Multibranched Polyunsaturated and Very-Long-Chain Fatty Acids of Freshwater Israeli Sponges

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Very-long-chain and multibranched polyunsaturated fatty acids of three freshwater sponges, *Ephydatia syriaca*, *Nudospongilla* sp., and *Cortispongilla barroisi*, were studied by silver TLC, GC−MS, UV, IR, HRMS, and NMR methods. One hundred and eighty-five conventional fatty acids were identified by GC−MS, out of which five were new multibranched polyunsaturated fatty acids. The freshwater sponges belonging to the family Spongillidae (class Demospongia) were shown to contain novel di-, tri-, and tetramethyl substituted dienoic, tetraenoic, and hexaenoic fatty acids. The compounds gave positive results in a brine shrimp toxicity assay.

Marine and freshwater sponges are relatively primitive multicellular organisms, which are known to contain in their membrane lipids a variety of distinctive fatty acids in unusual molecular species.[1−4] These include the demospongic acids, i.e., C_{24} to C_{36} fatty acids with 5,9-diunsaturation and frequently another double bond in the terminal part of the molecule. Since most of the published work has dealt with marine species,[3−7] limited information is available on freshwater sponges belonging to the family Spongillidae; however, they are known to contain demospongic acids.[8−13] The biology of freshwater sponges inhabiting Galilean Lakes was studied by Barrois[14] in the 19th century, and the endemic species were subsequently reviewed.[15,16] Our recent study of the lipids of three freshwater sponges from the Jordan River, Lake Hula, and the Sea of Galilee established the presence of 20 main fatty acids;[17] all were "classical" acids, i.e., 16:0, 16:1, 18:0, 18:ln−9, 18:2n−6, and 18:3n−3, that are found commonly in invertebrates.[18,19] Only one demospongic acid, namely, 5,9,17−26:3 (from 1.8 to 3.7%), was found in all sponge species.[14]

In this study, we continue our investigation of the fatty acid composition of three freshwater sponges collected in the Jordan River, Lake Hula, and the Sea of Galilee.[14]

Results and Discussion

In our study, high-resolution GC−MS detected 185 different fatty acids (see Supporting Information). The most frequent are common fatty acids. In addition to these common acids all three sponge species under study contain unique polyunsaturated fatty acids. Both the *iso*- and *anteiso*-type and also pristanic and phytanic acids were found among the saturated fatty acids. Among dienoic acids, the 6,9−14:2 is one of the shortest dienoic fatty acids and 5,9−31:2 is the longest. Trienoic and even tetraenoic fatty acids containing C_{24} to C_{30} carbons were also found. Marine sponges have been shown to contain demospongic acids, i.e., nonmethylene-interrupted fatty acids, mainly 5,9,17−26:3, 5,9,21−28:3, 5,9,22−29:3, and 5,9,23−30:3, and classical very-long-chain acids[1] (30:4n−6, 30:5n−3).

In addition to the above FA usually present in nature we identified five fatty acids (see Table 1) that, to our best

Table 1. Content of Multibranched Polyunsaturated Fatty Acids in Israeli Sponges (in mg/100 g of dry sample)

fatty acid	*E. syriaca*	*N.* sp.	*C. barroisi*
1		5.3	
2	2.8		
3	3.8		
4	3.9		
5			3.4

knowledge, have not been previously described in the literature.

The Ag^{+}-TLC separation was done according to Nikolova-Damyanova.[20] We used a combination of two solvent systems and a two-dimensional development. Small spots corresponding to chains with two, four, and six conjugated double bonds, respectively, were detected between large spots of fatty acids having from 0 to 6 double bonds. The appropriate spots were scraped off, and the compounds were further (after additional purification by RPHPLC) identified by physicochemical methods, i.e., UV, IR, ^{1}H NMR, ^{13}C NMR, and HRMS.

The molecular formula of **1** was determined to be $C_{21}H_{34}O_2$ on the basis of high-resolution EIMS data. The IR spectrum showed absorption bands at 1722 cm^{-1}, indicating the presence of an ester group. A conjugated diene system was suggested by the absorption bands at 1655 cm^{-1} in the IR spectrum and at 229 nm in the UV spectrum.

The ^{1}H and ^{13}C NMR spectra of **1** in CDCl$_3$ are summarized in Tables 2 and 3. The ^{13}C NMR spectrum of **1** showed 21 carbon signals, which were assigned to six methyl groups (including one methoxyl), six methylenes, seven methines, and three quaternary carbons by DEPT experiments. Two proton sequences, i.e., from H-4 to H-6 and H-18 and from H-8 to H-16, were established by the [1]H−^{1}H DQFCOSY and HOHAHA analysis of spectral data of **1**. The [1]H−[13]C long-range couplings were observed from H-2 (δ 5.63) to C-1 (δ 170.2), C-3 (δ 150.2), C-4 (δ 48.7), and C-17 (δ 19.0) and from H-17 (δ 2.12) to C-2 (δ 118.4), C-3, and C-4 in the HMBC spectrum. These data revealed the presence of a butenoic acid structure connected to C-4. The [1]H−[13]C long-range couplings observed between the terminal methylene protons H-19 (δ 4.71 and 4.86) and C-6−C-8 and from H-6 (δ 1.75 and 2.08) to C-7, C-19 (δ 111.6), and C-8 showed the presence of a vinylidene moiety bound to C-6 and C-8.

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Table 2. ^{1}H NMR of Compounds **1**−**5**

	1	2	3
OCH$_3$	3.67 (3H, s)	3.65 (3H, s)	3.64 (3H, s)
2	5.63 (1H, s)	5.86 (1H, d, J = 15.1)	5.91 (1H, d, J = 10.4)
3		6.89 (1H, dd, J = 15.1, 10.3)	6.92 (1H, dd, J = 10.4, 10.6)
4	1.91 (1H, m); 2.15 (1H, m)	6.22 (1H, dd, J = 15.0, 10.3)	6.31 (1H, dd, J = 15.4, 10.6)
5	1.93 (1H, m)	5.94 (1H, dd, J = 15.0, 7.0)	6.57 (1H, d, J = 15.4)
6	1.75 (1H, dd, J = 13.5, 8.6); 2.08 (1H, m)	2.44 (1H, m)	
7	-	2.28 (2H, m)	6.02 (1H, s)
8	2.20 (2H, m)	5.42 (1H, d, J = 10.8)	
9	2.00 (2H, m)		5.60 (1H, d, J = 10.2)
10	5.82 (1H, dt, J = 14.1, 7.2)	6.18 (1H, d, J = 15.2)	2.31 (1H, m)
11	6.27 (1H, dd, J = 14.1, 10.3)	5.46 (1H, dd, J = 15.2, 7.6)	2.17 (2H, m)
12	6.23 (1H, dd, J = 14.1, 10.3)	2.14 (1H, m)	5.54 (1H, dd, J = 9.7, 12.1)
13	5.87 (1H, dt, J = 14.1, 7.2)	1.31 (2H, m)	
14	1.90 (1H, m); 2.10 (1H, m)	0.96 (3H, t, J = 7.3)	6.28 (1H, dd, J = 10.4, 16.1)
15	2.06 (1H, m)	1.08 (3H, d, J = 7.6)	5.31 (1H, d, J = 10.4); 5.40 (1H, d, J = 16.1)
16	0.95 (3H, d, J = 6.7)	1.71 (3H, s)	2.06 (3H, s)
17	2.12 (3H, s)	1.14 (3H, d, J = 7.8)	2.09 (3H, s)
18	1.01 (3H, d, J = 5.5)		1.00 (3H, d, J = 7.8)
19	4.71 (1H, s); 4.86 (1H, s)		1.72 (3H, s)
20	0.95 (3H, d, J = 6.7)		

	4	5
OCH$_3$	3.69 (3H, s)	3.66 (3H, s)
2	5.79 (1H, d, J = 15.2)	5.92 (1H, d, J = 15.3)
3	6.72 (1H, d, J = 15.2)	6.87 (1H, dd, J = 15.3, 12.3)
4		6.25 (1H, dd, J = 16.1, 12.3)
5	5.67 (1H, d, J = 9.6)	6.07 (1H, dt, J = 16.1, 8.6)
6	2.15 (2H, m)	2.28 (2H, m)
7		2.42 (1H, m)
8		5.41 (1H, d, J = 9.5)
9		
10		6.11 (1H, d, J = 15.8)
11	1.2−1.4 (18H, m)	5.67 (1H, dt, J = 15.8, 8.1)
12		2.09 (2H, m)
13		1.32 (2H, m)
14		2.02 (1H, m)
15		1.37 (2H, m)
16	0.98 (3H, t, J = 7.4)	0.98 (3H, t, J = 7.5)
17	1.65 (3H, s)	1.15 (3H, d, J = 6.9)
18	1.08 (3H, d, J = 6.8)	1.65 (3H, s)
19		1.08 (3H, d, J = 7.6)
20		

Table 3. ^{13}C NMR of Compounds **1**−**5**

	1	2	3	4	5
1	170.2	169.1	168.7	167.7	168.4
2	118.4	124.1	124.3	122.4	117.4
3	150.2	146.2	145.8	144.9	149.8
4	48.7	126.8	133.4	128.7	133.1
5	30.8	142.0	126.4	132.0	134.6
6	44.1	34.2	138.5	38.4	33.0
7	148.1	33.9	126.8	29.8	36.8
8	36.5	138.8	140.4	132.8	31.8
9	32.2	135.2	131.8	138.1	30.7
10	130.7	123.1	30.2	132.3	
11	131.2	139.1	37.9	137.4	29.1−29.5
12	133.9	36.8	127.5	32.2	
13	134.2	29.8	142.8	37.0	30.0
14	42.8	12.8	136.9	34.8	32.1
15	28.0	19.3	120.0	26.1	21.9
16	18.1	16.8	23.6	12.4	14.2
17	19.0	20.0	13.5	19.2	17.5
18	19.5		19.2	17.8	18.6
19	111.6		16.7	19.5	
20	18.1				
OCH$_3$	51.2	51.3	51.3	51.4	51.3

The geometry of the trisubstituted double bond (C-2) was established as *E* owing to the presence of an NOE between H-2 and H$_a$-4 (δ 2.15) observed in the NOESY spectrum and the chemical shift of methyl carbon C-17 (δ 19.0) of **1**.[21,22] The geometries of the two disubstituted double bonds, C-10 and C-12, were determined to be 10*E*

and 12*E* by the coupling constants $J_{10,11}$ = 14.1 Hz and $J_{12,13}$ = 14.1 Hz. A deshielded chemical shift of C-9 (δ 32.2) was also measured. The resulting structure of **1** is shown in Figure 1.

Acid **2** showed a strong absorption at 231 nm (log ε 4.55). The IR spectrum of **2** suggested the presence of an ester (1720 cm^{-1}) and conjugated dienes (1655 cm^{-1}). The molecular formula of **2** was established as C$_{18}$H$_{28}$O$_2$.

Analysis of the ^{1}H NMR, ^{13}C NMR, DEPT, and HMQC spectra revealed the presence of two quaternary carbons (one carboxyl carbon and one olefinic carbon) and 16 proton-bearing carbons (seven olefinic methine, two methine, two methylene, and five methyl carbons). A combination of [1]H−^{1}H DQFCOSY and ^{1}H-HMBC experiments revealed the partial structure of the unsaturated branch of **2**. In the [1]H−^{1}H DQFCOSY spectrum, the protons at 5.86 and 6.22 ppm (denoted H-2 and H-4, respectively) revealed couplings to the proton at 6.89 (H-3). In the HMBC spectrum the proton at 5.86 ppm (H-2) showed cross-peaks with C-1. The [1]H−^{1}H DQFCOSY spectrum also revealed the couplings of protons at 6.18 (H-10) and 5.42 ppm (H-8) with the proton at 5.46 (H-11). HMBC revealed the coupling of H-12 with C-13, C-14, and C-17, and both H-8 and H-10 showed couplings with C-9. The geometry of double bonds Δ^{2,3}, Δ^{4,5}, and Δ^{10,11} was confirmed to be all *E* by the coupling constants of $J_{2,3}$ = 15.1, $J_{4,5}$ = 15.0, and $J_{10,11}$ = 15.2 Hz. Also the chemical shift (δ 16.8)
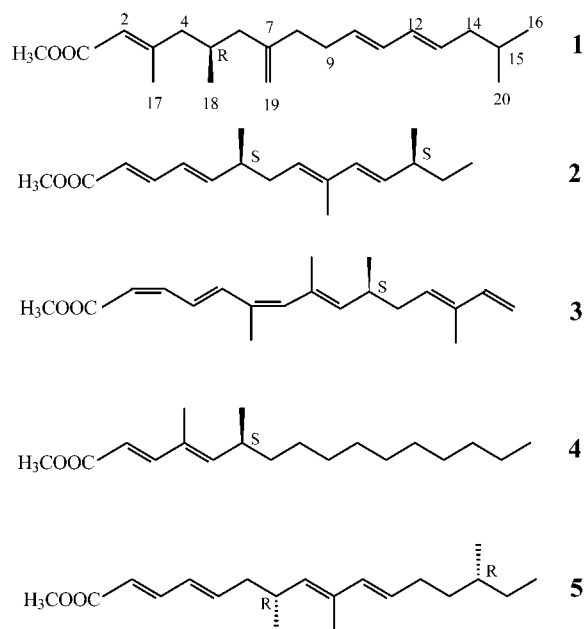
Figure 1. Structures of very-long-chain and multibranched polyunsaturated fatty acids (**1**–**5**) from three Israeli freshwater sponges: *Ephydatia syriaca*, *Nudospongilla* sp., and *Cortispongilla barroisi*.

of CH_3-16 suggested $8E$ geometry. Methyl signals at 1.08 (3H, d), 1.71 (3H, s), and 1.14 (3H, d) were typical for the ^1H NMR spectrum of E-oriented carotenoids.[23] The resulting structure of **2** is shown in Figure 1. The absolute configuration of **2** was determined as reported below.

On the basis of HREIMS and NMR data, compound **3** was shown to have the molecular formula of $C_{20}H_{28}O_2$, indicative of seven degrees of unsaturation. A resonance at 174.1 ppm in the ^{13}C NMR spectrum of **3** indicated the presence of one carbonyl carbon, probably an α,β-unsaturated ester on the basis of IR absorption data (1716 and 1624 cm^{-1}). The UV absorption spectrum in methanol showed a maximum at 301 nm.

The ^1H and ^{13}C NMR data (Tables 2 and 3) and HMQC spectra showed five methyls (including one methyl ester), two methylenes, nine methines, and four quaternary carbons. HMBC experiments were carried out to ascertain the connectivity of the partial structures. We assigned the observed correlation signals in the HMBC spectrum to olefinic protons H-2–H-5 and H-9–H-12, respectively. The correlations revealed connectivity from C-1 to C-6 and C-8–C-13, including four methyls (C-16–C-19). The correlations between C-13 and H-12, H-14, and H-19 and between C-12 and H-14 and H-15 indicated the connectivity from C-12 to C-15. Further connectivities were supported by COSY results (i.e., C-11–C-12 and C-14–C-15). The substitution patterns at C-6 and C-8 were deduced from HMBC correlation signals.

The geometries of the two disubstituted double bonds, C-2 and C-4, were determined to be $2Z$, $4E$ by the coupling constants $J_{2,3} = 10.4$ Hz and $J_{4,5} = 15.4$ Hz. The geometries of three trisubstituted double bonds (C-6, C-8, and C-12) were assigned as Z, E, and E because of the presence of an NOE between H-5 and H-7 observed in the NOESY spectrum and the chemical shift of methyl carbon C-16 (δ 23.6),[21] and according to the NOE and the chemical shift of C-17 at δ 13.5.[24]

From the above results, acid **3** was determined to be a novel hexaene type compound (Figure 1). The determination of the absolute configurations at the C-10 chiral carbon is described below.

methyl ester	RT of products after degradation (min^{-1})					
	standards	1	2	3	4	5
2R-Me-butyric	3.02					
2S-Me-butyric	3.15		3.16			
2R-Me-succinic	14.22	14.19				14.21
2S-Me-succinic	14.71		14.69	14.70		
4R-Me-hexanoic	14.93					14.91
4S-Me-hexanoic	15.42					
2S-Me-dodecanoic	63.21				63.18	

[a] The formic, acetic, and oxalic acids (after methylation) were present in all five reaction mixtures after oxidation. In the case of oxidation of **1** were presented also isovaleric, malonic, methylmalonic, and succinic acids (all determined as methyl esters).

The UV and IR spectra of **4** were very similar to **2**. The molecular weight was determined by HREIMS as $C_{19}H_{34}O_2$. A NOESY experiment established the stereochemistry of the olefinic bonds of the 4,6-dimethylhexadeca-2,4-dienoyl chain as $2E$, $4E$, consistent with the coupling constant of 15.2 Hz between H-2 and H-3 and the value of C-17 (δ 19.2). The ^1H–^{13}C correlations observed by HMBC and NOE interactions observed in the NOESY defined the structure of ($2E$)-4,6-dimethyl-2,4-hexadecadienoic acid methyl ester.

The UV and IR spectra of **5** were also practically identical with those of **2**. Its molecular weight was determined by HREIMS as $C_{20}H_{32}O_2$. The structure of the fatty acid chain was elucidated by analyzing the ^1H–^1H COSY, 2D-HOHAHA, and HMQC spectra of **5**. The geometry of C-2, C-4, and C-10 was confirmed to be all E by coupling constants analysis. The trisubstituted double bonds (C-8) were assigned as E on the basis of the NOESY data and the ^{13}C NMR value of C-18 (δ 18.6).

Oxidative splitting and chromatography of appropriate methyl esters on a chiral capillary column determined the absolute configuration of all five compounds. Baseline separations were obtained on the chiral capillary column (the conditions are described in the Experimental Section) with an appropriate stationary phase. First, the standards of all stereoisomers were chromatographed as methyl esters. Second, the oxidation products were chromatographed also as methyl esters, and the retention times of the corresponding peaks were compared (Table 4); see also Stritzel.[25] As shown in Table 4, many nonchiral and chiral compounds were isolated from the reaction mixture, i.e., (2S)-2-methylsuccinic acid dimethyl ester (from **3**), (2S)-2-methylsuccinic acid dimethyl ester and (2S)-2-methylbutyric acid methyl ester (from **2**), and (2R)-2-methylsuccinic acid dimethyl ester and (4R)-4-methylhexanoic acid methyl ester (from **5**). This last acid was prepared as a standard from (S)-(−)-citronellal according to Djerassi.[26] The degradation of **1** is very complicated; after oxidation by ozonolysis, further oxidation of the intermediate(s) gave a mixture of (2R)-2-methylsuccinic acid dimethyl ester with many further nonchiral compounds (see Table 4). Acid **4** yielded only one optically active compound, viz., the methyl ester of (2S)-2-methyldodecanoic acid.

The toxicity of compounds **1**–**5** was tested in the *Artemia salina* shrimp bioassay. The methyl esters (**1**–**5**) showed bioactivity (see Table 5). Table 5 also indicates that the acids were active only against Gram-positive bacteria and they were inactive against Gram-negative bacteria and yeast.

Very-long-chain acids are quite rare in nature, occurring in only a few animals, usually in specialized tissues,[3,27–29]

Table 5. Bioactivities of Fatty Acid Derivatives (**1**−**5**)

test organism	**1**	**2**	**3**	**4**	**5**
Staphylococcus aureus[a]	84.1	49.0	62.4	56.7	148.4
Bacillus subtilis[a]	74.9	117.6	89.0	81.0	78.1
Escherichia coli[a]	0	0	0	0	0
Saccharomyces cerevisiae[a]	0	0	0	0	0
Artemia salina[b,c]	1.7	10.2	1.1	4.9	5.0

[a] Samples (10 μg) were applied on 50.8 mm paper disks; values are diameters (mm) of inhibitory zones. [b] In μg/mL (minimum lethal doses). [c] The details are in the Experimental Section.

and also in marine dinoflagellates.[30] Both branched and polyunsaturated fatty acids are widespread in nature, but few sources, including sponges,[3] contain branched polyunsaturated fatty acids. These were found in different marine species[31,32] (e.g., 24-methyl-5,9-pentacosadienoic acid or (6*Z*)-2-methoxy-13-methyl-6-tetradecenoic acid); polyunsaturated isoprenoid fatty acids were found in a freshwater sponge.[13] An interesting correlation was discovered between the geographical occurrence and the chirality of compounds **1**−**5**. While the river sponge *E. syriaca* synthesized only *S* isomers (acids **1**, **3**, and **4**), the two sponges living in lakes synthesized only *R* isomers (i.e., acid **2** and **5**). It is remarkable that acid **5** probably has the "unnatural" 2*R*-2-methylbutyric acid as a starter unit, which is presumably synthesized from D-isoleucine or L-allo-isoleucine.

This study has demonstrated that Galilean freshwater sponges contain a broader spectrum of total fatty acids than both marine and freshwater sponges from other localities.

Experimental Section

General Experimental Procedure. UV spectra were measured by a Cary 118 (Varian) apparatus in EtOH in the range 200−350 nm. A Perkin-Elmer Model 1310 (Perkin-Elmer, Norwalk, CT) infrared spectrophotometer was used for scanning infrared spectroscopy of methyl esters as neat film. NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^{1}H) and 125.7 MHz (^{13}C). Both low-resolution mass spectra were recorded using a VG 7070E-HF spectrometer (Micromass, Manchester, UK). The ionizing condition was 70 eV.

Collection, Extraction, and Isolation. Animal Material. The freshwater sponge *Ephydatia syriaca* Topsent (ES07/060893) was collected in August 1993, in the spring of the Jordan river (Banias, near the Hermon Mountain), the freshwater sponge *Nudospongilla* sp. Topsent (N11/070594) was collected in May 1994 in Lake Hula, and the freshwater sponge *Cortispongilla barroisi* Topsent (CB02/230794) was collected in July 1994 in the Sea of Galilee (Lake Tiberias, Lake Kinneret, Israel). The voucher specimens are deposited in the collection under the curatorship of the second author (V.M.D.). All sponges (family Spongillidae) were washed in water, carefully cleaned of all debris, and cut into small pieces. Lipid extraction was done immediately after collection as described elsewhere.[13,14]

Briefly, the samples were separately extracted with a mixture of CH_2Cl_2−MeOH (1:1, v/v). The extracts were concentrated under N_2. The total lipid extracts, viscous dark oils, were subjected separately to Sephadex LH-20 column chromatography with CH_2Cl_2−MeOH (1:5). The appropriate fraction of free acids was treated with a solution of CH_2N_2 in diethyl ether. The crude methyl esters were further purified by RPHPLC (Supelco, RP-18, 95% MeOH−H_2O).

Ag$^+$-TLC Analysis.[20] Glass plates (20 × 20 cm) were coated with silica gel G (~0.8 mm), dried overnight at room temperature, impregnated by dipping into methanolic silver nitrate solution (0.7%), dried in air for 90 min, and activated at 110 °C for 10 min prior to use. A hexane solution of the FAME of the samples was applied in spots. The plates were developed by the mobile phase (CHCl$_3$−MeOH, 100:1 v/v, in the first and

hexane−acetone, 100:3 v/v, in the second dimension). The plates (after a two-dimensional development) were placed in an oven at 50 °C for 90 min to evaporate the solvents. The spots were sprayed with fluorescein and detected under UV light. The appropriate spots were scraped off, and compounds **1**−**5** were extracted from the silica gel by diethyl ether and used for further purification by semipreparative RPHPLC. The compounds with 2, 4, or 6 conjugated double bonds from the scraped-off spots after Ag$^+$-TLC were further purified by RPHPLC on a C18-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 mL/min) with ACN−H_2O (2:3) to yield compounds **1**−**5**.

GC−MS Analysis. Gas chromatography−mass spectrometry of a fatty acid picolinyl ester mixture (prepared according to Christie[33]) was done on a Finnigan 1020 B single-state quadruple GC−MS instrument in the electron ionization mode. Injection temperature (splitless injection) was 100 °C, and a fused-silica capillary column (Supelcowax 10; 60 m × 0.25 mm i.d., 0.25 μm film thickness; Supelco, PA) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm s^{-1}. All spectra were scanned within the range m/z 70−650.

Standards. The compounds formic, acetic, (2*S*)-2-methylbutyric, isovaleric, pyruvic, oxalic, malonic, methylmalonic, succinic, (2*S*)-2-methylsuccinic, and (2*R*)-2-methylsuccinic acids and (*S*)-(+)-citronellal, (*R*)-(−)-citronellal, L-allo-isoleucine, and L-amino acid oxidase *(Crotalus adamanteus* venom, EC 1.4.3.2) were purchased from Sigma-Aldrich (Prague, Czech Republic). (2*S*)-2-Methyldodecanoic acids were purchased from Interchim (Montlucon, France). (4*S*)-4-Methylhexanoic and (4*R*)-4-methylhexanoic acids were prepared from (*R*)-citronellal or from (*S*)-citronellal.[26] (2*R*)-2-Methylbutyric acid was prepared by oxidase L-amino acid (EC 1.4.3.2)-catalyzed oxidation of (2*S*,3*R*)-2-amino-3-methylpentanoic acid (L-allo-isoleucine) by a previously described method.[34]

Oxidative Splitting[35,36] **and Preparation of Standards.**[26] A stream of 4% ozone was passed through a solution of the given compound (~1 mg) in dichloromethane (2 mL) at −78 °C for 5 min. The solution was flushed with nitrogen and concentrated. The residue was dissolved in 90% HCOOH (0.7 mL), and 30% hydrogen peroxide (0.3 mL) was added. After gentle heating the mixture was heated under reflux for 70 min. The mixture was concentrated, and the residue was dissolved in methanol (0.5 mL) and treated with etheral diazomethane.

The mixture of oxidation products after ozonolysis of **2** was added gradually to a mixture containing 1.5 parts of nitric acid, 1 part of water, and 0.0125 part of ammonium metavanadate. The mixture was then heated to 110−114 °C for 60 min and treated with etheral diazomethane solution.

A mixture of 50 mg of (*R*)-citronellal, 50 μL of 85% hydrazine hydrate, 40 mg of KOH, and 5 mL of diethylene glycol was heated under reflux with stirring for 4 h at 175 °C. Ten milliliters of H_2O and 5 mL CH_2Cl_2 were added (after cooling) to the mixture, and the lower layer was separated to give a solution of olefin ((6*S*)-2,6-dimethyl-2-octene). The ozonolysis of olefin was performed exactly as described above. Methyl ester was obtained in nearly quantitative yield by methylation of the acid with diazomethane in diethyl ether solution. The yield of the methyl ester of (4*S*)-4-methylhexanoic acid, which was used directly for chiral chromatography, was 13.5 mg. The methyl ester of (4*R*)-4-methylhexanoic acid was prepared from *S*-citronellal in the same manner as above.

A solution of L-allo-isoleucine (10 mg, 0.076 mmol) in Tris-hydrochloride buffer (0.1 M, pH 7.2, 3 mL) was filtered. L-Amino acid oxidase *(Crotalus adamanteus* venom, EC 1.4.3.2, 0.33 units/mg, 20 mg) and sodium azide (0.3 mg) were added to the mixture, and it was vigorously stirred for 1 day at 30 °C. A stream of oxygen was continuously bubbled through the solution (7 mL/min). L-Amino acid oxidase (20 mg) and sterilized H_2O (1 mL) were added to the mixture, and it was further stirred for 1 day at 30 °C with oxygen bubbling. The consumption of the starting material was detected with ninhydrin reagent. The oxidase was removed from the

mixture by ultrafiltration, and the filtrate was concentrated in a vacuum. The crude mixture was cooled at 0 °C and acidified to pH 1 with concentrated hydrochloric acid. The ethereal extract was concentrated under atmospheric pressure, and an etheral solution of diazomethane was added. The resulting methyl ester of (2*R*)-2-methylbutyric acid was injected into the chiral column.

Chiral Chromatography. FS capillary column HYDRO-DEX β-3P (i.d. 0.25 mm, length 25 m) with the stationary phase [heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrine] from Macherey-Nagel GmbH & Co. KG, Düren, Germany, was used. Oven temperature: 50 to 150 °C at 2 °C/min, then to 240 °C at 5 °C/min, carrier gas helium, 20 cm/s, detector FID, 300 °C, injection of 1 μL mixture in methylene chloride (for standards: containing 0.5 mg/mL of each analyte), split (100: 1), 300 °C.

Methyl (2*E*,5*R*,10*E*,12*E*)-3,5,15-trimethyl-7-methylene-hexadeca-2,10,12-trienoate (1): colorless oil; [α]$_D$ +7.48 (*c* 0.11, MeOH); UV λ$_{max}$ (EtOH) (log ε) 229 (3.36) (nm); IR (KBr) ν$_{max}$ 1722, 1655 cm^{-1}; HREIMS (*m/z*) 318.22563 (calcd for C$_{21}$H$_{34}$O$_2$ 318.2559); NMR data, see Tables 2 and 3.

Methyl (2*E*,4*E*,6*S*,8*E*,10*E*,12*S*)-6,9,12-trimethyltetradeca-2,4,8,10-tetraenoate (2): slightly yellow oil, [α]$_D$ +8.36 (*c* 0.13, MeOH); UV λ$_{max}$ (EtOH) (log ε) 231 (4.55) (nm); IR (KBr) ν$_{max}$ 1720, 1655 cm^{-1}; HREIMS (*m/z*) 276.2094 (calcd for C$_{18}$H$_{28}$O$_2$ 276.2089); NMR data, see Tables 2 and 3.

Methyl (2*E*,4*E*,6*Z*,8*E*,10*S*,12*E*)-6,8,10,13-tetramethyl-pentadeca-2,4,6,8,12,14-hexaenoate (3): pale brown oil; [α]$_D$ +12.3 (*c* 0.14, MeOH); UV λ$_{max}$ (EtOH) (log ε) 301 (2.91) (nm); IR (KBr) ν$_{max}$ 1716, 1624 cm^{-1}; HREIMS (*m/z*) 300.2091 (calcd for C$_{20}$H$_{28}$O$_2$ 300.2089); NMR data, see Tables 2 and 3.

Methyl (2*E*,4*E*,6*S*)-4,6-dimethylhexadeca-2,4-dienoate (4): pale yellow oil, [α]$_D$ +9.24 (*c* 0.15, MeOH); UV λ$_{max}$ (EtOH) (log ε) 230 (3.27) (nm); IR (KBr) ν$_{max}$ 1724, 1660 cm^{-1}; HREIMS (*m/z*) 294.2562 (calcd for C$_{19}$H$_{34}$O$_2$ 294.2559); NMR data, see Tables 2 and 3.

Methyl (2*E*,4*E*,7*R*,8*E*,10*E*,14*R*)-7,9,14-trimethylhexadeca-2,4,8,10-tetraenoic acid (5): colorless oil, [α]$_D$ +10.18 (*c* 0.17, MeOH); UV λ$_{max}$ (EtOH) (log ε) 228 (3.75) (nm); IR (KBr) ν$_{max}$ 1724, 1658 cm^{-1}; HREIMS (*m/z*) 276.2406 (calcd for C$_{20}$H$_{32}$O$_2$ 304.2402); NMR data, see Tables 2 and 3.

Brine Shrimp Toxicity Bioassay. The sample (~0.05 mg) was dissolved in 50 μL of DMSO and added to a vial of artificial seawater (3.0 mL). Approximately 20 brine shrimp, *Artemia salina*, were added to the vial. The brine shrimp were observed periodically over a 24 h period. A positive assay was the death of all brine shrimp.

Antibacterial Tests. The test organisms were *Bacillus subtilis* (CCM 2216) and *Staphyloccocus aureus* (CCM 2551) (CCM−Czechoslovak Collection of Microorganisms, Brno). Antibacterial assays were carried out according to the literature.[37] The amounts of the compounds were 50 μg per test disk (see Table 5).

Supporting Information Available: Five Tables summarizing the composition of fatty acids (185 different fatty acids identified by GC−MS as methyl esters from saturated to polyenoic) detected in Israeli sponges. This material is available free of charge via the Internet at http://pubs.acs.org.

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