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IDENTIFICATION OF A NEW 6-BROMO-5,9-EICOSADIENOIC ACID FROM THE ANEMONE *CONDYLACTIS GIGANTEA* AND THE ZOANTHID *PALYTHOA CARIBAEORUM*

NÉSTOR M. CARBALLEIRA* and MORAYMA REYES

Department of Chemistry, University of Puerto Rico, P.O. Box 23346, San Juan, Puerto Rico 00931-3346

ABSTRACT.—A new brominated fatty acid, 6-bromo-5,9-eicosadienoic acid [**1**] was identified in the phospholipids (mainly phosphatidylethanolamine) of the anemone *Condylactis gigantea* and the zoanthid *Palythoa caribaeorum*. The $\Delta^{5,9}$ fatty acids, 5,9-octadecadienoic acid, 5,9-eicosadienoic acid, 5,9-docosadienoic acid, and 5,9-tetracosadienoic acid, were also identified in both organisms. Structural elucidation was accomplished by spectroscopic and chemical means. Our results further corroborate that $\Delta^{5,9}$ phospholipid fatty acids are not unique to sponges, as recognized previously, but can be found in other marine invertebrates such as anemones and zoanthids. An improved procedure for the synthesis of picolinyl esters is also described.

Marine invertebrates are the only known sources of naturally occurring phospholipids with brominated fatty acids (1–6), and 6-bromo-$\Delta^{5,9}$ fatty acids, which were found originally in sponges and were also recently reported from the anemone *Stoichactis helianthus* (7), are the only known examples (8–10). Brominated phospholipids, such as 1-oleoyl-2-(9,10-dibromostearoyl)-*sn*-glycero-3-phosphocholine, have been used to study protein insertion into membranes (11,12). The bromine atom provides a unique probe for fluorescence quenching experiments and X-ray diffraction studies (13).

In the present work we report the occurrence of a previously unrecognized naturally occurring 6-bromo-$\Delta^{5,9}$ fatty acid, 6-bromo-5,9-eicosadienoic acid [**1**], which was found in the phospholipids (mainly phosphatidylethanolamine) of the anemone *Condylactis gigantea* Weinland (Cnidaria, Anthozoa) and the zoanthid *Palythoa caribaeorum* Duchassaing (Hexacorallia, Zoanthidea). These results confirm that brominated $\Delta^{5,9}$ fatty acids are more common in anemones than previously recognized, and can be found in other marine invertebrates such as zoanthids.

RESULTS AND DISCUSSION

The phospholipids from *P. caribaeorum* and *C. gigantea* were isolated and separated as described in the Experimental. The main phospholipids were identified as phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylcholine (PC). These results are in agreement with a recent ^{31}P-nmr phospholipid study profile of these anemones (14). Transesterification of the total and individual phospholipids with 1 N HCl/MeOH allowed the characterization of the principal fatty acids as methyl esters by gc-ms. Tables 1 and 2 show the fatty acid distribution in phospholipids from *P. caribaeorum* and *C. gigantea*, respectively. Both fatty acid profiles were similar. The principal fatty acids in *C. gigantea* were characterized as hexadecanoic (16:0), 5,8,11,14-eicosatetraenoic (20:4 *n*-6), 5,8,11,14,17-eicosapentaenoic (20:5 *n*-3), and 7,10,13,16-docosatetraenoic (22:4 *n*-6). In fact, polyunsaturated fatty acids accounted for 38% of the total fatty acid composition of the anemone. On the other hand, the principal fatty acids in *P. caribaeorum* were characterized as hexadecanoic (16:0), 9,12,15-octadecatrienoic

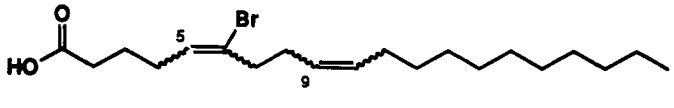

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TABLE 1. Fatty Acids from the Total Lipids and Major Phospholipids of *Palythoa caribaeorum*.

Fatty Acid	Abundance (wt %)			
	Total Lipids[a]	PE	PC	PS
Dodecanoic (12:0)	0.7	0.5	1.0	—
4,8,12-Trimethyltridecanoic (16:0)	0.7	—	0.8	—
Tetradecanoic (14:0)	2.4	3.2	5.6	1.0
9-Hexadecenoic (16:1)	3.3	3.3	3.0	—
Hexadecanoic (16:0)[b]	43.0	9.5	33.4	49.0
7-Methyl-6-hexadecenoic (17:1)[c]	3.7	3.7	1.9	—
Heptadecenoic (17:1)	4.5	2.2	—	—
Heptadecanoic (17:0)[b]	0.7	2.2	2.6	0.7
6,9,12,15-Octadecatetraenoic (18:4)	4.9	0.3	—	—
9,12,15-Octadecatrienoic (18:3)	0.7	0.4	0.2	—
5,9-Octadecadienoic (18:2)[d]	1.0	3.4	2.8	1.0
9-Octadecenoic (18:1)	1.4	29.2	25.0	16.2
11-Octadecenoic (18:1)	1.2	10.2	1.8	0.2
Octadecanoic (18:0)[b]	6.2	4.2	13.0	16.9
Nonadecanoic (19:0)[b]	0.6	—	0.4	0.7
5,8,11,14-Eicosatetraenoic (20:4)	5.2	4.3	0.4	1.0
Eicosatrienoic (20:3 *n*-6)	2.1	2.9	—	0.5
5,9-Eicosadienoic (20:2)[d]	2.0	1.4	—	1.0
13-Eicosenoic (20:1)	0.8	2.4	2.2	0.4
Eicosanoic (20:0)	0.8	1.5	0.5	0.5
Heneicosanoic (21:0)	0.8	0.8	0.6	—
7,10,13,16-Docosatetraenoic (22:4)	4.8	1.5	—	3.5
7,10,13,16,19-Docosapentaenoic (22:5)	3.0	—	—	2.0
5,9-Docosadienoic (22:2)[d]	0.9	0.9	—	1.5
Docosenoic (22:1)	0.5	11.6	3.3	2.3
5,9-Tetracosadienoic (24:2)[d]	0.7	—	—	—
Tetracosanoic (24:0)	0.6	—	—	—
6-Bromo-5,9-eicosadienoic [**1**] (20:2)[d]	0.3	0.1	—	—

[a]These are the total phospholipids. Other minor fatty acids identified in trace amounts were 9-tetradecenoic acid (14:1), pentadecanoic acid (15:0), and 9,12-octadecadienoic acid (18:2).

[b]The corresponding aldehydes were also detected in the fatty acid mixture as dimethyl acetals, probably arising from plasmalogens.

[c]Both (*E*) and (*Z*) isomers were observed in the total phospholipids.

[d]Occurrence of these acids in zoanthids is novel.

(18:3 *n*-3), 5,8,11,14-eicosatetraenoic (20:4 *n*-6), 7,10,13,16-docosatetraenoic (22:4 *n*-6), and 7,10,13,16,19-docosapentaenoic (22:5 *n*-3). Polyunsaturated fatty acids accounted for 20% of the total fatty acid composition of *P. caribaeorum*. The polyunsaturated profile of this zoanthid is similar to that reported for five *Palythoa* spp. from the Senegalese coast (15).

Of particular interest was the identification of five $\Delta^{5,9}$ fatty acids in *P. caribaeorum* accounting for 5% of the total phospholipid fatty acid composition of the zoanthid. We recently reported the occurrence of four of these $\Delta^{5,9}$ fatty acids in the phospholipids of the anemone *S. helianthus* (7). However, this is the first time that they have been isolated from a zoanthid. We were able to identify six $\Delta^{5,9}$ fatty acids accounting for 5.5% of the total phospholipid fatty acid composition of *C. gigantea*. The methyl ester derivatives of these acids were initially identified by a base peak at *m/z* 81, characteristic for $\Delta^{5,9}$ fatty acid methyl esters (7). The double-bond positions were rigorously confirmed by two independent methods. The method of choice was to

TABLE 2. Fatty Acids from the Total Lipids and Major Phospholipids of *Condylactis gigantea*.

Fatty Acid	Abundance (wt %)			
	Total Lipids[a]	PE	PC	PS
Dodecanoic (12:0)	0.1	—	—	0.5
9-Tetradecenoic (14:1)	1.1	—	—	—
Tetradecanoic (14:0)	11.8	2.4	1.7	5.0
Pentadecanoic (15:0)[b]	0.2	1.0	0.4	4.5
9-Hexadecenoic (16:1)	6.4	3.6	6.7	—
Hexadecanoic (16:0)[b]	21.4	6.8	15.1	26.6
15-Methylhexadecanoic (*i*-17:0)	0.1	—	3.4	1.2
9-Heptadecenoic (17:1)	0.1	0.5	—	—
Heptadecanoic (17:0)[b]	0.4	11.0	3.8	6.9
2-Hydroxyhexadecanoic (*h*-16:0)	1.0	—	—	—
6,9,12,15-Octadecatetraenoic (18:4)	6.7	—	—	—
5,9-Octadecadienoic (18:2)	0.8	2.3	—	—
9-Octadecenoic (18:1)	0.8	20.0	37.7	16.2
11-Octadecenoic (18:1)	1.1	6.0	4.0	—
Octadecanoic (18:0)[b]	7.6	7.9	11.4	16.8
Nonadecanoic (19:0)	0.2	—	—	2.2
5,8,11,14-Eicosatetraenoic (20:4)	12.0	8.2	3.0	2.6
5,8,11,14,17-Eicosapentaenoic (20:5)	9.2	10.6	3.0	2.8
5,9-Eicosadienoic (20:2)	3.0	3.8	—	1.0
9-Eicosenoic (20:1)	1.1	0.2	—	1.8
11-Eicosenoic (20:1)	1.0	0.2	—	—
13-Eicosenoic (20:1)	0.1	—	—	—
Eicosanoic (20:0)	1.0	0.2	2.0	2.6
5,9-Heneicosadienoic (21:2)	0.1	—	—	—
7,10,13,16-Docosatetraenoic (22:4)	5.6	1.6	—	2.6
4,7,10,13,16,19-Docosahexaenoic (22:6)	4.9	2.2	—	1.8
5,9-Docosadienoic (22:2)	0.8	0.7	—	—
Docosenoic (22:1)	0.1	8.8	6.5	—
Docosanoic (22:0)	0.3	—	—	1.3
5,9-Tetracosadienoic (24:2)	0.1	—	—	—
6-Bromo-5,9-eicosadienoic [**1**] (20:2)[c]	0.7	1.0	—	—

[a]These are the total phospholipids. Other minor fatty acids identified in trace amounts were 13-methyltetradecanoic acid (*i*-15:0), 7-methyl-6-hexadecenoic acid (17:1), and 9,12-octadecadienoic acid (18:2).

[b]The corresponding aldehydes were also detected in the fatty acid mixture as dimethyl acetals, probably arising from plasmalogens.

[c]Occurrence of this acid in nature is novel.

synthesize the corresponding pyrrolidides (16). The eims of the corresponding pyrrolidide derivatives displayed a strong peak at *m/z* 180, due to allylic cleavage between C-7 and C-8, and this confirmed the double bond arrangements. The second approach was conversion to the corresponding picolinyl derivatives. For example, the eims of the corresponding picolinyl derivatives displayed a strong peak at *m/z* 219, due also to allylic cleavage between C-7 and C-8, and this confirmed the double-bond arrangements (17). Despite the fact that there are several reported methods for the synthesis of picolinyl derivatives in the literature (17), we developed a more convenient synthetic route to convert fatty acid methyl esters to picolinyl derivatives. The steps are hydrolysis of the methyl esters with LiOH followed by DCC-assisted coupling of the resulting fatty acids with 3-hydroxymethylpyridine.

The cis-double bond stereochemistry in the most abundant fatty acid methyl esters

was confirmed by ^{13}C-nmr spectroscopy. The two methylene carbons adjacent to cis-double bonds resonate at a higher field (ca. 26–27 ppm) than those bonded to trans-double bonds at 31–34 ppm (18). In our fatty acid methyl ester mixture no absorbance between 31–34 ppm was observed with the exception of the C-2 carbons at 34 ppm and the ω-3 carbons at 31.9 ppm, but many absorptions in the 25–29 ppm region were detected. Therefore, the most abundant fatty acids in the mixture have the cis-stereochemistry.

The new brominated fatty acid 6-bromo-5,9-eicosadienoic acid [**1**] was identified in the phospholipids (mainly PE) from both *P. caribaeorum* and *C. gigantea*. Until recently this type of 6-bromo-$\Delta^{5,9}$ diunsaturation has only been recognized in sponges (1–4). Methyl ester **1** presented spectral characteristics very similar to those acids reported previously and this permitted its rapid characterization (7). In fact, **1** is the shortest 6-bromo-$\Delta^{5,9}$ fatty acid yet identified in a phospholipid and hence completes a fatty acid biosynthetic scheme for 6-bromo-$\Delta^{5,9}$ fatty acids in marine invertebrates ranging from C_{20} to C_{28}. The gc-ms data of the brominated fatty acid methyl ester showed strong mass spectral peaks at *m/z* 74 (due to the McLafferty rearrangement typical of fatty acid methyl esters) and a $[M-Br]^+$ peak at *m/z* 321 as the molecular ion, indicating facile loss of bromine under electron impact. Other fragmentations of interest were observed at *m/z* 289 $[M-Br-CH_3OH]^+$, *m/z* 81, and *m/z* 180 $[C_{13}H_{24}]^+$. The gc-ms of the corresponding brominated fatty acid pyrrolidide was critical for identifying the bromine substituent and the double-bond positions. The *N*-6-bromoeicosa-5,9-dienoylpyrrolidide afforded a strong peak at *m/z* 360 due to the loss of bromine, a base peak at *m/z* 113 resulting from the McLafferty rearrangement, an intense peak at *m/z* 180 $[C_{11}H_{18}NO]^+$ corresponding to a double allylic fragmentation between C-6 and C-9 with the loss of bromine, and a strong doublet of equal intensity at *m/z* 258 and *m/z* 260 due to the same fragmentation with the bromine substituent intact (2–5). Catalytic hydrogenation (PtO_2) of this brominated methyl ester yielded methyl eicosanoate and hence excluded the possibility of any methyl branching. The small amounts of this compound available precluded us from assigning unequivocally the double-bond stereochemistry.

The 6-bromo-5,9-eicosadienoic acid [**1**] presented in this work displays a type of substitution previously identified only in the phospholipids of sponges. We can only speculate as to its origin, but it is not clear at this point if **1** has an invertebrate origin, or arises from symbiotic zooxanthella (9). Nevertheless, it is probable that **1** originated from the action of a marine haloperoxidase (19) on the analogous 5,9-eicosadienoic acid which was also found in both *P. caribaeorum* and *C. gigantea*. In fact, the biosynthesis of 6-bromo-5,9-hexacosadienoic acid was previously investigated in sponges by Djerassi's group utilizing radiolabeled precursors, and they concluded that bromination was the terminal step in the biosynthesis of these unusual acids (2). Therefore, it is possible that the biosynthesis of the brominated acid **1** follows the biosynthetic route shown below.

$$16{:}0 \rightarrow 20{:}0 \rightarrow \Delta^5\text{- and } \Delta^9\text{-}20{:}1 \rightarrow \Delta^{5,9}\text{-}20{:}2 \rightarrow 6\text{-Br-}\Delta^{5,9}\text{-}20{:}2$$

The results presented in this work are important not only from the point of view of a new structure, but moreso from a fatty acid biosynthetic and comparative biochemistry standpoint. Our results indicate that the $\Delta^{5,9}$-diunsaturation is more common in anemones than previously recognized, and can be found in other marine invertebrates such as zoanthids. Moreover, anemones and zoanthids seem to biosynthesize $\Delta^{5,9}$ fatty acids between 16 and 23 carbons, in contrast to the greater chain-lengths of between 24 and 30 carbons that are normally predominant in sponges.

Work continues with other marine organisms in order to expand our present knowledge of brominated $\Delta^{5,9}$ fatty acids in marine invertebrates.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Fatty acid methyl esters were analyzed by gc-ms using a 5972A MS ChemStation (Hewlett-Packard, Palo Alto, CA) equipped with a 30-m×0.25-mm special performance capillary column (HP-5MS) crosslinked with 5% phenyl methylpolysiloxane. The temperature program was as follows: 130° for 2 min, then increased at 3°/min to 270° and maintained for 40 min. The carrier gas was He at a pressure of 10 psi. ^{1}H- and ^{13}C-nmr spectra were recorded on a GE 300 MHz spectrometer.

ANIMAL MATERIAL.—*Palythoa caribaeorum* was collected in June 1993, near Cayo Enrique, La Parguera, Puerto Rico, at a depth of 60 cm and *Condylactis gigantea* was collected in June 1994 at the same site. The animals were frozen immediately after collection and freeze-dried or lyophilized before analysis. Voucher specimens of *P. caribaeorum* and *C. gigantea* are available from the Department of Marine Sciences, University of Puerto Rico, Mayagüez Campus, Puerto Rico.

EXTRACTION AND ISOLATION.—The animals (50–60 g dry wt) were carefully cleaned of all debris and cut into small pieces. Extraction with 2×250 ml of $CHCl_3$-MeOH (1:1) yielded a total of ca. 5 g of lipids. The neutral lipids, glycolipids, and 30 mg of phospholipids were separated by cc on Si gel (60–200 mesh) using the procedure of Privett *et al.* (20). The phospholipid classes were fractionated by prep. tlc using Si gel 60 and $CHCl_3$-MeOH-NH_4OH (65:35:5) as solvent. Ninhydrin was used to specifically identify PE and PS, while Dragendorff reagent was used to visualize PC. The separated phospholipids were scraped off the plate and individually esterified with methanolic HCl.

The fatty acyl components of the phospholipids were obtained as their methyl esters by reaction of the phospholipids with methanolic HCl (21) followed by cc purification eluting with *n*-hexane-Et_2O (9:1). The double-bond positions of the mono- and dienoic fatty acids were determined by preparing the corresponding *N*-acylpyrrolidide derivatives or picolinyl derivatives. The *N*-acylpyrrolidide derivatives were prepared by direct treatment of the methyl esters with pyrrolidine-HOAc (10:1) in a capped vial for 93 h at 100° followed by ethereal extraction from the acidified solution and purification by prep. tlc. The picolinyl derivatives were prepared by dissolving the methyl esters (3–4 mg) in $CHCl_3$-MeOH-THF (1:1:3) together with a three-fold molar excess of LiOH. After stirring at room temperature for 4 h (or until all the methyl esters hydrolyzed as judged by tlc), the mixture was neutralized to pH 7 with HCl. The solvents were removed and the H_2O layer acidified to pH 2. It was then extracted with 2×10 ml of CH_2Cl_2, the layers separated, and the organic layer dried over $MgSO_4$ and filtered. After solvent removal the fatty acids were dissolved in 5 ml of CH_3CN and a 1.3 molar excess of 1,3-dicyclohexylcarbodiimide (DCC) was added together with an equimolar amount of 3-hydroxymethylpyridine. Catalytic amounts of 4-dimethylaminopyridine (DMAP) were also added. After all the fatty acids reacted, as judged by tlc, the solvent was removed *in vacuo* and the crude mixture was purified by prep. tlc. Hydrogenations were carried out in 10 ml of MeOH and catalytic amounts of PtO_2. Spectral data for the key fatty acid in this discussion follow.

Methyl 6-bromo-5,9-eicosadienoate.—Gc-ms (70 eV) *m/z* $[M-Br]^+$ 321 (8), $[M-Br-CH_3OH]^+$ 289 (4), 247 (4), 205 (3), $[C_{13}H_{24}]^+$ 180 (15), 167 (4), 160 (7), 159 (7), 149 (12), 147 (7), 141 (16), 139 (15), 135 (11), 133 (6), 131 (5), 121 (11), 119 (12), 111 (11), 109 (21), 107 (13), 105 (11), 97 (28), 95 (22), 91 (22), 85 (11), 83 (43), 81 (44), 79 (44), 74 (34), 71 (18), 69 (57), 67 (46), 57 (59), 55 (100).

N-*6-Bromoeicosa-5,9-dienoylpyrrolidine.*—Gc-ms (70 eV) *m/z* $[M-Br]^+$ 360 (20), 260 (9), $[C_{11}H_{17}NOBr]^+$ 258 (7), 194 (1), $[C_{11}H_{18}NO]^+$ 180 (5), 168 (3), 126 (26), $[C_6H_{11}NO]^+$ 113 (100), 98 (23), 85 (11).

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