3.09. Found: C, 41.05; H, 3.54; N, 3.02. Resonances for CH_3OH and $CHCl_3$ appear in the ^1H NMR spectrum.

The free amine was prepared by stirring the amine salt with 100 mL of CH_2Cl_2 and 100 mL of 10% aqueous K_2CO_3 for 2 h. The organic phase was separated, washed with water, and dried over Na_2SO_4. Evaporation of the solvent left 2.45 g (67%) of a white powder: mp ca. 150 °C soften; ^1H NMR ($CDCl_3$) δ 7.73 (s, 16, BrArH), 6.40 (s, 8, Ar H), 3.77 and 2.47 (pair d, 8, $ArCH_2Ar$), 2.69 (t, 8, $ArCH_2C$), 2.39 (t, 8, CH_2N), 2.0-1.8 (br s, 8, NH_2); ^{13}C NMR ($CDCl_3$) δ 142.9, 137.7, 134.9, 134.0, 132.3, 130.7, and 129.2 (Ar), 43.3, 39.1, and 31.1 ($ArCH_2CH_2N$ and $ArCH_2Ar$). Anal. Calcd for $C_{60}H_{56}N_4O_{12}S_4Br_4 \cdot CH_2Cl_2$: C, 47.01; H, 3.73; N, 3.59. Found: C, 47.37; H, 3.58; N, 3.52. The resonance for CH_2Cl_2 was observed in the ^1H NMR spectrum.

4-[(Dimethylamino)methyl]-2,6-dimethylphenol (9) was prepared from 2.44 g (0.02 mol) of 2,6-dimethylphenol, 3.38 g (0.03 mol) of 40% aqueous dimethylamine, 2.43 g (0.03 mol) of 37% aqueous HCHO, and 2 mL of acetic acid and was obtained in 86% yield as colorless crystals after recrystallization from hexane: mp 116-117 °C (lit.[35] mp 120-122 °C); ^1H NMR ($CDCl_3$) δ 6.87 (s, 2, Ar H), 6.0 (br s, 1, OH), 3.31 (s, 2, $ArCH_2N$), 2.22 and 2.18 (2 s, 12, NCH_3 and $ArCH_3$); ^{13}C NMR ($CDCl_3$) δ 151.7, 129.6, and 123.6 (Ar), 63.8 and 45.1 ($ArCH_2N$ and NCH_3), 16.1 ($ArCH_3$).

Palladium Complex of 11. A 0.259-g sample of $[Pd(CH_3CN)_2Cl_2]$ (0.001 mol) was dissolved in 20 mL of warm CH_3CN, 0.415 g (0.002 mol) of $AgClO_4$ in 5 mL of CH_3CN was added, and the mixture was stirred at room temperature for 1 h. The AgCl was removed by filtration, and the filtrate was added to 1.47 g (0.001 mol) of **11** in 30 mL of THF with stirring. After 2 h a copious white precipitate had formed, which was collected by filtration, triturated with 20 mL of THF, and dried under vacuum for 5 days at 69 °C to give 1.12 g (71%) of a gray powder: mp ca. 209 °C dec; IR (KBr) 3550 and 3280 (NH), 1380 and 1190 cm^{-1} (SO_2); ^1H NMR (DMSO-d_6) δ 7.94 and 7.58 (pair d, 16, BrArH), 7.27 and 6.94 (2 s, 8, Ar H), 3.80, 3.71, 2.80, and 2.65 (two pair d, 8, $ArCH_2Ar$), 3.04 (br s, 16, $ArCH_2CH_2Ar$), 5.00 and 2.21 (2 br s, 6, NH and NH_2). Anal. Calcd for $C_{60}H_{54}N_4S_4Br_4Pd$: C, 45.69; H, 3.43; N, 3.55. Found: C, 45.01; H, 3.38; N, 3.21.

6-Cetyl-1,4,8,11-tetraazaundecane was prepared as described in the literature[36] and obtained as a waxy solid; mp 98-100 °C (lit.[36] mp 98-100 °C).

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The Tunichromes. A Class of Reducing Blood Pigments from Sea Squirts: Isolation, Structures, and Vanadium Chemistry

Eugene M. Oltz,[†1] Reimar C. Bruening,[†2] Mitchell J. Smith,[†3] Kenneth Kustin,[‡] and Koji Nakanishi*[†]

Contribution from the Departments of Chemistry, Columbia University, New York, New York 10027, and Brandeis University, Waltham, Massachusetts 02254.
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Abstract: Marine tunicates ("sea squirts") display a remarkable propensity to sequester and reduce vanadium (or iron) in specialized blood cells termed vanadocytes (or ferrocytes). Characterization of the reducing blood pigments designated as tunichromes (TC's) suggested a plausible mechanism for accomplishing this. TC refers to a class of hydroxy-Dopa-containing peptides whose purification entailed several unusual chromatographic techniques, all performed anaerobically. The first TC characterized from *Ascidia nigra* (*An*-1) is one such modified tripeptide (**1a**).[4] The structural elucidation of two other major TCs from *Ascidia nigra* (*An*-2 and *An*-3), as well as two additional TC's from an iron-sequestering tunicate, *Molgula manhattensis* (*Mm*-1 and *Mm*-2), is reported here. Aqueous *An*/V complexation reactions exhibited a preferred stoichiometry of 2-3:1. Moreover, *A. nigra* blood cells afforded a green fraction possessing the spectroscopic features of an *An*/V complex. These and other findings regarding tunichrome-vanadium complexation chemistry are presented.

Organisms possess a variety of mechanisms for sequestering metal ions. One such metal, vanadium, displays a wide spectrum of biochemical properties.[5] Animal feeding studies suggest that vanadium may be essential for normal mammalian growth and development, yet, this conclusion awaits definitive verification,[6] and a physiological role in humans has not been established. To date, only two low molecular weight vanadium complexes have been isolated from natural sources;[7,8] both are from fungi and are unlikely to reflect vanadium interactions present in mammalian systems.[7-10] With the discoveries that vanadium is found at the active sites of an alternative nitrogenase from the Gram-negative bacterium *Azotobacter chroococcum*[9] and a bromoperoxidase from the marine algae *Ascophyllum nodosum*,[11] true biological roles for vanadium are now manifest.

In the animal kingdom, certain species of the marine organisms known as tunicates (phylum *chordata*) accumulate vanadium to 0.15 M, a level 10^7-fold greater than that present in sea water.[12] Other species sequester iron specifically. A widespread view is

[†] Columbia University.
[‡] Brandeis University.

(1) 1985-1986 Eli Lilly/American Chemical Society Fellow.
(2) Present address: Department of Chemistry, University of Hawaii at Manoa, 2545 The Mall, Honolulu, HI 96822.
(3) NIH Postdoctoral Fellow.
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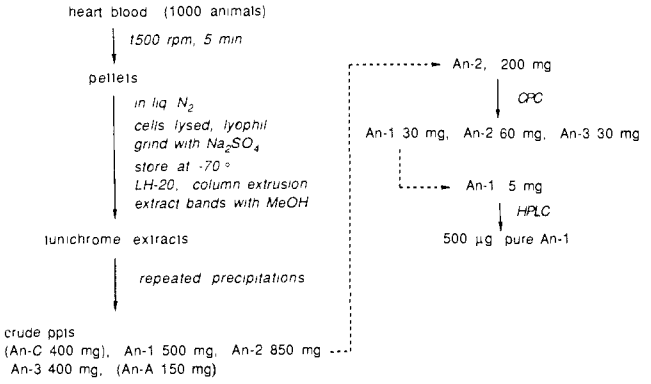


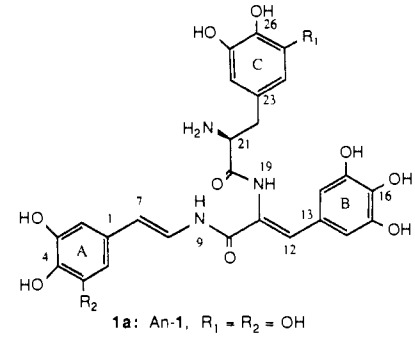
Figure 1. Tunichrome isolation scheme (entire process under Ar).

that vanadium is sequestered in electron-dense vacuoles, termed vanadophores, of the berry-shaped morula cells, primarily as the oxidation sensitive V(III) state.[13-19] Its function remains unknown but does not involve O_2 transport.[20] In the 1970s it was reported that a yellow chromogen, termed tunichrome, was present in high concentrations in the blood cells of *Ascidia nigra*, a vanadium collector. The pigment was a potent reductant, reducing V(V) to V(IV) and Fe(III) to Fe(II) in vitro.[12,21,22] Consequently, tunichrome was incorporated into a model for vanadium sequestration,[22] and attempts to isolate it were initiated.

An-1.[4,23] Early attempts by us and others to isolate a pure, characterizable tunichrome (TC) were not successful.[12,22,24] Our initial endeavors suggested that (1) TC is oxidized rapidly, (2) aqueous purifications give poor yields, (3) TC is unstable to mild alkaline conditions, and (4) TC adheres tenaciously to most solid-phase packing materials such as silica, alumina, cellulose, reversed-phase (RP), and lipophilic matrices. Initially, isolating derivatized TC was avoided in order to study its coordination chemistry. A second reason was concern over artifact generation during derivatization. TC detection was initially based on its yellow color.

All steps involved in the isolation[23] of *An*-1 (Figure 1) were performed under an atmosphere of dry, prepurified argon, using degassed solvents containing either methyl *tert*-butylhydroxyphenyl sulfide (MBHPS)[25] or the less efficient but volatile *tert*-butyl sulfide (*t*-BS) as an antioxidant. Specimens of *A. nigra* were collected off the coast of Key Biscayne, FL, flown to New York City (in lots of 1000–2000), and immediately processed. Uncontaminated blood was obtained from healthy individuals (as determined by olfactory inspection) by shaving the tunic, thereby exposing the heart for puncture. The bright yellow/green blood was drained and centrifuged. Blood pellets were lysed by immersion in liquid nitrogen and lyophilized to dryness. This protocol avoided the pitfall of exposing TC to an aqueous solution of lysed cellular components, including vanadium. The lyophilized blood pellet was subsequently ground with an equal amount of sodium sulfate, to facilitate grinding and serve as dehydrant, yielding a free-flowing green powder, which could be stored under argon at −70 °C.[26]

The second purification step involved extracting the blood powder, loaded directly onto a Sephadex LH-20 column, with solvents of increasing polarity (containing 0.2% MBHPS). By this procedure numerous bright fluorescent bands (see photographs in ref 23) were eluted; these were followed by three TC zones, thus providing the first indication that "tunichrome" was a mixture of closely related compounds. Complete elution of the TC's with ethanol (EtOH)/methanol (MeOH) mixtures was a slow process leading to considerable decomposition loss, while use of more polar solvents led to poor resolution. This problem was circumvented by chromatographing the TC's until the first yellow band reached the column end. The column contents were then extruded into a glovebag and sliced into portions according to color intensities. Each TC band was extracted with MeOH (containing 1% of the volatile *tert*-butyl sulfide), and each fraction was isolated as a dry powder by repeated precipitations. Five TC fractions designated *An*-A, *An*-3, *An*-2, *An*-1, and *An*-C (originally[23] TA, TBs-3,2,1, and TC) in order of increasing polarity were thus obtained and stored under argon at −20 °C. TC yields appear to be season dependent as noted earlier.[24] An isolation of 1000 animals collected in September 1984 gave 150 mg of *An*-A, 500 mg of *An*-1, 850 mg of *An*-2, 340 mg of *An*-3, and 400 mg of *An*-C, whereas the yield from specimen collected in February 1984 was roughly half as much.



1a: An-1, $R_1 = R_2 = OH$
b: An-2, $R_1 = H$, $R_2 = OH$
c: An-3, $R_1 = R_2 = H$

Extensive streaking of the TC's rendered their analysis by thin-layer chromatography (TLC) problematic. Simple spot tests had revealed the phenolic character of *An*-1 (staining with fast blue salt[27] and $FeCl_3$[27]) and were supported by UV observations; adding NaOAc to methanolic solutions of *An*-1 produced irreversible batho- and hypochromic shifts in its UV spectrum, indicative of conjugated phenols, especially catechols.[27] Consequently, a TLC solvent system based on those used in flavonoid studies[27] was developed: toluene (6)/2-butanone (24)/ethyl acetate (50)/formic acid (10)–water (10). Although decomposition of the compounds occurred immediately after TLC development, each sample was found to be a mixture of compounds. *An*-1, -2, and -3 were also resolvable by analytical reversed-phase (RP) HPLC in an acetonitrile/formate buffer system, but with only ca 20% recoveries.

Semipreparative separation of *An*-1, -2 and, -3 was finally accomplished by centrifugal partition chromatography (CPC).[23,28] For example, 200 mg of the *An*-2 fraction was separated in the normal-phase elution mode[29] until *An*-3, retention time 3–4 h, 30 mg, and *An*-2, 5–6 h, 60 mg, were recovered. The elution mode

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(29) "Normal" phase refers to the mode where the organic layer of a biphasic solvent system is used as the mobile phase, in "reversed"-phase mode the mobile phase is the aqueous layer.

Table I. ^{1}H NMR Data for TC-Ac's[a]

proton(s)	*An*-1 Ac	*An*-2 Ac	*An*-3 Ac	*Mm*-1 Ac	*Mm*-2 Ac
N^9	9.16 (d, 10.5)	9.17 (d, 10.3)	9.16 (d, 10.5)	9.12 (d, 11.0)	9.07 (d, 10.3)
N^{19}	7.93 (s)	7.87 (s)	8.07 (s)	7.66 (s)	7.45 (s)
8	7.49 (dd, 10.5, 14.6)	7.48 (dd, 10.3, 14.5)	7.48 (dd, 10.5, 14.6)	7.52 (dd, 11.0, 14.9)	7.53 (dd, 10.3, 14.6)
12	7.37 (s)	7.37 (s)	7.31 (s)	7.38 (s)	7.53 (s)
2/6	7.07 (s)	7.10–7.05	7.22–7.04	7.27–7.06	7.29–7.07
14/18	7.16 (s)	overlapping	7.15 (s)	overlapping	overlapping
24/28	7.02 (s)	resonances	7.22–7.04	multiplets	multiplets
N^{21}	6.61 (d, 5.7)	6.47 (d, 5.8)	6.54 (d, 5.9)	6.53 (t, 5.3)	6.11 (d, 5.0)
7	6.31 (d, 14.6)	6.29 (d, 14.5)	6.32 (d, 14.6)	6.36 (d, 14.5)	6.39 (d, 14.6)
21	4.39 (ddd, 3.8, 5.7, 10.7)	4.33 (ddd, 4.4, 5.8, 10.7)	4.43 (ddd, 3.9, 5.9, 10.6)	3.86 (d, 5.3)	4.22 (m)
22	3.30 (dd, 3.8, 14.2), 3.04 (dd, 10.7, 14.2)	3.28 (dd, 4.4, 14.2), 2.99 (dd, 10.7, 14.2)	3.27 (dd, 3.9, 14.2), 2.98 (dd, 10.6, 14.2)	none	1.78–1.48 (m)
O-Ac	~2.2 (s's)	~2.25 (s's)	~2.25 (s's)	~2.27 (s's)	~2.8 (s's)
N-Ac	1.92 (s)	1.92 (s)	1.90 (s)	2.02 (s)	2.06 (s)
misc	none	none	none	none	1.78–1.48 (m, H-23)
	none	none	none	none	1.00 (H-24/5, d, 5.7), 0.95 (H-24/5, d, 5.6)

[a] Chemical shifts are reported in ppm relative to an internal TMS standard; multiplicities and coupling constants (in hertz) are given in parentheses.

was then reversed, thereby eluting *An*-1, 30 mg, in a sharp peak (N = 1800), trailing more polar materials—presumably *An*-C and decomposition products. The *An* samples obtained from CPC were sufficiently pure for most purposes and were used for preliminary measurements of spectroscopic data. However, for final purification, 5 mg of the CPC *An*-1 fraction was submitted to a semipreparative HPLC employing two columns in a series; this yielded, with shaving of the peak, 500 μg of pure *An*-1, m/z 556 (M + H) by secondary-ion mass spectrometry (SIMS).

Purified *An*-1 was much more stable than when present in cell lysates, and an aqueous solution could be kept without noticeable decomposition for a few days. However, not unexpectedly, this polyphenolic compound underwent slow decomposition. Reaction of LH-20 purified *An*-1 with diazomethane afforded a nonamethyl ether (*An*-1 Me, MW 781), <20% yield, which was still unstable, however. Other permethylation reactions were even less suitable, as were several alternative derivatizations, e.g., formation of borate ester and silyl ether. In view of the stable nature of the decaacetate (below), the instability of the nonamethyl ether might be attributable to an intramolecular Michael addition of the free amino group ($N^{21}H_2$) at C-12. *An*-1 peracetate (*An*-1 Ac) was obtained with Ac_2O/pyridine, 1:1 [MW 975, orange/yellow fluorescence (UV_{366})], amongst 20–30 minor UV active and fluorescent components, as judged by TLC. The acetylation procedure served as the basis for an assay of *free* tunichromes (see the Experimental Section).

Pure *An*-1 Ac was ultimately obtained by extracting the highly purified *An*-1 CPC fraction into butanol and concentrating it to dryness; the residue was acetylated and then purified by preparative TLC (SiO_2, 4% 2-propanol (*i*-PrOH) in dichloromethane (CH_2Cl_2), followed by semipreparative, normal-phase (NP) HPLC (SiO_2, 4% *i*-PrOH/CH_2Cl_2). Peak shaving was required to separate it from *An*-2 Ac. MS analysis of *An*-1 derivatives suggested the presence of nine aromatic hydroxyls (nonamethyl ether) and an amine (decaacetate). High-resolution SIMS of *An*-1 decaacetate clarified the molecular formula of *An*-1 as being $C_{26}H_{25}N_3O_{11}$. An IR band at 1683 cm^{-1} indicated the presence of amides.

UV, circular dichroism (CD), and ^{13}C and ^{1}H NMR (Table I and ref 4) analyses of *An*-1 Ac suggested that the structural integrity of the *An* skeleton had remained intact through acetylation. The UV spectrum of *An*-1 is depicted in Figure 2. NMR revealed the presence of a trans enamide (CH=CHNHCO), an additional lowfield vinyl proton, three aromatic singlets (two protons each, pyrogallol acetates), a singlet amide NH, and a phenylalanine type ABM system (Ar–CH_2CHNHAc). Together with experiments described below, the data led to the proposed structure **1a** for *An*-1, a modified tripeptide derived from three hydroxydopa residues.[4] All major chemical-ionization (CI) MS fragmentations could be accounted for by **1a** Ac (Table II). The number and multiplicities of carbons in **1a** Ac was consistent with

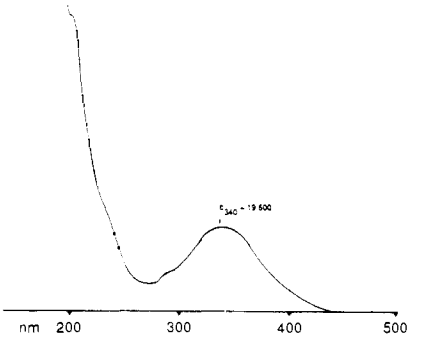
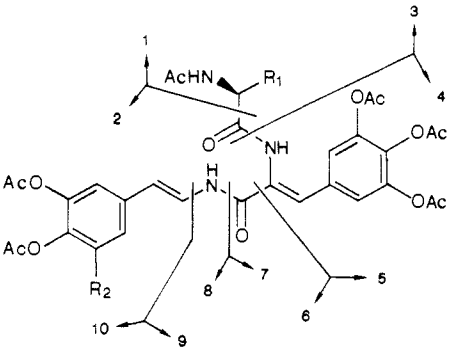


Figure 2. UV/visible spectrum of *An*-1.

Table II. Mass Spectral Data (DCI/CH_4) for TC-Ac's[a]



fragment	*An*-1 Ac	*An*-2 Ac	*An*-3 Ac	*Mm*-1 Ac	*Mm*-2 Ac
M + 1	976	918	860	596	652
M − 41	934	876	818	554	610
1	336	278	278	n.o.	128
2	641	641	583	n.o.	n.o.
3	364*	306	306*	100*	156*
4	613	612	555	496	497
5	655	597	597	334	391
6	322	n.o.	264	264	n.o.
7	683	625*	625	361	417
8	294	294	236	236	236
9	700	642	n.o.	n.o.	n.o.
10	n.o.	n.o.	n.o.	220	n.o.

[a] Fragments correspond to those illustrated in the accompanying structure. M − 42 is a prominent fragment observed in all spectra and corresponds to the loss of ketene (from a phenolic acetate); * = parent ion; n.o. = not observed.

those observed in ^{13}C broad band decoupled and DEPT experiments: 0 CH_3, 1 CH_2, 10 CH's, and 15 C's—discounting acetate carbons. Difference nuclear Overhauser effect (NOE) experi-

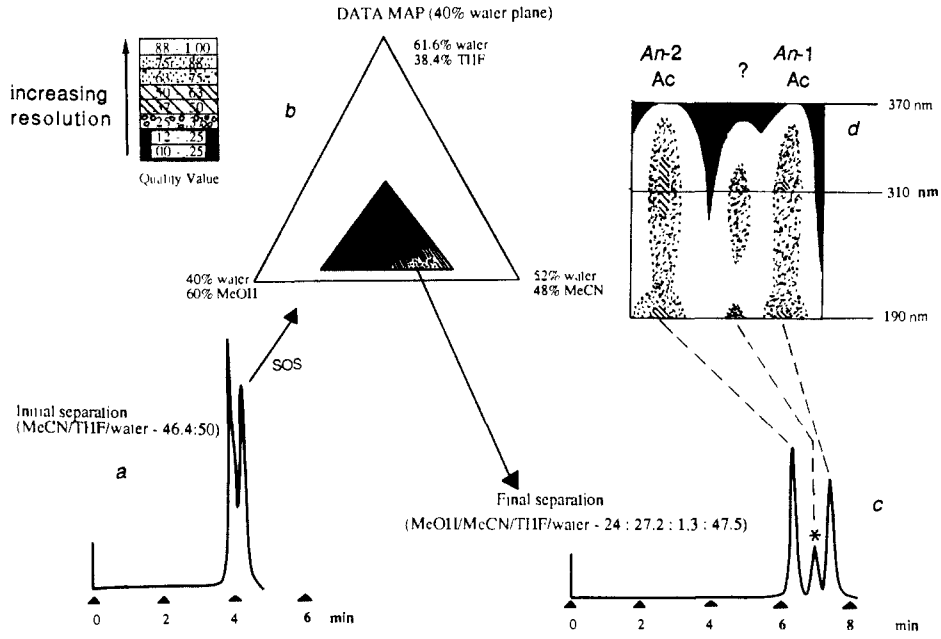


Figure 3. RP-HPLC solvent optimization via PESOS. (a) Typical separation prior to PESOS. (b) Data map at 40% water plane. (c) Optimal separation from PESOS survey; * denotes a peak corresponding to a new (uncharacterized) TC. (d) Contour plot of the separation by using diode array detection.

ments[4] supported the proposed connectivities and elucidated the C-11/C-12 olefin stereochemistry.

Several additional chemical transformations clarified the remaining ambiguities.[4] Ozonolysis of *An*-1 Ac generated 1.5 equiv of triacetoxygallaldehyde, on the basis of UV comparisons with synthetic standards. Methylation of *An*-1 (CH_2N_2) followed by acid hydrolysis of the reaction mixture (4 N HCl/Δ) yielded a complex mixture of polar compounds detected by RP-HPLC. The presence of both 3,4,5-trimethoxyphenylalanine (TMP) and 3,4-dimethoxyphenylalanine (DMP, from *An*-2, see below) was demonstrated by co-injection of synthetic samples (see the Experimental Section); ^{1}H NMR spectra of peak specimens displayed the requisite aromatic and methoxy resonances present in synthetic standards. Lastly, hydrogenation of *An*-1 Ac (H_2/Pd–C) produced the corresponding tetrahydro derivatives (4 H *An*-1 Ac, MW 979, data not shown). COSY analysis of one diastereomer displayed a second ABM system corresponding to a AcNHC^{11}HC^{12}H$_2$ system, as well as the original C-21/C-22 ABM system. The ^{13}C NMR spectrum of this derivative exhibited the required number of aliphatic CH's (2) and CH_2's (4) and lacked the corresponding number of olefinic resonances. The absolute stereochemistry at C-21 was ascertained by isolating a sample of TMP from the *An*-1 Me acid hydrolysate and preparing its methoxydiphenylfluorenone (MDPF)[30] derivative; the positive Cotton effect at 380 nm in its CD spectrum indicated an *S* configuration.

***An*-2 Ac and *An*-3 Ac.** Purification of *An*-2 Ac by normal phase (NP) HPLC proved inefficient due to contamination by *An*-1 Ac. Consequently, a variety of RP-HPLC systems were surveyed and rated by using a Perkin-Elmer solvent optimization system (PESOS); a wide variety of solvent systems—when employed in conjunction with an autoinjector and a miniature high-resolution cartridge column—can be screened overnight. The resultant chromatograms are assigned a quality value according to the number and resolution of peaks, color-rated, and displayed as a data map. When applied to the separation of a *An*-1,2 Ac mixture, a solvent system consisting of MeOH, acetonitrile (MeCN), tetrahydrofuran (THF), and water (H_2O) (24:27.2:1.3:47.5) was deemed optimal (partial data map and chromatograms are shown in Figure 3). A new TC was detected by using this system in conjunction with diode array monitoring (Figure 3, asterisked peak). Attempts to scale up separations led to significant losses in resolution. However, the fact that the elution order of *An*-1 and -2 Ac was reversed in RP, compared to NP-HPLC systems, suggested that RP-HPLC (with peak shaving) could provide pure *An*-2 Ac. Contrary to expectation, extensive decomposition occurred upon solvent evaporation (irreversible by reacetylation). Deacetylation by H_2O or MeOH, followed by oxidation from trace peroxides in the THF, may occur. Consequently, RP-HPLC was reserved for analytical separations.

Initial spectroscopic investigations of nearly pure *An*-2 Ac, obtained by NP-HPLC peak shaving ($ClF_2CCCl_2F/CH_2Cl_2/i$-PrOH, 25:69:6), confirmed its structural homology to *An*-1 Ac. The presence of one asymmetrically substituted aromatic ring was inferred from *An*-2 Ac's ^{1}H NMR (Table I). Correspondingly, the ^{13}C-DEPT spectrum of *An*-2 Ac exhibited two additional aromatic CH resonances compared to *An*-1 Ac. NOE studies indicated that ring C was altered; signals for 7-H and 8-H were enhanced by irradiation of one aromatic singlet, while 21-H displayed a small NOE to the aromatic multiplet. The structure of *An*-2 Ac was confirmed by MS [desorption CI (DCI)/CH_4, MW 917]. The fragmentation pattern (Table II) revealed that the asymmetry arose from two acetates (OAc's) on ring C, compared with three in *An*-1 Ac. All fragments containing this ring displayed a loss of 58 mass units (−OAc, +H) when compared with analogous *An*-1 Ac fragments (i.e. 364 becomes 306). The structure of *An*-2 was thereby deduced as **1b**. The presence of DMP in the *An*-1 Me hydrolysate could now be reconciled as due to *An*-2 Me contamination.

An-3 from the corresponding LH-20 fraction (ca. 90% pure) was acetylated and isolated by NP-HPLC. Spectral data indicated that *An*-3 Ac possessed two asymetrically substituted rings. An NOE from 12-H to the two-proton aromatic singlet suggested that rings A and C were disubstituted (**1c**). This inference was substantiated by MS (DCI/CH_4, MW 859, Table II); fragments possessing either ring A or ring C displayed the expected decrease of 58 mass units when compared with *An*-1 Ac.

***Mm*-1 and -2 Ac.** All ascidians examined to date exhibit some degree of metal-specific sequestration.[31] Since the tunichromes possess the requisite structural features for this process (see below), a TC survey of diverse tunicate species was undertaken. Only one of the 12 species screened, *Molgula manhattensis*, an iron accumulating *Stolidobranch*,[31] provided new TC's (*Mm*-1 and -2), while *An*-1 was detected as the sole TC pigment in two other vanadium-collecting species (*Ascidia ceratodes* and *Perophora viridis*). Approximately four dozen *Molgula* collected from Cape

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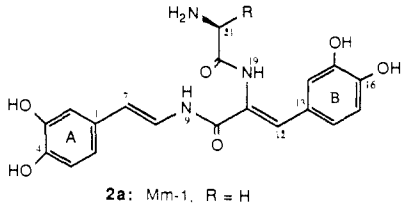
Cod Canal were bled, the blood was centrifuged, and the pellet was lyophilized and acetylated. *Mm*-1 and -2 Ac's were obtained by preparative TLC (5% *i*-PrOH/CH_2Cl_2) and NP-HPLC (8% *i*-PrOH/CH_2Cl_2 for *Mm*-1 Ac and ClF_2CCCl_2F/CH_2Cl_2/*i*-PrOH, 49:45:6 for *Mm*-2 Ac). ^1H NMR analysis of the *Mm*'s indicated extensive structural homology to the *An*'s. Specifically, the A and B rings were intact, whereas the hydroxydopa ^1H resonances of the C ring were absent. Insufficient amounts were obtained for ^{13}C NMR analyses.

^1H NMR data obtained for *Mm*-1 Ac, the major *free* tunichrome (>80%), are provided in Table I. The missing AMX system appeared to have been replaced by a two spin system consisting of a triplet amide NH coupled to a two proton doublet, hence CH_2NHAc. Substitution of glycine for the hydroxy-Dopa unit of *An*-1 Ac accounted for the difference. The absence of two proton singlets in the aromatic region suggested that the A and B rings were both disubstituted, thus indicating **2a** to be the structure of *Mm*-1. Additional support for structure **2a** was provided by MS of the *Mm*-1 peracetate; it displayed a molecular ion at *m/z* 596 (M + 1) and a parent fragment ion at *m/z* 100 (glycine, Table II). The fact that the CD spectrum of *Mm*-1 Ac did not exhibit a Cotton effect was compatible with **2a**.



2a: Mm-1, R = H
b: Mm-2, R = i-Bu
c: R = sec-Bu

The ^1H NMR spectrum of *Mm*-2 Ac exhibited a doublet NHAc coupled to a CH multiplet. The CH multiplet was also coupled to a multiproton multiplet, which, in turn, was coupled to two 3 H doublets (Table I). This pattern suggested that the hydroxydopa C ring residue in the *An*'s had been substituted with a leucine or an isoleucine (**2b,c**). The α-proton resonance (C*H*-NDAc) in the D_2O-exchanged spectrum was observed as a doublet of doublets. This is consistent with **2b** (Leu); the analogous isoleucine resonance would have appeared as a doublet. Comparison of CD data (in MeCN) of *An*-1 and *Mm*-2 peracetates, nm ($\Delta\epsilon$): 233 (+1.5)/260 (−0.9)/300 (−0.9); cd 229 (+1.7)/290 (−0.5), respectively, indicated that the absolute configuration of the two are identical.

Of the TC's characterized thus far, the feature shared by all is the presence of two catecholic ring systems bridged by a rigid enamide backbone. Further investigations may clarify whether this represents a minimum functional framework.

Structural Implications. The relative abundance of *free* (i.e., uncomplexed) *An*'s was estimated by acetylating lyophilized blood cells, initially purifying the *An*'s by preparative TLC, followed by semiquantitative analysis with RP-HPLC. The following percent composition was assessed from tunicates collected in September 1984: *An*-1 Ac (30%), *An*-2 Ac (50%), *An*-3 Ac (17%), minor components (ca. 1–2% each). Although probably not the biosynthetic end product, the major *free* TC was *An*-2. The total amount of *free An*'s and vanadium in *A. nigra* blood were also estimated (see the Experimental Section). Lyophilized blood (September 1984) contained ca. 0.16 μmol of vanadium and ca. 0.3–0.4 μmol of *An* per mg. Thus, *free An*'s constitute roughly 20% of the dry weight of *A. nigra* blood. A nearly equimolar presence of TC and vanadium had previously been demonstrated and was assumed to reflect a functional relationship.[22] However, since only *free* TC is detectable by the acetylation assay, the equimolar content may simply be fortuitous.

The *An*'s are structurally related to the celenamides[32] and clionamide,[33] compounds isolated from sponges (Figure 4). More recently, a series of compounds termed lamellarins have been isolated from a marine mollusc.[34] The skeletons of the lamellarins resemble those of the *An*'s (Figure 4) and interestingly, the authors note that colonial tunicates are a staple of molluscs closely related to *Lamellaria*. The above data explain many of the observed properties of the tunichromes; TC's are (i) prone to oxidation, (ii) difficult to separate due to their differing degrees of hydroxylation, and (iii) possibly biosynthesized via sequential hydroxylations of a tripeptide precursor.

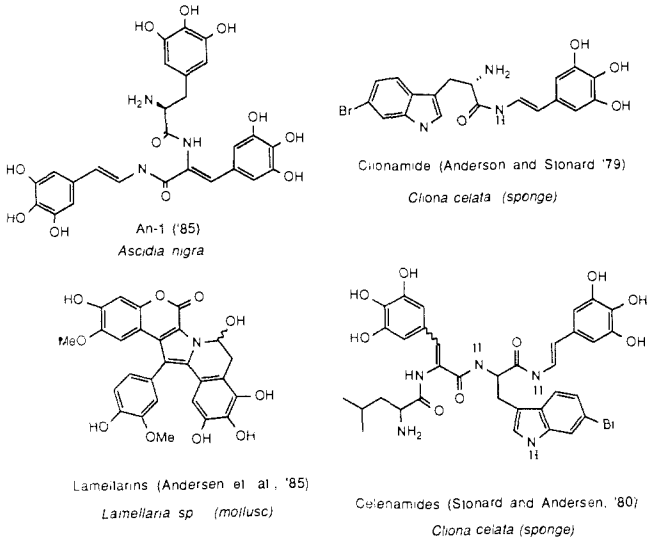


Figure 4. Structural comparison of *An*-1 with other reported marine natural products. (a) *An*-1. (b) Clionamide (ref 33). (c) Lamellarin A (ref 34). (d) Celenamide A (ref 32).

Tunichrome–Vanadium Complexation Chemistry. Several techniques, including magnetic susceptibility measurements,[15] cytological staining,[16] EPR,[35] NMR,[17] EXAFS,[18] and SQUID,[19] have identified vanadic [V(III), with up to 10% V(IV)] as the predominant oxidation state present in blood cells of *A. nigra* and related species. The fact that both V(III) and V(IV) are unstable above pH 3 if present in millimolar concentrations or greater appears paradoxical. Numerous mechanisms have been invoked to explain V(III) stability; one such claim deserving further scrutiny is that the *intravanadophoric* milieu is strongly acidic. Evidence supporting the abnormally low *intracellular* pH was first provided by Henze,[36] who reported that upon lysis, ascidian blood cells produced a highly acidic, red-brown solution (Henze's solution), which formed a white precipitate upon addition of barium salts. He estimated that the cytoplasm of vanadocytes was 1.8 N H_2SO_4. Counter proposals suggest that the observed acidity might be generated during cell lysis and that barium vanadate might account for the white precipitate. Ascidian blood cells harbor extraordinarily high concentrations of sulfur in the form of sulfate and possibly sulfonate.[18] Carlson, Hodgson, and coworkers have recently reported a series of studies (^1H NMR,[17] EPR,[35a] and X-ray absorption[18]), which suggest a largely hydrated environment for the metal, being coordinated by only one sulfate (or sulfonate) ion, [i.e. $V^{III}(SO_4)(H_2O)_{4-5}^{+(or\ 2+)}$]. Such coordination would require an acidic *intravanadophoric* milieu, and these data may represent the most compelling evidence in support of this postulate. X-ray and neutron diffraction studies on $[V(H_2O)_6][H_5O_2](CF_3SO_3)_4$ crystals have been reported by Cotton et al.[37]

On the other hand, several studies indicate that unfractionated ascidian blood cells possess a normal *intracellular* pH (6.9–7.2).[38]

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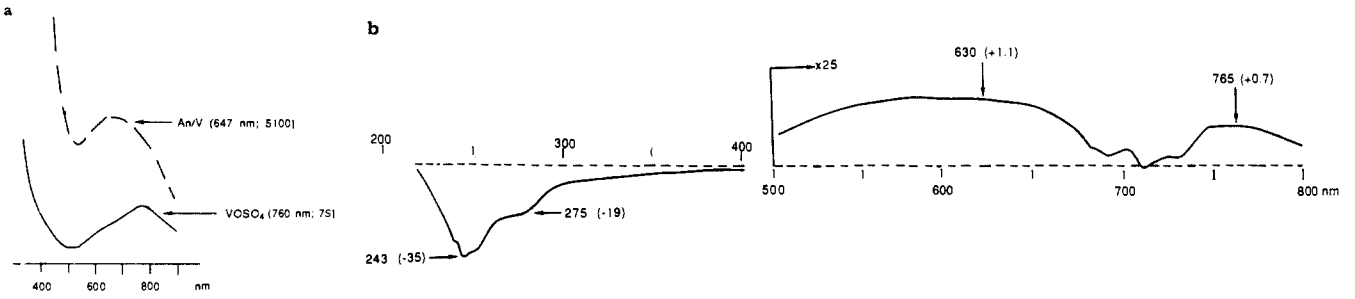

Figure 5. (a) UV/visible spectra of $VOSO_4$ (solid line) and *An*/V complex (broken line). Specific λ_{max}'s and their ϵ's are provided in parentheses. All spectra were obtained with aqueous solutions at pH 3.5. The *An*/V complex was prepared by slow addition of 16 mM vanadate to 4mM *An*-1 (~1:4 volume); the UV spectrum of *An*/V complex at pH 7 is indistinguishable from that at pH 3.5. Spectra obtained for analogous complexes prepared from vanadyl sulfate [V(IV)] were indistinguishable from the one shown. (b) CD spectrum ($\Delta\epsilon$'s in parentheses) of *An*/V complex prepared at pH 7 by methods described in the Experimental Section (Job's analysis).

These include radiolabeled base equilibrations;[38] fluorescent base equilibrations;[39] and cytological staining with non redox sensitive indicators.[40] Lastly, microscopic observations indicate that vanadocytes possess few mitochondria;[41] they may thus lack the normal machinery for generating a large proton gradient. The pH controversy outlined above revolves around the selection of a valid pH indicator; regarding the vanadium milieu, it should presumably reflect the pH of the *vanadophore*. If the pH around V(III) is not abnormally low, an alternative mechanism is necessarily responsible for stabilizing the metal.

In light of the capacity of TC to complex and reduce vanadium and iron, its large content in *A. nigra* blood cells may not be coincidental. The vicinal di- and trihydroxybenzene substituents in the *An*'s and *Mm*'s confer strong chelating and reducing properties toward these transition metals.[42,43] The reported overall formation constants[44] (K_f) of these substituents for VO(IV), V(III), Fe(III), and Fe(II) are over 10^{10}, while several surpass 10^{28}. The most powerful natural iron chelator known, enterobactin,[45,46] possesses three such dihydroxybenzene substituents and exhibits a $K_f = 10^{52}$ for Fe(III).[47] It is also noteworthy that Fe(III)-binding dopa pigments, termed adrenochromes, have been obtained from the branchial heart of *Octopus vulgaris*.[48] If tunichrome and vanadium are present in the same blood cell, barring the presence of a stronger ligand, physical barrier, or low pH, a TC–V complex (possibly transient) is anticipated. Yet, little persuasive evidence supporting this possibility has been forthcoming.

As a first approximation of the postulated TC–vanadium complex in vivo complexation chemistry studies in vitro were initiated. Slow addition of dilute aqueous solutions of either V(V) (16 mM NH_4VO_3; no significant absorption above 500 nm) or V(IV) (16 mM $VOSO_4$) to aqueous *An*-1 (LH-20 fraction, 4 mM, yellow) at pH 3, anaerobically or aerobically, immediately generated a dark olive green solution. At these concentrations a colloidal, dark green precipitate formed over a period of 30 min. Prior to precipitation, the two solutions formed from $VOSO_4$ and NH_4VO_3 exhibited identical visible spectra, λ_{max} 647 nm, 765 (sh) (Figure 5a); see below for comments on CD (Figure 5b) When a suspension containing the precipitated complex was centrifuged, the supernatant fluid was found to contain the excess uncomplexed *An* (by UV). The green precipitate was insoluble in common solvents, except aqueous acid, which yielded a yellow solution and a corresponding reappearance of the *An* maxima at 290–320 nm, suggesting that the ligand could be liberated in a state reminiscent of *free* TC. However, if the acid solution was lyopholized and acetylated, no *An* acetate was formed *unless doped with excess ligand*. The fact that the acetylation assay detects only added *free* TC may reflect the K_f of TC–V complexes and/or ligand oxidation (see ref 42 and 43).

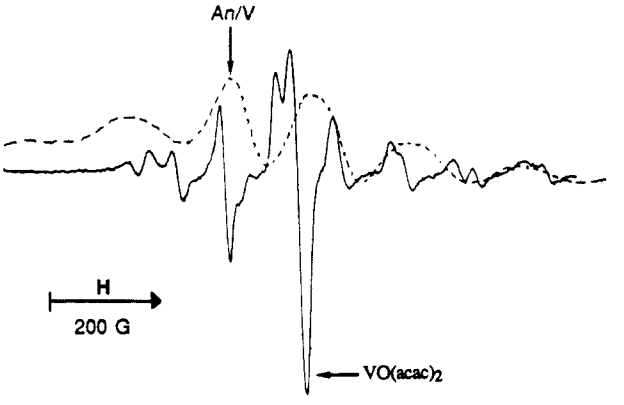

Figure 6. EPR spectra of *An*/V complex (not precipitated, dashed line) and 0.01 M $VO(acac)_2$ (solid line) in MeOH (77 K). Complex sample was prepared by slow addition of saturated ammonium vanadate (in MeOH) to 1.3 mM *An*-1. The two spectra were recorded with different gain settings (3.2 for $VOSO_4$ and 800 for the complex).

Because *An*-1 has at least six coordination groups, a totally intramolecular octahedral complex is possible; however, molecular models indicate this to be unlikely for steric reasons. Instead, it is anticipated that the metal bridges *An* molecules, forming intermolecular polynuclear complexes (i.e., network polymers) that possess peculiar solubility properties.[49] The poor resolution in the IR and EPR spectra of such reconstituted vanadium complexes is compatible with the presence of such polymers (see below). Molecular weight determinations on vanadium complexes extracted from tunicate blood cells lead to the same conclusion (see below).

The stoichiometry for the reconstituted *An*/V complexes was estimated to be 2:1 (elemental analysis, most reliable); 2.5:1 (analysis for *An* by UV and V by atomic absorption spectrometry); and 2.5:1 (Job's analysis at pH 7).[50] The FTIR spectrum (KBr) of the H_2O-rinsed and dried green precipitate displayed numerous weak, broad absorbances of no diagnostic value between 2000 and

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800 cm^{-1}. EPR spectra (77 K) of the vanadium complex produced from mixing methanolic NH_4VO_3 (V^V), and *An*-1 displayed at least seven broad lines (Figure 6), indicating the presence of V(IV). Spectra taken under analogous conditions by other workers[51] have exhibited the following parameters: $A_0 = 94.1$, $A_\parallel = 162.9$, and $A_\perp = 59.7$ G; $g_0 = 1.977$, $g_\parallel = 1.953$, $g_\perp = 1.990$. These parameters do not resemble those for aqueous vanadyl ion[35] or any reported for a tunicate blood sample.[35] This may reflect the differing conditions used for spectral observation (MeOH vs water).

The UV/visible spectrum of a reconstituted *An*/V solution (at pH 7 or 3.5) corresponding to a point slightly on the downward side of the Job's plot (slight excess V) displayed a maximum at 630 nm (ϵ 5100, per mole of bound V) and a shoulder at 765 nm (Figure 5a). The 765-nm shoulder is resolved in the CD spectrum (Figure 5b) of the same solution, 765 nm ($\Delta\epsilon$ +0.7); 630 (+1.1); 275 (−19); 243 (−35). This data discounted the possibility that the 765-nm shoulder reflected absorbance by residual (uncomplexed) vanadyl (λ_{max} 760 nm), as this would not display a Cotton effect. The Cotton effect at $\lambda > 500$ nm indicated that the metal was under the influence of the chiral environment of C-21. Complexation reactions run at higher dilutions at pH 7 (1 mM NH_4VO_4, with or without Hepes buffer) provided dark green solutions with no precipitation [λ_{max} 765 nm (sh), 650 nm (ϵ 5100, based on moles of V); determining whether concentration, pH, or differing counterions lead to the lack of precipitation requires further work. The magnitude of this ϵ is suggestive of a ligand–metal charge transfer band (ϵ's for d–d transitions, $\sim 10^2$).

The fact that the rigid backbone of the tunichromes should predispose them to generate polynuclear metal complexes is consonant with the environmental constraints imposed on tunicates. Intracellular sequestration of metals using polymers synthesized by organisms is a common mechanism utilized to remove and trap metal ions such as cadmium, nickel, and copper from solution.[52,53] Macara et al. have accumulated considerable evidence for a vanadium "trapping" mechanism in tunicates.[22] Alternative metal assimilatory systems such as siderophore-based high affinity iron transport[54] are likely to be inefficient due to problems of ligand diffusion in marine environments.

As a second approximation of vanadium coordination in vivo we sought to isolate the vanadium complex(es) from *A. nigra* blood cells. Sephadex LH-20 chromatography of lyopholized blood cells yields, besides *free* tunichrome, a green fraction containing ca. 1% vanadium, designated *An*-C (Figure 1). A specimen of *An*-C washed repeatedly with degassed water (until *An* absorption was no longer observed) and dissolved in 0.1 N HCl generated the typical *An* 290–320 nm UV doublet; yet the presence of extraneous, firmly adsorbed *free* TC cannot be formally ruled out. Our initial attempts to isolate the vanadium complex have been reported elsewhere.[49] Aqueous 50% polyethylene glycol (PEG) 1000/polypropylene glycol (PPG) 425/mercaptoethanol (49:49:2), in conjunction with CPC enabled us to isolate a vanadium complex. Following vanadium removal[49] and acetylation (Ac_2O/pyridine, 1:1), material possessing the spectroscopic features of tunichrome was detected [290–320 nm UV doublet; ring protons by NMR; and orange/yellow fluorescent TLC bands comigrating with *An* Ac standards (data not shown)]. However, it became apparent that this approach could not clarify the make up of the indigenous vanadium complex for three reasons: (1) potential ligand exchange during cell lysis; (2) neither PEG nor mercaptoethanol could be removed completely from the material as isolated, possibly due to their metal-binding properties; (3) TC rearrangements (e.g., polymerization reactions in the absence of an antioxidant).

Phenols are reported to exhibit strong affinities toward PEG and LH-20.[55] Ether-containing compounds like ethoxyethanol (EE) reduce such adsorptions.[56] Consequently, EE and methoxyethanol (ME) were tested and found to facilitate elution of the *An*-C from LH-20, cellulose, and PEG-bound polystyrene. Approximately 20% of the total vanadium in *A. nigra* blood cell pellets (from ca. 70 mL of whole blood, collected under argon) could be extracted with ca. 10 mL of degassed ME, as determined by atomic absorption spectrometry; importantly, vortexing the cell pellet in ME led to cytolysis and generated a dark olive green solution. This procedure would certainly enable *free* TC to displace any weaker ligands on the indigenous vanadium complex. The unextracted vanadium might be present as an insoluble metal polymer, amongst numerous other possibilities. LH-20 chromatography of the vanadium complexes so extracted was carried out in ME plus 1% *t*-BS as an antioxidant; Approximately 85% of the total vanadium could be eluted from the column, of which ca. 60% eluted as a sharp, olive green peak with an apparent MW >4000, consistent with the notion that vanadium is present in the form of a polymer network [void volume elution point, as determined by using 24000 MW polyvinylpyrrolidone (data not shown)]. UV spectra recorded in ME plus 1% *t*-BS showed that all V-containing fractions possessed a 320–330-nm UV absorbance band, indicative of TC, in addition to a second band at ca. 275 nm.

In the absence of *t*-BS, LH-20 chromatography in ME yielded a series of broad, olive green vanadium peaks eluting well after the void volume, thereby exhibiting reduced apparent MWs; the vanadium complexes are either lower in MW and/or interact with the resin more strongly. Approximately 50% of the total vanadium loaded had adsorbed onto the resin as a blue precipitate. It was not possible to further characterize this V-containing polymer. Hawkins and co-workers[35c] have noted that when phleobranch blood cells are lysed in O_2-free water, neither vanadium, acid, nor pigment susceptible to oxidation is detected in the lysate; in the presence of oxygen, such cell lysates contained vanadium, acid, and oxidizable pigment. Conceivably then, O_2 might liberate vanadium and the pigment via oxidation of the actual or potential metal ligands.

As a third approximation of vanadium coordination in vivo, we have endeavored to identify the tunicate blood cell types that contain vanadium and/or tunichrome.[57] Tunicates possess several types of blood cells, the lymphocytes (LC), leucocytes (LU), pigment cells, and vacuolated cells, i.e., signet ring cells (SRC), morula cells (MR), and compartment cells (CC). The metal, function unknown, resides in these vacuolated cells (vanadocytes or ferrocytes); MR's are generally regarded as the primary vanadocyte,[58] but recent evidence, e.g., X-ray microanalysis[59–61] and density separation of cells[62] suggested that SRC's and CC's also contain V. Taking advantage of the inherent fluorescence of TC, we have separated *A. nigra* blood cells by fluorescence-activated cell sorting (FACS) into three predominant fractions: (i) a population comprised almost totally of MR's, (ii) an LC + LU mixture, and (iii) a mixture of SRC + CC. Each fraction was analyzed for *free* TC's by acetylation with subsequent HPLC detection of TC peracetates, while V was analyzed by atomic absorption spectrometry.[57] Results of these semiquantitative analyses showed that V was predominantly associated with the SRC + CC fraction (ca. 110 nmol of $V/10^6$ cells) and absent in the LC + LU fraction; the MR fraction contained significantly less V (<3 nmol of $V/10^6$ cells) but practically all of the *free* TC present in the blood. Namely, the bulk of V appears to be present in the SRC + CC while virtually all of the *free* TC is detected in the morula cells.

(51) We are grateful to Professor Y. Sugiura, Kyoto University, for measurements of these data.
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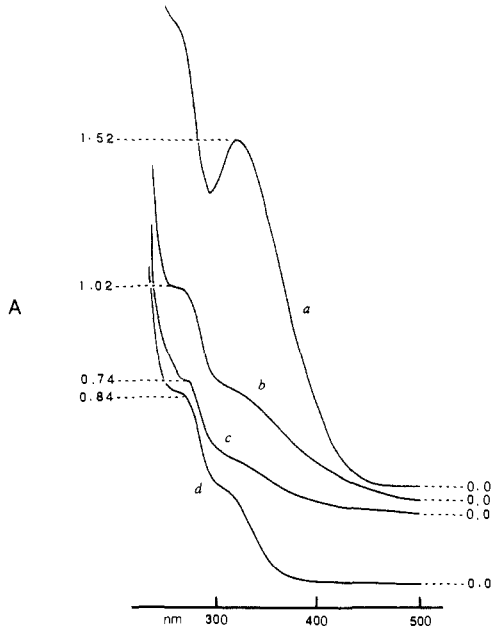

Figure 7. UV absorption spectra of the following solutions: (a) Approximately 1.1 million FACS-fractionated MRs lysed in 2 mL of Ar-purged 0.1 N HCl/H_2O, time zero. (b) A typical LH-20 peak fraction of reconstituted *An*-2/V in Ar-purged ME + 1% *t*-BS. (c) Approximately 4.8 million FACS-fractionated SRC's + CC's lysed in 2 mL of Ar-purged 0.1 N HCl/H_2O, time zero. (d) Lysed MR solution (a) after 3 days. UV absorbancies depicted on the ordinate represent values normalized to the concentrations cited.

Significantly, evidence suggestive of a tunichrome–vanadium complex in vivo, although indirect, is that UV absorption curves (Figure 7) of the FACS-purified SRC + CC fraction (which lacks *free* TC) lysed in 0.1 N HCl (Figure 7c) is strikingly similar to the following absorption curves: (1) reconstituted tunichrome–vanadium complexes fractionated by LH-20 with ME plus 1% *t*-BS (Figure 7b); (2) ME-extracted vanadium complexes from fresh blood cells; (3) FACS-purified MR fractions lysed in 0.1 N HCl for 3 days at ambient temperature (Figure 7d), which initially exhibited the characteristic tunichrome UV curve (see Figures 2 and 7a); and (4) oxidative rearrangement products of 1,2-dehydro-*N*-acetyldopamine.[63] The change in the MR fraction UV occurring during the 3-day period is presumably due to oxidation of the TC chromophore by oxygen or traces of transition metals, thus giving rise to a new chromophore similar to that existing in curves 7b and 7c. Namely, it is possible that signet ring and/or compartment cells do indeed possess an oxidized form of tunichrome altered by its interaction with vanadium (Figure 7c). Yet if one grants merit to the above interpretation, the question as to why TC's native fluorescence is masked in the SRC + CC fraction (isolated by their virtual absence of red fluorescence) ensues. It may thus be pertinent that paramagnetic metals effectively quench chromophore fluorescence.[64]

Concluding Remarks

The tunichromes appear to be important biomolecules in *A. nigra* and certain other species (ca. 20 and 50% dry weight of *A. nigra* blood and MR's, respectively) and are harbored in cells that take part in a variety of physiological responses (e.g., immunological reactions and wound healing; see ref 65). The body of *A. nigra*, like all ascidians, is invested by a thick, rubberlike coating, the tunic, which primarily consists of a cellulose-like polysaccharide.[66] One newly defined postulate is that TC serves to cross-link tunicin fibers (cellulose) to each other, or to proteins in the tunic ground substance.[67,68] This would be analogous to the well-documented involvement of catecholic amino acids in the sclerotization of insect cuticle, where chitin (a mucopolysaccharide consisting primarily of poly-*N*-acetylglucose) is crosslinked to proteins.[69,70] If true, TC would be involved in organizing or anchoring random microfibrils. This, in fact, represents one of the major functions proposed for morula cells and is being investigated presently.

The decreases in TC yields observed when aqueous-based purification schemes were used may reflect TC oxidation and/or metal chelation. The disparate pH measurements after anaerobic cell lysis[18,35] might also hinge on such reactions; e.g., chelation of vanadium from the signet ring and/or compartment cells by *free* TC from the morula cells. A similar interpretation regarding the acidity of certain tunicate blood cell lysates has been offered by Hawkins et al.[40] and is supported by Hodgson and co-workers.[35a] Frank et al. have calculated that oxidation/hydrolysis of V(III) to V(IV) can account for only a fraction of the observed acidity ($1H^+/V$).[35] Specifically, blood cell lysates of some V-sequestering tunicates (*Phallusia julinea*)[35] exhibit a neutral pH, whereas others (*Ascidia ceratodes*, which produces large quantities of *free An*-1)[60] exhibit an acidic pH. Since exposure of free tunichrome to vanadium is expected to generate protons via oxidation/chelation, such discrepancies could reflect the differences in the level of chelated/oxidized TC existing in the various specimen submitted to pH measurements. Quantitation of free TC in such samples would clarify these issues. Careful consideration of the putative tunichrome–vanadium complex in vivo must account for its conditional formation constant (K_f^{cond}), which, in turn, hinges on pH and ligand stability. Well-devised experiments employing FACS could resolve many of the issues outlined above.

Carrano and co-workers[71] have reported an acid-catalyzed disproportionation of V(IV), promoted by the phenolate ligand salen, generating V(III) and V(V) in organic solvents. Recent work in our group[72] has demonstrated that pyrogallol, when reacted with $VO(acac)_2$ produces a crystalline dimeric V(III) complex. By analogy, tunichrome itself in blood cells may be capable of reducing V(IV) to V(III) under certain conditions. Elucidation of the relationship between tunichrome and vanadium requires further investigation. In particular, does *free* TC fulfill a key role in tunicate physiology itself or function simply as a V sequestering agent? Addressing such questions may provide an insight to the role of vanadium for tunicates and other animals. We expect to answer some of these basic questions upon completion of syntheses of unprotected tunichromes.[77]

Experimental Section

Materials and Methods. ^1H and ^{13}C NMR spectra were recorded on a Bruker WM-250 spectrometer (^{13}C at 62.9 MHz). $CDCl_3$ was passed through a short plug of basic alumina prior to use in order to remove traces of DCl that promote deacetylation of the *An*-Ac's. EPR spectra were measured on a Varian E-line spectrometer at 9.12 GHz and 10 MW power. DCI mass spectra were measured on a Ribermag R-10-10 spectrometer (CH_4 as the carrier gas unless stated otherwise); high-resolution FAB spectra were measured on a Kratos M-50 spectrometer (Xe ionizing gas, thioglycerol matrix) and high-resolution CI spectra were measured on a VG-70EQ spectrometer (CH_4 carrier gas). FTIR

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measurements were performed on an IBM IR-85 spectrophotometer equipped with an MCT detector, operating at 2-cm^{-1} resolution. Spectra were obtained as single-beam interferograms, Fourier transformed and corrected for KBr (blank), water vapor, and CO_2 absorption. UV–visible spectra were recorded on a Perkin-Elmer Model 320 spectrophotometer and were baseline corrected. CD spectra were obtained on a JASCO 500A spectropolarimeter operating in the single beam mode, driven by a JASCO DP500N data processor, and were corrected for baseline errors.

All solvents used in the isolation of underivatized *An*'s were HPLC grade and were dried by distillation from appropriate dehydrating reagents (P_4O_{10} for CH_2Cl_2, K_2CO_3 for *i*-PrOH and Mg for MeOH and EtOH). All chromatography solvents were degassed by successive cycles of sonication, evacuation, and purging with argon. All steps in the separation of *An*-1 were carried out under inert atmospheres (prepurified Ar). HPLC analyses were performed on a system composed of the following: a Perkin-Elmer Series 4 liquid chromatography pump, a PE 7500 professional computer, a Kratos Spectroflow 773 detector and a Spectra-Physics SP4100 computing integrator. Diode array analysis was performed by an LKB 2140 rapid spectral detector (spectral width 200–370 nm, scanning interval 0.4 s) linked to an IBM-PC for data analysis. CPC separations were accomplished with a Sanki CPC-B92-N instrument in tandem with a Sanki CPC-UVM-I detector. Vanadium atomic absorption analyses were performed on a Varian Model AA-875 instrument operating in the oven mode, with 2% HNO_3. Matrix effects were corrected for by the method of standard addition.[73] Elemental analyses were performed by Galbraith laboratories (Knoxville, TN).

Isolation of *An*-1 and *An*-1 Ac. Detailed descriptions of this work are available as supplemental material (see also ref 4 and 23).

Preparation of *An*-1 Me. A number of conditions (i.e. Mitsonobu, dimethyl sulfate, DBU/MeI, and methyl-*p*-toluatriazene/Al(O-*t*-Bu)$_3$) were employed in unsuccessful attempts to prepare *An*-1 Me. Only diazomethane/MeOH successfully provided the desired product, but all attempts to improve the yield of this reaction with various catalysts failed (i.e. SiO_2, BF_3·Et_2O, and $ZnCl_2$).

An-1 (LH-20 fraction, 18.3 mg) was dissolved in dry, degassed MeOH (8 mL) under an Ar atmosphere. Addition of an ethereal solution of CH_2N_2 (8 mL, 40 mmol) was accompanied by immediate evolution of N_2 and a color change from yellow to orange. The solution was stirred at room temperature for 45 min, at which time a second aliquot of CH_2N_2 (8 mL, 40 mmol) was added and the solution allowed to stir for an additional hour. Excess CH_2N_2 was quenched by careful addition of a 10% AcOH/Et_2O solution until N_2 evolution ceased. Purification by preparative TLC (SiO_2, 5% *i*-PrOH/CH_2Cl_2) and subsequent respotting of the *An*-1 Me fraction (orange fluorescent, 4.1 mg, 18%, R_f 0.48), revealed a reformation of many of the original impurities, indicating decomposition. The mass spectrum (DCI/NH_3) of this fraction displayed only one prominent peak at m/z 682 (M + 1), corresponding to an addition of nine methyls. The ^1H NMR spectrum ($CDCl_3$) confirmed the presence of a mixture, but all expected resonances were observed including numerous methoxy resonances (δ 3.8–4).

Preparation of *An*-1 Ac. Freshly distilled acetic anhydride (Ac_2O, 15 mL) was added to *An*-1 (CPC fraction, 101 mg, 182 mmol) with stirring. Pyridine (15 mL) was added slowly, accompanied by a solubilization of the material. The yellow solution was stirred at room temperature for 1 h and evaporated in vacuo to yield a yellow solid, which was taken up in CH_2Cl_2 and purified by preparative TLC (SiO_2, 4 × 500 μm plates, 5% i-PrOH/CH_2Cl_2). The product mixture is composed of numerous UV quenching and fluorescing bands. The *An*-Ac bands (R_f 0.34) were identified by their visible yellow color and yellow/orange fluorescence (UV_{366} detection). Prepurified *An*-1 Ac (83 mg, 50%) was separated from *An*-2 Ac by NP-HPLC (YMC gel SI-5μ, 1.5 × 40 cm, 2 mL/min, 4% i-PrOH/CH_2Cl_2, 300-nm detection) to yield analytically pure *An*-1 Ac (t_R 22.5 min).

An-1 Ac displayed the following characteristics: FAB/HRMS, $C_{46}H_{45}N_3O_{21}$; DCI/CH_4, MS fragmentations given in Table II; UV (MeOH) 280 nm (sh), 319 (ϵ 26 000); CD (MeOH) 233 nm ($\Delta\epsilon$ +1.5), 260 (–0.9), 300 (–0.9); IR (KBr) 3285 cm^{-1} (br), 1777, 1651, 1540, 1497; ^1H NMR ($CDCl_3$) Table I; ^{13}C NMR ($CDCl_3$, ^1H-decoupled and DEPT) δ 172.5 (s, N^{29}-acetyl C=O), 170.7 (s, 20), 168–167 (9 s, *O*-acetyl C=O's), 161.6 (s, 10), 143.5–143.3 (3 s, 3/5, 15/17, 25/27), 135.7–135.0 (3 s, 4, 16, 26), 133.4–131.7 (3 s, 1, 13, 23), 130.3 (d, 12), 129.0 (s, 11), 125.0 (d, 8), 121.6–121.5 (2 d, 2/6, 14/18), 117.7 (d, 24/28), 112.4 (d, 7), 55.6 (d, 21), 35.0 (t, 22), 22.9 (q, *N*-acetyl CH_3), 20.5–20.1 (9 q, *O*-acetyl CH_3's).

Ozonolysis of *An*-Acs. A mixture consisting of 50% each of *An*-1 and -2 Ac (560 μg, 0.60 μmol of *An*) was dissolved in CH_2Cl_2 (2 mL) and cooled to –78 °C. A steady stream of ozone (electrically generated) was bubbled through the solution for 5 min, followed by a stream of O_2 (2 min). The reaction mixture was quenched with Me_2S (1.5 mL) and evaporated (in vacuo). The presence of gallaldehyde triacetate (GAT) in the resulting mixture was confirmed by TLC and NMR comparisons with a synthetic sample. The GAT was purified by preparative TLC (SiO_2, 250 μ, 50% EtOAc/hexane, R_f 0.23, (dinitrophenyl)hydrazine positive) and dissolved in MeCN (15 mL). The UV spectrum of this solution exhibited an A_{249} = 0.73. Synthetic GAT has an ϵ_{249} 12 000, thus 0.92 μmol of GAT was produced, corresponding to 1.5 equiv of GAT/*An*-Ac. The 0.5 equiv missing might be due to incomplete ozonolysis and/or purification losses.

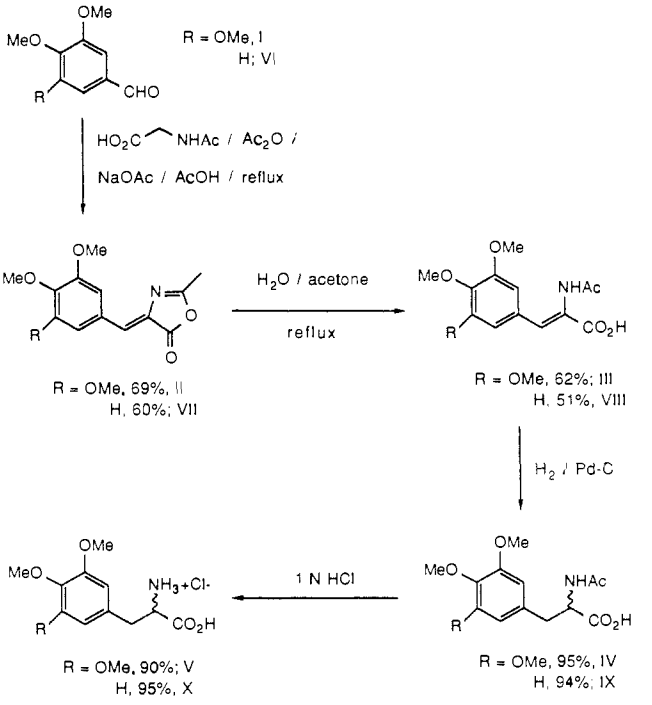

Figure 8. Syntheses of TMP and DMP·HCl.

Hydrolysis of *An*-1 Me. An ethereal solution of excess diazomethane (0.6 M, 5 mL, 3 mmol) was added to a MeOH (7.5 mL) solution of an *An*-1,2 mixture (19 mg, ca. 35 μmol, see above for reaction details). Subsequent to quenching and evaporation, the crude product mixture was refluxed in 4 N HCl overnight. TLC analysis of the hydrolysate (SiO_2, BuOH/AcOH/H_2O, 4:1:1, ninhydrin detection) indicated the presence of both 3,4-dimethoxy- and 3,4,5-trimethoxyphenylalanine (DMP and TMP, compared with synthetic standards, see below). RP-HPLC analysis (Chrompak CP Spher C18, 3 × 100 mm, 15% MeOH in 0.01 M NaOAc buffer, pH 4, 0.5 mL/min, 213-nm detection) yielded a complex chromatogram, which included two peaks with retention times identical with those of synthetic DMP and TMP (22.8 and 48.5 min, respectively). Co-injection of the hydrolysate with synthetic DMP and TMP confirmed their presence. Small amounts of both compounds were collected for ^1H NMR analysis (CD_3OD). Although the NaOAc resonance dominated the spectra and masked the aliphatic resonances, the methoxy and aromatic protons were observed easily and occurred at chemical shifts identical with those found in the synthetic amino acids: DMP δ 3.81, 3.84 (OMe's), 6.81, 6.85, 6.89, 6.92 (Ar's); TMP δ 3.74, 3.85 (OMe's), and 6.62 (Ar).

Synthesis of TMP·HCl and DMP·HCl (Figure 8). Preparation of 3,4,5-Trimethoxyphenyl Azalactone (TMPAL, II).[74] 3,4,5-Trimethoxybenzaldehyde (I, 2.95 g, 15.0 mmol), *N*-acetylglycine[75] (1.33 g, 11.4 mmol), and NaOAc (1.88 g, 22.3 mmol) were dissolved in a mixture of acetic anhydride (7.0 g, 38.3 mmol) and acetic acid (0.2 g) by stirring at 80 °C (20 min), producing an orange/brown solution. The reaction mixture was refluxed (143 °C) for 1 h to yield a dark red solution, which was stored at 0 °C overnight. The resultant brown solid mass was broken up with small amounts of ice water, suction filtered, and rinsed thoroughly (cold water). The yellow/orange solid was dried (in vacuo) to yield TMPAL (2.20 g, 69%, mp 152–153 °C): MS (DCI/NH_3) 278 (M + 1), 295 (M + 1 + H_2O), 312 (M + 1 + H_2O + NH_3); ^1H NMR (CD_3OD) δ 7.54 (s, 2 H, Ar H's), 7.09 (s, 1 H, Ar CH=C), 3.90 (s, 6 H, OCH_3's), 3.85 (s, 3 H, OCH_3), 2.41 (s, 3 H, N=CCH_3).

Preparation of α-Acetamido-3,4,5-trimethoxycinnamic Acid (ATMC, III).[74] Acetone (15 mL) and water (5 mL) were added to TMPAL (0.95 g, 3.4 mmol). The suspension was heated to reflux (with stirring). Acetone was added in small aliquots until dissolution of the solid was effected. The orange solution was refluxed overnight. Acetone was

removed by heating to leave an orange slurry from which the product was recrystallized (water added as needed) to yield pure ATMC (0.63 g, 62%, mp 202–203 °C): UV (MeOH) 294 (ε 17 500), 223 (31 000); ^{1}H NMR (CD_3OD) δ 7.47 (s, 1 H, Ar CH=C), 6.95 (s, 2 H, Ar), 3.86 (s, 6 H, OCH_3's), 3.80 (s, 3 H, OCH_3), 2.13 (s, 3 H, NAc).

Preparation of TMP-*N*-acetate (IV).[76] Methanol (10 mL) and catalyst (5% Pd–C, 10 mg) were added to ATMC (102.2 mg, 0.35 mmol) and stirred under an atmosphere of H_2 (ca. 1.5 atm) overnight. The reaction mixture was filtered through a pad of Celite ($^1/_4$ in.) and rinsed thoroughly with MeOH. Solvent was evaporated (in vacuo) to yield pure TMP-*N*-acetate (98.3 mg, 95%, white plates): ^{1}H NMR (CD_3OD) δ 6.52 (s, 2 H, Ar), 4.65 (dd, *J* = 8.8, 5.1 Hz, 1 H, CHNAc), 3.81 (s, 6 H, OCH_3's), 3.72 (s, 3 H, OCH_3), 3.14 (dd, *J* = 13.9, 5.1, 1 H, Ar CH_2), 2.88 (dd, *J* = 13.9 8.8, 1 H, Ar CH_2), 1.93 (s, 3 H, NAc).

Preparation of TMP·HCl (V).[76] TMP-*N*-acetate (84 mg, 0.28 mmol) was refluxed in 1 N HCl (5 mL) overnight. The water was evaporated (in vacuo) to yield pure TMP·HCl (73 mg, 90%): TLC (SiO_2, BuOH/H_2O/AcOH, 5:1:1, ninhydrin detection) R_f 0.25; MS (DCI/NH_3) 256 (M + 1), 273 (M + 1 + NH_3), 238 (M − NH_3), 212 (M − CO_2); ^{1}H NMR (CD_3OD) δ 6.59 (s, 2 H, Ar), 4.24 (dd, *J* = 7.9, 5.0 Hz, 1 H, $CHND_3^+$), 3.84 (s, 6 H, OCH_3's), 3.26 (dd, *J* = 14.5, 5.0, 1 H, Ar CH_2), 3.07 (dd, *J* = 14.5, 7.9, 1 H, Ar CH_2).

Preparation of 3,4-Dimethoxyphenyl Azalactone (DMPAL, VII). 3,4-Dimethoxybenzaldehyde (VI, 2.90 g, 17.5 mmol), *N*-acetylglycine[75] (1.53 g, 13.1 mmol), and sodium acetate (2.15 g, 26.2 mmol) were dissolved in a mixture of Ac_2O (7.0 g, 38.3 mmol) and AcOH (0.2 g) by stirring at 80 °C (10 min). The reaction mixture was refluxed (143 °C) for 1 h followed by evaporation (in vacuo) to yield nearly pure DMPAL (<10% 3,4-dimethoxybenzaldehyde) as an orange solid (2.08 g, 60%): ^{1}H NMR (CD_3OD) δ 8.00 (d, Ar H_2), 7.58 (dd, 1 H, Ar H_5), 7.08 (s, 1 H, Ar CH=C), 7.02 (d, 1 H, Ar H_4), 3.88 (s, 3 H, OCH_3), 3.83 (s, 3 H, OCH_3), 2.39 (s, 3 H, N=CCH_3).

Preparation of α-Acetamido-3,4-dimethoxycinnamic Acid (ADMC, VIII). Acetone (15 mL) and H_2O (5 mL) were added to DMPAL (1.8 g, 6.79 mmol). The suspension was heated to reflux, and acetone (ca. 25 mL) was added until a homogeneous solution was produced. The orange solution was allowed to reflux overnight. Acetone was removed by heating, and water (75 mL) was added. The solution was saturated with NaCl, extracted with EtOAc (3 × 25 mL), dried (brine, Na_2SO_4), filtered, and evaporated (in vacuo) to yield ADMC (0.92 g, 51%) as an orange solid: ^{1}H NMR (CD_3OD) δ 7.48 (s, 1 H, Ar CH=C), 7.28 (d, 1 H, Ar H_2), 7.19 (dd, 1 H, Ar H_5), 6.98 (d, 1 H, Ar H_4), 3.87 (s, 3 H, OCH_3), 3.84 (s, 3 H, OCH_3), 2.13 (s, 3 H, NAc).

Preparation of DMP-*N*-acetate (IX). Methanol (10 mL) and catalyst (5% Pd–C, 10 mg) were added to ADMC (85 mg, 0.32 mmol) and stirred under an atmosphere of H_2 (ca. 1.5 atm) for 2 days. The reaction mixture was filtered through a pad of Celite ($^1/_4$ in.) and rinsed thoroughly with MeOH. The solvent was evaporated (in vacuo) to yield nearly pure DMP-*N*-acetate (contaminated with ca. 5% ADMC, 82 mg, 94%): ^{1}H NMR (CD_3OD) δ 6.9–6.7 (m, 3 H, Ar's), 4.61 (dd, CHNDAc), 3.81 (s, 3 H, OCH_3), 3.79 (s, 3 H, OCH_3), 3.14 (dd, 1 H, Ar CH_2), 2.88 (dd, 1 H, Ar CH_2), 1.91 (s, 3 H, NAc).

Preparation of DMP·HCl (X). DMP-*N*-acetate (71 mg, 0.21 mmol) was refluxed overnight in 1 N HCl (5 mL). Water was evaporated (in vacuo) to yield pure DMP·HCl (67 mg, 95%): TLC (SiO_2, BuOH/H_2O/AcOH, 5:1:1, ninhydrin detection) R_f 0.06; ^{1}H NMR (CD_3OD) δ 7.0–6.8 (m, 3 H, Ar's), 4.24 (dd, 1 H, $CHND_3^+$), 3.26 (dd, 1 H, Ar CH_2), 3.10 (dd, 1 H, Ar CH_2).

Preparation of 4 H *An*-1 Ac. *An*-1 Ac (8 mg, 8.2 μmol) was dissolved in EtOAc (20 mL), and a catalytic amount of 5% Pd–C was added. The resulting suspension was stirred overnight under an atmosphere of H_2 (ca. 1.5 atm), filtered through a pad of Celite, and evaporated (in vacuo). TLC analysis (SiO_2, 6% *i*-PrOH/CH_2Cl_2) revealed that the product was present as a mixture of diastereomers (A, R_f 0.20; B, R_f 0.14), which were separated by preparative TLC (SiO_2, 6% *i*-PrOH/CH_2Cl_2) to yield pure 4 H *An*-1 Ac (A) (2.1 mg, 26%) [^{1}H NMR ($CDCl_3$) δ 6.97 (s, 2 H, Ar), 6.91 (s, 2 H, Ar), 6.84 (s, 2 H Ar), 6.51 (t, 1 H, N-9-H), 6.42 (d, 1 H, CHN*H*), 6.40 (d, 1 H, CHN*H*), 4.72 (dd, 1 H, C*H*NH), 4.46 (dd, 1 H, C*H*NH), 3.53 (m, 1 H, C-8-H), 3.25 (m, 1 H, C-8-H), 2.16 (dd, 1 H, Ar CH), 3.05–2.9 (m, 3 H, Ar CH's), 2.70 (dd, 2 H, C-7-H_2), 2.2 (s's, 27 H, OAc's), 1.75 (s, 3 H, NAc)] and pure 4 H *An*-1 Ac (B) (2.2 mg, 28%) as white solids: MS (DCI/NH_3) 980 (M + 1), 997 (M + 1 + NH_3); ^{1}H NMR ($CDCl_3$, assignments confirmed by COSY) δ 6.95 (s, 2 H, Ar), 6.91 (s, 2 H, Ar), 6.89 (s, 2 H, Ar), 6.79 (t, 1 H, N-9-H), 6.68 (d, 1 H, N-19-H), 6.23 (d, 1 H, N-21-H), 4.60 (dd, 1 H, C-8-H), 4.27 (dd, 1 H, C-21-H), 3.57 (m, 1 H, C-9-H), 3.26 (m, 1 H, C-8-H), 3.05–2.80 (m, 4 H, C-12 and C-22-H_2), 2.74 (t, 2 H, C-7-H_2), 2.2 (s's, 27 H, OAc's), 1.79 (s, 3 H, NAc); the broad band decoupled ^{13}C NMR spectrum of B displayed six aliphatic resonances as expected; NOE's were observed from the NAc methyl to N-21-H and the two C-22-H's and from a 2 H aromatic singlet to C-11-H.

MDPF Derivatization. Due to the small sample size (ca. 50 μg) and the large excess of NaOAc present in the TMP (from HPLC) obtained via hydrolysis and HPLC of *An*-1 Me, the MDPF reaction was initially performed with synthetic TMP, which was doped with a large excess of NaOAc. All conditions and data outlined below refer to this blank run (except where otherwise indicated).

A MeOH/H_2O (1:2, 1 mL) solution of TMP (2.8 mg, 9.5 μmol), NaOAc (6 mg) and Et_3N (10 μL) was warmed to 55 °C. Upon addition of MDPF (5.1 mg, 19.2 μmol) in warm solvent (0.2 mL), the solution immediately became yellow. The solution was stirred for 15 min (55 °C), and a second portion of MDPF (2.5 mg, 9.5 μmol) in warm solvent (0.1 mL) was added. The reaction mixture was stirred 10 additional min and cooled, and the solvent was evaporated (in vacuo). The yellow, fluorescent derivative (UV_{366} detection) was purified by preparative TLC (SiO_2, 20% MeOH/$CHCl_3$, R_f 0.3 for the natural derivative). The pure derivative displayed λ_{max}'s at 381, 284, and 270 (sh) nm and the following ^{1}H NMR spectrum: ^{1}H NMR (CD_3OD) δ 8.96 (s, 1 H, Ar C=CHN), 7.75 (dd, 2 H, Ar), 7.5–6.9 (m, 8 H, Ar's), 6.37 (s, 2 H, TMP Ar), 4.05 (m, 1 H, TMP-H$^\alpha$), 3.76 (s, 3 H, OCH_3), 3.64 (s, 6 H, OCH_3's), 3.3–3.0 (m, 2 H, Ar CH_2). The CD spectrum of the naturally derived TMP·MDPF displayed the following long wavelength CE's (MeOH): 380 nm (Δε +2.3, indicating S) and 327 (−0.8).

***An*-2 Ac.** *An*-2 (LH-20 fraction, 105 mg) was acetylated in the same manner as *An*-1 and initially purified by preparative TLC (SiO_2, 4% *i*-PrOH/CH_2Cl_2) to yield 106 mg (60%) of an *An*-1,2 Ac mixture. An attempt to find a suitable RP system was made with the aid of PESOS. The following conditions were employed in the overnight search: solvents (isoelutropic ratio, minimum/maximum %) MeOH (1.0, 20/58), MeCN (0.80, 20/58), THF (0.64, 2/20), H_2O (–, 40/58); search increment, 2% for both strong and weak solvents; minimum % height, 5%; minimum % area, 12%; lockout time, 0 min; analysis time (max), 15 min; equilibration time, 7.5 min; column, PE HS-3μ C_{18}, 0.5 × 3.5 cm; flow rate, 0.4 mL/min; detection, 320 nm. The optimal solvents resided in the 40% H_2O plane. Due to problems described above, the RP system was abandoned. Instead, *An*-1,2 Ac mixture was further purified by NP-HPLC (YMC gel, 3 μm SiO_2, 1.5 × 40 cm, 2 mL/min, *i*-PrOH/CH_2Cl_2/ClF_2CCCl_2F, 6:69:25, 300 nm detection) to yield nearly pure *An*-2 Ac (t_R 15.2 min, *An*-1 Ac 14.2 min, contaminated with <10% of a minor, uncharacterized *An*-Ac): MS (DCI/CH_4) 918 (M + 1), fragmentations in Table II; UV (MeCN) 321 nm (ε 27 000), 282 (26 500), 220 (sh); CD (MeCN) 304 nm (Δε −1.3), 286 (−1.9), 230 (+5.4); IR (KBr) 3265 cm^{-1} (br), 1774, 1651, 1501, 1371; ^{1}H NMR ($CDCl_3$) Table I; ^{13}C NMR ($CDCl_3$, broad band decoupled and DEPT) δ 172.4 (s, *N*-acetyl C=O), 170.9 (s, C-20), 168.2–166.8 (8 s, *O*-acetyl C=O's), 161.4 (s, C-10), 143.6–143.5 (2 s), 142.3 (s), 141.2 (s), 135.7 (2 s), 131.8 (s), 130.4 (d), 129.2 (s), 127.1 (d), 125.0 (d), 124.3 (d), 123.8 (d), 121.5 (d, 2 C's), 117.7 (d, 2 C's), 112.5 (d, C-7), 56.0 (d, C-21), 35.0 (t, C-22), 23.0 (q, *N*-acetyl CH_3), 20.6–20.0 (8 q's, *O*-acetyl CH_3's).

***An*-3 Ac.** *An*-3 (100 mg) was acetylated in the same manner as *An*-1 and initially purified by preparative TLC (SiO_2, 4% *i*-PrOH/CH_2Cl_2) to yield 72 mg (46%) of nearly pure *An*-3 Ac, which was further subjected to NP-HPLC (same as for *An*-2 Ac) to yield analytically pure *An*-3 Ac (t_R 12.5 min): MS (DCI/CH_4) 860 (M + 1), fragmentations in Table II; UV (MeCN) 319.5 nm (ε 24 500), 280 (sh, 20 500); CD (MeCN): 303 nm (Δε −1.6), 285 (−1.2), 272 (−1.3), 232 (+5.1); IR (KBr) 3295 cm^{-1} (br), 1775, 1652, 1503, 1429, 1372; ^{1}H NMR ($CDCl_3$) Table I; ^{13}C NMR ($CDCl_3$, broad band decoupled and DEPT) δ 172.3 (s, *N*-acetyl C=O), 171.0 (s, C-20), 168.2–166.8 (s's, *O*-acetyl C=O's), 161.5 (s, C-10), 143.4 (s), 142.2 (2 s), 141.1 (s), 140.5 (s), 135.8 (2 s), 134.8 (s), 131.8 (s), 130.1 (d), 129.2 (s), 127.1 (d), 124.3 (d), 123.7 (2 d), 123.4 (d), 121.6 (d), 120.4 (d), 117.7 (d), 113.0 (d), 55.8 (d, C-21), 35.0 (t, C-22), 22.9 (q, *N*-acetyl CH_3), 20.5–20.1 (s, *O*-acetyl CH_3's).

***Free* Tunichrome Assay (Natural Composition).** Acetic anhydride (e.g., 15 mL) and pyridine (e.g., 15 mL) were added sequentially to crude, lyophilized blood cells (e.g., 100 mg) and stirred at room temperature for 1 h. The resultant green solution was evaporated (in vacuo), taken up in CH_2Cl_2, and filtered. The *An*-Ac's were obtained by partial purification (preparative TLC, SiO_2, 4% *i*-PrOH/CH_2Cl_2) and analyzed by RP-HPLC (column: IBM-C_{18}, 1 × 25 cm, MeCN/MeOH/THF/H_2O, 27.2:24:1.3:47.5, 2.5 mL/min, 320-nm detection) and indicated the following composition of uncomplexed TC's: *An*-1 Ac (30%), *An*-2 Ac (50%), *An*-3 Ac (17%), minor components (2%).

Quantitation of *Free* Tunichrome and V in *A. nigra* Blood. Blood powder (22.7 mg, sept 1984) was acetylated in the usual manner. The *An*-Ac's were purified by preparative TLC (SiO_2, 4% *i*-PrOH/CH_2Cl_2, *An*-Ac's, orange/yellow fluorescence), and the dry *An*-Ac's weighed (4.8 mg). With use of the observed natural composition, a normalized molecular weight for the *An*-Ac's is calculated to be 925. Thus, the blood contains 0.3–0.4 μmol of *free tunichrome* per mg, assuming the normally

observed acetylation yield of 50-60%.

A separate sample of blood powder (2.805 mg, sept 1984) was incubated overnight in concentrated HNO_3 (glass distilled, Aldrich, 0.5 mL). HNO_3 was removed from the orange solution by heating overnight in a sand bath (130-140 °C). The residue was dissolved in 2% HNO_3 (10 mL) and a "spiked" sample consisting of this solution (3 mL) and a 1002 μg/mL V standard (Aldrich, 4 μL, spike 1.33 μg/mL) were prepared. These samples analyzed by AA to contain 2.10 and 3.35 μg V/mL. The spiked sample allowed correction of the blood sample for matrix contributions[73] (m = 0.94), yielding a final value of 2.23 μg V/mL. This corresponded to 0.16 μmol of V per mg of blood.

Composition of Precipitated Complex (*An*/V). The following solutions were prepared in degassed water (sparged with N_2 overnight) and adjusted to pH 3 with small amounts of 0.1 N HCl (unnecessary for III): I, 4.3 mM *An*-1 (LH-20 fraction); II, H_2O; III, 5.6 mM $VOSO_4 \cdot 3H_2O$. Five precipitate samples and three controls were prepared (order of addition I, II, and then III). The mixtures were incubated at room temperature (20 min) and centrifuged (6000 rpm, 30 min). Supernatants were withdrawn by pipette, and precipitates were washed with II (3 mL) and recentrifuged. Supernatants were combined, a portion of each was diluted (1:10 with solution A), and UV spectra obtained for each. Precipitates were lyophilized and weighed by microbalance; a portion was dissolved in 0.1 N HCl (5 mL, solution B) and then diluted (1:3) and subjected to UV analysis. Standardized solutions of *An*-1 provided the following UV parameters: pH 3 (ϵ_{330} 15 000), pH 1 (ϵ_{320} 11 300). Samples were prepared for atomic absorption analysis as follows: supernatant, 1 mL of A + 3 mL of 2% HNO_3; precipitate, 1.5 mL of B + 3 mL of 2% HNO_3. Matrix effects were determined on samples 2 and 6 spiked with 0.75 μg/mL vanadium (for both supernatant, m = 2.7, and precipitate, m = 2.8).

The sample destined for elemental analysis was prepared by addition of $VOSO_4$ (20 mL, 5.5 mM, pH 3) to *An*-1 (LH-20 fraction, 30 mL, 3.85 mM, pH 3). The precipitate was centrifuged (6000 rpm, 30 min), washed (H_2O, 15 mL, pH 3) and lyophilized to dryness.

Precipitate samples for FTIR analysis were prepared by addition of an excess of either NH_4VO_3 (16 mM, pH 3) or $VOSO_4$ (16 mM, pH 3) to *An*-1 (4 mM, pH 3) followed by centrifugation, washing and lyophilization.

Job's Analyses. A representative analysis is described. The following solutions were prepared in degassed water (sparged with N_2 overnight), and the pH adjusted to 7 with small amounts of 0.1 N NaOH, 1.00 mM *An*-3 (LH-20 fraction), 0.99 mM NH_4VO_3. Various amounts of *An*-3 and V(V) were combined and allowed to incubate 15 min (room temperature, under N_2) and the A_{630} was recorded.

***An*-C Analysis.** The green *An*-C (20 mg) was washed with water and centrifuged (4 × 5 mL). The pellet was dissolved (over a period of 1 h) in 0.1 N HCl and analyzed by UV-visible spectroscopy.

***Mm*-1 Ac.** Blood was obtained from *M. manhattensis* (collected from Cape Cod Canal by the Marine Biological Laboratories, Woods Hole, MA) by removal of a portion of the tunic from the animals' posterior followed by gentle squeezing to drain their body fluids. Lyophilized pellets were acetylated and preparative TLC (5% *i*-PrOH/CH_2Cl_2, R_f 0.1) followed by NP-HPLC (column: YMC gel 3μ SiO_2, 1.5 × 40 cm; 8% *i*-PrOH/CH_2Cl_2, 2 mL/min, 320-nm detection, t_R 22 min) provided pure *Mm*-1 Ac: MS (DCI/CH_4, Table II) 596 (M + 1), 624 (M + 1 + 28), 638 (M + 1 + 42, reaction with ketene), 100 (O=CCH_2NHAc); UV (MeCN), 320 nm (ϵ 12 100), 285 (10 900), 225 (sh, 13 100); ^1H NMR ($CDCl_3$) Table I.

***Mm*-2 Ac.** Acetylated *Molgula* blood was subjected to preparative TLC (5% *i*-PrOH/CH_2Cl_2, R_f 0.30) to yield pure *Mm*-2 Ac: MS (DCI/CH_4, Table II) 652 (M + 1), 680 (M + 1 + 28), 694 (M + 1 + 42, reaction with ketene), 156 [O=$CCHNHAcCH_2CH(CH_3)_2$]; UV (MeCN) 225 (sh, 12 700), 285 (13 400), 320 nm (ϵ 15 600); CD (MeCN) 290 nm ($\Delta\epsilon$ −0.50), 229 (+1.7); ^1H NMR ($CDCl_3$, Table I).

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Supplementary Material Available: Detailed descriptions for the isolation of *An*-1 and *An*-1 Ac, as well as NOE data (5 pages). Ordering information is given on any current masthead page.

Biomimetic Syntheses of Pretetramides. 1. Synthesis of Pretetramide by Tandem Extension of a Polyketide Chain

S. Gordon Gilbreath, Constance M. Harris, and Thomas M. Harris*

Contribution from the Department of Chemistry, Vanderbilt University, Nashville, Tennessee 37235. *Received January 11, 1988*

Abstract: Pretetramide (**4**), the fully aromatic naphthacenic precursor in the biosynthesis of 6-demethyltetracycline (**2**), has been synthesized in biomimetic-type fashion using a [3 + (2 × 2) + 1 + 2] strategy. Tandem additions of *tert*-butyl acetoacetate dianion to the bis(*N*-methyl-*N*-methoxyamide) (**9**) of 3-(1-pyrrolidinyl)glutaric acid followed by two spontaneous aldol cyclizations produced dihydroxynaphthalene diester **7b**. Conversion of **7b** to anhydride **14** was followed by addition of *tert*-butyl lithioacetate; dehydration gave enol-lactone **16**. The dilithium salt of 3-hydroxy-5-methylisoxazole was condensed with **16** to give the anthracene–isoxazole **23** after acidic workup. Treatment of **23** with a refluxing mixture of acetic and hydriodic acids containing red phosphorus produced pretetramide (**4**). The overall yield from **9** was 9.3%.

The tetracycline antibiotics, e.g., tetracycline (**1**) and 6-demethyltetracycline (**2**), have broad-spectrum activity against Gram-positive and Gram-negative bacteria and constitute one of the major classes of antimicrobials in use today. Their polyketide origin was first suggested by Robinson;[1] biosynthetic experiments with ^{14}C-labeled acetate gave labeling consistent with tetracyclines being formed by head-to-tail assembly of acetate units.[2,3] Later studies with [1,2-^{13}C]-, [1-^{13}C,$^2H_3$]-, and [1-^{13}C,$^{18}O_2$]acetate confirmed this finding and defined the folding pattern of the putative decacarbonyl acyclic precursor of the tetracyclic ring system (Scheme I).[4-6] Polyketide metabolites commonly arise by polymerization of malonyl CoA with acetyl-CoA serving as the initiator; the tetracyclines are unusual in that the chain initiator is a malonate unit, although uncertainty remains as to whether

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