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Chemistry

Acyl tunichlorins: A new class of nickel chlorins isolated from the Caribbean tunicate *Tridemnum solidum*

[ascidian/sea squirt/nickel(II)/marine natural product]

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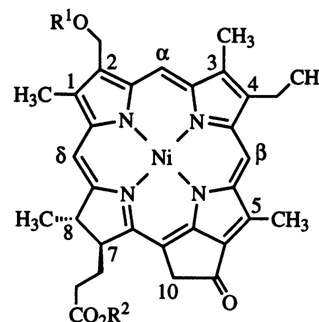
ABSTRACT **A new class of nickel-containing chlorins (acyl tunichlorins) has been isolated from the Caribbean tunicate *Trididemnum solidum*. The structures of 28 of these nickel(II) hydroporphyrins were elucidated using mass spectrometry, one- and two-dimensional NMR spectroscopy, and chemical degradation/derivatization. Unique structural features of these compounds include the diversity of aliphatic side chains, which are derived from $C_{14:0}$ to $C_{22:6}$ fatty acids, and their location at an unprecedented position at C-2a on the hydroporphyrin nucleus. No chlorins with ester-linked acyl side chains at C-2a have been reported previously. Although the exact biological role that these compounds play in *T. solidum* remains unknown, acyl tunichlorins represent the only nickel-containing chlorins to be isolated from a living system and are the C-2a acyl derivatives of tunichlorin, a nickel chlorin reported by this laboratory in 1988.**

Among transition metals known to be of biological significance are vanadium, chromium, manganese, iron, cobalt, copper, zinc, molybdenum, tungsten, and nickel (1). Nickel was one of the last to be accepted, primarily because its abundance in the lithosphere and biosphere prevents natural nickel deficiency (1). Although initially viewed as a toxin with no biological merit, nickel has since been identified as an essential trace element in bacteria, plants, and animals (2, 3). In the marine environment, nickel uptake has been reported in phytoplankton, oysters, mussels, scallops, and tunicates (4). Tunicates have been found to contain fixed nickel/cobalt ratios, suggesting that nickel may play an important metabolic role in these marine urochordates (5).

In spite of the accumulating data on the chemistry and function of nickel within biological systems, only one nickel-containing metabolite has been previously isolated from a marine organism. Tunichlorin, Chart 1, a blue-green pigment produced by the Caribbean tunicate *Trididemnum solidum*, was reported from this laboratory in 1988 and identified as nickel(II) 2-devinyl-2-hydroxymethylpyropheophorbide a by chemical and spectroscopic method (6). Underivatized tunichlorin could not be sufficiently purified to provide good NMR data, so structural work was carried out on the more stable and more easily purified methanolysis derivative, dimethyltunichlorin (6). More recently, tunichlorin has been reported from the South Pacific sea hare *Dolabella auricularia* (7). Although the exact role that tunichlorin plays in these invertebrates is unknown, its presence in two different phyla suggests that it has biological relevance rather than being just an unusual degradation product.

In our investigation of the role that tunichlorin may play in *T. solidum*, a search for tunichlorin-related compounds has now identified a number of novel nickel-containing chlorins. These compounds are structurally unique in that they exist as a mixture of esters derived from different fatty acids in the C-2a position. No naturally occurring chlorins with ester-linked acid side chains at this position have been reported previously. The present compounds are designated acyl tunichlorins, because tunichlorin itself appears to be the biosynthetic precursor of these uniquely substituted nickel chlorins. Isolation and structure elucidation of these compounds are described in the present manuscript.



1 tunichlorin	$R^1 = R^2 = H$
2 dimethyltunichlorin	$R^1 = R^2 = CH_3$
3a-30a acyl-tunichlorins	R^1 (see Table 1); $R^2 = H$
3b-30b acyl-tunichlorin methyl esters	R^1 (see Table 1); $R^2 = CH_3$

Chart I

MATERIALS AND METHODS

General. HPLC separations were conducted with a Waters HPLC apparatus employing a photodiode array UV detector (420 nm) and Altech semipreparative HPLC columns. Analytical thin-layer chromatography was performed on Merck silica gel plates with F-254 indicator. The acyl tunichlorins appeared as brilliant, blue-green bands. Column chromatographic separations were carried out on 0.05–0.2-mm mesh silica gel eluted with CH_2Cl_2-MeOH or hexane-EtOAc-Et_3N mixtures. IR spectra were recorded in $CHCl_3$ with a Mattson Galaxy 5020 spectrophotometer. Radial chromatography was performed using a Harrison Research Chromatotron, model 7924T. Methyl ester standards were purchased from Sigma.

NMR Spectroscopy. One- and two-dimensional NMR spectra were recorded on either a General Electric GN-500 or a Varian VXR-500 NMR spectrometer in $CDCl_3$ using residual $CHCl_3$ (δ = 7.26 ppm, ^1H; 77.0 ppm, ^{13}C) as an internal

Abbreviations: HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond coherence; COSY, correlation spectroscopy; GC/MS, gas chromatography/mass spectrometry; FABMS, fast atom bombardment mass spectrometry; HRFABMS, high-resolution FABMS; DMDS, dimethyl disulfide; EI, electron ionization; m/z, mass to charge ratio.

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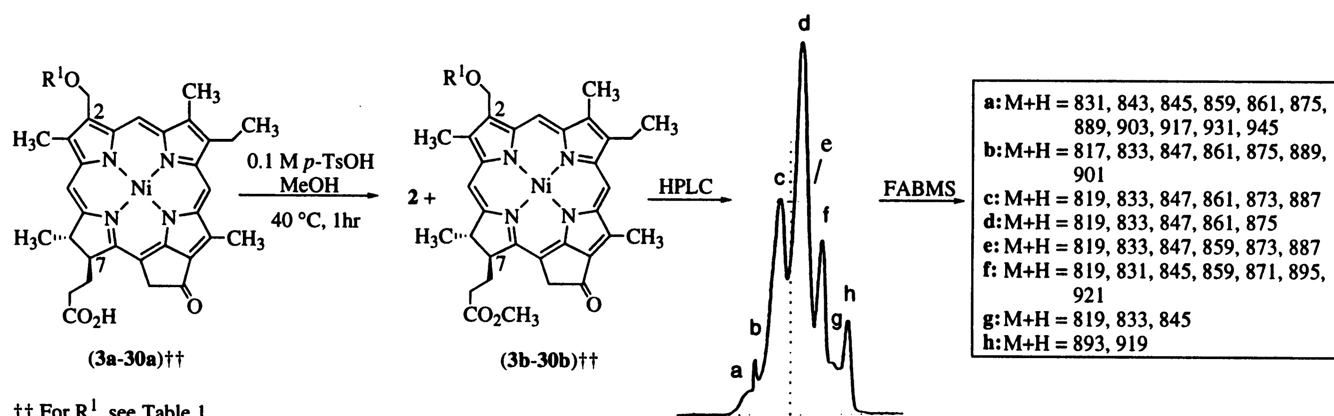


Scheme I

standard. Heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond coherence (HMBC), and correlation spectroscopy (COSY) experiments were performed on the VXR-500 using a Zens 5-mm Inverse Detection Probe. Standard Varian pulse sequences and parameters were used. Chemical shifts (δ) are in ppm; multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Coupling constants (J) are reported in Hz.

Gas Chromatography and Mass Spectrometry (GC/MS). Mass spectrometry was performed by the Mass Spectrometry Lab, School of Chemical Sciences, University of Illinois. Fast atom bombardment mass spectra were obtained on a ZAB SE mass spectrometer with a dithiothreitol/dithioerythritol [3:1 (vol/vol); "magic bullet"] (8) matrix. GC/MS analysis for saturated methyl esters (**3c–12c**) was performed on a Hewlett Packard (HP) GC/MS model 5809 [electron impact (EI), 70 eV] mass spectrometer attached to an HP-5 capillary column (10 m × 0.2 mm I.D.). GC/MS analysis for unsaturated methyl esters (**13c–30c**) was performed on a VG-70 VSE (EI, 70eV) mass spectrometer attached to a J & W Scientific DB-5 capillary column (30 m × 0.25 mm I.D.). The injection volumes were 1 μl (2–10 ng of material). Mass spectra were recorded with an Opus 3.1X data system (repetitive scans, m/z 50–500, approximately 0.8 s per scan).

Tunicate Collection and Acyl Tunichlorin Isolation. The tunicate, *T. solidum*, was collected by SCUBA at −10 to −40 m off St. George's Cay, Belize, Central America. Frozen tunicate (sample IKE89 I-VI-89-1-1, 20 kg) was homogenized in batches in a Waring blender with methanol/toluene [3:1 (vol/vol)]. The homogenates were filtered, combined with 1 M sodium nitrate, and partitioned into aqueous and toluene layers. The organic layer was removed and evaporated, and the residue was subjected to repeated silica gel column chromatography using either a MeOH-CH_2Cl_2 gradient (0–100% MeOH) or hexane-EtOAc with 5% triethylamine (0–100% EtOAc) to yield a crude acyl tunichlorin mixture, which gave FABMS M+H ions at m/z = 805, 819, 833, 847, and 861 for **3a–7a**. Acyl tunichlorins represented approximately 0.01% of the crude residue.

Acyl Tunichlorin Methyl Esters. The crude acyl tunichlorin mixture (242 mg) above was dissolved in 0.01 M methanolic *p*-toluenesulfonic acid (10 ml; Scheme I). The solution was stirred at 40°C for approximately 1 h until the acyl tunichlorin spot (R_f = 0.20) disappeared completely [R_f (product) = 0.84, 1:1 hexane-EtOAc]. Methylene chloride was added to the reaction mixture, and the blue-green organic phase was washed with water, dried over magnesium sulfate, and evaporated. Purification using silica gel (2:1 hexane-EtOAc) gave a mixture (15 mg) of acyl tunichlorins esterified at C-7c and as a side product dimethyltunichlorin (**2**) (R_f = 0.50, 8.5 mg). The acyl tunichlorin methyl ester mixture was then subjected to normal phase HPLC (7:3 hexane-EtOAc, flow rate 1.8 ml/min, 420 nm). FABMS of the chromatographic fractions obtained gave M+H ions corresponding to the methyl esters of acyl tunichlorins (**3b–30b**; Scheme I).

Acyl Tunichlorin Methyl Esters: Mass Spectrometry and NMR Studies on 3b–7b (m/z = 819, 833, 847, 861, 875). The acyl tunichlorin methyl ester mixture appeared as a blue-green glass-like solid: UV(CH_2Cl_2) λ_{max} 389 (33,960), 416 (41,612), and 641 nm (43,478). High resolution FABMS calculated for $C_{47}H_{61}N_4NiO_5$ M_r 819.3995 (M + H), found 819.3947; for $C_{48}H_{63}N_4NiO_5$ M_r 833.4151 (M + H), found 833.4087; for $C_{49}H_{63}N_4NiO_5$ M_r 847.4308 (M + H), found 847.4250; for $C_{50}H_{65}N_4NiO_5$ M_r 861.4464 (M + H), found 861.4420; and for $C_{51}H_{67}N_4NiO_5$ M_r 875.4621 (M + H), found 875.4595. ^{1}H NMR ($CDCl_3$): 9.34 (s, 1H, HC(α)), 9.11 (s, 1H, HC(β)), 8.21, (s, 1H, HC(δ)), 6.12 (d, J = 12.5, 1H, H_aC(2a)), 6.08 (d, J = 12.5, 1H, H_bC(2a)), 4.88 (d, J = 19, 1H, H_aC(10)), 4.83 (d, J = 19, 1H, H_bC(10)), 4.29 (q, J = 7.0, 1H, HC(8)), 4.02(m, 1H, HC(7)), 3.61 (m, 2H, H_2C(4a)), 3.59 (s, 3H, H_3C(7c)), 3.49 (s, 3H, H_3C(5a)), 3.17 (s, 3H, H_3C(1a)), 3.15 (s, 3H, H_3C(3a)), 2.45 - 2.08 (m, 4H, H_2C(7a, 7b)), 2.39 (t, J = 7.5, 2H, H_2C(2c)), 1.61 (t, J = 7.5, 3H, H_3C(4b)) 1.44 (d, J = 7.0, H_3C(8a)), 1.5–0.7 (acid side chains).

Isolation and GC/MS Analysis of the Acyl Tunichlorin Acid Side Chains. Following HPLC purification (above) the acyl tunichlorin methyl esters (**3b–30b**; 1–10 mg) were combined with 0.5 M NaOH in MeOH (2 ml) and heated at 65°C for 5 min under nitrogen. The reaction mixture was cooled to room temperature, evaporated under a stream of nitrogen, and then combined with 2 ml of 14% $BF_3 \cdot Et_2O$ in MeOH (2 ml), heated for 20 min at 65°C, and cooled to room temperature. Hexane (3 ml) was added, followed by water (3 ml). The aqueous layer was extracted twice more with hexane (3 ml), and the organic layers were combined and evaporated under nitrogen. The resulting mixture was separated by preparative thin-layer chromatography into two fractions, one containing the esterified acid side chains (**3c–30c**) (R_f = 0.5, 9:1 hexane/EtOAc) and the other containing cleaved acyl tunichlorin (R_f = 0.0, 9:1 hexane/EtOAc).

The methyl esters **3c–12c** were analyzed using the Hewlett Packard GC/MS. The samples were introduced without a split, and the column temperature was programed as follows: 2 min isothermal at 50°C, 40°C/min to 200°C. They were identified as straight chain, saturated methyl esters by coinjection with their corresponding standards.

Dimethyl Disulfide (DMDS) Derivatization. The DMDS derivatives of **13c–20c** were prepared following the procedure of Buser *et al.* (9) and were analyzed on the VG 70-VSE. The samples were introduced with a split ratio of 40:1 with the

column temperature programed from 80 to 250°C at 20°C/min.

Analysis of Polyunsaturated Acid Side Chains: 21c-26c and 28c. The polyunsaturated methyl esters were separated from the monounsaturated and saturated methyl esters using radial argentation chromatography. A 1-mm silver nitrate-impregnated radial chromatography plate was prepared as follows. Silver nitrate (5 g) was dissolved in water (90 ml). Silica gel 60 PF-245 with calcium sulfate (45 g) was then added to this solution, and the silica/silver nitrate mixture was used to prepare a 1-mm radial chromatography plate. The plate was stored in the dark until use. The mixture of methyl esters (5 mg) was applied to the plate and eluted with a 10–100% EtOAc in hexane gradient. The fractions obtained were analyzed using the VG 70-VSE. The samples were introduced without a split, and the column temperature was programed from 80 to 250°C at 15°C/min.

4,4-Dimethyloxazoline Derivatives. The oxazoline derivatives of the polyunsaturated methyl esters were prepared following the procedure of Fay and Richli (10) and analyzed on the VG 70-VSE. The samples were introduced without a split, and the column temperature was programed from 80 to 250°C at 15°C/min.

Analysis of Polyunsaturated Acid Side Chains: 27c, 29c, and 30c. The methyl esters **27c**, **29c**, and **30c** could not be detected after argentation chromatography. It is presumed that, due to their low abundance, they adhered to the plate. The oxazoline derivatization was therefore performed on the entire mixture of methyl esters. The samples were analyzed on the VG 70-VSE. The samples were introduced without a split, and the column temperature was programed from 80 to 250°C at 15°C/min. The resulting compounds were difficult to resolve; however, the oxazoline adducts for compounds **27c**, **29c**, and **30c** were easily detected.

RESULTS

FABMS of the blue-green pigment isolated from *T. solidum* revealed a homologous series of high molecular weight nickel-containing chlorins, acyl tunichlorins, with observed M+H ions at m/z = 805, 819, 833, 847, and 861 (ratio 1:1:3:2:5). The extreme polarity of these hydroporphynoids made purification difficult, and contamination by paramagnetic nickel could be inferred in the NMR spectra. Therefore, structural work was carried out after preparing the methyl ester of the propionic acid side chain at C-7c, Scheme I (6). A singlet at 3.59 ppm (3H) in the ^{1}H NMR spectrum, an increase of 14 Da in the molecular masses, and a shift in R_f from 0.20 to 0.84 (1:1 hexane-EtOAc), implied esterification of the free acid had occurred. Final purification was then achieved using silica gel.

Complete separation of the esterified acyl tunichlorin congeners proved to be extremely arduous. Argentation chromatography (11), radial chromatography on a silica-coated rotor, MgO·celite (12), and powdered sugar (13) failed to completely separate these compounds. Only partial separation was achieved using normal phase HPLC with 7:3 hexane/EtOAc as the mobile phase (Scheme I). FABMS of the chromatographic fractions obtained from the HPLC separation revealed several additional nickel-containing chlorins that had not been seen in the original mass spectra due to their minute concentrations. Based upon the molecular formulae established by high resolution FABMS, the acyl tunichlorins appeared to differ from tunichlorin itself by an aliphatic side chain containing one oxygen atom.

One- and two-dimensional NMR experiments were performed on acyl tunichlorin mixtures, since the compounds could not be completely separated. To determine the location of the aliphatic side chain, the NMR spectra (^{1}H, COSY, HMBC, HMQC) of a mixture of acyl tunichlorins with M+H ions at m/z = 819, 833, 847, 861, and 875 (**3b–7b**), was compared with that of dimethyltunichlorin, **2**. Aside from the presence of many aliphatic proton resonances (δ = 0.7–1.5 ppm), only one critical difference in the NMR of these compounds was noted. In the acyl tunichlorin mixture, the 2a methylene AB quartet was found at δ 6.10 (J = 12.5 Hz), and the ^{13}C NMR resonance for C-2a was observed at δ 56.0. This was in contrast to the 2a methylene AB quartet of dimethyltunichlorin, which was centered at δ 5.43 (J = 12.5 Hz) and was attached to a carbon that appeared at δ 65.5. The difference in chemical shift of the 2a-methylene protons in acyl tunichlorin suggested that the side chain was located at C-2a. This was unequivocally established using long range heteronuclear NMR correlations. HMBC data clearly indicated the presence of two carbonyl carbons in the ester region (^{13}C, δ = 173.8, 174.5). The 7a,b protons were correlated to the ester carbonyl at δ 173.8, while the α protons in the acyl group at δ 2.39 and the methylene protons on C-2a were correlated to the ester carbonyl at δ 174.5, confirming that the side chains were located on C-2a (Fig. 1). No naturally occurring chlorins with ester linked aliphatic side chains have been reported, nor has substitution of any sort been previously found at C-2a.

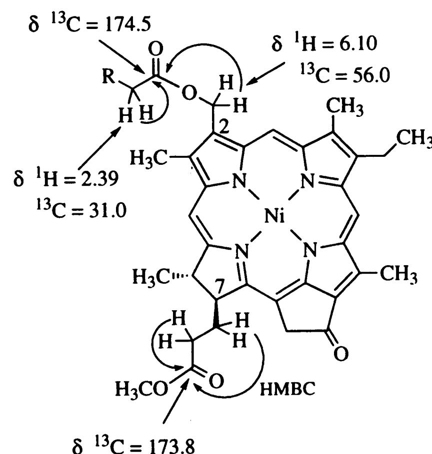
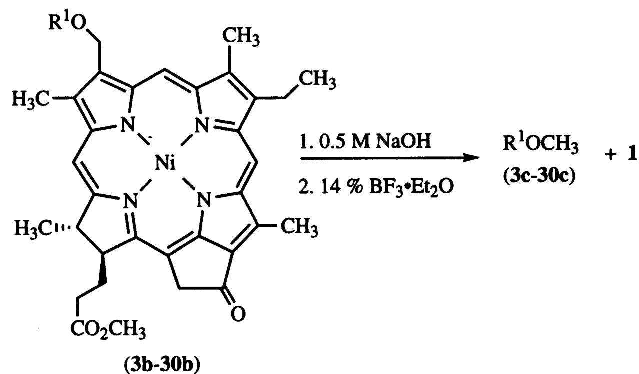


FIG. 1. Selected ^{1}H NMR and ^{13}C chemical shifts and HMBC correlations for **3b–7b** (M+H = 819, 833, 847, 861, and 875).

To further investigate the nature of the aliphatic side chains, the ester linkages of the acyl tunichlorin mixture were cleaved (Scheme II). The aliphatic acids obtained were then esterified with $BF_3{\cdot}Et_2O$ in MeOH (for a review of esterification methods of fatty acids for gas chromatography see ref. 14) and examined by GC/MS. The GC trace for the methyl esters obtained (**3c–30c**) is shown in Fig. 2. The EI mass spectra of these peaks revealed 28 methyl esters, which could be divided into three distinct groups based on the degree of unsaturation present in the molecular formulae. Group I consisted of saturated methyl esters (**3c–12c**); Group II consisted of esters



Scheme II

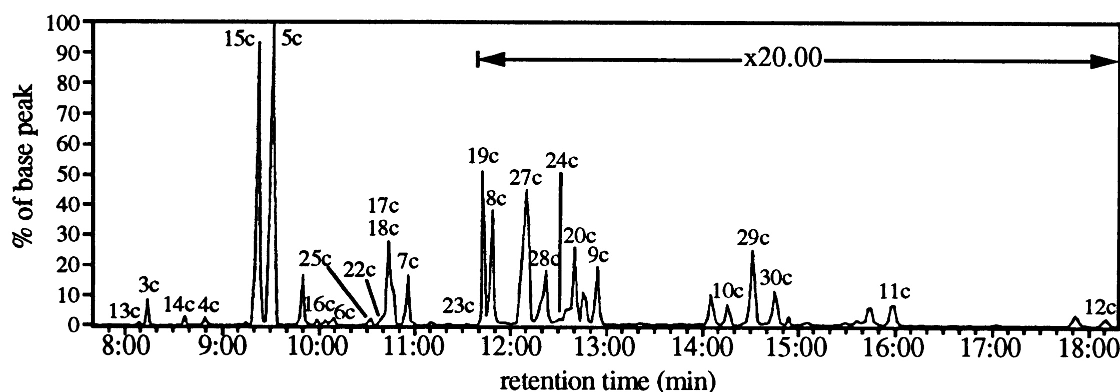


FIG. 2. GC trace of the esterified aliphatic side chains (**3c–30c**) obtained from hydrolysis of the ester linkages of the acyl tunichlorin methyl esters (**3b–30b**). The methyl esters **21c** and **26c** were observed after radial argentation chromatography.

possessing one degree of unsaturation (**13c–20c**); and Group III consisted of polyunsaturated esters (**21c–30c**; Table 1, Fig. 2).

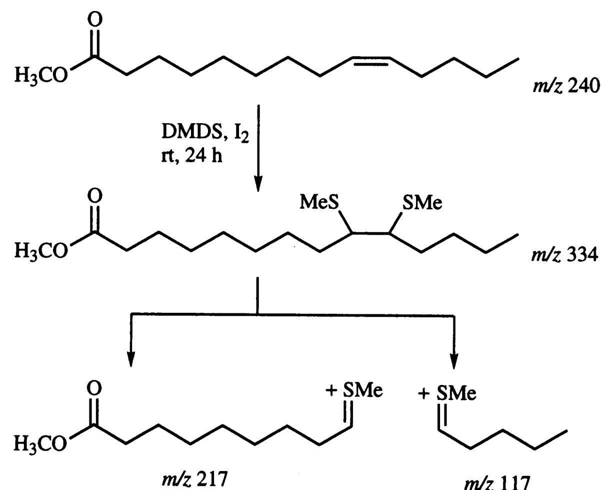
Structures of the Group I Acyl Tunichlorins. The aliphatic methyl esters from the saturated Group 1 series gave characteristic fragmentation ions at m/z 74 and 87 and molecular ions at m/z 242, 256, 270, 284, 298, 312, 326, 340, 354, and 368. These esters were found to be derived from unbranched $C_{14:0}$ to $C_{23:0}$ fatty acids by coelution with the corresponding methyl ester standards, establishing the structures of acyl tunichlorins **3a–12a**.

Structures of the Group II Acyl Tunichlorins. The aliphatic methyl esters of the monounsaturated series, **13c–20c**, required chemical modification to locate the positions of the double bonds. Double bonds show a pronounced tendency to migrate along the aliphatic chain under electron ionization mass spectrometry conditions, making it impossible to locate the point of unsaturation directly by mass spectrometry (15).

Table 1. Mass and molecular formulae of the acyl tunichlorin methyl esters (**3b–30b**) and molecular formulae of the aliphatic side chains as determined by HRFABMS and GC/MS data.

	m/z	Formula	R^1 (Chart I)
3b	819	$C_{47}H_{61}N_4NiO_5$	$C_{14}H_{27}O$ = tetradecanoate
4b	833	$C_{48}H_{63}N_4Nio_5$	$C_{15}H_{29}O$ = pentadecanoate
5b	847	$C_{49}H_{65}N_4NiO_5$	$C_{16}H_{31}O$ = hexadecanoate
6b	861	$C_{50}H_{67}N_4NiO_5$	$C_{17}H_{33}O$ = heptadecanoate
7b	875	$C_{51}H_{69}N_4NiO_5$	$C_{18}H_{35}O$ = octadecanoate
8b	889	$C_{52}H_{71}N_4NiO_5$	$C_{19}H_{37}O$ = nonadecanoate
9b	903	$C_{53}H_{73}N_4NiO_5$	$C_{20}H_{39}O$ = eicosanoate
10b	917	$C_{54}H_{75}N_4NiO_5$	$C_{21}H_{41}O$ = heneicosanoate
11b	931	$C_{55}H_{77}N_4NiO_5$	$C_{22}H_{43}O$ = docosanoate
12b	945	$C_{56}H_{79}N_4NiO_5$	$C_{23}H_{45}O$ = tricosanoate
13b	817	$C_{47}H_{59}N_4NiO_5$	$C_{14}H_{25}O$ = 9-tetradecenoate
14b	831	$C_{48}H_{61}N_4NiO_5$	$C_{15}H_{27}O$ = 9-pentadecenoate
15b	845	$C_{49}H_{63}N_4NiO_5$	$C_{16}H_{29}O$ = 9-hexadecenoate
16b	859	$C_{50}H_{65}N_4NiO_5$	$C_{17}H_{31}O$ = 9-heptadecenoate
17b	873	$C_{51}H_{67}N_4NiO_5$	$C_{18}H_{33}O$ = 9-octadecenoate
18b	873	$C_{51}H_{67}N_4NiO_5$	$C_{18}H_{33}O$ = 11-octadecenoate
19b	887	$C_{52}H_{69}N_4NiO_5$	$C_{19}H_{35}O$ = 10-nonadecenoate
20b	901	$C_{53}H_{71}N_4NiO_5$	$C_{20}H_{37}O$ = 13-eicosenoate
21b	843	$C_{49}H_{61}N_4NiO_5$	$C_{16}H_{27}O$ = 9,12-hexadecadienoate
22b	871	$C_{51}H_{65}N_4NiO_5$	$C_{18}H_{31}O$ = 9,12-octadecadienoate
23b	885	$C_{52}H_{67}N_4NiO_5$	$C_{19}H_{33}O$ = 9,14-nonadecadienoate
24b	899	$C_{53}H_{69}N_4NiO_5$	$C_{20}H_{35}O$ = 10,12-eicosadienoate
25b	869	$C_{51}H_{63}N_4NiO_5$	$C_{18}H_{29}O$ = 9,12,15-octadecatrienoate
26b	897	$C_{53}H_{67}N_4NiO_5$	$C_{20}H_{33}O$ = 7,10,13-eicosatrienoate
27b	893	$C_{53}H_{63}N_4NiO_5$	$C_{20}H_{29}O$ = 5,8,11,14,17-eicosapentaenoate
28b	895	$C_{53}H_{65}N_4NiO_5$	$C_{20}H_{31}O$ = 5,8,11,14-eicosatetraenoate
29b	919	$C_{55}H_{65}N_4NiO_5$	$C_{22}H_{31}O$ = 4,7,10,13,16,19-docosahexaenoate
30b	921	$C_{55}H_{67}N_4NiO_5$	$C_{22}H_{33}O$ = 7,10,13,16,19-docosapentaenoate

DMDS had been used previously for derivatization of complex mixtures of monounsaturated alkenes, allowing for the determination of double bond positions by electron ionization mass spectrometry (9). The mass spectra of the DMDS adducts show molecular ions (M^+) and major fragment ions produced from cleavage of the carbon–carbon bond between the methylthio substituents, which indicates the site of unsaturation and confirms the acid chain length, as demonstrated in Scheme III using methyl 9-tetradecenoate as a representative example.



Scheme III

The GC trace of the methyl esters after derivatization is shown in Fig. 3. As one can see, the DMDS derivatives separate quite easily, allowing for facile structure determination of the monounsaturated olefinic compounds. For example, the methyl esters **17c** and **18c** are positional isomers of methyloctadecenoate, which were not resolved under the GC conditions employed (Fig. 2). On the other hand, DMDS derivatization allowed these isomers to be distinguished, further confirming the utility of this method. This method established that the aliphatic side chains of acyl tunichlorins **13a–20a** are derived from the monounsaturated fatty acids listed in Table 1.

Structures of the Group III Acyl Tunichlorins. The structures of the acyl tunichlorins containing polyunsaturated side chains were more difficult to assign since most of the standard derivatization methods fail to locate double bond positions in highly unsaturated compounds (15). Furthermore, several of the polyunsaturated methyl esters were barely detectable, making analysis of a complex mixture problematic. The methyl esters were separated into mono- and polyunsaturated com-

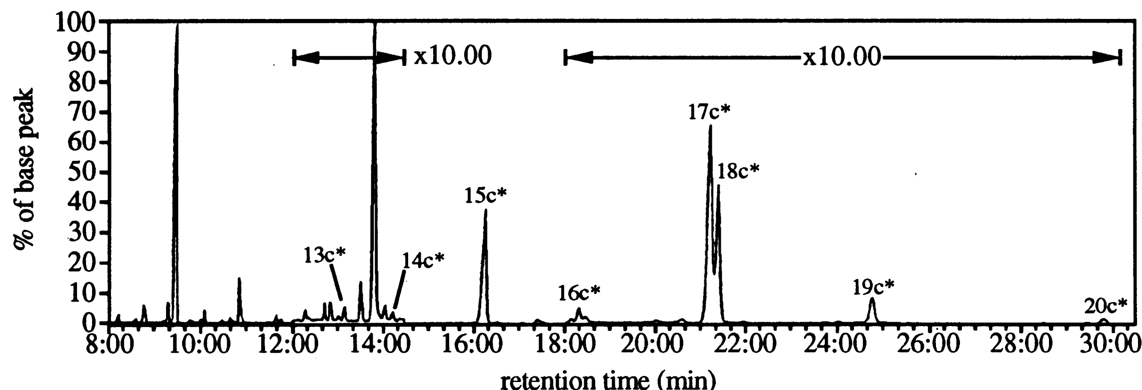


FIG. 3. GC trace of the esterified aliphatic side chains (**3c–30c**) after DMDS derivatization. The DMDS derivatives of the monounsaturated series (**13c–20c**) are indicated by asterisks.

pounds using radial argentation chromatography and converted into the 4,4-dimethyloxazoline derivatives (10). The oxazoline introduces a unit with low ionization potential, which localizes charge and minimizes double-bond migration. The location of the double bonds can then be determined directly by mass spectrometry when an interval of 12 Da, rather than 14, is observed between intense peaks in the series, as demonstrated in Fig. 4 with methyl 9,12-hexadecadienoate as a representative example. This method argued that the acid side chains for acyl tunichlorins **21a–30a** were derived from the polyunsaturated fatty acids listed in Table 1.

All of the mono and polyunsaturated compounds appear to contain only the usual cis double bonds. The IR spectra for these methyl esters did not show the diagnostic band at 960 cm^{-1} for trans di-substituted olefins. The presence of only cis double bonds in the acid side chains is not surprising, as most biologically important fatty acids contain cis alkenes (16).

DISCUSSION

The remarkable number, diversity, and unusual location of the acyl tunichlorin side chains raise many questions about the biosynthesis and biological function of these hydroporphyrins. It appears that the enzyme responsible for the esterification of tunichlorin is extremely nonselective in choosing an aliphatic group. This is in contrast to other known hydroporphyrins, which contain only one esterifying alcohol such as phytol or farnesol (17). Furthermore, very few hydroporphyrins exist as a series of homologues. Two known examples are the bacteriochlorophylls c, d, and e, which exist as mixtures of different alkyl homologues in the 4 and 5 positions, and chlorophyll c, with its three components (c_1, c_2, and c_3), which differ at the 3 and 4 positions (14, 18). The effect of these substituents is a shift in the electronic absorption spectra of these molecules (17). However, unlike the bacteriochlorins and chlorophylls, the different acyl side chains in the acyl tunichlorins do not change their absorption spectra since they are well removed from the chromophore.

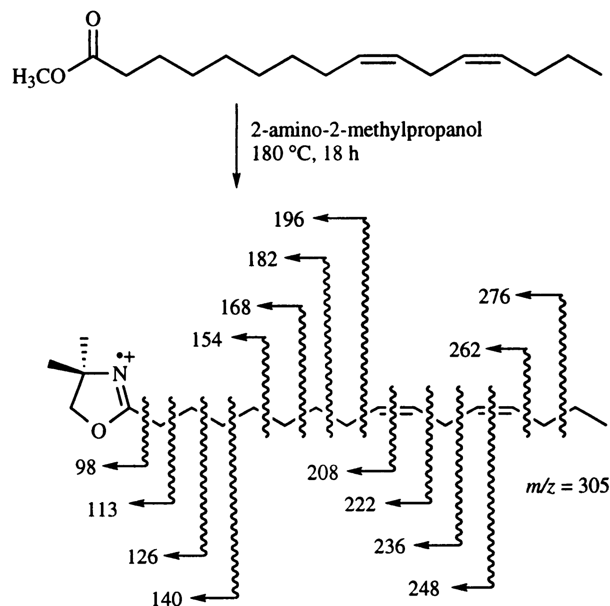


FIG. 4. 4,4-Dimethyloxazoline derivatization and EIMS fragmentation pattern for methyl 9,12-hexadecadienoate.

T. solidum is symbiotically associated with the blue-green alga *Synechocystis trididemni* (19), and the presence of symbiotic microalgae raises questions as to the origin of the acyl tunichlorins. In a study to ascertain whether the acyl tunichlorins are produced by the alga or the tunicate, *S. trididemni* cells were separated from frozen and freeze dried *T. solidum* cells, and the blue-green pigments of the isolated algal and ascidian cells were examined (20). Acyl tunichlorins were present in the tunicate-containing fractions but were absent from the isolated alga, arguing that the acyl tunichlorins are localized in the tunicate. In other experiments freeze-dried, frozen, and 2-propanol-preserved *T. solidum* were assayed for the absence or presence of tunichlorin and acyl tunichlorins (20). The freeze-dried and frozen samples contained nearly exclusively acyl tunichlorins, with no or little tunichlorin, while *T. solidum* preserved in 2-propanol for 3 weeks contained mainly tunichlorin. We conclude therefore that the acyl tunichlorins are the natural state of these chlorins, while the tunichlorin previously isolated from alcohol-stored tunicate is mainly a degradation product resulting from solvolysis of the ester linkage.

It is reasonable to suggest that the acyl tunichlorins are tunicate-modified algal products, as ascidians do not contain photosynthetic pigments of their own and no nickel porphynoids have been reported as algal natural products. Of the hydroporphyrins that have been isolated from *T. solidum*, acyl tunichlorins most closely resemble pyropheophorbide a, a degradation product of chlorophyll a (6). Acyl tunichlorins and pyropheophorbide a differ in only two respects: (i) acyl tunichlorins are nickel chelates, while pyropheophorbide a exists as the free base, and (ii) the C-2 substituent of the acyl tunichlorins is an esterified hydroxymethyl group while that of pyropheophorbide is a vinyl group. The other two chlorins identified in *T. solidum* and *S. trididemni* cells were chlorophyll a, which was found exclusively in the algal fraction, and pheophytin a, which was in both the algal (26%) and tunicate (74%) fractions. From its presence in both fractions, we conclude that pheophytin a is biosynthesized by the alga, which

also converts it to chlorophyll a. A portion of the pheophytin a is, however, transferred to the tunicate, which converts it to the acyl tunichlorins, perhaps via pyropheophorbide a and tunichlorin intermediates. A biosynthetic study will be necessary to establish whether or not pheophytin a and/or tunichlorin are biosynthetic precursors of the acyl tunichlorins.

The other tunichlorin-producing marine species, the sea hare *D. auricularia*, is an alga-consuming mollusk, which may also be using the porphynoid pigments of the alga to produce tunichlorin. An examination of the algae consumed by this sea hare could be informative. It is interesting to note that the only two marine organisms known to produce nickel chlorins also produce potent antitumor cyclic peptides that are currently in clinical trials: the dolastatins (21) (from *Dolabella*) and the didemnins (22) (from *Trididemnum*).

The isolation of the acyl tunichlorins provides some suggestions into the role of these unusual nickel chlorins in *T. solidum*. The long acid side chains suggest that the acyl tunichlorins may serve as membrane-associated enzymatic cofactors. The acyl tunichlorins contain a hydroporphyrin attached to a hydrophobic unit, which should be capable of spanning a phospholipid bilayer, as in the chlorophylls and bacteriochlorins. The incorporation of nickel also provides some insight into the reactivity of these molecules. Studies on Factor F_{430} (23–24) show that the presence of nickel(II) produces conformational strain in the macrocycle (25). The nitrogen atoms are pulled toward the metal center ito accommodate the Ni—N bond lengths required for a tetracoordinate nickel(II) ion. The strain energy associated with this deformation can be interpreted as a driving force toward the addition of axial ligands, a process that leads to longer Ni—N bonds and allows the macrocycle to relax to an unstrained conformation. Studies on synthetic nickel-containing chlorins show that Ni—N distances in chlorins are also shorter than the optimal Ni—N length, causing deformation of the macrocycle (26). By analogy, the nickel center in acyl tunichlorin should have reduced Ni—N bond lengths and, hence, increased reactivity. A biosynthetic study of the acyl tunichlorins could provide valuable information on the role of these unusual compounds in *T. solidum* and on selective accumulation of nickel in this tunicate.

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