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J. Nat. Prod., **1993**, 56 (11), 1898-1904• DOI:
10.1021/np50101a005 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 56, No. 11, pp. 1898-1904, November 1993

ISOPRENOID POLYUNSATURATED FATTY ACIDS FROM FRESHWATER SPONGES

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ABSTRACT.—Fourteen novel isoprenoid polyunsaturated fatty acids of two homologous series [i.e., based on 3,7,11,15-tetramethylhexadecanoic acid (phytanic) and 4,8,12-trimethyltridecanoic acid] were characterized in the fresh-water sponges *Baicalospongia intermedia, Baicalospongia bacilifera,* and *Lubomirskia baikalensis* (from 3 and 11 m) from Lake Baikal, Russia. The acids were isolated and identified by means of tlc, reversed-phase hplc, ms, ^{1}H nmr, and ^{13}C nmr.

Isoprenoid fatty acids are a frequently studied group of natural compounds, especially in the context of elucidation of the origin of fossil fuels (1). Various hypotheses have been proposed concerning the transformation of the two most abundant isoprenoic acids, phytanic and pristanic, into isoprenoid hydrocarbons (2). The precursor of both acids is phytol, a constituent of both aquatic and nonaquatic photosynthesizing organisms. The former acid is contained in sea fishes, mammals, and sponges as well as in the milk of herbivores (3) and in the tissue of patients suffering from Refsum's syndrome (4). Another isoprenoid acid, 4,8,12-trimethyltridecanoic acid, was found to be formed in marine fishes and in sheep by phytanic acid degradation. Marine sponges were found to contain both phytanic and pristanic acid (5,6); *Aplysia fistularis* contains 3% phytanic acid and *Petrosia ficiformis* up to 4% pristanic acid. The conclusion of both studies (5,6) and a later study (7) indicates that some marine sponges contain only pristanic acid, others only phytanic acid. However, most of them were found to contain no isoprenoid acids, probably because of failure to identify all chromatographic peaks or because the concentrations of these acids are too low. We used fractionation and enrichment methods, hplc, nmr, and ms to isolate and identify isoprenoid acids from the freshwater sponges *Baicalospongia intermedia* Lub., *Baicalospongia bacilifera* Lub., and *Lubomirskia baikalensis* Lub. All three sponges are from the class Demospongiaea, order Cornacuspongida, family Lubomirskiidae. To our knowledge, these acids have not been described in natural material.

RESULTS AND DISCUSSION

Our results are summarized in Table 1. In contrast to the above papers (3–7), we succeeded, by using physico-chemical methods, in identifying two homologous series of isoprenoid fatty acids. The basic problem was to acquire sufficiently enriched fractions of the fatty acid methyl esters under study. Classical Ag^{+}-tlc was used to obtain a fraction containing compounds with 1 to 3 double bonds. Further enrichment was achieved by crystallization of fatty acid methyl esters as inclusion compounds with urea. The resulting mixture enriched with methyl esters of isoprenoid polyunsaturated fatty acid was divided into two parts (1:10). The smaller part of the enriched fraction was converted into picolinyl esters and subjected to gc-ms. Individual peaks were identified (Table 1). As seen from the ms of picolinyl esters, both homologous groups consisted of multi-branched isoprenoid polyunsaturated fatty acids. Analysis of the larger part by reversed-phase hplc yielded fourteen peaks whose ^{13}C-nmr, ^{1}H-nmr, ir, and mass spectra were determined.

TABLE 1. Polyisoprenoid Fatty Acid Composition from the Freshwater Sponges *Lubomirskia baikalensis*, *Baicalospongia bacilifera*, and *Baicalospongia intermedia* (% of total fatty acids).

Fatty acid	Peak no.[a]	$[M]^+$ (Me ester)	ECL[b]	*L. baikalensis* 3 m depth	*L. baikalensis* 11 m depth	*B. bacilifera* 2 m depth	*B. intermedia* 2 m depth
18:1	1	338	16.27	0.018	0.024	0[c]	0
20:1	2	370	18.33	0.035	0.041	0	0
20:2	3	368	18.53	0.037	0.014	0.028	0.056
20:3	4	366	19.04	0	0	0.067	0.039
22:1	5	408	19.67	0.006	0.008	0	0
22:1	6	394	20.46	0.060	0.042	0.005	0.003
22:2	7	392	20.62	0.029	0.017	0.021	0.012
22:3	8	390	21.09	0	0	0.230	0.157
24:1	9	436	21.80	0.004	0.003	0.008	0.014
24:2	10	434	22.07	0.015	0.010	0.085	0.071
24:3	11	432	22.49	0	0	0.127	0.094
24:3	12	422	23.16	0	0	0.098	0.107
26:1	13	464	23.93	0.042	0.031	0	0
26:2	14	462	24.12	0.019	0.012	0.008	0.012

[a] 1: 9,13,17-trimethyl-5-octadecenoic acid.
2: 11,15,19-trimethyl-5-eicosenoic acid.
3: 11,15,19-trimethyl-5,9-eicosadienoic acid.
4: 11,15,19-trimethyl-5,9,17-eicosatrienoic acid.
5: 9,13,17,21-tetramethyl-5-docosenoic acid.
6: 13,17,21-trimethyl-5-docosenoic acid.
7: 13,17,21-trimethyl-5,9-docosadienoic acid.
8: 13,17,21-trimethyl-5,9,19-docosatrienoic acid.
9: 11,15,19,23-tetramethyl-5-tetracosenoic acid.
10: 11,15,19,23-tetramethyl-5,9-tetracosadienoic acid.
11: 11,15,19,23-tetramethyl-5,9,17-tetracosatrienoic acid.
12: 15,19,23-trimethyl-5,9,17-tetracosatrienoic acid.
13: 13,17,21,25-tetramethyl-5-hexacosenoic acid.
14: 13,17,21,25-tetramethyl-5,9-hexacosadienoic acid.

[b] ECL = Equivalent chain length.

[c] Detection limit was 0.001% of total fatty acids.

The mass spectra always contained molecular ions and exhibited fragmentation indicating the presence of double bonds (gaps of 26 amu or 40 amu) (8) and of a multibranched chain (gap of 28 amu) (8). The spectrum of the picolinyl ester of the 11,15,19,23-tetramethyl-5,9,17-24:3 acid may serve as an example (see Figure 1). The $[M]^+$ was clearly present, and the 28 amu gap between *m/z* 494 and 466 indicated the presence of the first methyl from the Me end. Another gap of 28 amu (between ions *m/z* 424 and 396) indicated chain branching by another methyl group. The gap between *m/z* 396 and 370 attested to the presence of a double bond in position 17 (this was confirmed by the high abundance of the ion with *m/z* 356, i.e., a gap of 40 amu). A very small ion at *m/z* 342 and the consequent gap of 28 amu pointed to the presence of the third methyl group. A fourth methyl group is bound to the chain in position 11 (a gap of 28 amu between ions at *m/z* 286 and 258). The position of another two double bonds was determined by two 26 amu gaps (ions at *m/z* 258 and 232, or at *m/z* 204 and 178). Two gaps of 40 amu were found for ions with higher abundance (*m/z* 258 and 218, or *m/z* 204 and 164).

The complete structure of this methyl ester of 11,15,19,23-tetramethyl-5,9,17-tetracosatrienoic acid was provided by nmr. The ^{1}H-nmr spectrum featured signals for the five methyl groups. Signals in the interval of 1.3–2.3 ppm belong to individual hydrogen atoms in the vicinity of double bonds, or to methine groups (CH), and the presence of three double bonds was indicated by signals of intensity CH around 5.3 ppm. The spectra of the other thirteen compounds were practically identical. The ^{13}C-nmr spectra (Tables 2 and 3) also exhibited the above structural features (3×C=C and 5×Me). The structures were further substantiated by detailed analysis in the region around 30 ppm, which showed distinct signals for CH groups (32.0, 31.8, 32.9, and 28.1 ppm). A common method for the determination of the configuration of double bonds involves ir spectroscopy. Peaks in the range 960–980 cm^{-1}, characteristic for trans isomers, were not identified.

Hrms was employed to determine the exact mass of the molecular ion, and the $[M-MeO]^+$ and $[M-C_3H_7]^+$ ions, confirming the proposed composition. The composition of the major peak, i.e., the 22:3 acid (8), in the second homologous series was determined analogously. Similarities between the mass spectra of picolinyl esters of the

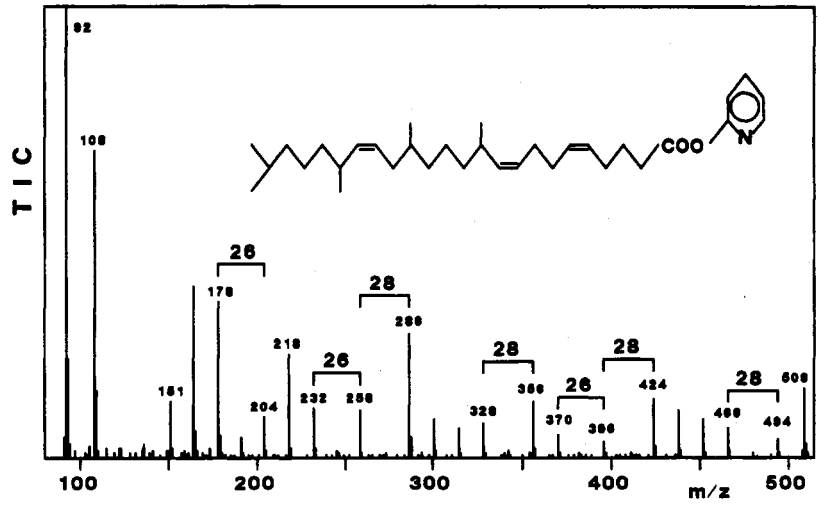

FIGURE 1. Structure and mass spectrum of picolinate of 11,15,19,23-tetramethyl-5,9,17-tetracosatrienoic acid.

TABLE 2. ^{13}C-nmr Data of Tetramethyl Fatty Acid Methyl Esters.

Carbon	Fatty acid methyl ester					
	22:1	24:1	24:2	24:3	26:1	26:2
C-1	174.3	174.2	174.3	174.3	174.4	174.3
C-2	34.3	34.3	34.4	34.3	34.4	34.3
C-3	24.8	24.7	24.8	24.8	24.7	24.8
C-4	27.1[a]	27.0[a]	27.1[a]	27.1[a]	27.1[a]	27.0[a]
C-5	129.9[b]	129.9[b]	129.9[b]	129.9[b]	129.8[b]	129.8[b]
C-6	129.3[b]	129.3[b]	129.3[b]	129.3[b]	129.2[b]	129.2[b]
C-7	27.5[a]	27.5[a]	27.5[a]	27.5[a]	27.5[a]	27.5[a]
C-8	27.4[a]	27.3[a]	27.3[a]	27.3[a]	27.4[a]	27.4[a]
C-9	31.8[c]	29.6[a]	129.1[b]	129.1[b]	29.8[d]	129.1[b]
C-10	29.8[d]	29.7[d]	128.9[b]	128.9[b]	29.7[d]	128.8[b]
C-11	29.8[d]	32.1[c]	31.9[c]	32.0[c]	29.5[d]	29.6[d]
C-12	29.5[d]	29.5[d]	29.6[d]	29.5[d]	29.5[d]	29.6[d]
C-13	32.0[c]	29.5[d]	29.7[d]	29.8[d]	32.1[c]	32.3[c]
C-14	29.5[d]	29.6[d]	29.6[d]	29.5[d]	29.6[d]	29.5[d]
C-15	29.5[d]	32.0[c]	32.0[c]	31.8[c]	29.5[d]	29.7[d]
C-16	29.5[d]	29.6[d]	29.7[d]	29.5[d]	29.5[d]	29.5[d]
C-17	31.7[c]	29.6[d]	29.5[d]	130.1[b]	32.0[c]	32.1[c]
C-18	29.5[d]	29.4[d]	29.5[d]	129.5[b]	29.6[d]	29.7[d]
C-19	29.6[d]	32.4[c]	32.3[c]	32.9[c]	29.5[d]	29.5[d]
C-20	29.5[d]	29.7[d]	29.6[d]	29.5[d]	29.5[d]	29.5[d]
C-21	28.3	29.7[d]	29.7[d]	29.8[d]	29.8[d]	29.8[d]
C-22	23.1	29.5[d]	29.5	29.5[d]	29.5[d]	29.5[d]
C-23	—	28.3	28.2	28.1	29.5[d]	29.5[d]
C-24	—	23.2	23.1	23.1	29.4[d]	29.4[d]
C-25	—	—	—	—	28.2	28.1
C-26	—	—	—	—	23.1	23.1
C-27	19.6[e]	19.6[e]	19.6[e]	19.6[e]	19.6[e]	19.7[e]
C-28	19.7[e]	19.7[e]	19.7[e]	19.8[e]	19.6[e]	19.6[e]
C-29	19.7[e]	19.8[e]	19.6[e]	19.7[e]	19.8[e]	19.6[e]
C-30	23.1	23.2	23.1	23.1	23.2	23.1

[a–e]Assignments designated with the same letter may be interchanged. Last four values are signals of primary carbons (methyls).

two major peaks (8,11) and the spectra of other compounds (minor peaks) were used as a basis for proposing analogous structures shown in Table 1.

The freshwater sponges were found to contain phytanic and pristanic acid in relative proportions of 0.48% and 0.15% of total fatty acids, respectively. As mentioned in the introduction, some marine sponges contain one of the two acids (phytanic or pristanic). This is the first time that both acids were found in the same sponge. However, the situation is further complicated by the presence of 4,8,12-trimethyltridecanoic acid.

Isoprenoid acids may arrive from many sources, such as nutrition or symbiotic organisms, or they can be synthesized and degraded by the organisms's own cells. We assume that both homologous series (Table 1) are biosynthesized from phytanic acid arriving from food, i.e., from photosynthesizing microorganisms. The other homologous series based on the acid 4,8,12-trimethyltridecanoic acid (a biodegradation product of phytanic acid) arises by chain extension and desaturation of this acid. A third homologous series based on pristanic acid as the starting unit was not found, whether because of the low sensitivity of the analytical methods (below one thousandth of a per cent of total fatty acids) or because of the absence of appropriate enzymes participating in the biosynthesis of such a homologous series. We assumed on the basis of literature precedent

TABLE 3. ^{13}C-nmr Data of Trimethyl Fatty Acid Methyl Esters.

Carbon	Fatty acid methyl ester							
	18:1	20:1	20:2	20:3	22:1	22:2	22:3	24:3
C-1	174.4	174.4	174.5	174.5	174.4	174.5	174.5	174.6
C-2	34.1	34.2	34.2	34.1	34.1	34.1	34.1	34.1
C-3	24.8	24.8	24.9	24.9	24.9	24.8	24.9	24.8
C-4	27.2[a]	27.1[a]	27.1[a]	27.2[a]	27.2[a]	27.1[a]	27.2[a]	27.2
C-5	129.8[b]	129.8[b]	129.8[b]	129.9[b]	129.8[b]	129.9[b]	129.8[b]	129.9
C-6	129.4[b]	129.4[b]	129.4[b]	129.4[b]	129.3[b]	129.3[b]	129.4[b]	129.3
C-7	27.5[a]	27.4[a]	27.4[a]	27.4[a]	28.4[a]	27.5[a]	27.4[a]	27.5
C-8	28.3[b]	28.3[b]	27.4[a]	27.3[a]	28.3[a]	27.3[a]	27.3[a]	27.4
C-9	31.9[c]	29.7[a]	129.2[b]	129.1[b]	29.8[d]	129.2[b]	129.2[b]	129.8
C-10	29.8[d]	29.8[d]	129.0[b]	128.9[b]	29.8[d]	128.9[b]	128.9[b]	130.1
C-11	29.7[d]	32.0[c]	32.0[c]	32.1[c]	29.6[d]	29.5[d]	29.7[d]	29.7
C-12	29.8[d]	29.6[d]	29.7[d]	29.6[d]	29.7[d]	29.7[d]	29.6[d]	29.8
C-13	32.1[c]	29.7[d]	29.6[d]	29.7[d]	32.0[c]	32.2[c]	32.3[c]	29.6
C-14	29.5[d]	29.6[d]	29.7[d]	29.7[d]	29.7[d]	29.6[d]	29.6[d]	29.6
C-15	29.5[d]	32.1[c]	32.1[c]	31.9[c]	29.6[d]	29.7[d]	29.6[d]	32.1
C-16	29.5[d]	29.7[d]	29.8[d]	29.5[d]	29.6[d]	29.7[d]	29.7[d]	29.5
C-17	28.3	29.6[d]	29.6[d]	30.0[d]	32.1[c]	32.0[c]	32.0[c]	129.7
C-18	23.2	29.6[d]	29.6[d]	29.5[d]	29.7[d]	29.6[d]	29.6[d]	130.1
C-19	—	28.3	28.4	28.3	29.6[d]	29.6[d]	130.0[b]	29.5
C-20	—	23.1	23.3	23.2	29.7[d]	29.7[d]	129.8[b]	29.8
C-21	—	—	—	—	28.2	28.4	28.3	29.6
C-22	—	—	—	—	23.2	23.4	23.3	30.3
C-23	—	—	—	—	—	—	—	28.4
C-24	—	—	—	—	—	—	—	23.2
C-25	19.6[e]	19.7[e]	19.6[e]	19.6[e]	19.6[e]	19.7[e]	19.7[e]	19.7
C-26	19.7[e]	19.7[e]	19.7[e]	19.8[e]	19.6[e]	19.6[e]	19.6[e]	19.8
C-27	23.2	23.1	23.1	23.1	23.2	23.1	23.1	23.2

[a–e]Assignments designated with the same letter may be interchanged. Last three values are signals of primary carbons (methyls).

(9) that the biosynthesis proceeds analogously as with palmitic acid, by chain elongation by C_2 units and desaturation at positions 5 and 9. The presence of the double bond in positions 17 or 19 can be explained either by the presence of a double bond in the starting unit, as previously observed (10), or by a heretofore unknown mechanism of desaturation occurring in the final (elongated) molecule.

To our best knowledge the isoprenoid polyunsaturated fatty acids identified in lake freshwater sponges have not yet been found in nature, nor have they been synthesized. The biosynthesis of these extraordinary acids with an unusual structure is still a matter of conjecture. Several possibilities can be envisaged concerning the significance of isoprenoid polyunsaturated fatty acid for the sponge. They may represent a specific feature of endemic fungi which have been living separately from their marine relatives for tens of millions of years. Alternatively, by a more exotic hypothesis, these acids may arise by biotransformations of primary metabolites and may serve as agents for changing membrane permeability as a consequence of the freshwater environment, or for detoxification of isoprenoid acids. Whether this is a species-specific feature or a special case of a freshwater endemic trait is not yet clear, although analyses performed so far seem to substantiate the first (endemic) hypothesis.

To be able to answer these questions and to generalize the features described in this paper, we intend to analyze other sponges, including marine sponges, for the presence of these branched acids.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The semipreparative Gradient LC System G-I (Shimadzu, Kyoto, Japan) with two LC-6A pumps (2 ml/min), an SCL-6A system controller, an SPD ultraviolet detector (210 nm), an SIL-1A sample injector, and a C-R3A data processor, with an analytical column 250 mm×4 mm i.d., packed with SGX 18 with 5 μm particles (Tessek, Prague, Czech Republic) were used for reversed-phase hplc. The ^{13}C-nmr (75 MHz) and ^{1}H-nmr (300 MHz) spectra were measured by means of a Bruker AM 300 instrument in $CDCl_3$. For scanning ir spectroscopy of the methyl esters after reversed-phase hplc (in CCl_4), a Perkin-Elmer Model 1310 (Perkin Elmer, Norwalk, CT, USA) infrared spectrophotometer was used. The hrms were determined by the Finnigan MAT 90 mass spectrometer with ei mode. All gc-ms separations were performed using a Finnigan MAT (San Jose, CA) 1020 B apparatus with electron impact. Injection temperature (splitless injection) was 100°, and a fused-silica capillary column (Supelcowax 10) (60 m×0.25 mm i.d., 0.25 μm film thickness) was used. The temperature program was as follows: 100° for 1 min, then increased at 20°/min to 180° and at 2°/min to 280°, which was maintained for 1 min. The carrier gas was H_2 at the linear velocity of 60 cm/sec. All mass spectra were scanned with the range *m/z* 70–550. With the exception of gc-ms, which used picolinyl esters, the other values were measured for fatty acid methyl esters.

SPONGE MATERIAL.—*B. intermedia, B. bacilifera* (at depth of 2 m), and *L. baikalensis* (from depth 3 and 11 m) were collected August 21, 1992, in Lake Baikal, Russia. The sponges were classified by V.M. Dembitsky. Voucher specimens of these sponges are on file at the Institute of Ecology of the Volga River Basin.

EXTRACTION AND ISOLATION OF ACIDS.—The total lipids were extracted by the method of Bligh and Dyer (11). The glycolipids were separated from neutral lipids and phospholipids on silicic acid column as described by Kates (12). The methyl esters were prepared from the glycolipid fractions by base catalyzed transesterification according to Christie (8).

The total methyl esters (from the glycolipid fraction) were chromatographed [hexane-Et_2O (80:20)] on 20×20 cm tlc plates coated with a 0.5 mm layer of Si gel G with 10% $AgNO_3$. After visualization with 2′,7′-dichlorofluorescein (under uv), the band corresponding to components having one to three double bonds was scraped and extracted by Et_2O. The extract was dissolved in 3 ml MeOH, and urea (500 mg) was added and dissolved by warming. The solution was crystallized at −15°, 3 h. After filtration, the filtrate was acidified and extracted by hexane to isolate the methyl-branched fatty acid esters. An aliquot of this extract was identified by gc-ms as the picolinyl esters, prepared as described by Harvey (13). The remainder of this extract (90%) was used for the isolation of pure compounds, as described below.

SEPARATION OF THE INDIVIDUAL COMPONENTS.—Elution was performed with a gradient of MeCN-H_2O (90:10) to pure MeCN during 50 min. The effluent (single peaks) was manually collected. The appropriate esters (13,17,21-trimethyl-5,9,19-22:3 and 11,15,19,23-tetramethyl-5,9,17-24:3) had retention times 20.5 min and 30.4 min, respectively, and were obtained at 92% and 94% purity (checked by gc-ms), respectively.

Structures of all 14 peaks were confirmed on the basis of their ms and ^{13}C nmr (Tables 2 and 3).

Methyl 11,15,19,23-tetramethyl-5,9,17-tetracosatrienoate.—^{1}H nmr δ 0.87 m (3×isoprenoid CH_3), 1.22 d (2×CH_3 on C-23), 1.29–1.32 m (bulk methylenes), 1.36 m (probably H-15), 1.47 m (H-23), 1.57 m (H-3), 2.07 m (H-4, -7, -8, -16), 2.14 m (H-11, -19), 2.23 t (H-2), 3.63 s (OCH_3), 5.28–5.34 m (H-5, -6, -9, -10, -17, -18); hrms *m/z* $[M]^+$ 432.7382 (calcd for $C_{29}H_{52}O_2$, 432.7366), $[M-MeO]^+$ 401.7037 (calcd for $C_{28}H_{49}O$, 401.7021), $[M-C_3H_7]^+$ 389.6488 (calcd for $C_{26}H_{45}O_2$, 389.6474). Gc-ms of picolinate *m/z* (%) $[M]^+$ 509 (14), 494 (3), 480 (1), 466 (6), 452 (8), 438 (10), 424 (12), 396 (3), 370 (5), 356 (12), 342 (1), 328 (7), 314 (6), 300 (8), 286 (27), 258 (9), 232 (10), 218 (22), 204 (8), 191 (4), 178 (31), 164 (34), 151 (10), 108 (78), 92 (100).

Methyl 13,17,21-trimethyl-5,9,19-docosatrienoate.—^{1}H nmr δ 0.87 m (2×isoprenoid CH_3), 1.22 d (2×CH_3 on C-21), 1.29–1.32 m (bulk methylenes), 1.47 m (H-21), 1.57 m (H-3), 2.07 m (H-4, -7, -8, -11, -18), 2.14 m (H-21), 2.23 t (H-2), 3.63 s (OCH_3), 5.28–5.34 m (H-5, -6, -9, -10, -19, -20); hrms *m/z* $[M]^+$ 390.6549 (calcd for $C_{26}H_{46}O_2$, 390.6553), $[M-MeO]^+$ 359.6210 (calcd for $C_{25}H_{43}O$, 359.6206), $[M-C_3H_7]^+$ 347.5676 (calcd for $C_{23}H_{39}O_2$, 347.5661). Gc-ms of picolinate *m/z* (%) $[M]^+$467 (16), 452 (4), 438 (1), 424 (2), 410 (1), 398 (9), 384 (18), 370 (2), 356 (7), 342 (8), 328 (7), 314 (9), 286 (10), 272 (7), 258 (3), 232 (2), 218 (14), 204 (27), 191 (3), 178 (34), 164 (41), 151 (12), 108 (78), 92 (100).

The methyl esters of the other twelve acids have ^{1}H-nmr spectra very similar to those of the two major acids. The structures of the minor esters were deduced from their ^{13}C-nmr spectra (Tables 2 and 3) and ms (Table 1).

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Received 22 February 1993