-tyrosine, *N*-acetlydopamine (=NADA), *N*-acetly tyrosine ethyl ester, dopa methyl ester and routine laboratory reagents were provided by Sigma-Aldrich Chemicals (St. Louis, MO, USA). High-performance Thin Layer Chromatography plates were obtained from VWR International Ltd (Bridgeport, NJ, USA). Acetonitrile, acetic acid, and methanol were supplied by Fisher Chemical (Pittsburgh, PA, USA). Protein markers were purchased from Biorad (Hercules, CA, USA). *S. aureus* (ATCC 33591) and *E. coli* strain ML-35p were obtained from American Type Culture Collection (Manassas, VA, USA). Trypticase soy broth and agar powder were provided by Becton, Bickinson and Company (Sparks, MD, USA). Microtox® reagent, diluent, and reconstitution solution were purchased from Strategic Diagnostics (Newark, DE, USA). *N*-acetyl dopa, *N*-acetyl dopa methyl ester, *N*-acetyl dehydrodopa methyl ester, 3,4-dihydroxyphenyl glycine, *N*-acetyl

dehydrodopa, ketocatechol, *N*-acetyl arterenone, and *N*-acetyl dehydrodopamine (=dehydro NADA) were synthesized in our laboratory (Dali and Sugumaran, 1988; Sugumaran and Ricketts, 1995; Sugumaran et al., 1996).

2.2. Methods

2.2.1. Isolation and identification of tunichromes

Blood was obtained from healthy *A. nigra* by cutting through the tunic of the ventral base of the body, exposing the body cavity. The bright yellow/green blood was drained into 50 mL centrifuge tubes on ice and subsequently centrifuged for 20 min at 800 *g* and 4 °C. The plasma was decanted and discarded. Tunichrome was isolated from the blood cell pellet following the method of Taylor et al. (1995). In brief, the blood cell pellet was 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 C18 cartridges (6 mL; 1 g of packing material) and eluted with 60% aqueous solution of acetonitrile containing 0.09% trifluoracetic acid (TFA) after a 20 mL 0.1% aqueous TFA wash. Eluent was lyophilized, resuspended in 0.1% TFA, and separated by Reverse Phase Thin Layer Chromatography (10 cm × 10 cm plates), using 50% acetonitrile (containing 0.09% TFA) as the mobile phase. The tunichrome band was identified by its distinctive pumpkin-colored fluorescence under long wavelength UV light (364 nm; Robinson et al., 1996). The TLC plates were stained with 2% $FeCl_3$ in order to identify phenol groups (Wagner et al., 1983). All the major bands were scraped off the TLC plates and eluted from the silica gel with 60% aqueous solution of acetonitrile containing 0.09% TFA, and evaporated to dryness to collect the purified products (Oltz et al., 1988; Robinson et al., 1996). Subsamples of the dried products were redissolved in 0.1% TFA and the UV absorbance-spectra were determined on a Perkin-Elmer 552A spectrophotometer.

2.2.2. Polymerization studies

The participation of tunichrome and two model compounds (NADA and dehydro NADA) in polymerization reactions was investigated using three test proteins, lysozyme (M_r 14,300), cytochrome *c* (M_r 12,300) and ovalbumin (M_r 44,300), under oxidative conditions. Mushroom tyrosinase was used to generate the oxidative derivatives of tunichrome that may be involved in polymerization (Sugumaran et al., 1987). The progress of the polymerization reaction was easily monitored by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

A standard reaction mixture (500 μL) containing 1 mg of test protein (e.g. lysozyme, cytochrome *c* or ovalbumin), 50 μg mushroom tyrosinase, and 500 nmol of catecholic compound (tunichromes or model compounds), in 0.01 M sodium phosphate buffer, pH 7.0, were incubated in an Eppendorf tube at 30 °C for 2 h. The reaction was usually initiated by the addition of substrate and stopped by the addition of 500 μL of denaturing buffer (0.125 M Tris-HCl; 20% glycerol; 4% SDS; 10% β-mercaptoethanol and 0.025% bromophenol blue). After the reaction was stopped, the tubes were heated in a boiling water bath for 3 min, and the samples were subject to electrophoresis on either a 10% SDS-PAGE gel (3.5% stacking gel; 10% running gel) or a 4%–15% gradient SDS-PAGE gel. Protein bands were stained with 0.2% Coomassie brilliant blue R. The size of the polymerized products was determined by comparison of their mobilities with those of standard protein molecular weight markers. All polymerization experiments were repeated three times.

2.2.3. Microtox® assay and antimicrobial assays

Tunichromes, and 12 model compounds (*N*-acetyldopa, *N*-acetyl dehydrodopa, dopa methylester, *N*-acetyldopa ester, *N*-acetyl tyrosine ethyl ester, *N*-acetyl dehydrodopa methyl ester, 3,4-dihydroxyphenyl glycine, *N*-acetyldopamine (=NADA), 1,2-dehydro *N*-acetyldopamine (=dehydro NADA), glycine–glycine–tyrosine, ketocatechol, and *N*-acetyl arterenone) were tested for antibacterial properties using Microtox® (Strategic Diagnostics, Newark, DE, USA), a rapid, routine bacterial toxicity test system that measures the reduction in light output by the chemiluminescent bacteria, *Photobacterium phosphorium* (gram-negative) upon exposure to antimicrobial compounds. Tunichrome and all twelve model compounds were tested on 3 separate occasions using standard procedures (Azur Environmental, 1995). By using various dilutions of the tested compounds (0.1 mM to1 mM), dose-response was quantified as EC_{50} (i.e. the effective concentration causing a 50% decrease in light output). EPA aquatic toxicity criteria were used to classify the toxicity of tunichromes and the other model compounds (EPA, 2002): highly toxic if $EC_{50} < 1$ mM; moderately toxic if 1 mM $< EC_{50} <$ 10 mM; slightly toxic if 10 mM $< EC_{50} <$ 100 mM.

Tunichromes and model compounds were also tested against two test microorganisms, *S. aureus* (gram-positive) and *E. coli* (gram-negative), using a standard two-stage radial diffusion assay (Steinberg and Lehrer, 1997). Organisms were grown to mid-logarithmic phase at 37 °C in trypticase soy broth. After they were washed with 10 mM phosphate buffer (pH 7.4), approximately 4×10^6 bacterial colony-forming units were spread onto the underlay gel mixture (1% agarose, 10 mM sodium phosphate buffer, pH 7.4, and 0.3 mg/mL trypticase soy broth powder). Sample wells, 6 mm in diameter and 1.2 mm deep, were punched into the underlay gel and filled with 10 μL of test sample (0.1 mM to 2 mM). After the plates were incubated for 3 h at 37 °C, a nutrient-rich overlay gel (60 mg/mL trypticase soy broth, 1% agarose cooled to 40 °C) was poured onto the plates and allowed to harden. Plates were incubated overnight to allow surviving organisms to form microcolonies. The diameters of completely clear zones were measured to the nearest 0.5 mm and expressed in units (1 unit = 1 mm), after first subtracting the well diameter. The relationship between zone diameter and the $\log_{10}$ of the peptide concentration was found to be linear. Slopes of the regression lines for tunichrome, dopa methyl ester and *N*-acetyl dehydrodopa methyl ester were compared using Student's *t*-test (Zar, 1984). $P \leq 0.05$ was accepted as being significant. Because these regressions were linear, the *x*-intercept of the least mean squares regression line was considered to represent the minimal effective concentration (MEC), according to the convention used in similar studies on antimicrobial compounds in tunicates (Lee et al., 1997a,b; Taylor et al., 2000; Tincu et al., 2000, 2003). All experiments were repeated three times.

3. Results

3.1. Tunichrome isolation and identification

The blood cell extracts (5% acetic acid) were bright yellow/green. After they were subjected to solid phase extraction on Waters Sep-Pak Vac C18 cartridges, a light yellow eluent was obtained. Final tunichrome separation by reverse phase TLC revealed over ten differently colored bands, five of which were major bands (Fig. 2), with the remainder too weak or variable to clearly differentiate. The five major bands consisted of two yellow bands (Rf = 0.15 and 0.20), a bright orange band (Rf = 0.6), a black band (Rf = 0.26) and a pumpkin orange band (Rf = 0.37) (Fig. 2). Staining with $FeCl_3$ revealed that the pumpkin-orange band was the only band with phenolic characteristics.

All major bands were scraped off, eluted with 60% acetonitrile, and checked for UV absorbance. Spectral studies revealed that only the pumpkin-orange band exhibited a UV absorbance spectrum characteristic of tunichrome An-1 (Oltz et al., 1988). This band exhibited a broad absorbance peak at 340 nm and a tiny shoulder peak at 300 nm (Fig. 2). The previously published spectrum for An-1 contained a 340 nm peak that was narrower and higher than our purified product, probably indicating that our product contained a mixture of tunichrome An-1, An-2 and An-3. The identities of the remaining TLC bands are unknown. Approximately 10 mg of purified tunichromes were obtained from the whole blood extracts of 75 medium-sized *A. nigra*.

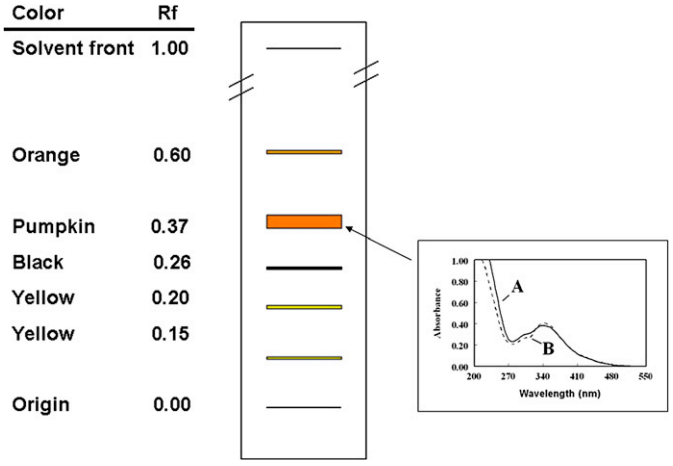

Fig. 2. Diagram of a typical thin layer chromatographic separation of tunichromes from *Ascidia nigra* hemocytes. Following a 30-min separation, bands were visualized by long-wavelength UV (364 nm). Fluorescing color and relative mobilities (Rf) are presented for each band. Insert shows UV/visible absorbance spectrum of the pumpkin-orange band with an Rf of 0.37, identifying it as the tunichrome band. Solid line (A) is the absorption spectrum of our isolated product; dashed line (B) is the spectrum of purified tunichrome An-1 published by Oltz et al. (1988), rescaled to match the experimental data.

3.2. Polymerization of test proteins and either model compounds or tunichromes

Initial studies with two model compounds, NADA and dehydro NADA, demonstrated that these compounds were capable of cross-linking all three of the test proteins (Fig. 3 depicts lysozyme, a typical example). No polymerization was evident at zero time for any of the three proteins. In the case of lysozyme, dimers (~25 kDa) formed after 5 min of incubation with both model compounds. As incubation time was increased, the concentrations of dimers increased and the trimer (~40 kDa) and tetramer (~60 kDa) gradually appeared. The degree of polymerization increased with increasing incubation time. A similar pattern of dimer, trimer and tetramer formation was observed when the model compounds were incubated with cytochrome *c* (data not shown). However, in the polymerization of ovalbumin, only the monomer and dimer could be observed after 1 hour incubation with either NADA or dehydro NADA (data not shown).

Tunichrome was also capable of crosslinking lysozyme, cytochrome *c* and ovalbumin. Reactions with either lysozyme or cytochrome *c* resulted

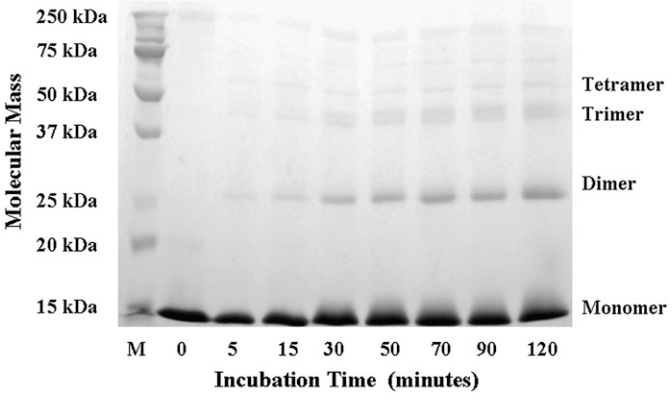

Fig. 3. A 10% SDS-PAGE gel depicting the polymerization production of *N*-acetyl dehydrodopamine (=dehydro NADA) and lysozyme following incubation at 30 °C for up to 2 hours (0–120 min). Incubation solution contained 1 mg lysozyme, 50 μg mushroom tyrosinase and 500 nmol dehydro NADA, in 0.01 M sodium phosphate buffer (pH 7.0). After incubating for indicated time intervals, samples were subject to electrophoresis and stained with 0.2% Coomassie brilliant blue M: protein molecular weight markers.

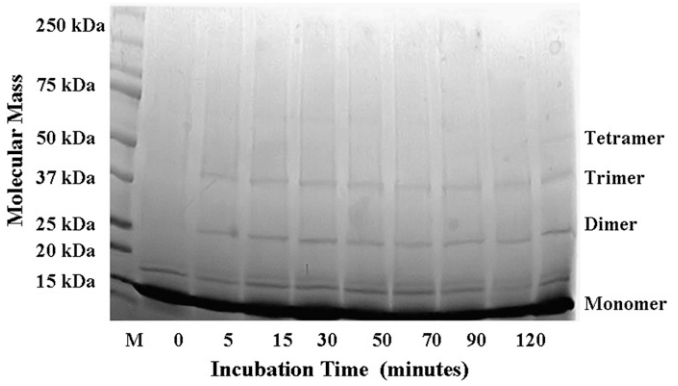

Fig. 4. A 4%–15% gradient SDS-PAGE gel depicting the polymerization production of tunichrome and lysozyme following incubation at 30 °C for the indicated time intervals (0–120 min). Incubation solution contained 1 mg lysozyme, 50 μg mushroom tyrosinase and 500 nmol tunichrome, in 0.01 M sodium phosphate buffer (pH 7.0). After incubation, samples were subjected to electrophoresis and stained with 0.2% Coomassie brilliant blue. M: protein molecular weight markers.

in the formation of dimer (~25 kDa), trimer (~40 kDa) and tetramer (~60 kDa), and followed a similar time course as exhibited for the model compounds (e.g. Fig. 4). As with the experiments using the model dopa compounds, the incubations of tunichrome and ovalbumin only resulted in dimer formation after the one-hour incubation period (data not shown). Control incubations where either mushroom tyrosinase or tunichrome were omitted from the incubation mixture exhibited no evidence of polymerization.

3.3. Microtox® test of tunichromes and model compounds

Microtox® toxicity tests indicated that tunichromes and several model compounds exhibited toxicity to the gram-negative *P. phosphorium* (Table 2). According to their EC_{50} values, *N*-acetyl arterenone is the most toxic model compound. The toxicity of tunichromes is between that of NADA and dehydro NADA. Ketocatechol is the least toxic model compound among the four model compounds that exhibited toxicity. Based on EPA's aquatic toxicity criteria (EPA, 2002), *N*-acetyl arterenone, dehydro NADA and NADA can be classified as "highly" toxic. Ketocatechol showed "moderate" toxicity. The rest of the model compounds did not show toxicity at 1 mM concentration, the highest concentration used in the test. Tunichrome was classified as "highly" toxic, with an EC_{50} of 0.46±0.02 mM for the 5 min test and 0.27±0.01 mM for the 15 min test.

Table 2
Microtox® EC_{50} values (mean±SD, n=3) of tunichromes and twelve model compounds

Compound name	EC_{50} value	Toxic evaluation
N-acetyl arterenone	5 min: 0.20±0.01 mM	Highly toxic
	15 min: 0.20±0.01 mM	Highly toxic
NADA	5 min: 0.40±0.02 mM	Highly toxic
	15 min: 0.20±0.01 mM	Highly toxic
Tunichrome	5 min: 0.46±0.02 mM	Highly toxic
	15 min: 0.27±0.01 mM	Highly toxic
Dehydro-NADA	5 min: 0.60±0.03 mM	Highly toxic
	15 min: 0.40±0.02 mM	Highly toxic
Ketocatechol	5 min: 2.8±0.14 mM	Moderately toxic
	15 min: 1.7±0.10 mM	Moderately toxic
N-acetyl dopa	CNC	Nontoxic at 1 mM
Dopa methyl ester	CNC	Nontoxic at 1 mM
N-acetyl dopa ester	CNC	Nontoxic at 1 mM
N-acetyl tyrosine ethyl ester (ATEE)	CNC	Nontoxic at 1 mM
N-acetyl dehydrodopa	CNC	Nontoxic at 1 mM
3,4-dihydroxyphenylglycine	CNC	Nontoxic at 1 mM
N-acetyl dehydrodopa methyl ester	CNC	Nontoxic at 1 mM
Glycine–glycine–tyrosine	CNC	Nontoxic at 1 mM

Separate EC_{50}s were calculated for 5 min and 15 min Microtox® incubation periods.
CNC: could not calculate EC_{50} values (EC_{50}>1 mM).

3.4. Two-stage radial diffusion assay results of tunichromes and model compounds

Two-stage radial diffusion assays on tunichrome and the other twelve model compounds revealed a different pattern of toxicity than shown in the Microtox® tests. Tunichrome inhibited the growth of *E. coli* and formed a clear zone (Fig. 5). Among all 12 model compounds, only dopa methyl ester (Fig. 5) and *N*-acetyl-dehydrodopa methyl ester (data not shown) showed inhibition on *E. coli*. Neither of these two compounds had exhibited toxicity effects in the Microtox® test. Two-stage radial diffusion assays using different concentrations of tunichromes, dopa methyl ester and *N*-acetyl dehydrodopa methyl ester exhibited a linear dose-response relationship between the zone diameter and the $\log_{10}$ of the compound concentration (Fig. 6). The *x*-intercept of the regression can be considered to represent the MEC. (minimal effective concentration; Tincu et al., 2003). Tunichrome and *N*-acetyl dehydrodopa methyl ester exhibited similar slopes (Student's *t*-test for comparison of slopes; $P>0.05$; Zar, 1984) and apparently similar MECs (49.8 μM vs 49.9 μM respectively), whereas the slope of dopa methyl ester was significantly steeper ($P<0.05$) and the MEC was apparently greater (61.1 μM). This implies that the mechanism of toxicity is similar for tunichrome and *N*-acetyl dehydrodopa, but probably slightly different for dopa methyl ester.

In contrast to the result for the gram-negative *E. coli*, neither tunichromes nor any other model compounds inhibited the growth of *S. aureus* (data not shown). No clear zones were observed compared to the positive control (0.1 mM ampicillin).

4. Discussion

The initial purification of tunichrome Ans and Mms (Bruening et al., 1985, 1986; Oltz et al., 1988) involved protection of the unstable molecule by acetylation and Sephadex LH-20 column chromatography. Instead, we used a new procedure first established by Taylor et al. (1995) and used in a subsequent study to isolate tunichrome Sp-1 (Tincu and Taylor, 2002). The final purified product we obtained using this new method exhibited a very similar UV absorbance spectrum to the one represented for pure tunichrome An-1 (Oltz et al., 1988). Compared to the old method, this protocol is more straightforward and can yield large amounts of stable tunichrome in a relatively short time.

Although the structures of tunichrome Ans and Mms have been known since the late 1980's (Bruening et al., 1985, 1986; Oltz et al., 1988), their biological functions remain unknown. The present study clearly demonstrates that tunichromes are capable of crosslinking proteins *in vitro* once they are enzymatically oxidized by tyrosinase, and they have

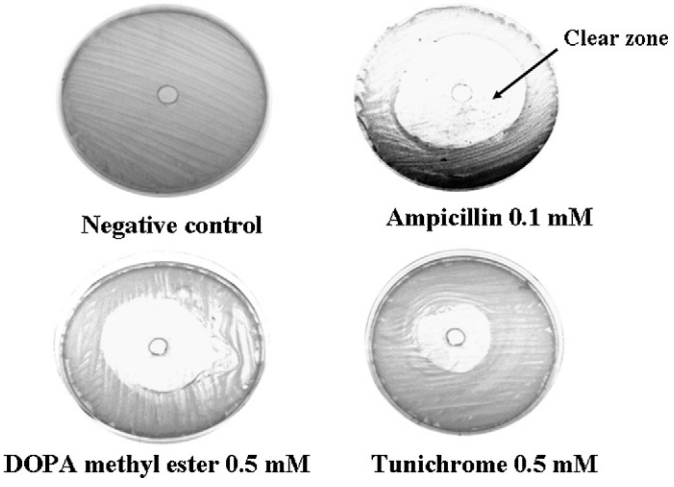

Fig. 5. The two-stage radial diffusion assay of different compounds using *E. coli*. A: negative control; B: positive control=ampicillin (0.1 mM); C: dopa methyl ester (0.5 mM); D: purified tunichromes (0.5 mM).

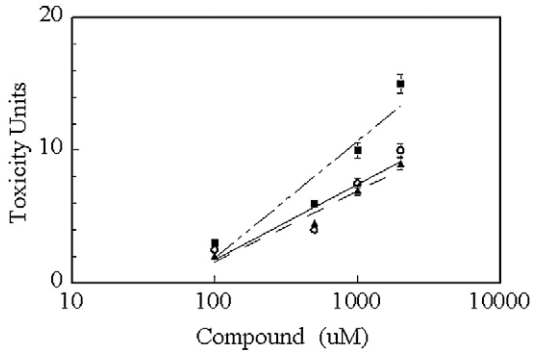

Fig. 6. Dose response curve for tunichromes (▲), dopa methyl ester (■) and *N*-acetyl dehydrodopa methyl ester (○) obtained from two-stage radial diffusion assays. Regression equations (least mean squares fit): $y=2.3\ \log_{10}(x)-9.1$, $r^2=0.962$; $y=3.8\ \log_{10}(x)-15.7$, $r^2=0.893$; $y=2.5\ \log_{10}(x)-9.7$, $r^2=0.876$ respectively. Toxic units are defined as the diameters (mm) of completely clear zones after subtracting the well diameter. Data points are mean±SD, n=3.

in vitro antimicrobial activity against two gram-negative bacteria (*E. coli* and *P. phosphorium*). Whether these *in vitro* properties are manifested *in vivo* has yet to be determined.

If tunichromes have crosslinking activity *in vivo* as well as *in vitro*, it is possible that they participate in tunic formation and repair processes. The tunic is the extracellular matrix covering the epidermis of tunicates, and has been reported to consist of a variety of compounds, including a fibrous matrix composed of a type of cellulose (called tunicin), elastins, collagen and other proteins, as well as a ground substance composed of mucopolysaccharide, glycoproteins, proteins, inorganic compounds and hemocytes (Hall and Saxl, 1961; Deck et al., 1966; De Leo et al., 1977; Patricolo and De Leo, 1979; Robinson et al., 1983, 1996). However, it is not known how these compounds interact to form the tunic matrix. Since the present study demonstrates that tunichrome could crosslink three very different model proteins *in vitro*, it is possible that tunichrome could crosslink structural proteins, glycoproteins, or structural carbohydrates in the same way and form the tunic *in vivo*. The occurrence of adduct formation between quinonoid compounds and polymeric carbohydrates such as chitin in the insect cuticle has been confirmed by solid state NMR studies (Schaefer et al., 1987), although the exact chemical nature of the adduct remains to be established. The amino groups (generated by partial hydrolysis of *N*-acetylamino sugars) as well as the hydroxyl groups could form potential reactive sites on the structural carbohydrate polymers.

The most likely mechanism involved in tunichrome polymerization should involve quinone methide imine amide generated by the oxidation of *N*-acetyl dehydrodopamine derivatives (Sugumaran, 2000; Sugumaran et al., 1992). Based on the logical expectation that the oxidation of a catechol directly yields a quinone, one might expect the dehydrodopamine quinone to be the initial product of dehydrodopamine oxidation. However, *N*-acetyl 1,2-dehydrodopamine, the monomer found in tunichrome, upon oxidation directly yields the quinone methide imine amide as the first observable two electron oxidation product (Sugumaran, 2000; Sugumaran et al., 1992). The normally expected isomeric quinone is formed only under conditions of extreme acidity. Quinone methides will exhibit non-enzymatic Michael-1,6-nucleophilic addition with any available nucleophiles including side chains of proteins, chitin and even water molecules (Sugumaran, 1998, 2002). The Shiff's base imine amide will react with another nucleophile, simultaneously and independently forming another adduct or a cross-link (Sugumaran, 1998, 2002). Quinone methide reactions with nucleic acids are responsible for the antitumor activity of antibiotics such as mitomycin C, adriamycin and daunomycin (reviewed in Thompson et al., 1993). If no other molecules are available, quinone methides can simply self-dimerize or self-oligomerize to form a variety of polymeric product groups (Wagner and Grompper, 1974; Sugumaran, 1998). In addition, the side chains of a number of amino acids could potentially react with

quinone methides and form adducts (Sugumaran, 1998). The hydroxyl group of the chitin polymer can also react with quinone methide under very mild conditions, making them very attractive candidates for adduct formation and crosslinking.

Dehydrodopamine is the active component of tunichromes. Therefore, its enzymatic oxidation by a phenoloxidase (=tyrosinase) will generate the quinone methide imine amide derivative that can readily crosslink with proteins and carbohydrate polymers nonenzymatically. The presence of phenoloxidase has been reported in the morula cells of several species of tunicates (Chaga, 1980; Smith and Söderhäll, 1991; Arizza et al., 1995; Cammarata et al., 1996; Ballarin et al., 1994; Frizzo et al., 2000; Parrinello et al., 2003). Since tunichrome has also been reported to be located in morula cells (Oltz et al., 1989), the co-location of enzyme and substrate supports the hypothesis that tunichromes could be oxidized enzymatically. Using tunichrome Mm-1 (gly-dedopa-dehydrodopamine) as an example, the possible mechanisms of crosslinking reactions are depicted in Fig. 7. Since tunichrome Mm-1 has two dehydrodopamine units, both can be independently oxidized. Earlier work from our laboratory has established that two electron oxidation of a dehydodopamine unit by tyrosinase directly produces quinone methide imine amide as the initial product (Sugumaran, 2000; Sugumaran et al., 1992). Thus, tyrosinase (A) catalyzes the oxidation of tunichrome Mm-1 producing two different quinone methide imine imide derivatives (one internal and the other external) that can now form adducts and crosslinks. This transient intermediate is unstable and immediately reacts forming adducts and crosslinks. Such a reaction with one of the dehydrodopamine units of Mm-1 is enough to produce adducts and crosslinks. Subsequent oxidation of the second dehydrodopamine unit of Mm-1 will generate additional crosslinks and adducts, as shown in Fig. 7.

Tunichromes exhibited antibacterial activity against two gram-negative bacteria, *E. coli* and *P. phosphorium*, but not against the gram-positive bacteria, *S. aureus* (at the concentrations tested). Similarly, several of the model compounds exhibited antimicrobial activity against *E. coli* and *P. phosphorium*, but not against *S. aureus*. The most obvious difference between gram-positive and gram-negative bacteria is the nature of the cell wall. All bacteria have peptidoglycans in their cell walls. These peptidoglycans are heteropolymers of glycan strands crosslinked through short peptides consisting of both L- and D-amino acids (Coley et al., 1978; Reusch, 1984; Alouf and Muller-Alouf, 2003). In gram-positive bacteria, the peptidoglycans form a thick, multilayered structure, while in gram-negative bacteria the peptidoglycans are simple in structure and form a thinner layer within

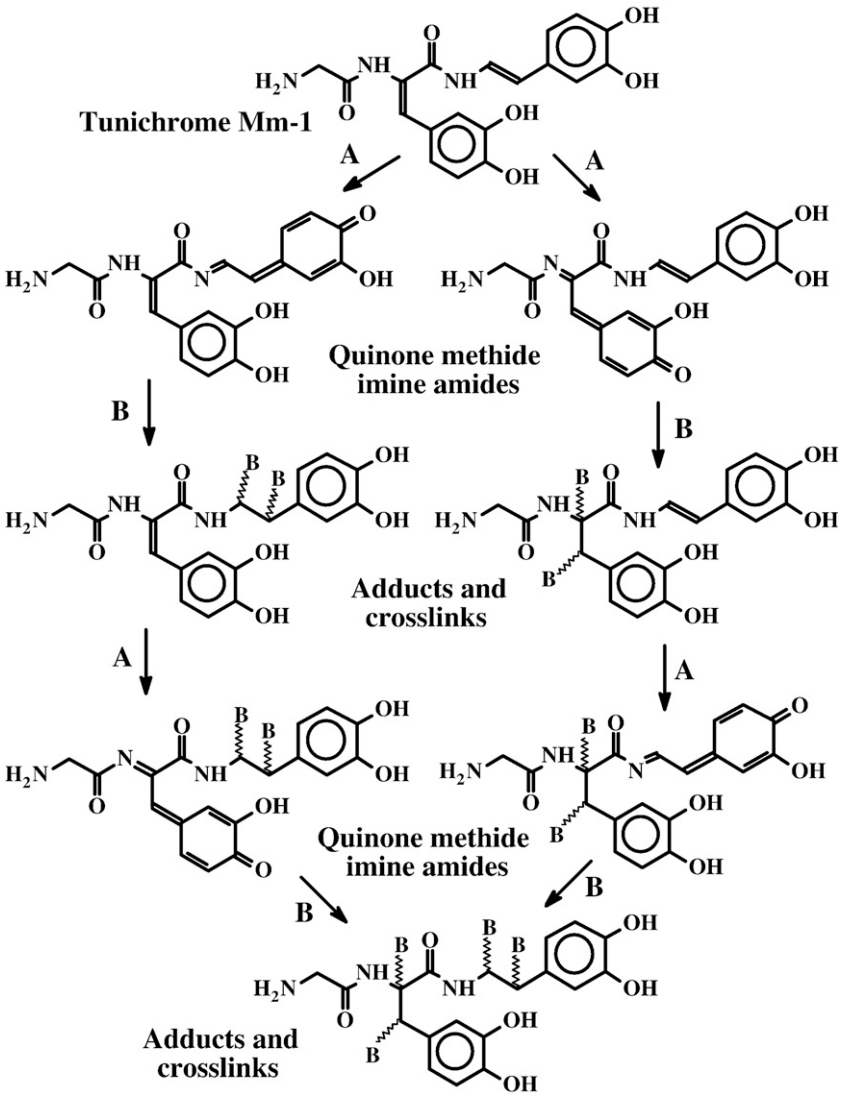

Fig. 7. The possible mechanism of polymerization between tunichrome Mm-1 and test proteins. Tunichrome Mm-1 is oxidized by phenoloxidase (A) to the corresponding quinone methide that forms adducts and crosslinks with protein and other macromolecular side chains (B). Further oxidation of these products by phenoloxidase generates quinone methide imine imide at a different locus on the same molecule. Coupling of these reactive intermediates with proteins and other substrata generates additional adducts and crosslinks.

the cell wall that is relatively uniform throughout most gram-negative genera (Vollmer and Holtje, 2001). Tunichromes might exhibit antibacterial activity by crosslinking the structural proteins of the bacteria and changing the permeability of the cell wall. The damaged cell wall could result in the inhibition of growth and cell division. In gram-negative bacteria, the thin layer of simple peptidoglycans might be easily compromised by tunichrome and the model compounds, dopa methyl ester and *N*-acetyl dehydrodopa methyl ester. In gram-positive bacteria, however, the thickness of the peptidoglycan layer might offer some protection, even if the outer layer of peptidoglycan is damaged. This is consistent with the observation that styelin D acts on the outer and inner membranes of *E. coli*, making these membranes more permeable to some antibiotics (Taylor et al., 2000). Similarly, Lehrer et al. (2003) observed disruption of the cell walls of both *E. coli* (gram-negative) and *S. aureus* (gram-positive) that resulted in the extrusion of cytoplasm upon exposure to styelin D.

Alternatively, tunichrome and its derivatives could be toxic in their own right. Essential metals, for example, could be chelated by the catechol and pyrogallol moieties of the tunichrome molecule, thereby inhibiting bacterial metabolism. Quinone methides, if present as key intermediates in the process of tunichrome crosslinking, are electrophilic and strong oxidants. A number of naturally occurring quinone methides have been shown to be toxic (Thompson et al., 1993). In addition, the catechol and pyrogallol groups of the tunichromes might be involved in the generation of reactive oxygen species (ROS) due to their ease of oxidation and reaction with molecular oxygen. The subsequent cycling between quinones and semiquinones could produce significant quantities of damaging free redicals. Since *S. aureus* produces catalase, it might offer some protection from the attack of electrophiles and the production of ROS (Over et al., 2000), and might explain why *S. aureus* can resist tunichrome and model compound toxicity.

While tunichrome's antibacterial property was consistently shown in both Microtox® and two-stage radial diffusion assays with gram-negative bacteria (*P. phosphorium* and *Escherichia coli*, respectively), the model coumpounds used in this study did not exhibit consistent toxicity in these two assays. Only four model compounds exhibited toxicities in the Microtox® test, while the other eight model compounds did not exhibit any toxicity at the concentrations tested. However, in the two-stage radial diffusion assay, these four model compounds did not exhibit toxicity. Instead, two other compounds, *N*-acetyl dehydrodopa methyl ester and dopa methyl ester, inhibited the growth of *E. coli.* The difference in model compound response in the two assays may indicate that there are exhibiting separate mechanisms of toxicity in the Microtox® and two-stage radial diffusion assay. The short term (5 to 15 min exposure) inhibition of light output (*P. phosphorium*) may not be related to the long term (24 h exposure) pattern of clear zones in the radial diffusion assay. Neither of the toxic responses necessarily indicate that the bacteria have been killed. The two-stage radial diffusion assay appears to reflect the inhibition of growth and reproduction of bacteria. Microtox® testing is probably not as integrative. While Microtox® inhibition could also be due to inhibition of cell growth or the prevention of cell division, it could also indicate damage to a single metabolic pathway that is linked to *P. phosphorium* light output. Given the uncertainty concerning the interpretation of the Microtox® tests, it seems that the two-stage radial diffusion assay represents a more integrative measure of toxicity compared to the Microtox® test.

With the exception of the tunichromes (this study), all dopa-containing peptides in tunicates so far examined for antimicrobial activity appear to act on both gram-negative and gram-positive bacteria, although there is some indication that the compounds exhibit slightly less potency against gram-positive species (Lee et al., 1997a,b; Taylor et al., 2000; Tincu et al., 2000, 2003; Lehrer et al., 2003). Had we used higher concentrations of tunichromes against the gram-positive *S. aureus*, we would likely have measured a toxic response. Based on *E. coli*'s response, tunichromes' antibacterial potency appears to be more similar to that of halocyamine than it is to the other antibacterial dopa-containing molecules plicatamide, styelins, and clavanins (Table 3). The latter three classes of substances exhibited one to two orders of magnitude higher potency against *E. coli* in similarly conducted two-stage radial diffusion assays at neutral pH (7.4; Lee et al., 1997a,b; Taylor et al., 2000; Tincu et al., 2000, 2003). While inhibition of culture growth instead of a radial diffusion assay was used in the halocyamine experiments (Table 3), the MEC for the tunichromes was within the range of the minimum inhibition concentration for halocyamine (Azumi et al., 1990a,b). Although antimicrobial potency of these compounds appears to be proportional to the molecular weight of the compounds, it is not related to the number of modified phenylalanine or tyrosine amino acid residues in each of the compound's structures. The lower potency of tunichromes compared to these other compounds may indicate that the *primary* role of tunichrome is not antibacterial, but may instead be the crosslinking of tunic components. However, more work is needed to assess the potential role of tunichromes as antibacterial compounds.

The results of this study strongly lend support to the hypothesis that tunichromes are potential crosslinkers for the formation of tunic as well as antibacterial agents of the tunicate defense system. Crosslinking and antibacterial activity appear to be related characteristics that would make tunichrome a highly useful compound for the biomedical industry. Tunichromes could find applications in accident triage, emergency medical treatment and surgery, as well as for minor wound repair. In addition, nonmedical uses could entail the production of antifouling panels for marine and freshwater construction. Moreover, the detailed mechanisms that we have learned from our studies can be directly applicable for the “Green” formulation of environmentally safe products with inherent antipredator, antibacterial and antifouling properties.

Table 3
Comparison of antimicrobial potencies of several dopa-containing compounds isolated from tunicates

Compound	Mol. mass (Da)	# a.a. residues	% modified phe	pH	MEC		Reference
					μg/mL	μM	
Tunichromes							
An-1, 2 and 3	556[a]	3	100%	7.4	27.7	49.8	this study
Halocyamines	668 and 730[b]	4	25%	?	25–100	36–147	Azumi et al., 1990a,b
Plicatamides	984–116	8	50%	7.4	1–2.5	1–2.5	Tincu et al., 2000, 2003
Clavanins	2666–2695	23	17–22%	7.4	~1	~0.4	Lee et al., 1997a
Styelin A & B	~3690[c]	>23	21–22%	7.4	1–10	0.3–2.7	Lee et al., 1997b
Styelin D	4081–4144[d]	32	17–22%	7.4	~6	~1.5	Taylor et al., 2000

Information on molecular mass (Da), the number of amino acid residues (# a.a. residues) and the molar percentage of modified phenylalanine residues (% modified Phe) are presented for each compound. Data from experiments conducted at comparable pH were selected. Minimal Effective Concentrations (MEC) were determined from radial diffusion assays for all compounds except halocyamine (where a Minimum Inhibition Concentration was determined from cell culture experiments). All responses are for the gram-negative bacterium, *Escherichia coli*, except for the reported MEC for Styelin D (where the gram-positive *Staphylococcus aureus* was used as the test organism).
[a] Molecular mass of tunichrome An-1 (Oltz et al., 1988); [b]Molecular mass of halocyamine A=668; B=730; 4:1 mixture of A:B.
[c] Partial amino acid sequence; [d]Compound exhibits microheterogeneity.

Acknowledgement

Research was funded by the Massachusetts Institute of Technology Sea Grant Program, under grant number RB-56 subaward 5710001422.

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