Two Novel *Iso*-Branched Octadecenoic Acids from a *Micrococcus* Species

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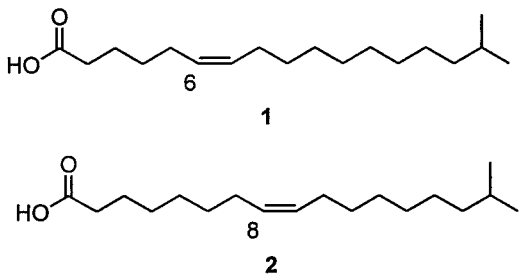
The novel fatty acids 16-methyl-6(*Z*)-heptadecenoic acid and 16-methyl-8(*Z*)-heptadecenoic acid were identified for the first time in nature in a species of the bacterium *Micrococcus* isolated from Lake Pomorie in Bulgaria. The principal fatty acids in this bacterium were a series of *iso*−*anteiso* fatty acids with chain lengths between C_{14} and C_{24}, while the most interesting series of monounsaturated fatty acids was a family of Δ^6 fatty acids with chain lengths between C_{14} and C_{17}. The novel compounds were characterized using a combination of GC−MS and chemical transformations, such as dimethyl disulfide derivatization and catalytic hydrogenation. The results established for the first time a bacterial origin for some of these Δ^6 fatty acids.

Monounsaturated even-chain fatty acids with Δ^6 and Δ^8 unsaturations are less common than the corresponding fatty acids with Δ^5 and Δ^9 double bonds.[1−4] Normal-chain Δ^6 fatty acids with C_{14}−C_{17} chain lengths have been identified in a variety of natural sources such as sponges,[1] opisthobranchs,[2] human skin,[3] and seed oil.[4] On the other hand, Δ^8 monounsaturated fatty acids, such as the 8-octadecenoic acid, have also been identified in nature, for example, in the exocrinology of the queen bumble bee *Bombus terrestris* (Hymenoptera).[5]

Branched-chain *iso*−*anteiso* Δ^6 and Δ^8 monounsaturated fatty acids are less ubiquitous than their corresponding normal-chain analogues. Perhaps the best known example is the 14-methyl-6(*Z*)-pentadecenoic acid (*i*-16:1 Δ^6), which was identified in several marine organisms, for example, in the phospholipids of the sponge *Tethya aurantia*[6] and in the freshwater mussel *Unio tumidus*.[7] 14-Methyl-6(*Z*)-pentadecenoic acid has also attracted the attention of synthetic chemists, inasmuch as its total synthesis was accomplished in seven steps starting with hexane-1,6-diol.[8]

Despite all of these efforts there are no reports in the literature with respect to the identification of either Δ^6 or Δ^8 *iso*-branched octadecenoic acids, in particular the compounds 16-methyl-6(*Z*)-heptadecenoic acid (**1**, *i*-18:1 Δ^6) and the two-carbon biosynthetic elongation of *i*-16:1 Δ^6, namely, the 16-methyl-8(*Z*)-heptadecenoic acid (**2**, *i*-18:1 Δ^8). In this paper we describe the identification of these two novel Δ^6 and Δ^8 *iso*-$C_{18:1}$ fatty acids from a *Micrococcus* sp., a bacterium isolated from Lake Pomorie in Bulgaria. We establish a bacterial origin for these novel Δ^6 fatty acids and suggest new biosynthetic possibilities for these *iso*-$C_{16:1}$−$C_{18:1}$ even-chain fatty acids.

The *Micrococcus* sp. isolated from Lake Pomorie in Bulgaria presented a rather complex fatty acid composition of around 44 identifiable fatty acids, as shown in Table 1. Fatty acid chain lengths ranged between C_{12} and C_{26}, mainly consisting of saturated and monounsaturated fatty acids. The *iso*−*anteiso* methyl-branched fatty acids were particularly abundant in this bacterium; they made up 70.4% of the total fatty acid composition. For example, a whole series of *iso* methyl-branched fatty acids with chain lengths between C_{14} and C_{24} was identified in this bacterium, but the predominant fatty acid was an *anteiso*-15:0 (35.8%). All of these fatty acids were characterized as methyl esters by GC−MS as well as by gas chromatographic comparison with authentic standards.



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2

In addition to the saturated fatty acids, an unusual series of monounsaturated fatty acids with chain lengths between C_{14} and C_{17} was also identified in which the rare Δ^6 monounsaturation predominated. The double-bond positions in the corresponding methyl esters were determined by dimethyl disulfide (DMDS) derivatization.[9] In particular, our attention was centered on two methyl octadecenoates that had almost identical GC retention times with equivalent chain-length (ECL) values of 17.33−17.35 in nonpolar capillary gas chromatography. The low fractional chain length (FCL) values of 0.33−0.37 compared favorably with that of the known methyl 14-methyl-6(*Z*)-pentadecenoate (ECL = 15.37), which was also present in this bacterium, thus supporting the *iso*-methyl branching in addition to one unsaturation for both unknowns. In fact, the *iso*-methyl branching was confirmed by catalytic hydrogenation (PtO_2) as both methyl esters of **1** and **2** were transformed into methyl 16-methylheptadecanoate, as confirmed by GC coelution with an authentic sample.

Mass spectrometry was the spectrometric technique of choice used to fully elucidate the structures of **1** and **2** because of their low abundance in the bacterium and almost identical GC retention times. The methyl esters of **1** and **2** displayed a molecular ion peak [M^+] at *m*/*z* 268

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Table 1. Identified Fatty Acids from *Micrococcus* sp.

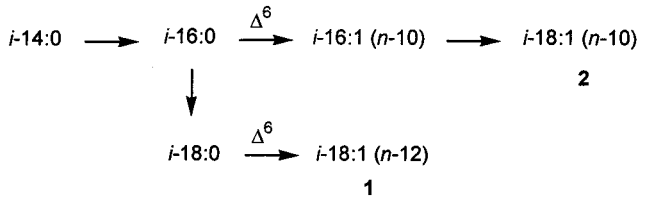
fatty acids	abundance (wt %)
dodecanoic (12:0)	0.2
tridecanoic (13:0)	0.3
(*Z*)-6-tetradecenoic (14:1)	3.1
12-methyltridecanoic (*i*-14:0)	0.4
tetradecanoic (14:0)	1.3
13-methyltetradecanoic (*i*-15:0)	3.0
12-methyltetradecanoic (*ai*-15:0)	35.8
(*Z*)-6-pentadecenoic (15:1)	0.3
pentadecanoic (15:0)	0.9
(*Z*)-14-methyl-6-pentadecenoic (*i*-16:1)	0.2
(*Z*)-6-hexadecenoic (16:1)	0.4
hexadecanoic (16:0)	3.3
2-methylhexadecanoic (17:0)	0.2
15-methylhexadecanoic (*i*-17:0)	2.8
14-methylhexadecanoic (*ai*-17:0)	15.0
(*Z*)-6-heptadecenoic (17:1)	0.2
heptadecanoic (17:0)	0.9
(*Z*)-16-methyl-6-heptadecenoic (*i*-18:1)[a]	0.2
(*Z*)-16-methyl-8-heptadecenoic (*i*-18:1)[a]	0.2
16-methylheptadecanoic (*i*-18:0)	2.0
(9*Z*,12*Z*)-9,12-octadecadienoic (18:2)	0.4
(*Z*)-8-octadecenoic (18:1)	0.9
(*Z*)-9-octadecenoic (18:1)	0.8
(*Z*)-11-octadecenoic (18:1)	0.4
octadecanoic (18:0)	4.2
17-methyloctadecanoic (*i*-19:0)	1.7
16-methyloctadecanoic (*ai*-19:0)	6.2
nonadecanoic (19:0)	1.8
18-methylnonadecanoic (*i*-20:0)	1.0
(*Z*)-11-eicosenoic (20:1)	0.3
(*Z*)-13-eicosenoic (20:1)	0.4
eicosanoic (20:0)	5.1
19-methyleicosanoic (*i*-21:0)	0.3
18-methyleicosanoic (a*i*-21:0)	1.0
heneicosanoic (21:0)	0.4
20-methylheneicosanoic (*i*-22:0)	0.4
(*Z*)-13-docosenoic (22:1)	0.5
docosanoic (22:0)	0.7
21-methyldocosanoic (*i*-23:0)	0.4
tricosanoic (23:0)	0.4
22-methyltricosanoic (*i*-24:0)	0.4
tetracosanoic (24:0)	0.7
pentacosanoic (25:0)	0.3
hexacosanoic (26:0)	0.3

[a] Unprecedented in nature.

and typical fragmentations of a monounsaturated methyl ester at *m*/*z* 264 [$M^+ - 32$, loss of methanol], *m*/*z* 241 [$M^+ - 55$], *m*/*z* 222 [$M^+ - 74$, loss of the McLafferty ion], *m*/*z* 180 [$M^+ - 116$], and *m*/*z* 74 (McLafferty rearrangement ion). However, there were no ions that permitted the unequivocal location of the monounsaturation due to double-bond migration during ionization. For this purpose, DMDS adducts were used to "fix" the double bonds and permit their location by mass spectrometry.[9] For example, one of the unknowns yielded methyl 16-methyl-6,7-bis-(methylthio)heptadecanoate upon reaction with CH_3SSCH_3/I_2. In the mass spectrum of the latter [$M^+ = 390$] the principal cleavage occurred between the carbons that originally constituted the double bonds (C-6 and C-7), yielding two substantial fragment ions (one containing the terminal methyl group of the molecule at *m*/*z* 215 and a second at *m*/*z* 175 containing the ester group). A third prominent peak was observed at *m*/*z* 143 due to the loss of methanol from the *m*/*z* 175 ion. Therefore, one of the unknown fatty acids is 16-methyl-6(*Z*)-heptadecenoic acid (**1**).

Upon DMDS derivatization, the second methyl octadecenoate yielded 16-methyl-8,9-bis(methylthio)heptadecanoate. In this case, the DMDS adduct also presented a mass spectrum with a molecular ion peak at *m*/*z* 390 and key fragment ions at *m*/*z* 187 (methyl end) and *m*/*z* 203 (carboxy end), with the latter ion losing methanol to afford a third prominent ion at *m*/*z* 171. Therefore, the second unknown fatty acid was characterized as 16-methyl-8(*Z*)-heptadecenoic acid (**2**). The IR spectrum of the fatty acid methyl ester mixture displayed no significant absorption in the 960−980 cm^{-1} region, but rather an absorption in the 760−780 cm^{-1} region, bespeaking cis double bonds in all of the studied monounsaturated fatty acids.[10] Further structural confirmation, including the stereochemistry of both **1** and **2**, was achieved by GC coelution of the methyl esters with synthetic samples, which were made for antimicrobial bioassays.[11]

Scheme 1



Based on these results, some new biosynthetic pathways for *iso* even-chain fatty acids in marine bacteria can be postulated (Scheme 1). Due to the variety of Δ^6 fatty acids characterized we can conclude that there is a high Δ^6 desaturation activity in this *Micrococcus* sp. Our new *iso*-$C_{18:1}$ fatty acids could have very well originated from *i*-14:0. For example, chain elongation of *i*-14:0 to *i*-16:0 and desaturation at C-6 is a likely pathway for the known *i*-16:1 Δ^6. A subsequent two-carbon elongation of *i*-16:1 Δ^6 can then result in the novel *i*-18:1 Δ^8. The other new acid, *i*-18:1 Δ^6, could very well have originated from a four-carbon elongation of *i*-14:0 to *i*-18:0 followed by Δ^6 desaturation to *i*-18:1 Δ^6.

In addition to expanding the limits of Δ^6 desaturation in bacteria, we can also conclude from this work that all of the Δ^6 fatty acids in Table 1, many of which are known constituents of marine invertebrates, do have a bacterial origin. Work is in progress to elucidate the origin of unusual fatty acids in marine biota.

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Nicolet 600 FTIR spectrophotometer. Fatty acid methyl esters were analyzed by GC−MS 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 polymethylsiloxane cross-linked with 5% phenyl methylpolysiloxane. The temperature program was as follows: 130 °C for 1 min, then increased at a rate of 3 °C/min to 270 °C and maintained for 30 min at 270 °C.

Bacterial Material. The *Micrococcus* sp. was collected from the water of Lake Pomorie, partially connected to the Black Sea, during 1998. Samples were inoculated on 5-mL liquid YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) and cultivated overnight in test tubes. All cultivation experiments were performed at 37 °C. Different dilutions from each test tube were plated onto Petri dishes with solid YPD medium (containing 2% agar) and further cultivated for single colonies. Colonies with characteristic morphologies were isolated and studied. One strain was grown on liquid YPD medium supplemented with marine water to obtain 4% salt concentration in the stationary growth phase. Cells were harvested by centrifugation and washed twice. Characterization was performed by routine biochemical and antibiotic tests modified for marine bacteria.[12] The API 20E system was also used for characterization, as recommended by MacDonell et al. for marine isolates.[13] The microorganism in question was identified as a *Micrococcus* sp. Freeze-dried samples of the bacterium

(voucher no. 2−2) are available at the Chemistry Department of the University of Puerto Rico, Río Piedras Campus.

Lipid Isolation and Characterization. The lipids were extracted from the bacterium (8−16 g wet biomass) with chloroform/methanol (2:1 vol/vol) following the procedure of Bligh and Dyer.[14] Approximately 9 mg/mL of lipids were obtained for this bacterium, accounting for 40−45 mg of total lipids. Fatty acids were identified as methyl esters, which were prepared by direct methylation of the lipid extract with 1.5 N HCl/MeOH, as previously described.[15] In a typical run, 7−12 mg of fatty acid methyl esters were obtained from around 20−22 mg of total lipids.

Derivatives. The methyl esters were hydrogenated in 10 mL of absolute methanol and catalytic amounts of platinum oxide (PtO_2). The double-bond positions in these compounds were determined by DMDS derivatization following a procedure that was previously described.[9] Mass spectral data for the novel methyl esters and derivatives are presented below.

Methyl 16-methyl-6(*Z*)-heptadecenoate: GC−MS (70 eV) *m*/*z* 296 [M^+] (7), 264 (31), 253 (1), 249 (1), 235 (3), 222 (16), 194 (2), 191 (1), 180 (13), 166 (7), 155 (2), 153 (4), 152 (8), 151 (6), 141 (9), 139 (6), 127 (5), 125 (12), 123 (16), 113 (4), 111 (22), 99 (6), 97 (41), 85 (11), 84 (41), 83 (48), 74 (58), 71 (13), 69 (64), 67 (39), 57 (30), 55 (100).

Methyl 16-methyl-6,7-bis(methylthio)heptadecanoate: GC−MS (70 eV) *m*/*z* 390 [M^+] (6), 343 (1), 294 (1), 247 (1), 216 (3), 215 (19), 176 (2), 175 (13), 151 (5), 144 (4), 143 (36), 137 (6), 123 (27), 109 (17), 97 (39), 95 (40), 87 (66), 83 (51), 81 (41), 74 (95), 69 (68), 67 (40), 57 (68), 55(100).

Methyl 16-methyl-8(*Z*)-heptadecenoate: GC−MS (70 eV) *m*/*z* 296 [M^+] (3), 265 (11), 264 (15), 253 (1), 249 (2), 241 (6), 235 (1), 222 (9), 209 (4), 194 (1), 191 (2), 181 (1), 180 (5), 167 (2), 166 (4), 155 (1), 153 (2), 152 (4), 151 (4), 141 (6), 139 (4), 127 (3), 125 (8), 123 (14), 113 (3), 111 (17), 99 (4), 97 (37), 85 (11), 84 (36), 83 (42), 74 (56), 71 (13), 69 (72), 67 (46), 57 (40), 55 (100).

Methyl 16-methyl-8,9-bis(methylthio)heptadecanoate: GC−MS (70 eV) *m*/*z* 390 [M^+] (12), 343 (2), 327 (1), 294 (1), 281 (1), 264 (1), 256 (1), 245 (1), 204 (12), 203 (100), 188 (13), 187 (89), 172 (6), 171 (59), 137 (3), 123 (38), 109 (10), 97 (20), 95 (43), 87 (31), 83 (37), 81 (42), 74 (14), 69 (49), 67 (36), 57 (24), 55 (75).

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References and Notes

(1) Barnathan, G.; Doumenq, P.; Njinkoue, J. M.; Miralles, J.; Debitus, C.; Levi, C.; Komprobst, J. M. *Lipids* **1994**, *29*, 297−303.
(2) Carballeira, N. M.; Anastacio, E.; Salva, J.; Ortega, M. J. *J. Nat. Prod.* **1992**, *55*, 1783−1786.
(3) Shinohara, T. *Kagaku Keisatsu Kenkyusho Hokoku* **1970**, *23*, 18−23.
(4) Spencer, G. F.; Kleiman, R.; Miller, R. W.; Earle, F. R. *Lipids* **1971**, *6*, 712−714.
(5) Hefetz, A.; Taghizadeh, T.; Francke, W. Z. *Naturforsch., C: Biosci.* **1996**, *51*, 409−422.
(6) Zimmerman, M. P.; Hoberg, M.; Ayanoglu, E.; Djerassi, C. *Lipids* **1990**, *25*, 383−390.
(7) Stefanov, K.; Seizova, K.; Brechany, E. Y.; Christie, W. W. *J. Nat. Prod.* **1992**, *55*, 979−981.
(8) Hassarajani, S. A.; Mithran, S.; Mamdapur, V. R. *Indian J. Chem., Sect. B: Org. Chem. Incl. Med. Chem.* **1995**, *34B*, 429−431.
(9) Dunkelblum, E.; Tan, S. H.; Silk, R. J. *J. Chem. Ecol.* **1985**, *11*, 265−277.
(10) Doumenq, P.; Guiliano, M.; Bertrand, J. C.; Mille, G. *Appl. Spectrosc.* **1990**, *44*, 1355−1359.
(11) The details of the total syntheses of both **1** and **2**, together with other similar fatty acids, will be published in a separate paper.
(12) Oliver, J. D. *Deep-Sea Res.* **1982**, *29*, 795−798.
(13) MacDonell, M. T.; Singleton, F. L.; Hood, H. A. *Appl. Environ. Microbiol.* **1982**, *44*, 423−427.
(14) Bligh, E. G.; Dyer, W. J. *Can. J. Biochem. Physiol.* **1959**, *37*, 911−917.
(15) Christie, W. W.; Brechany, E. Y.; Stefanov, K.; Popov, S. *Lipids* **1992**, *27*, 640−644.

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