Characterization of Novel Methyl-Branched Chain Fatty Acids from a Halophilic *Bacillus* Species

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The 4-methylated fatty acids 4,9-dimethyldecanoic, 4,11-dimethyldodecanoic, 4,10-dimethyldodecanoic, and 4,13-dimethyltetradecanoic acid as well as the 2-methylated fatty acids 2,13-dimethyltetradecanoic and 2,12-dimethyltetradecanoic acid were identified for the first time in nature in the halophilic bacterium *Bacillus* sp. isolated from the salt pans of Burgas in Bulgaria. The principal fatty acids in this bacterium were a series of *iso-anteiso* fatty acids with chain lengths between C_{11} and C_{19}, but an interesting series of linear alkylbenzene fatty acids with chain lengths between C_{10} and C_{14}, such as 12-phenyldodecanoic acid, were also identified. The novel 4-methylated fatty acids were characterized using a combination of GC−MS and chemical transformations such as *N*-acylpyrrolidide derivatization. The 2-methylated fatty acids were also identified by GC−MS and gas chromatographic coelution with synthetic samples. The novel methyl-branched fatty acids probably originated from the selective incorporation of methylmalonyl-CoA by one of the fatty acid-synthesizing enzymes of the bacterium.

Methyl-branched fatty acids with methyl groups at even carbon atoms (methyl substituents on carbon 2, 4, 6, etc.) occur in several organisms and originate from the selective incorporation of methylmalonyl-CoA by the fatty acid synthase.[1] Some classic examples of these methyl-branched fatty acids include the 2- and 4-monomethylated fatty acids from the uropygial gland of ducks, such as *Anas platyrhynchos*,[1] as well as from the guinea pig Harderian gland.[2−5] These branched-chain fatty acids also occur at both the *sn*-1 and *sn*-2 positions of phosphatidylethanolamines and phosphatidylcholines from the guinea pig Harderian gland, an established source for this type of methylated fatty acids.[4] Triacylglycerols from barley-fed lambs also contain uncommon 4,8-dimethylated fatty acids such as 4,8-dimethyltridecanoic acid and 4,8-dimethylpentadecanoic acid.[6] However, their occurrence in bacteria has been practically limited to the mycobacteria, where the acid 2,4-dimethyltetradecanoic acid is constantly cited as characteristic for these microorganisms.[7,8] In addition, other unusual methylated fatty acids from mycobacteria include the long-chain fatty acids 2,4,6-trimethyldocosanoic acid and 2,4,6-trimethyltetracosanoic acid.[7] Therefore, fatty acid analyses have been proposed as useful to identify different mycobacterium species.[7,8] In this paper we report the identification of six novel dimethylated *iso-anteiso* fatty acids with methyl substituents at carbons 2 and 4 from a halophilic *Bacillus* sp. isolated from the salt pans of Burgas in Bulgaria. These methyl-branched fatty acids probably originated from the selective incorporation of methylmalonyl-CoA by the fatty acid synthase of the *Bacillus*. It is noteworthy that some of the novel dimethylated fatty acids that we describe herein have been synthesized for industrial applications, but were hitherto unknown from a natural source.[9−10]

The halophilic *Bacillus* sp. isolated from the salt pans of Burgas in Bulgaria presented a rather complex fatty acid composition of around 41 identifiable fatty acids, as shown in Table 1. Fatty acid chain lengths ranged between C_{10} and C_{24}, mainly consisting of saturated fatty acids. The *iso-anteiso* methyl-branched fatty acids were particularly abundant in this *Bacillus*, making up 69.4% of the total fatty acid composition. For example, a homologous series of *anteiso* odd-chain fatty acids with chain lengths between C_{11} and C_{19} were identified in the bacterium, but the predominant fatty acids in the series were an *anteiso*-15:0 (15.8%) and an *anteiso*-17:0 (11.2%). All of these fatty acids were characterized as methyl esters by GC−MS as well as by gas chromatographic comparison with authentic standards.

Our attention was centered on an interesting series of novel *iso-anteiso* dimethylated fatty acids between C_{12} and C_{16} with at least one methyl branching at either C-2 or C-4. The novel 4-methylated compounds were characterized as 4,9-dimethyldecanoic acid (**1**), 4,11-dimethyldodecanoic acid (**2**), 4,10-dimethyldodecanoic acid (**2a**), and 4,13-dimethyltetradecanoic acid (**3**) by the relative GC retention times of the methyl esters as well as by interpreting the MS of both their corresponding methyl esters and pyrrolidides.[11] For example, the *iso* 4-methylated methyl esters between C_{12} and C_{16} displayed rather low gas chromatographic equivalent chain length (ECL) values between 11.07 and 15.08, while the only *anteiso* 4-methylated methyl ester had an ECL value of 13.14. These ECL values clearly indicated dimethylation. Upon electron impact MS the base peak in the mass spectra of the four 4-methylated methyl esters was observed at *m*/*z* 87, instead of the usual base peak at *m*/*z* 74 (McLafferty rearrangement), supporting the methyl substitution at C-4.[2] The 4-methyl substitution as well as the *iso-anteiso* methyl branching in **1**−**3** were unequivocally assigned interpreting the MS of their corresponding pyrrolidide derivatives.[11] For example, the C-4 methyl substitution in **1**−**3** was confirmed since enhanced peaks were observed at *m*/*z* 126 and 154 leading

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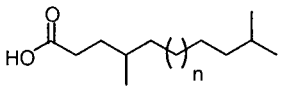
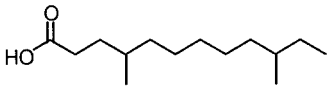
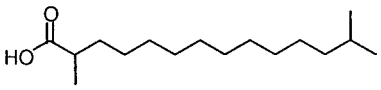
to a diminished fragmentation at *m/z* 140 in the MS of the pyrrolidide derivatives prepared from **1**−**3**. In addition, the *iso* methyl substitution in **1**−**3** became evident from the enhanced M^+-15 and M^+-43 fragmentations observed in the mass spectra of the *iso* pyrrolidides, leading to a diminished fragmentation at M^+-29.[11] The *anteiso* methyl substitution in **2a** was confirmed from the enhanced M^+-29 and M^+-57 fragmentations observed in conjunction with a diminished fragmentation at M^+-43 in the mass spectrum of the pyrrolidide derived from **2a**.[11]



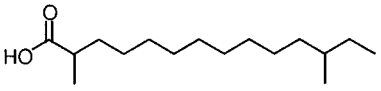
1: n = 1
2: n = 3
3: n = 5



2a



4



4a

The novel 2-methylated fatty acids were characterized as the *iso-anteiso* pair 2,13-dimethyltetradecanoic acid (**4**) and 2,12-dimethyltetradecanoic acid (**4a**). The methyl ester of **4** also displayed a rather small gas chromatographic ECL value of 14.97, while the methyl ester of **4a** had a slightly larger ECl value of 15.02. These ECL values indicated a different kind of dimethylation. The base peak in the MS of the methyl esters of **4** and **4a** was observed at *m/z* 88, instead of the usual base peak at *m/z* 74 (McLafferty rearrangement), supporting the α-methylation. Final structural confirmation was achieved by GC coelution of the methyl esters of **4** and **4a** with synthetic samples prepared by α-methylating commercially available methyl 13-methyltetradecanoate and methyl 12-methyltetradecanoate with CH_3I and LDA.

Another interesting finding in this *Bacillus* sp. was the identification of the linear alkylbenzene fatty acids 10-phenyldecanoic acid, 12-phenyldodecanoic acid, 13-phenyltridecanoic acid, and 14-phenyltetradecanoic acid. These linear alkylbenzene fatty acids were identified, as methyl esters, by MS comparing to reported mass spectral data (National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health mass spectral library from the U.S. Department of Commerce). The mass spectra of the corresponding methyl esters were all similar, i.e., a base peak at *m/z* 91 (tropylium ion), abundant fragmentations at *m/z* 55, 74, 104, and a strong M^+-32 peak. The straight alkyl chain was further confirmed by pyrrolidine derivatization, since the mass spectra of the pyrrolidide derivatives presented a continuous series of ion clusters, 14 amu apart, starting at the carbonyl end of the chain and finishing at the penultimate carbon before the benzene ring, where a gap of 91 amu was observed before the molecular ion. Moreover, a linear plot was observed when the capillary GC retention times of these C_{10}−C_{14} methyl phenylalkanoates were plotted against carbon chain length. Several *ω*-phenylalkanoic acids have been identified in marine bacteria such as *Alcaligenes* sp. and *Acinetobacter* sp.[12] In addition, the 13-phenyltridecanoic acid has been particularly abundant in seed lipids of the subfamily Aroideae under the Araceae and in *Typhonium flagelliforme*.[13,14]

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

fatty acid	abundance (wt %)
decanoic (10:0)	0.1
9-methyldecanoic (*i*-11:0)	0.1
8-methyldecanoic (*ai*-11:0)	0.1
4,9-dimethyldecanoic (12:0)[a]	0.2
10-methylundecanoic (*i*-12:0)	0.3
dodecanoic (12:0)	0.2
11-methyldodecanoic (*i*-13:0)	0.5
10-methyldodecanoic (*ai*-13:0)	0.1
4,11-dimethyldodecanoic (14:0)[a]	0.4
4,10-dimethyldodecanoic (14:0)[a]	0.4
12-methyltridecanoic (*i*-14:0)	4.2
tetradecanoic (14:0)	2.0
13-methyltetradecanoic (*i*-15:0)	14.7
12-methyltetradecanoic (*ai*-15:0)	15.8
2,13-dimethyltetradecanoic (16:0)[a]	2.8
pentadecanoic (15:0)	5.0
4,13-dimethyltetradecanoic (16:0)[a]	1.9
4,12-dimethyltetradecanoic (16:0)	1.8
2,12-dimethyltetradecanoic (16:0)[a]	0.4
14-methylpentadecanoic (*i*-16:0)	9.7
(*Z*)-9-hexadecenoic (16:1)	2.4
(*Z*)-11-hexadecenoic (16:1)	0.5
hexadecanoic (16:0)	8.7
15-methylhexadecanoic (*i*-17:0)	2.6
14-methylhexadecanoic (*ai*-17:0)	11.2
heptadecanoic (17:0)	0.8
16-methylheptadecanoic (*i*-18:0)	0.5
(9*Z*,12*Z*)-9,12-octadecadienoic (18:2)	0.9
(*Z*)-9-octadecenoic (18:1)	0.8
(*Z*)-11-octadecenoic (18:1)	0.2
octadecanoic (18:0)	3.7
17-methyloctadecanoic (*i*-19:0)	0.2
16-methyloctadecanoic (*ai*-19:0)	1.4
eicosanoic (20:0)	0.6
docosanoic (22:0)	0.4
tricosanoic (23:0)	0.1
tetracosanoic (24:0)	0.4
10-phenyldecanoic (10:0)	0.4
12-phenyldodecanoic (12:0)	3.1
13-phenyltridecanoic (13:0)	0.2
14-phenyltetradecanoic (14:0)	0.1

[a] Unprecedented as natural compounds.

Although fatty acids similar to the novel ones reported here have been isolated before from several terrestrial sources, in particular from the uropygial or Harderian glands of mammals,[1,2] this is the first report of these kinds of fatty acids from a *Bacillus* sp.[15−17] The most common related compounds of bacterial origin have been identified from the mycobacteria,[7] where the 2,4-dimethyltetradecanoic acid has taxonomic importance. The *iso-anteiso* branching in the novel methyl-branched fatty acids is most probably derived from leucine and isoleucine, respectively, followed by a series of elongations with malonyl-CoA. At either the last or penultimate elongation step methylmalonyl-CoA seems to be selectively incorporated by one of the fatty acid synthesizing enzymes from the bacterium resulting in the methyl-branched fatty acids described herein.[18,19] Whether this is a known or unknown methyl-branched fatty acid synthase in bacteria is at present a matter of speculation.[3,18]

Experimental Section

General Experimental Procedures. 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. Fatty acid methyl ester standards were obtained from Matreya, Inc.

Bacterial Material. The *Bacillus* sp. was collected from the salt pan of Burgas, Bulgaria, in August 1999. 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 in order to obtain 4% NaCl in the stationary growth phase (the use of 20% NaCl solution gave worse results). Cells were harvested by centrifugation and washed twice. Characterization was done by routine biochemical and antibiotic tests modified for marine bacteria.[20] The API 20E system was also used for characterization, as recommended by MacDonell et al. for marine isolates.[21] The microorganism in question was identified as a *Bacillus* sp. Freeze-dried samples of the bacterium (voucher no. 1-1) 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 (3.06 g wet biomass) with chloroform/methanol (2:1 vol/vol) following the procedure of Bligh and Dyer.[22] Approximately 60 mg of total lipids was obtained for this bacterium. Fatty acids were identified as methyl esters, which were prepared by direct methylation of the lipid extract with 1.5 M HCl/MeOH, as previously described.[23] In a typical run 7−12 mg of fatty acid methyl esters was 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.[24] The methyl branching in the branched fatty acids was determined by preparing the corresponding *N*-acylpyrrolidide derivatives. The *N*-acylpyrrolidide derivatives were prepared by direct treatment of the methyl esters with pyrrolidine−HOAc (10:1) in a capped vial for 24 h at 100 °C followed by ethereal extraction from the acidified solution and purification by preparative TLC. Mass spectral data for the novel methyl esters and derivatives are presented below.

Methyl 4,9-dimethyldecanoate: GC−MS (70 eV) *m*/*z* 214 $[M]^+$ (2), 183 (4), 173 (4), 171 (2), 158 (4), 157 (21), 149 (3), 143 (7), 141 (19), 132 (5), 129 (4), 127 (4), 115 (15), 109 (8), 101 (10), 99 (8), 97 (10), 95 (10), 88 (28), 87 (100), 85 (24), 83 (24), 81 (9), 74 (71), 71 (24), 69 (27), 67 (8), 59 (17), 57 (34), 55 (48).

***N*-4,9-Dimethyldecanoylpyrrolidine:** GC−MS (70 eV) *m*/*z* 253 $[M]^+$ (0.8), 238 (1.5), 224 (0.6), 210 (0.7), 196 (1), 182 (1.1), 168 (1), 154 (4), 140 (0.9), 127 (5.1), 126 (33), 114 (8), 113 (100), 98 (10), 97 (4), 95 (3), 85 (11), 83 (5), 81 (3), 71 (17), 69 (8), 59 (7), 57 (10), 55 (21).

Methyl 4,11-dimethyldodecanoate: ECL = 13.07, GC−MS (70 eV) *m*/*z* 242 $[M]^+$ (1), 211 (3), 195 (1), 186 (2), 185 (18), 171 (2), 169 (9), 157 (2), 143 (3), 129 (2), 119 (4), 115 (11), 111 (5), 105 (9), 99 (9), 97 (10), 87 (100), 83 (15), 74 (63), 71 (19), 69 (20), 59 (10), 57 (31), 55 (35).

***N*-4,11-Dimethyldodecanoylpyrrolidine:** GC−MS (70 eV) *m*/*z* 281 $[M]^+$ (0.5), 266 (1.2), 252 (0.3), 238 (0.4), 224 (0.7), 210 (0.3), 196 (0.3), 182 (0.9), 168 (0.6), 154 (3), 140 (0.6), 126 (32), 113 (100), 101 (2), 98 (8), 85 (8), 71 (13), 57 (6), 55 (15).

Methyl 4,10-dimethyldodecanoate: ECL = 13.14, GC−MS (70 eV) *m*/*z* 242 $[M]^+$ (2), 213 (2), 211 (3), 186 (3), 185 (20), 171 (2), 169 (9), 157 (2), 143 (2), 129 (2), 119 (3), 115 (9), 111 (5), 105 (6), 99 (10), 97 (10), 87 (100), 83 (15), 74 (56), 71 (20), 69 (19), 59 (10), 57 (34), 55 (35).

***N*-4,10-Dimethyldodecanoylpyrrolidine:** GC−MS (70 eV) *m*/*z* 281 $[M]^+$ (0.5), 266 (0.8), 252 (1.2), 224 (1), 210 (0.4), 196 (0.6), 182 (1.1), 168 (1), 154 (3), 140 (2), 127 (5), 126 (30), 114 (8), 113 (100), 101 (2), 98 (9), 97 (4), 85 (9), 83 (4), 76 (6), 71 (14), 69 (7), 67 (4), 57 (11), 55 (17).

Methyl 4,13-dimethyltetradecanoate: ECL = 15.08, GC−MS (70 eV) *m*/*z* 270 $[M]^+$ (3), 241 (1), 227 (1), 213 (16), 199 (4), 185 (1), 171 (2), 157 (3), 143 (10), 129 (3), 119 (1), 115 (12), 111 (9), 105 (2), 99 (5), 97 (12), 87 (100), 83 (20), 74 (90), 71 (18), 69 (24), 67 (6), 59 (11), 57 (42), 55 (41).

***N*-4,13-Dimethyltetradecanoylpyrrolidine:** GC−MS (70 eV) *m*/*z* 309 $[M]^+$ (1.4), 294 (2), 280 (0.2), 266 (0.7), 252 (0.5), 238 (0.5), 224 (0.5), 210 (0.6), 196 (1.5), 182 (2), 168 (0.8), 154 (4), 140 (2), 126 (23), 113 (100), 98 (8), 85 (6), 71 (9), 70 (12), 67 (2), 57 (5), 55 (14).

General Procedure for the Preparation of Methyl 2,13-Dimethyltetradecanoate and Methyl 2,12-Dimethyltetradecanoate. To a solution of either commercially available (Matreya, Inc.) methyl 13-methyltetradecanoate or methyl 12-methyltetradecanoate (0.02 g, 78 mmol) in 0.5 mL of tetrahydrofuran at −78 °C was added dropwise a solution of lithium diisopropylamide (100 mmol) in 0.5 mL of tetrahydrofuran prepared from diisopropylamine (14 μL, 100 mmol) and *n*-butyllithium (50 μL, 100 mmol, 2.0 M) in hexane. After the mixture was stirred at −78 °C for 30 min, methyl iodide (6 μL, 90 mmol) dissolved in hexamethylphosphoric triamide (18 μL, 100 mmol) was added, and the reaction mixture was stirred at −78 °C for 1 h. The reaction mixture was quenched with a saturated NH_4Cl solution (5 mL) and extracted with ether (3 × 5 mL). The organic phase was successively washed with a 0.1 M HCl solution (2 × 5 mL) and a saturated NaCl solution (2 × 5 mL) and finally dried over Na_2SO_4. After rotoevaporation the dimethylated ester was obtained in sufficient amounts (5% GC yield) for GC coelution experiments.

Methyl 2,13-dimethyltetradecanoate: ECL = 14.97, GC−MS (70 eV) *m*/*z* 270 $[M]^+$ (5), 256 (1), 239 (1), 227 (3), 213 (6), 199 (4), 185 (2), 171 (2), 157 (7), 143 (8), 129 (2), 115 (3), 101 (36), 97 (6), 95 (2), 88 (100), 87 (15), 83 (8), 81 (2), 74 (17), 71 (5), 69 (15), 67 (3), 57 (17), 55 (22).

Methyl 2,12-dimethyltetradecanoate: ECL = 15.02, GC−MS (70 eV) *m*/*z* 270 $[M]^+$ (4), 239 (1), 227 (2), 213 (9), 199 (2), 185 (2), 171 (2), 157 (8), 143 (8), 129 (3), 115 (5), 101 (40), 97 (10), 95 (4), 88 (100), 87 (5), 83 (12), 81 (5), 74 (2), 71 (9), 69 (19), 67 (4), 57 (24), 55 (24).

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