4-Methyl-7,11-heptadecadienal and 4-Methyl-7,11-heptadecadienoic Acid: New Antibiotics from Sporothrix flocculosa and Sporothrix rugulosa

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4-METHYL-7,11-HEPTADECADIENAL AND 4-METHYL-7,11-HEPTADECADIENOIC ACID: NEW ANTIBIOTICS FROM *SPOROTHRIX FLOCCULOSA* AND *SPOROTHRIX RUGULOSA*

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ABSTRACT.—4-Methyl-7,11-heptadecadienal [**1**] and 4-methyl-7,11-heptadecadienoic acid [**2**] were isolated as new compounds from liquid cultures of *Sporothrix flocculosa* and *Sporothrix rugulosa*. Compounds **1** and **2** inhibited the growth of *Fusarium oxysporum* f. sp. *lycospersici*, *Trichoderma viride*, and *Bacillus subtilis*.

Many species of *Sporothrix* Hektoen & Perkins are encountered as fungicolous hyphomycetes on sporocarps of other fungi (1). One example is *Sporothrix fungorum* de Hoog & de Vries, which was collected originally in Germany on an old basidiocarp of a bracket fungus, *Fomes* sp. (2). It was later associated with yeast-like strains that produced *Stephanoascus* teleomorphs (3). Subsequent studies confirmed a clear connection with the ascus-forming yeast, *Stephanoascus farinosus* de Hoog (4). In a search for fungicolous fungi in the phyllosphere, two new *Stephanoascus* species with *Sporothrix* anamorphs (conidial stages) were discovered on moldy leaves of maize and mildewed leaves of red clover (5). Antibiosis is an important mechanism of fungal antagonism in phyllosphere environments (6). We report here the isolation of two new secondary metabolites in culture filtrates of *Sporothrix flocculosa* Traquair, Shaw & Jarvis and *S. rugulosa* Traquair, Shaw & Jarvis. The antimicrobial activity of compounds **1** and **2** is reported based on agar diffusion assays with selected fungi and bacteria.

RESULTS AND DISCUSSION

Extraction of the yeast-malt-potato-dextrose (YMPD) broth from 28-day-old still liquid cultures of *Sporothrix flocculosa* and *S. rugulosa* with $CHCl_3$ gave the same compounds from both species. Two major compounds were separated by tlc. Compound **1** was eluted with CH_2Cl_2-hexane (1:9) and compound **2** with EtOAc-hexane (1:4). The characterization of the new structures was performed with ir, nmr (^1H, ^{13}C), and gc-ms, using procedures outlined by Apona and Nicolaides (7).

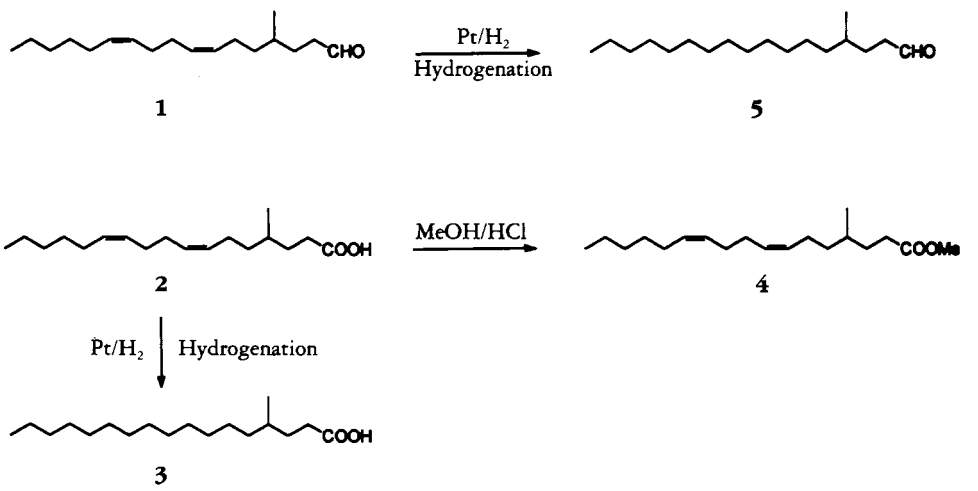
Compound **1** had a molecular formula of $C_{18}H_{32}O$ ([M^+], *m/z* 264.2451 requires 264.2453). The Ft-ir spectrum showed absorptions at 2700 and 1720 cm^{-1}, which indicated the presence of a -CHO group [^1H nmr δ 9.74]. Compound **2** had the molecular formula $C_{18}H_{32}O_2$ ([M^+], *m/z* 280.2403, requires 280.2402). Ir absorption maxima at 3300 cm^{-1} (-OH stretch) and 1710 cm^{-1} (C=O stretch), indicated the presence of a -COOH group [^1H nmr δ 11.60]. Compound **2** is therefore a fatty acid.

Further confirmation of the proposed structure of compound **2** was obtained by generation of pyrrolidine derivatives which were used in the location of double bonds and branching. According to Andersson (8,9), if an interval of 12 mass units, instead of the usual 14 mass units, is observed between the most intense peaks of clusters of fragments containing n and n−1 carbon atoms of the acid moiety, a double bond is indicated between carbons n and n+1 in the molecule. The pyrrolidine derivative of compound **2** had a mol wt of ([M]$^+$, *m/z* 333) and a base peak at *m/z* 113, confirming it to be the pyrrolidine derivative of 4-methyl-7,11-heptadecadienoic acid. The double bonds in the

molecule were located by observation of an interval of 12 mass units between fragments at *m/z* 182 (C-6) (n−1) and *m/z* 194 (C-7) (n), indicating a double bond at Δ^7 between C-7 (n) and C-8 (n+1). A second interval of 12 mass units between fragments at *m/z* 236 (C-10) and *m/z* 248 (C-11) confirmed the position of the second double bond at Δ^{11} between C-11 and C-12. The position of methyl branching was also determined from the pyrrolidine derivative. In general, a peak of lower intensity than the analogous peak in a straight-chain fatty acid pyrrolidide indicates a branched methyl at that position (8,9). This structure was further supported by ^{13}C and ^{1}H nmr of compounds **1** and **2**, which showed two methyl peaks, one at δ 14.1 (C-17) indicating a terminal methyl group [^{1}H nmr, δ 0.9 ppm, $-CH_3$], and another value at δ 19.8 (CH_3, at C-4) and 32.6 (C-4) indicating methyl branching at C-4 (10). The presence of branching was also confirmed by attached-proton-test (APT) ^{13}C nmr which showed singlets at δ 1.1 for [**1**] and at δ 1.08 for **2**.

To confirm the proposed structure, the methyl ester of acid [**4**] was cleaved with $KMnO_4/NaIO_4$, followed by esterification with HCl/MeOH. A mixture of two short-chain dimethyl esters and one monomethyl ester was obtained. One of these was the dimethyl ester of succinic acid (mol wt 146), which demonstrated the presence of two $-CH_2$ groups between the two double bonds. The other dimethyl ester was 4-methyl-1,7-dimethylheptanoate ester (mol wt 202). The third ester was methyl hexanoate with mol wt 130. Dimethyl esters of succinic acid and methyl hexanoate were compared with commercial authentic samples, and this ms fragmentation pattern supported the positioning of double bonds at $\Delta^{7,11}$. Ft-ir spectra of the methyl esters showed no absorption in the 960–980 cm^{-1} region, indicating the presence of cis unsaturation which was further supported by ^{13}C nmr [δ 27.4 (C-9, C-13), 27.3 (C-10) for compound **1**, and δ 27.35 (C-9, C-13), 27.4 (C-10) for compound **2**] (11). Based on these data, the structural formula of the new fatty acid (sporothricenoic acid) is 4-methyl-7,11-heptadecadienoic acid [**2**], which was further corroborated by catalytic hydrogenation with PtO_2 to produce 4-methyl-heptadecanoic acid [**3**]. The assimilation of four hydrogen atoms indicates the presence of two double bonds. This was confirmed using Ft-ir, ^{1}H-nmr, and ms. Similarly, the fatty aldehyde compound [**1**] (sporothricenal) upon catalytic hydrogenation with PtO_2 formed 4-methyl-heptadecanal (Scheme 1).

Compounds **1** and **2** were equally potent when bioassayed on two soil-borne fungi, *Fusarium oxysporum* Schlecht. f. sp. *lycospersici* and (Sacc.) W.C. Snyder & H.N. Hans. and



SCHEME 1

Trichoderma viride Pers. Fr., and a Gram-positive soil bacterium, *Bacillus subtilis* (Ehrenberg) Conn. by agar diffusion assay (12) at different concentrations and time intervals. Data are presented for compound **2** only (Figure 1). The fatty acid **2** was dissolved in $CHCl_3$ and the zone of inhibition of the test organisms growing on yeast-malt-potato-dextrose agar was measured over a two-day period. Addition of $CHCl_3$ alone, applied as a control treatment, did not inhibit growth of the test microorganisms.

The toxicity of saturated fatty acid compounds to insects is reported to increase with the number of carbon atoms to a maximum of C-10 or C-11 and then to decrease (13). In contrast to their insecticidal activity the unsaturated C-18 fatty acids, oleic and linoleic, are not reported to be toxic to fungi (14). Our studies show that the new unsaturated C-17 fatty acid [**2**] and C-17 fatty aldehyde [**1**] from *S. flocculosa* and *S. rugulosa* are toxic to two fungi and to a Gram-positive bacterium.

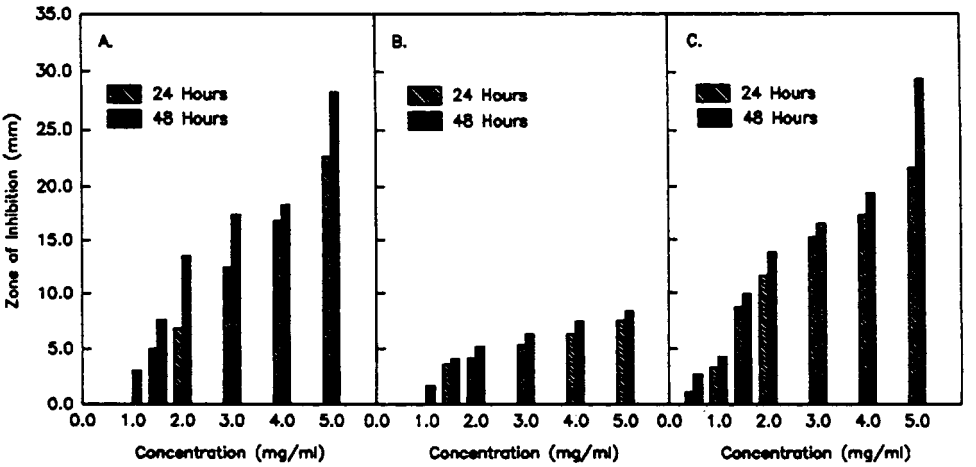


FIGURE 1. Inhibition of radial growth on agar of *Fusarium oxysporum* f. sp. *lycopersici* (A), *Bacillus subtilis* (B), and *Trichoderma viride* (C). Zone of inhibition was measured in mm and results are the mean of six replicates for each test organism. The standard error in all cases was less than ± 0.1. $CHCl_3$, when used as solvent, did not cause inhibition of test microorganisms in a control treatment.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H-Nmr spectra were recorded using a 200 MHz Varian XL-200 spectrometer with tetramethylsilane (TMS) as an internal standard, with $CDCl_3$ being used a solvent for recording all spectra. All chemical shifts were recorded in ppm downfield from TMS. Ir were recorded using a Bruker Ft-ir instrument with IBM Ft-ir/32 software; bands are stated in cm^{-1}. Uv spectra were obtained using a Shimadzu Uv/vis-160 spectrophotometer. A Waters Model 481 hplc was used for purification on a C18 reversed-phase column Spheri-5 RP-18, 5 μm, 250×7.0 mm (with a flow rate of 10 ml/min). Gc-ms (stationary phase) was performed with a Varian 3400 gas chromatograph coupled with a Finnigan MAT-8230 mass spectrometer. Baker 60–200 mesh Si gel was used for cc and Kieselgel 60 F_{254} plastic sheets (Merck) were used for tlc. Standard methods were used in preparation of broth cultures and agar diffusion bioassays of compounds **1** and **2** with various test organisms (15). Voucher cultures are maintained in the Patent Depository at the American Type Culture Collection (ATCC), Rockville, Maryland as ATCC 64874 (*S. flocculosa*) and ATCC 64875 (*S. rugulosa*).

EXTRACTION AND ISOLATION.—Liquid medium (YMPD) broth (1 liter), obtained from a 28-day-old still culture of *S. flocculosa*, was filtered to remove the mycelial mat, and extracted repeatedly with $CHCl_3$. The combined organic layer was dried over Na_2SO_4 and evaporated *in vacuo*. The crude extract (0.50 g) contained two major components, compounds **1** and **2**, with R_f 0.8 and R_f 0.1, respectively, as determined by tlc using the solvent systems CH_2Cl_2-hexane (1:9) for **1** and EtOAc-hexane (1:4) for **2**. Compound **1** was separated by cc on Si gel (60–200 mesh) (16), using CH_2Cl_2-hexane (1:9), which yielded 20 mg (4%). For hplc the culture broth was filtered to remove mycelium. The mycelium was washed six times with MeOH.

The MeOH-soluble fraction (0.53 g), with R_f 0.1 [**2**] was fractionated by two passes through a reversed-phase hplc column, using MeOH (100%) and 80% MeOH as solvents (eluting in 7.2 column volumes). The fraction was dried *in vacuo* and further purified by cc using EtOAc-hexane (1:4) as solvent, which provided pure 4-methyl-heptadecadienoic acid [**2**] (25 mg; 4.7%). The structures are shown in Scheme 1.

Compound **1**.—Hrfabms *m/z* $[M+H]^+$ 264.2451 ($C_{18}H_{32}O$ requires 264.2453); eims *m/z* $[M^+]$ 264 (80), 263 (60), 249 (40), 248 (35), 235 (2), 221 (3), 206 (10), 207 (12), 192 (4), 179 (30), 164 (14), 152 (12), 138 (4), 124 (45), 111 (15), 99 (13), 97 (60), 85 (32), 84 (50), 81 (89), 71 (100), 57 (24); ir ν max 2700, 1720, 1640, 1470, 1412, 996, 730 cm^{-1}; ^1H nmr δ 9.74 (1H, s, C*H*O), 5.3 (4H, m, C*H*=), 2.35 (2H, t, CH_2CHO), 1.2–1.5 (19H, m, CH_2), 0.9 (3H, t, CH_3), 1.1 (3H, br s, CH_3); ^{13}C nmr δ 14.1 (C-17), 19.7 (CH_3 at C-4), 22.7 (C-16), 24.8 (C-6), 27.3 (C-10), 27.4 (C-13 and C-9), 29.5 (C-14), 31.2 (C-3), 31.6 (C-15), 32.6 (C-4), 36.9 (C-5), 43.1 (C-2), 129.9 (C-8 and C-11), 130.0 (C-7 and C-12), 190.1 (C-1).

Compound **2**.—Hrfabms *m/z* $[M+H]^+$ 280.2403 ($C_{18}H_{32}O_2$ requires 280.2402); eims *m/z* $[M^+]$ 280 (95), 265 (74), 264 (62), 250 (10), 236 (14), 222 (18), 220 (32), 208 (45), 207 (28), 194 (12), 192 (70), 181 (65), 169 (60), 165 (30), 154 (41), 151 (25), 140 (16), 138 (22), 127 (10), 115 (14), 111 (48), 101 (50), 97 (25), 86 (11), 84 (18), 73 (84), 71 (100), 59 (70), 57 (30); ir ν max 3300, 2650, 1710, 1665, 1410, 1230, 920 cm^{-1}; ^1H nmr δ 11.60 (1H, s, COO*H*), 5.32 (4H, m, C*H*=), 2.33 (2H, t), 1.2–1.5 (19H, m, CH_2), 0.9 (3H, t, CH_3), 1.08 (3H, br s, CH_3); ^{13}C nmr δ 14.2 (C-17), 19.8 (CH_3 at C-4), 22.8 (C-16), 24.8 (C-6), 27.35 (C-13 and C-9), 27.45 (C-10), 29.4 (C-14), 31.8 (C-15), 32.1 (C-3), 32.6 (C-4), 34.1 (C-2), 37.1 (C-5), 129.97 (C-8 and C-11), 130.05 (C-7 and C-12), 180.0 (C-1).

PREPARATION OF 4-METHYL-HEPTADECANAL [**5**].—A catalytic amount of PtO_2 in MeOH was stirred in a stream of H_2. A quantity of compound **1** (26.4 mg, 0.01 mmol) dissolved in 10 ml of MeOH was added. After 0.5 h the reaction mixture was filtered and solvent was evaporated. A pure compound [**5**] was obtained (23.5 mg, 89%); eims *m/z* $[M^+]$ 268 (92), 253 (55), 238 (30), 224 (11), 211 (60), 210 (15), 196 (3), 183 (35), 182 (42), 168 (50), 155 (22), 154 (38), 141 (18), 126 (6), 112 (73), 99 (80), 85 (19), 84 (64), 70 (10), 57 (100); ir ν max 2750, 1722, 1470, 1412, 996, 733 cm^{-1}; ^1H nmr δ 9.76 (H, s, C*H*O), 2.33 (2H, t, CH_2CHO), 1.2–1.5 (27H, m, CH_2), 0.9 (6H, m, CH_3).

PREPARATION OF 4-METHYL-HEPTADECANOIC ACID [**3**].—A catalytic amount of PtO_2 in MeOH was stirred in a stream of H_2. Compound **2** (28.0 mg, 0.01 mmol) was dissolved in 10 ml of MeOH and added. After 0.5 h, the reaction mixture was filtered and evaporation of solvent under reduced pressure gave pure compound **3** (25.8 mg, 92.1%); eims *m/z* $[M^+]$ 284 (88), 283 (20), 269 (34), 268 (15), 254 (12), 240 (10), 226 (5), 224 (60), 212 (52), 211 (28), 198 (43), 196 (3), 184 (64), 170 (2), 155 (10), 142 (22), 128 (30), 115 (40), 113 (79), 101 (85), 86 (5), 73 (100), 71 (92), 59 (65), 57 (58); ir ν max 3300, 2658, 1710, 1415, 1238, 928 cm^{-1}; ^1H nmr δ 11.68 (1H, s, COO*H*), 2.36 (2H, t, CH_2-COOH), 1.2–1.5 (27H, m, CH_2), 0.99 (6H, m, CH_3).

PREPARATION OF 4-METHYL-7,11-HEPTADECADIENOIC ACID METHYL ESTER [**4**].—Compound **2** (28.0 mg, 0.01 mmol), in 10 ml of methanolic HCl, was stirred for 45 min. Finally, solvent was evaporated under reduced pressure. A pure methyl ester of the fatty acid (17) was obtained using cc by eluting with *n*-hexane and EtOAc (9:1), (22.7 mg, 81%); eims *m/z* $[M^+]$ 294 (82), 279 (70), 264 (45), 250 (32), 248 (20), 236 (19), 222 (65), 220 (5), 208 (10), 207 (25), 195 (30), 183 (28), 179 (40), 168 (15), 165 (10), 154 (55), 151 (60), 141 (10), 129 (16), 125 (35), 115 (48), 111 (66), 100 (5), 97 (45), 87 (95), 81 (76), 73 (54), 71 (100), 59 (90), 57 (22); ir ν max 3080, 1725, 1650, 1445, 1438, 998, 735 cm^{-1}; ^1H nmr δ 5.35 (4H, m, C*H*=), 3.67 (3H, s, COOCH_3), 2.30 (2H, t, CH_2-COOCH_3), 1.2–1.5 (19H, m, CH_2), 0.99 (6H, m, CH_3).

PYRROLIDIDE DERIVATIVE OF 4-METHYL-7,11-HEPTADECADIENOIC ACID.—For the location of double bonds and methyl branching (9), the *N*-pyrrolidide derivative was prepared by direct treatment of compound **4** (29.4 mg, 0.01 mmol) in 1 ml of freshly distilled pyrrolidine (Aldrich Chemical Co.) and 0.1 ml of HOAc. The mixture was heated at 100° in a sealed tube and cooled to room temperature. The amide so formed was taken up with CH_2Cl_2 and washed with H_2O. After drying with $MgSO_4$, solvent was evaporated under reduced pressure and purity was checked by tlc (25.2 mg, 85.7%); eims *m/z* $[M^+]$ 333 (80), 318 (20), 304 (12), 290 (18), 276 (10), 262 (5), 248 (3), 236 (4), 222 (14), 208 (30), 194 (5), 182 (3), 168 (40), 154 (50), 140 (2), 126 (80), 113 (100), 97 (28), 70 (35).

PERMANGANATE-PERIODATE OXIDATION OF THE METHYL ESTER OF **2**.—To confirm the double bond positions, a modified von Rudloff's oxidation (18) was performed on the methyl ester of compound **2**. A stock oxidant solution of sodium metaperiodate (2.09 g) and $KMnO_4$ (0.04 g) in H_2O (100 ml) was prepared. This solution (10 ml) together with K_2CO_3 solution (1 ml, 2.5 g/liter) was added to compound **4** (29.41 mg, 0.01 mmol) in *t*-BuOH (10 ml) in a test tube and the mixture was shaken thoroughly at room temperature (1 h). The solution was then acidified with one drop of concentrated H_2SO_4, and excess oxidant was destroyed with $NaHSO_3$. The solution was extracted thoroughly with Et_2O (3×4 ml), the organic layer was dried over

Na_2SO_4, and the Et_2O removed in a stream of N_2 at room temperature. The products were methylated with 1.2 N HCl/MeOH for 30 min at room temperature for gc-ms analysis, which was compared with appropriate authentic samples (dimethyl ester of succinic acid and methyl hexanoate) supplied by Aldrich Chemicals.

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