Isolation of Unstable Tunichromes from Tunicate Blood *via* Protection–Deprotection

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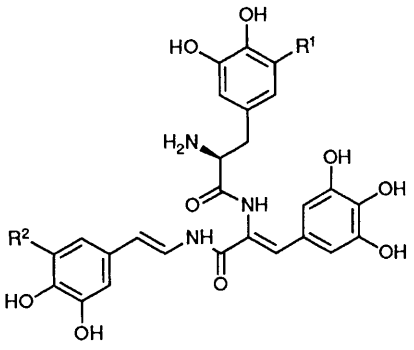
An isolation method based on protection–deprotection, with possible applicability to other unstable natural products, is described for tunichromes.

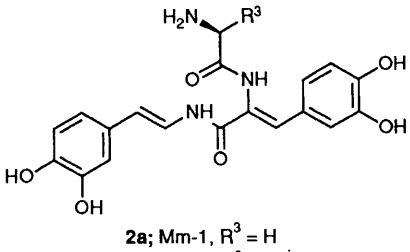
Frequently the isolation of labile natural products is hampered by the fact that in nature they exist in a stabilizing microenvironment, *e.g.*, in the presence of antioxidants, in membranes, *etc.*, and that during the isolation process the compounds become separated from such stabilizing factors. A typical case in the tunichromes An-1–3, **1a–c,** polyphenolic tripeptides with enamide moieties,[1–3] which are present in the blood of the vanadium-assimilating tunicates (or sea-squirts) *Ascidia nigra*, *A. ceratodes, etc.*[4,5] Exposure of collected blood to air results in decomposition of pigments and browning in colour. Although they coexist in the same blood cell with vanadium,[6] addition of V^{V} or V^{IV} to tunichromes leads to immediate precipitation. Thus, the initial isolation of unprotected tunichromes An-1–3 required special treatments,[2] such as carrying out the entire process, including collection of blood, under argon and anhydrous conditions, immediate lyophilization of collected blood and use of centrifugal partition chromatography (CPC).[7] Final purification was carried out with reverse phase HPLC with 80% loss.

Characterization of the second group of tunichromes, Mm-1 and 2, **2a** and **2b**,[3] from the Fe-assimilating tunicates *Molgula manhattensis*, thus included an acetylation step; however, since free tunichromes cannot be regenerated from peracetates (or permethylates), peracetylation (permethylation) is unsuited for ongoing studies aimed towards clarifying the biological roles of tunichromes, including reactions with V and Fe. In the recent total syntheses of unprotected tunichromes An-1,[8] Mm-1 and Mm-2,[9] the hydroxy and amino groups were protected with t-butyldimethylsilyl (TBDMS)[10] and butoxycarbonyl (Boc)[11] groups during the synthesis; the free unstable tunichromes were obtained by N,O-deprotection and fractional precipitation with a CH_2Cl_2 based solvent system. Application of these processes directly to the blood not only led to an efficient and greatly simplified isolation, but also gave additional unknown pigments related to tunichromes. Freeze-dried blood pellets from two specimens, *A. ceratodes* and *A. nigra*, were examined. The lyophilized blood pellet[2] (100 mg) of *A. ceratodes* containing An-1 **1a** was ground in a glove-bag with an equal amount of Na_2SO_4, treated with TBDMSCl and imidazole in CH_2Cl_2 and stirred under Ar for 36 h (Scheme 1).† As the TBDMS protection proceeded, tunichrome, which is insoluble in CH_2Cl_2, gradually dissolved in the solvent leading to an intensification of the green-yellow colour of the solution. This protection step converted the highly polar and unstable tunichrome to the stable nonpolar nonakis-TBDMS-An-1, which could be isolated by preparative TLC (hexane–EtOAc, 10 : 1; R_f 0.1). Subsequent protection of the amino group by $(Boc)_2O$ made the derivative more stable and nonpolar. A distinct yellow band was separated by

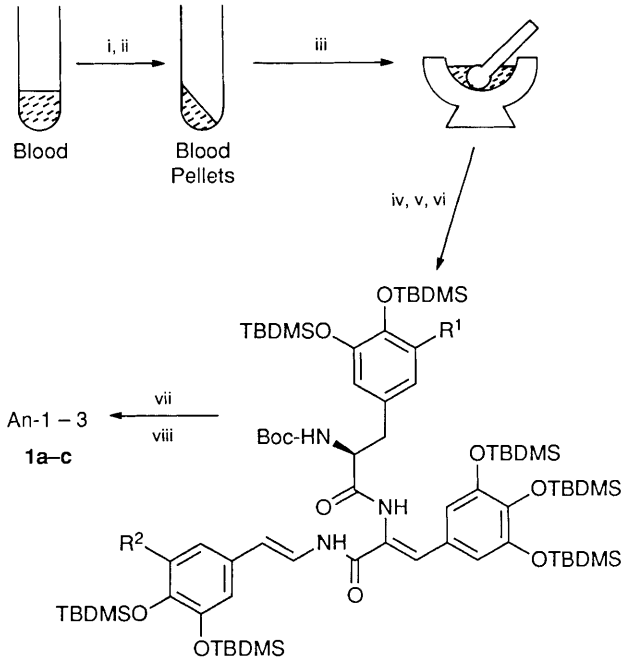
† *Experimental procedure:* The tunicate blood pellet (1.36 g) from *A. nigra* was ground in a mortar with anhydrous Na_2SO_4 (1.36 g) in a glove-bag, yielding a green powder (the role of the sulphate is to facilitate grinding and serve as dehydrant). To a mixture of the powder, TBDMSCl (12 g) and imidazole (5.5 g), placed in a 100 ml round bottom flask, was added dry CH_2Cl_2 (20 ml). After being stirred at room temperature for 44 h under Ar, greenish-black materials containing cell lysates were filtered off. The filtrate was diluted with EtOAc (50 ml), washed with water (3×) and saturated aq. $NaHCO_3$ (3×), and dried over $MgSO_4$. Concentration gave a mixture of TBDMS-protected Ans as a viscous oil, which was dissolved in dry CH_2Cl_2 (20 ml) and $(Boc)_2O$ (3 g) was added in one portion. The solution was stirred at room temperature for 8 h. After concentration, three distinct bright yellow bands were separated by flash chromatography (hexane–EtOAC, 10 : 1), followed by preparative TLC (3×: hexane–EtOAc, 10 : 1) to give the protected tunichromes An-1 (42 mg), An-2 (17 mg) and An-3 (10 mg). The protected An-1 (20 mg) was submitted to the known deprotection conditions[8] to yield natural An-1 (4.1 mg, 62%).



1a; An-1, $R^1 = R^2 = OH$
1b; An-2, $R^1 = H$, $R^2 = OH$
1c; An-3, $R^1 = R^2 = H$



2a; Mm-1, $R^3 = H$
2b; Mm-2, $R^3 = Bu^i$



3a; An-1, $R^1 = R^2 = OTBDMS$
3b; An-2, $R^1 = H$, $R^2 = OTBDMS$
3c; An-3, $R^1 = R^2 = H$

Scheme 1 Isolation of tunichromes An-1–3 from blood pellets by protection–deprotection. i, Centrifugation (1500 rpm, 5 min); ii, lyophilization (3 days); iii, anhydrous Na_2SO_4; iv, TBDMSCl, imidazole, CH_2Cl_2; v, $(Boc)_2O$, CH_2Cl_2; vi, separation; vii, TFA, CH_2Cl_2; viii, 48% aq. HF–Py

flash chromatography and subsequent preparative TLC to give the desired **3a** (5 mg). The ^1H NMR and mass spectra of the compound were identical to those of authentic protected (±)-An-1, prepared during the total synthesis of (±)-An-1.[8] The advantage of this method is demonstrated in the facile separation of the closely related tunichromes An-1, 2 and 3 (protected form) by preparative TLC (hexane–EtOAc, 10 : 1; R_f, An-1: 0.44; An-2: 0.4; An-3: 0.33). Deprotection of both BOC and TBDMS groups in protected An-1 was carried out as previously reported[8] by trifluoroacetic acid (TFA) and 48% aq. HF–pyridine (Py), respectively. Purification by fractional precipitation afforded An-1, as evidenced by comparisons of ^1H NMR, UV and mass spectra of unprotected and peracetylated samples with those of authentic samples.

This scheme has made it possible to isolate various tunichromes from stored blood pellets for further investigations.‡ Four new bright yellow pigments (less polar than **1a–c**) with UV absorptions similar to the tunichromes (by diiode array detection), λ_{max}/nm 340 (ε 19600), 285 (sh), 245 (sh), 210 (ε 68000 $dm^3\ mol^{-1}\ cm^{-1}$), were also separated by this method. They may correspond to the extremely labile compounds noted previously;[2] characterizations are in progress. The current scheme is applicable to cases where hydrolysis of peracetates or demethylation[2–4] are problematic, as for the tunichromes. It should be possible to apply the present method to the isolation of other classes of unstable natural products containing hydroxy and/or amino groups.

‡ The extraction yield cannot be determined since the tunichrome content is season (and animal) dependent. Protected tunichrome can be obtained in *ca.* 5–10 weight % of blood pellet, which corresponds to a tunichrome content of *ca.* 3 weight % of blood pellet.

For a typical experimental procedure for isolating An-1, 2 and 3 see footnote.†

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