New 2-Hydroxy Fatty Acids in the Caribbean Urchin Tripneustes esculentus

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NEW 2-HYDROXY FATTY ACIDS IN THE CARIBBEAN URCHIN *TRIPNEUSTES ESCULENTUS*

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ABSTRACT.—The novel α-hydroxy fatty acids 2-hydroxy-13-docosenoic acid [**1a**], 2-hydroxy-14-tricosenoic acid [**2a**], and 2-hydroxy-15-tetracosenoic acid [**3a**] were identified in the Caribbean urchin, *Tripneustes esculentus*. The double-bond positions of the novel α-hydroxy fatty acids were determined by derivatization with dimethyl disulfide and shown to correlate with the corresponding non-hydroxylated mono-unsaturated fatty acids, 13-docosenoic acid, 14-tricosenoic acid, and 15-tetracosenoic acid also present in *T. esculentus*. The total fatty acid composition of the urchin is also reported where cis-5-olefinic fatty acids such as 5,9-octadecadienoic acid and 5,11-eicosadienoic acid were found to predominate in the mixture. Cholesterol was the predominant sterol in *T. esculentus*.

The lipid chemistry of urchins has been the subject of numerous efforts in an attempt to discover novel metabolites with intriguing biological activities. Among the less familiar lipids isolated from sea urchins are unprecedented glycosphingolipids, which include novel fucosylglycolipids (1) and ceramide trihexosides (2) from the eggs of the urchin *Hemicentrotus pulcherrimus*, unusual gangliosides (3) and melibiosylceramides (4) from the eggs of *Anthocidaris crassispina*, and unprecedented sialosphingolipids from gonadal tissue of the sea urchin *Echinocardium cordatum* (5) and *Strongulocentrotus intermedius* (6). In all of the latter glycosphingolipids the principal fatty acids are 2-hydroxylated monounsaturated fatty acids of between 22 and 24 carbons, in addition to mono-unsaturated fatty acids of identical chain-length (3). However, in none of the above reports were the double-bond positions of the fatty acids determined. It would have been of interest to find out if the double-bond positions in the monounsaturated fatty acids from the glycosphingolipids of these urchins corresponded to the same positions as those of the 2-hydroxylated monounsaturated fatty acids. This could give valuable information as to the biogenesis of 2-hydroxy fatty acids, since the presence of an active lipoxygenase in urchins is a possibility.

As part of a continuing interest in the lipid biochemistry of marine invertebrates we studied the total lipids, in particular the phospholipid fatty acids and sterols, from the Caribbean urchin *Tripneustes esculentus* Leske, order Centrechinoida, family Echinidae. This organism is an important food item in the Caribbean in as much as the eggs are edible and consumed in several regions. The total lipids of *T. esculentus*, in particular the phospholipids, have not been studied before. Phospholipids from urchins have aroused considerable interest because sea urchin spermatozoa seem to use phosphatidylcholine (PC) as the energy source for swimming (7). It has also been reported that polyunsaturated fatty acids such as eicosapentaenoic acid (20:5) and arachidonic acid (20:4) induce larval settlement and metamorphosis in urchins (8).

RESULTS AND DISCUSSION

The phospholipids from *T. esculentus* were isolated as described in the Experimental and identified by tlc comparisons with authentic samples. The mixture consisted of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). In order to identify the fatty acids and aldehydes in the phospholipid fraction we esterified each individual phospholipid with MeOH/HCl to afford, after purification, fatty acid methyl esters and dimethyl acetals which were characterized by gc-ms. The total lipids, including the glycolipid and phospholipid

fractions, were also esterified for comparison. A complete list of fatty acids and aldehydes from *T. esculentus* is presented in Table 1. The principal fatty acids in the mixture were hexadecanoic acid (16:0), octadecanoic acid (18:0), 9- and 11-octadecenoic acids (18:1), and 5-eicosenoic acid (20:1). The polyunsaturated fatty acids eicosapentaenoic (20:5) and eicosatetraenoic (20:4) of the *n*-3 and *n*-6 families, respectively, were also characterized in the mixture. Previous work with the urchins *H. pulcherrimus* (9) and *S. intermedius* (10) has shown that saturated fatty acids are esterified at the 1-position of the phospholipids, while polyunsaturated fatty acids are principally found at the 2-position.

TABLE 1. The Fatty Acids and Aldehydes from *Tripneustes esculentus*.

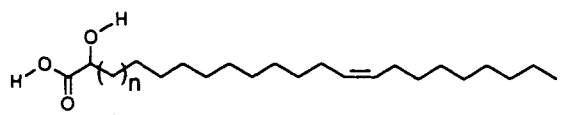
Compound	Abundance (wt %)			
	Total Lipids[a]	PE	PS/PI	PC
Aldehydes[b]				
Hexadecanal (16:0)	0.5		0.3	
Heptadecanal (17:0)	1.0			
Octadecanal (18:0)	10.3		0.6	1.5
Fatty Acids				
Tetradecanoic (14:0)	2.4	6.6	4.6	6.3
Pentadecanoic (15:0)	0.6	2.4	2.4	3.0
9-Hexadecenoic (16:1)	0.5	6.6	6.8	6.8
Hexadecanoic (16:0)	17.5	21.8	8.5	12.0
Heptadecenoic (17:1)	0.9	0.6	0.9	1.1
Heptadecanoic (17:0)	0.2	0.3	0.9	0.3
5,9-Octadecadienoic (18:2)	3.4	5.7	6.0	6.0
9-Octadecenoic (18:1)	0.5	43.2	44.0	43.0
11-Octadecenoic (18:1)	1.0	2.4	8.1	9.0
Octadecanoic (18:0)	5.4	3.7	2.8	2.5
5-Nonadecenoic (19:1)	1.6		0.5	
Nonadecanoic (19:0)	0.6			
Eicosapentaenoic (20:5 *n*-3)	8.0		0.3	0.5
Eicosatetraenoic (20:4 *n*-6)	9.0		0.6	0.6
5,11-Eicosadienoic (20:2)	8.4			0.5
5-Eicosenoic (20:1)	13.0		1.0	0.8
11-Eicosenoic (20:1)	1.1		0.4	
Eicosanoic (20:0)	1.1		0.3	
7-Heneicosenoic (21:1)	1.5			
12-Heneicosenoic (21:1)	0.7			
Docosahexaenoic (22:6 *n*-3)	0.6			
13-Docosenoic (22:1)	2.3	6.6	9.7	4.5
Docosanoic (22:0)	1.0		0.2	
14-Tricosenoic (23:1)	1.3			
Tricosanoic (23:0)	0.3			
15-Tetracosenoic (24:1)	1.9			
Tetracosanoic (24:0)	0.3			
2-Hydroxy Acids				
2-Hydroxy-13-docosenoic (*h*-22:1)[c]	0.3			
2-Hydroxydocosanoic (*h*-22:0)	0.1			
2-Hydroxy-14-tricosenoic (*h*-23:1)[c]	0.3			
2-Hydroxytricosanoic (*h*-23:0)	0.1			
2-Hydroxy-15-tetracosenoic (*h*-24:1)[c]	0.6			
2-Hydroxytetracosanoic (*h*-24:0)	0.2			

[a]Some minor components were not identified.
[b]Originated from plasmalogens and were characterized as dimethyl acetals.
[c]Occurrence of these acids in nature is novel.

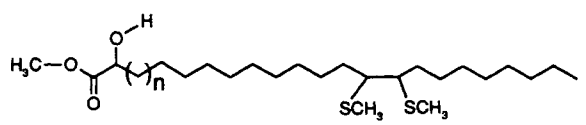
Noteworthy in *T. esculentus* is the abundance of cis-5-olefinic acids, i.e., considerable amounts of *cis*-5-nonadecenoic acid, *cis*-5-eicosenoic acid, all-*cis*-5,9-octadecadienoic acid, and all-*cis*-5,11-eicosadienoic acid were identified (Table 1). In fact, cis-5-olefinic acids have been proposed as distinctive lipid components of sea urchins (11).

The acids, 5,9-octadecadienoic acid (18:2) and 5,11-eicosadienoic acid (20:2), deserve special mention. The substance, 5,9-octadecadienoic acid (18:2), is a typical fatty acid of sponges belonging to the Demospongiae and the $\Delta^{5,9}$-diunsaturation is not common in other marine invertebrates (12). However, the fatty acid 5,11-eicosadienoic acid, is more common in marine invertebrates. The characterization of these acids was possible by means of gc-ms on their corresponding dimethyl disulfide derivatives, i.e., addition of two moles of dimethyl disulfide to methyl 5,9-octadecadienoate resulted in a rearranged thiophene (12) with clear-cut ms fragmentation patterns, while addition of two moles of dimethyl disulfide to methyl 5,11-eicosadienoate resulted in a tetrakis(methylthio) derivative that permitted the double-bond localization by its ms fragmentation (13).

The most interesting fatty acid methyl esters in the mixture were a series of 2-hydroxylated monounsaturated fatty acid methyl esters with molecular weights of $[M]^+$ 368, 382, and 396, accounting for fatty acid methyl esters of between 22 and 24 carbons. These fatty acids probably arise from glycosphingolipids (1–6). The 2-hydroxy substitution was confirmed by gc-ms and ^{1}H nmr. The ms of each of these fatty acid methyl esters displayed a strong $[M-COOCH_3]^+$ ion together with significant peaks at *m/z* 90 and *m/z* 103, derived by McLafferty rearrangement. ^{1}H-Nmr spectroscopy was used to identify the signal of the α hydrogen as a triplet at 4.35 ppm, as expected for α-hydroxy fatty acid methyl esters, while the signal for the methoxy group of the ester was observed at 3.78 ppm, in contrast to the signal of the methoxy group of a normal fatty acid methyl ester which appears at 3.66 ppm. The double-bond positions were again determined by dimethyl disulfide addition and the stereochemistry of the double bonds determined by gc-Ft-ir and ^{13}C nmr. Dimethyl disulfide addition to the 2-hydroxy monounsaturated fatty acid methyl ester with a molecular weight of $[M]^+$ 368 resulted in methyl 13,14-*bis*(methylthio)-2-hydroxydocosanoate [**1b**] with $[M]^+$ 462. The fragmentations at *m/z* 289 (49%) $[C_{15}H_{29}SO_3]^+$ and at *m/z* 173 (100%) $[C_{10}H_{21}S]^+$ confirmed the double-bond position as being between C-13 and C-14. The peak at *m/z* 289 fragmented further either by losing a mole of CH_3SH [*m/z* 241 (97%) $[C_{14}H_{25}O_3]^+$] or 60 mass units [*m/z* 229 (24%) $[C_{13}H_{25}SO]^+$] probably due to the loss of a $[C_2H_4O_2]^+$ fragment. Catalytic hydrogenation (PtO_2) resulted in the known methyl 2-hydroxydocosanoate, thus



1a n=1
2a n=2
3a n=3



1b n=1
2b n=2
3b n=3

excluding the possibility of methyl branching. Our results indicate that the original compound in question is the 2-hydroxy-13-docosenoic acid [**1a**], which has not been reported before. It is of interest to mention here that dimethyl disulfide addition to 2-hydroxy fatty acid methyl esters has not been documented previously.

Addition of dimethyl disulfide to the 2-hydroxy monounsaturated fatty acid methyl ester with molecular weight of $[M]^+$ 382 resulted in methyl 14,15-*bis*(methylthio)-2-hydroxytricosanoate [**2b**] with $[M]^+$ 476. The fragmentations at *m/z* 303 (36.6%) $[C_{16}H_{31}SO_3]^+$ and at *m/z* 173 (95%) $[C_{10}H_{21}S]^+$ confirmed the double-bond position to be between C-14 and C-15. The peak at *m/z* 303 fragmented further either by losing a mole of CH_3SH [*m/z* 255 (81%)] or 60 mass units [*m/z* 243 (19%)] $[C_{14}H_{27}SO]^+$ probably also due to the loss of the $[C_2H_4O_2]^+$ fragment. Catalytic hydrogenation (PtO_2) of the original ester resulted in the known methyl 2-hydroxytricosanoate, thus excluding the possibility of methyl branching. These results indicate that the second compound in question is 2-hydroxy-14-tricosenoic acid [**2a**], which has not been reported before.

Finally, addition of dimethyl disulfide to the longest 2-hydroxy monounsaturated fatty acid methyl ester with molecular weight of $[M]^+$ 396 resulted in methyl 15,16-*bis*(methylthio)-2-hydroxytetracosanoate [**3b**] with $[M]^+$ 490. The fragmentations at *m/z* 317 (30%) $[C_{17}H_{33}SO_3]^+$ and at *m/z* 173 (98%) $[C_{10}H_{21}S]^+$ confirmed the double-bond position to be between C-15 and C-16. The peak at *m/z* 317 fragmented further either by losing a mole of CH_3SH [*m/z* 269 (89%)] or 60 mass units [*m/z* 257 (18.4%) $[C_{15}H_{29}SO]^+$ probably due to the loss of the $[C_2H_4O_2]^+$ fragment. Once again, catalytic hydrogenation (PtO_2) resulted in the known methyl 2-hydroxytetracosanoate, thus excluding the possibility of methyl branching. These results indicate that the third compound in question is 2-hydroxy-15-tetracosenoic acid [**3a**]. Once the double-bond positions were unequivocally determined in the 2-hydroxy acids, the double-bond stereochemistry in all of the unsaturated fatty acids was established by both Ft-ir and ^{13}C nmr. The ir spectra of all of the methyl esters exhibited an absorption around 719 cm^{-1} (out-of-plane bending vibration) and not even traces of an absorption in the 960–980 cm^{-1} region, thus indicating cis rather than trans stereochemistry (12). Furthermore, the ^{13}C-nmr spectrum of all monounsaturated methyl esters presented the allylic carbons in the chain at δ 27.21 (typical of methylenes adjacent to cis double bonds) but no absorptions around δ 32.6 where the allylic carbons adjacent to trans double bonds in fatty acids normally resonate (14). Therefore, all fatty acids in the mixture had a cis stereochemistry. Whether these compounds are the same as the 2-hydroxy acids reported before from other urchins (1–6) we can only speculate at this point. It is also of interest to mention here that cholesterol was found to be the major sterol in *T. esculentus*.

The results obtained in this work are of significance since we have been able to demonstrate, at least in *T. esculentus*, that the double-bond positions in the monounsaturated fatty acids 22:1, 23:1, and 24:1 are the same as those in the 2-hydroxylated *h*-22:1, *h*-23:1, and *h*-24:1. All of these acids belong to the *n*-9 series. These novel hydroxylated fatty acids could have arisen from glucosylceramides since they were not found in the common phospholipids. As to their biogenesis, it is a matter of speculation at this time. These 2-hydroxy fatty acids could originate from a monounsaturated fatty acid oxygenase, possibly lipoxygenase, that converts the free fatty acids to hydroxy fatty acids (15). In fact, a calcium-stimulated lipid peroxidizing system has been identified in the eggs of the sea urchin *Strongylocentrotus purpuratus* (15). Another possibility is that they originated as integral parts of fatty acid chain elongation and/or shortening, possibly by peroxisomes. Work is in progress with the lipids of other Caribbean urchins.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Fatty acid methyl esters were analyzed by gc-ms using a

59970 MS ChemStation (Hewlett-Packard, Palo Alto, CA) equipped with a 30 m×0.32 mm nonpolar fused silica column (Supelco, Bellefonte, PA) with SPB™-1 as the bonded phase. Gc-Ft-ir spectra were recorded on a Nicolet (Madison, WI) 740 Ft-ir spectrometer. ^{1}H- and ^{13}C-nmr spectra were recorded on a GE 300 MHz spectrometer. The urchins were freeze-dried or lyophilized before analysis.

COLLECTION OF *TRIPNEUSTES ESCULENTUS*.—This organism was collected in July 1993, near Cayo Enrique, La Parguera, Puerto Rico, at a depth of 1 ft. A voucher specimen of the urchin has been deposited at the Department of Marine Sciences, University of Puerto Rico, Mayagüez campus, Mayagüez, Puerto Rico.

EXTRACTION AND ISOLATION OF PHOSPHOLIPIDS.—The whole urchins (ca. 80 g, dry wt) were carefully cleaned of all debris and cut into small pieces (including the egg masses). Extraction with 250 ml of $CHCl_3$-MeOH (1:1) yielded the total lipids (ca. 250 mg). 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.* (16). The phospholipid classes were fractionated by prep. tlc using Si gel 60 and $CHCl_3$-MeOH-NH_4OH (65:35:5) as solvent. The separated phospholipids were scraped off the plate and individually esterified. The total glycolipid and phospholipid fractions were also esterified for comparison.

PREPARATION AND ISOLATION OF FATTY ACID DERIVATIVES.—The fatty acyl components of the glycolipids and phospholipids were obtained as their methyl esters by reaction of the phospholipids with methanolic HCl (17) followed by cc purification eluting with *n*-hexane-Et_2O (9:1). The double-bond positions of the mono- and dienoic fatty acids were elucidated by preparing the corresponding dimethyl disulfide derivatives by dissolving the esters (2 mg) in dimethyl disulfide (0.2 ml) and adding a solution (0.05 ml) of I_2 in Et_2O (60 mg/ml), heating the solution at 50° for 24 h, followed by the standard workup (18). Hydrogenations were carried out in 10 ml of MeOH and catalytic amounts of PtO_2. Spectral data for the key fatty acids for this discussion follow.

Spectral data for all fatty acid methyl esters.—Ir ν max 3010, 2924, 2853, 1743, 1461, 1435, 1304, 1244, 1196, 1169, 1122, 719 cm^{-1}; ^{1}H nmr (300 MHz, $CDCl_3$) δ 0.88 (t, CH_3CH_2-), 1.26 (m, -CH_2-), 1.60 (m, -CH=CH-CH_2-CH_2-), 2.05 (m, -CH_2-CH=CH-), 2.30 (t, -CH_2-CO_2CH_3), 2.82 (m, -CH=CH-CH_2-CH=CH-), 3.66 (s, CH_3O), 3.78 (s, -CH(OH)CO_2CH_3), 4.35 (t, -CH(OH)CO_2CH_3), 5.35 (m, CH=CH-); ^{13}C nmr (75 MHz, $CDCl_3$) δ 14.09 (q, ω-1), 22.67 (t, ω-2), 24.60–24.95 (t, C-3), 27.21 (t, CH_2-CH=CH), 31.91 (t, ω-3), 34.11 (t, C-2), 51.40 (q, CH_3O), 52.56 (q, CH_3O), 127.50–131.18 (d, CH=CH), 174.32 (s, C-1).

Methyl 2-hydroxy-13-docosenoate.—Ms *m/z* [M]$^+$ 368 (36), [M−59]$^+$ 309 (28), 207 (1), 142 (2), 137 (6), 135 (9), 127 (9), 123 (14), 121 (14), 111 (19), 109 (29), 107 (9), 103 (6), 97 (39), 95 (64), 90 (19), 87 (52), 79 (30), 77 (11), 74 (67), 69 (97), 67 (88), 65 (8), 59 (36).

Methyl 2-hydroxy-14-tricosenoate.—Ms *m/z* [M]$^+$ 382 (17), [M−59]$^+$ 323 (35), 207 (2), 142 (2), 137 (9), 135 (10), 127 (9), 125 (9), 123 (18), 121 (11), 111 (20), 109 (34), 107 (6), 103 (6), 97 (36), 95 (70), 90 (30), 87 (15), 79 (26), 77 (10), 74 (21), 69 (100), 67 (89), 65 (7), 59 (23).

Methyl 2-hydroxy-15-tetracosenoate.—Ms *m/z* [M]$^+$ 396 (11), [M−59]$^+$ 337 (36), 207 (1), 142 (2), 137 (8), 135 (9), 127 (8), 125 (7), 123 (17), 121 (14), 111 (16), 109 (33), 103 (7), 97 (39), 95 (69), 90 (24), 83 (60), 79 (26), 77 (10), 74 (10), 69 (100), 67 (83), 65 (6), 59 (19).

Methyl 13,14-bis(*methylthio*)-*2-hydroxydocosanoate.*—Ms *m/z* [M]$^+$ 462 (20), 403 (0.6), 400 (4), [$C_{22}H_{43}SO$]$^+$ 355 (24), 309 (0.8), 307 (0.6), 290 (8), [$C_{15}H_{29}SO_3$]$^+$ 289 (49), 265 (0.5), 259 (1), 242 (15), [$C_{14}H_{25}O_3$]$^+$ 241 (97), 239 (3), 230 (0.7), 229 (24), 191 (5), [$C_{10}H_{21}S$]$^+$ 173 (100), 157 (6), 147 (4), 127 (4), 121 (11), 117 (6), 97 (29), 95 (47), 85 (21), 79 (29).

Methyl 14,15-bis(*methylthio*)-*2-hydroxytricosanoate.*—Ms *m/z* [M]$^+$ 476 (16), [$C_{23}H_{45}SO$]$^+$ 369 (20), [$C_{16}H_{31}SO_3$]$^+$ 303 (37), [$C_{15}H_{27}O_3$]$^+$ 255 (81), 243 (19), 239 (1), 227 (14), 213 (2), 201 (10), [$C_{10}H_{21}S$]$^+$ 173 (95), 169 (2), 155 (3), 129 (4), 105 (5), 97 (20), 95 (41), 85 (12), 79 (19), 74 (15), 71 (13).

Methyl 15,16-bis(*methylthio*)-*2-hydroxytetracosanoate.*—Ms *m/z* [M]$^+$ 490 (13), 443 (4), [$C_{24}H_{47}SO$]$^+$ 383 (21), [$C_{17}H_{33}SO_3$]$^+$ 317 (30), 270 (15), [$C_{16}H_{29}O_3$]$^+$ 269 (89), 257 (18), 241 (16), 237 (5), 215 (10), 174 (13), [$C_{10}H_{21}S$]$^+$ 173 (98), 172 (20), 151 (4), 145 (5), 123 (10), 109 (19), 97 (31), 95 (42), 87 (34), 83 (65), 81 (52), 79 (23), 74 (15), 69 (100), 61 (89), 57 (37).

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