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Microbial production of a novel trihydroxy unsaturated fatty acid from linoleic acid

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A bacterium isolated from a dry soil sample collected from McCalla, AL, USA, converted linoleic acid to a novel compound, 12,13,17-trihydroxy-9 (*Z*)-octadecenoic acid (THOA). The organism is a Gram-positive, non-motile rod (0.5 μm × 2 μm). It was identified as a species of *Clavibacter* ALA2. The product was purified by high pressure liquid chromatography, and its structure was determined by ^{1}H and ^{13}C nuclear magnetic resonance and Fourier transform infrared spectroscopies, and by mass spectrometer. Maximum production of THOA with 25% conversion of the substrate was reached after 5–6 days of reaction. THOA was not further metabolized by strain ALA2. This is the first report of a 12,13,17-trihydroxy unsaturated fatty acid and its production by microbial transformation. Some dihydroxy intermediates were also detected. THOA has a structure similar to those of known plant self-defense substances.

Keywords: unsaturated fatty acids; trihydroxy fatty acid; bioconversion; *Clavibacter*; linoleic acid; 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid

Introduction

Microbial conversions of unsaturated fatty acids to their hydroxy products have been widely exploited. Microbial production of monohydroxy fatty (10-hydroxy stearic) acid was first reported by Wallen *et al* in 1962 [25]. The literature reveals that oleic acid is converted to 10-hydroxy- and 10-keto-stearic acids by hydratases in many bacteria and fungi. Recently, this topic was extensively reviewed by Hou [10]. The hydratases from *Flavobacterium* sp DS5 and others [7,9] are C10 positional-specific enzymes. We also reported the first microbial production of dihydroxy unsaturated fatty acid from oleic acid [13]. Oleic acid was converted to 7,10-dihydroxy-8(*E*)-octadecenoic acid [11,13,14] via a 10-hydroxy-8(*Z*)-octadecenoic acid as intermediate [12]. Microbial oxidation of di-, and poly-unsaturated fatty acids has also 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* sp [20] and *Flavobacterium* sp [8,9]. Dihydroxy-unsaturated fatty acids have been synthesized chemically from oleic acid by utilizing selenium dioxide as catalyst [19].

In our ongoing search for microorganisms capable of converting fatty acids to value-added products, a new microbial culture, strain ALA2, isolated from a dry soil sample collected from Alabama was found to convert linoleic acid to a new trihydroxy unsaturated fatty acid (TUFA). Microbial production of TUFAs are rare. 8,9,13-Trihydroxy docosaenoic acid was produced by yeast as an extracellular lipid [23]. 9,10,13-Trihydroxy-11(*E*)- and 9,12,13-trihydroxy-10(*E*)-octadecenoic acids were detected in beer [5]. It has been suggested that these trihydroxy fatty acids were formed from linoleic acid during the processes of malting and mashing of barley [1]. However, TUFAs were found in many plants. Enzymatic conversion of lipid hydro-peroxides, products of reactions catalyzed by lipoxygenase, has been reported in many higher plants [26]. Hydroperoxide isomerase converts lipid hydroperoxides to trihydroxy fatty acids. Hydroperoxide isomerase from flaxseed was the first enzyme found that could metabolize lipid hydroperoxides [27] to α- and β-ketols. Kato *et al* [15,18] 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 infected with rice blast, and were shown to be active against the fungus [16]. 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 [17,24]. 9,12,13-Trihydroxy-10 (*E*)-octadecenoic acid was isolated from *Colocasia antiquorum* inoculated with *Ceratocystis fimbriata*, and showed anti-black rot fungal activity [21]. However, these plant self-defense substances were found only in trace amounts. Gardner *et al* [3] reported the production of diastereomeric (*Z*)-11,12,13-trihydroxy-9-octadecenoic acids and four isomers of (*E*)-9,12,13 (9,10,13)-trihydroxy-10(11)-octadecenoic acids by acid-catalyzed transformation of 13(*S*)-hydroperoxylinoleic acid. Regio- and stereochemical analyses of such trihydroxy unsaturated fatty acids were carried out by Hamberg's method [4,6].

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In this paper, we describe the identification of a new microbial isolate, the isolation and the structure determination of a new compound, 12,13,17-trihydroxy-9 (*Z*)-octadecenoic acid (THOA) produced from linoleic acid and the optimum conditions for the production of THOA. Other than extraction from plant materials, 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 [2] agar plates was grown at 30°C aerobically in a 125-ml Erlenmeyer flask (shaken 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 pH of the medium was adjusted to 7.0 with dilute phosphoric acid. Cultures were maintained on agar slants with the above-mentioned medium except for the addition of 3% agar. Microbial isolates were identified by the Biolog automated microbial identification system (Microstation, Hayward, CA, USA).

Chemicals

Linoleic and oleic acids (purity >99% by gas chromatography (GC)) were purchased from NuChek-Prep Inc (Elysian, MN, USA). 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, USA).

Bioconversion

Bioconversions were carried out by adding 0.4 ml (0.35 g) linoleic acid to 24-h-old culture flasks and the flasks were shaken again at 200 rpm at 30°C for 4–5 days. At the end of the incubation 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 by rotary evaporation. Control runs without microbial culture showed no production of THOA.

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, USA) and methylene chloride/methanol (92 : 8 v/v) as solvent were used with a DuPont Instruments (Wilmington, DE, USA) chromatographic pump equipped with a Waters Model 403 reflective index detector (Marlborough, MA, USA) and an ISCO Inc (Lincoln, NE, USA) 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 [11,13]. Toluene/dioxane/acetic acid (79 : 14 : 7, v/v/v) was the TLC solvent system. For GC, the samples were methylated with diazomethane. GC of these methyl esters was analyzed with a Hewlett Packard 5890 gas chromatograph (Wilmington, DE, USA) 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 as described previously [11,13].

The 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 are 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 ARX400 spectrometer (Billerica, MA, USA), operating at a frequency of 400 and 100 MHZ, respectively. FTIR analysis of both free acid and methyl ester of the product was run on a Perkin Elmer infrared Fourier Transform Model 1750 spectrometer (Perkin Elmer, Oak Brook, IL, USA).

Results and discussion

Identification of microorganism

Of the many water and soil samples screened, only one culture, designated ALA2, 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-positive, nonmotile rod (0.5 μm × 2 μm). Comparison with known strains using the Biolog System indicated that strain ALA2 had the characteristics of the genus *Clavibacter*, and has a 58% similarity to the closest matching species, *C. michganese*. Strain ALA2 is therefore designated *Clavibacter* sp ALA2 and has been deposited in the ARS Culture Collection as NRRL B-14943.

Structure determination

The main reaction product (GC retention time at 24 min), purified by HPLC is a colorless, oily liquid. It appeared as a single spot (R_f = 0.14) on TLC and was 93% pure by GC analysis.

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 [22]. Large fragments corresponding to α-cleavage with ions m/z 227 (25%) and 129 (100%) place two hydroxy groups at the C-12 and C-13 positions and the third hydroxy group at a position higher than carbon 13 (Figure 1). This was further confirmed by GC/MS of the TMS-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 two hydroxy groups at C-12 and C-13, and the third hydroxy group at a position higher than carbon 13.

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

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 three hydroxy groups at C-12, C-13 and C-17 and further confirmed the identity of the bioconversion product as

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.

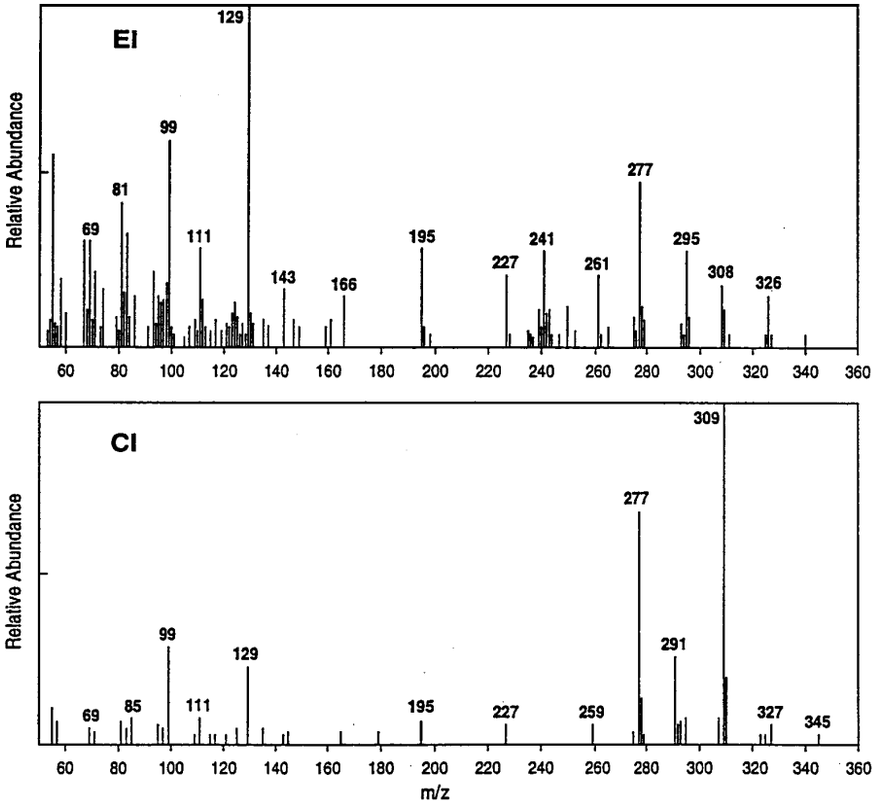
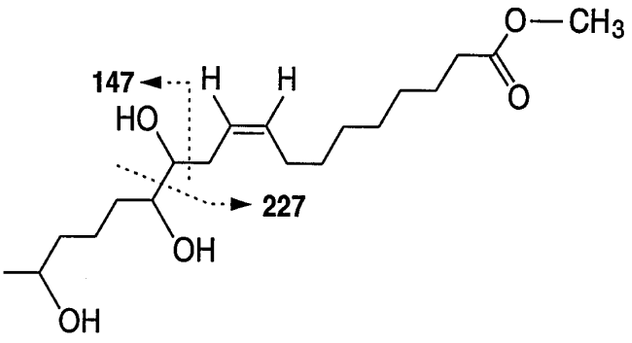


Figure 1 CI and EI mass spectra of the methyl ester of product from strain ALA2.



Scheme 1 Methyl ester of the microbial conversion product generated from linoleic acid by strain ALA2 and its mass fragmentation.

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 (Scheme 1). As far as we are aware, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid has not been reported.

Other reaction products

Typical reaction products analyzed by GC are shown in Figure 2. In addition to the main reaction product (retention time 24 min), there were small amounts of products at Rt 13 and 17 min (possible intermediates; mass spectral fragments indicated the presence of dihydroxy structure. Unpublished data). The structures of these compounds as well as those others having GC retention time at Rt 7.9, Rt 10.3 and Rt 19.8 (Figure 2) are currently under investigation.

In order to develop a production process, the following variables of the bioconversion of linoleic acid to THOA were studied.

Effect of pH

The effect of pH on the production of THOA was studied using 0.1-M buffer solutions. Potassium phosphate buffer was used for pH 5.5–7.0 and Tris buffer was used for pH 7.5–8.5. The production of THOA was found in pH between 6.5 and 8.0 with a peak at 7.0.

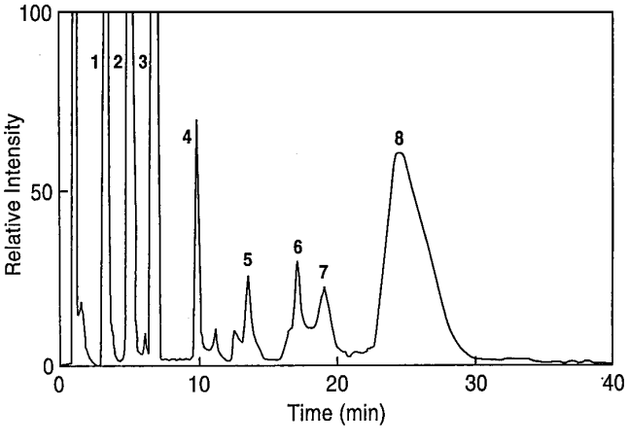


Figure 2 A typical gas chromatography of strain ALA2 reaction products. 1: Internal standard, palmitic acid. 2: Substrate, linoleic acid. 3–7: Unknown. 8: product, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid.

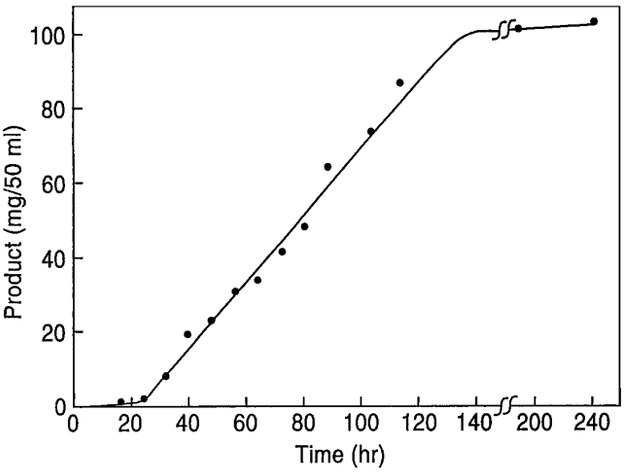


Figure 3 Time course of the production of THOA from linoleic acid by strain ALA2.

Effect of temperature

The effect of temperature on the production of THOA was studied between 15 and 45°C. THOA production was found at temperatures between 25 and 35°C with a peak at 30°C.

Time course

The reaction was carried out 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 5–6 days of reaction (Figure 3). 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.

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