Identification of the Novel Antimicrobial Fatty Acid (5*Z*,9*Z*)-14-Methyl-5,9-pentadecadienoic Acid in *Eunicea succinea*

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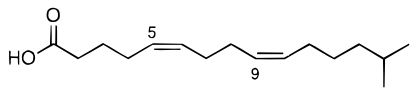
The phospholipid fatty acid composition of *Eunicea succinea* was investigated, and the novel (5*Z*,9*Z*)-14-methyl-5,9-pentadecadienoic acid was identified. Structural characterization was accomplished by means of mass spectrometry of its pyrrolidide derivative, NMR, FTIR, and total synthesis. Other interesting phospholipid fatty acids in *E. succinea* were the tetracosapolyenoic acids 6,9,12,15,18,21-tetracosahexaenoic acid (24:6) and 6,9,12,15,18-tetracosapentaenoic acid (24:5). The title compound was particularly active against Gram-positive bacteria such as *Staphylococcus aureus* (MIC 0.24 μmol/mL) and *Streptococcus faecalis* (MIC 0.16 μmol/mL).

Marine invertebrates are the source of unusual $\Delta^{5,9}$ fatty acids with no counterpart in the terrestrial world.[1] Some of these unusual fatty acids are known to arise from a symbiotic relationship of associated bacteria with the host cells of the marine invertebrate, as was demonstrated for the very long-chain $\Delta^{5,9}$ fatty acids 25-methyl-5,9-hexacosadienoic acid and 24-methyl-5,9-hexacosadienoic acid, constituents of the phospholipids of the Australian sponge *Jaspis stellifera*.[2] In other sponges bacteria have been implicated as the only source of these $\Delta^{5,9}$ fatty acids. For example, the shortest $\Delta^{5,9}$ fatty acid known to date, namely 5,9-hexadecadienoic acid, was first isolated from the sponge *Chondrilla nucula*, and it was postulated to have arisen only from a bacterial symbiont.[3] Just recently, it was demonstrated that sponges are not the only source of these unusual $\Delta^{5,9}$ fatty acids, but other marine organisms, such as zoanthids and anemone, can metabolize, although in trace amounts, intermediate chain-length $\Delta^{5,9}$ fatty acids such as 5,9-heneicosadienoic acid.[4] In this paper, we wish to expand the origin of these $\Delta^{5,9}$ acids to the Gorgoniidae by reporting the identification and antimicrobial activity of the novel (5*Z*,9*Z*)-14-methyl-5,9-pentadecadienoic acid (**1**) from the Caribbean gorgonian *Eunicea succinea* Pallas (phylum Coelenterata, class Anthozoa, subclass Octocorallia, order Gorgonacea). It is interesting to mention that both the Δ^5 and Δ^9 monounsaturated analogues of **1**, namely 14-methyl-5(*Z*)-pentadecenoic acid and 14-methyl-9(*Z*)-pentadecenoic acid, were previously identified in two sedimentary marine bacteria from a mangrove stand on the Low Isles, North Queensland, Australia.[5] Therefore, the novel acid **1** could have arisen from associated bacteria within *E. succinea*. The fatty acid composition of the Gorgoniidae is also of interest because these invertebrates, in contrast to other marine organisms, can extend their polyunsaturated fatty acid biosynthetic sequence to the longer-chain methylene-interrupted tetracosapolyenoic fatty acids, particularly of the *n*-3 and *n*-6 series.[6]



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The main phospholipids in *E. succinea* were identified by TLC as phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine as judged by TLC comparison with authentic standards. Preparation of the fatty acid methyl esters from these phospholipids afforded the fatty acid methyl esters presented in Table 1. Polyunsaturated fatty acids of the *n*-3 and *n*-6 series predominated in the mixture and accounted for almost 50% of the total fatty acids from the gorgonian. Noteworthy in this series were the tetracosapolyenoic acids 6,9,12,15,18,21-tetracosahexaenoic acid (24:6) and 6,9,12,15,18-tetracosapentaenoic acid (24:5), characteristic chain-lengths of the Gorgoniidae.[6] The most abundant saturated fatty acid was palmitic (ca. 19%). Essential for the characterization were GC retention times of the fatty acid methyl esters, MS data on the methyl esters and their corresponding pyrrolidide derivatives, and catalytic hydrogenation of the whole fatty acid methyl ester mixture for easier elucidation of methyl branching.

Our primary interest was centered on the new compound 14-methyl-5,9-pentadecadienoic acid (**1**), which was initially characterized as the methyl ester. The GC retention time of the methyl ester of **1** displayed an equivalent chain-length value of 15.21, implying diunsaturation and methyl branching. The mass spectrum of the methyl ester of **1** displayed a molecular ion peak at *m*/*z* 266 and a base peak at *m*/*z* 81. This information revealed a methyl branched $\Delta^{5,9}$ hexadecadienoic acid methyl ester inasmuch as the base peak at *m*/*z* 81 is characteristic of the $\Delta^{5,9}$ diunsaturation.[4] The double-bond positions were confirmed by pyrrolidide derivatization.[7] The mass spectrum of *N*-14-methyl-5,9-pentadecadienoylpyrrolidine displayed a strong peak at *m*/*z*

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Table 1. Total Phospholipid Fatty Acids and Aldehydes from *E. succinea*[a]

compd	abundance (wt %)
fatty acids	
dodecanoic (12:0)	0.2
9-tetradecenoic (14:1)	0.4
tetradecanoic (14:0)	2.0
13-methyltetradecanoic (*i*-15:0)	0.1
14-methyl-5,9-pentadecadienoic (16:2 *n*-6)[b]	0.3
4,7,10-hexadecatrienoic (16:3 *n*-6)	0.2
9-hexadecenoic (16:1)	0.2
hexadecanoic (16:0)	18.8
7-methyl-6Z-hexadecenoic (17:1)	0.4
7-methyl-6E-hexadecenoic (17:1)	0.7
heptadecanoic (17:0)	0.1
6,9,12-octadecatrienoic (18:3 *n*-6)	9.3
6,9,12,15-octadecatetraenoic (18:4 *n*-3)	14.3
9,12-octadecadienoic (18:2 *n*-6)	0.6
9-octadecenoic (18:1)	1.3
octadecanoic (18:0)	1.1
5,8,11,14-eicosatetraenoic (20:4 *n*-6)	10.6
5,8,11,14,17-eicosapentaenoic (20:5 *n*-3)	0.6
8,11,14-eicosatrienoic (20:3 *n*-6)	0.2
eicosanoic (20:0)	0.2
4,7,10,13,16,19-docosahexaenoic (22:6 *n*-3)	5.3
7,10,13,16-docosatetraenoic (22:4 *n*-6)	1.3
2-hydroxyeicosanoic (20h:0)	1.0
docosanoic (22:0)	0.2
6,9,12,15,18-tetracosapentaenoic (24:5 *n*-6)	3.0
6,9,12,15,18,21-tetracosahexaenoic (24:6 *n*-3)	1.8
2-hydroxydocosanoic (22h:0)	2.1
aldehydes	
hexadecanal (16:0)	2.2
heptadecanal (17:0)	1.3
octadecanal (18:0)	16.0
nonadecanal (19:0)	0.8

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

180 (15%), due to allylic cleavage between C-7 and C-8, and differences of 12 amu between peaks at *m/z* 140 (C_4) and *m/z* 152 (C_5), as well as between peaks at *m/z* 194 (C_8) and *m/z* 206 (C_9), thus confirming the $\Delta^{5,9}$ double-bond arrangement.[7] The double-bond stereochemistry was established by FTIR. Both double bonds were *cis* because the IR spectrum of **1** exhibited absorption at 723 cm^{-1} and no absorption in the 960−980 cm^{-1} region.[8] This stereochemistry was further confirmed by ^{13}C NMR, since the allylic carbons C-4, C-7, C-8, and C-11 resonated between 26 and 27 ppm.[9] Catalytic hydrogenation (PtO_2) was used to establish methyl branching, for the diunsaturated methyl ester was transformed into the known 14-methylpentadecanoic acid methyl ester, which coinjected nicely in GC with an authentic standard.[5] The terminal *iso* branching in **1** was further corroborated by ^1H NMR because a doublet at 0.86 ppm was observed. All of our spectral data support 14-methyl-5,9-pentadecadienoic acid (**1**) as the unknown acid. Final structural confirmation was achieved by GC co-injection, of the methyl ester, with a synthetic sample that was previously synthesized by us for antimicrobial bioassays.[10]

The title compound **1** displayed antimicrobial activity, specifically against Gram-positive bacteria (Table 2) such as *Staphylococcus aureus* (MIC 0.24 μmol/mL) and *Streptococcus faecalis* (MIC 0.16 μmol/mL). It was not, however, active against such Gram-negative bacteria as *Pseudomonas aeruginosa* and *Escherichia coli*. It is important to mention that hexadecanoic acid (16:0) showed no activity (MIC > 100 μg/mL) against any of these four microorganisms.

Table 2. Antimicrobial Activity of **1**

bacteria	MIC (μmol/mL)	IC_{50} (μg/mL)
S. aureus	0.24	36
S. faecalis	0.16	<10
P. aeruginosa[a]		
E. coli[a]		

[a] Not active (MIC > 100 μg/mL).

The identification of **1** in *E. succinea* is of interest because it expands the occurrence of $\Delta^{5,9}$ fatty acids to the Gorgoniidae. The origin of **1** is most likely bacterial and presents interesting biosynthetic considerations, in particular with respect to the order of double-bond introduction, as compared to marine sponges.[1] The specificity of **1** against Gram-positive bacteria is also of interest.

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 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 polymethyl siloxane crosslinked with 5% phenyl methylpolysiloxane. IR spectra were run neat in a Magna-IR 750 Nicolet spectrometer. HRMS data were recorded on a VG AutoSpec high-resolution mass spectrometer. NMR data were collected in a Bruker Avance DPX 300 spectrometer. *J* values are given in Hz.

Sample Collection. *E. succinea* was collected in November 1992, at Mona Island, Puerto Rico, at a depth of between 5 and 8 m. The gorgonian was washed in sea water, carefully cleaned of all non-coral debris, and lyophilized. A voucher specimen (IP-1−4) is available at the Department of Chemistry of the University of Puerto Rico, Río Piedras campus.

Extraction and Isolation of Phospholipids. One specimen of *E. succinea* (45 g) was carefully cleaned and cut into small pieces. Extraction with 2 × 150 mL of $CHCl_3$−MeOH (1:1) yielded the total lipids (ca. 5 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.*[11] 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's 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 in the polyunsaturated fatty acids were determined by preparing the corresponding *N*-acylpyrrolidide derivatives, which 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. Hydro-

genations were carried out in 10 mL of MeOH and catalytic amounts of PtO_2. Spectral data for **1** and its derivatives are presented below.

(5*Z*,9*Z*)-14-Methyl-5,9-pentadecadienoic acid (1): IR (neat) υ_{max} 3500−2500 (OH), 3007 (=CH), 2954, 2930, 2869, 1711 (C=O), 1459, 1435, 1413, 1384, 1366, 1241, 938, 723 (HC=CH, *cis*) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.30−5.44 (4H, m, H-5,6 and H-9,10), 2.35 (2H, t, J = 7.5, H-2), 1.97−2.13 (8H, m, H-4, H-7, H-8, and H-11), 1.72−1.75 (2H, m, H-3), 1.52−1.57 (1H, m, H-14), 1.16−1.23 (4H, m, H-12, H-13), 0.86 (6H, d, J = 6.6, H-15, H-16); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 179.60 (s, C-1), 130.55 (d, C-6 and C-9), 128.91 (d, C-5), 128.60 (d, C-10), 38.63 (t, C-13), 33.38 (t, C-2), 27.88 (d, C-14), 27.48 (t, C-7 and C-8), 27.38 (t, C-11), 27.26 (t, C-12), 26.47 (t, C-4), 24.58 (t, C-3), 22.61 (q, C-15 and C-16); MS (70 eV) m/z $[M]^+$ 252 (3), 237 (0.01), 209 (0.01), 196 (1), 181 (1), 167 (3), 149 (4), 136 (6), 127 (17), 109 (31), 95 (23), 81 (61), 69 (100), 55 (65); HRMS calcd for $C_{16}H_{28}O_2$ 252.2073, found 252.2089.

Methyl 14-methyl-5,9-pentadecadienoate: GC−MS(70 eV) m/z $[M]^+$ 266 (3), 234 (2), 217 (1), 192 (3), 141 (3), 136 (10), 109 (38), 95 (28), 81 (100), 79 (29), 74 (18), 69 (50), 67 (63), 55 (67).

***N*-14-Methyl-5,9-pentadecadienoylpyrrolidine**: GC−MS (70 eV) m/z $[M]^+$ 305 (1), 290 (0.1), 262 (0.2), 248 (0.1), 234 (0.4), 220 (0.4), 208 (0.1), 206 (0.2), 194 (0.3), 180 (15), 166 (0.6), 154 (0.3), 152 (0.4), 140 (0.7), 126 (12), 113 (100), 98 (16), 85 (12), 81 (4), 72 (11), 70 (16), 67 (7), 55 (27).

Antibacterial Activity. Antibacterial activity against *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and *S. faecalis*-group D (ATCC 29212) was determined following National Committee of Clinical Laboratory Standards (NCCLS).[12] A 200-μL solution of **1** in Mueller−Hinton broth was inoculated with 10^5 colony-forming units in a 96-well plate. The minimal inhibitory concentration (MIC) was determined after an overnight incubation of **1** and the microorganisms at 37 °C. The MIC was determined by observing the highest dilution of the fatty acid that inhibited growth when compared to an uninoculated chemical-control well. The generated data were taken from at least three separate experiments.

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