Biosynthesis of Tetrahydrofuranyl Fatty Acids from Linoleic Acid by *Clavibacter* sp. ALA2

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ABSTRACT: *Clavibacter* sp. ALA2 converts linoleic acid into many novel oxygenated products including hydroxy FA and tetrahydrofuranyl unsaturated FA (THFA). One of them was tentatively identified by GC–MS as 12,13,16-trihydroxy-9(*Z*)-octadecenoic acid (12,13,16-THOA) (Hou, C.T., H.W. Gardner, and W. Brown, *J Am. Oil Chem. Soc. 78*:1167–1169, 2001). We have separated and purified 12,13,16-THOA from its isomer, 12,13,17-THOA, by silica gel column chromatography and by preparative TLC. Its structure was then confirmed by proton and ^{13}C NMR analyses. Purified 12,13,16-THOA was used as a substrate to study the biosynthesis of THFA. Within 24 h of incubation, cells of strain ALA2 converted 12,13,16-THOA to both 12-hydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid (12-hydroxy-THFA) and 7,12-dihydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid (7,12-dihydroxy-THFA). The relative abundance of 7,12-dihydroxy-THFA increased with incubation time, whereas that of 12,13,16-THOA and of 12-hydroxy-THFA decreased. Therefore, the biosynthetic pathway of THFA from linoleic acid by strain ALA2 is as follows: linoleic acid → 12,13-dihydroxy-9(*Z*)-octadecenoic acid → 12,13,16-THOA → 12-hydroxy-THFA → 7,12-dihydroxy-THFA.

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KEY WORDS: Bioconversion, biosynthetic pathway, *Clavibacter* sp. ALA2, 13,16-epoxy-9(Z)-octadecenoic acids, linoleic acid, 12,13,16-trihydroxy-9(*Z*)-octadecenoic acid.

Clavibacter sp. ALA2 is a unique microbe that produces a variety of hydroxy FA from linoleic acid (1). In previous studies, we identified eight novel hydroxy FA produced from linoleic acid as follows (2–5): 12,13-dihydroxy-9(*Z*)-octadecenoic acid (12,13-DHOA); 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (12,13,17-THOA), 12,13,16-trihydroxy-9(*Z*)-octadecenoic acid (12,13,16-THOA); 12-hydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid (12-hydroxy-THFA); 7,12-dihydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid (7,12-dihydroxy-THFA); 12,17;13,17-diepoxy-9(*Z*)-octadecenoic acid (DEOA); 7-hydroxy-12,17;13,17-diepoxy-9(*Z*)-octadecenoic acid (7-hydroxy-DEOA); 16-hydroxy-12,17;13,17-diepoxy-9(*Z*)-octadecenoic acid (16-hydroxy-DEOA). Hydroxy FA can be used not only as specialty chemicals but also as bioactive agents such as antifungal agents (6–8). The structures of tetrahydrofuranyl FA (THFA) resemble those of known anticancer agents (9,10). The diepoxy bicyclic FA are new chemical entities with many functional groups in their molecules. Their applications in the biomedical and specialty chemical industry are anticipated.

To develop an industrial process for the production of these hydroxy FA by strain ALA2, it is important to clarify their biosynthetic pathways. Iwasaki *et al.* (11) reported the biosynthetic pathway of diepoxy bicyclic FA as follows: linoleic acid → 12,13-DHOA → 12,13,17-THOA → DEOA → 7-hydroxy-DEOA. Recently, we reported (5) the identification of a minor product, 12,13,16-THOA, by GC–MS analyses. 12,13,16-THOA is a possible intermediate in the biosynthesis of THFA from linoleic acid by strain ALA2. Therefore, it is important to further confirm its chemical structure. In this paper, we isolated and purified 12,13,16-THOA from the crude extract and confirmed the structure by proton and ^{13}C NMR analyses. We predicted that 12,13,16-THOA is transformed to THFA such as 12-hydroxy-THFA and 7,12-dihydroxy-THFA by cyclization. By using the purified 12,13,16-THOA as substrate, we found that 12,13,16-THOA is cyclized to form THFA by the strain ALA2 enzyme system.

MATERIALS AND METHODS

Chemicals. Linoleic acid was purchased from Sigma (St. Louis, MO). A mixture of trimethylsilylimidazole (TMSI) and pyridine (1:4, vol/vol) was purchased from Supelco Inc. (Bellefonte, PA). Silica Gel 60 F254 TLC plates and Kieselgel 60 were obtained from EM Science (Cherry Hill, NJ). All other chemicals were of reagent grade and were used without further purification.

Microorganisms. *Clavibacter* sp. ALA2 was isolated from a dry soil sample collected from McCalla, Alabama (3). Strain ALA2 was cultivated aerobically in a 125-mL Erlenmeyer flask containing 50 mL of medium with shaking at 200 rpm, 30°C. The medium composition (per liter) was 5 g dextrose, 15 g yeast extract, 10 g tryptone, 5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.014 g $ZnSO_4$, 0.008 g $MnSO_4$, and 0.01 g nicotinic acid. The medium was adjusted to pH 6.8 with diluted phosphoric acid.

Production of 12,13,16-THOA. Strain ALA2 was incubated aerobically in 50 mL of the medium described above for 1 d at 30°C, 200 rpm. A quantity of 125 μL (100 mg) of linoleic acid was added to the 1-d-old culture and the culture was then incubated for an additional 2 d. At the end of incubation, the

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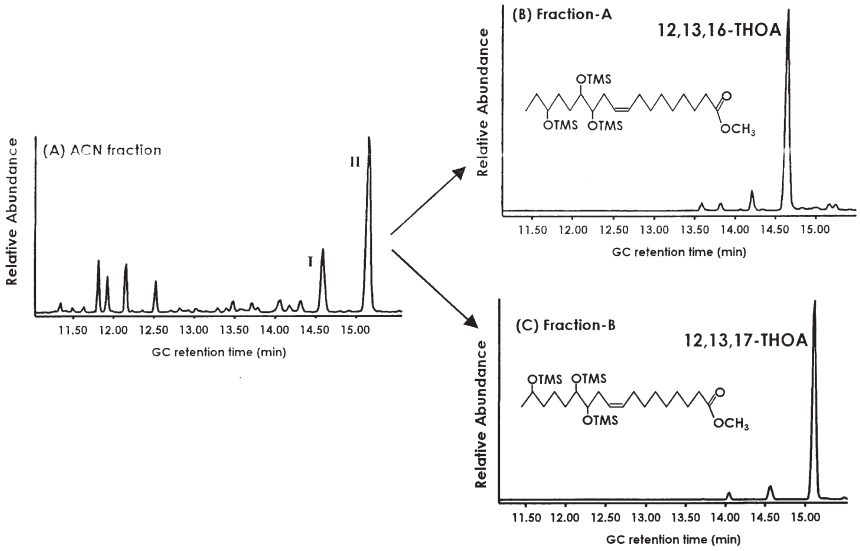


FIG. 1. Gas chromatograms of fractions separated by silica gel column chromatography. (A) Acetonitrile (ACN) fraction of crude extracts (silylated methyl esters) obtained from the culture with strain ALA2 and linoleic acid. I: 12,13,16-trihydroxy-9(*Z*)-octadecenoic acid (12,13,16-THOA). II: 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (12,13,17-THOA). (B) Fraction A was eluted by the first 500 mL of methylene chloride/MeOH (95:5, vol/vol). (C) Fraction B was eluted by the second 500 mL of methylene chloride/MeOH (95:5, vol/vol).

culture was acidified to pH 2 with 6N HCl, and the lipid fraction was extracted twice with 100 mL ethyl acetate followed by 100 mL diethyl ether. The solvent was evaporated from the combined extracts with a rotary evaporator.

Bioconversion. 12,13,16-THOA (188 μL) was added into a 2-d-old culture of strain ALA2 and incubated aerobically for an additional 2 d at 30°C, 200 rpm. A portion (0.5 mL) of the culture was withdrawn into microcentrifuge tube after 24 and 48 h of incubation. Then 20 μL of 6N HCl and 10 μL of palmitic acid (30 mg/mL) in ethyl acetate were added as previously reported (11). The lipid fraction was extracted twice with 0.5 mL ethyl acetate and then once with 0.5 mL diethyl ether. The solvent was removed with nitrogen gas.

Analyses of products. Reaction products were analyzed by GC–MS and NMR. For GC–MS, silylation of the methyl-esterified extract was achieved with the mixture of $TMSI^+$ pyridine (1:4, vol/vol) for 30 min at room temperature. GC–MS analysis was performed with a Hewlett-Packard Model 5890 gas chromatograph (Palo Alto, CA) interfaced with a Model 5971 mass selective detector operating at 70 eV. The capillary column used was a Hewlett-Packard HP-5-MS cross-linked with 5% phenyl methyl silicone, 30 m × 0.25 mm i.d., film thickness 0.25 μm. The carrier gas was helium and its flow rate was 0.65 mL/min. The GC column was programmed from 65 to 260°C at a rate of 20°C/min and then held at 260°C for 20 min. NMR spectra were obtained with a Bruker model ARX-400 spectrometer (Billerica, MA) equipped with a 5-mm $^{13}C/^{1}H$ dual probe (^{13}C NMR, 100 MHz; ^{1}H NMR, 400 MHz). NMR spectra were recorded with $CDCl_3$ as internal standard and solvent.

RESULTS AND DISCUSSION

Purification of 12,13,16-THOA. We failed to note 12,13,16-THOA in our previous investigation (5). Here we have purified 12,13,16-THOA as follows. To concentrate THOA in the crude extract, partitioning between *n*-hexane and acetonitrile (ACN) was carried out. GC analysis of the silylated methyl esters of

TABLE 1
Proton and ^{13}C NMR Signals and Molecular Assignments for 12,13,16-Trihydroxy-9(*Z*)-octadecenoic Acid[a]

	Resonance signals (ppm)	
Carbon number	Proton	^{13}C
1	—	176.5
2	2.29 *t* (*J* = 7.4 Hz)	34.0
3	1.60 *m*	24.9
4	1.30 *m*	29.0
5	1.30 *m*	29.0
6	1.30 *m*	29.0
7	1.30 *m*	29.4
8	2.06 *m*	27.2
9	5.49 *m*	132.3
10	5.49 *m*	125.4
11	2.20 *m*	31.3
12	3.50 *m*	73.0
13	3.50 *m*	73.8
14	1.50–1.60 *m*	29.5
15	1.50–1.60 *m*	32.6
16	3.50 *m*	73.7
17	1.50 *m*	29.9
18	0.94 *t* (*J* = 7.4 Hz)	9.7
OH	3.80 *s*	—

[a]*t*, triplet; *m*, multiplet; *s*, singlet.

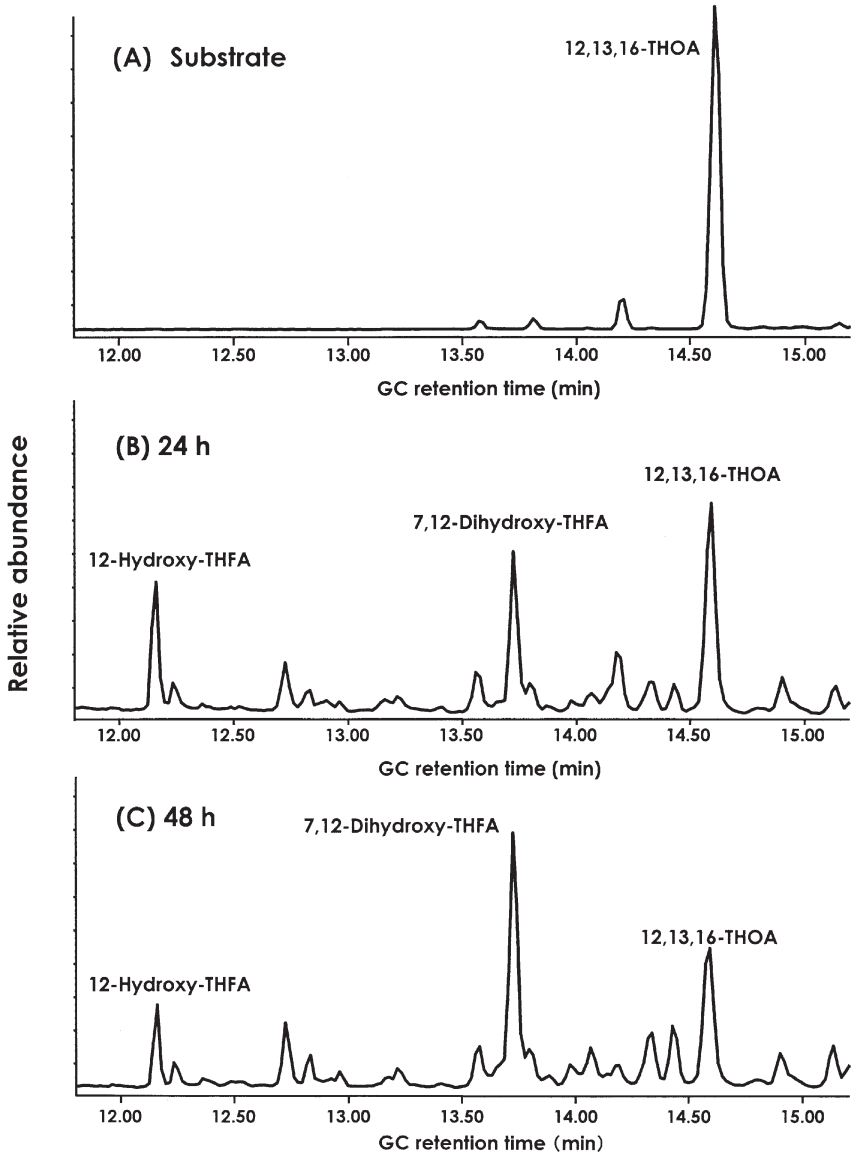
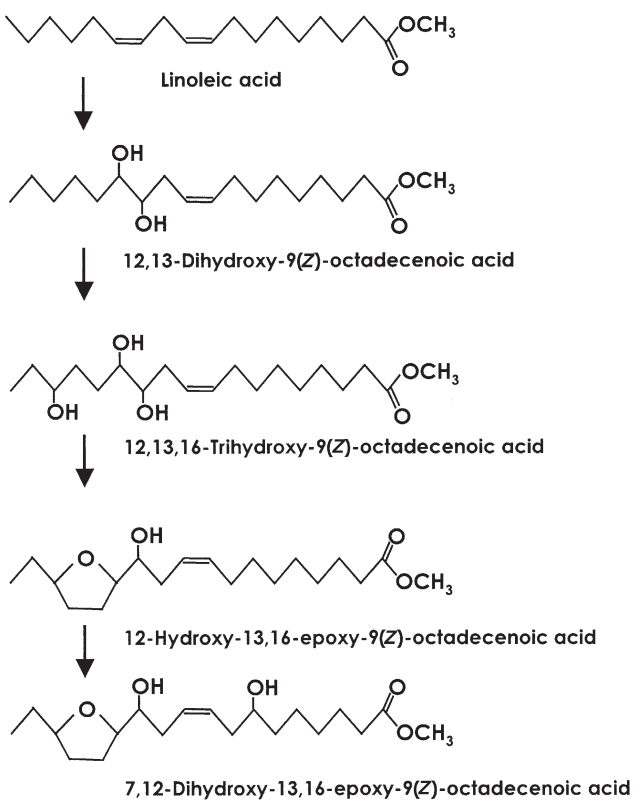


FIG. 2. GC analyses of OTMS/methyl ester derivative of lipid fractions extracted through bioconversion of 12,13,16-THOA by strain ALA2. (A) Substrate only; (B) 24 h after substrate was incubated with ALA2 cells; (C) 48 h after substrate was incubated with ALA2 cells. 12-Hydroxy-THFA, 12-hydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid; 7,12-dihydroxy-THFA, 7,12-dihydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid; for other abbreviations see Figure 1.

the ACN fraction showed many components (Fig. 1A). To purify 12,13,16-THOA, the ACN fraction (16.5 g) was applied onto a column (4 cm i.d. × 50 cm height) packed with Kieselgel 60 (70–230 mesh) that was then eluted with a methylene chloride/MeOH solvent system. After washing the column with 500 mL methylene chloride and then with 500 mL methylene chloride/MeOH (97:3, vol/vol), the column was eluted with 500 mL methylene chloride/MeOH (95:5, vol/vol) to obtain fraction A (2.0 g). A majority of the materials were eluted by an additional 500 mL of methylene chloride/MeOH (95:5, vol/vol) as fraction B. When the OTMS/methyl ester derivative of fraction A was analyzed by GC–MS, a major peak was observed at retention time of 14.6 min (Fig. 1B). The mass spectrum of the major peak was interpreted as follows: EI–MS: *m/z* (relative intensity) 455 $[M - CH_3 - TMSOH]^+$ (0.2), 441 $[M - CH_2CH_3 - TMSOH]^+$ (0.6), 363 $[M - C_{11}H_{18}O_2CH_3]^+$ (3), 351 $[M - CH_2CH_3 - 2TMSOH]^+$ (0.7), 299 $[M - C_6H_{11} - 2(OTMS)]^+$ (11), 273 $[363 - TMSOH]^+$ (9), 270 [rearrangement TMS + $C_{10}H_{18}COOCH_3]^+$ (4), 261 (8), 217 (4), 171 $[261 - TMSOH]^+$ (92), 159 (2), 131 $[C_3H_6OTMS]^+$ (25), 129 (24), 117 (4), 73 (100), 55 (11). Since the mass spectrum was the same as that of 12,13,16-THOA reported previously (5), this major peak was identified as the OTMS/methyl ester derivative of 12,13,16-THOA. 12,13,16-THOA was further purified



SCHEME 1

from fraction A by preparative silica gel TLC with ethyl ether/acetone (4:1, vol/vol) as solvent. 12,13,16-THOA was detected on the plate by iodine vapor at $R_f = 0.41$. The purity of the resulting product, 12,13,16-THOA, was greater than 87% by GC analysis.

NMR analyses. The structure of the purified product was determined by ^{1}H NMR and ^{13}C NMR analyses. The ^{13}C NMR signals at 73.0, 73.8, and 73.7 ppm corresponded to hydroxy groups at the C-12, C-13, and C-16 positions in the molecule, respectively (Table 1). These assignments of the hydroxyl groups were also supported by ^{1}H NMR signals at 3.5 ppm. ^{13}C NMR signals of 132.3 and 125.4 ppm indicated the presence of a double bond between C-9 and C-10. Resonance signals of the olefinic proton were also observed at 5.49 ppm. The double bond was determined to be a *cis* configuration because of the ^{13}C NMR chemical shift at C-8 of 27.2 ppm (12). From these NMR data, the purified product was confirmed to be 12,13,16-THOA. On the other hand, fraction B was analyzed by GC–MS and NMR and confirmed to be 12,13,17-THOA (3) (Fig. 1C). Thus, isomers of 12,13,16-THOA and 12,13,17-THOA were separated, keeping FFA on the silica gel column.

Biosynthesis of THFA. We predicted that THFA were biosynthesized from cyclization of 12,13,16-THOA by the strain ALA2 enzyme system (5). To support this theory, we performed the bioconversion using purified 12,13,16-THOA as substrate. The GC chromatogram of the TMS/methyl ester derivative of an extract obtained after 24 h incubation is shown in Figure 2. The mass spectrum of the peak at a retention time of 12.1 min showed characteristic signals at *m/z* (relative intensity) 299 (16), 270 (13), 201 (34), and 99 (15) as well as other ions [e.g., *m/z* 185 (5), 155 (7), 129 (20), 109 (21), 73 (100), 55 (32)]. These fragments were identical to those reported previously for 12-hydroxy-THFA (4). On the other hand, the peak at retention time 13.7 min was identified as 7,12-dihydroxy-THFA because the characteristic fragment ions at *m/z* (relative intensity) 358 (2), 297 (3), 231 (61), 201 (11), 99 (11), and 73 (100) were identical to those reported previously (4). The relative abundance of 12-hydroxy-THFA, 7,12-dihydroxy-THFA, and 12, 13,16-THOA was 1.6, 3.6, and 4.8, respectively, after 24 h incubation. Afterward, the relative abundance of 7,12-dihydroxy-THFA increased, and that of 12,13,16-THOA and of 12-hydroxy-THFA decreased (12-hydroxy-THFA, 7,12-dihydroxy-THFA, and 12,13,16-THOA were 1.0, 5.5, and 3.2, respectively, after 48 h incubation). These results indicated that 12,13,16-THOA is a precursor in the biosynthesis of 12-hydroxy-THFA and 7,12-dihydroxy-THFA. In addition, the ratio of the peak area of 7,12-hydroxy-THFA to that of palmitic acid (added as internal standard) increased from 8 (24 h incubation) to 15% (48 h incubation), whereas those of 12-hydroxy-THFA were 5% at 24 h and 4% at 48 h incubation, respectively. These results suggest that 12-hydroxy-THFA was converted to 7,12-dihydroxy-THFA by hydroxylation at the C-7 position, analogous to the biosynthesis of diepoxy bicyclic FA (11). It also indicated that the conversion of 12-hydroxy-THFA to 7,12-dihydroxy-THFA was very fast and was not a rate-limiting step.

In conclusion, strain ALA2 converts linoleic acid to 12,13-DHOA first. 12,13-DHOA is then converted not only to 12,13,17-THOA but also to 12,13,16-THOA to a lesser extent. In this paper we showed that 12,13,16-THOA was transformed to 12-hydroxy-THFA by cyclization, and then to 7,12-dihydroxy-THFA by hydroxylation at the C-7 position. Therefore, the biosynthetic pathway for THFA from linoleic acid by strain ALA2 is as shown in Scheme 1.

Moghaddam *et al.* (13) reported that linoleic acid and arachidonic acid can be metabolized to their dihydroxy-THFA (tetrahydrofuran diols) *in vitro* by microsomal cytochrome P-450 epoxidations followed by the reaction of microsomal epoxide hydrolase. In their metabolic pathways, saturated dihydroxy-THFA are produced because the 9,10(12,13)-dihydroxy-12,13(9,10)-epoxy octadecenoates converted from linoleic acid methyl ester are cyclized (14). These saturated dihydroxy-THFA isolated from corn exhibit cytotoxic activity and mitogenic activity for breast cancer and prostate cancer cells (15,16). Furanoid FA have also been found in cod liver (17) and other marine and freshwater species (18). However, their structures are different from those described here. Strain ALA2 produces unsaturated hydroxy-THFA from linoleic acid through a biosynthetic pathway, which is different from the metabolic pathway in mouse liver microsomes. The biological functions of unsaturated hydroxy-THFA are currently under investigation.

REFERENCES

1. Hou, C.T., Biotransformation of Unsaturated Fatty Acids to Industrial Products, *Adv. Appl. Microbiol. 47*:201–220 (2000).

2. Gardner, H.W., C.T. Hou, D. Weisleder, and W. Brown, Biotransformation of Linoleic Acid by *Clavibacter* sp. ALA2: Heterocyclic and Heterobicyclic Fatty Acids, *Lipids 35*:1055–1060 (2000).
3. Hou, C.T., A Novel Compound, 12,13,17-Trihydroxy-9(*Z*)-Octadecenoic Acid, from Linoleic Acid by a New Microbial Isolate *Clavibacter* sp. ALA2, *J. Am. Oil Chem. Soc. 73*:1359–1362 (1996).
4. Hou, C.T., H. Gardner, and W. Brown, Production of Polyhydroxy Fatty Acids from Linoleic Acid by *Clavibacter* sp. ALA2, *Ibid. 75*:1483–1487 (1998).
5. Hou, C.T., H.W. Gardner, and W. Brown, 12,13,16-Trihydroxy-9(*Z*)-Octadecenoic Acid, a Possible Intermediate in the Bioconversion of Linoleic Acid to Tetrahydrofuranyl Fatty Acids by *Clavibacter* sp. ALA2, *Ibid. 78*:1167–1169 (2001).
6. Hou, C.T., and R.J. Forman III, Growth Inhibition of Plant Pathogenic Fungi by Hydroxy Fatty Acids, *J. Ind. Microbiol. Biotechnol. 24*:275–276 (2000).
7. Kato, T., Y. Yamaguchi, N. Abe, T. Uyehara, T. Nakai, S. Yamanaka, and N. Harada, Unsaturated Hydroxy Fatty Acids, the Self-Defensive Substances in Rice Plant Against Blast Disease, *Chem. Lett. 25*:409–412 (1984).
8. Masui, H., T. Kondo, and M. Kojima, An Antifungal Compound, 9,12,13-Trihydroxy-(*E*)-Octadecenoic Acid, from *Colocasia antiquorum* Inoculated with *Ceratocytis fimbriata, Phytochemistry 28*:2613–2615 (1989).
9. Kawagishi, H., M. Ando, T. Mizuno, H. Yokota, and S. Konish, A Novel Fatty Acid from Mushroom *Hericium erinaceum, Agric. Biol. Chem. 54*:1329–1331 (1990).
10. Stadler, M., A. Mayer, H. Anke, and O. Sterner. 1994. Fatty Acids and Other Compounds with Nematicidal Activity from Cultures of *Basidiomycetes, Plant. Med. 60*:128–132 (1994).
11. Iwasaki, Y., W. Brown, and C.T. Hou, Biosynthetic Pathway of Diepoxy Bicyclic Fatty Acids from Linoleic Acid by *Clavibacter* sp. ALA2, *J. Am. Oil Chem. Soc. 79*:369–372 (2002).
12. Lee, K.J., and A.K.L. Cheng, Confirmation of the Carbon Chemical Shifts of Ethylenic Carbon Atoms in Methyl Ricinoleate and Methyl Ricinelaidate, *Nat. Prod. Lett. 3*:65–69 (1993).
13. Moghaddam, M.F., K. Matoba, B. Borhan, F. Pinot, and B.D. Hammock, Novel Metabolic Pathways for Linoleic and Arachidonic Acid Metabolism, *Biochim. Biophys. Acta 1290*:327–339 (1996).
14. Halarnkar, P.P., J. Nourooz-Zadeh, E. Kuwano, A.D. Jones, and B.D. Hammock, Formation of Cyclic Products from the Diepoxide of Long-Chain Fatty Ester by Cytosolic Epoxide Hydrolase, *Arch. Biochem. Biophys. 294*:586–593 (1992).
15. Markaverich, B.M., M.A. Alejandro, D. Markaverich, L. Zitzow, N. Casajuna, N. Camarao, J. Hill, K. Bhirdo, R. Faith, J. Turk, and J.R. Crowley, Identification of an Endocrine Disrupting Agent from Corn with Mitogenic Activity, *Biochem. Biophys. Res. Commun. 291*:692–700 (2002).
16. Markaverich, B., S. Mani, M.A. Alejandro, A. Mitchell, D. Markaverich, T. Brown, C. Velez-Trippe, C. Murchison, B. O'Malley, and R. Faith, A Novel Endocrine-Disrupting Agent in Corn with Mitogenic Activity in Human Breast and Prostatic Cancer Cells, *Environ. Health Perspect. 110*:169–177 (2002).
17. Scrimgeour, C.M. Quantitative Analysis of Furanoid Fatty Acids in Crude and Refined Cod Liver Oil, *J. Am. Oil Chem. Soc. 54*: 210–211 (1977).
18. Gunstone, F.D., R.C. Wijesundera, and C.M. Scrimgeour, The Component Acids of Lipids from Marine and Freshwater Species with Special Reference to Furan-Containing Acids, *J. Sci. Food Agric. 29*:539–550 (1978).

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