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Chemistry

Tunichlorin: A nickel chlorin isolated from the Caribbean tunicate *Trididemnum solidum**

[ascidian/sea squirt/nickel(II) 2-devinyl-2-hydroxymethylpyropheophorbide a/marine natural products/semisynthesis]

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ABSTRACT **Tunichlorin, a blue-green pigment isolated from the Caribbean tunicate *Trididemnum solidum*, has been identified as nickel(II) 2-devinyl-2-hydroxymethylpyropheophorbide a by chemical and spectroscopic methods, with confirmation by partial synthesis of dimethyl tunichlorin from chlorophyll a. Nickel chlorins have been reported from geological sources but not from living organisms. Its occurrence in a living system suggests a metabolic role for tunichlorin and may clarify the selective accumulation of nickel by marine tunicates. Because *Trididemnum* tunicates are associated with algal symbionts, tunichlorin may arise directly from the tunicate, from symbiotic algae, or from tunicate modification of an algal chlorin.**

Until the mid-1960s, nickel was viewed as a toxin with no significance in metabolic processes (1). It has since been found necessary for optimal activity of several enzyme systems (1, 2) and has been identified as an essential micronutrient for microorganisms (1), higher plants (2), and animals, including humans (3).

Although there is much interest in the chemistry and specific function of nickel within enzyme systems (1), they remain poorly understood. Factor F430 (1, 4), a yellow porphynoid pigment associated with methanogenic bacteria, has recently been found to be bound to methyl coenzyme M reductase and to be involved in the final reduction step in methanogenesis. No other fully characterized nickel compounds have been shown to participate in enzymatic processes, although several enzyme systems contain nickel (1).

The selective accumulation of metals (e.g., vanadium, iron) by marine tunicates (ascidians) has been studied for many years (5). Identification of tunichrome B-1 (6), a labile polyphenolic compound implicated in tunicate vanadium sequestration, provided the first real understanding of metal sequestration and accumulation in tunicates. Recently, marine tunicates have been shown to accumulate nickel selectively and to contain fixed nickel/cobalt ratios (7), suggesting a metabolic role for the two micronutrients in tunicates.

This report describes tunichlorin (Structure **1**), nickel(II) 2-devinyl-2-hydroxymethylpyropheophorbide a, isolated from the Caribbean tunicate *Trididemnum solidum* (family Didemnidae). Tunichlorin appears to be the second nickel porphynoid reported from a living system (see F430 above) and the only nickel chlorin yet isolated from a living organism.

MATERIALS AND METHODS

Chromatography. HPLC separations were conducted with Beckman and Waters HPLC pumps and UV detectors (254 nm) and Altech, Waters, and DuPont preparative and semi-preparative HPLC columns. TLC was done with both silica gel and reversed-phase (RP) C_{18} precoated glass plates (0.25 mm; Merck) containing UV indicators. Column chromatographic separations were carried out by using 0.05- to 0.2-mm mesh silica solid support (Brinkmann) and methanol, chloroform, hexane, and ethyl acetate mobile-phase mixtures.

Spectroscopy. ^1H and ^{13}C NMR spectra were recorded on Varian, Nicolet, and General Electric Fourier transform NMR spectrometers at 200, 300, 360, 400, and 500 MHz; typically, "100%" C^2HCl_3 was used as a solvent. Mass spectra were obtained with VG 70 SE and ZAB SE mass spectrometers in the fast atom bombardment ionization mode with a dithiothreitol/dithioerythritol (5:1, vol/vol; "magic bullet") matrix (8). Electronic absorption spectra were obtained on a Perkin–Elmer Lambda-3 UV-VIS absorption spectrometer, with spectroscopic grade methanol, chloroform, and dichloromethane as solvents. IR spectra were obtained with a Perkin–Elmer model 30S FTIR spectrophotometer; samples were examined neat after application to a thin-film cell. Optical rotatory dispersion (ORD)–CD spectra were recorded in methanol on a JASCO J-20 ORD–CD spectrometer.

Tunicate Collection and Tunichlorin Isolation. The tunicate classified as *Trididemnum solidum* (9) was collected by scuba techniques at −10 to −40 m off St. George's Cay, Belize, Central America, and preserved in isopropyl alcohol until work-up. Preserved tunicate (≈82.6 kg) was homogenized, in batches, in a Waring Blendor with isopropyl alcohol preservative (82.6 liters) and methanol/toluene (≈296 liters; 3:1, vol/vol). The homogenates were filtered, combined with 1.0 M sodium nitrate (132.2 liters), and allowed to partition between aqueous (larger) and toluene (smaller) fractions.

The aqueous layer was then extracted with dichloromethane, and the organic solvent was removed at reduced pressure. The residue (148.7 g, 0.18%) was subjected to normal-phase preparative HPLC [silica gel, 0–40% MeOH-$CHCl_3$ (vol/vol) elution with $CHCl_3$/MeOH, 3:2 (vol/vol)] to give a solid (9.25 g).

The column eluate above [1.25 g, $CHCl_3$/MeOH, 3:2 (vol/vol)] was purified by repeated C18 RP-HPLC [MeOH/H_2O, 4:1 (vol/vol); then, MeOH/H_2O/Et_3N, 80:20:0.1 (vol/vol), pH 7.5 with HOAc buffer]. Final purification by hexane trituration to remove nonpolar hydrocarbon contaminants, precipitation from methanol by water addition, and silica gel Sep-Pak chromatography (loading with chloroform, washing with hexane and acetone, and eluting with methanol) gave 1.25 mg (1.11×10^{-5}%) of **1**.

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Abbreviations: RP-HPLC, reversed-phase high-performance liquid chromatography; LR- and HRFABMS, low- and high-resolution fast atom bombardment mass spectrometry; NOE, nuclear Overhauser effect; ORD, optical rotatory dispersion.

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Analysis. Calculated for $C_{32}H_{33}N_4NiO_4$: M_r, 595.1855. Found: 595.1829 [M + H, high-resolution fast atom bombardment mass spectrometry (HRFABMS)].

Methanolysis Product Isolation. The column eluate above [8.00 g, $CHCl_3$/MeOH, 3:2 (vol/vol)] was subjected to C18 RP-HPLC [MeOH/H_2O, 4:1 (vol/vol)]. Hexane trituration of the material obtained (698 mg) gave partially purified solid tunichlorin [nickel(II) 2-devinyl-2-hydroxymethylpyropheophorbide a; Structure 1], which was dissolved in 0.07 M methanolic *p*-toluenesulfonic acid (50 ml); the solution was stirred in the dark under nitrogen at 40°C for ≈30 min (4) with monitoring by silica gel TLC [$CHCl_3$/MeOH, 15:1 (vol/vol)] until the reactant spot (R_f = 0.20) disappeared completely [R_f (product) = 0.84]. The reaction was stopped by cooling the mixture to 20°C, adding dichloromethane (150 ml), and washing the organic phase with 0.1 M aqueous sodium hydroxide and water (both 3 × 50 ml). The blue-green organic layer was taken to dryness at reduced pressure to give 496 mg of methanolysis product. Purification by C18 RP-HPLC [MeOH/H_2O, 24:1 (vol/vol)] gave 7.36 mg, and final purification by normal-phase silica gel HPLC [C_6H_{14}/EtOAc, 1:1 (vol/vol)] gave 6.4 mg (8.50×10^{-6}%) of dimethyl tunichlorin [methyl nickel(II) 2-devinyl-2-methoxymethylpyropheophorbide a; Structure 2].

Analysis. Calculated for $C_{34}H_{37}N_4NiO_4$: M_r, 623.2167. Found: 623.2147 (M + H, HRFABMS).

Deuteriomethanolysis to give heptadeuteriodimethyl tunichlorin [Structure 3; *m/z* 629 (M) by low-resolution electron ionization mass spectrometry] was conducted in an analogous fashion, except that all fluid volumes were reduced by 90% and methanol-d_4 was used instead of methanol.

Deuterium Exchange. A solution of 2 (0.9 mg) in 3% deuteriosulfuric acid in methanol-d_4 (5.6 ml) was stirred in the dark under nitrogen at 22°C for 22 hr (10), diluted with chloroform (3 ml), and washed with 1 M sodium bicarbonate (3 ml) and water (3 × 3 ml). Repeated Sep-Pak silica gel chromatography of the dried chloroform extract [loading with $CHCl_3$, washing with C_6H_{14}, $CHCl_3$, and $CHCl_3$/EtOAc, 1:1 (vol/vol), and eluting with EtOAc] gave 0.9 mg (100%) of decadeuteriodimethyl tunichlorin (Structure 4): LRFABMS *m/z* 633 (M + H).

Monomethyl Demetallotunichlorin (Structure 5). A solution of **2** (0.9 mg) in concentrated sulfuric acid (1.0 ml) was stirred for 4 hr at 22°C (11), then diluted carefully with water (19 ml), and extracted with dichloromethane (3 ml). Purification by Sep-Pak silica gel chromatography as for **4** above gave 0.2 mg (22%) of **5**.

Analysis. Calculated for $C_{33}H_{37}N_4O_4$: M_r, 553.2814. Found: 553.2812 (M + H, HRFABMS).

Remetalation to Dimethyl Tunichlorin (Structure 2). A solution of **5** (0.2 mg) in chloroform (1.0 ml), combined with saturated methanolic nickel(II) acetate solution (5 ml), was stirred in the dark under nitrogen at 70°C for 2 hr (12). Complete remetalation was assessed by the disappearance of the absorption spectroscopy band at 665 nm and the reappearance of a band at ≈645 nm. The reaction mixture was extracted with water (3 × 3 ml), and the organic layer was taken to dryness at reduced pressure to give **2** (0.2 mg; 90%): LRFABMS *m/z* 623 (M + H).

Dimethyl Copper(II) Demetallotunichlorin (Structure 6). The demetalation product **5** was treated as described above, except that copper(II) acetate replaced nickel acetate, and the temperature and reaction time were decreased (40°C, 30 min) to give **6** (0.2 mg; 90%).

Analysis. Calculated for $C_{34}H_{37}CuN_4O_4$: M_r, 628.2112. Found: 628.2079 (M + H, HRFABMS).

Pheophytin a (Structure 8). A solution of chlorophyll a (Structure 7; 0.5 mg; Sigma) in methanol/chloroform [2 ml; 1:1 (vol/vol)] was acidified by dropwise addition of concentrated sulfuric acid until its color changed from blue- to gray-green (11). The chloroform layer was washed with water (3 × 3 ml) and then evaporated to dryness to give **8** (0.5 mg; 100%) (11, 13): LRFABMS *m/z* 872 (M + H).

Methyl Pheophorbide a (Structure 9). A solution of **8** [87 mg, from spinach (11)] in 3% methanolic sulfuric acid (3 ml) was stirred in the dark under nitrogen at 22°C for 22 hr (10, 11), diluted with chloroform (2 ml), washed with water (3 × 2 ml), and purified by silica gel column chromatography to give **9** (80 mg; 92%) (11): LRFABMS *m/z* 607 (M + H).

Methyl Pyropheophorbide a (Structure 10). A solution of **9** (80 mg) in collidine (3 ml) was stirred in the dark under nitrogen at 165°C for 100 min, evaporated to dryness, and purified by silica gel column chromatography to give **10** (75 mg; 93%) (11).

Analysis. Calculated for $C_{34}H_{37}N_4O_3$: M_r, 549.2865. Found: 549.2862 (M + H, HRFABMS).

Methyl Nickel(II) Pyropheophorbide a (Structure 11). A solution of **10** (1.0 mg) in chloroform/methanol saturated with nickel(II) acetate [2 ml; 1:1 (vol/vol)] was stirred in the dark under nitrogen at 70°C for 2 hr (12), washed with water (3 × 3 ml), and purified by RP-HPLC to give **11** (0.4 mg; 40%): ^{1}H NMR 9.33 (s), 9.10 (s), 8.17 (s), 7.76 (m), 6.00 (m), 4.85 (dd), 4.29 (q), 4.00 (m), 3.62 (q), 3.60 (s), 3.51 (s), 3.16 (s), 3.14 (s), 2.43 (m), 2.24 (m), 2.14 (m), 1.62 (t), 1.56 (d).

Analysis. Calculated for $C_{34}H_{35}N_4NiO_3$: M_r, 605.2062. Found: 605.2032 (M + H, HRFABMS).

Methyl 2-Devinyl-2-formylpyropheophorbide a (Structure 12). A solution of **10** (76.5 mg) in dioxane/water [1:1 (vol/vol); 4 ml] was stirred with osmium tetroxide (2.0 mg in 135 μl of CCl_4, 0.075 M) and sodium periodate (59.8 mg, 2 M) at 20°C for 4 hr. The mixture was combined with chloroform (3 ml) and washed with dilute aqueous acetic acid and then with water (both 3 × 3 ml). Purification by silica gel column chromatography [C_6H_{14}/EtOAc, 7:3 (vol/vol)] gave **12** (35.1 mg; 46%).

Analysis. Calculated for $C_{33}H_{35}N_4O_4$: M_r, 551.2658. Found: 551.2670 (M + H, HRFABMS).

Methyl 2-Devinyl-2-hydroxymethylpyropheophorbide a (Monomethyl Demetallotunichlorin; Structure 5). A solution of **12** (15.0 mg) in methanol (3 ml) was stirred with sodium borohydride (6.0 mg) at 20°C for 1 min. The reaction was stopped by the rapid addition of chloroform (3 ml) and repeated vigorous washing with 5% aqueous acetic acid and then with water (both 3 × 3 ml). Purification by silica gel column chromatography gave **5** (13.2 mg; 88%).

Analysis. Calculated for $C_{33}H_{37}N_4O_4$: M_r, 553.2815. Found: 553.2789 (M + H, HRFABMS).

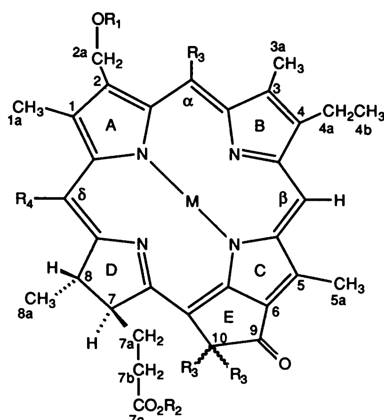
Methyl Nickel(II) 2-Devinyl-2-hydroxymethylpyropheophorbide a (Structure 13). A solution of **5** (6.0 mg) in methanol/chloroform saturated with nickel(II) acetate [1:1 (vol/vol); 4 ml] was stirred at 70°C for 3 hr, cooled to room temperature, combined with 2 ml of chloroform, washed with water (3 × 5 ml), and purified by silica gel column chromatography [C_6H_{14}/EtOAc, 7:3 (vol/vol] to give **13** (2.7 mg; 41%).

Analysis. Calculated for $C_{33}H_{35}N_4NiO_4$: M_r, 609.2011. Found: 609.1969 (M + H, HRFABMS).

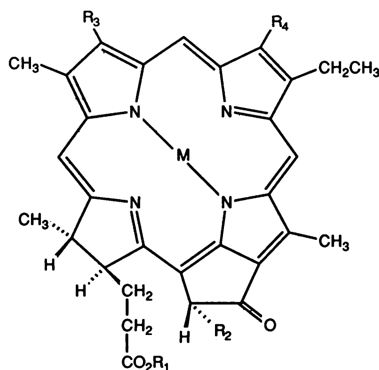
Methyl Nickel(II) 2-Devinyl-2-methoxymethylpyropheophorbide a (Dimethyl Tunichlorin; Structure 2). A solution of crude **13** (1.3 mg) in 0.07 M methanolic *p*-toluenesulfonic acid was stirred at 40°C for 10 min, combined with chloroform (3 ml), and washed with water (4 × 5 ml). Purification by C18 RP-HPLC [MeOH/H_2O, 9:1 (vol/vol)] gave semisynthetic **2** (0.4 mg, 30%): ^{1}H NMR 9.30 (s), 9.13 (s), 8.17 (s), 5.42 (dd), 4.83 (dd), 4.28 (m), 3.99 (m), 3.59 (s), 3.59 (s), 3.47 (s), 3.13 (s), 2.42 (m), 2.25 (m), 2.12 (m), 1.60 (t), 1.54 (d); LRFABMS *m/z* 623 (M + H).

Isolation of Other *Trididemnum* Pigments. Eight other green pigments found during tunicate processing were purified by repeated silica gel column chromatography ($CHCl_3$, MeOH,

C_6H_{14}, and EtOAc combinations as mobile phases), followed by C18 RP-HPLC, and those compounds having free carboxylic acid substituents were isolated as their methyl esters. Five pigments were obtained from the toluene extract: chlorophyll a [Structure **7**, in variable amounts; UV and chromatographic properties identical with authentic sample (11)]; pheophytin a [Structure **8**, 0.127% wet weight of tunicate; UV, ^1H, and ^{13}C NMR, chromatographic properties, and high-resolution mass spectrometry (HRMS) identical with authentic sample (11, 13); HRFABMS 871.5784 (M + H)]; 10-hydroxypheophytin a [4.71×10^{-3}%; UV, ^1H NMR, and HRMS structure determination (10–12); HRFABMS 887.5662 (M + H)]; pyropheophytin a [tentative identification; 3.79×10^{-3}%; UV, chromatographic properties, and low-resolution mass spectrometry (LRMS) identical with authentic sample (11); FABMS *m/z* 813]; isopropyl pheophorbide a [3.17×10^{-3}%; UV, ^1H NMR, and HRMS structure determination; HRFABMS 635.3224 (M + H)]. The chloroform/methanol (3:2) HPLC fraction yielded pheophorbide a [2.80×10^{-6}%; melting point, UV, chromatographic properties, and HRFABMS identical with authentic sample (11, 13); HRFABMS 607.2917 (M + H) for methyl ester]; 10-hydroxypheophorbide a [tentative identification; 5.60×10^{-7}%; UV, chromatographic properties, and HRMS identical with authentic sample (11); HRFABMS 623.2863 (M + H) for methyl ester]; and an apparent tunichlorin isomer [not identified; 1.12×10^{-7}%; LRFABMS *m/z* 595 (M + H)].



		R_1	R_2	R_3	R_4	M
1:	tunichlorin	H	H	H	H	Ni
2:	dimethyl tunichlorin	CH_3	CH_3	H	H	Ni
3:	heptadeuteriodimethyl tunichlorin	CD_3	CD_3	H	D	Ni
4:	decadeuteriodimethyl tunichlorin	CD_3	CD_3	D	D	Ni
5:	monomethyl demetallotunichlorin	H	CH_3	H	H	H_2
6:	dimethyl copper(II) demetallotunichlorin	CH_3	CH_3	H	H	Cu



	R_1	R_2	R_3	R_4	M
7:	phytyl	CO_2CH_3	—CH=CH$_2$	CH_3	Mg
8:	phytyl	CO_2CH_3	—CH=CH$_2$	CH_3	H_2
9:	CH_3	CO_2CH_3	—CH=CH$_2$	CH_3	H_2
10:	CH_3	H	—CH=CH$_2$	CH_3	H_2
11:	CH_3	H	—CH=CH$_2$	CH_3	Ni
12:	CH_3	H	—CHO	CH_3	H_2
13:	CH_3	H	—CH$_2$OH	CH_3	Ni
14:	phytyl	CO_2CH_3	—CH=CH$_2$	CHO	Mg
15:	H	H	—CH=CH$_2$	CH_3	H_2

RESULTS

Tunichlorin (Structure 1). Underivatized tunichlorin (**1**) could not be sufficiently purified to provide good NMR data, but a typical "chlorin" porphynoid absorption spectrum [$\lambda_{max}(CH_2Cl_2)$ 389 nm (ε, 33,960), 416 nm (ε, 41,612), 641 nm (ε, 43,478)] was observed (14). The similar intensities of the UV and visible absorption maxima confirmed the reduced form of at least one of its four pyrroles (14). The split "Soret" band, diagnostic of an isocyclic cyclopentanone (E) ring (14), suggested a porphynoid system similar to that of chlorophylls a (**7**) and b (**14**).

Dimethyl Tunichlorin (Structure 2). Because purification of underivatized tunichlorin was arduous and decomposition was rapid even with low-temperature storage under nitrogen, structural work was carried out on the more stable and more easily purified methanolysis derivative. The major methanolysis product **2** showed FABMS ions at *m/z* 623 (+ ion) and 621 (− ion) and an M + H ion (HRFABMS, see above) consistent with the formation of two methoxyl groups during methanolysis. This suggested at least two carboxyl or reactive alcohol functionalities, as in dimethyl tunichlorin (**2**).

The presence of nickel, originally suggested by mass spectral data, was confirmed by atomic absorption spectroscopy (0.9-mg sample: observed, 8.1% Ni; calculated, 8.9%) and by demetalation and remetalation of **2** (see above).

The absorption spectrum of **2** is indistinguishable from that of unmodified tunichlorin, indicating that methanolysis had not disrupted the extended aromaticity of the tunichlorin porphynoid nucleus. ^{13}C APT (attached proton test) (Fig. 1) and DEPT (distortionless enhancement by polarization transfer) spectra of **2** show the presence of 34 carbons (ketone, carboxyl, and 15 other quaternary carbons; three aromatic and two aliphatic methines; four methylenes and one oxymethylene; five methyls and two methoxyls).

The ^1H NMR spectrum of **2** (Fig. 2) is typical of a chlorin (13). The appearance of only three downfield methine peaks (1 H each) suggests that one of the compound's four bridging methines is functionalized, consistent with an isocyclic cyclopentanone (E) ring. The 9.31, 9.13, and 8.17 ppm peaks were assigned as β, α, and δ, respectively, on the basis of

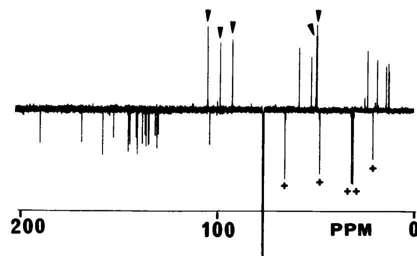


FIG. 1. ^{13}C NMR APT spectrum of dimethyl tunichlorin (Structure **2**). Assignments of CH_3 vs. CH (▼) (up) and of CH_2 (+) vs. C (down) were reached based on DEPT results (not shown).

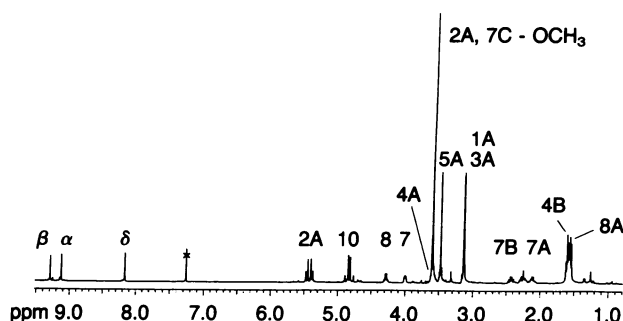
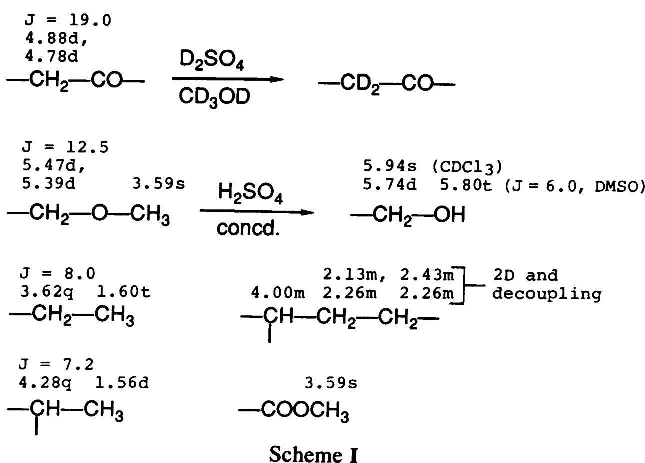


FIG. 2. ^{1}H NMR spectrum of dimethyl tunichlorin (Structure 2).

deuterium exchange (11) and by analogy to NMR spectra of known chlorophylls (11, 13). An AB quartet centered at 4.82 ppm (2 H, J = 19.0 Hz) disappeared on deuterium exchange (Scheme I, 2→4), confirming both the E ring ketone and the C-10 methylene. [Scheme I shows functional groups and their ^{1}H NMR absorptions in dimethyl tunichlorin (2). J is in Hz; D, deuterium; DMSO, dimethyl sulfoxide.]

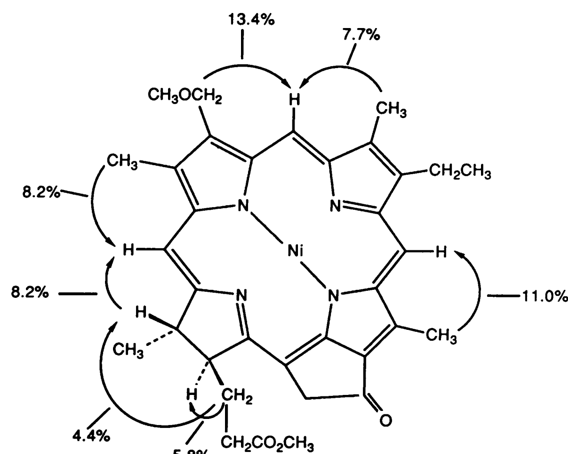


Scheme I

A quartet at 4.28 ppm (1 H), coupled to the doublet at 1.56 ppm (3 H), indicates a $CHCH_3$ unit (Scheme I). The multiplet at 4.00 ppm (1 H), coupled to the multiplets at 2.13 and 2.26 ppm (1 H, 2 H), and the coupling of the multiplets at 2.43, 2.26, and 2.13 ppm (1 H, 2 H, 1 H) suggest a $CHCH_2CH_2$ unit (Scheme I). These data and the appearance of the 4.00- and 4.28-ppm multiplets characteristic of H-7 and H-8 of chlorophyll a (13) confirm the presence of a reduced D ring as in chlorophyll a. Moreover, irradiation of the 4.28-ppm proton showed nuclear Overhauser effect (NOE) enhancement of the δ proton at 8.17 ppm.

Five sharp singlets between 3.00 and 4.00 ppm suggest three aryl-substituted methyls as well as two methoxyls added during methanolysis. The ^{1}H NMR spectrum of deuteriomethanolysis product 3 lacked the two sharp singlets near 3.59 ppm, confirming them as methoxyls added during methanolysis. The other three sharp singlets were confirmed as methyl porphynoid substituents by NOE studies of their proximity to the adjacent porphynoid methines (Scheme II). [Scheme II shows dimethyl tunichlorin (2) and NOE data.] Thus, irradiation of the 3.48- and 3.14-ppm methyls showed NOE enhancement of the β and α/δ protons, respectively, locating methyls on C-1, C-3, and C-5 as in chlorophyll a.

The ^{1}H NMR spectrum of 2 also contains a quartet at 3.62 ppm (2 H) coupled to a triplet at 1.60 ppm (3 H), indicating an aryl ethyl substituent (Scheme I). With methyl groups at C-1, C-3, and C-5, the ethyl group should be at either C-2 or C-4; it was located at C-4 by NOE studies (Scheme II). An asymmetrically substituted methylene was suggested by an AB quartet centered at 5.43 ppm (2 H, J = 12.5 Hz), which collapsed to a singlet (2 H) on hydrolysis to give 5. Coupling of this methylene to the adjacent hydroxyl proton was demonstrated by ^{1}H NMR spectroscopy in deuteriodimethyl sulfoxide, confirming the hydroxymethyl functionality (Scheme I). Assignment of the hydroxymethyl unit to C-2 was made from its NOE enhancement of the α proton (Scheme II).

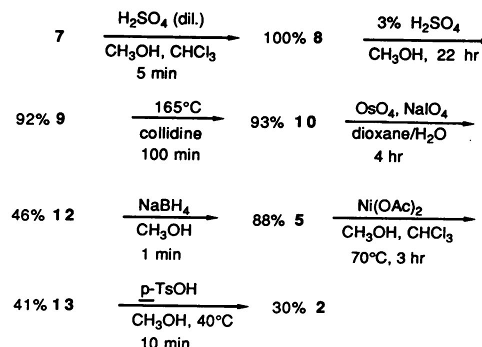


Scheme II

The relative stereochemistry at C-7 and C-8 was established by ^{1}H NMR spectroscopy: H-7 and H-8, with no coupling, must be trans; the 7a methylene protons showed NOE enhancement of H-8, to confirm them as cis. Comparison of the ORD spectra of tunichlorin and of methyl pyropheophorbide a (15) established an absolute stereochemistry identical with that of chlorophyll a.

All NMR assignments reported were confirmed by the proton–carbon heterocorrelation spectrum of tunichlorin, and the ^{1}H NMR chemical shifts of dimethyl tunichlorin (Fig. 1) are strikingly close to those of the related semisynthetic model compound methyl nickel(II) pyropheophorbide a (11) (see *Materials and Methods*).

Finally, the ^{1}H NMR, IR, ORD, CD, and LRFABMS spectra and chromatographic properties (C18 RP-HPLC and silica gel TLC) are identical for natural dimethyl tunichlorin and semisynthetic methyl nickel(II) 2-devinyl-2-methoxymethylpyropheophorbide a, prepared in 5% overall yield from chlorophyll a by the route shown in Scheme III. [Scheme III shows conversion of chlorophyll a (7) to dimethyl tunichlorin (2). Ts, tosyl.]



Scheme III

Other *Trididemnum* Green Pigments. Chlorophyll a, pheophytin a, and pheophorbide a from tunicate extracts

were compared by chromatography, NMR spectroscopy, and mass spectrometry with authentic samples (11, 13). Both isopropyl pheophorbide a and 10-hydroxypheophytin a were identified by their respective UV, ^{1}H NMR, and mass spectral properties (10, 12). Pyropheophytin a and 10-hydroxypheophorbide a were assigned tentative structures on the basis of UV, chromatographic, and mass spectral data and their similarity to authentic samples produced during the course of (spinach) chlorophyll a derivatization (11). A small quantity of a blue-green pigment resembling tunichlorin in UV, chromatographic, and mass spectral behavior appears to be a tunichlorin isomer.

No additional green pigments were isolated from *Trididemnum* processing; no chlorophyll b or chlorophyll b degradation products, no other metalated chlorins, no 2-hydroxymethylchlorins, and no tunichlorin phytyl ester were found.

DISCUSSION

Tunichlorin (**1**) resembles pyropheophorbide a (**15**), a degradation product of chlorophyll a (11), but it differs in two respects. First, tunichlorin is the nickel chelate, while pyropheophorbide a exists as the free base; second, the C-2 substituent of tunichlorin is a hydroxymethyl group, while that of pyropheophorbide a is a vinyl group.

Because *T. solidum* coexists with an algal symbiont (16–19), the possibility must be considered that tunichlorin is an algal rather than a tunicate pigment. Algal chlorophylls and other pigments have been examined in great detail (11, 17), however, and no nickel porphynoid has previously been reported as an algal natural product. It seems unlikely, therefore, that tunichlorin is a primary algal product. It appears to be a tunicate-modified algal chlorophyll, and indeed tunicate utilization of algal products has been demonstrated for CO_2-derived photosynthesized molecules (18, 19).

The identification of tunichlorin may represent an important step toward understanding the role of nickel in tunicate and animal metabolism. Many metals chelate more easily with porphynoids than does nickel, and vigorous synthetic conditions are usually necessary to facilitate nickel chelation in chlorins (12), making serendipitous nickel incorporation in intact organisms highly unlikely. "Natural" nickel chelation with porphynoids has previously been reported only under "geological" conditions of high temperature and/or pressure (20–22). Furthermore, there are no known nickel chlorin natural products other than tunichlorin, and only F430 (4, 7) has been identified as a nickel porphynoid natural product. Thus, tunichlorin is probably not a serendipitous porphynoid degradation product. Rather, it appears reasonable that tunichlorin has a specific metabolic function in tunicates. This conclusion is reached in spite of the remarkably low concentration of tunichlorin in the tunicate (10^{-5}%), which is apparently an order of magnitude lower than that of tunichrome B-1, already regarded (6) as strikingly low.

An extensive search for compounds related to tunichlorin in *T. solidum* extracts yielded only chlorophyll a and known chlorophyll a degradation products, including pheophytin and pheophorbide a. No tunichlorin phytyl ester, no tunichlorin analogues containing other metals, and no other chlorins with hydroxymethyl functionalities were found, suggesting that tunichlorin itself may be a biologically "functional" compound.

The occurrence of hydroxymethyl chlorin substituents is quite unusual and has apparently only been reported in one other chlorin natural product—acrylochlorin, an iron-containing cofactor in the nitrate reductases of *Pseudomonas aeruginosa* (23, 24). It is intriguing that F430, the only other known nickel porphynoid natural product, also plays a role in a reductive process—that is, in the final reduction of the methyl-sulfur bond in methylcoenzyme M to yield methane and the corresponding thiol (1, 4). Tunichromes also take part in reductive processes (6). By analogy to such reducing pigments, tunichlorin may be involved in a reductive process.

Two distinct algal symbionts of tunicates have been reported (16–19, 25). Blue-green algae (cyanophytes) produce chlorophyll a but not chlorophyll b (16), and *Prochlorons* (obligate tryptophan auxotrophs) produce both chlorophylls a and b (17–19, 25). The isolation of chlorophyll a, but not chlorophyll b, from *T. solidum* confirms that its associated alga is a cyanophyte rather than a *Prochloron*. Establishment of independent and combined laboratory cultures of *T. solidum* and its associated cyanophyte would allow detailed studies of the biosynthesis of tunichlorin and provide further insight into both nickel utilization by tunicates and alga-tunicate symbiosis.

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