New Perspectives in the Chemistry and Biochemistry of the Tunichromes and Related Compounds

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Contents

I. Introduction

The tunichromes constitute a series of compounds isolated from the blood cells of ascidians, marine invertebrates that form the major class of the subphylum Urochordata (Tunicata). Early this century it was discovered by the German chemist Martin Henze that the Mediterranean ascidian *Phallusia mammillata* accumulated high concentrations of vanadium in its blood cells.[1] Later it was found that the metal was predominantly stored in the oxidatively sensitive trivalent state.[2−4] Other examples of vanadium enrichment in biological systems can be found in certain mushrooms, where levels approach 250 ppm (dry weight)[5] and the metal is stored as a vanadium(IV) complex known as amavadin,[6] and most recently, in a polychaete worm, where the metal is stored in the +3 oxidation state in epidermal cells in concentrations comparable to those found in ascidian blood cells (5600 ppm, dry weight).[7] However, ascidians provide the oldest known example of vanadium sequestration in nature, and despite 85 years of research since Henze's discovery, a definitive function for the metal remains obscure. The tunichromes were discovered in the quest for compounds that are involved in vanadium accumulation and reduction in ascidian blood cells. Their structures are strongly indicative of such a role, containing modified TOPA (3,4,5-trihydroxyphenylalanine) and DOPA (3,4-dihydroxyphenylalanine) residues, reducing amino acids with potent metal-chelating potential through their catechol and pyrogallol moieties, respectively (Figure 1).[8−12]

In this review, we will cover the literature in the five years since the last comprehensive review of tunichrome chemistry[12] and discuss a mounting body of data that may shed light on the origins of these novel compounds, in particular as to their possible derivation from TOPA and DOPA polypeptide precursors. Recent innovations in synthetic strategies for both the tunichromes and DOPA/TOPA peptides will be discussed and, in addition, literature dealing with their possible biological functions will be reviewed. The vanadium chemistry of the tunichromes has recently been reviewed elsewhere,[11] and we do not intend to concentrate on aspects already covered in that review. However, material either omitted from that review, or very recently published on tunichrome−vanadium chemistry, will be discussed. But first it is pertinent to briefly introduce ascidians to readers not familiar with these animals and aspects of their blood cell physiology which are relevant to understanding the possible biological roles for the tunichromes and related compounds.

II. Ascidian Biology

Ascidians are ubiquitous filter-feeders, commonly called "sea squirts" because they release jets of water through their siphons when disturbed. Ascidians vary in size from microscopic colonial forms that form encrusting sheets to large individuals 20−30 cm in length. All species have basically the same simple structure consisting of two apertures, atop a sac-like body, from which they get their name (the word "ascidian" comes from the Greek word *askidion*, meaning "small wine-skin") (Figure 2).

The subphylum Tunicata, to which ascidians belong, also includes the free-swimming thaliceans (salps) and larvaceans. Tunicates get their name from their tough, often translucent, tunic-like body covering. The tunic is composed of a material called "tunicin" which is composite of carbohydrate and acid mucopolysaccharide apparently secreted by the epithelial cells of the mantle wall and interspersed with proteinaceous microfibrils from the blood cells.[13]

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Steven Taylor was born in 1966 in Brisbane, Australia. He obtained his B.S. in 1987 and a Ph.D. in 1993 at the University of Queensland under the direction of Cliff Hawkins. During his Ph.D. he also performed research in the laboratory of Jim Swinehart during a one-year stay at the University of California, Davis. He returned to Davis as a postdoctoral fellow with Ted Molinski and later with Herb Waite at the University of Delaware. Currently, he is an Alexander von Humboldt research fellow at the University of Tübingen with Professor Bayer. His research interests include isolation and characterization of DOPA and TOPA peptides from ascidian blood cells and metal-binding studies on DOPA proteins in general.

Bernd Kammerer was born in Rottweil, Germany, and received his Diploma in Chemistry at the University of Tübingen in 1993. The thesis topic was "Total synthesis of fully protected Tunichrome Pm-1". This work is continuing in Professor Bayer's laboratory at Tübingen. The focus of his doctoral studies is the enantioselective synthesis of the tunichromes found in *Phallusia mammillata*, synthesis of potential precursors of the tunichromes, and screening of natural products by ion-spray–MS and LC–MS coupling.

Recent studies on tunicin from the ascidian *Halocynthia roretzi* by NMR confirmed that it is predominantly composed of celluloses similar to those found in bacteria and algae.[14]

Species of the class Ascidiacea are divided into two orders, the Pleurogona and the Enterogona, and three suborders, the Aplousobranchia, the Phlebobranchia and the Stolidobranchia. Vanadium-accumulating species are found in the former two suborders.[15,16] Some stolidobranch ascidians have been found to accumulate iron but not in concentrations comparable with vanadium levels in the blood cells of certain phlebobranch and aplousobranch ascidians.[15−20]

In their larval stage ascidians are free swimming "tadpoles" (Figure 3). They possess a dorsal, hollow nerve chord and notochord-like cells in their larval tail which are lost on metamorphosis to the sedentary adult form. These characteristics, together with the

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pharyngeal gill slits which are retained in the branchial basket of the adult ascidian place ascidians, and tunicates in general, in the phylum *Chordata* to which all higher vertebrates belong. While clearly in their adult form these animals lack a backbone and are invertebrates, they occupy a unique niche in the animal kingdom by simultaneously being classified as chordates.

III. Ascidian Blood Cells

Ascidians have an open circulatory system consisting of a tubular heart enclosed in a pericardium. Blood is pumped by peristaltic contractions of the heart which reverses blood flow periodically. A network of blood vessels, spaces and channels, allow circulation of blood throughout the body and tunic of the animal.

Ascidian blood cells bear little resemblance to vertebrate blood cells (Figure 4). They are all actively amoeboid and are not confined to the vascular system. Blood cells can be observed migrating across the walls of vessels and penetrating the tunic.

There appear to be six to nine different blood cell types, but this is highly variable. Large variations may be observed both in the number and proportions of the circulating blood cells, not only between different species, but also between different individuals of the same species. Age, health, size, and seasonal influences are just some of the factors that may effect the blood cell population. Blood cells make up from 1% to 2% of the blood volume in *Pyura stolonifera*[17] and *Ascidia nigra*,[21] respectively. By far the most work has concentrated, however, on the chemical constituents of the vacuolated cells which comprise the compartment, signet ring, and morula cells. These are generally the predominant cell types, the repositories of accumulated metals and source of the tunichromes.

The distribution of vanadium and tunichrome among different cell types has been extensively

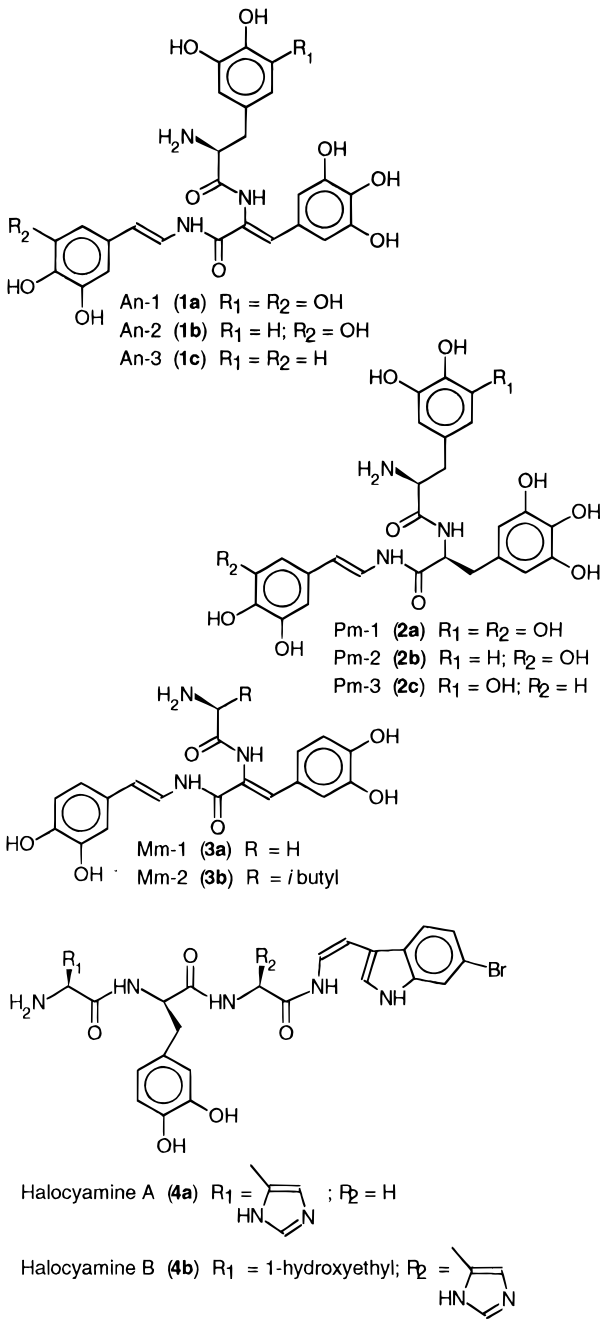

Figure 1. Structures of the tunichromes and halocyamines.

studied in recent years by analytical electron microscopy,[22−29] cell sorting,[30,31] and 2,2′-bipyridine staining for vanadium(III).[28] With the exception of one recent report[32] it appears that the signet ring and compartment cells contain the bulk of the vanadium while the morula cells are the source of the tunichromes.

The major roles of the vacuolated cells are believed to be in tunic formation, coagulation, and wound repair. At the site of vascular injury blood cells are observed sticking together, forming sheets and threads, then cytolyzing and releasing a fiber-producing intraglobular material which eventually seals the wound.[33] The predominant cell within these aggregates is the morula cell. Similarly, the morula cell predominates in the fibrous matrix of the tunic. Morula cells can be observed at various stages of disintegration within the tunic. The vacuoles break free and are found associated with microfibrils,

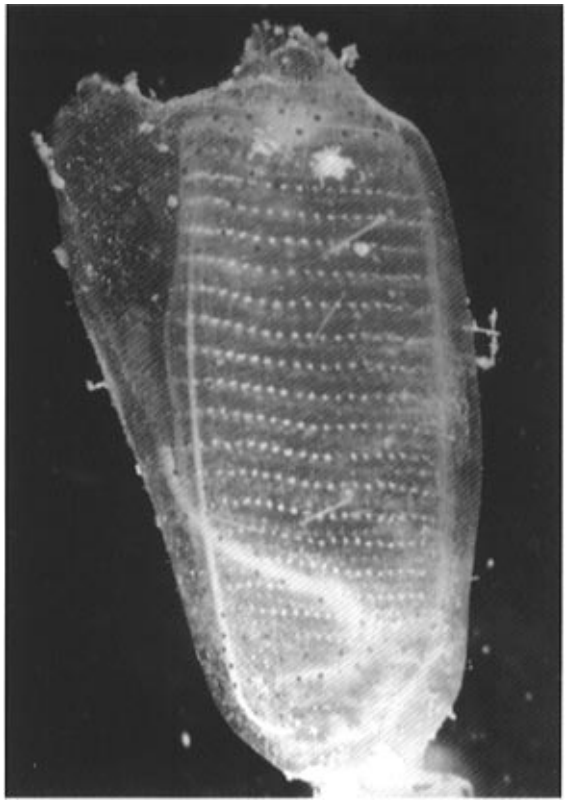

Figure 2. Adult *Ecteinascidia diaphanis*, a typical solitary ascidian.

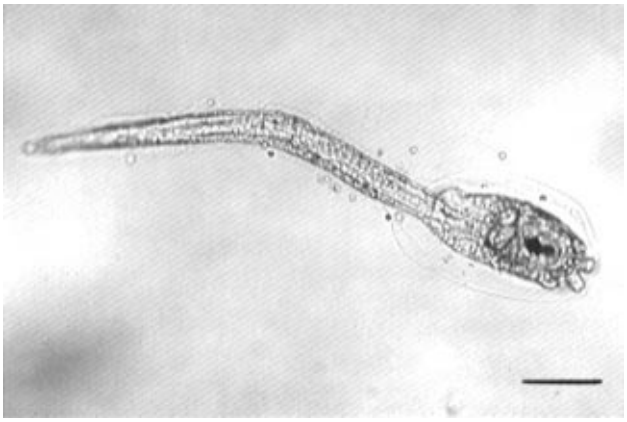

Figure 3. Free-swimming *Phallusia julinea* larva (scale bar 100 μm).

suggesting a relationship between fiber production and morula cell disintegration.[33] Vacuolated cells are also important in the defense mechanisms of ascidians and have been implicated in immunogenic responses to foreign substances and bacteria.[34] The characterization of the tunichromes and halocyamines from ascidian blood cells allows us to correlate structure and chemical properties to the physiological processes described above.

IV. The Tunichromes and Halocyamines

A. Isolation and Characterization

The original tunichrome, An-1, was isolated from the phlebobranch ascidian *A. nigra*.[8] Its structure consisted of a modified tripeptide of three TOPA units (Figure 1, **1a**). Later, the structures of An-2 and An-3 were determined, differing only in the degree of hydroxylation (Figure 1, **1b**,**c**).[9] The isolation was

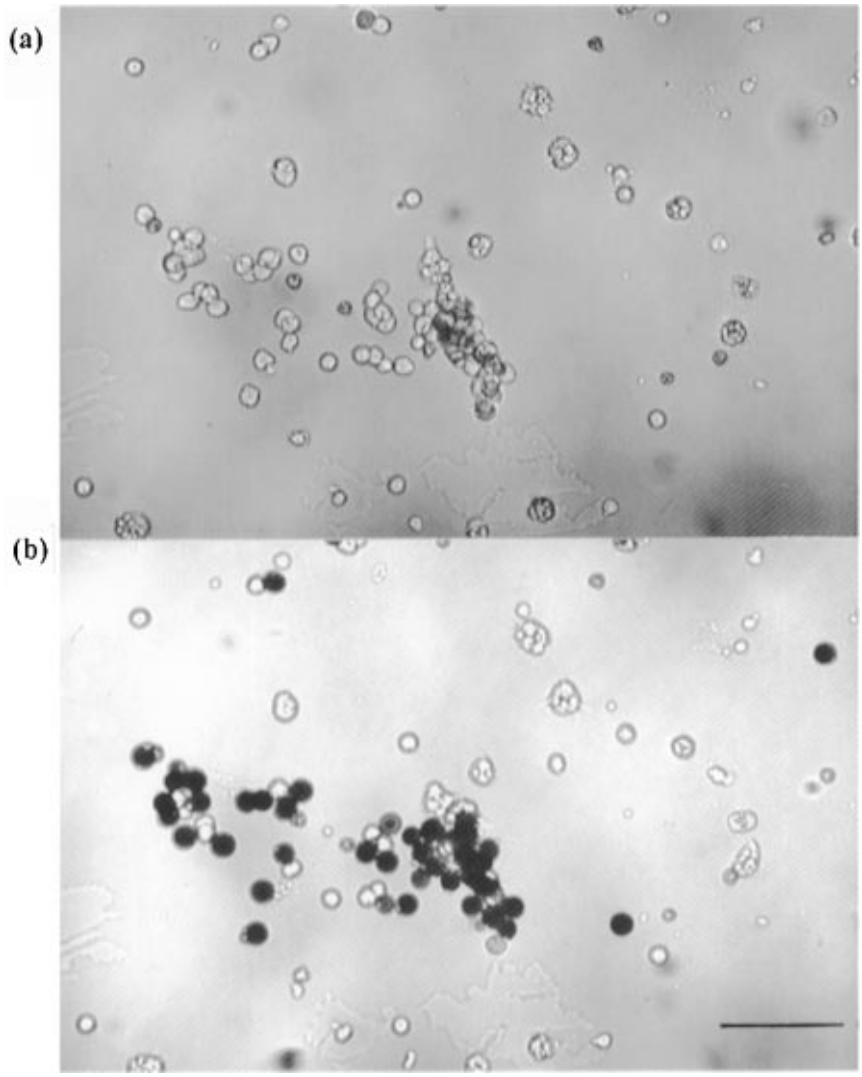

Figure 4. *P. julinea* blood cells (a) in the presence and (b) absence of 2,2′-bipyridine. Signet ring cells stain deep purple due to complexation of vanadium(III) by the diimine chelate (scale bar 20.0 μm).

difficult owing to the air sensitivity, and reactivity, of the compounds. Kim et al. subsequently reported a more efficient means of tunichrome isolation via protection−deprotection.[35] More recently, it has been found that aqueous acetic acid−urea extraction of *A. ceratodes* blood cells incorporating 0.1 M EDTA allows ready isolation of tunichrome An-1 by reversed-phase HPLC.[36] Once isolated in the pure form, the tunichromes were found to be much more stable. It is these compounds, and not vanadium, which are suggested to be responsible for the yellow color of the morula cells. The oxidatively sensitive pyrogallol moieties of TOPA are susceptible to cross-linking reactions. These factors, along with the physiological behavior of the morula cells and the vacuoles in which the tunichromes are stored, implicate a role for the tunichromes in tunic biogenesis and wound repair. This is further discussed below.

More recently, three tunichromes were identified from the blood cells of *P. mammillata* and were designated as "Pm-1, Pm-2, and Pm-3" (Figure 1, **2a**−**c**).[10] The structural difference between these tunichromes and those from *A. nigra* is that the "Pm" tunichromes are saturated at the C11−C12 bond.

Two tunichromes, Mm-1 and -2, have been isolated from the blood cells of the iron-accumulating stolidobranch ascidian *Molgula manhattensis*.[9] Also tripeptides, they differ from the An tunichromes by containing two DOPA derivatives and a glycine (Mm-1) (Figure 1, **3a**), or leucine (Mm-2) (Figure 1, **3b**), residue.

The halocyamines were isolated from acetone-extracted blood cells of the stolidobranch ascidian *Halocynthia roretzi*.[37] They are both tetrapeptide derivatives and contain DOPA. Halocyamine A (Figure 1, **4a**) consists of DOPA, histidine, glycine, and a bromotryptophan derivative, while in halocyamine B (Figure 1, **4b**) the histidine and glycine are replaced by threonine and histidine in the sequence, respectively. The compounds were found to have antimicrobial activity and were suggested to play roles in the defense mechanisms of *H. roretzi*. Preliminary investigations suggest that the halocyamines can reduce ferric iron to ferrous iron.[38]

An assay has been developed to screen ascidian blood for the presence of tunichromes.[39] These compounds have now been identified in blood cell extracts of numerous phlebobranchs but no other stolidobranchs.

B. Synthesis

The first total synthesis of a racemic tunichrome, the tunichrome An-1 from *A. nigra*, was described

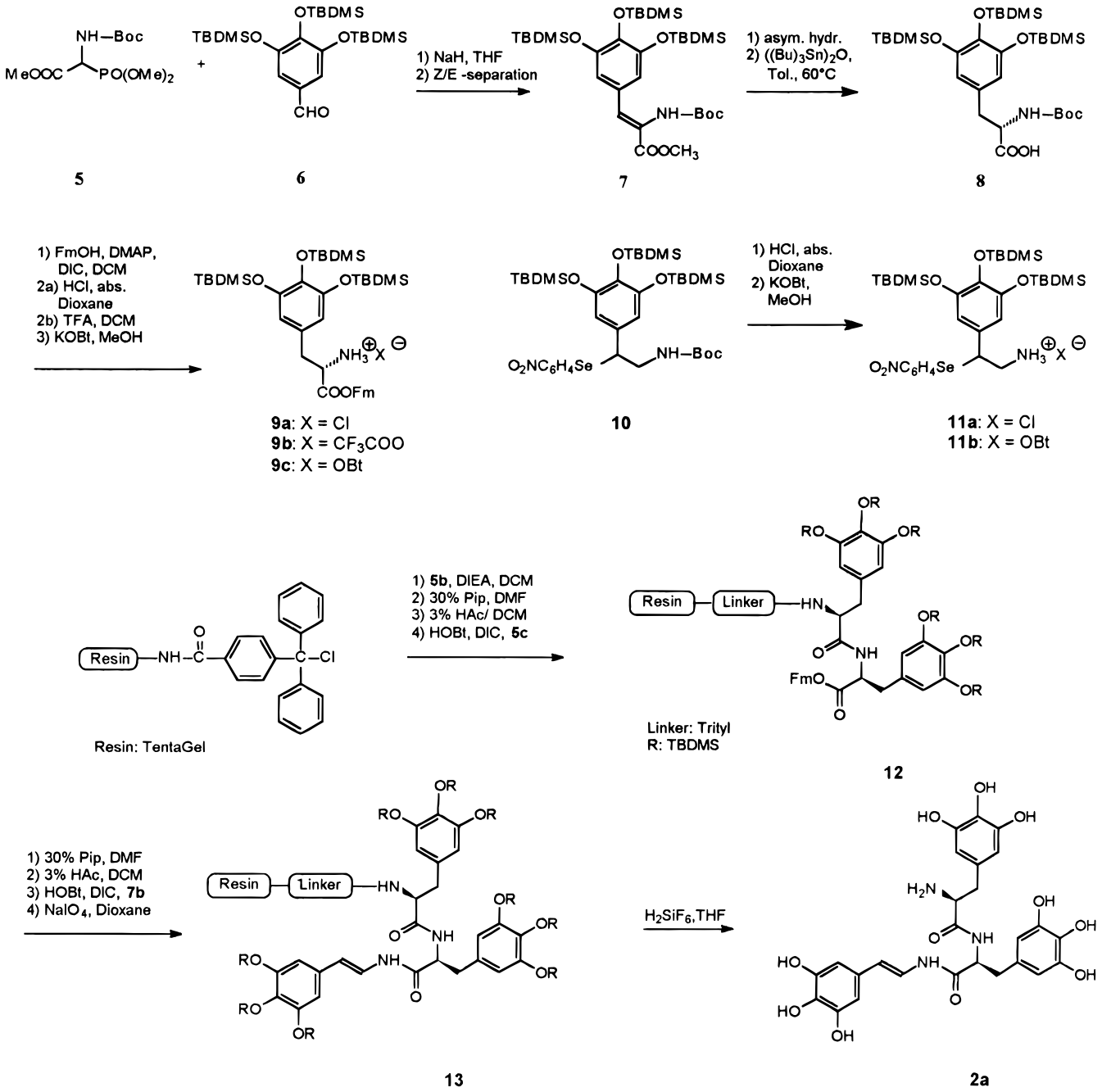

Figure 5. Synthetic route for tunichrome Pm-1.

by Horenstein and Nakanishi 1989 and has been reviewed elsewhere,[12,40] as has the synthesis of free Mm-1 and Mm-2 racemates.[12,41]

Recently, Pm-1 and Pm-2 from the *P. mammillata* tunichrome family were synthesized in our laboratory.[42] As for the An tunichromes, the method of U. Schmidt et al. to prepare dehydroamino acids by Horner−Emmons−Wittig condensation was employed.[43−45] Fully protected L-TOPA was produced following asymmetric hydrogenation using a Wilkinson-type homogenous catalyst (enantiomeric excess 97%). This is in contrast to the An synthetic strategy where the racemate was synthesized.[40] The synthetic route for the remainder of the tunichrome Pm-1 molecule utilized a solid-phase strategy.[42] Clearly, the usual synthesis in the C−N direction was not possible as there is no terminal carboxylate available for coupling to the linker. Therefore we employed an inverse synthetic strategy from N- to C-terminus[46] by using trihydroxyphenylalanine-fluorenenylmethyl ester with *tert*-butyldimethylsilyl protecting groups (Figure 5). Cleavage from the resin and complete deprotection was achieved in a one-step reaction using fluorosilic acid providing the desired (+)-tunichrome Pm-1.

V. DOPA and TOPA Proteins and Peptides as Potential Precursors for the Tunichromes and Halocyamines

Since the isolation of the tunichromes, another class of compounds has been identified in ascidian blood cell extracts with features that implicate them in having a role in metal sequestration and reduction. They are a series of higher molecular weight polypeptides and proteins that are related to the tunichromes by possessing DOPA and/or TOPA functionalities, and are isolated from the morula cells of diverse

species. DOPA proteins have been previously identified among invertebrates;[47] however, those from ascidian blood cells are the first examples among chordates and possess novel properties which, while having frustrated their total characterization, have given tantalizing new insights into a new class of proteins and particularly into the possibility that they may act as precursors to the tunichromes.

A. Isolation and Characterization

Ferreascidin was the first DOPA protein isolated from the blood cells of the stolidobranch ascidian *P. stolonifera*.[48] The isolation procedure is comparatively simple involving acetate extraction followed by chromatography on Sephadex G50 and then on a phenylboronate affinity column. It is a novel protein with a very high aromatic amino acid content, including 42% tyrosine and 17% DOPA. These amino acids also give the protein strong iron-binding capacity, from which its name derives.[49,50] Ferreascidin is basic, has a molecular weight of 10 000, and contains two glucose units as well as an uncharacterized oxygen-sensitive 360 nm chiral chromophore which gives the protein a yellow pigmentation.[48] Protein sequence information is largely lacking because of the protein's apparent lack of susceptibility to proteolytic enzymes. The protein is coisolated with an enzyme which catalyzes the oxidation of the DOPA moieties.[51] Together they are believed to make up a system important in the processes of test formation and adhesion.

A series of five DOPA-containing peptides were isolated from *H. roretzi* blood cells by gel filtration on Sephadex G-50 followed by HPLC on boronate-5PW and reversed-phase columns.[38] Two peptides consisted only of DOPA and another, as yet unidentified, amino acid. The other three contained DOPA, proline, phenylalanine, histidine, and arginine. Molecular weights were estimated to be less than 6500 by SDS−polyacrylamide gel electrophoresis however no sequence information was obtained.

Recently, using aqueous acetic acid urea extraction protocols, a series of DOPA- and TOPA-containing proteins and polypeptides were isolated from the blood cells of *Ascidia ceratodes* and *M. manhattensis*, sources of the An- and Mm-tunichromes, respectively.[36] The molecular weights of the two *M. manhattensis* proteins were measured by both MALDI and electrospray ionization mass spectrometry and gave $[M + H]^+$ peaks at *m/z* 9560 and a cluster of peaks at 10 836 and 10 740. They were basic, consisted largely of four amino acids: DOPA, Ala, Phe, and Val and gave identical N-terminal sequences, Ala-Phe-Tyr, before Edman degradation ceased. Proteolytic resistance hampered further sequence acquisition. The DOPA levels of these peptides were very high for a molecule of this size ranging from 25% to 39% of the amino acid composition. Intriguingly, the Tyr levels of the protein were very low which is surprising since this amino acid usually serves as a parent for DOPA which is derived as a posttranslational modification. Either conversion is extremely efficient in this system or the observation of relatively high concentrations of phenylalanine in the system lends itself to the speculative conclusion that peptidyl DOPA in this system may be derived from Phe (this is further discussed below).

Four novel polypeptides, or rather polypeptide families, were isolated from *A. ceratodes*.[36] Molecular masses were estimated by MALDI-MS to be between 3500 and 5300 Da and each contained high levels of TOPA and DOPA ranging from 15% to 36% and 7% to 20% of the total composition of amino acids, respectively. In fact, most of the composition of the peptides could be solely accounted for by these amino acids together with Ala and either Ser or Thr. A complementary relationship was observed between DOPA and TOPA contents across their elution profiles from HPLC on C-18, suggesting that the former could act as a precursor for the latter amino acid; however, no such relationship was observed between Tyr, or Phe, with DOPA. Each of the four peptides exhibited extensive microheterogeneity reflected by their large molecular weight distribution as determined by MALDI, the variation in the ratio of their 280 nm absorption band to chromophores of unknown structure (reminiscent of ferreascidin) and, in the case of two polypeptides, N-terminal sequence analysis which was determined to be predominantly X-TOPA-TOPA-TOPA-Ala-Ala-Ala-Ala for *Ascidia* blood cell polypeptide A (AbcpA) and X-TOPA-Ala-DOPA-Ala-Ala for AbcpB (where X = Asp or TOPA). All were resistant to proteolytic cleavage (like all other ascidian blood cell proteins and polypeptides yet examined) and no further sequence information could be obtained.

B. Relevance to the Biosynthetic Origins of the Tunichromes

Nothing is really known about the biosynthetic origins of the tunichromes. Either they represent the products of secondary metabolism and are assembled from the free amino acids or their synthesis is ribosomally controlled. In the latter case, the precursor peptides would be extensively posttranslationally modified after their synthesis to produce the tunichromes. These modifications could be either enzymatic or nonenzymatic. In this section we review available data and propose a speculative mechanism of how the DOPA/TOPA peptides may fulfill the the role of precursor peptides based on biomimetic chemistry and the isolation of putative intermediates.

He et al. studied the biosynthesis of tunichrome An-1 from *A. ceratodes* by examining the *in vivo* incorporation of [^{14}C]phenylalanine.[52] They observed appearance of the radiolabeled amino acid into newly sythesized tunichrome molecules after 1 day of exposure of the specimens to the amino acid in their surrounding seawater. Higher incorporation was observed after 20 days of exposure.

During the course of their investigations into the fate of peptidyl DOPA analogues upon oxidation, Rzepecki and co-workers observed an unusual oxidative tautomerization of *N*-acetyl-DOPA ethyl ester to the corresponding enamide (Figure 6).[53,54] By using NMR it was found that the dehydro-DOPA enamide adopted a z-configuration like the internal TOPA and DOPA residues of the An and Mm tunichromes.[55] The discovery of this biomimetic reaction led to the speculation that peptidyl DOPA sequences may

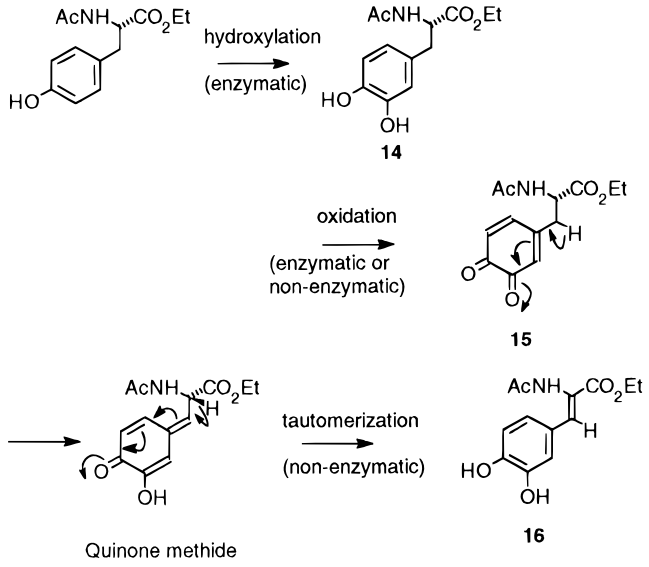

Figure 6. Formation of a peptidyl DOPA analogue **14** and oxidation to an orthoquinone **15** with subsequent tautomerization to *N*-acetyl-ΔDOPA ethyl ester **16** (from ref 55).

provide a source for the tunichromes. This observation gained further significance with the discovery of the Pm tunichromes which were proposed to potentially act as precursors for the An tunichromes by a similar reaction.[10]

The distribution of the DOPA polypeptides from *H. roretzi* was found to be identical to that of the halocyamines among sorted blood cell populations.[38] Although none of these peptides had amino acid compositions that suggested they might be precursors to the halocyamines, it was suggested that high molecular weight DOPA-containing precursors may exist in the morula cells in small amounts.

In contrast, all of the *A. ceratodes* polypeptides gave amino acid analyses strongly suggestive of poly-TOPA/DOPA sequences which was confirmed by N-terminal sequence analysis in the case of two of the peptides discussed above.[36] While these provide good candidates for tunichrome precursors not enough material has been isolated as yet to allow studies on the conditions that might be favorable the production of tunichromes from these peptides. In that regard, it is hoped that synthetic sequences of TOPA and DOPA prepared as described above may shed further light onto this question.

In recent developments, a 10 kDa DOPA protein called ferreascidin associated protein (FAP) has been purified from *P. stolonifera* which shows an N-terminal sequence with two aromatic regions separated by a potential protease cleavage site.[56] FAP is a minor component in the blood cell extracts and is proposed to act as a possible precursor to ferreascidin by being subsequently modified to produce the 360 nm chromophore.[56] A putative Pm tunichrome precursor has been identified in low concentrations in the form of the tripeptides TOPA-TOPA-DOPA by tandem HPLC−MS−MS of *P. mammillata* blood cell lysates.[57a] Most recently, a 3830 Da TOPA/DOPA polypeptide has been isolated from methanolic and acetic acid−urea extracts of *P. mammillata* blood cell lysates.[58] The other major amino acids are Ser, Leu, Phe, and Ala and the N-terminal tripeptide fragment has been isolated from chymotryptic digests and has been identified as 6-bromotryptophan-Leu-Phe.

On the basis of the cumulative data a putative scheme can be hypothesized for the biosynthesis of the tunichromes from polyPhe or polyTyr sequences in larger peptides (Figure 7). After hydroxylation to polyDOPA/TOPA sequences and subsequent cleavage from the parent peptide to produce DOPA/TOPA tripeptides, oxidative decarboxylation would yield tunichromes such as the Pm's which could be further oxidatively unsaturated to produce the An-1 tunichromes. For this scheme to be valid the following questions have to be answered. What enzymes or other factors (or perhaps vanadium) are involved in the proposed (1) hydroxylation of Tyr and Phe, (2) the cleavage of tripeptides from their postulated precursors, and (3) oxidative decarboxylation and unsaturation of DOPA or TOPA? While enzymatic and nonenzymatic precedents exist for most of these processes in natural and model systems[53−55,59−63] their relevance to the system in ascidian blood cells remains to be assessed.

C. Synthesis

To answer the above questions, peptides containing polyDOPA/TOPA sequences obviously would be of great value. Until recently, only a few peptides containing DOPA have been synthesized. The syntheses require protecting groups to protect the labile phenolic hydroxyl groups against oxidation, especially under basic conditions. The application of different acetyl- and carbamate-protecting groups has been described for the synthesis of different prodrug forms of L-DOPA.[64] The coupling of the protected DOPA residues was achieved with DIC in solution by conventional peptide synthesis. Free peptides were obtained by alkaline hydrolysis, under conditions where DOPA is susceptible to both oxidation and racemization.

H. Yamamoto and Hayakawa made the first concerted attempt to produce synthetic polypeptides containing DOPA as models for adhesive DOPA peptides from marine mollusks.[65−68] PolyDOPA, random and sequential copolypeptides of DOPA with glutamate and lysine, as well as synthetic polypeptides based on a tandemly repetitive decapeptide sequence in a DOPA protein isolated from the marine mussel *Mytilus edulis*,[69] were prepared. Their approach was to couple the amino acids in solution as Boc derivatives employing benzyl protecting groups to protect the hydroxyl functions of DOPA. Y. Yamamoto and co-workers described solid-phase synthesis of DOPA peptides utilizing Fmoc strategy.[70] DOPA was protected with phenylboronic acid and Fmoc chloride was introduced under basic conditions. Recently, (*tert*-butyldimethylsilyl)catechol-protecting groups were described for the synthesis of DOPA dipeptides in solution from DOPA, Boc-DOPA, and DOPA esters.[71] Optimal conditions for the deprotection with TFA, HCl, and $HClO_4$ in acetic acid were also presented.

No synthesis of polypeptides containing TOPA has been previously described to our knowledge. In our laboratory, we have synthesized some smaller

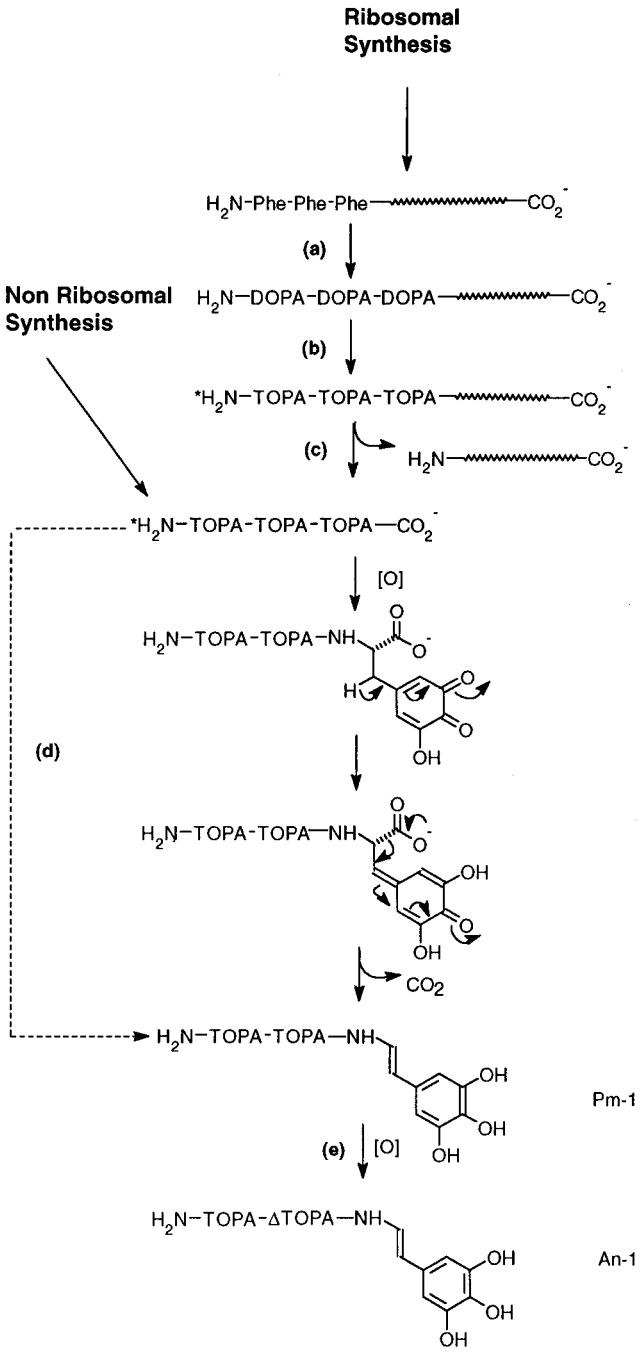

Figure 7. Hypothetical derivation of tunichromes Pm-1 and An-1 from polypeptide precursors (adapted from ref 55) (a) Hydroxylation 1 (phenylalanine 3,4-dihydroxylase??): He et al. (1992) found that *A. ceratodes* incorporated [^{14}C]phenylalanine into tunichome An-1.[52] (b) Hydroxylation 2 (5-DOPA hydroxylase??):[59,60] Taylor et al. (1995) found a complementary relationship between DOPA and TOPA compositions of HPLC fractions of various *A. ceratodes* blood cell polypeptides[36] (* indicates that hydroxylation is not always complete yielding also mixed DOPATOPA peptides). (c) Hydrolysis (enzymatic??): Polypeptides with polyTOPA/DOPA N-terminal sequences isolated from *A. ceratodes* blood cell extracts;[36] TOPA-TOPA-DOPA isolated from *P. mammillata* blood cell extracts.[57a] (d) Decarboxylation (enzymatic, e.g. TOPA decarboxylase + α,β-TOPAamine desaturase[61] or spontaneous, e.g. via the mechanism for oxidative decarboxylation of 3,4-dihydroxymandelic acid).[62,63] (e) Oxidative tautomerization (see Figure 6).

peptides containing TOPA using Fmoc strategy on solid phase.[57b] More work in this field is in progress.

VI. Biological Roles for the Tunichromes and Related Compounds

While no definitive biological role for the tunichromes, halocyamines, and DOPA and TOPA polypeptides and proteins is known with certainty, their cellular distribution, structural characteristics and chemical reactivity give insights into their possible physiological functions. In this section, we briefly review evidence for the existence of tunichromes in ascidian blood cells, give a new assessment of evidence for their proposed role in vanadium accumulation, reduction and storage. Literature exploring possible roles for the halocyamines and ferreascidin is reviewed and, finally, the formation of a hemostat when the different components mix at the site of vascular injury is discussed based on physical, chemical, and cytochemical studies on ascidian blood cells and their lysates.

A. Evidence for the Tunichromes in Ascidian Blood Cells

An area of controversy recently raised is evidence for the existence of tunichromes in ascidian blood cells. The major observations that have been reported to prove the existence of the An tunichromes in intact blood cells have been that the tunichromes are (1) yellow (λ_{max} 340 nm in methanol[8]) and (2) fluorescent, as are the morula cells when observed by light and fluorescence microscopy.[11] The Pm tunichromes are colorless (λ_{max} 301 nm in methanol[10]) and reside in nonfluorescent,[72] faint yellow-green morula cells.[73]

Early attempts that were made to spectroscopically correlate the properties of An-1 to those of intact blood cells have recently come into question. Cell spectra obtained from *A. nigra* blood cells suspended in glycerol have been reported to show a 335 nm maximum, characteristic of tunichrome An-1 in acid.[74] In a recent study the 335 nm absorption band in the blood cell spectra of *A. ceratodes* and *Phallusia julinea* could only be observed on deliberate cell lysis followed by high dilution.[75] Intact blood cells only showed characteristic vanadium chromophores consistent with previous studies.[18,29] Spectrofluorometric emission peaks observed in *Ascidia callosa* blood cells fixed in 2.5% glutaraldehyde were found to correspond to those of purified tunichrome in 0.1 M HCl.[76] However, it was subsequently found that the fluorescence spectra could be reproduced by a 0.1 M HCl solution alone on excitation at the same wavelength.[75] In that regard, a convincing fluorescence emission spectrum of tunichrome An-1 is yet to be reported even though the purified solid gives a pumpkin yellow fluorescence under UV radiation.[8] This reflects the sensitivity of the fluorescence to the environment. This, coupled with the fact that non-tunichrome-containing blood cells of other species of ascidian contain fluorescing components, suggests that caution should be exercised when drawing conclusions about the presence or absence of tunichromes in ascidian blood cells from fluorescence observations alone.

More direct evidence for Pm-1 in lyophilized *P. mammillata* blood cells was obtained by solid-state NMR, with good correlation between ^{13}C signals from

a purified Pm-1 sample and the cells.[10] Immunoelectronmicroscopic investigations hold promise for detecting tunichromes and DOPA/TOPA polypeptides in blood cell sections as a reflection of the environment *in vivo*.

B. The Tunichromes and Vanadium Chemistry

The tunichromes and other DOPA- and TOPA-containing molecules, at first sight, appeared to be the key to answering the question of vanadium accumulation and reduction in ascidian blood cells. But, despite their structural features being strongly indicative of such a role, little supporting evidence has been forthcoming since their discovery. Here we attempt to complement the previous review on tunichrome−vanadium complexation chemistry[11] by including material omitted from that review, or very recently published, on the biological relevance of tunichrome−vanadium interactions, measurements on the ability of tunichromes to reduce vanadium to the trivalent oxidation state and proposals of how tunichrome may be involved in vanadium accumulation.

Tunichromes from synthetic and natural sources generally form intractable green or blue violet precipitates when reacted with vanadium, restricting attempts at characterizing discrete tunichrome−vanadium complexes.[9,77,78] More success has been achieved with model compounds having pyrogallol and catechol ligands.[79,80] These studies were recently reviewed[11] and will not be reiterated here. It is interesting, however, to compare early results of Oltz et al. for vanadium complexes of An-1[9] with those of Schiefer for Pm-1 tunichrome complexes.[78] Job analysis of the vanadium−An-1 complex revealed a stoichiometry of 2 to 2.5 An-1 molecules per vanadium atom, suggesting that, even though there are six phenolic oxygens available for totally intramolecular coordination of vanadium(III), intermolecular coordination is favored.[9] This is most probably the result of the rigid internal stryamide bond. However, this bond is absent from Pm-1 and as a result there are less steric constraints. Accordingly, Schiefer found a stoichiometry of 1−2 Pm-1 molecules per vanadium atom by Job analysis.[78] Force field calculations (MMX)[81] revealed that, in contrast to tunichrome An-1, totally intramolecular coordination of vanadium(III) sulfate is possible (Figure 8).[78]

Evidence for tunichrome−vanadium complexes, such as described above, in ascidian blood cells has not been forthcoming. In fact, investigations into the intracellular form of vanadium(III) in ascidian blood cells is an area fraught with controversy. Two major theories have prominence (1) that vanadium(III) is stabilized by a strongly acidic intracellular mileu[82−86] or (2) that vanadium(III) is stabilized by chelation to an endogenous ligand.[87−90] The TOPA/DOPA-containing tunichromes and polypeptides would provide good candidates for such ligands, but as stated above, they reside in the morula cells which appear not to contain as much vanadium as the vanadium-rich compartment and signet ring cells. The reader is directed to a recent review which summarizes the experimental basis for those viewpoints.[11]

Alternative hypotheses have been also been offered. Michibata proposes that vanadium is bound by

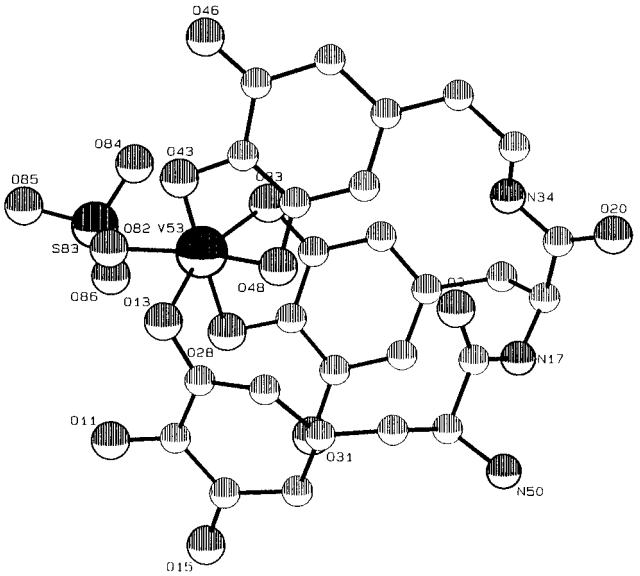

Figure 8. Structure for a putative Pm-1 vanadium(III) sulfate complex using force field calculations showing that totally intramolecular coordination is possible (from ref 78).

vanadium-binding proteins in the signet ring cells and vacuolated amoebocytes.[91] Meier et al. proposed an equilibrium in ascidian blood cells between monomeric, oligomeric, and membrane-bound vanadium(III) based on speciation studies by spectrophotometric titrations.[92] Details for both proposals can be found in the respective reviews. A very recent report on the magnetic properties of ascidian blood cells using a superconducting quantum interference device (SQUID) suggested that the predominant form of vanadium(III) storage was as a ferromagnetically coupled dimer with a putative unprotonated μ-oxo bridge.[93] Combined, these studies provide confusing, and sometimes contradictory, models for the form vanadium in ascidian blood cells. However, all studies share a common element in that none yield evidence of tunichrome complexation of vanadium. This was demonstrated in the case of tunichrome Pm-1 by the lack of evidence for vanadium complexation in the solid state NMR of lyophilized *P. mammillata* blood cells.[10]

A recent cytochemical investigation yielded yet a different picture for the intracellular environment and should be noted as it attempts to localize vanadium, tunichrome, and related substances in ascidian blood cells by employing a variety of staining reagents.[32] This was complemented with analytical electron microscopy which gave an indication of the distribution of vanadium, oxygen, and sulfur. Air-dried or cyrofixed smears of *Phallusia fumigata*, *P. mammillata*, and *Ascidia sydneiensis* blood cells showed increasing levels of vanadium in going from the signet ring to the compartment cells to the morula cells, respectively. This is in contrast to the other investigations.[22−31] High levels of sulfur were identified in all cell types. The authors propose thiolate coordination of vanadium in the signet ring and compartment cells and storage of vanadium as an oxo bridged polymeric complex in the morula cells, physically separated from the tunichromes. While the histological approaches merit consideration, caution should be exercised in the overinterpretation of data based on cytochemical staining which is very prone to giving false negatives and positives, espe-

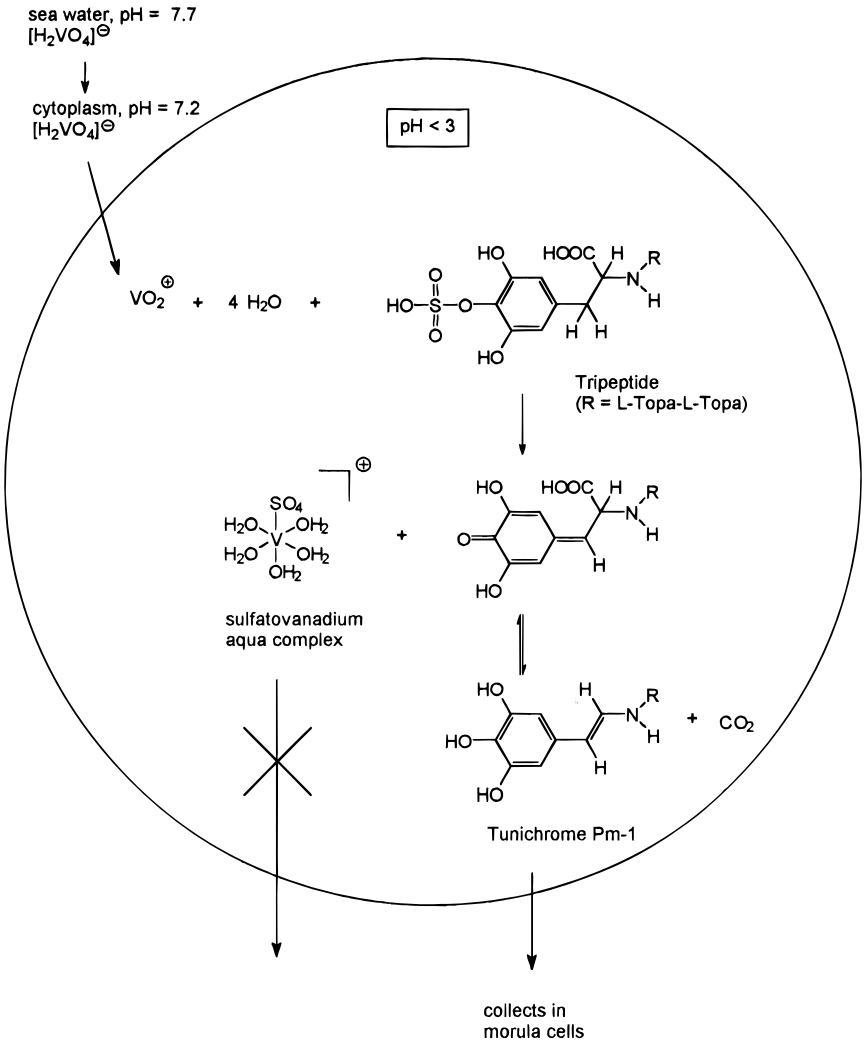

Figure 9. Proposed scheme for vanadium accumulation in the blood cells of *P. mammillata*. (Reprinted with permission from ref 10. Copyright VCH Verlagsgesellschaft mbH)

cially in biological tissue as unusual as ascidian blood cells. In addition, it is not clear to which blood cells the analyses refer as corresponding micrographs are not supplied.

Despite the lack of evidence for tunichrome complexation in the morula cells, investigators have still proposed that tunichrome may be instrumental in vanadium accumulation in the signet ring cells and other vanadocytes. In the last comprehensive review of tunichrome chemistry a "vanadium-trapping" model was proposed for tunichrome's role in vanadium accumulation.[12] The reader is directed to that review for further details.[12] This model was refined by Bayer et al. who observed (1) no evidence for the presence of tunichrome-complexed vanadium(III) by solid-state NMR and (2) a putative sulfoconjugate of tunichrome Pm-1 by MS of a crude methanolic extract.[10] A scheme was proposed where vanadate in an acidic intravacuolar environment acts as a two-electron acceptor and induces oxidation of a sulfoconjugate of a putative tunichrome Pm-1 tripeptide precursor to the corresponding quinone methide with transfer of the sulfate group to aquated vanadium(III) (Figure 9). The quinone methide was then suggested to undergo an oxidative decarboxylation to form tunichrome Pm-1. The resultant cationic sulfatovanadium complex cannot pass through the anionic channels while the tunichrome Pm-1 can leave the cell (or subcellular compartment?) via peptide transport mechanisms and accumulate in the morula cells.

However, the ability of any of the tunichromes to reduce vanadate from seawater down to the trivalent state remains to be proven. A report that tunichrome Mm-1 had the ability to reduce vanadium(V) to vanadium(III)[77] was very recently refuted by the same laboratory[94] and tunichrome An-1 generated mainly vanadium(IV) complexes in neutral to moderately basic aqueous media.[95]

C. Biological Roles for the Halocyamines and Ferreascidin

The halocyamines have been found to be localized in one specific blood cell type in *H. roretzi* by cell sorting using density gradient centrifugation.[96] This cell type has a "morula-like" vaculoated appearence and was found to also contain a hemagglutinin.[34] In cellular defense mechanisms, these vacuolated cells have been suggested to act together with other blood cell types which have protease activity.[96] The halocyamines role has been proposed to be antimicrobial. They have been shown that they have the ability to inhibit the growth of certain Gram-positive bacteria, fungi, and marine bacteria as well as some fish RNA viruses.[37,97] The molecular basis for the halo-

cyamines' biological activity, a property not as yet demonstrated to be shared by any of the tunichromes, is unknown. The presence of a bromine atom, as observed in the biologically active "eudistomins" from another ascidian species,[98,99] may be significant.[37]

Ferreascidin was discovered in the search for iron-accumulating agents from *P. stolonifera* blood cells. It was subsequently found to form strong complexes with iron(III) at pH 7.0 *in vitro*, adopting a predominantly biscatecholato coordination mode through the DOPA residues.[49] Later EPR and Mössbauer investigations revealed that ferreascidin bound iron(III) as novel binuclear and trinuclear antiferromagetically coupled clusters, either added directly to the protein or formed after the protein bound mononuclear iron.[50] In the latter case it is interesting to speculate that ferreascidin may act as a nucleating agent for ferritin-like iron oxide deposits. However, the morula cells, designated as "ferrocytes" by Endean,[17] have not been found to contain high concentrations of iron by analytical electron microscopy[100] and do not have the blue or purple appearence associated with ferric complexes at the slightly acidic or neutral pH values reported to exists *in vivo*.[88] Instead, the biological significance of ferric ion−ferreascidin interactions was suggested to be post-cellular lysis in the process of wound repair.[49,50] Furthermore, this may prove to be a common theme for the interaction of tunichrome and all related compounds with metal ions as discussed in the next section.

D. Reactions upon Cellular Lysis and Their Relevance to Wound Repair and Tunic Formation

The process of ascidian blood cell lysis has intrigued investigators since Henze's initial discovery. When blood cells from vanadium-accumulating ascidians release their contents a host of reactive possibilities avail. The characterization of the tunichromes and related compounds allows some conjecture as to the physiological implications of their concomitant release with vanadium, sulfate, and acid, particularly to the mechanism of wound repair. Cellular lysis in water was initially observed by Henze and has been extensively studied. The so-called "Henze solution" is a red-brown acidic solution that turns blue on standing in air. Its primary component was suggested to be a pigment called "hemovanadin", initially thought to be involved respiration, but later found to be a complex of a nitrogenous organic component (Pm tunichrome?), a protein, vanadium, and sulfate held together by pH-dependent interactions.[73] For a summary of the early literature the reader is directed to another review.[16]

The reactions that occur upon lysis of *A. ceratodes* and *P. julinea* blood cells have been studied by EPR spectroscopy.[101] Although most of the vanadium in ascidian blood cells exists as the trivalent ion, a small percentage is present in the EPR-active +4 state. This vanadium(IV) may represent either (1) an intermediate in the reduction of vanadium(V) from seawater or (2) a small amount of oxidized vanadium(III). Alternatively, the coordination of vanadium(IV) may not reflect the resting state of the bulk of the vanadium(III) and caution should be borne in mind when drawing conclusions based on the EPR measurements alone. Nevertheless, vanadium(IV) EPR provides the most convenient spectroscopic probe and thus a basis for a survey of inter- (and intra-) species variation in vanadium coordination by comparing data accumulated from many research groups around the world.

In keeping with previous investigations,[85] whole blood from individual *A. ceratodes* specimens was found to give EPR spectra characteristic of aquated vanadyl ion, however the signals were much broader than those reported earlier until the cells were lysed.[101,102] It was proposed that the EPR signal observed from *A. ceratodes* whole blood has its origin in a small proportion of vanadium from lysed cells which is in an environment which gives broad signals (i.e. higher pH, low sulfate).[101] When the blood cells lyse they release acid and sulfate resulting in a narrowing of the signals. Blood from *P. julinea* specimens gave EPR parameters indicative of a weakly coordinated vanadyl ion.

A comparison was made with EPR data obtained from tissue samples prepared from whole ascidians in specially designed tubes.[28] EPR signals can be attributed to the blood cells suspended in the tissue because the amount of vanadium in tissue is negligible compared with that in the isolated blood cells.[16] It was found that tissue samples gave EPR spectra characteristic of complexed vanadium(IV) while blood samples gave EPR parameters progressively reflecting a weaker coordination sphere. An examination was made of the EPR data for all ascidians and relevant model complexes reported in the literature for blood and tissue samples. Tissue samples were proposed to provide a better reflection of the resting state of vanadium in living blood cells than packed cells or whole blood. In whole blood there is less physiological material to provide a pH buffering effect against blood cell lysis. In packed cells from many individual ascidian specimens there is a strong possibility of allogenic interactions such as recently observed with *H. roretzi* blood cells which degranulate[37] and release phenoloxidase on mixing.[103]

A scheme was proposed for the processes involved in cellular lysis based on available EPR. *In vitro* vanadium released from lysed cells may undergo a series of chelate exchange reactions each involving a release of protons and an accumulation of acid resulting in progressively weaker vanadium coordination. In water or at neutral pH, this soup is the red-brown "Henze solution" (see above), which has a maximum in its visible spectrum at 425 nm[73] which has good correspondence to EPR silent vanadium(III) hydrolysis products such as $(VOV)^{4+}$.[104]

Recently, intact and lysed cells from *A. ceratodes* were examined by low temperature vanadium K-edge X-ray adsorption spectroscopy (XAS) and EXAFS. Both techniques have the advantage that they directly measure vanadium(III).[86] Packed cells were reported to give XAS spectra very similar to that of vanadium(III) in aqueous sulfuric acid solution. EXAFS data for both the intact and lysed cells gave no evidence for coordination by an endogenous chelate or $(VOV)^{4+}$ dimers. Since the integrity of the cells seems to have been carefully monitored, it appears that the population of *A. ceratodes* from Monterey Bay, CA, examined in that investigation contain

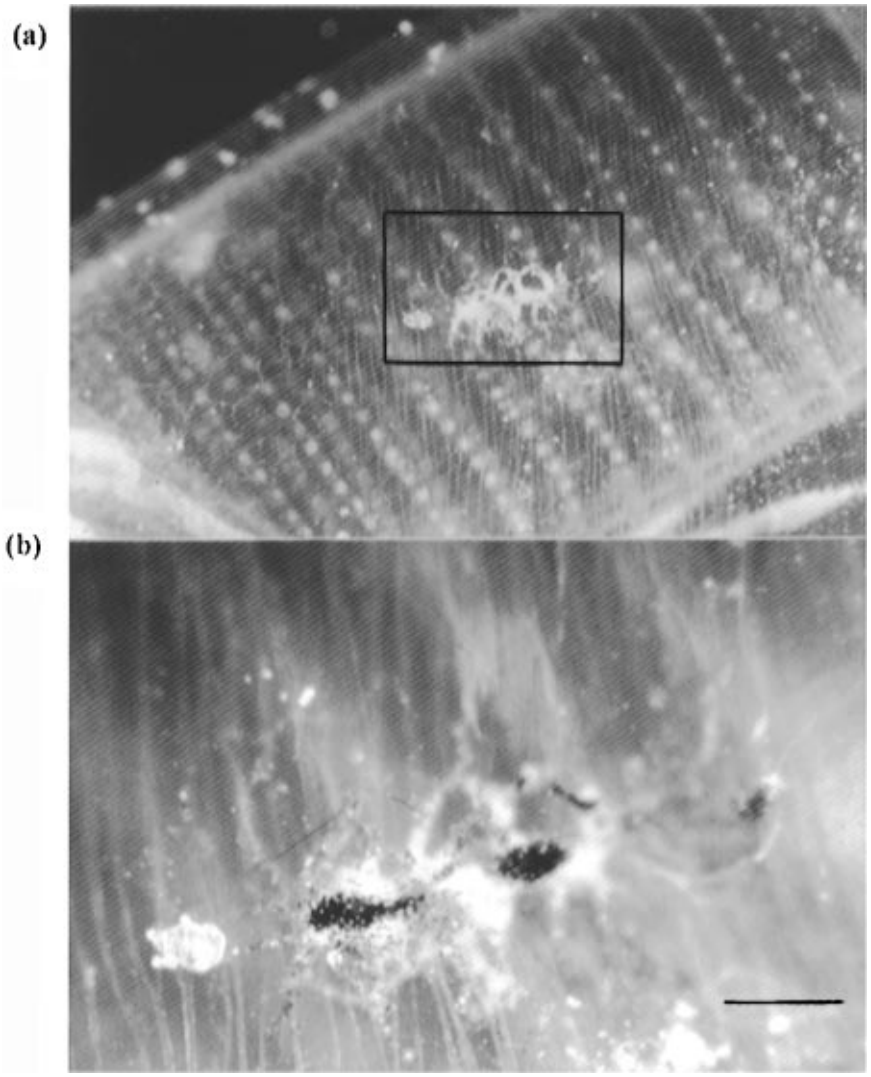

Figure 10. Wound repair in *E. diaphanis*: (a) blood cells aggregate at a wounded area of the tunic of the individual shown in Figure 2 and (b) expanded view of injury site (boxed area in a) after staining with 2,2′-bipyridine for vanadium-(III) (scale bar 0.2 mm).

blood cells with aquated vanadium(III) as the dominant form with a large reservoir of endogenous acid. However, this is apparently in conflict with both the blood cell spectra studies on *A. ceratodes* specimens collected from Bodega Bay, CA[29] (on which the EPR studies described above were also performed) and also the very recent investigation on *A. ceratodes* (also collected at Monterey Bay) by SQUID which showed the $(VOV)^{4+}$ dimer to be the predominant form of vanadium.[93] Thus it remains to be seen whether any one general scheme can be applied to all ascidians (even within a single species under different geographical and seasonal influences).

Regardless of the source of the acidity, when lysis occurs at the site of vascular injury, the acid can diffuse away and tunichrome and DOPA/TOPA peptides are exposed to the slightly basic pH of seawater. Under these conditions, the catechol and pyrogallol moieties of the DOPA and TOPA residues, respectively, can oxidize leading to a host of cross-linking reactions via the quinone-tanning reactions proposed by Waite for peptidyl DOPA.[47] However, very recent studies by Waite and co-workers on marine mussel byssal threads, thought to be a classical example of an extracellular, DOPA protein, quinone-tanned matrix in seawater, failed to identify any of the cross-links previously proposed such as Michael adducts[105] or diphenyl-linked aromatic rings by solid-state NMR of labeled byssus.[106] Instead, DOPA was suggested to exert a stabilizing effect through multiple noncovalent, pH, and redox-sensitive interactions.[106] Similarly, strong noncovalent cross-links may predominate in the hemostat of vanadium-accumulating ascidians. The DOPA and TOPA residues of the tunichromes and polypeptides, released from the morula cells on cellular lysis, may stabilize the hemostat by chelation to both the vanadium(III) concomitantly released from signet ring and compartment cells, and higher valencies of vanadium produced after oxidation.

The potential relevance of this chemistry to the process of wound repair is demonstrated by the phlebobranch ascidian *Ecteinascidia diaphanis*. This small ascidian has a delicate, translucent tunic and its response to a small injury is readily observed under a dissecting microscope (Figure 10). Furthermore, staining with 2,2′-bipyridine[28] allows visualization of the distribution of vanadium(III) in the hemostat. At the site of injury aggregation of many blood cells is observed. Although the predominant cell type in these aggregates is the morula cell, many cells stained purple with 2,2′-bipyridine-indicating

the presence of large amounts of vanadium(III). The absence of plasma-clotting factors in ascidian blood necessitates coagulation to maintain hemostasis;[33] however, the mechanism of blood cell coagulation remains unknown. Hecht showed that blood cell coagulation was triggered on contact with seawater.[107] In vanadium-accumulating phlebobranch ascidians, tunichrome−vanadium interactions may play an important role in a primitive clotting mechanism.

Covalent and/or noncovalent interactions of the tunichromes with the cellulose component of tunicin may also be important in wound repair. Additionally, they may play a role in tunic formation, as suggested by preliminary solid-state NMR results.[108] The morphology of the tunic of *P. mammillata* was described in detail in early work by Endean.[109] "Vanadocytes" (later identified as morula cells and lacking large quantities of vanadium but the source of large quantities of tunichrome) where observed releasing intraglobular material in the tunic matrix producing fiber-like processes which span areas devoid of tunic substance. These microfibrils may consist of polymerized tunichrome and were proposed to act as frameworks for cellulose synthesis. Kaneko et al. recently studied the distribution of different blood cell types in the tissues of another phlebobranch ascidian, *Ascidia sydneiensis samea*.[110] Vanadium-containing signet ring cells were immunochemically localized around the alimentary canal while autonomously fluorescing morula cells localized beneath the epidermis of the mantle, ideally situated for tunic formation.

Ascidian blood cells of several different non-vanadium-accumulating species were recently found to contain proteases while vanadium-containing *P. mammillata* blood cell extracts did not.[111] However, when a pure population morula cells from *P. mammillata* was extracted, protease activity was observed.[112] Since vanadium appeared to have an inhibitory effect on these proteases it was suggested that the metal may be important in regulating their activity. These interactions may have relevance to the mechanism of coagulation but the significance is not yet clear. Further studies are anticipated to see whether putative DOPA and TOPA polypeptide precursors to the tunichromes are acted upon by these enzymes.

With our current knowledge of ascidian blood cell components, the hemostat of phlebobranch ascidians can be envisaged to be made up of a cross-linked tunichrome, DOPA/TOPA polypeptide matrix interspersed with large quantities of chelated vanadium with possible covalent or noncovalent cross-links to the tunicin matrix material of the tunic, thus forming an intractable and resilient protective barrier during the period required for tunic regeneration.

VII. Conclusion

In the last five years two seemingly separate foci of research into ascidian blood cell chemistry, the tunichromes and DOPA proteins, have started to merge. Their relationship, although tenuous at this early stage, is gathering more substance. Synthesis of both tunichromes and DOPA/TOPA peptides will undoubtedly prove invaluable in determining whether such a link exists. The role vanadium may play holds promise for discovery of new chemical and biological functions. It is hoped that the current review will stimulate research in these aspects of ascidian blood cell chemistry.

VIII. Abbreviations

Boc	*N*-*tert*-butoxycarbonyl
ΔDOPA	dehydroDOPA
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DCM	dichloromethane
DIC	*N*,*N*′-diisopropylcarbodiimide
DIEA	*N*,*N*′-diisopropylethylamine
DMAP	4-dimethylaminopyridine
EtOAc	ethyl acetate
EPR	electron paramagnetic resonance
Fm	9-fluorenylmethyl
Fmoc	[(9-fluorenylmethyl)oxy]carbonyl
HOBt	1-hydroxybenzotriazole
KOBt	potassium 1-hydroxybenzotriazoate
MeOH	methanol
Pip	piperidine
TBDMS	*tert*-butyldimethylsilyl
TFA	trifluoroacetic acid
ΔTOPA	dehydroTOPA

IX. Acknowledgments

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