The First Naturally Occurring α-Methoxylated Branched-Chain Fatty Acids from the Phospholipids of *Amphimedon complanata*

Néstor M. Carballeira* and Johanna Alicea

Department of Chemistry, University of Puerto Rico, Río Piedras, Puerto Rico 00931

ABSTRACT: The phospholipid fatty acid composition of the sponge *Amphimedon complanata* was reinvestigated, and the 2-methoxy-13-methyltetradecanoic acid, 2-methoxy-14-methylpentadecanoic acid, and 2-methoxy-13-methylpentadecanoic acid were identified for the first time in nature. Structure characterization was accomplished by means of gas chromatographic retention times and gas chromatography–mass spectrometry. These acids could have originated from bacteria in symbiosis with the sponge.

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The only naturally occurring α-methoxy fatty acids known to date are those from the phospholipids of sponges (1–3). Earlier examples include normal-chain saturated 2-methoxy fatty acids between C_{19}–C_{24} carbons and very long chain monounsaturated fatty acids such as (2*R*,21*Z*)-2-methoxy-21-octacosenoic acid, which was the first-reported α-methoxy fatty acid from a phospholipid (2). All of these acids were reported to have the *R* configuration at the chiral center. Recent efforts have concentrated on the C_{16} family of α-methoxylated fatty acids, such as the 2-methoxy-5(*Z*)-hexadecenoic acid, 2-methoxy-6(*Z*)-hexadecenoic acid, and the 2-methoxyhexadecanoic acid, which were identified in the phospholipids of several Caribbean sponges such as *Amphimedon compressa* (4). Both 2-methoxy-5(*Z*)-hexadecenoic acid and 2-methoxyhexadecanoic acid occur more often in the phospholipids of sponges than their Δ6 analogs (5).

Despite these isolation efforts there are no reports in the literature as to any natural source for branched-chain α-methoxylated fatty acids although some mid-chain methoxylated fatty acids, such as (4*E*,8*E*)-7-methoxy-9-methylhexadeca-4,8-dienoic acid from *Lyngbya majuscula*, are known (6). We have now investigated the sponge *A. complanata* (7), collected at a different site, and found a novel series of iso and anteiso C_{15}–C_{16} α-methoxylated phospholipid fatty acids that we describe herein. These novel compounds could have originated from the phospholipids of a novel bacterium in symbiosis with *A. complanata*.

*To whom correspondence should be addressed at Department of Chemistry, University of Puerto Rico, P.O. Box 23346, San Juan, Puerto Rico 00931-3346. E-mail: ncarball@upracd.upr.clu.edu

Abbreviations: DMDS, dimethyl disulfide; ECL, equivalent chain length; FCL, fractional chain length; GC–MS, gas chromatography–mass spectrometry; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Instrumentation. Gas chromatography–mass spectrometry (GC–MS) data were collected at 70 eV with a Hewlett-Packard 5972A MS ChemStation (Palo Alto, CA) equipped with a 30 m × 0.25 mm special performance capillary column (HP-5MS) cross-linked with 5% phenyl methylpolysiloxane. The temperature program for the analyses was as follows: 130°C for 2 min, then increased at 3°C/min to 270°C and maintained for 40 min.

Sample collection. *Amphimedon complanata* was collected on February 27, 2000, at El Natural, Aguadilla, Puerto Rico, at a depth of 25 ft (7.6 m). The sponge was washed in seawater, carefully cleaned of all nonsponge debris, and lyophilized. A voucher specimen is available at the Department of Chemistry of the University of Puerto Rico, Río Piedras campus.

Extraction and isolation of phospholipids. The freeze-dried sponge (48.2 g) was extracted with 2 × 250 mL of chloroform/methanol (1:1, vol/vol) yielding the total lipids (11.9 g). The neutral lipids, glycolipids, and phospholipids (1.2 g) were separated by column chromatography on silica gel (60–200 mesh) using the procedure of Privett *et al*. (8). The phospholipid classes were investigated by preparative thin-layer chromatography (TLC) using silica gel and $CHCl_3$/MeOH/NH_4OH (65:35:5) as solvent and comparing with authentic samples. The principal phospholipids were phosphatidylethanolamine and phosphatidylserine, as previously described (7).

Preparation and isolation of fatty acid derivatives. The fatty acyl components of the phospholipids were obtained as either their methyl or ethyl esters by reaction of the phospholipids with methanolic or ethanolic HCl followed by column chromatography (9). The double-bond positions in the monounsaturated acids were determined by dimethyl disulfide (DMDS) derivatization following the standard procedure previously described (10). *N*-Acylpyrrolidide derivatives were prepared by direct treatment of the methyl esters with pyrrolidine-HOAc (10:1) in a capped vial (24 h at 100°C). The methyl esters were hydrogenated in 10 mL absolute methanol in the presence of catalytic amounts of platinum oxide (PtO_2). Mass spectral data for the novel methoxylated acids and some of their derivatives follow.

Methyl 2-methoxy-13-methyltetradecanoate. Equivalent chain length (ECL) = 15.86; GC–MS *m/z* (relative intensity) M^+ 286 (1), 270 (1), 236 (2), 228 (17), 227 (100), 213 (1),

199 (1), 185 (1), 171 (1), 153 (1), 152 (2), 139 (3), 138 (3), 125 (11), 123 (6), 117 (1), 111 (27), 109 (10), 104 (6), 99 (4), 97 (58), 95 (21), 87 (17), 85 (16), 83 (68), 81 (31), 71 (61), 69 (68), 67 (37), 58 (15), 57 (50), 55 (70).

Ethyl 2-methoxy-13-methyltetradecanoate. ECL = 15.79 (calculated using ethyl esters); GC–MS *m/z* (relative intensity) M^+ 300 (1), 270 (1), 237 (3), 235 (1), 228 (1), 227 (100), 213 (1), 211 (1), 194 (4), 185 (1), 171 (1), 155 (3), 152 (4), 139 (5), 138 (3), 125 (13), 118 (6), 111 (33), 109 (13), 101 (13), 97 (69), 95 (25), 88 (14), 85 (23), 83 (83), 81 (35), 71 (69), 69 (82), 67 (40), 58 (17), 57 (68), 55 (84).

N-*2-Methoxy-13-methyltetradecanoylpyrrolidine*. GC–MS *m/z* (relative intensity) M^+ 325 (1), 310 (10), 295 (30), 266 (2), 252 (4), 228 (8), 227 (33), 196 (2), 168 (2), 156 (2), 143 (55), 128 (15), 126 (24), 113 (22), 111 (29), 97 (63), 95 (12), 85 (14), 83 (61), 82 (12), 72 (19), 69 (64), 67 (20), 57 (47), 55 (100).

Methyl 2-methoxy-14-methylpentadecanoate. ECL = 16.86; GC–MS *m/z* (relative intensity) M^+ 300 (1), 250 (2), 242 (9), 241 (55), 167 (1), 153 (2), 152 (2), 139 (2), 125 (8), 111 (24), 109 (10), 104 (4), 99 (5), 97 (59), 95 (21), 85 (24), 83 (55), 81 (27), 68 (22), 67 (30), 58 (15), 57 (61), 55 (73).

Methyl 2-methoxy-13-methylpentadecanoate. ECL = 16.94; GC–MS *m/z* (relative intensity) M^+ 300 (1), 250 (3), 242 (12), 241 (70), 239 (2), 180 (2), 167 (1), 153 (2), 152 (3), 139 (5), 138 (5), 125 (10), 123 (7), 111 (30), 104 (10), 99 (5), 97 (66), 95 (37), 87 (9), 85 (23), 83 (81), 81 (37), 70 (13), 69 (81), 67 (38), 58 (18), 57 (100), 55 (94).

RESULTS

As previously reported, the main phospholipids from *A. complanata* were phosphatidylethanolamine and phosphatidylserine, with lesser amounts of phosphatidylcholine (7). Transesterification with either 1.0 N HCl/MeOH or HCl/EtOH permitted the characterization of the fatty acids as methyl or ethyl esters and the aldehydes as dimethyl or diethyl acetals using GC–MS. A total of 72 phospholipid fatty acids were identified. DMDS derivatives were used to locate the double bonds in the monounsaturated methyl esters, whereas pyrrolidides were mainly used to locate methyl branching. The total phospholipid fatty acid composition of *A. complanata* is presented in Table 1. Saturated normal-chain fatty acids between C_{12} and C_{29} predominated in these phospholipids (42%), in particular the acids 16:0 and 18:0, which were the most abundant in this family (12.3%). Monounsaturated fatty acids between C_{16} and C_{30} were the second-most abundant series of compounds (26.8%) and noteworthy among these was vaccenic acid. Branched-chain iso and anteiso fatty acids accounted for only 7% of the total composition, but most of these acids had chain lengths between C_{15} and C_{19}, which are the typical chain-lengths of iso and anteiso bacterial fatty acids.

Attention was centered on six α-methoxylated fatty acids that constituted 1.7% of the total phospholipid fatty acid composition. Three of these acids, namely, 2-methoxy-5(*Z*)-hexadecenoic acid, 2-methoxy-6(*Z*)-hexadecenoic acid and 2-

TABLE 1
Identified Phospholipid Fatty Acids from *Amphimedon complanata*

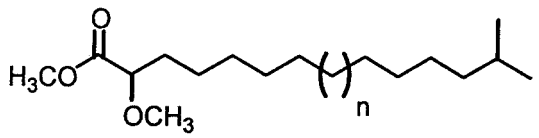
Fatty acids	Relative abundance[a] (wt%)
Dodecanoic (12:0)	0.4
Tetradecanoic (14:0)	4.0
12-Methyltetradecanoic (i-15:0)	1.5
13-Methyltetradecanoic (ai-15:0)	0.9
Pentadecanoic (15:0)	1.0
11-Hexadecenoic (16:1n-5)	1.1
2-Methoxy-13-methyltetradecanoic (2-OMe-i-15:0)[b]	0.9
Hexadecanoic (16:0)	6.2
2-Methoxy-5-hexadecenoic (2-OMe-5-16:1)	0.01
2-Methoxy-6-hexadecenoic (2-OMe-6-16:1)	0.01
2-Methoxy-14-methylpentadecanoic (2-OMe-i-16:0)[b]	0.2
2-Methoxy-13-methylpentadecanoic (2-OMe-ai-16:0)[b]	0.4
Heptadecanoic (17:0)	1.0
2-Methoxyhexadecanoic (2-OMe-16:0)	0.2
7-Methyl-6-hexadecenoic (17:1)	1.3
15-Methylhexadecanoic (i-17:0)	1.9
14-Methylhexadecanoic (ai-17:0)	0.8
9-Heptadecenoic (17:1n-8)	0.4
Heptadecanoic (17:0)	2.4
9,12-Octadecadienoic (18:2n-6)	0.5
9-Octadecenoic (18:1n-9)	0.8
11-Octadecenoic (18:1n-7)	4.1
Octadecanoic (18:0)	6.1
Methyloctadecanoic (19:0)	1.5
11-Methyloctadecanoic (19:0)	1.1
17-Methyloctadecanoic (i-19:0)	1.0
16-Methyloctadecanoic (ai-19:0)	0.7
Nonadecenoic (19:1)	0.8
Nonadecenoic (19:1)	1.1
Nonadecanoic (19:0)	2.4
5,8,11,14-Eicosatetraenoic (20:4n-6)	2.8
Eicosadienoic (20:2)	1.9
11,14-Icosadienoic (20:2n-6)	2.7
11-Eicosenoic (20:1n-9)	1.9
13-Eicosenoic (20:1n-7)	1.4
Eicosanoic (20:0)	1.9
Methyleicosanoic (21:0)	0.5
Heneicosanoic (21:0)	1.9
4,7,10,13,16,19-Docosahexaenoic (22:6n-3)	0.9
7,10,13,16-Docosatetraenoic (22:4n-6)	2.4
4,7,10,13,16-Docosapentaenoic (22:5n-6)	1.4
13-Docosenoic (22:1n-9)	0.5
15-Docosenoic (22:1n-7)	1.0
17-Docosenoic (22:1n-5)	0.3
Docosanoic (22:0)	7.5
16-Tricosenoic (23:1n-7)	1.4
17-Tricosenoic (23:1n-6)	1.8
18-Tricosenoic (23:1n-5)	0.7
Tricosanoic (23:0)	4.0
2-Hydroxydocosanoic (2-OH-22:0)	1.1
17-Tetracosenoic (24:1n-7)	1.3
18-Tetracosenoic (24:1n-6)	1.4
19-Tetracosenoic (24:1n-5)	1.8
Tetracosanoic (24:0)	3.0
18-Pentacosenoic (25:1n-7)	0.8
19-Pentacosenoic (25:1n-6)	0.8
5,9-Hexacosadienoic (26:2n-17)	0.7
17-Hexacosenoic (26:1n-9)	0.5
19-Hexacosenoic (26:1n-7)	0.9
Heptacosenoic (27:1)	0.1
5,9,19-Octacosatrienoic (28:3n-9)	0.2
5,9,21-Octacosatrienoic (28:3n-7)	0.1
5,9-Octacosadienoic (28:2n-19)	0.1
19-Octacosenoic (28:1n-9)	0.1
21-Octacosenoic (28:1n-7)	0.3
Octacosanoic (28:0)	0.01
Nonacosanoic (29:0)	0.01
5,9,21-Nonacosatrienoic (29:3n-8)	0.02
5,9,23-Nonacosatrienoic (29:3n-6)	0.1
5,9,23-Triacontatrienoic (30:3n-7)	4.8
21-Triacontenoic (30:1n-9)	0.1
23-Triacontenoic (30:1n-7)	0.1

[a]The following aldehydes were also identified in the sponge: 16:0, 17:0, 18:0, 19:0, 20:0, and 25:0.
[b]These acids are unprecedented in nature.

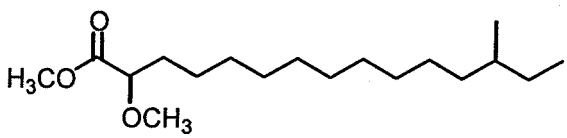
methoxyhexadecanoic acid, were previously reported by us from several Caribbean sponges (3,4). The finding in *A. complanata* that has never been observed before is that all three of these acids were present (at low levels) in the same sponge. Characterization of the remaining three saturated methoxylated acids (as either methyl or ethyl esters) was possible using GC–MS as well as gas chromatographic ECL values as compared to synthetic standards (4). For example, the mass spectrum of one of these methyl esters, **1a** (Scheme 1), displayed a molecular ion peak at *m/z* 286 and a strong $M^+ - 59$ peak at *m/z* 227 (100%), together with a small peak at *m/z* 104 (McLafferty rearrangement) typical of an α-methoxylated saturated methyl ester (4). On the other hand, both methyl esters **1b** and **2** yielded molecular ion peaks at *m/z* 300 and strong $M^+ - 59$ peaks at *m/z* 241, together with small peaks at *m/z* 104, confirming that both were α-methoxylated C_{16} saturated methyl esters (4). The natural origin of the α-methoxy substitution was further confirmed when the corresponding ethyl esters were directly prepared from the phospholipids, since α-methoxylated ethyl esters were obtained. For example, in the mass spectrum of the ethyl ester of **1a**, the molecular ion peak shifted to *m/z* 300 and the McLafferty rearrangement peak to *m/z* 118, but the *m/z* 227 peak remained the same. In the mass spectrum of ethyl esters **1b** and **2**, the M^+ shifted to *m/z* 314 and the McLafferty rearrangement peak to *m/z* 118, but the *m/z* 241 peak remained the same. Therefore, this simple transesterification reaction (HCl/EtOH) proved that the methoxylated fatty acids are not artifacts arising from the HCl/MeOH reaction.

Although the α-methoxy substitution in these novel C_{15}–C_{16} methyl esters was confirmed, the GC retention times suggested that all of these methoxylated methyl esters were branched. The position of the methyl branching was determined from ECL values and mass spectral data. For example, methyl esters **1a** and **1b** presented ECL values of 15.86 and 16.86, respectively, whereas **2** had an ECL value of 16.94 on the nonpolar capillary column. These values suggested methyl branching near the end of the chain, in particular when compared to a calculated ECL value of *ca.* 16.2 expected for a normal-chain methyl 2-methoxypentadecanoate and an ECL value of 17.2 reported for methyl 2-methoxyhexadecanoate (4). From the ECL values it was also clear that **1a** and **1b** had the same methyl substitution, while the methyl substitution in **2** was different. Additional data further favored an iso branching for **1a** and **1b**, and an anteiso branching for **2**. For example, the methyl branching in methyl 2-hydroxy-13-methyltetradecanoate was determined by actually synthesizing the methoxylated ester **1a** and further looking in its mass spectrum at the intensity of the *m/z* 43 fragment (11). Comparing the intensity of the *m/z* 43 fragment (loss of a propyl unit) with that of other fragments and further comparing their respective intensities to those observed in the mass spectrum of a normal-chain α-methoxy methyl ester are good ways to confirm the iso branching (11). In fact, the intensity of the *m/z* 43 fragments in **1a** and **1b** was increased compared to other mass peaks, such as the *m/z* 57 fragments. For this comparison the mass spectrum (measured under the same conditions as **1a** and **1b**) of methyl 2-methoxyhexadecanoate, previously synthesized and also present in this sponge, was used. In the case of **2,** a distinctive ion at $[M - 61]^+$ (*m/z* = 239) was observed, indicative of anteiso branching. Another piece of evidence favoring the iso branching for **1a** and **1b** and the anteiso branching for **2** was obtained from their GC retention times when gas chromatographic ECL values were calculated using only the α-methoxy methyl esters as standards. In such a calculation **1a** and **1b** present fractional chain-length (FCL) values of 0.67, while **2** has an FCL of 0.74, in reasonable agreement with those values reported for the traditional iso and anteiso fatty acid methyl esters (12). Therefore, it can be concluded that the α-methoxylated methyl esters in question are the iso methyl 2-methoxy-13-methyltetradecanoate (**1a**) and methyl 2-methoxy-14-methylpentadecanoate (**1b**), as well as the anteiso methyl 2-methoxy-13-methylpentadecanoate (**2**).

Pyrrolidide derivatives were also synthesized in an attempt to determine the methyl branching in the methoxylated acids by GC–MS, but fragmentations involving the polar methoxy group obscured the typical fragmentation pattern expected from a branched pyrrolidide (13). For example, in the mass spectrum of *N*-2-methoxy-13-methyltetradecanoylpyrrolidine a molecular ion peak (M^+) at *m/z* = 325 was observed together with strong fragmentations at *m/z* = 295 ($M^+ - 30$), *m/z* = 227 ($M^+ - 98$), and the typical McLafferty rearrangement at *m/z* = 143 confirming the α-methoxy functionality. However, a strong ($M^+ - 15$) peak at m/z = 310 was also identified, and the intensity of this ($M^+ - 15$) peak was higher than the corresponding peak in the mass spectrum of *N*-2-methoxyhexadecanoyl pyrrolidine, which we also synthesized. This observation also supported the iso-branching for **1a**. Therefore, pyrrolidide derivatization has limited usefulness in locating methyl branching in α-methoxylated fatty acids.



1a, n=1
1b, n=2



2

SCHEME 1

DISCUSSION

This specimen of *A. complanata* contains a very complex phospholipid fatty acid profile typical of sponges belonging

to the genus *Amphimedon* (4,5,7). Noteworthy is its ability to extend the n-7 and n-9 families of even-numbered chain monounsaturated fatty acids from 9-18:1 and 11-18:1 to 21-30:1 and 23-30:1, respectively. In fact, this is the first report of both 21-triacontenoic acid and 23-triacontenoic acid from the phospholipids of an *Amphimedon* sponge. These C_{30} fatty acids were reported before from sponges such as *Trikentrion loeve* (14). The n-6 and n-7 families predominated in the odd-numbered chain monounsaturated fatty acid series, but they mainly reached C_{25}–C_{27} chain lengths.

The interesting feature of this *Amphimedon* is that it contains all of the C_{16} short-chain α-methoxylated fatty acids reported to date plus a series of novel iso-anteiso α-methoxylated C_{15}–C_{16} fatty acids. These are the first branched α-methoxylated phospholipid fatty acids from any natural source. An analogy to the corresponding iso-anteiso α-hydroxylated C_{15}–C_{16} fatty acids is unavoidable, and in fact the methoxylated acids could have originated, although not necessarily, from the corresponding α-hydroxylated fatty acids. For example, the 2-hydroxy-13-methyltetradecanoic acid is known as a characteristic lipid constituent of gliding bacteria such as *Flexibacter elegans* (11); it has been identified in the phospholipids of Gram-positive Flavobacteria such as *Flavobacterium meningosepticum* and also, in addition to α-OH iso-C_{16}, in the Actinomycetales such as *Arthrobacter simplex* (15–17). The occurrence of α-OH anteiso-C_{16} is rare, but it is known to be a constituent of the ceramide dihexosides from the spermatozoa of the starfish *Asterias amurensis* and a phospholipid constituent of *Arthrobacter simplex* (18,19). In addition, α-OH anteiso-C_{16} has been identified in Antartic lake sediment (19).

The identification of small amounts of α-methoxylated iso-anteiso C_{15}–C_{16} fatty acids in *A. complanata* suggests that these acids are probably constituents of a novel bacterium associated with the sponge. There are several pieces of information that point in this direction. One is their low abundance in the sponge, which indicates that they are not major sponge phospholipid constituents. The typical sponge fatty acids are the Δ5,9 acids, and several biosynthetic experiments tend to indicate that the shorter-chain C_{15}–C_{19} fatty acids are actually of bacterial origin (21). A structural comparison of these α-methoxylated fatty acids with known α-hydroxylated fatty acids also suggests a bacterial origin, as most of the α-OH iso and anteiso C_{15}–C_{17} acids are indeed bacterial in nature (22). In fact, some myxobacteria contain phosphatidylethanolamines as the major phospholipid with α-hydroxy iso-$C_{17:0}$ fatty acids in the 2-position and nonhydroxy fatty acids in the 1-position (22). Therefore, it is very likely that in *A. complanata* a novel symbiotic marine bacterium could actually contain novel phosphatidylethanolamines with iso and anteiso branched α-methoxylated fatty acids at the 2-position, similar to what is known for the α-hydroxy iso-$C_{17:0}$ fatty acids in myxobacteria (22).

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