Production of Polyhydroxy Fatty Acids from Linoleic Acid by *Clavibacter* sp. ALA2

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ABSTRACT: Hydroxy fatty acids are important industrial materials. We isolated a microbial culture, *Clavibacter* sp. ALA2, which converts linoleic acid to many polyhydroxy fatty acids. Structures of the products were determined as: 12,13,17-trihydroxy-9(*Z*)-octadecenoic (THOA, main product), 12-[5-ethyl-2-tetrahydrofuranyl]-7,12-dihydroxy-9(*Z*)-dodecenoic (ETDDA), and 12-[5-ethyl-2-tetrahydrofuranyl]-12-hydroxy-9(*Z*)-dodecenoic (ETHDA) acid. The yield of THOA was 25% and the relative amount of the products were THOA/ETDDA/ETHDA = 9:1.3:1. The structures of the hydroxy unsaturated fatty acids resemble those of plant self-defense substances.

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KEY WORDS: Biocatalysis, biotransformation, enzymes, hydroxy unsaturated fatty acids, microbes.

Plant systems are known to produce hydroxy fatty acids, which are important industrial materials. The hydroxy group gives a fatty acid special properties, such as higher viscosity and reactivity compared with other fatty acids. Presently, imported castor oil and its derivatives are the only commercial source of these industrial hydroxy fatty acids.

Because of their special chemical attributes, hydroxy fatty acids are used in a wide range of products, including resins, waxes, nylons, plastics, corrosion inhibitors, cosmetics, and coatings. Furthermore, they are used in grease formulation for high-performance military and industrial equipment. Ricinoleic and sebacic acids, two of the castor derivatives, are classified by the Department of Defense as strategic and critical materials.

Production of hydroxy fatty acids through biocatalysis has drawn more attention recently. There are many reports on the production of monohydroxy fatty acids by various microbial species (1–6). Conversion of oleic acid to a new dihydroxy unsaturated fatty acid by *Pseudomonas aeruginosa* PR3 was also demonstrated (7–10). Microbial oxidation of unsaturated fatty acids was reviewed recently (11).

We have been investigating the production of value-added products from soybean oil. Most recently, we have isolated a new microbial culture, *Clavibacter* sp. ALA2 (12), which converts linoleic acid to a trihydroxy and other hydroxy unsaturated fatty acid products. We have identified structures of two additional products as: 12-[5-ethyl-2-tetrahydrofuranyl]-12-hydroxy-9(*Z*)-dodecenoic and 12-[5-ethyl-2-tetrahydrofuranyl]-7,12-dihydroxy-9(*Z*)-dodecenoic acids. Other than extraction from plant materials, this is the first report on the production of trihydroxy unsaturated fatty acids and tetrahydrofuranyl hydroxy fatty acids by microbial transformation. In this paper, we report the isolation and structure determination of these two new hydroxy unsaturated fatty acid products.

MATERIALS AND METHODS

Microorganisms. Strain ALA2 (12) and other microorganisms were maintained on agar plates with the following composition (per L): 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 grown at 30°C aerobically in a 125-mL Erlenmeyer flask (shaker at 200 rpm) that contained 50 mL of medium with the same composition as above.

Chemicals. Linoleic and oleic acids [purity >99% by gas chromatography (GC)] were purchased from Nu-Chek-Prep Inc. (Elysian, MN). All solvents used were high-performance liquid chromatography (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 0.4 mL (0.35 g) linoleic acid to a 24-h-old culture and shaking the flasks at 200 rpm at 30°C for 4–5 d. At the end of the reaction, 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 (EA) followed by 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 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 hexane/ethyl acetate (gradient) as solvent were used with a Hewlett-Packard (Wilmington, DE) Model 1100 HPLC chromatography system equipped with refractive index (RI) de-

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tector and variable wavelength detector. Gradient elution with hexane and EA was performed as follows: 0 to 50 min (20% to 30% EA), 50 to 200 min (30 to 35% EA), 200 to 250 min (35 to 50% EA), 250 to 270 min (50 to 100% EA), and held at 100% EA until 350 min. The flow rate was 8 mL per min. Fractions were collected based on the response on RI and absorption spectra. 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 (12). Toluene/dioxane/acetic acid (79:14:7, vol/vol/vol) was the TLC solvent system. For GC, the samples were methylated with diazomethane. GC of these methyl esters were analyzed with a Hewlett-Packard 5890 gas chromatograph equipped with flame-ionization detector, a Supelco (Belletone, PA) SPB-1 capillary column (15 m; 0.32 mm i.d.; 0.25 μm thickness) and a Hewlett-Packard 3392A integrator. 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.

$KMnO_4$ oxidation of double bonds was used as outlined by Hamberg *et al.* (13). Alcohols were oxidized by pyridinium chlorochromate as described by Corey and Suggs (14). Other chemical procedures are described by Gardner (15). GC–mass spectrometry (MS) was accomplished with a Hewlett-Packard Model 5890 gas chromatograph interfaced with a Model 5971 mass selective detector operating at 70 eV. The capillary column used was a Hewlett-Packard HP-5MS cross-linked 5% phenyl methyl silicone, 0.25 mm × 30 m, film thickness 0.25 μm. The methyl esterified-OTMSi derivatives were separated by temperature programming from 160 to 260°C at 5°C/min, then held at 260°C for 10 min (He flow rate = 0.67 mL/min). Dimethyl nonanedioate [retention time (Rt) = 11.9 min] was separated by temperature programming from 65 to 260°C at 10°C/min.

RESULTS AND DISCUSSION

Isolation of reaction products. Figure 1 shows GC analysis of the crude extracts. In addition to the main product, retention time (Rt) 23 min, there are several minor products with Rt of 10, 13, and 17 min. These products were separated by HPLC in fractions around the following HPLC running time: product Rt 10, 78–85 min; product Rt 13, 125–150 min; product Rt 17, 185–193 min; and product Rt 23, 330–340 min. Purity analysis of these products by GC showed 75% purity for Rt 10 and Rt 13 products, 85% purity for Rt 17, and 93% purity for Rt 23.

Structure analyses of products. Chemical structure of the main product (Rt 23) was identified through MS, nuclear magnetic resonance (NMR) spectroscopy, and Fourier transform infrared measurement as described previously (12). It was identified as 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (Scheme 1). Mass spectral analysis of the additional products Rt 17 and Rt 10 are shown in Figure 2A and B, respectively. These products were methyl ester and trimethylsilane ether derivatives. The structure of Rt 13 is currently under investigation.

Product with GC Rt 17 min. The product with a GC Rt of 17 min was shown to be 12-[5-ethyl-2-tetra-hydrofuranyl]-7,12-dihydroxy-9(*Z*)-dodecenoic acid (ETDDA). ETDDA afforded mass spectra indicative of either a terminal oxopentyl, epoxypentyl, ethyltetrahydrofuranyl, or methylpyranyl. This compound was resistant to 14% BF_3 in MeOH (10 min at room temperature), and hydrogenated derivatives were stable to BF_3-MeOH under more stringent conditions (15 min at 65°C), ruling out the presence of epoxide. ETDDA was unchanged after $NaBH_4$ exposure, ruling out an oxopentyl group. Because the

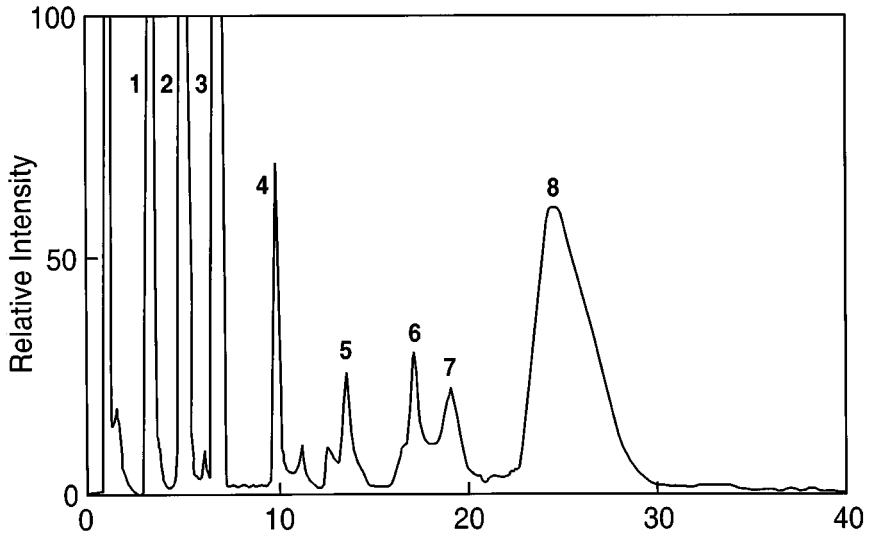
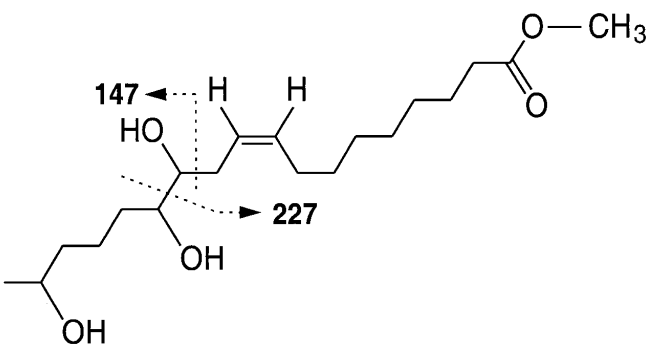
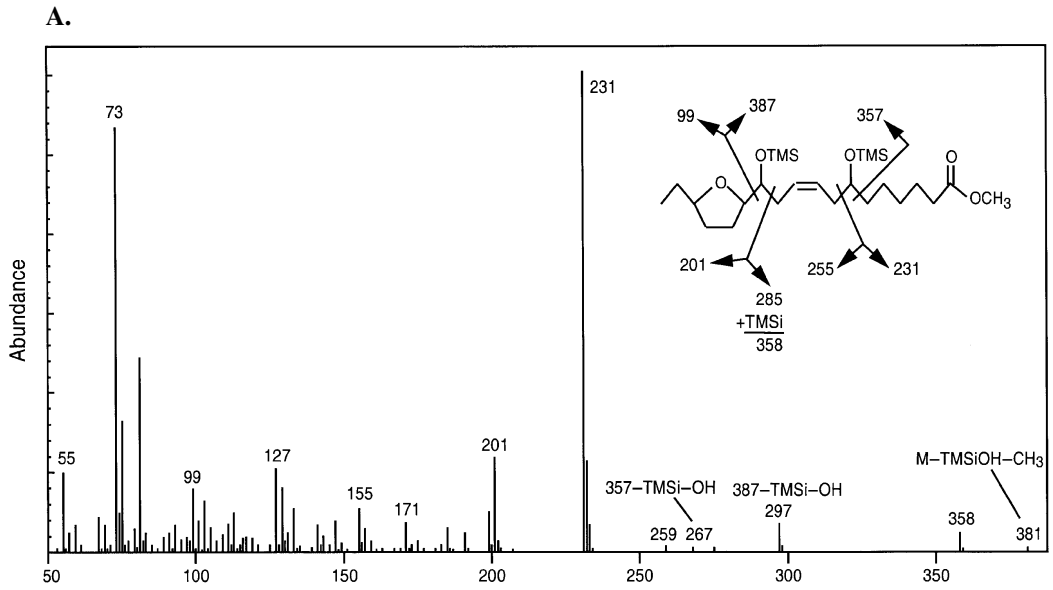


FIG. 1. A typical gas chromatograph of strain ALA2 reaction products. 1: Internal standard, palmitic acid. 2: Substrate, linoleic acid. 3–7: Unknown. 8: main product, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid. See the Materials and Methods section for GC conditions.



SCHEME 1

terminal methyl had ^{1}H NMR triplet multiplicity, this fatty acid was determined to have a terminal ethyltetrahydrofuranyl group. Other features of the ^{1}H-NMR confirm the structure (data not shown). The mass spectrum of the Me ester/OTMSi ether (Fig. 2A) was interpreted as follows; electron ionization mass spectrometry (EIMS) *m/z* (relative intensity): 381 [M – TMSiOH – Me]$^+$ (0.5), 358 [rearrangement TMSi + CH_2CH=$CHCH_2$ $CHOTMSi(CH_2)_5COOMe]^+$ (3), 297 [M – ethyltetrahydrofurany l – TMSiOH]$^+$ (6), 267 [M – $(CH_2)_5COOMe$ – TMSiOH]$^+$ (1), 259 (1), 231 [$CHOTMSi(CH_2)_5COOMe]^+$ (100), 201 [ethyltetrahydrofuranyl – CHOTMSi]$^+$ (19), 199 (9), 171 (8), 155 (14), 129 (17), 127 (19), 99 [ethyltetrahydrofuranyl]$^+$



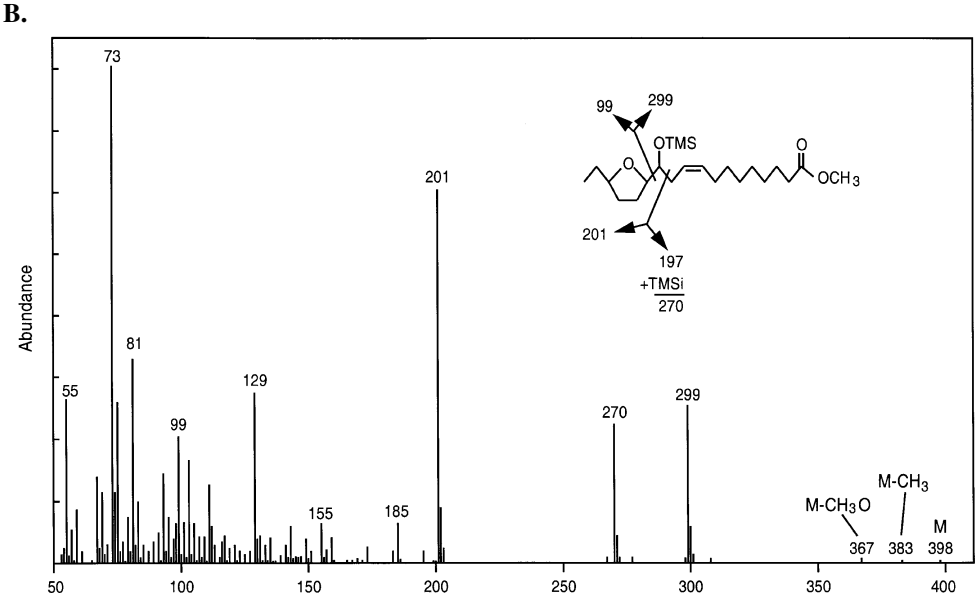


FIG. 2. Mass spectral analyses of products at GC retention time: Rt 17 (A) and Rt 10 (B).

(18), 81 (40), 73 [TMSi]$^+$ (93), and 55 (16). The mass spectrum of the OTMSi ester/OTMSi ether confirmed the above assignments of the fragments containing the carboxylate function by the presence of an *m/z* ion + 58; otherwise the mass spectrum was largely similar to the previous spectrum; EIMS *m/z* (relative intensity): 416 [rearrangement TMSi + $CH_2CH{=}CHCH_2CHOTMSi\ (CH_2)_5\ COOTMSi]^+$ (3), 355 [M – ethyltetrahydrofuranyl – TMSiOH]$^+$ (3), 289 [CHOTMSi $(CH_2)_5$ COOTMSi]$^+$ (57). Hydrogenation furnished a fatty acid (Me ester/OTMSi ether) with the following EIMS *m/z* (relative intensity): 389 [M – ethyltetrahydrofuranyl]$^+$ (19), 357 [M – $(CH_2)_5COOMe]^+$ (3), 299 [M – ethyltetrahydrofuranyl – TMSiOH]$^+$ (6), 259 (3), 231 [CHOTMSi$(CH_2)_5COOMe]^+$ (28), 209 [M – ethyltetrahydrofuranyl – 2TMSiOH]$^+$ (23), 177 [M – $(CH_2)_5COOMe$ – 2TMSiOH]$^+$ (24), 159 (22), 142 (24), 129 (50), 99 [ethyltetrahydrofuranyl]$^+$ (31), 81 (51), 73 [TMSi]$^+$ (100), and 55 (24). Oxidation of the 7,12-dihydroxyl groups with pyridinium chlorochromate gave three isomers which separated by GC–MS (as methyl esters) with similar spectra, presumably due to double-bond isomerization. Products from incomplete oxidation were identified by preparing the OTMSi ethers. The following representative spectrum of the diketone showed a strong ion due to the ethyltetrahydrofuranyl moiety from facile cleavage between the C-12 oxo and ethyltetrahydrofuranyl group; EIMS *m/z* (relative intensity): 338 [M]$^+$ (0.6), 310 (0.5), 307 [M – MeO]$^+$ (0.4), 241 (0.7), 207 (1), 193 (1), 166 (2), 157 $[CO(CH_2)_5COOMe]^+$ (2), 125 (7), 99 [ethyltetrahydrofuranyl]+ (100), 81 (56), and 55 (26).

Product with GC retention time 10 min. The product with a GC retention time of 10 min was shown to be 12-[5-ethyl-2-tetrahydrofuranyl]-12-hydroxy-9*Z*-dodecenoic acid (ETHDA). Like ETDDA, ETHDA was resistant to 14% BF_3 in MeOH, and did not change with exposure to $NaBH_4$. Additional mass spectral features described below were indicative of a similar terminal ethyltetrahydrofuranyl moiety in its structure. The mass spectrum of the Me ester/OTMSi ether (Fig. 2B) was interpreted as follows; EIMS *m/z* (relative intensity): 398 [M]$^+$ (0.6), 383 [M – Me]$^+$ (0.7), 367 [M – MeO]+ (0.9), 299 [M – ethyltetrahydrofuranyl]$^+$ (37), 270 [rearrangement TMSi + $CH_2CH{=}CH(CH_2)_7COOMe]^+$ (28), 201 [ethyltetrahydrofuranyl – CHOTMSi]$^+$ (77), 185 (11), 155 (12), 129 (48), 99 [ethyltetrahydrofuranyl]$^+$ (28), 81 (45), 73 [TMSi]$^+$ (100), and 55 (35). The mass spectrum of the OTMSi ester/OTMSi ether confirmed the above assignments of the fragments containing the carboxylate function by the presence of an *m/z* ion + 58; otherwise the mass spectrum was very similar to the previous spectrum (except for the absence of M$^+$, M – Me$^+$, and M – MeO$^+$); EIMS *m/z* (relative intensity): 357 [M – ethyltetrahydrofuranyl]$^+$ (26), 328 [rearrangement TMSi + $CH_2CH{=}CH(CH_2)_7COOTMSi]^+$ (20). Hydrogenation furnished a fatty acid (Me ester/OTMSi ether) with the following EIMS *m/z* (relative intensity): 385 [M – Me]$^+$ (1.1), 369 [M – MeO]$^+$ (0.7), 301 [M – ethyltetrahydrofuran]$^+$ (100), 285 (6), 201 [ethyltetrahydrofuranyl – CHOTMSi]$^+$ (2), 197 (5), 161 (7), 143 (9), 129 (35), 99 [ethyltetrahydrofuranyl]$^+$ (30), 95 (36), 81 (46), 73 [TMSi]$^+$ (84), and 55 (33). Oxidation of the 12-hydroxyl group with Corey's reagent (pyridinium chlorochromate) gave five isomers (three major and two minor) which separated by GC–MS (as methyl esters) with similar spectra presumably due to double-bond isomerization. The following representative spectrum showed a strong ion due to the ethyltetrahydrofuranyl moiety from facile cleavage between the C-12 oxo and ethyltetrahydrofuranyl group; EIMS *m/z* (relative intensity): 324 [M]$^+$ (0.7), 296 (1.3), 293 [M – MeO]$^+$ (1.2), 99 [ethyltetrahydrofuranyl]$^+$ (100), 81 (48), and 55 (21). Hydrogenation of the pyridinium chlorochromate oxidation products gave one GC–MS peak, as expected. The hydrogenated derivative continued to show the strong ethyltetrahydrofuranyl fragment ion, as well as several fragment ions of very low intensity, including a few of diagnostic significance (shown here); EIMS *m/z* (relative intensity): 326 [M]$^+$ (1.4), 298 (1.0), 295 [M – MeO]$^+$ (3), 227 $[CO(CH_2)_{10}COOMe]^+$ (0.7), 142 [ethyltetrahydrofuranyl-$COCH_2$ + H]$^+$ (1.1), 99 [ethyltetrahydrofuranyl]$^+$ (100), 81 (44), 74 (8), and 55 (27). The double bond was localized by subjecting the fatty acid to oxidation by KMnO4 in acetic acid. The predominant product after methyl esterification was shown to be diMe nonanedioate by comparison of its GS–MS retention time and spectrum to a standard.

The amount of the main product THOA in the culture media increased with time and reached a maximum after 5–6 days of reaction with a yield of 35%. Further incubation did not reduce THOA content in the medium, indicating that strain ALA2 did not metabolize THOA. The relative amount of products were: main product/Rt 17/Rt 10 = 9:1.3:1.

It is interesting to note that the structures of these products resemble those of the plant self-defense substances (16,17). Other than extraction from plants suffering from fungal diseases, these compounds are for the first time produced by microbial transformation.

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