Phospholipid Studies of Marine Organisms: 14. Ether Lipids of the Sponge *Tethya aurantia*[1]

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The novel unesterified alkyl glycerol monoethers, (2S)-1-(hexadecyloxy)-2,3-propanediol (*1*), (2S)-1-(16-methylheptadecyloxy)-2,3-propanediol (*2*) and (2S)-1-(15-methylheptadecyloxy)-2,3-propanediol (*3*) were isolated from the marine sponge *Tethya aurantia* and were characterized by spectroscopic methods. These three saturated ethers as well as a series of alk-1′-enyl glycerol monoethers were also encountered in the phospholipids of the same sponge after reduction with $LiAlH_4$. Incorporation experiments with dissociated cells of *T. aurantia* indicated that [1-^{14}C]-hexadecanol was incorporated into the unesterified alkyl glycerol monoethers.
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Sponges are the most primitive of the multicellular animals and have been found to be rich sources of many unusual compounds (1,2), including lipids (3). Recently, a number of reports have appeared on the direct isolation of alkyl and alk-1′-enyl glycerol monoethers from marine sponges (4–7). To date, such compounds have only been isolated in the unesterified form from marine sponges, although glycerol ethers in the esterified form are commonly encountered in a wide variety of organisms (8).

Our interest in *Tethya aurantia* stems from studies currently underway in this laboratory concerning the biosynthesis of unusual fatty acids found in this sponge (9). Here we report the direct isolation of novel alkyl glycerol monoethers from *T. aurantia* without any prior hydrolysis. Alkyl and alk-1′-enyl glycerol monoethers were also found in the phospholipids of *T. aurantia* after reduction with $LiAlH_4$. Incorporation experiments utilizing [1-^{14}C]-hexadecanol were carried out to establish whether the glycerol monoethers were of dietary origin or were biosynthesized in the sponge. The possible role of the unesterified alkyl glycerol monoethers is also discussed.

MATERIALS AND METHODS

Materials. [1-^{14}C]Hexadecanoic acid was obtained from Amersham Corp. (Arlington Heights, Illinois) and 1-O-hexadecyl-glycerol from Sigma Chemical Co. (St. Louis, Missouri).

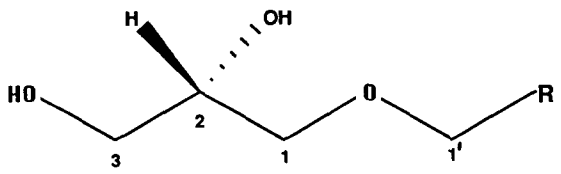
General methods. T. aurantia was collected from Carmel River Beach, Carmel, California, in February and April, 1986, at depths of 5 to 10 m. After freeze-drying, the sponges were extracted by the method of Folch et al. (10), and the resulting lipid extract was fractionated by flash chromatography (11) by elution with hexane/ether mixtures, acetone and methanol. The unesterified alkyl glycerol monoethers, which were eluted in the ether fraction by flash chromatography, were purified further by normal phase high performance liquid chromatography (HPLC), which was performed on a Waters Associates HPLC system (M-45 pump, R-401 differential refractometer) using an Altex Ultrasil-Si column (25 cm × 10 mm ID) with hexane/ether (3:7, v/v) as the mobile phase at 2 ml/min. The methanol-eluting fraction from flash chromatography, containing the phospholipids, was reduced with $LiAlH_4$ as described previously (12). The resulting mixture, which consisted predominantly of fatty alcohols and glycerol ethers, was separated by normal phase HPLC as described above.

^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Nicolet NT 300 WB (300 MHz) spectrometer using 5 mm ID spinning sample tubes. All samples were recorded in $CDCl_3$ and are expressed as ppm downfield from tetramethylsilane (TMS), the primary reference being chloroform, which resonates at 7.26 ppm relative to TMS.

Gas chromatography/mass spectrometry (GC/MS) analyses were performed with either a Ribermag R10-10 quadrupole mass spectrometer connected to a Carlo Erba series 4160 Fractovap chromatograph or a Hewlett Packard 5970 quadrupole mass spectrometer coupled to a Hewlett Packard 5890 gas chromatograph.

The identity of the alkyl glycerol ether hydrocarbon side chains (Fig. 1) was determined by GC/MS of both the nitrile and the pyrrolidide derivatives generated from the corresponding alkyl iodides after cleavage of the ether linkage. The cleavage was accomplished by refluxing the alkyl glycerol ethers with concentrated hydriodic acid for 3 hr (13) and by converting the resulting iodides to the nitriles by heating for 30 min at 90 C with NaCN in dimethylsulfoxide (14). The nitriles were hydrolyzed by refluxing in 6% KOH/ethanol for 16 hr, and the resulting acids were transformed into the methyl esters by refluxing with HCl/methanol for 30 min. The pyrrolidides were then synthesized by heating the methyl esters with pyrrolidine/acetic acid (10:1, v/v) at 100 C for 30 min (15).

The identity of the hydrocarbon side chains of the alk-1′-enyl glycerol monoethers (Fig. 2) was established by GC/MS of the resulting pyrrolidide derivatives after hydrolysis of the alk-1′-enyl ether linkage and derivatization. The vinyl ether linkage was hydrolyzed with



1 R = 15:0

2 R = 16:0 iso

3 R = 16:0 anteiso

FIG. 1. Structures of the alkyl glycerol monoethers isolated from *T. aurantia*.

[1]For preceding paper, see Ayanoglu, E., Düzgünes, N., Wijekoon, W.M.D., and Djerassi, C. (1986) *Biochim. Biophys. Acta 863,* 110–114.

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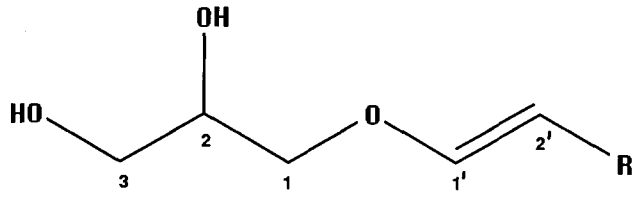

4 R = 14:0

5 R = 16:0

6 R = 16:1

7 R = 16:2

FIG. 2. Structures of the alk-1′-enyl glycerol monoethers isolated after reduction of *T. aurantia* phospholipids.

concentrated HCl for 2 min (16), and the resulting aldehydes were oxidized to the corresponding acids with Jones reagent (17). The methyl esters and pyrrolidides were synthesized from the resulting acids as described above.

Gas liquid chromatography (GLC) was carried out using a Hewlett Packard (HP) 5790 Series gas chromatograph equipped with an HP Ultra 2 (5% Ph Me Silicone) capillary column (25 m × 0.2 mm), temperature-programmed from 170 to 300 C at 5 C/min. Peak areas were calculated using an HP 3392A integrator.

Infrared spectra were recorded on a Perkin-Elmer 1310 infrared spectrophotometer.

Incorporation experiments. [1-^{14}C]Hexadecanol was synthesized by$LiAlH_4$ reduction of [1-^{14}C]hexadecanoic acid. Biosynthetic incorporation experiments were carried out using both intact sponges and dissociated sponge cells. [1-^{14}C]Hexadecanol (10 μCi in 0.25 ml 95% ethanol) was incorporated into intact sponges (ca. 6 cm in diameter) by injection and aquarium incubation in a minimum volume (ca. 200 ml) of seawater with aeration for 24 hr. The aquarium was then flushed continually with filtered seawater, with aeration, for a further 6 or 16 days, after which time the sponge was processed.

Dissociated sponge cells were produced by initially cutting the sponge into small pieces (ca. 1 cm × 1 cm), which were then pressed through a fine nylon cloth. To the filtrate (ca. 75 ml) was added [1-^{14}C]hexadecanol (10 μCi in 0.25 ml 95% ethanol), and the cell suspension was incubated in a 250-ml flask closed with a cotton plug, with shaking at 15 C for 16 hr. Isolation and analysis of the glycerol ethers were carried out as described above. Small aliquots (usually 1/100 or 1/50) of the ^{14}C samples were dissolved in 10 ml of organic counting scintillant, and the radioactivity was measured with a Beckman LS 7500 liquid scintillation counter. All results were corrected for background radiation and calculated as dpm by using a ^{14}C standard solution.

Identification of compounds: unesterified alkyl glycerol monoethers (Fig. 1). ^{1}H NMR (300 MHz, $CDCl_3$) of the alkyl glycerol ethers (s, d, t and m indicate singlet, doublet, triplet and multiplet): δ/ppm: 3.84 (1H, m, H-2), 3.70 (1H, dd, J = 3.5 Hz, J = 11 Hz, H-3a), 3.63 (1H, dd, J = 5 Hz, J = 11 Hz, H-3b), 3.50 (2H, t, J = 3.5 Hz, H-1a,1b), 3.44 (2H, dt, J = 1 Hz, J = 6 Hz, H-1′), 1.55 (2H, m, CH_2CH_3), 1.23 (s, CH_2), 0.85 (m, CH_3).

Mass spectra (GC/MS) of the nitrile derivatives of the hydrocarbon side chains: m/z [$CH_3(CH_2)_{15}CN$]: 251 (M^+, 1%), 236 (9%), 222 (36%), 208 (44%), 194 (31%), 180 (24%), 166 (24%), 152 (24%), 138 (35%), 124 (58%), 110 (83%), 96 (78%), 82 (56%), 68 (14%), 55 (100%); m/z [$(CH_3)_2CH(CH_2)_{14}CN$]: 265 (M^+, 1%), 250 (23%), 236 (3%), 222 (40%), 208 (19%), 194 (18%), 180 (18%), 166 (18%), 152 (21%), 138 (24%), 124 (41%), 110 (70%), 96 (55%), 82 (42%), 68 (10%), 55 (100%); m/z [$CH_3CH_2CH(CH_3)(CH_2)_{13}CN$]: 265 ($M^+$, 1%), 250 (7%), 236 (51%), 222 (5%), 208 (15%), 194 (16%), 180 (29%), 166 (33%), 152 (32%), 138 (32%), 124 (34%), 110 (42%), 96 (40%), 82 (33%), 68 (10%), 55 (100%).

Mass spectra (GC/MS) of the pyrrolidide derivatives of the hydrocarbon side chains: m/z [$CH_3(CH_2)_{15}CONC_4H_8$]: 323 (M^+, 2%), 308 (1%), 294 (1%), 280 (1%), 266 (1%), 252 (1%), 238 (1%), 224 (1%), 210 (1%), 196 (1%), 182 (2%), 168 (3%), 154 (1%), 140 (3%), 126 (19%), 113 (100%); m/z [$(CH_3)_2CH(CH_2)_{14}CONC_4H_8$]: 337 ($M^+$, 2%), 322 (2%), 308 (<1%), 294 (2%), 280 (1%), 266 (1%), 252 (1%), 238 (1%), 224 (1%), 210 (1%), 196 (1%), 182 (2%), 168 (3%), 154 (1%), 140 (3%), 126 (20%), 113 (100%); m/z [$CH_3CH_2(CH_3)CH(CH_2)_{13}CONC_4H_8$]: 337 ($M^+$, 2%), 322 (1%), 308 (2%), 294 (<1%), 280 (2%), 266 (<1%), 252 (<1%), 238 (1%), 224 (1%), 210 (1%), 196 (1%), 182 (2%), 168 (3%), 154 (2%), 140 (3%), 126 (19%), 113 (100%).

IR (CCl_4) of the alkyl glycerol ethers: ν_{max} = 3400, 2920, 2860, 1460, 1380, 1120, 1060.

$[\alpha]_{589}^{20}$ + 2.9° (c, 0.01 in $CHCl_3$).

Alk-1′-enyl glycerol monoethers (Fig. 2). ^{1}H NMR (300 MHz, $CDCl_3$) of the alk-1′-enyl glycerol monoethers derived after reduction of phospholipids with $LiAlH_4$: δ/ppm: 5.94 (1H, d, J = 6 Hz, H-1′), 5.39 (t, J = 5 Hz, CH=CH), 4.41 (1H, q, J = 6 Hz, J = 10 Hz, H-2′), 3.92 (1H, m, H-2), 3.80 (2H, dd, J = 2 Hz, J = 5 Hz, H-1a,1b), 3.74 (1H, q, J = 3 Hz, J = 12 Hz, H-3a), 3.66 (1H, q, J = 6 Hz, J = 12 Hz, H-3b), 2.02 (m, $CH_2CH{=}CHCH_2$), 1.27 (s, CH_2), 0.88 (3H, t, J = 6 Hz, CH_3).

Mass spectra (GC/MS) of the pyrrolidide derivatives of the hydrocarbon side chains: m/z [$CH_3(CH_2)_{14}CONC_4H_8$]: 309 (M^+, 1%), 294 (1%), 280 (1%), 266 (1%), 252 (1%), 238 (1%), 224 (1%), 210 (1%), 196 (1%), 182 (1%), 168 (2%), 154 (1%), 140 (3%), 126 (20%), 113 (100%); m/z [$CH_3(CH_2)_{16}CONC_4H_8$]: 337 (M^+, 2%), 308 (1%), 294 (1%), 266 (1%), 252 (1%), 238 (1%), 224 (1%), 210 (1%), 196 (1%), 182 (2%), 168 (3%), 154 (2%), 140 (3%), 126 (19%), 113 (100%); m/z [$CH_3(CH_2)_7CH{=}CH(CH_2)_7CONC_4H_8$]: 335 ($M^+$, 2%), 278 (1%), 264 (1%), 250 (1%), 236 (2%), 222 (1%), 208 (1%), 196 (1%), 182 (2%), 168 (1%), 154 (1%), 140 (3%), 126 (70%), 113 (100%); m/z [$CH_3(CH_2)_2CH{=}CH(CH_2)_4CH{=}CH(CH_2)_6CONC_4H_8$]: 333 ($M^+$, 2%), 276 (1%), 264 (1%), 250 (1%), 236 (1%), 222 (1%), 208 (1%), 194 (1%), 182 (1%), 168 (1%), 154 (1%), 140 (2%), 126 (60%), 113 (100%).

IR (CCl_4) of the alk-1′-enyl glycerol ethers: ν_{max} = 3400, 2920, 2860, 1660, 1460, 1120.

RESULTS AND DISCUSSION

Alkyl glycerol monoethers were isolated after flash chromatography and HPLC of total lipid extracts of *T. aurantia*, without any prior derivatization or hydrolysis

steps, and represented 2% of the total lipid and 0.02% of the sponge dry weight.

The free alkyl glycerol monoethers were identified by a combination of ^{1}H NMR, GC/MS and chemical derivatization. ^{1}H NMR experiments indicated the presence of the glycerol monoether moiety (see Fig. 1). A multiplet at 3.84 ppm assignable to the H-2 proton was coupled to two double doublets at 3.70 and 3.63 ppm that are assignable to the two H-3 protons and also to a triplet at 3.50 ppm assignable to the H-1 protons. A double triplet at 3.44 ppm assignable to the H-1′ protons was coupled to the methylene resonance at 1.55 ppm. The ^{1}H NMR spectrum was consistent with the ^{1}H NMR spectrum of commercial 1-0-hexadecylglycerol and with earlier literature data (18). The ^{1}H NMR methyl resonance was observed as a complex multiplet, which was suggestive of methyl branch points on the hydrocarbon side chain.

Mass spectroscopy (direct insertion) of the intact alkyl glycerol monoethers and GC/MS of the isopropylidene derivatives identified three species of glycerol ethers with hydrocarbon chains corresponding to 16:0 (1) and 17:0 (2 and 3). The mass spectra of the isopropylidene derivatives gave a characteristic base peak at m/z 101, which is supportive of the glycerol monoether structure (19). The mass spectra, however, did not give any information on the existence or position of any methyl branching in the hydrocarbon side chain.

To establish the position of methyl branching, the ether linkage was cleaved and the resulting alkyl iodide was derivatized to the pyrrolidide derivative for mass spectral determination of the branch position(s). N-Acylpyrrolidides have been shown to be suitable derivatives for the mass spectral localization of methyl branches and unsaturation in fatty acids (15,20). GC/MS of both the nitrile and the pyrrolidide derivatives produced diagnostic fragments indicating the presence and position of the methyl branching. The three hydrocarbon chains were identified as 17:0, 18:0 iso and 18:0 anteiso, an extra carbon atom having been added during the transformation via the nitrile derivative. Thus, the hydrocarbon side chains of compounds 1, 2 and 3 correspond to 16:0, 17:0 iso and 17:0 anteiso, respectively (Fig. 1). The position of the methyl branch was indicated by fragments of higher intensity 14 amu above and below a low intensity fragment. The mass spectra of the nitrile derivatives, however, proved to be superior to those of the pyrrolidides, showing significantly larger intensity differences between the diagnostic fragments. For example, the 17:0 iso nitrile derivative gave fragments of mass 250 (23%), 236 (3%) and 222 (40%), while the pyrrolidide derivative showed 322 (2%), 308 (0.05%) and 294 (2%). The mass spectra of aliphatic nitrile compounds have been described previously (21).

Quantification of the individual species was achieved by GLC of the isopropylidene derivatives of compounds 1, 2 and 3; the results are shown in Table 1.

The specific rotation, $[\alpha]_{598}^{20}$ + 2.9°, of the alkyl glycerol monoether mixture is in numerical agreement with that found for other natural glycerol monoethers (4,22,23), all of which possess the (2S)-absolute configuration.

The reduction of phospholipids with $LiAlH_4$ or $NaAlH_2(OCH_2OCH_3)_2$ has been suggested (24) as an efficient method of isolating alkyl and alk-1′-enyl glycerol monoethers from complex lipids. In view of the presence of the unesterified glycerol monoethers in *T. aurantia*, it was of interest to examine the presence of glycerol ethers in the phospholipids of this sponge.

Reduction of *T. aurantia* phospholipids with $LiAlH_4$ allowed the isolation of both alkyl and alk-1′-enyl glycerol monoethers, which were identified by ^{1}H NMR, GC/MS and chemical derivatization. The compositions of the alkyl glycerol monoethers derived from the phospholipids were found to be identical to the unesterified alkyl glycerol monoethers. The proportion of the three species, however, was markedly different, as shown in Table 1.

The alk-1′-enyl glycerol ether moiety (see Fig. 2) was identified by ^{1}H NMR (23). A doublet at 5.94 ppm was assigned to the H-1′ proton and a quartet at 4.41 ppm to the H-2′ proton. The chemical shift and coupling constant of the H-1′ are consistent with the *cis* isomer and similar to other naturally occurring alk-1′-enyl glycerol ethers (25). The resonances between 4.0 ppm and 3.6 ppm were assignable to the protons of the glycerol moiety. Specifically, a multiplet at 3.92 ppm was assigned to the

TABLE 1

Composition of Glycerol Ethers Isolated from *T. aurantia*

Hydrocarbon side chain	Alkyl glycerol monoethers		Alk-1′-enyl glycerol monoethers
	Unesterified[a]	Phospholipids[b,c]	Phospholipids[b,d]
16:0	44	92	52
17:0 iso	32	6	—
17:0 anteiso	24	2	—
18:0	—	—	30
18:1	—	—	10
18:2	—	—	8

[a]Isolated directly without any prior chemical step. Represent 2% of the total lipids of the sponge.

[b]Isolated after reduction of phospholipids (40% of total lipids of the sponge) with $LiAlH_4$.

[c]Represent 32% of *T. aurantia* phospholipids.

[d]Represent 26% of *T. aurantia* phospholipids.

H-2 proton, the double doublets at 3.80 ppm were assigned to the H-1 protons and the two quartets at 3.74 and 3.68 ppm were assigned to the H-3 protons. Assignment of the glycerol protons was confirmed by acetylation of the mixture, which resulted in a downfield shift of ca. 1 ppm of the proton resonance(s) attached to the O-acetylated carbons. A triplet at 5.39 ppm indicated additional unsaturation in the alk-1′-enyl hydrocarbon side chain, while the methyl resonance was a clean triplet indicating no detectable methyl branching.

From the quantities of the compounds isolated, the proportions of the three species of phospholipid—diacyl, monoalkylacyl and monoalk-1′-enylacyl glycerides—were 42%, 32% and 26%, respectively. Thus, about half of the phospholipids in *T. aurantia*, which represent 40% of the total lipids extracted, contain an ether linkage.

To establish the identities of the hydrocarbon side chains of the alk-1′-enyl glycerol ethers, the ether bond was hydrolyzed with HCl to yield aldehydes and glycerol (16). The aldehydes were then oxidized to the corresponding acids and subsequently transformed into pyrrolidides. GC/MS of the pyrrolidide derivatives and GLC of the methyl esters enabled identification and quantification of the hydrocarbon side chains as 16:0, 18:0, 18:1 and 18:2 (see Fig. 2 and Table 1). The positions of unsaturation were identified by GC/MS by an interval of 12 amu in the fragmentation instead of the regular 14 amu spacing and suggested the unsaturation positions to be Δ^9-18:1 and $\Delta^{8,14}$-18:2. For example, Δ^9-18:1 showed characteristic fragments at m/z 222, 208, 196 and 182, while the $\Delta^{8,14}$-18:2 acid displayed characteristic fragments at m/z 276, 264 and 250 and at 208, 194, 182 and 168. The suggested unsaturation positions at $\Delta^{8,14}$-18:2 are to our knowledge previously unknown. Insufficient material was available to provide more secure evidence for the location of these double bonds.

Incorporation experiments were carried out with *T. aurantia* using both intact sponges and dissociated sponge cells. Different incorporation results were obtained using [1-^{14}C]hexadecanol with the two systems. Significant incorporation (1.1% of the total activity added) into the unesterified alkyl glycerol monoethers was obtained with dissociated sponge cells, whereas the esterified (i.e., phospholipid-derived) alkyl and alk-1′-enyl glycerol ethers contained essentially no radioactivity (<0.03% of the total activity added).

The incorporation experiments with intact sponges resulted primarily in the oxidation of the [1-^{14}C]hexadecanol to the acid and incorporation of the acid into the phospholipids; thus 1.7% and 1.1% of the total activity added was recovered, after 7 and 17 days, respectively, as fatty alcohols after reduction of the phospholipids. The incorporation of fatty alcohols primarily into complex lipids after being oxidized has also been observed for various mammalian tissues (26). The glycerol ether fractions contained essentially no radioactivity (<0.08% of the total activity added).

Screening a number of marine sponges of the class Demospongiae—*Aplysina fistularis* (Order Verongida), *Microciona prolifera* (O. Poecibsclerida), *Pseudaxinyssa* sp., Australian Museum Specimen #Z4988 (O. Axinellida) and *Reneira* sp. (O. Haplosclerida)—did not uncover any detectable level of unesterified alkyl glycerol monoethers, thus showing that this type of ether lipid is not widely distributed among sponges. No evidence of any di- or triglycerides of any form was obtained.

The unesterified alkyl glycerol monoethers **2** and **3**, isolated from *T. aurantia*, are novel compounds. The ether **1**, although previously reported, so far has not been encountered in unesterified form. Their biosynthesis can be explained on the basis of known enzymatic pathways (27) and represents a branch of the usual biosynthetic pathway to esterified glycerol ethers. The hydrocarbon side chains of the alkyl glycerol ethers, especially the 17:0 iso and anteiso chains, and possibly 16:0, are probably of bacterial origin, being either assimilated from dietary sources, which consist largely of bacteria (28), and/or contributed from the presumably symbiotic microorganisms residing in the sponge matrix. (*T. aurantia* contains numerous microorganisms as shown by electron microscopy; unpublished results.) Recently it has been shown that short-chain straight and branched fatty acids are elongated and desaturated by the sponges *Jaspis stellifera* (29) and *Aplysina fistularis* (30). The ^{14}C incorporation experiment with dissociated sponge cells suggests that the unesterified alkyl glycerol monoethers are, however, biosynthesized by *T. aurantia* and are not of dietary origin. The difference between the hydrocarbon side chain composition of the esterified alkyl and alk-1′-enyl glycerol monoethers (see Table 1) is surprising in view of the fact that alkyl glycerol ethers are considered (26) to be the precursors of the alk-1′-enyl glycerol ethers.

Alkyl glycerol monoethers have been found to possess potent antimicrobial activity, affecting both glycerolipid and lipoteichoic acid biosynthesis in *Streptococcus mutans* (31). Thus, it is possible that the alkyl glycerol monoethers isolated from *T. aurantia* may play a defensive role as antimicrobial agents.

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