

Antineoplastic unsaturated fatty acids from Fijian macroalgae

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ABSTRACT

Phytochemical analysis of Fijian populations of the green alga *Tydemania expeditionis* led to the isolation of two unsaturated fatty acids, 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*),15(*Z*)-trienoic acid (**1**) and 3(ζ)-hydroxy-hexadeca-4(*E*),6(*Z*)-dienoic acid (**2**), along with the known 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*)-dienoic acid (**4**). Investigations of the red alga *Hydrolithon reinboldii* led to identification of a glycolipid, lithonoside (**3**), and five known compounds, 15-tricosenoic acid, hexacos-5,9-dienoic methyl ester, β -sitosterol, 10(*S*)-hydroxypheophytin A, and 10(*R*)-hydroxypheophytin A. The structures of **1–3** were elucidated by spectroscopic methods (1D and 2D NMR spectroscopy and ESI-MS). Compounds **1**, **2**, and **4**, containing conjugated double bonds, demonstrated moderate inhibitory activity against a panel of tumor cell lines (including breast, colon, lung, prostate and ovarian cells) with IC₅₀ values ranging from 1.3 to 14.4 μ M. The similar cell selectivity patterns of these three compounds suggest that they might act by a common, but unknown, mechanism of action.

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1. Introduction

Unsaturated fatty acids are widely distributed in human foods including vegetable oils, meat, milk, and soy products (Jacobsen, 2004), fulfilling important physiological functions. Docosahexaenoic acid and arachidonic acid are important constituents in mammalian cell membranes (Sala-Vila et al., 2006) and are crucial to brain and eye development in human infants (Birch et al., 1997). Consumption of omega-3 and omega-6 fatty acids has been associated with reduced mortality from cardiovascular disease, suppressed arthritis-associated inflammation, and decreased risk of cancer (Simopoulos 2002). Specifically, arachidonic acid was shown to inhibit proliferation of chronic myeloid leukemia cells by inducing apoptotic cell death (Rizzo et al., 1999). However, the proliferation of prostate cancer cell lines (PC3 and LNCap) was stimulated by arachidonic acid due to the increased formation of 5-HETE, a 5-lipoxygenase product important to prostate cancer cells (Ghosh and Myers, 1998). In contrast, conjugated linoleic acids were reported to inhibit human prostate cancer and to reduce metastases of cancers to lung tissue (Cesano et al., 1998). These fatty acids were also reported to block growth and systemic spread of human breast cancer via mechanisms independent of the

host immune system (Visonneau et al., 1997), perhaps by peroxidation of intracellular lipids (Devery et al., 2001).

Glycolipids, which incorporate both fatty acid and carbohydrate moieties, have also been shown to be active against tumor cells. For example, galactolipids with octadecatrienoyl residues were found to possess potent activity against murine leukemia and human colon adenocarcinoma cells (Jung et al., 1996), and nigriganosides A and B, ether-linked glycolipids, showed antimitotic activity against human breast and colon cancer cells (Williams et al., 2007). Glycolipids also have ecological functions such as herbivore deterrence in the brown seaweed *Fucus vesiculosus* (Deal et al., 2003) and inhibition of bacterial pathogens in the terrestrial plant *Arabidopsis thaliana* (Andersson et al., 2006).

Marine algae are a rich source of unsaturated fatty acids (Khotimchenko et al., 2002). During our ongoing effort to search for novel natural products as pharmaceutical leads, extracts of the green alga *Tydemania expeditionis* and of the red alga *Hydrolithon reinboldii* were found to be potently cytotoxic in an invertebrate toxicity assay. Herein, we report the isolation and structural elucidation of two novel unsaturated fatty acids (**1–2**) and a novel glycolipid (**3**) from these two species, and their inhibitory effects on 11 cancer cell lines.

2. Results and discussion

Guided by a rotifer (invertebrate) toxicity assay, investigation of the ethyl acetate-soluble fraction of *T. expeditionis* led to the

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isolation of two new fatty acids, 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*),15(*Z*)-trienoic acid (**1**) and 3(ζ)-hydroxy-hexadeca-4(*E*),6(*Z*)-dienoic acid (**2**), along with the known 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*)-dienoic acid (**4**). Similarly, investigation of the cytotoxic hexane-soluble fraction of *H. reinboldii* led to a new glycolipid, lithonoside (**3**), along with five known compounds, 15-tricosenoic acid, hexacos-5,9-dienoic acid methyl ester, β -sitosterol, 10 (*S*)-hydroxyphoeophytin A, and 10 (*R*)-hydroxyphoeophytin A.

Compound **1** was isolated as a colorless oil. HRESIMS analysis indicated a quasimolecular ion $[M-H]^-$ at m/z 293.2069, corresponding to a molecular formula of $C_{18}H_{30}O_3$, with four units of unsaturation.

The 1H NMR spectrum of **1** (Table 1) indicated four conjugated olefinic protons at δ 5.62 (1H, *dd*, J = 6.5, 15.5 Hz, H-4), 6.49 (1H, *dd*, J = 11, 15.5 Hz, H-5), 5.95 (1H, *t*, J = 11 Hz, H-6) and 5.41 (1H, *dt*, J = 8.0, 11 Hz, H-7), two non-conjugated olefinic protons at δ 5.35 (1H, *m*, H-15) and 5.46 (1H, *m*, H-16), an oxymethine proton at δ 4.10 (1H, *dd*, J = 6.5, 13 Hz, H-3), a methylene envelope at 1.32, and a terminal methyl group at δ 0.95 (3H, *t*, J = 7.5 Hz, H-18), which suggested hydroxylated and unsaturated fatty acid skeleton. The optical rotation for **1** was zero, indicating that **1** is likely present as a racemic mixture, consistent with fatty acids previously reported (Stavri et al., 2006). The large coupling constant between H-4 and H-5 (15.5 Hz) indicated that these two protons were *trans*-oriented. In contrast, the 11 Hz coupling for both $\Delta^{6,7}$ and $\Delta^{15,16}$ indicated they were *cis*-oriented. Analysis of the ^{13}C NMR and DEPT spectra of **1** indicated presence of six olefinic carbon atoms, a methyl group, an oxymethine, and nine methylenes. The carboxyl group was not seen in the ^{13}C NMR spectrum, but a clear correlation of H-2 at δ 2.25 to a carbonyl at 179.0 ppm was observed in the HMBC spectrum. Therefore, the carbon spectral data further confirmed the skeleton revealed by 1H NMR spectroscopy.

The full NMR assignments and connectivities of **1** were determined by 1H 1H COSY, HSQC, and HMBC spectroscopic data analysis. Some key HMBC correlations are shown in Fig. 1. The proton at δ 4.10 (H-3), attached to a carbon at δ 72.3, showed HMBC correlations to C-2, C-4, and C-5, indicating the location of the oxymethine at C-3. In the 1H 1H COSY spectrum, H-5 showed cross-peaks with H-6 and H-7, and H-8 was correlated to H-7 as well as to the methylene envelope at H-9–H-13. The location of the third double bond at C-15 and C-16 was established by the following HMBC correlations: H-18 $\rightarrow$ C-17 (δ 20.7) and C-16 (δ 133.6), and H-17 $\rightarrow$ C-16

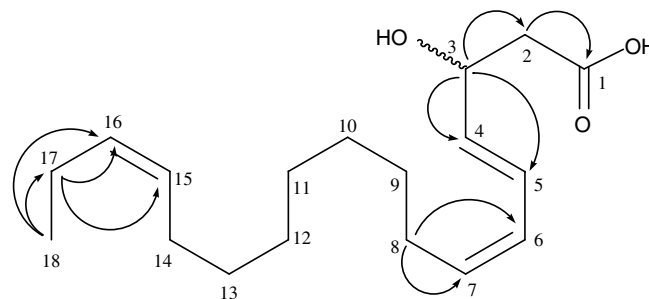


Fig. 1. Key HMBC correlations for **1**.

and C-15 (δ 124.5). Accordingly, the structure of compound **1** was established to be 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*),15(*Z*)-trienoic acid.

Compound **2** was also isolated as a colorless oil. The HRESIMS analysis indicated a quasimolecular ion $[M-H]^-$ at m/z 267.1966, corresponding to a molecular formula of $C_{16}H_{28}O_3$, possessing three units of unsaturation. Compared to the one separated and two conjugated double bonds of **1**, the 1H NMR spectroscopic data of **2** only suggested two conjugated double bonds, with olefinic proton signals at δ 5.60 (1H, *dd*, J = 6.5, 15 Hz, H-4), 6.49 (1H, *dd*, J = 11, 15 Hz, H-5), 5.96 (1H, *t*, J = 11 Hz, H-6) and 5.41 (1H, *m*, H-7), which were confirmed by the four olefinic carbon resonances at δ 136.3 (C-4), 125.5 (C-5), 128.4 (C-6) and 131.9 (C-7). The other signals were similar to those of **1**. As with **1**, the carboxyl group was not visible in the ^{13}C NMR spectrum, but was observed in the HMBC spectrum by a clear correlation of H-2 at δ 2.34 to a carbon at 177.0 ppm. Accordingly, the structure of compound **2** was established to be 3(ζ)-hydroxy-hexadeca-4(*E*),6(*Z*)-dienoic acid.

Compound **3** was isolated as a white powder from the cytotoxic hexane-soluble extract of the coralline red alga *H. reinboldii*. HRESIMS analysis indicated a quasimolecular ion $[M+Na]^+$ at m/z 801.5503, corresponding to a molecular formula of $C_{45}H_{78}O_{10}$. The 1H NMR spectrum (Table 2) showed four geminal protons attached to carbon atoms bearing an oxygen functionality at δ 4.38 (*dd*, J = 3.5, 12 Hz, H-1a), 4.19 (*dd*, J = 6.5, 12 Hz, H-1b), 3.73 (*dd*, J = 6.5, 11 Hz, H-3a), and 3.88 (*dd*, J = 5.5, 11 Hz, H-3b) and a methine at 5.30 (1H, *m*, H-2), indicating the presence of a glycerol moiety, which was also confirmed by ^{13}C and DEPT signals at δ 63.4 (*t*, C-1), 70.5 (*d*, C-2) and 68.8 (*t*, C-3).

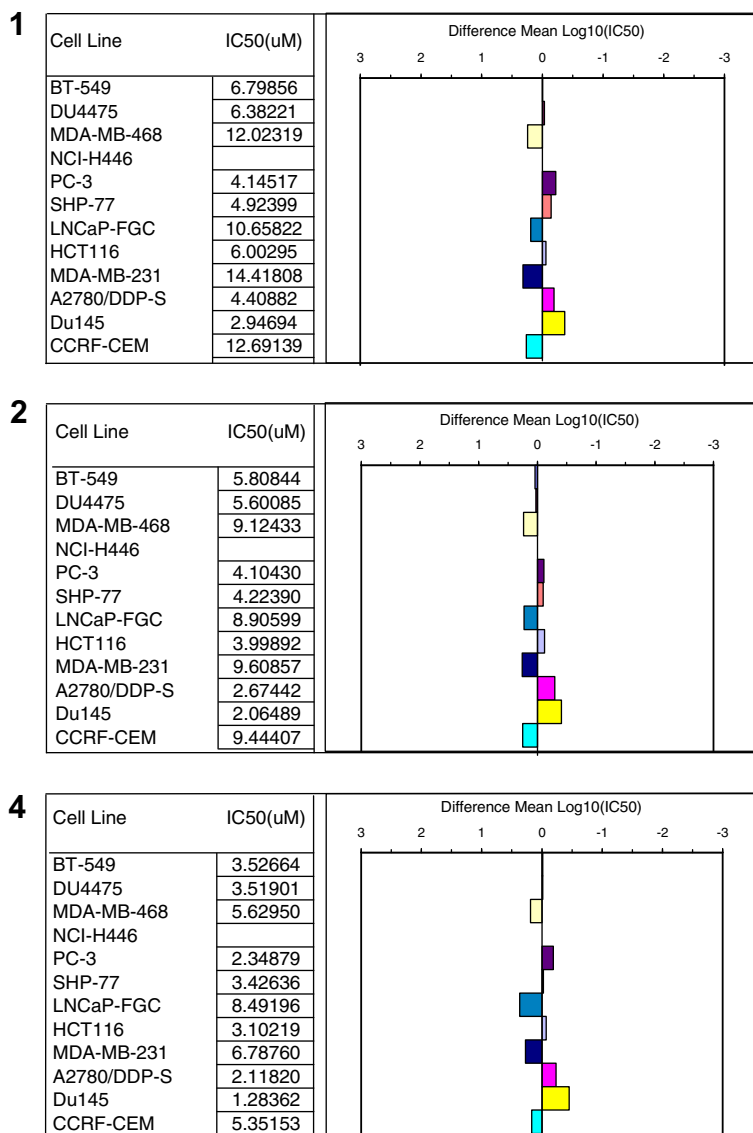
The appearance of a triplet at δ 0.87 (J = 7.0 Hz, H-20'') in the 1H NMR spectrum of **3** was assigned to a terminal methyl of a fatty acid chain, and the multiplets at δ 2.31 (H-2''), 1.69 (H-3'') and 2.09 (H-4'') were due to the α , β , γ -methylenes, respectively, linked to the ester carbonyl functionality. These signals, together with the broad multiplet at δ 2.80 arising from allylic methylene protons and olefinic methine proton resonances overlapping at δ 5.35, were assigned to an unsaturated acyl fatty chain. The eight carbon signals in the low-field region (δ 129.4, 128.2, 129.2, 129.0, 128.7, 128.5, 127.9 and 130.9) indicated four separated double bonds in this chain. The small coupling constants (<11 Hz) revealed by the homonuclear J -spectrum (Derome, 1987; Sánchez-Sampedro et al., 2007) suggested a *Z*-configuration for all double bonds. Similarly, a triplet at δ 0.85 (J = 7.0 Hz, H-16') belonging to the terminal methyl group, broad methylene resonances at δ 1.20–1.30 due to $(CH_2)_n$ of the aliphatic chain, and a multiplet at δ 2.05 (H-2') associated with the α -methylene linked to the ester carbonyl functionality, were attributed to a saturated fatty acyl chain. The presence of two acyl fatty acids was reinforced by the MS/MS sodium adduct fragments m/z 497, which represented loss of eicosatetraenoic acid, and m/z 545, which represented loss of palmitic acid. Thus, the two chains were concluded to consist of eicosatetraenoyl and palmitoyl

Table 1
 1H and ^{13}C NMR spectroscopic data of **1–2** (in CD_3OD)

Position	1		2	
	δ_C DEPT	δ_H (mult., J in Hz)	δ_C DEPT	δ_H (mult., J in Hz)
1	179.0, s		177.0, s	
2	35.3, t	2.25 (2H, <i>m</i>)	37.4, t	2.34 (1H, <i>m</i>) 1.52 (1H, <i>m</i>)
3	72.3, t	4.10 (1H, <i>dd</i> , 6.5, 13)	72.4, d	4.06 (1H, <i>dd</i> , 6.5, 13)
4	135.7, d	5.62 (1H, <i>dd</i> , 6.5, 15.5)	136.3, d	5.60 (1H, <i>dd</i> , 6.5, 15)
5	125.7, d	6.49 (1H, <i>dd</i> , 11, 15.5)	125.5, d	6.49 (1H, <i>dd</i> , 11, 15)
6	128.3, d	5.95 (1H, <i>t</i> , 11)	128.4, d	5.96 (1H, <i>t</i> , 11)
7	132.1, d	5.41 (1H, <i>dt</i> , 8.0, 11)	131.9, d	5.41 (1H, <i>m</i>)
8	27.6, t	2.18 (2H, <i>m</i>)	27.6, t	2.20 (2H, <i>m</i>)
9	25.7, t	1.59 (2H, <i>m</i>)	25.3, t	1.60 (1H, <i>m</i>) 1.41 (1H, <i>m</i>)
10	29.7, t	1.32 (2H, <i>m</i>)	29.8, t	1.33 (2H, <i>m</i>)
11	29.4, t	1.32 (2H, <i>m</i>)	29.6, t	1.33 (2H, <i>m</i>)
12	29.3, t	1.32 (2H, <i>m</i>)	29.5, t	1.33 (2H, <i>m</i>)
13	29.2, t	1.32 (2H, <i>m</i>)	29.1, t	1.33 (2H, <i>m</i>)
14	20.7, t	2.05 (2H, <i>m</i>)	32.0, t	1.33 (2H, <i>m</i>)
15	124.5, d	5.35 (1H, <i>m</i>)	22.7, t	1.33 (2H, <i>m</i>)
16	133.6, d	5.46 (1H, <i>m</i>)	13.4, t	0.91 (3H, <i>t</i> , 7.5)
17	20.7, t	2.05 (2H, <i>m</i>)		
18	13.5, q	0.95 (3H, <i>t</i> , 7.5)		

Table 2¹H and ¹³C NMR spectroscopic data of **3** (in CDCl₃)

Position	δ_H (mult., J in Hz)	δ_C DEPT	Position	δ_H (mult., J in Hz)	δ_C DEPT
1	4.38 (1H, <i>dd</i> , 3.5, 12)	63.4, <i>t</i>	10''	2.80 (2H, <i>m</i>)	25.3, <i>t</i>
	4.19 (1H, <i>dd</i> , 6.5, 12)		11''	5.35 (1H, <i>m</i>)	128.7, <i>d</i>
2	5.30 (1H, <i>m</i>)	70.5, <i>d</i>	12''	5.35 (1H, <i>m</i>)	128.5, <i>d</i>
3	3.88 (1H, <i>dd</i> , 5.5, 11)	68.8, <i>t</i>			
	3.73 (1H, <i>dd</i> , 6.5, 11)		13''	2.80 (2H, <i>m</i>)	25.1, <i>t</i>
1'		175.0, <i>s</i>	14''	5.35 (1H, <i>m</i>)	127.9, <i>d</i>
2'	2.05 (2H, <i>m</i>)	34.5, <i>t</i>	15''	5.35 (1H, <i>m</i>)	130.9, <i>d</i>
3'	1.66 (2H, <i>m</i>)	26.0, <i>t</i>	16''	2.01 (2H, <i>m</i>)	27.6, <i>t</i>
4'–14'	1.2–1.3 (22H, <i>m</i>)	29.5–30.1, <i>t</i>	17''	1.67 (2H, <i>m</i>)	30.6, <i>t</i>
15'	1.34 (2H, <i>m</i>)	23.0, <i>t</i>	18''	1.27 (2H, <i>m</i>)	32.3, <i>t</i>
16'	0.85 (3H, <i>t</i> , 7)	14.5, <i>q</i>	19''	1.36 (2H, <i>m</i>)	23.1, <i>t</i>
1''		174.0, <i>s</i>	20''	0.87 (3H, <i>t</i> , 7)	14.5, <i>q</i>
2''	2.31 (2H, <i>m</i>)	34.7, <i>t</i>	1'''	4.26 (1H, <i>d</i> , 7.5)	104.4, <i>d</i>
3''	1.69 (2H, <i>m</i>)	33.9, <i>t</i>	2'''	3.63 (1H, <i>m</i>)	72.1, <i>d</i>
4''	2.09 (2H, <i>m</i>)	26.9, <i>t</i>	3'''	3.99 (1H, <i>m</i>)	69.9, <i>d</i>
5''	5.35 (1H, <i>m</i>)	129.4, <i>d</i>	4'''	3.53 (1H, <i>m</i>)	74.9, <i>d</i>
6''	5.35 (1H, <i>m</i>)	128.2, <i>d</i>	5'''	3.59 (1H, <i>m</i>)	73.8, <i>d</i>
7''	2.80 (2H, <i>m</i>)	25.4, <i>t</i>	6'''	3.96 (1H, <i>m</i>)	63.2, <i>d</i>
8''	5.35 (1H, <i>m</i>)	129.2, <i>d</i>		3.84 (1H, <i>m</i>)	
9''	5.35 (1H, <i>m</i>)	129.0, <i>d</i>			

**Fig. 2.** Cancer cell line selectivity patterns of **1**, **2**, and **4**.

moieties. In the HMBC spectrum, the glycerol methylene proton at δ 4.19 (H-1b) was correlated to the ester carbonyl at δ 175.0 (C-1'), indicating linkage of the palmitoyl moiety to the glycerol portion at C-1, and the eicosatetraenoyl moiety was inferred to be connected at C-2.

The existence of a galactopyranosyl moiety was inferred from the anomeric protons at δ 4.26 (d, J = 7.5 Hz, H-1'''), and confirmed by the presence of a set of galactose carbon signals at δ 104.4 (d, C-1'''), 72.1 (d, C-2'''), 69.9 (d, C-3'''), 74.9 (d, C-4'''), 73.8 (d, C-5'''), and 63.2 (d, C-2'''). The 7.5 Hz coupling of the anomeric proton supported the β -configuration for this sugar unit. In the HMBC spectrum, this anomeric proton was correlated to C-3 at δ 66.8, indicating the linkage of this sugar to the glycerol moiety.

The structure of **3** is reminiscent of galactolipids which have been found in both terrestrial plants (Catani et al., 2003; Jung et al., 1996) and marine algae (Shao et al., 2002; Deal et al., 2003). The most closely related structure to **3** was 1-O-(palmitoyl)-2-O-(5,8,11,14,17-eicosapentaenoyl)-3-O- β -D-galactopyranosyl-glycerol, identified by spectroscopic analysis of a glycolipid mixture from the red alga *Chondria armata*, with one *trans* and four *cis* double bonds in the acyl fatty chain (Al-Fadhli et al., 2006). Thus, compound **3** is a new galactosyl glycolipid identified to be 1-O-(palmitoyl)-2-O-(5Z, 8Z, 11Z, 14Z-eicosatetraenoyl)-3-O- β -D-galactopyranosyl-glycerol, and was accorded the trivial name lithonoside.

The structures of three known fatty acids, 3(ζ)-hydroxy-octadeca-4(E),6(Z)-dienoic acid (**4**) (Stavri et al., 2006), 15-tricosenoic acid (Carballeira et al., 1998), and hexacos-5,9-dienoic methyl ester (Lam et al., 1989), and three non-fatty acids, β -sitosterol (Shang et al., 2000), 10(S)-hydroxypheophytin A (Nakatani et al., 1981), and 10(R)-hydroxypheophytin A (Nakatani et al., 1981), were identified by comparing their spectroscopic data with those of reported values.

by a common mechanism of action. In contrast, galactolipid **3** showed more modest activity with a mean IC_{50} value of 19.8 μ M, and 15-tricosenoic acid and hexacos-5,9-dienoic methyl ester, with only separated double bonds, were inactive with mean IC_{50} values larger than 60 μ M (individual cell line data not shown). In contrast to the antineoplastic activity, **3–4** demonstrated weak antimalarial activity (with IC_{50} values of 72 and 77 μ M, respectively). All other compounds tested had IC_{50} values larger than 90 μ M (data not shown).

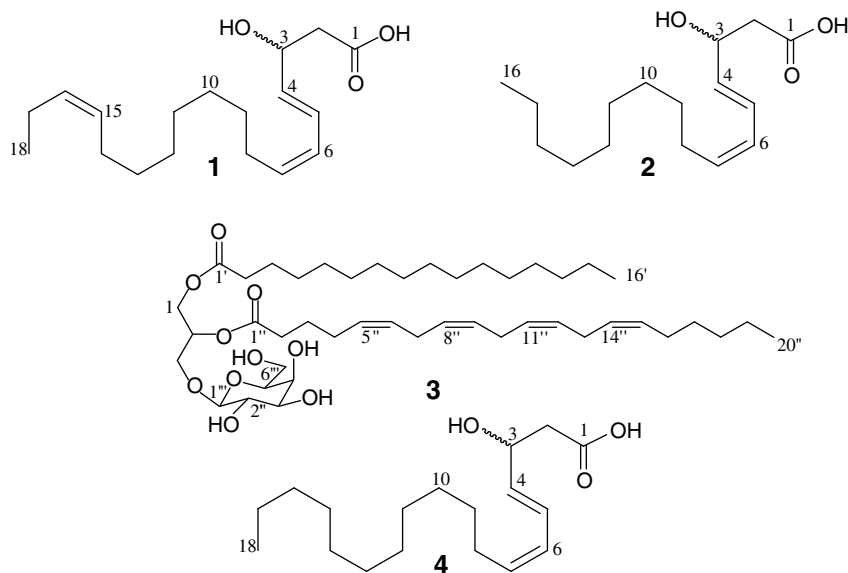
3. Conclusions

From the green alga *T. expeditionis*, we identified two novel antineoplastic fatty acids (**1–2**) as well as one structurally-related, previously-known fatty acid (**4**). Lithonoside (**3**), a novel glycolipid, is the first natural product reported from the coralline red algal genus *Hydrolithon*, possessing modest antineoplastic activity. Compounds **1**, **2**, and **4**, containing conjugated double bonds and a hydroxyl group at C-3, demonstrated moderate inhibitory activity against a panel of tumor cell lines with IC_{50} values ranging from 1.3 to 14.4 μ M, considerably more potent than conjugated linoleic acids (Ochoa et al., 2004; Kim et al., 2005). In contrast, **3** with a mean antineoplastic IC_{50} value of 19.8 μ M, was less potent than recently discovered ether-linked glycolipids, e.g., nigricanosides A and B (Williams et al., 2007).

4. Experimental

4.1. General

UV spectra were recorded with online HPLC-DAD. Optical rotations were measured on a Rudolph automatic polarimeter. NMR



Fatty acids **1**, **2**, and **4**, containing conjugated double bonds, demonstrated modestly potent inhibitory activity against a panel of 12 breast, colon, lung, prostate, and ovarian tumor cells, with IC_{50} values in the ranges 1.3 to 14.4 μ M (Fig. 2). Close examination of their cell selectivity patterns (Fig. 2) showed that all three compounds were more active against SHP-77 (lung), HCT-116 (colon), A2780/DDP-S (ovarian), and PC-3 and Du145 (both prostate) cell lines than against CCRF-CEM (leukemia), LNCap-FGCM (prostate), and MDA-MB-468 and MDA-MB-231 (both breast) lines. The similar selectivity patterns of these compounds suggest they might act

spectra (1H , ^{13}C , DEPT, COSY, HSQC, and HMBC) were acquired on a Bruker DRX-500 instrument using a 5 mm broadband probe, and were referenced to the residual light solvent (δ 7.24 and 77.0 for chloroform; δ 3.31 and 49.0 for methanol). High-resolution mass spectra were generated by electrospray ionization with an Applied Biosystems QSTAR-XL hybrid quadrupole-time-of-flight tandem mass spectrometer and Analyst QS software. All commercial chemicals were reagent grade, except for the HPLC and Optima grade solvents used for HPLC and LC-MS, respectively (Fisher Scientific Co.). NMR solvents were purchased from Cambridge Isotope Laboratories.

4.2. Plant materials

T. expeditionis was collected at 7–26 m depth at Herald Pass, Kadavu Province, Fiji (18° 46.370' S, 178° 27.746' E) and was frozen at –20 °C until extraction (collection G-2004-06-45). Collection of *H. reinboldii* was made at 2 m depth in Denny's reef, Rewa Province, Fiji (18° 10.038' S, 178° 25.211' E); this plant was immediately immersed in MeOH (collection G-2004-06-13). Specimens were identified by Dr. Posa Skelton at University of South Pacific, by comparison with morphological traits previously described (Littler and Littler, 2003), stored in aqueous formaldehyde, and deposited at the South Pacific Center for Biodiversity Conservation and Economic Development, at the University of the South Pacific (Suva, Fiji).

4.3. Rotifer (invertebrate) toxicity assay

The experiments were performed using procedures described previously (Snell, 2005).

4.4. Extraction and isolation guided by rotifer toxicity assay

The whole *T. expeditionis* plant (500 g, wet mass) was exhaustively extracted with MeOH and MeOH–CH₂Cl₂ (1:1). The solutions were combined, filtered, and concentrated under reduced pressure to afford a crude extract (17.9 g, yield 3.6% of wet mass), which was then partitioned between MeOH/H₂O (9:1) and hexane. Next, the aqueous fraction was rotary evaporated to remove MeOH, and successively partitioned against EtOAc and *n*-BuOH to eventually result in four fractions (hexane-, EtOAc, *n*-BuOH- and H₂O-soluble). The bioactive EtOAc-soluble fraction was further separated by semi-preparative HPLC to afford compounds **1** (4.6 mg), **2** (3.2 mg), and **4** (5.5 mg). Purification of these compounds was performed on a Waters 2695 system equipped with 2996 diode-array UV detection and an Agilent Zorbax SB-C₁₈ reversed-phase column (5 µm, 9.4 × 250 mm) with a gradient mobile phase (A: H₂O, B: CH₃CN): 0–53 min, 10 → 70% B; 53–60 min: 70 → 100% B; and 60–63 min, 100 → 10% B; flow rate 3 ml/min and detection wavelengths 215 and 235 nm.

The MeOH crude extract (2.0 g) of *H. reinboldii* was successively partitioned as described above to afford hexane- (0.193 g), EtOAc- (0.067 g), *n*-BuOH- (0.222 g), and H₂O-soluble fractions (1.088 g). The hexane-soluble fraction showed the most potent cytotoxicity, and so further fractionation was carried out (on 60 mg) by semi-preparative reversed-phase HPLC, affording lithonoside (**3**) (2.3 mg), 15-tricosenoic acid (1.0 mg), hexacos-5,9-dienoic methyl ester (3.3 mg), β-sitosterol (5.6 mg), 10(*S*)-hydroxyphoeophytin A (1.3 mg), and 10(*R*)-hydroxyphoeophytin A (1.2 mg), respectively. Purification of these compounds was performed on the same system as above but an Alltech Alltima-C₁₈ column (5 µm, 10 × 250 mm) with a gradient mobile phase. (A: H₂O/MeOH/EtOAc 20:65:15; B: H₂O/MeOH/EtOAc 10:30:60): 0–15 min, 60% B; 15–20 min: 60 → 100% B; and 20–35 min, 100% B; 35–40 min 100 → 60% B; flow rate 3 ml/min and detection wavelengths 215 and 265 nm.

3(ζ)-Hydroxy-octadeca-4(E),6(Z),15(Z)-trienoic acid (1). Colorless oil; UV (acetonitrile/H₂O) γ_{max} 236 nm; for ¹H and ¹³C NMR spectroscopic data, see Table 1; ESI-MS *m/z* 293 [M–H][–], 275 [M–H–H₂O][–]; HRESI-MS *m/z* 293.2069 [M–H][–]; required 293.2117.

3(ζ)-Hydroxy-hexadeca-4(E),6(Z)-dienoic acid (2). Colorless oil; UV (acetonitrile/H₂O) γ_{max} 234 nm; for ¹H and ¹³C NMR spectroscopic data, see Table 1; ESI-MS *m/z* 267 [M–H][–], 249 [M–H–H₂O][–]; HRESI-MS *m/z* 267.1966 [M–H][–]; required 267.1960.

Lithonoside (3). Colorless powder; [α]_D²⁰ –66.0° (c 0.03 in CH₃OH); for ¹H and ¹³C NMR spectroscopic data, see Table 2; ESI-MS/MS *m/z* 497 [M–C₂₀H₃₂O₂+Na]⁺, *m/z* 545 [M–C₁₆H₃₂O₂+Na]⁺,

801 [M+Na]⁺, HRESI-MS *m/z* 801.5499 [M+Na]⁺; required 801.5493.

4.5. Cancer cell growth assay

Natural products were tested against a panel of tumor cell lines: breast cancer (BT-549, DU4475, MDA-MB-468, MDA-MB-231), colon cancer (HCT116), lung cancer (NCI-H446 and SHP-77), prostate cancer (PC-3, LNCap-FGC, Du145), ovarian cancer (A2780/DDP-S) and leukemia (CCRF-CEM). The cells were seeded in 96-well plates and compounds added 24 h later. After 72 h of exposure, the cells were stained with (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), leading to a reduced product from live cell metabolism, which was quantified spectrophotometrically (Lee et al., 2001). Dose response curves were generated and IC₅₀ values calculated for each compound and cell line. A mean IC₅₀ value was determined for the 12 cell line panel and converted to a log value, then the log of each individual IC₅₀ value was subtracted from the mean log IC₅₀ and that difference was plotted. Bars projecting to the right indicate cell lines more sensitive than the mean, whereas bars projecting to the left indicate cell lines less sensitive than the mean.

4.6. Malarial parasite growth inhibition assay

Antimalarial activity was tested using a SYBR Green-based whole-parasite proliferation assay, adapted from Smilkstein et al. (2004) and Bennett et al. (2004), as described by Lane et al. (2007). *Plasmodium falciparum* (3D7 strain MR4/ATCC, Manassas, VA) were cultured in human O+ erythrocytes as previously described (Trager and Jensen, 1976).

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