A Novel Compound, 12,13,17-Trihydroxy-9(*Z*)-Octadecenoic Acid, from Linoleic Acid by a New Microbial Isolate *Clavibacter* sp. ALA2

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ABSTRACT: A novel compound, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (THOA), was produced from linoleic acid by microbial transformation at 25% yield. The newly isolated microbial strain that catalyzed this transformation was identified as *Clavibacter* sp. ALA2. The product was purified by high-pressure liquid chromatography, and its structure was determined by ^{1}H and ^{13}C nuclear magnetic resonance, Fourier transform infrared, and mass spectroscopy. Maximum production of THOA was reached after 85 h of reaction. THOA was not further metabolized by strain ALA2. This is the first report on 12,13,17-trihydroxy unsaturated fatty acid and its production by microbial transformation.
JAOCS 73, 1359–1362 (1996).

KEY WORDS: Bioconversion, *Clavibacter*, linoleic acid, trihydroxy fatty acid, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid, unsaturated fatty acids.

Microbial conversions of unsaturated fatty acids have been widely exploited, especially at our Center. The literature reveals that oleic acid is converted to 10-hydroxy- and 10-ketostearic acids by hydratases in many bacteria and fungi. Recently, Hou reviewed this topic (1). The hydratases from *Flavibacterium* sp. DS5 and others (2,3) are C10-positional specific enzymes. Production of dihydroxy unsaturated fatty acids has also been reported. Oleic acid was converted to 7,10-dihydroxy-8(*E*)-octadecenoic acid (4–6) *via* a 10-hydroxy-8(*Z*)-octadecenoic acid as intermediate (7). Microbial oxidation of di-, and polyunsaturated fatty acids has been reported. Linoleic and linolenic acids were converted to 10-hydroxy-12(*Z*)-octadecenoic and 10-hydroxy-12(*Z*),15(*Z*)-octadecadienoic acid, respectively, by *Nocardia* (8) and *Flavobacterium* (3,9). Dihydroxy-unsaturated fatty acids have also been synthesized from oleic acid with selenium dioxide as catalyst (10).

Oxygenated metabolites of unsaturated fatty acids play a variety of important roles in biological systems. Enzymatic conversion of lipid hydroperoxides, products of reactions catalyzed by lipoxygenase, has been reported in many higher plants (11). Hydroperoxide isomerase converts lipid hydroperoxides to trihydroxy fatty acids. Hydroperoxide isomerase from flaxseed was the first enzyme found that could metabolize lipid hydroperoxides (12) to α- and β-ketols. 8,9,13-Trihydroxy docosaenoic acid was produced by yeast as an extracellular lipid (13). 9,10,13-Trihydroxy-11(*E*)- and 9,12,13-trihydroxy-10(*E*)-octadecenoic acids were detected in beer (14). It has been suggested that these trihydroxy fatty acids are formed from linoleic acid during the processes of malting and mashing of barley (15). Gardner *et al.* reported the production of diastereomeric 11,12,13-trihydroxy-9(*Z*)-octadecenoic acids and four isomers of 9,12,13(9,10,13)-trihydroxy-10(11)(*E*)-octadecenoic acids by acid-catalyzed transformation of 13(*S*)-hydroperoxylinoleic acid (16). Kato *et al.* (17,18) reported that hydroxy and epoxy unsaturated fatty acids, present in some rice cultivars, acted as antifungal substances and were active against rice blast fungus. It was postulated that these fatty acids were derivatives of linoleic and linolenic acid hydroperoxides. Recently, mixed hydroxy fatty acids were isolated from the *Sasanishiki* variety of rice plant which suffered from the rice blast disease, and were shown to be active against the fungus (19). Their structures were identified as 9*S*,12*S*,13*S*-trihydroxy-10-octadecenoic acid and 9*S*,12*S*,13*S*-trihydroxy-10,15-octadecadienoic acid (20–22). 9,12,13-Trihydroxy-10(*E*)-octadecenoic acid was also isolated from *Colocasia antiguorum* inoculated with *Ceratocystis fimbriata*, and it showed anti-black rot fungal activity (23). Hamberg (24) developed a method for regio- and stereochemical analyses of trihydroxy unsaturated fatty acids.

In this report, we described the isolation and the structure determination of a new compound, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (THOA), produced from linoleic acid by a new microbial isolate, *Clavibacter* sp. ALA2. Other than extraction from plant materials and chemical synthesis, this is the first report on production of trihydroxy unsaturated fatty acids by microbial transformation.

MATERIALS AND METHODS

Microorganisms. Microorganisms from soil and water samples were screened for their ability to modify linoleic acid. Each isolate from a single colony on TGY (25) agar plates was grown at 30°C aerobically in a 125-mL Erlenmeyer flask (shaker at 200 rpm) that contained 50 mL of medium with the following composition (per liter): dextrose, 10 g; K_2HPO_4, 5 g; yeast extract, 5 g; soybean meal, 5 g, $FeSO_4 \cdot 7H_2O$, 0.5 g;

$ZnSO_4$, 0.014 g; $MnSO_4 \cdot H_2O$, 0.008 g; and nicotinic acid, 0.01 g. The medium was adjusted to pH 7.0 with dilute phosphoric acid. Cultures were maintained on agar slants with the above-mentioned medium except the addition of 3% agar. Microbial isolates were identified by the Biolog automated bacteria and yeast identification system (Microstation, Hayward, CA).

Chemicals. Linoleic and oleic acids [purity >99% by gas chromatography (GC)] were purchased from Nu-Chek-Prep Inc. (Elysian, MN). All solvents used were HPLC-grade and were obtained from commercial sources. Kieselgel 60 and thin-layer precoated Kieselgel $60F_{254}$ plates were obtained from EM Science (Cherry Hill, NJ).

Bioconversion. Bioconversions were carried out by adding 0.25 mL (0.22 g) linoleic acid to a 24-h-old culture, and the flasks were shaken again at 200 rpm at 30°C for 2–3 d. At the end of this time, the culture broth was acidified to pH 2 with 6 N hydrochloric acid. The culture broth was then extracted with an equal volume of ethyl acetate and then diethyl ether. The solvent was removed from the combined extracts with a rotary evaporator.

Purification of products. Crude extracts of reaction products were subjected to high-performance liquid chromatography (HPLC) to isolate pure material for further identification. A Dynamax-60A silica column (25 cm × 21.4 mm i.d., Rainin Instrument Co. Emeryville, CA) and methylene chloride/methanol (92:8 vol/vol) as solvent were used with a DuPont Instruments (Wilmington, DE) chromatographic pump equipped with a Waters Model 403 refractive index detector (Marlborough, MA) and an ISCO Inc. (Lincoln, NE) V^4 variable wavelength detector. Purity of fractions was analyzed with thin-layer chromatography (TLC) and GC.

Analyses of products. The reaction products were analyzed by TLC and GC as described previously (4,5). Toluene/dioxane/acetic acid (79:14:7, vol/vol/vol) was the TLC solvent system. For GC, the samples were methylated with diazomethane, and then analyzed with a Hewlett-Packard 5890 gas chromatograph (Wilmington, DE), equipped with flame ionization detector, a Supelco SPB-1 capillary column (15 m; i.d. 0.32 mm; 0.25 μm thickness) and a Hewlett-Packard 3392A integrater. GC was run isothermally at 210°C. For quantitative analysis, palmitic acid was added as internal standard prior to the solvent extraction. Methyl palmitate was used as a standard to establish a linear relationship between mass and peak area and to calculate the amount of analyte in samples assayed by GC.

Chemical structure of the product was identified through mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and Fourier transform infrared (FTIR) measurement. Electron impact (EI) and chemical ionization (CI) mass spectra were obtained with a VG 70-VSE high-resolution mass spectrometer. Electron energy and emission current for EI and CI were 70 and 130 eV and 100 and 200 μA, respectively, Ion source temperature was 200°C, and probe temperature was a gradient from 25 to 175°C. Data acquisition and processing were controlled by the VG OPUS data system running on a VAX station 4000 computer. Proton and ^{13}C NMR spectra were determined in deuterated chloroform with a Bruker WM-300 spectrometer (Rheins, Tepten, Germany), operating at a frequency of 300 and 75.5 MHz, respectively. FTIR analysis of both free acid and methyl ester of the product as run in smear on a Mattson infrared Fourier transform CYGNUS 25 spectrometer (Mattson Instruments, Inc., Madison, WI).

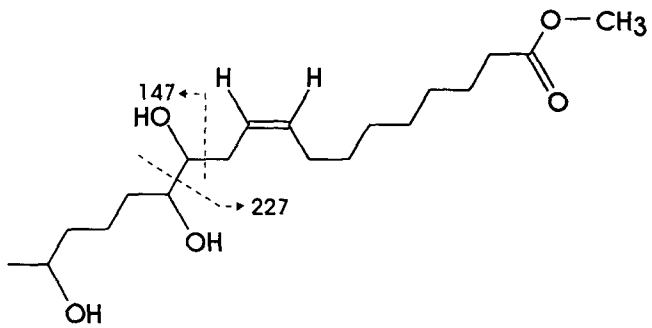
RESULTS AND DISCUSSION

Identification of microorganism. Of the many water and soil samples screened, only one culture, designated ALA2 and isolated from a dry soil sample collected from McCalla, Alabama, converted linoleic acid to more polar compounds at greater than trace amounts. Strain ALA2 is a Gram (+), nonmotile rod (0.5 μm × 2 μm). Further identification with the Biolog automated bacteria identification system showed that strain ALA2 belongs to genus *Clavibacter* and has a 59% biochemical similarity to the closest species of *c. michiganese.* Therefore, strain ALA2 has been assigned as *Clavibacter* sp. ALA2 (Hou, unpublished data).

Structure determination. The main reaction product, purified by HPLC, is a colorless, oily liquid. It showed a single spot (Rf = 0.14) on TLC and 93% purity by GC.

The chemical ionization spectrum of the methyl ester prepared with diazomethane gave a molecular ion of *m/z* 345. Fragments of 327 (M-18), and 309 (M-2 × 18) were also seen. The electron impact spectrum of the methylated product provided more fragments for structural analysis. Ions formed from α-cleavage with respect to the hydroxy group give characteristic fragmentation patterns that provide sufficient information to determine the position of the hydroxy group (26). Large fragments corresponding to α-cleavage with ions *m/z* 227 (25%) and 129 (147–18. 100%) place hydroxy groups at the C-12 and C-13 positions, and the third hydroxy group at a position between carbon 14 and 18 (Scheme 1). This was further confirmed by GC/MS of the trimethylsilyl-derivative of the product, which gave large fragments at *m/z* 299 (18%), 273 (14%) and 171 (100%). Therefore, the product is likely a trihydroxy monoene C_{18} fatty acid with hydroxy groups at C-12 and C-13.

FTIR of the free acid showed absorption of the acid hydroxy group around 2800–3200 cm^{-1} and the alkyl hydroxy



SCHEME 1

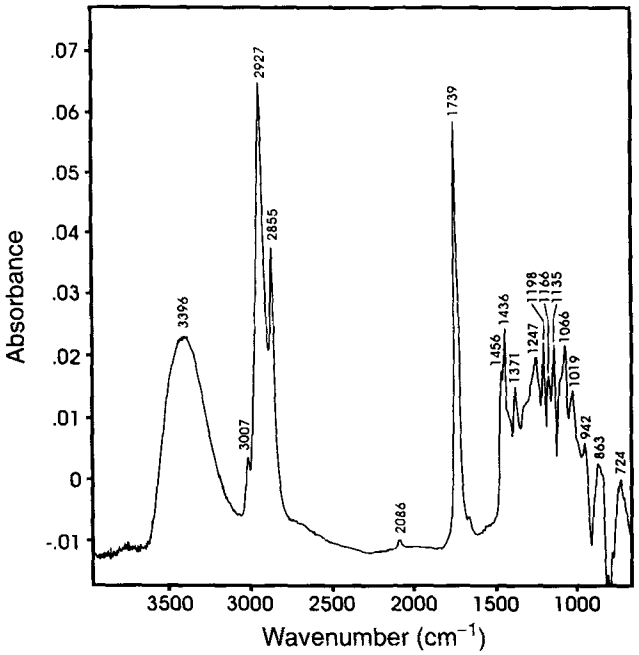


FIG. 1. Fourier transform infrared of the methyl ester of product from strain ALA2.

groups at 3410 cm^{-1}. The FTIR of the methyl ester lacked the absorption for the acid hydroxy group at 2800–3200 cm^{-1} but retained the alkyl hydroxy group at 3397 cm^{-1} (Fig. 1). As expected, the carbonyl at 1710 cm^{-1} for the acid shifted to 1739 cm^{-1} for the ester. Strong CH stretches were seen at 2856 and 2928 cm^{-1}. No keto carbonyl was detected. Unsaturation was seen at 3007 cm^{-1}. In the absence of significant absorbance at 970 cm^{-1} (evidence of *trans* double bonds), the unsaturation seen at 3007 cm^{-1} is presumed to be *cis*.

TABLE 1
Proton and ^{13}C Nuclear Magnetic Resonance Signals and Molecular Assignments for Bioconversion Product

Carbon number	Resonance Chemical shifts (ppm)/coupling (Hz)	
	^{13}C	Proton
1	174.4	—
2	34.1	2.29 *t* J2,3 = 7.4[a]
3	24.9	1.60 *m*
4	29.0	1.30 *bs*
5	29.0	1.30 *bs*
6	29.0	1.30 *bs*
7	29.5	1.30 *bs*
8	27.3	2.04 *m* J8,9 = 7.0
9	133.8	5.55 *m* J9,10 = 10.7
10	124.6	5.40 *m* J10,11 = 7.2
11	31.7	2.29 *m*
12[b]	73.7	3.48 *m*
13[b]	73.8	3.48 *m*
14	33.5	1.48 *m*
15	21.7	1.30 *bs*
16	39.1	1.45 *m*
17	68.0	3.82 *m* J17,18 = 6.1
18	23.5	1.18 *d*
OCH_3	51.5	3.65 *s*

[a]Coupling constant J in Hz.

[b]Shift may be interchanged.

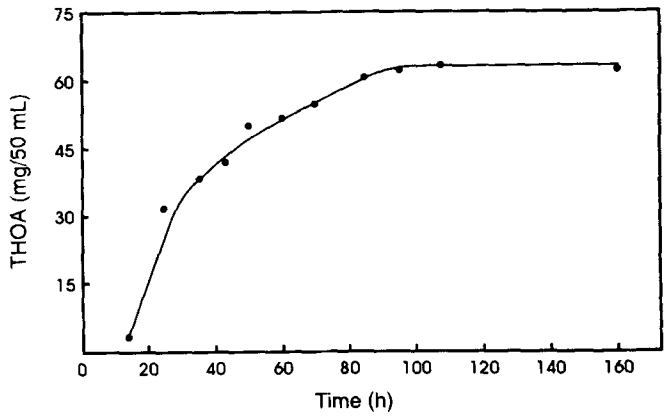


FIG. 2. Time course of the production of 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (THOA) from linoleic acid by strain ALA2.

The reaction product was also subjected to proton and ^{13}C NMR analyses. Resonance signals (ppm) and corresponding molecular assignments given in Table 1 located hydroxy groups at C_{12}, C_{13}, and C_{17} and identified the bioconversion product as 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid. The coupling constant of 10.7 Hz at $C_{9,10}$ confirmed our infrared data that the unsaturation is in *cis* configuration. As far as we are aware, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid has not been reported.

Time course. To develop a production process, the time course of the bioconversion of linoleic acid to THOA was studied. The reaction was conducted at 30°C for the time specified. The amount of the product THOA in the culture media increased with time and reached a maximum after 85 h of reaction (Fig. 2). Further incubation did not reduce THOA content in the medium, indicating that strain ALA2 did not metabolize THOA. The biological activity of THOA is currently under investigation.

ACKNOWLEDGMENTS

The author gratefully acknowledges the excellent technical assistance of W. Brown and her contribution of soil and water samples for screening. The author also thanks Dr. D. Weisleder for NMR, Dr. D. Labeda for the identification of strain ALA2, Dr. T. Abbott for FTIR measurements, and Dr. H. Gardner for his useful discussion. High-resolution mass spectra were obtained through the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, Urbana, IL.

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[Received January 12, 1996; accepted April 16, 1996]