New extracellular fatty acids in culture filtrates of *Sporothrix flocculosa* and *S. rugulosa*

Streeti R. Choudhury, James A. Traquair, and William R. Jarvis

Abstract: Two new and rare unsaturated, extracellular fatty acids were identified in the culture filtrates of the biocontrol fungi, *Sporothrix flocculosa* and *S. rugulosa*. 16-Methyl-9*E*-nonadecenoic acid (**1**) and (*Z*,*Z*)-10,14-eicosadienoic acid (**2**) were characterized on the basis of infrared (IR) and nuclear magnetic resonance spectra (^{13}C and ^{1}N NMR), and gas chromatographic – mass spectrometric data (GC–MS). The structure of compounds **1** and **2** was confirmed by oxidative degradation to the known standards, dimethyl azelate, dimethyl succinate, dimethyl sebacate, and methyl caproate.

Key words: unsaturated fatty acids, fungi.

Résumé : On a identifié les nouveaux et peu abondants acides gras insaturés : les acides 16-méthyl-9*E*-nonadecénoique (**1**) et (*Z*,*Z*,)-10,14-eicosadiénoique (**2**) dans les filtrats de culture des champignons de biocontrôle, *Sporothrix flocculosa* et *S. rugulosa*, et on les a caractérisés par spectroscopie IR et de RMN du ^{13}C et du ^{1}H et par les données de chromatographie en phase gazeuse couplée à un spectromètre de masse (GC–MS). On a confirmé les structures des composés **1** et **2** par dégradation oxydante de standards connus : l'azélate de diméthyle, le succinate de diméthyle, le sébacate de diméthyle et la caproate de méthyle.

Mots clés : acides gras insaturés, champignons.

[Traduit par la rédaction]

Long-chained, unsaturated fatty acids with 14–24 carbon atoms and one or more double bonds are characteristic of the fungi, particularly the yeast and yeastlike species. The major unsaturated fatty acids associated with the cellular fractions of these fungi are oleic (C18:1) and linoleic (C18:2) acids (1, 2). A few rare and unusual fatty acids in the fungi include methyl-branched fatty acids and hydroxy fatty acids (1). Most studies deal with cellular fatty acids, which are involved in membrane structure and function. For example, linoleic acid is implicated in the structural integrity and activity of mitochondria (3). Linoleic acid has been reported as a morphogenetic factor in the sexual reproduction of the phytopathogens *Ophiostoma ulmi* (4) and *Nectria haematococca* (5). In general, fatty acids are also implicated in the synthesis of other secondary metabolites such as cyclopentanes, polyacetylenes, and polyketide aromatic compounds, which can be potent antibiotics and mycotoxins (1, 3). Four fungitoxic C18:2 hydroxy-unsaturated fatty acids have been identified in the stroma of the endophytic phytopathogen, *Epichloe typhina* (6). Little is known about extracellular fatty acids in the fungi, but acetylated and hydroxy fatty acids have been reported (1).

Recently, we identified a new antimicrobial, methyl-substituted C18:2, extracellular fatty acid in the culture filtrates of the yeastlike, biocontrol fungi, *Sporothrix flocculosa* and *S. rugulosa* (7). In the present report we describe two new and rare extracellular C20 fatty acids that are not antimicrobial.

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Results and discussion

The new unsaturated, C20 extracellular fatty acids were identified from filtrates of both *Sporothrix flocculosa* and *S. rugulosa* grown for 28 days on yeast–malt extract broth in static liquid culture. Compound **1** is an oil with molecular formula $C_{20}H_{38}O_2$. An FT-IR spectrum contained absorption bands at 3300 cm^{-1} (-OH stretch) and 1710 cm^{-1} (C=O stretch), indicating the presence of a spectrum -COOH group. The presence of this group was also revealed in the ^{1}H NMR spectrum at 11.65 ppm, while in the ^{13}C NMR spectrum resonances at 34.1 (C-2) and 180.0 ppm (C-1) were expected for attachment of this group to a methylene (8). The mass spectrum of compound **1** shows fragmentation at 293 [M – 17] and 266 [M – 44], which indicates the presence of an acid group at the terminal end in a chain (9). The ^{1}H NMR shows $\delta = 5.30$ ppm (t, 2H, $J = 17.1$ Hz, -CH=), indicating the presence of one double bond. Normally for *E* isomers the *J* values range between 12.0 and 18.0 Hz (10). We note that the coupling constant values thus indicate an *E* isomer, which was supported by ^{13}C NMR. The DEPT ^{13}C NMR showed two peaks at 130.35 and 130.45 ppm, which indicates the presence of a double bond. The position of the double bond was identified by following the technique given by Bus et al. (11), for the ^{13}C values of the neighbouring carbons of the double bonds. The *trans* unsaturations possess higher values in the range 29.5–38.0 ppm, whereas *cis* unsaturations have values of 26.0–28.5. The ^{13}C

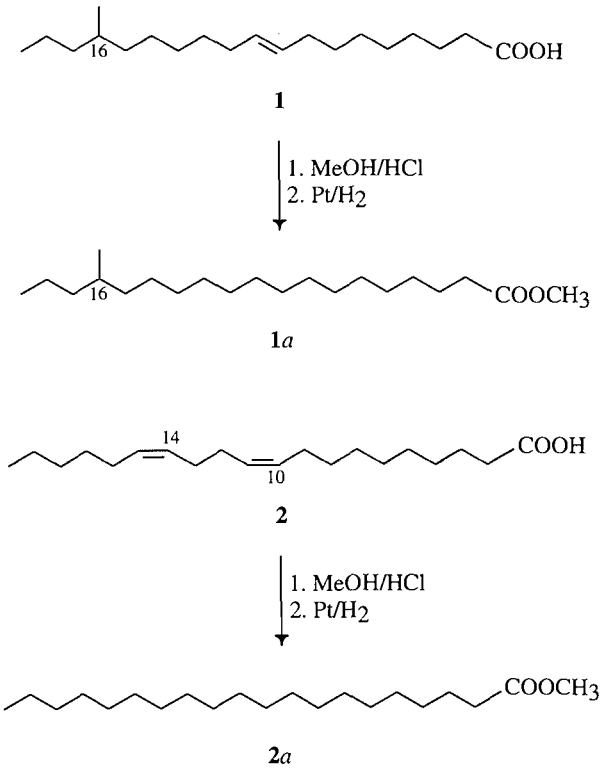
values of compounds **1** are 32.6–32.65 for C-8 and C-11, which implies that the position of the *trans* unsaturation is between C-9 and C-10. This confirms the position of the double bond between C-9 and C-10.

The presence of two methyl groups was revealed by ^{13}C and ^{1}H NMR: one, at 14.5 (C-19), indicating a terminal methyl group that is shielded by methyl branching at the γ-position (^{1}H nmr δ = 0.9 (t, J = 6 Hz, 3H, -CH_3)) and another value at 19.8 (-CH_3 at C-16), indicating methyl branching at C-16 (ref. 8, p. 56). The triplet at 1.15 ppm (J = 6.3 Hz, 3H, -CH_3) is observed in ^{1}H NMR, and a singlet with a downfield shift in APT ^{13}C NMR indicating the presence of branching (12). In general, the APT ^{13}C NMR shows downfield shifts for both the CH_3 and CH, whereas in DEPT both CH_3 and CH give upfield shifts (ref. 10, p. 276). The ^{13}C NMR of the carbon bearing the methyl group shows resonance at 32.2 (C-16), which confirms the position of methyl branching at C-16 (13). The carbons next to the methyl branching show values 36.5 (C-15) and 38.7 (C-17). These values are deshielded by the methyl branching at C-16. The position of methyl branching was further supported by the fragmentation pattern of 16-methyl methylnonadecanoate **1***a*. Two large peaks are observed, one at 282 [M – 44] (50%) and the second at 255 (42%), with a small peak at 269 (5%) that carried the methyl group. The peaks at 251 and 233, observed by the loss of a methanol and a water molecule simultaneously from the fragment $^{+}CH(CH_3)(CH_2)_{14}$-$COOCH_3$, are also characteristic of the branching at C-16 (9).

To confirm the structure of compound **1** its methyl ester was oxidized using the Lemieux–von Rudloff reagent (14). This method was used to indicate the position of the double bond. Esterification of the acid fragments with HCl/MeOH after von Rudloff's oxidation gave two major components: one is 7-methyl methyldecanoate (Mol. Wt. 200). The *m/e* fragmentation pattern has two major peaks at 156 (40%) and 129 (45%) along with a small peak at 143 (7%). The smaller peak is the indication of the carbon bearing the methyl group; the peaks observed at 125 and 107 indicate the simultaneous loss of methanol and water from the fragment $^{+}CH(CH_3)(CH_2)_5$-$COOCH_3$. This confirms the methyl branching at C-7. The second major component is nonanedioic acid dimethyl ester (Mol. Wt. 216). The *m/e* fragmentation pattern shows the base peak at 98 (100%), which indicates the presence of dimethyl ester. This establishes the position of the double bond between C-9 and C-19. The catalytic hydrogenation of the methyl ester of compound **1** with PtO_2 resulted in formation of 16-methyl methylnonadecanoate **1***a*. From all these data it was concluded that this new fatty acid **1** is 16-methyl-9*E*-nonadecenoic acid.

Compound **2** is an oil with molecular formula $C_{20}H_{36}O_2$. An FT-IR spectrum contained broad absorption bands at 3310 cm^{-1} (-OH stretch) and 1710 cm^{-1} (-C=O). The ^{1}H NMR resonances at 11.60 (s, H, -COO*H*) as well as ^{13}C NMR signals at 180.1 (C-1) and 34.1 (C-2) indicate the presence of a -COOH group (8). The terminal acid group was also determined with the help of fragmentation patterns at 291 [M–17] and 264 [M – 44] (9). The ^{1}H NMR resonances at 5.32 (m, 4H, J = 10.2 Hz, -CH=) indicate the presence of two double bonds. Normally for *Z* isomers the range of J values is 6.0–12 Hz, but we have observed that the coupling constant of both the double bonds is 10.2 Hz, which indicates *Z,Z* isomers. The presence of two unsaturations in compound **2** was supported by the DEPT ^{13}C NMR (6), which gave four upfield signals at 129.30, 129.45, 130.22, and 130.35, indicating that double bonds are present between C-10 and C-11 and between C-14 and C-15. Compound **2** was reported previously as a principal component of corn oil, but without stereochemistry (15). The *cis* unsaturation of both the double bonds was confirmed in our study with the help of ^{13}C NMR, which shows a range of values between 27.2 and 27.5 for the neighboring carbons 9, 12, 13, and 16 (11).

Scheme 1



The lack of branching was confirmed by ^{1}H NMR. A triplet appeared for 3*H* at 0.8 ppm (J = 6.0 Hz) and DEPT ^{13}C NMR: 14.1 ppm (C-20) with an upfield shift indicating the presence of a terminal methyl group in the chain. Oxidation of compound **2** was done by Lemieux–von Rudloff's reagent (14) to establish the position of the double bonds. A mixture of three short-chain dimethyl esters was obtained, one of which was the dimethyl ester of succinic acid. Production of this ester indicates the presence of two -CH_2 groups between the two double bonds in the fatty acid **2**. The second dimethyl ester was decane-1,10-dioic acid dimethyl ester, whose formation located the position of the first double bond at Δ^{10}. The third ester was methyl hexanoate, whose formation indicated the position of a second double bond at Δ^{14}. On the basis of these data, compound **2** is *Z,Z*-10,14-eicosadienoic acid. Upon catalytic hydrogenation using PtO_2, the methyl ester derivative of this acid (2) gave eicosanoic acid methyl ester **2***a*. The ^{1}H NMR showed resonance at 3.6 ppm (s, 3H, -COOCH_3) and ^{13}C NMR signals were at 51.40 (-COOCH_3) and 174.45 (-$COOCH_3$).

Experimental

General experimental procedures

Proton nuclear magnetic resonance (^{1}H NMR) and ^{13}C (APT

and DEPT) spectra were recorded using $CDCl_3$ as solvent on a 200 MHz Varian XL-200 spectrometer with tetramethylsilane (TMS) as internal standard. All chemical shifts were recorded in ppm downfield from TMS. All infrared spectra were recorded on films using a Bruker FT-IR instrument with IBM FT-IR/32 software; intensities of all the bands are stated in cm^{-1}. UV spectra were obtained using $CHCl_3$ as solvent on a Shimadzu UV/VIS-160 spectrophotometer. Gas chromatographic (GC) analysis was carried out on a Varian 3400 GC fitted with a 10 m microbore glass column (0.53 mm diameter, 2.65 μm film thickness SE-30 stationary phase), using nitrogen (20 mL/min) as the carrier gas under temperature-programmed conditions (initial temperature of 140°C, final temperature 250°C, rate 5°C/min) or isothermally (200°C) and a flame ionization detector. For GC–MS analysis, gas chromatography on a Varian 3400 GC coupled with a MS Finnigan MAT-8230 mass spectrometer, with a mass selective detector fitted with a 12 m capillary glass column (0.2 mm internal diameter, 0.33 μm film thickness, cross-linked methyl silicone gum), and helium (ca. 2 mL/min) as a carrier gas were used under temperature-programmed conditions (initial temperature 140°C, final temperature 240°C, rate 5°C/min). Thin-layer chromatography (TLC) was performed on microscope glass plates coated with silica (about 0.1 mm thickness). A mixture of *n*-hexane–chloroform and *n*-hexane – ethyl acetate in various concentrations was used as developer. Preparative TLC was carried out on Kieselgel 60 F_{254} plastic sheets (Merck) coated with silica. Column chromatography was performed on Baker 60–200 mesh silica gel using gradient elution of mixtures of *n*-hexane–chloroform for compound **1** and *n*-hexane – ethyl acetate for compound **2**. Collected fractions were checked by TLC before pooling. Dimethyl succinate, methyl caproate, dimethyl azelate, and dimethyl sebacate acid (Aldrich Chemicals) were used as standards.

Growth of the fungi and extraction of free fatty acids

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*). The fungi were grown on yeast–malt–potato–dextrose (0.5:1.0:2.0) broth (7) in 10 250-mL cotton-plugged Erlenmeyer flasks each containing 100 mL of broth. The mycelium from 28-day-old static liquid cultures grown at 23°C in the dark was filtered through a Buchner funnel using Whatman filter paper no. 2 to remove the mat. Remaining spores were removed by millipore filtration (0.22 μm) and then the cell-free filtrate was extracted seven times with $CHCl_3$ in a separatory funnel. The extracts were combined and dried over anhydrous Na_2SO_4 (1.89 g). Further purification was done by column chromatography on silica gel (60–200 mesh) using a procedure similar to that of Privett et al. (16). The two new fatty acids were investigated by preparative TLC using $CHCl_3$–hexane (3:7) as eluting solvent for compound **1** (yield 75 mg, 3.968%) and hexane–EtOAc (1:4) as eluting solvent for compound **2** (yield 82 mg, 4.33%).

*Compound **1** (oil)*: IR ν_{max}: 3300, 1710, 1660, 1410, and 920 cm^{-1}. ^1H NMR: 11.65 (s, 1H, COO*H*), 5.30 (5, 2H, J = 17.1 Hz, C*H*=), 2.3 (t, 2H, J = 6.5 Hz, CH_2COOH), 1.2–1.5 (m, 27H, CH_2), 1.15 (5, 3H, J = 6.3, CH_3, br), and 0.9 (t, 3H, J = 6 Hz, CH_3). ^{13}C NMR: 14.5 (C-19), 19.8 (C-20), 20.8 (C-18), 24.8 (C-3), 26.9 (C-14), 29.2 (C-6), 29.4 (C-5), 29.5 (C-4, 12), 29.6 (C-7), 29.8 (C-13), 32.2 (C-16), 32.60 (C-8), 32.65 (C-11), 34.1 (C-2), 36.5 (C-15), 38.7 (C-17), 130.35 (C-9), 130.45 (C-10), and 180.0 (C-1). HRFABMS *m/z* $\{M\}^+$ 310.2870 ($C_{20}H_{38}O_2$ requires 310.2871). EIMS, *m/z* $\{M^+\}$: 310(14), 295(22), 293(57), 281(17), 267(36), 266(28), 252(8), 250(10), 239(42), 236(40), 225(25), 222(47), 211(52), 209(38), 197(65), 195(55), 183(82), 181(75), 169(32), 167(30), 156(44), 154(32), 143(62), 141(15), 129(20), 127(34), 115(72), 112(56), 101(80), 98(76), 87(78), 74(100), 59(68), and 56(49).

*Compound **2** (oil)*: IR ν_{max}: 3300, 2650, 1710, 1665, 1412, 1230, and 920 cm^{-1}. ^1H NMR: 11.60 (s, H, COO*H*), 5.32 (m, 4H, J = 10.2 Hz, C*H*=), 2.3 (t, 2H, J = 6.8 Hz, CH_2COOH), 1.2–1.5 (m, 26H, CH_2), and 0.8 (t, 3H, J = 6 Hz, CH_3,). ^{13}C NMR: 14.1 (C-20), 22.7 (C-19), 24.9 (C-3), 29.4 (C-17), 27.3 (C-9), 27.45 (C-12), 27.5 (C-13), 27.2 (C-16), 29.2–29.8 (C-4–C-9), 31.6 (C-18), 34.1 (C-2), 129.30 (C-11), 129.45 (C-14), 130.20 (C-15), 130.35 (C-10), and 180.1 (C-1). HRFABMS *m/z* $\{M\}^+$ 308.2712, ($C_{20}H_{36}O_2$ requires 308.2715). EIMS, *m/z* $\{M^+\}$: 308(45), 293(24), 291(13), 264(42), 250(48), 236(20), 224(15), 220(18), 211(52), 207(38), 197(40), 194(42), 183(26), 179(41), 170(44), 165(70), 157(53), 151(68), 143(62), 138(27), 129(18), 125(56), 115(35), 111(74), 101(64), 98(72), 87(58), 84(34), 74(100), 60(77), and 57(60).

*16-Methyl-9*E*-nonadecenoic acid methyl ester*

A solution of **1** (31.0 mg, 0.01 mmol) in 10 mL of methanolic HCl was boiled under reflux for 60 min (17). The solvent was removed under reduced pressure and the pure methyl ester was obtained by column chromatography, using *n*-hexane and ethyl acetate (1:1) (25.0 mg, 77.1%). IR ν_{max}: 3010, 1725, 1680, 1375, and 996 cm^{-1}; ^1H NMR: 5.30(t, J = 17.2 Hz, 2H), 3.60(s, 3H), 2.33(t, J = 7 Hz, 2H), 1.3–1.5 (m, 27H), and 0.92(t, J = 6 Hz, 6H). HRFABMS *m/z* (rel. int.) $\{M\}^+$ 324.3019 ($C_{21}H_{40}O_2$ requires 324.3028). EIMS, *m/z* $\{M\}^+$: 324(6), 309(12), 306(24), 295(54), 293(22), 292(30), 281(58), 265(5), 253(60), 250(54), 240(32), 238(28), 225(57), 222(40), 211(46), 208(66), 196(25), 183(40), 181(76), 170(24), 168(35), 156(45), 153(52), 142(58), 141(85), 129(15), 127(77), 115(40), 113(68), 101(50), 98(44), 87(56), 84(70), 74(100), 59(80), and 43(52).

*16-Methyl methylnonadecanoate **1**a*

A catalytic amount of PtO_2 in MeOH was stirred in a stream of hydrogen. The methyl ester of compound **1** (32.4 mg, 0.01 mmol) was dissolved in MeOH (10 mL) and added to the reaction mixture. After 1 h this was filtered and evaporation of solvent gave 31.2 mg (95.75%) of pure compound **1***a*. IR ν_{max}: 2960, 1740, 1460, and 1340 cm^{-1}. ^1H NMR: 0.9 (m, 6H, CH_3), 1.2–1.8 (m, 31H, CH_2), 2.3 (t, *J = 6.1 Hz, 2H,* CH_2COOCH_3), and 3.66 (s, 3H, COOCH_3). ^{13}C NMR: 14.4 (C-19), 19.3 (C-20), 20.6 (C-18), 25.0 (C-3), 26.8 (C-14), 29.2–29.8 (C-4–C-13), 32.3 (C-16), 34.2 (C-2), 36.4 (C-15), 38.7 (C-17), 51.40 (OCH_3), and 174.35 (C-1). HRFABMS *m/z* (rel.int.) $\{M\}^+$ 326.3185 ($C_{21}H_{42}O_2$ requires 326.3184). EIMS, *m/z* $\{M\}^+$: 326(10), 311(32), 297(31), 295(12), 282(50), 269(5), 255(42), 253(30), 251(52), 241(42), 233(38), 227(20), 213(19), 199(15), 185(60), 171(45), 157(35), 143(32), 129(18), 115(24), 101(55), 87(80), 74(100), 71(86), 59(72), and 43(54).

(Z,Z)-10,14-Eicosadienoic acid methyl ester
A solution of **2** (30.8 mg, 0.01 mmol) in 10 mL of methanolic HCl was boiled under reflux for 60 min (17). The solvent was removed under reduced pressure and the pure methyl ester was obtained by column chromatography using *n*-hexane and ethyl acetate (1:2) (26.5 mg, 82.8%). IR ν_{max}: 2980, 2820, 1735, 1450, 1154, and 998 cm^{-1}. ^1H NMR: 5.34 (m, J = 10 Hz, 4H), 3.68 (s, 3H), 2.33 (t, J = 6.5 Hz, 2H), 1.3–1.5 (m, 26H), and 0.9 (t, J = 6 Hz, 3H). HRFABMS *m/z* (rel.int.) $\{M\}^+$ 322.2869 ($C_{21}H_{38}O_2$ requires 322.2871). EIMS, *m/z* $\{M\}^+$: 322(5), 307(10), 293(2.4), 291(8), 280(30), 265(27), 263(42), 251(39), 250(57), 249(40), 238(31), 235(23), 225(29), 221(44), 206(47), 197(28), 192(25), 184(15), 179(6), 172(46), 166(16), 157(16), 152(18), 142(28), 138(54), 130(32), 125(20), 115(40), 112(35), 101(74), 97(45), 88(58), 84(60), 74(100), 71(90), 59(85), 57(78), and 43(82).

Methyl eicosanoate **2**a
Compound **2** (32.2 mg, 0.01 mmol) was dissolved in MeOH (10 mL) and added to a catalytic amount of PtO_2 in MeOH and stirred in a stream of hydrogen for 1 h. The suspension was filtered and solvent was removed under reduced pressure to give **2***a* (29.7 mg, 91.1%). IR ν_{max}: 1740 and 1460 cm^{-1}. ^1H NMR: 0.8 (t, J = 6 Hz, 3H, CH_3), 1.2–1.8 (m, 34H, CH_2), 2.3 (t, J = 6.9 Hz, 2H, CH_2COOCH_3), and 3.6 (s, 3H, $COOCH_3$). ^{13}C NMR: 14.1 (C-20), 23.3 (C-19), 29.2–29.8 (C-3–C-17), 32.8 (C-18), 34.2 (C-2), 51.40 (OCH_3), and 174.45 (C-1). HRFABMS *m/z* (rel.int.) $\{M\}^+$ 326.3179 ($C_{21}H_{42}O_2$ requires 326.3184). EIMS, *m/z* $\{M\}^+$: 326(35), 311(20), 283(15), 255(6), 227(12), 198(42), 171(22), 143(8), 115(24), 87(38), 74(100), and 59(66).

Lemieux–von Rudloff oxidation
To establish the position of the double bonds a modified Lemieux–von Rudloff oxidation (14) was performed on the methyl esters. A stock oxidant solution of sodium metaperiodate (2.09 g) and $KMnO_4$ (0.04 g) in water (100 mL) was prepared. This solution (1 mL) together with K_2CO_3 solution (1 mL; 2.5 g/L) was added to the methyl ester (1 mg) in *t*-BuOH (1 mL) in a test tube and the mixture was shaken thoroughly at room temperature for 1 h. At the end of this time, the solution was acidified with one drop of concentrated sulphuric acid, and excess oxidant was destroyed with $NaHSO_3$. The solution was extracted with diethyl ether (3 × 4 mL). The organic layer was dried over sodium sulphate and removed in a stream of nitrogen at room temperature. The products were methylated with 1.2 N HCl/MeOH for GC–MS analysis. The oxidative compounds obtained from compound **1** are nonanedioic acid dimethyl ester (azelaic acid dimethyl ester) and 7-methyl methyldecanoate. Decane-1, 10-dioic acid dimethyl ester (sebacic acid dimethyl ester), succinic acid dimethyl ester, and methyl hexanoate (methyl caproate) are the oxidative products of compound **2**. The spectroscopic data were compared with authentic samples supplied by Aldrich Chemicals. The following MS data were collected for the oxidative compounds.

7-Methyl methyldecanoate: m/e (rel.int %): 200(44), 169(20), 157(30), 156(40), 143(7), 141(72), 129(45), 125(32), 107(22), 115(12), 101(25), 87(35), 74(100), and 59(42).

Dimethylazelate: m/e (rel.int %): 216(54), 185(32), 152(24), 143(55), 124(72), 111(60), 98(100), 92(42), 84(38), 73(40), 64(50), 55(35), and 41(22).

Dimethyl sebacate: m/e (rel.int %): 230(50), 199(34), 166(45), 157(62), 138(55), 124(40), 106(58), 98(100), 92(65), 73(44), 64(33), 55(28), and 41(60).

Dimethyl succinate: m/e (rel.int %): 146(65), 115(32), 98(100), 91(72), 86(54), 83(48), 73(40), 55(35), 63(58), and 60(54).

Methyl caproate: m/e (rel.int %): 130(35), 129(72), 115(45), 101(32), 99(62), 87(54), 74(100), 71(58), and 59(66).

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