# Identification of the Novel 7-Methyl-6-octadecenoic Acid in *Holothuria mexicana*

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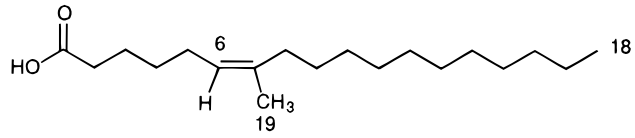
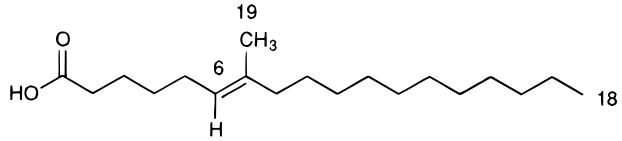
The phospholipid fatty acid composition of *Holothuria mexicana* was investigated, and the novel 7-methyl-6-octadecenoic acid was identified. Structural characterization was accomplished by means of pyrrolidide derivatization and total synthesis. Other interesting phospholipid fatty acids in *H. mexicana* were 7-eicosenoic acid, 13-tricosenoic acid, 7-methyl-6-hexadecenoic acid, and 2-hydroxy-15-tetradecenoic acid.

Natural products from holothurians have received considerable attention. A large variety of biologically intriguing triterpene glycosides has been isolated from several species, as, for example, frondoside A from *Cucumaria frondosa*, pseudostichoposide A from *Pseudostichopus trachus*, and holothurins A and B from *Holothuria mexicana*.[1−3] Fatty acids from holothurians have also been studied. An earlier report by Kaneniwa *et al.* identified the occurrence of considerable amounts of *cis*-14-tricosenoic acid in *Holothuria leucospilota* and *Stichopus japonicus*.[4] A later study by Svetashev *et al.* revealed that the main fatty acids in holothurians from tropical and temperate waters, such as *Holothuria impatients* and *Stichopus japonicus*, are 16:0, 18:0, 20:1, 20:4 (*n*-6), and 20:5 (*n*-3).[5] The double bond position in the eicosenoic acid (20:1) was not determined. These fatty acids were found in phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). The phospholipid fatty acid composition of *H. mexicana* H. L. Clark (phylum Echinodermata, class Holothuroidea) has not been thoroughly investigated. Therefore, as part of our continuing study of fatty acids from marine organisms we studied the phospholipid fatty acid composition of *H. mexicana* and herein report the identification of the novel 7-methyl-6(*Z*)-octadecenoic acid (**1a**).




**1a**




**1b**

The main phospholipids in *H. mexicana* were identified by TLC as phosphatidylethanolamine and phosphatidylcholine. Preparation of the fatty acid methyl esters from these phospholipids afforded the fatty acid methyl esters and aldehydes presented in Table 1. As expected, the principal fatty acids in *H. mexicana* were hexadecanoic acid, octadecanoic acid, arachidonic acid, and 7-eicosenoic acid. The double bond positions in the monounsaturated fatty acid methyl esters were determined by dimethyl disulfide derivatization as previously described.[6] It should be noted that Δ7 monounsaturation predominated in *H. mexicana* as exemplified by 7-nonadecenoic acid, 7-eicosenoic acid, and 7-heneico-

**Table 1.** Total Phospholipid Fatty Acids and Aldehydes from *H. mexicana*[a]

| compound | abundance (wt %) |
|---|---|
| fatty acids | |
| tetradecanoic (14:0) | 1.2 |
| pentadecanoic (15:0) | 0.8 |
| methylpentadecanoic (16:0) | 0.3 |
| 9-hexadecenoic (16:1) | 3.1 |
| 11-hexadecenoic (16:1) | 0.5 |
| hexadecanoic (16:0) | 18.6 |
| methylhexadecanoic (17:0) | 0.7 |
| 15-methylhexadecanoic (*i*-17:0) | 0.8 |
| 7-methyl-6-hexadecenoic (17:1) | 0.4 |
| methylhexadecanoic (17:0) | 0.5 |
| heptadecanoic (17:0) | 0.7 |
| 9,12-octadecadienoic (18:2) | 1.0 |
| 9-octadecenoic (18:1) | 2.0 |
| 11-octadecenoic (18:1) | 6.0 |
| 13-octadecenoic (18:1) | 0.6 |
| octadecanoic (18:0) | 10.9 |
| 7-methyl-6-octadecenoic (19:1)[b] | 0.7 |
| methyloctadecanoic | 0.5 |
| 5-nonadecenoic (19:1) | 0.8 |
| 7-nonadecenoic (19:1) | 0.8 |
| nonadecanoic (19:0) | 1.0 |
| 2-hydroxyoctadecanoic (18h:0) | 0.6 |
| 5,8,11,14-eicosatetraenoic (20:4 *n*-6) | 5.8 |
| 7-eicosenoic (20:1) | 8.3 |
| 13-eicosenoic (20:1) | 0.6 |
| eicosanoic (20:0) | 2.5 |
| 7-heneicosenoic (21:1) | 0.9 |
| heneicosanoic (21:0) | 1.1 |
| 15-docosenoic (22:1) | 1.1 |
| docosanoic (22:0) | 1.4 |
| 14-tricosenoic (23:1) | 1.4 |
| tricosanoic (23:0) | 0.6 |
| 2-hydroxydocosanoic (22h:0) | 0.8 |
| 15-tetracosenoic (24:1) | 1.4 |
| 2-hydroxytricosanoic (23h:0) | 0.6 |
| 2-hydroxy-15-tetracosenoic (24h:1) | 1.2 |
| 2-hydroxytetracosanoic (24h:0) | 0.5 |
| aldehydes | |
| octadecanal (18:0) | 15.2 |
| nonadecanal (19:0) | 0.5 |

[a] Some minor acids were not identified. [b] Unprecedented in nature.

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senoic acid. In a previous paper, Δ7 monounsaturation was not recognized to exist in holothurians.[5] The characteristic 14-tricosenoic acid of holothurians was identified in *H. mexicana* in 1.4% relative abundance.[4] Monounsaturated fatty acids accounted for 29.8% of the total fatty acid composition of *H. mexicana*.

A methyl branched monounsaturated fatty acid methyl ester in *H. mexicana* was of considerable interest since it presented an equivalent chain length (ECL) value of 18.08 in nonpolar capillary gas chromatography. The mass spectrum of this methyl ester presented a $[M]^+$ at *m/z* 310, a M − 31 peak at *m/z* 279 (2%), and a characteristic intense fragment at *m/z* 138 (49%), which was present in the mass spectra of methyl 7-methyl-6-hexadecenoate, also identified in this holothurian.[7] This initial data suggested methyl 7-methyl-6-octadecenoate, which has not been recognized to exist in nature before, as the probable structure. In order to secure this structure two experiments were performed with the whole fatty acid methyl ester mixture, i.e., pyrrolidide derivatization[8] and catalytic hydrogenation. The pyrrolidide derivative of **1a** displayed a prominent peak at *m/z* 208 (15%) and a diminished intensity peak at *m/z* 180 (1%). This implies methyl substitution at C-7, which was corroborated by hydrogenation to the known methyl 7-methyloctadecanoate.[9] The C-7 methyl substitution in methyl 7-methyloctadecanoate is identified by an intense fragment at *m/z* 157 in its mass spectrum.[9] The double-bond position in **1a** was determined to be at C-6 by a difference of 12 mass units between fragments at *m/z* 154 ($C_5$) and *m/z* 166 ($C_6$) in the pyrrolidide derivative.[8] It was not possible to accurately determine the double bond stereochemistry by mass spectrometry.

Final structural confirmation of **1a** was accomplished by total synthesis. Wittig reaction of (6-carboxyhexyl)-triphenyl phosphonium bromide with 2-tridecanone afforded a 1:1 mixture of 7-methyl-6(*Z*)-octadecenoic acid and 7-methyl-6(*E*)-octadecenoic acid. Separation of the stereoisomers proved difficult, but it was possible to distinguish some characteristic NMR absorptions for each isomer. The $^1$H NMR spectrum of both stereoisomers presented a broad triplet at 5.09 ppm (H-6), but the vinyl methyl group (Me-19) of 7-methyl-6(*Z*)-octadecenoic acid (**1a**) appeared as a broad doublet at 1.66 ppm, while the vinyl methyl group (Me-19) of 7-methyl-6(*E*)-octadecenoic acid (**1b**) appeared as a broad singlet at 1.57 ppm. The NOESY spectrum of the mixture showed a through-space correlation between the broad doublet at 1.66 ppm (Me-19) and H-6, while in **1b** no such correlation was observed. The $^{13}$C NMR spectrum of 7-methyl-6(*Z*)-octadecenoic acid presented olefinic absorptions at 124.35 ppm (C-6) and at 136.12 ppm (C-7), while the 7-methyl-6(*E*)-octadecenoic acid had the olefinic absorptions at 123.65 ppm (C-6) and at 135.85 ppm (C-7). These assignments were made on the basis of COSY experiments.

Acids **1a** and **1b** were further methylated with HCl/MeOH for GC−MS analysis. In our nonpolar capillary column fatty acid methyl esters with the *trans* stereochemistry elute after their *cis* counterparts,[10] and the GC retention time of the earlier eluting methyl 7-methyl-6(*Z*)-octadecenoate corresponded to the methyl ester of our unknown, as did the mass spectra. The new acid is thus assigned as 7-methyl-6(*Z*)-octadecenoic acid (**1a**). We have recently identified both the *E* and *Z* isomers of **1a** in a strain of *Vibrio alginolyticus*.[11] This recent finding supports a possible bacterial origin for **1a**.

Other fatty acids and aldehydes from *H. mexicana* deserve a special mention. Arachidonic acid (5.8%) was the principal polyunsaturated fatty acid in *H. mexicana*, but 20:5 (*n*-3), known to be present in other holothurians,[5] was not identified. However, interesting was the identification of a series of 2-hydroxy fatty acids, in particular 2-hydroxy-15-tetracosenoic acid, which was characterized as previously described.[12] The latter acid is common in sea urchins and is known to occur in glycosphingolipids.[12] However, this is the first time that it has been identified in a holothurian. Octadecanal (18:0) was also identified in considerable amounts (15%), and it most likely arises from PE plasmalogens.

Other lipids, such as sterols and waxes, were found in *H. mexicana*. The sterol composition of *H. mexicana* was previously studied, and $\Delta^7$-sterols were found to predominate.[13] A wax, namely hexadecyl hexadecanoate, was also identified in *H. mexicana*. Its mass spectrum presented a molecular ion peak at *m/z* 480 and a base peak at *m/z* 257.[14]

The identification of **1a** in *H. mexicana* is of interest since it expands the occurrence of the 7-methyl-6-ene functionality to the Holothuroidea. From a fatty acid biosynthetic standpoint is also of interest since **1a** suggests, especially when compared to its shorter-chain analogue 7-methyl-6-hexadecenoic acid, that the 7-methyl-6-ene functionality could be introduced in fatty acyl chains after chain elongation. The origin of **1a** is most likely bacterial. In fact, just recently the isomeric 10-methyl-9(*Z*)-octadecenoic acid was isolated from the marine fungus *Microsphaeropsis olivacea*.[15] Work is in progress elucidating the origin of unusual fatty acids in marine invertebrates.

## Experimental Section

**General Experimental Procedures.** Fatty acid methyl esters were analyzed by gas chromatography in a Hewlett-Packard 5890A Series II gas chromatograph equipped with a fused silica capillary column (30 m × 0.32 mm i.d.) containing either SE-54 or SPBTM$^{-1}$ (carrier gas He). Analyses were performed using the following conditions: initial temperature,130 °C; rate, 3 °C/min; final temperature, 260 °C. Samples were also analyzed by gas chromatography−mass spectrometry at 70 eV using a Hewlett-Packard 5972A MS ChemStation equipped with a 30 m × 0.25 mm special performance capillary column (HP-5MS) of polymethyl siloxane cross-linked with 5% phenyl methylpolysiloxane. NMR data were collected in a Bruker Avance DPX 300 spectrometer. *J* values are given in Hz.

**Sample Collection.** *H. mexicana* was collected in May 1992 at Ahogado Reef in La Parguera, Puerto Rico, at a depth of 35 ft. The holothurian was kept in dry ice until transportation to the University of Puerto Rico. A voucher specimen is available at the Department of Marine Sciences of the University of Puerto Rico, Mayagüez campus.

**Extraction and Isolation of Phospholipids.** One specimen of *H. mexicana* (200−300 g) was carefully cleaned and cut into small pieces. Extraction with 2 × 250 mL of $CHCl_3$−MeOH (1:1) yielded the total lipids (ca. 3 g). The neutral lipids, glycolipids, and 20 mg of

phospholipids were separated by column chromatography on Si gel (60−200 mesh) using the procedure of Privett *et al.*[16] The phospholipid classes were fractionated by preparative 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.

**Preparation and Isolation of Fatty Acid Derivatives.** The fatty acyl components of the phospholipids were obtained as their methyl esters by reaction of the phospholipids with methanolic HCl followed by column chromatography eluting with *n*-hexane−diethyl ether (9:1). The double bond positions of the monounsaturated fatty acids was determined by preparing the corresponding dimethyl disulfide derivatives as previously described.[7] *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 °C followed by ethereal extraction from the acidified solution and purification by preparative TLC. Hydrogenations were carried out in 10 mL of MeOH and catalytic amounts of $PtO_2$. Mass spectral data for derivatives of **1a** are presented below.

**Methyl 7-methyl-6-octadecenoate**: GC−MS (70 eV) *m*/*z* $[M]^+$ 310 (2), 280 (1), 279 (2), 263 (1), 249 (1), 236 (1), 222 (1), 196 (3), 195 (5), 194 (3), 180 (2), 169 (3), 155 (4), 151 (9), 143 (3), 139 (9), 138 (49), 137 (12), 133 (2), 128 (4), 125 (11), 124 (7), 123 (15), 117 (4), 115 (23), 111 (20), 110 (21), 109 (21), 97 (33), 95 (39), 83 (50), 82 (40), 81 (39), 75 (40), 74 (23), 71 (68), 69 (67), 57 (50), 55 (100).

***N*-(7-Methyl-6-octadecenoyl)pyrrolidine**: GC−MS (70 eV) *m*/*z* $[M]^+$ 349 (18), 348 (2), 334 (2), 320 (1), 306 (1), 292 (1), 278 (1), 264 (2), 250 (2), 236 (3), 222 (5), 208 (15), 194 (6), 180 (1), 168 (5), 166 (8), 154 (10), 140 (8), 127 (19), 126 (48), 113 (100), 98 (25), 95 (7), 85 (11), 81 (9), 70 (27), 69 (21), 67 (13), 57 (15), 56 (14), 55 (47).

**Synthesis of 7-Methyl-6(*Z*)-octadecenoic Acid (1a).** A solution of 6-bromohexanoic acid (3.95 g, 20 mmol), triphenylphosphine (5.30 g, 20 mmol), and dry benzene (75 mL) was refluxed for 20 h. After the solution was cooled to rt, the solvent was rotoevaporated and the remaining solid was washed thoroughly with ether. After drying, 8.4 g (85% yield) of (6-carboxyhexyl)triphenylphosphonium bromide was recovered. To a mixture of 115 mg (0.58 mmol) of 2-tridecanone and 278 mg (0.61 mmol) of (6-carboxyhexyl)triphenylphosphonium bromide in 10 mL of dry THF was added dropwise 1.1 mL of 1.2 M *t*-BuOK in THF at 0 °C. After being stirred for 1.5 h, the reaction mixture was quenched at 0 °C with 15 drops of 1.0 N HCl. The solvent and excess HCl was rotoevaporated. Silica gel column chromatography of the crude mixture, with hexane−ether (1:1) as eluent, afforded 91 mg (53% yield) of a colorless oil consisting of a cis−trans (1:1) mixture of 7-methyl-6-octadecenoic acid (**1a** and **1b**).

**7-Methyl-6(*Z*)-octadecenoic acid (1a) and 7-methyl-6(*E*)-octadecenoic acid (1b)**: IR (dry film) $\nu_{max}$ 3500−3100 (OH, *E* and *Z*), 3020 (=CH, olefinic, *E* and *Z*), 2858 (*E* and *Z*), 2935 (*E* and *Z*), 1725 (C=O, acid, *E* and *Z*), 1380 (*E* and *Z*), 1080 (*E* and *Z*), 1130 (*E* and *Z*) $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz) $\delta$ 5.09 (1H, brt, $J$ = 7.0, H-6, *E* and *Z*), 2.35 (2H, t, $J$ = 7.4, H-2, *E* and *Z*), 1.90−2.05 (4H, m, H-5,H-8, *E* and *Z*), 1.66 (3H, brd, $J$ = 1.2, Me-19, Z), 1.57 (3H, brs, Me-19, *E*), 1.55 (2H, m, H-3, *E* and *Z*), 1.33 (2H, m, H-4, *E* and *Z*), 1.26 (18H, m, *E* and *Z*), 0.87 (3H, t, $J$ = 6.4, Me-18, *E* and *Z*); $^{13}C$ NMR ($CDCl_3$, 75.4 MHz) $\delta$ 178.67 (s, C-1, *E* and *Z*), 136.12 (s, C-7, *Z*), 135.88 (s, C-7, *E*), 124.35 (d, C-6, *Z*), 123.61 (d, C-6, *E*), 33.77 (t, C-2, *E* and *Z*), 31.91 (t, C-16, *E* and *Z*), 31.77 (t, *E* and *Z*), 29.68 (t, *E* and *Z*), 29.64 (t, *E* and *Z*), 29.55 (t, *E* and *Z*), 29.45 (t, *E* and *Z*), 29.35 (t, *E* and *Z*), 29.30 (t, *E* and *Z*), 29.24 (t, *E* and *Z*), 28.04 (t, *E* and *Z*), 27.97 (t, *E* and *Z*), 27.44 (t, *E* and *Z*), 27.33 (t, *E* and *Z*), 24.38 (t, *E* and *Z*), 23.41 (q, C-19, *Z*), 22.68 (t, *E* and *Z*), 15.90 (q, C-19, *E*), 14.11 (q, C-18, *E* and *Z*).

**7-Methyl-6(*Z*)-octadecenoic acid (1a):** GC−MS (70 eV) *m*/*z* $[M]^+$ 296 (9), 278 (11), 196 (7), 181 (6), 168 (4), 157 (6), 156 (10), 138 (61), 125 (14), 123 (21), 110 (31), 101 (29), 97 (47), 95 (40), 83 (50), 81 (45), 69 (80), 67 (36), 57 (56), 55 (100).

**7-Methyl-6(*E*)-octadecenoic acid (1b):** GC−MS (70 eV) *m*/*z* $[M]^+$ 296 (12), 278 (1), 196 (9), 181 (7), 168 (5), 157 (8), 156 (12), 138 (80), 125 (17), 123 (26), 110 (40), 101 (36), 97 (53), 95 (49), 83 (53), 81 (48), 69 (82), 67 (38), 57 (56), 55 (100).

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