Phospholipid Studies of Marine Organisms: V. ^1
New \( \alpha \) -Methoxy Acids from Higginsia tethyoides

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**ABSTRACT**

The phospholipids of the demosponge Higginsia tethyoides are shown to have at least 16 long-chain \( \alpha \)-methoxy acids, which represent a new class of fatty acids. Among them are the saturated \( \alpha \)-methoxy acids containing 19-24 carbon atoms. The monounsaturated compounds are 2-OMe-A^17-24:1, 2-OMe-A^18-25:1, 2-OMe-A^19-26:1 and 2-OMe-A^21-28:1. The major diunsaturated ones were shown to be 2-OMe-A^19-26:2 and 2-OMe-A^21-28:2. Small amounts of 2-OMe-23:1, 2-OMe-26:3, 2-OMe-27:1 and 2-OMe-28:3 were also encountered. Structures of the minor monounsaturated compounds were tentatively assigned as 2-methoxy-16-tricosenoic acid and 2-methoxy-20-heptacosenoic acids. The double bonds of the fatty acids show all-cis configuration. Circular dichroism measurements indicate an R-configuration for the \( \alpha \)-methoxy acids. The major component of the total phospholipid acid mixture is 5,9,23-triacontatrienoic acid. Possible biosynthetic routes to these unusual phospholipid acids are discussed. The major phospholipids were phosphatidylethanolamine, phosphatidylglycerol and phosphatidylserine. The distribution of fatty acids among the phospholipids was also investigated.

**INTRODUCTION**

The reports of a large number of novel sterols with unusual side-chain elongation and branching in marine invertebrates and algae (2,3) raise interesting questions concerning membrane structures of these organisms. Recent research carried out in various laboratories has shown that some other lipophilic compounds such as carotenoids and tetracyclic sesterpenoids are present in primitive marine organisms (4-6). In addition, Litchfield and coworkers (7-11) have encountered new fatty acids in different species of sponges. These “demospongiac” acids contain 24-30 carbons and usually feature A^19 diunsaturation, sometimes together with one more double bond near the chain terminus. Our investigation of marine organisms has already revealed a large number of straight chain and methyl branched C_24-30 phospholipid acids featuring typical A^19 diunsaturation or A^\( \gamma \) monounsaturation in the sponges Aplysina fistularis (12), Petrosia ficiformis (13) and Strongylophora durissima (Dasgupta, A., Ayanoglu, E., and Djerassi, C., manuscript in preparation), all of which also contain high levels of sterols with “unusual” side chains (12,14-17). Another unusual feature is that 5,9-hexacosadienoic acid (A^\( \gamma \)-26:2) was found to be virtually the sole acid in the PS and PE fractions of the Mediterranean sponge Axinella verrucosa (Ayanoglu, E., and Djerassi, C., unpublished data), in contrast to conventional phospholipids in which the two acyl fragments are different.

In a recent communication, we have also reported (1) the occurrence of a new compound, (2R,21Z)-2-methoxy-21-octacosenoic acid, as the second major nonpolar component in the phospholipids of the sponge Higginsia tethyoides, following 5,9,23-triacontatrienoic acid (A^\( \gamma \)-30:3), a previously reported (8) demospongic acid. Consequently, we undertook a more extensive search for such \( \alpha \)-methoxy acids, which do not appear to have been encountered previously in nature, and report here-with the isolation and identification of an entire series of such acids in the phospholipids of the African sponge Higginsia tethyoides.

**EXPERIMENTAL**

H. tethyoides sponge colonies were collected near Joal (ca. 100 km from Dakar, Senegal). The lyophilized samples were extracted with cold CHCl\(_3\)/MeOH (1:1, v/v). Separation of the total phospholipids from other lipids using silicic acid column chromatography has already been described (12). The samples were kept under argon at -10°C in solutions containing 0.002% BHT. Silica Gel H was used as thin layer chromatography (TLC) adsorbent for separations of fatty acid derivatives as well as phospholipids. Assignments based on the comparisons with codeveloped standards were verified by the use of ninhydrin (PE, PS), Dragendorff (PC, SM) and periodate-Schiff (DPG, PG, PI) reagents. Molybdenum Blue and ceric sulfate

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were the general reagents for the phospholipids and fatty acid derivatives, respectively, and preparative TLC plates were sprayed with Rhodamine 6G as the nondestructive color reagent (18,19). Quantitative estimation of phospholipids was carried out using a spectrometric phosphorus assay (20). Phospholipids were chromatographed in chloroform/methanol/28% NH4OH (65:35:8, v/v/v). General developing solvent systems for the fatty acid derivatives were hexane/ether (8:2, v/v) and (3:7, v/v). For argentie TLC, 75 μ layers of silica gel were impregnated with 15% (by weight) silver nitrate, and the plates were developed in hexane/dichloromethane (8:2).

The following instruments and conditions were used for other chromatographic separations and for obtaining physical data.

**Capillary gas chromatography (GC).** Carlo Erba series 4160 Fractovap chromatograph equipped with a fused silica column (30 m × 0.32 nm) coated with SE-54 (J & W scientific, Inc.), a Model 400 LT programmer, a cooled on-column injection system and a flame ionization detector were used. The initial oven temperature was 70°C or 140°C for fatty acid methyl esters and 200°C for pyrrolidides. Final temperature was 290°C. The program temperature was either set to 3.0 or 10.0°C/min.

**High performance liquid chromatography (HPLC).** A 50 cm × 9 mm Whatman ODS-2 reversed-phase column or a 25 cm × 10 mm Altex Ultrasphere column, a Waters M-6000A pump, a Valco loop injector and a Waters R401 refractometer detector were used.

**Gas chromatography-mass spectrometry (GC-MS).** Either a Finnigan MAT-44 GC-MS system using a spiral glass column (1.80 m × 2.0 mm), coated with 3% OV-17 on GCQ, or a Ribermag GC-MS-DS system, combining a Ribermag R 10-10 quadrupole mass spectrometer with a Carlo Erba series 4160 Fractovap chromatograph containing a fused silica column (28 m × 0.32 mm) with SE-54 (J & W Scientific, Inc.) was used.

**Nuclear magnetic resonance (NMR) spectra.** A Varian Associates HA-100 NMR instrument was used. Deuterated chloroform was used as solvent and shift values are given in ppm (δ).

**Infrared (IR) spectra.** A Beckman Acculab spectrophotometer was used. The samples were measured in chloroform.

**Circular dichroism (CD) spectra.** A Jasco Model J-40 instrument was used, with all measurements being conducted in hexane or in cyclohexane.

Transesterification of the phospholipid acids to obtain methyl esters was carried out by sodium hydroxide in methanol and hydrochloric acid in methanol (21). Boron trifluoride in methanol was used for the same purpose (22). The individual phospholipid classes were analyzed for the fatty acid content by preparative TLC. Each phospholipid band was scraped into a test tube, digested with methanolic boron trifluoride for transesterification (23) and the resulting methyl esters then analyzed by GC. Aliquots of fatty acid methyl esters were hydrogenated in methanol with platinum (IV) oxide (8 hr, normal pressure, room temperature). The methyl esters were converted to N-acetylpyrrolidine derivatives in pyrroline/acetic acid (10:1, v/v, 1 hr, 100°C) for the location of double bonds as well as branching. LiAlH4 reduction was carried out in dry tetrahydrofuran (2 hr, reflux temperature). NaIO4/KMnO4 oxidative degradation was achieved in tert-butanol (24) (5-7 hr, room temperature). Ozonization in BF3/MeOH was also performed (25) for the conversion of double bonds to methyl esters and gave almost quantitative yields. For this purpose, the sample was subjected to ozonization using 14% BF3 in methanol as reactive solvent for 1 min at room temperature and the reaction mixture was heated at 100°C in a capped vial for 1 hr. The standard fatty acid methyl ester samples were obtained from Supelco (Supelco Park, Bellefonte, PA) or from Applied Science (Milton Ray Co. Laboratory Group, State College, PA) for comparison purposes.

**RESULTS AND DISCUSSION**

Quantitative analysis of the phospholipids using solvent elution and spectrophotometric analysis of each TLC spot indicated that the 4 classes listed in Table 1 comprise ca. 86% of the mixture and that PC was essentially absent. The remaining 14% was distributed among several minor spots on the chromatogram.

The capillary GC analysis of the total phospholipid fatty acid mixture allowed equivalent chain length (ECL) values to be assigned to each major peak, on the basis of their retention times, relative to methyl esters (26). This analysis indicated the presence of ca. 61 detectable peaks, of which 21 comprised 87% of the total (Table 2). Many of the peaks, especially the unsubstituted "conventional" ones, were simply identified by comparing their

<table>
<thead>
<tr>
<th>Phospholipid class</th>
<th>Mol %</th>
</tr>
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<tbody>
<tr>
<td>Phosphatidylethanolamine</td>
<td>31</td>
</tr>
<tr>
<td>Phosphatidyleglycerol</td>
<td>24</td>
</tr>
<tr>
<td>Phosphatidylycerine</td>
<td>26</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>5</td>
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</tbody>
</table>

*Average of 3 replicates; percentages are based on relative phosphate content.

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