Microbial Transformation of 12-Hydroxyoctadecanoic Acid to 5-*n*-Hexyl-Tetrahydrofuran-2-Acetic Acid[1]

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ABSTRACT: Stationary-phase cells of a corynebacterium (FUI-2) and a bacillus (NRRL B-14864) isolate, when grown aerobically in 1% YE medium at 25°C, converted 12-hydroxystearic acid to a major compound, 5-*n*-hexyl-tetrahydrofuran-2-acetic acid, and other intermediate and minor compounds (6-hydroxydodecanoic acid, 4-hydroxydecanoic acid, 4-ketodecanoic acid and γ-decanolactone). The yields of 5-*n*-hexyl-tetrahydrofuran-2-acetic acid, 4-hydroxydecanoic acid, and γ-decanolactone, by *Bacillus lentus* NRRL B-14864 were 43%, 18% and 5%, respectively, after 2.5 d of incubation.
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KEY WORDS: *Bacillus lentus*, corynebacterium, D-(+)-12-hydroxy-octadecanoic acid, D-(+)-12-hydroxystearic acid, γ-decanolactone, 4-hydroxydecanoic acid, 6-hydroxydodecanoic acid, 4-ketodecanoic acid, 5-*n*-hexyl-tetrahydrofuran-2-acetic acid.

A microorganism capable of producing small amounts of 2-hydroxymethylfuran-5-carboxylic acid was first reported by Sumiki in 1929 (1). Since then, naturally occurring furan or tetrahydrofuran fatty acids have been reported in *Exocarpus cupressiformis* seed oil, *Hevea brasiliensis* latex, human urine, human plasma, different organs of marine and freshwater fish (2–4), and in a variety of *Streptomyces* cultures (5). Chemically synthesized tetrahydrofuran fatty acid derivatives have been claimed to possess some biological activities of both prostaglandin E (6) and insect juvenile hormone activity (7,8), and also serve as constituents of the antibiotic Nonactin (9).

During efforts to screen microorganisms for biotransformation of oleic acid (10–13), ricinoleic acid (14), or 12-hydroxystearic acid (12-HSA) to value-added product(s), we isolated two bacterial strains, a corynebacterium, FUI-2 and a bacillus, 5d2, that convert 12-HSA to a major compound, 5-*n*-hexyl-tetrahydrofuran-2-acetic acid, and other intermediate and minor compounds.

In this paper, we describe the isolation and identification of products, and bacteria which transform 12-HSA.

EXPERIMENTAL PROCEDURES

Materials. Chemicals and solvents were ACS-grade obtained from commercial sources. The purities of R-(+)-12-hydroxystearic acid, (R,S)-3-hydroxydecanoic acid, γ-decanolactone and palmitic acid were greater than 98% by gas chromatography (GC). 10-Hydroxystearic and 10-ketostearic acids were purified (>95%) from microbial transformation products at our laboratory. Yeast extract and dehydrated skim milk were from Difco Laboratories (Detroit, MI). Sterile disposable tissue culture filters were obtained from Nalge Company (Rochester, NY).

Microorganisms. Six bacterial strains [isolated from fish and from lake water, Macomb, IL (15)] were screened for the bioconversion of 12-hydroxystearic acid. One active bacterial strain, FUI-2, was originally identified as *Acinetobacter* strain FUI-2 (15). Further identification during this investigation concluded that the isolate should be placed within the coryneform group (see results). Also during cultivation of *Corynebacterium* FUI-2, three distinctive contaminant isolates (*Bacillus* species 5d1, 5d2 and 5d4) were obtained. Microorganisms were maintained on Tryptone soy agar at 4°C for short-term storage. Bacteria were reinoculated onto fresh Tryptone agar plates monthly. For long-term storage, fresh growing bacteria were suspended in 10% skim milk and kept at −80°C. *Bacillus lentus* 5d2 (NRRL B-14864) is deposited in the Agricultural Research Service culture collection, USDA (Peoria, IL).

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Media preparation. The 1% YE medium contained 1% yeast extract, 0.2% KH_2PO_4, 0.4% Na_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.01% $CaCl_2 \cdot H_2O$ and 0.001% $FeSO_4 \cdot 7H_2O$, pH 7.2. Freshly prepared media were filtered sequentially through filter paper, 0.45-μm and 0.2-μm sterile tissue culture filters and then stored at 4°C.

Cell growth. Cells were grown aerobically in 10 mL of 1% YE medium at 25°C in 50-mL sterile conical tubes, which were placed in an orbital shaker set at 250 rpm. Samples (0.5 mL) were taken at various time intervals for an OD_{560} reading in a Beckman DU-70 spectrophotometer.

Microbial conversion studies. When OD_{560} reached 2.1, 12-hydroxystearic acid, dissolved in 100% ethanol, was added to 10 mL of the above culture to a final concentration of 0.2% (wt/vol). The final concentration of ethanol was 2% (vol/vol). Incubation continued at 25°C and 250 rpm.

Samples (0.5 mL) were removed at various intervals and acidified to pH 3 with 3 N HCl. Palmitic acid (50 μg), in 100 μL of hexane, was added as internal standard, and then the samples were extracted twice by vortexing 15 s at 4,000 rpm with equal volumes of diethyl ether. Solvent phases were separated by centrifuging in 1.5-mL microfuge tubes at 12,800 × *g* for 10 s. The combined ether layers were washed once with equal volumes of deionized water prior to solvent removal under nitrogen.

For large-scale culture, cells were grown aerobically in 500-mL Erlenmeyer flasks containing 100 mL or 150 mL of 1% YE medium until OD_{560} reached 1.6. 12-HSA was added (final concentration 0.2%, wt/vol), and incubation was continued for 2.5 d. Products were extracted with diethyl ether and worked up in the usual manner.

Mass spectra [GC–mass spectrometry (MS)]. The extracted samples were esterified with diazomethane, and then the solvent was removed under nitrogen. Esters (1 μL of 1% solution in ether) were chromatographed (1 to 50 split) with a Hewlett-Packard (Avondale, PA) model HP 5890A series II gas chromatograph equipped with a flame-ionization detector. Unless mentioned elsewhere, esters were separated on an SPB-1 column (15 m × 0.32 mm i.d. and 0.25 μm thickness; Supelco Co., Bellefonte, PA) either isothermally at 200°C (temperatures of injection port and detector were 230 and 250°C, respectively) or temperature programmed (initially 3 min at 100°C and then increased linearly to 270°C at 10°C/min). Peak areas were determined with a Hewlett-Packard 3396A electronic integrator. Linear standard curves were established for quantitative analyses with methyl palmitate as a standard. Electron-impact mass spectra (70 eV) of methyl esters and trimethyl silyl (TMS-) derivatives (prepared with Tri-Sil-TBT at 80°C for 10 min) were obtained on a gas chromatograph (equipped with DB-1 capillary column, 15 m × 0.25 mm i.d.; J&W Scientific Co., Folsom, CA) coupled to a quadrupole mass spectrometer. Unless mentioned elsewhere, samples were injected (splitless) at 100°C. The temperature was maintained at 100°C for 3 min and then programmed to 270°C at 10°C/min.

Preparative high-performance liquid chromatography (HPLC). Preparative chromatography was conducted isocratically on a Delta Prep 3000 HPLC (Millipore Corp., Milford, MA) with a 99:1 (vol/vol) mixture of methylene chloride and methanol pumped at 25 mL/min. Solvent (25 mL) containing 0.251 g of converted products was injected onto a Dynamax-60A silica column (25 cm × 41.4 mm i.d.; Rainin Instrument Co, Woburn, MA). Effluent was monitored serially by ultraviolet absorbance (245 nm, 0.2 AUFS) and refractive index (64× range; Waters Model 403). Solvent was removed from collected fractions with a rotary evaporator. Samples of each fraction were methylated with diazomethane and analyzed by GC.

GC-Fourier transform infrared (GC–FTIR) spectroscopy. Esters (3–40 μL) were separated on a 15 m × 0.75 mm DB-1 Megabore column (J&W Scientific) with a GC programmed from 130 to 240°C at 10°C/min and held at 240°C for 15 min. The column was connected by transfer line to a Mattson GC-IR interface, consisting of a light pipe and a Sirius 100 spectrophotometer (Analytical Technology Inc., Madison, WI). The light pipe was heated to 220°C, and the MCT detector measured infrared absorbance of eluted components. Spectra of individual GC peaks, collected at 8 cm^{-1} resolution and a scan speed of 80 KHz, were ratioed to adjacent backgrounds. The effluent was returned to the GC flame-ionization detector *via* a light pipe. Liquid-phase spectra were determined as neat films between NaCl crystals on the same spectrophotometer.

Nuclear magnetic resonance (NMR) spectra. Proton and ^{13}C NMR spectra of 5-*n*-hexyl-tetrahydrofuran-2-acetic acid in $CDCl_3$ were obtained with a Bruker WM-300 WB spectrometer equipped with a 5-mm dual probe operating at frequencies of 300 and 75 MHz , respectively.

RESULTS AND DISCUSSION

Identification of microorganisms. Four bacterial strains—5d1, 5d2, 5d4 and FUI-2—transformed R-(+)-12-hydroxystearic acid to significant amounts of converted compounds. Strains 5d2 and FUI-2 were characterized further. Strain FUI-2 was not precisely identified, but because it is an aerobic gram-positive bacterium with short or irregular rods, possessing catalase activity, positive for citrate utilization and negative for indole production, it appears to be a corynebacterium (16,17). Strain 5d2, a gram-positive, spore-forming obligate aerobe, was identified as a *B. lentus* according to its morphological and biochemical characteristics: spore formation (morphological group 1); positive catalase activity; negative for production of acid in glucose, arabinose and xylose; Voges-Proskauer reaction; starch hydrolysis; nitrate reduction; citrate utilization; and indole production (16,17).

Identification of conversion products. Corynebacterium FUI-2 and *B. lentus* NRRL B-14864 transformed 12-HSA to several identical or similar compounds, although their relative abundance varied (Fig. 1). A similar major product (1A3 and 1B4) was identified by analysis of the free acid by GC–MS and NMR and the methyl ester by GC–MS. Ester fragment ions at *m/z* 228 (M, 1%), 111 (71%), 116 (74%) and 143 (100%) were identical to those reported previously (18).

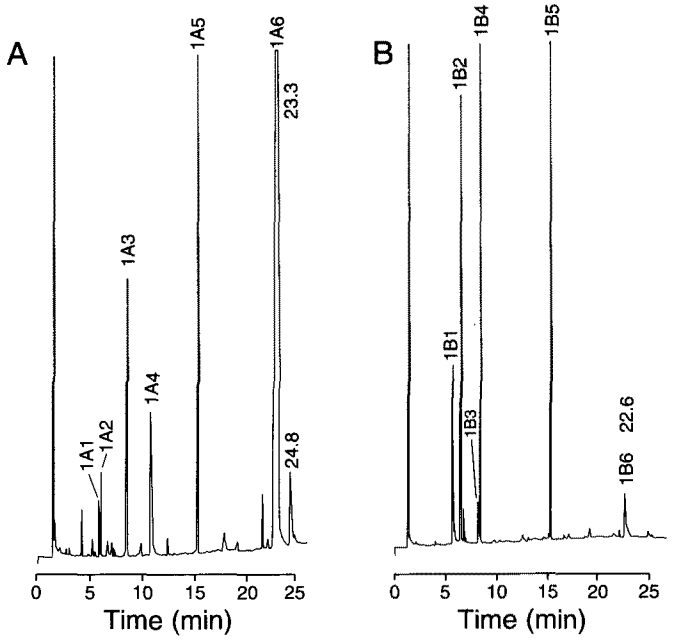

FIG. 1. Capillary gas chromatograms of bioconversion product methyl esters. A: Products from 12-hydroxystearic acid (HSA) by a stationary-phase culture of strain FUI-2 (OD_{560} reached 2.1) after 10 d incubation. Peak 1A1: γ-decanolactone; 1A2: methyl 4-ketodecanoate; 1A3: methyl 5-*n*-hexyl-tetrahydrofuran-2-acetate; 1A4: methyl 6-hydroxydodecanoate; 1A5: methyl palmitate (internal standard); 1A6: methyl 12-hydroxystearate. B: Products from 12-HSA by a stationary-phase culture of *Bacillus lentus* NRRL B-14864 (OD_{560} reached 2.1) after 4 d incubation. Peak 1B1: γ-decanolactone; 1B2: methyl 4-hydroxydecanoate; 1B3 and 1B4 are two diastereomers of methyl 5-*n*-hexyl-tetrahydrofuran-2-acetate; 1B5: methyl palmitate (internal standard); 1B6: methyl 12-hydroxystearate. Peaks 1A1 to 1A4, 1B1 to 1B4 were not present in the absence of substrate. Esters were separated on SPB-1 column as mentioned in Experimental Procedures section, except temperature was increased linearly 5°C/min. The times required for peak 1A6 and 1B6 to emerge were 23.3 and 22.6 min, respectively.

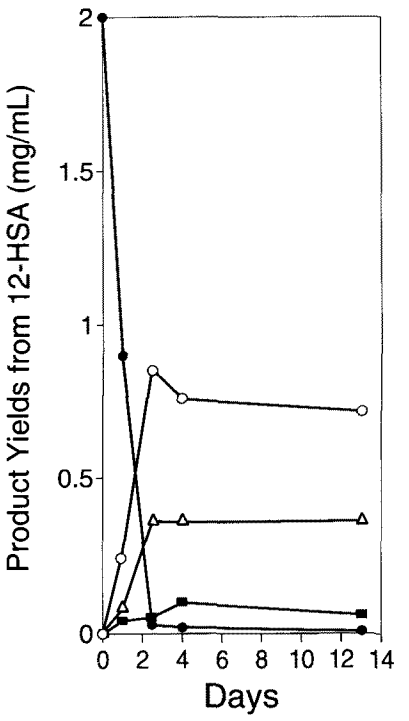

FIG. 2. Typical time course for the transformation of 12-hydroxystearic acid by *Bacillus lentus* B-14864 in 1% YE medium. Product yields are expressed as mg relative to 12-HSA added. γ-Decanolactone (-■-■-■-); 4-hydroxydecanoic acid (-△-△-△-); 5-*n*-hexyl-tetrahydrofuran-2-acetic acid (-○-○-○-) and 12-HSA (-●-●-●-) See Figure 1 for abbreviation.

The base ions from both the acid and ester (*m/z* 214 and 228, respectively) resulted from loss of the hexyl substituents.

Proton NMR spectrum of the free acid showed -C<u>H</u>$_2$-CH-O- at 2.61 ppm (C_{2a}), 2.48 ppm (C_{2b}) at 0.85 ppm (J/Hz 2a,b is 15.3; 2a,3 is 7.1; and 2b,3, is 6) and 2.08 ppm (C_7); two -C<u>H</u>-O- at 3.97 and 4.34 ppm; six -C<u>H</u>$_2$- ranging from 1.25 to 1.56 ppm ($C_{4,5,8-11}$); and a terminal -C<u>H</u>$_3$ (C_{12}) at 0.85 ppm. ^{13}C NMR data also provided information that the compound was a 12-carbon fatty acid and confirmed the presence of -<u>C</u>OOH (C_1) at 176 ppm; two -<u>C</u>H-O- at 79.4 (C_3) and 74.4 ppm (C_6); two -<u>C</u>H$_2$-C-O- at 35.7 ppm (C_7) and 40.5 ppm (C_2); six -<u>C</u>H$_2$-, ranging from 22.6 to 31.8 ppm ($C_{4,5,8-11}$); and -<u>C</u>H$_3$ at 14.0 ppm (C_{12}). ^{1}H and ^{13}C NMR spectra indicated that there were two "-C-O-" linkages located at C_3 and C_6 of the molecule in addition to the carboxyl group at C_1. Because there were no hydroxyl groups (results from NMR of free acid and GC–MS of TMS-derivatized), the molecule is a nonaromatic cyclic ether fatty acid. NMR and MS data clearly identify the compound as 5-*n*-hexyl-tetrahydrofuran-2-acetic acid (maximum yield 43%, Fig. 2).

With two chiral centers in the molecule, four isomers might exist. We observed two peaks for 5-*n*-hexyl-tetrahydrofuran-2-acetic acid (98% vs. 2%) by GC on both polar (DB-FFAP), and nonpolar (SPB-1 or DB-1) capillary columns (Fig. 1B, peaks 1B3 and 1B4). Mass spectra of the two diastereomers were identical. The isomers were not resolved on a cyclodextran-B chiral capillary column. Thus, we do not know which peak represents the *cis* and which represents the *trans* isomer. Maume and Cheetham (19) reported that appreciable amounts of *cis* and *trans* diastereomers of 5-*n*-hexyl-tetrahydrofuran-2-acetic acid were produced by yeast (*Rhodotorula glutinis* and *Sporobolomyces odorus)* during transformation of castor oil to 4-hydroxydecanoic acid.

Peaks (1A1 and 1B1) were identified as γ-decanolactone (maximum yield 5%, Fig. 2) by comparison of FTIR (1812 cm^{-1}, lactone) and GC–MS results with those of commercial γ-decanolactone and literature values (20). Similar microbial conversions to γ-decanolactone and γ-dodecanolactone have been reported from castor, soybean and copra oils or from 10-hydroxy-8(Z)-hexadecenoic and 10-hydroxy-8(Z)-octadecenoic acids (21).

Other products identified by FTIR and GC–MS were 4-ketodecanoic acid (peak 1A2 as methyl ester), 6-hydroxydodecanoic acid (peak 1A4) and 4-hydroxydecanoic acid (peak 1B2; maximum yield 18%, Fig. 2). Microbial conversions of

oleic acid and its ester derivatives to 4-ketodecanoic acid were demonstrated with *Sarcina lutea* (22).

Precedents have been reported for nonenzymatic formation of 5-methyl-tetrahydrofuran-2-acetic acid from (*E*)-2,6-heptadienoic acid or 6-hydroxy-2(*E*)-heptenoic by acid catalysis (23), for tetrahydrofuran fatty acid derivatives under low pH (24) or by Pd (II) activation of hydroxyalkenes (25) and for 5-*n*-hexyl-tetrahydrofuran-2-acetic acid from *R*-(-)-3-hydroxy-5(*Z*)-dodecenoic acid under extremely low pH (18). In all examples, two double bonds or a hydroxy group and a double bond are common structures of those starting materials, and high temperature and low pH are essential for reaction to occur. During ß-oxidation of 12-hydroxystearic acid, similar intermediate structures could lead to formation of 5-*n*-hexyl-tetrahydrofuran-2-acetic acid. However, in this work, cells were grown in pH 7.2 medium at 25°C; afterward, the pH was 8.2. Second, extraction of bioconversion products with or without acidification gave the same amount of 5-*n*-hexyl-tetrahydrofuran-2-acetic acid. Third, *B. lentus* did not survive in 1% YE medium of pH < 3. Conversion did not occur when cells were subjected to acid and temperature treatments (20 min in boiling water), followed by incubation for one or five days. Therefore, nonenzymatic formation of 5-*n*-hexyl-tetrahydrofuran-2-acetic acid is unlikely.

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