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Microbial Oxidation of Oleic Acid

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Resting cells of *Saccharomyces cerevisiae* (baker's yeast, type II; Sigma) were used to convert oleic acid into 10-hydroxyoctadecanoic acid with a 45% yield. *Nocardia aurantia* (ATCC 12674), *Nocardia* sp. (NRRL 5646), and *Mycobacterium fortuitum* (UI 53378) all converted oleic acid into 10-oxo-octadecanoic acid with 65, 55, and 80% yields, respectively. Structures of all metabolites were suggested by ^{1}H and ^{13}C nuclear magnetic resonance and by infrared and mass spectrometry. Structures of isomeric hydroxystearate and oxostearate derivatives and the stereochemical purity of hydroxystearates are difficult to prove unambiguously unless authentic standard compounds are available for spectral comparison. We describe the use of the chemical Baeyer-Villiger oxidation technique with 10-oxo-octadecanoic acid followed by mass spectral analysis of neutral extracts as a simple method to confirm the position of oxo-functional groups in the structures of fatty acid ketones. We further introduce a simple method based on ^{1}H nuclear magnetic resonance analysis of diastereomeric *S*-(+)-*O*-acetylmandelate esters of hydroxystearates as a means of ascertaining stereochemical purities of hydroxy fatty acids.

There is considerable interest in utilizing abundantly available natural resources such as corn oil as renewable feedstocks in the preparation of useful chemicals. Plant oils are rich sources of many types of naturally occurring compounds, including mixtures of glycerides, fatty acids, glycerol, tocopherols, and various sterols, as potential chemical feedstocks (9). We are investigating microbiological transformations of corn oil and compounds that are readily derived from this renewable lipid feedstock as a means of generating value-added products.

Oleic acid (*cis*-Δ^9-octadecenoic acid) is the major component of corn oil, representing nearly 30 to 40% of the fatty acids present in triglyceride mixtures (9). As a useful carbon source, oleic acid has been envisioned as a substrate capable of conversion to a variety of potentially valuable derivatives by both chemical and microbiological means. Chemical treatment of oleic acid with sulfuric acid and subsequent hydrolysis leads to mixtures of 9- and 10-hydroxystearic acids (36). Such hydroxystearic acids and the corresponding keto fatty acids are useful as lubricants (23); as surfactants and plasticizers (26); as components in detergent, coating, and paint industries (35); and in the synthesis of resins (20).

Microbial transformations have been widely exploited in the preparation of many types of useful chemical products (5, 7, 10, 14, 15, 19, 27–30, 39). Microbial transformation reactions expected with oleic acid would include hydration or epoxidation of the double bond (39); reduction of the carboxylic acid; hydroxylation at various positions in the saturated carbon chain, including those allylic to the double bond; degradative reactions in which oleic acid would be transformed into other classes of chemical products; and combinations of these reactions. The literature reveals that oleic acid is converted to ricinoleic acid [*R*-(*Z*)-12-hydroxy-*cis*-9-octadecenoic acid] by a soil bacterium (35) and to 10-hydroxyoctadecanoic and 10-oxo-octadecanoic acids by *Rhodococcus rhodochrous* (*Nocardia aurantia*) (20), *Nocardia cholesterolicum* (17, 18), and *Pseudomonas* sp. (8, 32–34, 39). The 10-hydroxyoctadecanoic acid formed by *Pseudomonas* sp. is optically active (33); it is formed by the stereospecific hydration of the 9,10 double bond (24). Low yields of 9-hydroxyoctadecanoic acid have been reported with chemical (3) or microbial (20) hydration of the oleic acid double bond. Hou et al. recently described 7,10-dihydroxy-8-(*E*)-octadecenoic acid as a novel product of oleic acid biotransformation (12, 13).

For this report we describe microbial transformation screening and preparative-scale experiments concerned with the bioconversion of oleic acid into 1-hydroxyoctadecanoic and 10-oxo-octadecanoic acids. Screening experiments revealed that oleic acid is hydrated by resting cells of *Saccharomyces cerevisiae* with excellent yields and identified genera of fungi, yeasts, and bacteria previously unknown to achieve the hydration reaction. We introduce the use of the chemical Baeyer-Villiger oxidation of fatty acid ketones as a simple chemical oxidation technique that permits the unambiguous assignment of the position of oxygenation in long-chain fatty acid ketones. We describe a simple ^{1}H nuclear magnetic resonance (NMR) spectral technique that measures of stereochemical purity of hydroxy fatty acids.

MATERIALS AND METHODS

Chemicals. Oleic acid, 1-nonanoic acid, 1-decanoic acid, 1,9-nonandioic acid, 1,10-decanedioic acid, 1-octanol, 1-nonanol, dicyclohexylcarbodiimide, and *S*-(+)-*O*-acetylmandelic acid were obtained from Sigma Chemical Co. Their identities and purities were confirmed by thin-layer chromatography (TLC), proton and carbon NMR spectroscopy, and mass spectrometry.

Instrumentation and general procedures. Melting points were recorded on a Thomas Hoover Unimelt capillary melting point apparatus and are uncorrected. Low-resolution mass spectra were obtained on a Trio-1 mass spectrometer linked with a 5890 Hewlett-Packard gas chromatograph equipped with a Quadram (IBM-compatible) computer at high ionization voltage (70 eV). Electron impact gas chromatography-mass spectrometry was achieved with an OV-1 3% column (6 m by 0.25 cm [outer diameter], 80-100 mesh) with a helium carrier gas flow of 20 ml/min; the temperatures

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for the injector, column, and detector were held at 220, 180, and 300°C, respectively. Chemical ionization mass spectral analyses were obtained by using a Nermag R 1010c instrument linked to a gas chromatograph (Girdel series 32, Delsi, France) with helium as the carrier gas and a 5% phenylmethyl silicon column (25 m by 0.2 mm [inner diameter]) with 0.33-mm film thickness. High-resolution electron impact mass spectra were obtained by using direct inlet sample introduction on a VG-ZAB-HF spectrometer. NMR spectra were obtained on a Bruker AMX-600 MHz or Bruker NM-360 MHz high-field spectrometer equipped with an IBM Aspect-2000 processor. ^{1}H- and ^{13}C NMR spectra were recorded at 360.134 or 600.136 MHz with tetramethylsilane as an external standard. Chemical shift values are reported in parts per million, and coupling constants (J values) are given in hertz. Abbreviations for NMR signals are as follows: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; and m, multiplet.

Chromatography. TLC was performed on 0.25-mm layers of silica gel GF_{254} (Merck) prepared on glass plates (5 by 20 cm or 20 by 20 cm) with a Quikfit Industries (London) spreader. Plates were air dried and activated at 120°C for 1 h before use. Plates were developed in a solvent mixture of petroleum ether-diethyl ether-glacial acetic acid (90:10:1, vol/vol/vol), and developed chromatograms were visualized under 365-nm UV light to observe compound fluorescence after spraying with a solution of 0.1% 2,7′-dichlorofluorescene (Aldrich) in methanol. Plates were then sprayed with *p*-anisaldehyde visualization reagent (60 ml of methanol, 0.5 ml of H_2SO_4, and 0.5 ml of *p*-anisaldehyde) before warming with a heat gun to develop a brown color. Gas chromatography was routinely performed on a Hewlett-Packard 5890A gas chromatograph equipped with a Supelco column (30 cm by 0.32 cm [inner diameter], 0.20-mm film thickness; Supelco Inc., Bellefonte, Pa.) and linked to a Hewlett-Packard 3390A integrator. Nitrogen was used as the carrier and makeup gas at flow rates of 30 and 10 ml/min, respectively, and eluting compounds were detected by flame ionization detection. The column, injector, and detector temperatures were maintained at 180 to 210 (5°C/min), 220, and 300°C, respectively. Both column head carrier gas and hydrogen pressures were held at 35 lb/in^2.

Microorganisms. The microorganisms used in this work are contained in the University of Iowa College of Pharmacy culture collection and were maintained on Sabouraud-dextrose agar or sporulation agar (ATCC no. 5 medium) (2). Medium no. 5 slants consisted of 0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, 1 mg of $FeSO_4$ per liter, 1% glucose, and 1.5% agar. The medium was adjusted to pH 7.2 before it was autoclaved.

Cultures screened for their abilities to transform oleic or 10-oxo-octadecanoic acid include dry baker's yeast (Sigma; catalog no. YSC-2) and representatives of the following genera: 2 *Absidia* spp., 1 *Armillaria* sp., 1 *Arthobacter* sp., 1 *Arthrobutrys* sp., 1 *Ascobolus* sp., 1 *Ascoidia* sp., 16 *Aspergillus* sp., 4 *Bacillus* spp., 1 *Botrytis* sp., 1 *Brevilegnia* sp., 1 *Caldariomyces* sp., 12 *Candida* spp., 1 *Cephalosporium* sp., 1 *Chaetomium* sp., 1 *Chrysosporium* sp., 1 *Circinella* sp., 1 *Cladosporium* sp., 1 *Corynebacterium* sp., 7 *Cunninghamella* spp., 1 *Curvularia* sp., 1 *Cyathus* sp., 2 *Cylindrocarpon* spp., 1 *Eurotium* sp., 2 *Hansenula* spp., 1 *Mycobacterium* sp., 4 *Nocardia* spp., 4 *Pseudomonas* spp., 3 *Rhodotorula* spp., 2 *Saccharomyces* spp., 1 *Schizosaccharomyces* sp., and 1 *Zygosaccharomyces* sp.

Fermentation and screening procedure. Cultures were grown by our standard two-stage fermentation protocol (4) in 25 ml of sterile soybean meal-glucose medium held in stainless steel-capped 125-ml DeLong culture flasks. Medium A contained 2% glucose, 0.5% yeast extract, 0.5% soybean meal, 0.5% NaCl, and 0.5% K_2HPO_4 in distilled water and was adjusted to pH 6.8 with 6 N HCl and then autoclaved at 121°C for 15 min. Medium B contained peptone instead of soybean meal but was otherwise the same as medium A. Cultures were incubated with shaking at 250 rpm at 28°C on New Brunswick Scientific G25 Gyrotory shakers. A 10% inoculum derived from 48-h-old stage I cultures was used to initiate stage II cultures, which were incubated for 24 h before receiving of oleic acid as a substrate. Substrate controls consisted of sterile medium containing the same amount of substrate and incubated under the same conditions but without microorganisms.

Substrate-containing cultures were sampled at various time intervals by removing 2 ml of the entire culture. Samples were adjusted to pH 2 with 6 N HCl and extracted with an equal volume of ethyl acetate-methanol (9:1, vol/vol), and the organic and aqueous layers were separated by centrifugation for 1 min in a desk-top centrifuge. The organic solvent layer was removed, evaporated to dryness, and reconstituted in 0.5 ml of methanol, and samples were spotted onto TLC plates for analyses.

RESULTS

Biotransformation of oleic acid (1) to 10-hydroxyoctadecanoic acid (2) by resting cells of *S. cerevisiae*. A 200-mg sample of dry *S. cerevisiae* cells (Sigma) was added to each of eight 125-ml DeLong flasks containing 25 ml of medium A. After 48 h, the activated and growing yeast cells were harvested by centrifugation, and the pelleted cells were evenly distributed among six 1-liter DeLong flasks, each containing 200 ml of 0.1 M phosphate buffer (pH 6.7). Oleic acid (0.5 g) was added to each flask, and incubations were conducted as usual. Samples were withdrawn at various time intervals, and the reactions were terminated at 60 h, at which time TLC indicated that the metabolite yield was at a maximum. The reaction mixtures were pooled, acidified to pH 2, and extracted three times with 2.5 liters of ethyl acetate-methanol (9:1, vol/vol). The combined extracts were washed with water, dried over anhydrous Na_2SO_4, and concentrated under vacuum at 40°C in a rotary evaporator to give 2.6 g of brown oily residue. The residue was dissolved in 1 ml of CH_3OH, adsorbed onto 2 g of silica gel, and placed on top of a silica gel column (120 g, 2.4 by 45 cm). The column was eluted with petroleum ether-ethyl acetate mixtures of increasing polarities. Fractions containing similar components (TLC) were pooled, concentrated, and crystallized from acetone.

The major spot at R_f 0.35 gave 1.35 g (45% yield) of colorless needles of 2, which gave the following physical properties: melting point, 74 to 75°C; ^{1}H NMR (dimethyl sulfoxide-d_6), δ, 1.21 (3H, t, CH_3), 2.09 to 2.50 [18H, m, $(CH_2)_9$], 2.71 (2H, t, CH_2), 4.60 (1H, m, H-10), 5.48 (4H, m, H-9,11), 10.20 (1H, s, COOH); ^{13}C NMR (dimethyl sulfoxide-d_6) δ, 18.81 (C-18), 20.20 (C-17), 24.60 (C-16), 26.00 (C-8 and C-13 to C-15), 28.10 (C-4 to C-7), 31.40 (C-3), 33.70 (C-2), 37.29 (C-11 and C-9), 69.54 (C-10), and 174.81 (C-1); infrared (IR) KBr disc, 3,420 cm^{-1} (OH, COOH); mass spectrometry, NH_3 CI, m/z (ion, percent relative abundance), 318 ($M^+ + 1 + NH_3$, 100%), 300 (M^+, 32%), 282 ($M^+ - H_2O$, 13%), 204, 186, 169, and 158. This compound was identical to an authentic sample of 10-hydroxyoctadecanoic acid (18).

A 5-mg sample of 2 was treated with 200 ml of a 12% borontrifluoride methanol complex (16), and the mixture was heated on a steam bath for 10 min. The reaction mixture was evaporated to dryness under a stream of nitrogen, extracted with 1 ml of ethyl acetate, and subjected to gas chromatography-mass spectrometry. The methyl ester (2a) gave the following high-resolution mass spectral properties: *m/z* 314.4687 (M^+, $C_{19}H_{38}O_3$, calculated 314.5062), 296 (M − H_2O), 283 (M^+ − CH_3O), 201 [CH_3OOC − $(CH_2)_8CHOH$], 172 [CH_3OOC − $(CH_2)_8$ + H^+], 169 (*m/z* 201 − CH_3OH).

Transformation of oleic acid to 10-oxo-octadecanoic acid (3) by cultures of *Mycobacterium fortuitum* (UI AM53378), *Nocardia* sp. (NRRL 5646), and *N. aurantia* (ATCC 12674). The standard two-stage fermentation procedure was used to grow *M. fortuitum*, except that 1% dextrose was used in stage II cultures. A total of 2.5 g of oleic acid was distributed among five 1-liter DeLong flasks; the reaction was terminated after 28 h, at which time all substrate had been consumed. The culture was extracted with 3 liters of ethyl acetate-methanol (9:1, vol/vol) after acidification to pH 2 with 6 N HCl. The organic extracts were washed with an equal volume of distilled water, dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation to 1.8 g of a viscous residue. Metabolite 3 was purified by silica gel column chromatography (120 g of silica gel, 2.5 by 45 cm) by elution with petroleum ether and petroleum ether-acetone mixtures of increasing polarity. Chromatographically similar fractions at R_f 0.4 were pooled and concentrated under vacuum to give 2 g (80% yield) of pure metabolite, which was characterized as described below.

With identical fermentation, isolation and purification procedures, *Nocardia* sp. (NRRL 5646) converted 3 g of 1 in 1,200 ml of medium to 1.3 g of 3 (55% yield) and *N. aurantia* (ATCC 12674) transformed 3.9 g of 1 to 2.1 g of 3 (65% yield).

Physical properties of 3 isolated from *Mycobacterium* and *Nocardia* biotransformation reaction mixtures. The isolated and purified metabolite (3) had the following physical properties: melting point, 79 to 80°C (the value in reference 39 is 83.5 to 84°C); NMR, 360 MHz ($CDCl_3$) δ_H, 0.88 (3H, t, H-18), 1.28 (2H, m, H-17), 1.55 to 2.34 [16H, m, $(CH_2)_8$], 2.35 (2H, m, H-12), 2.38 (2H, m, H-3), 2.96 (2H, m, H-8), 3.41 (2H, m, H-2), 4.53 (2H, m, H-11), 5.66 (2H, m, H-9), and 11.11 (1H, s, COOH); ^{13}C NMR (90 MHz) ($CDCl_3$) δ_C 13.99 (C-18), 22.65 (C-16 and C-17), 23.05 (C-15), 24.0 (C-14), 24.73 (C-6), 29.08 (C-4,5 and C-13), 29.26 (C-7), 29.36 (C-12), 29.41 (C-8), 31.88 (C-3), 34.08 (C-2), 42.76 (C-9), 42.84 (C-11), 179.55 (C-1), and 211.35 (C-10); IR KBr, cm^{-1}, 1720 (C-CO-C), 1,699, 1,471; low-resolution chemical ionization (NH_3) mass spectrum, *m/z* (percent relative abundance) 316 (25%, MH^+ + NH_3), 299 (50.4%, MH^+), 281 (10%, MH^+ − H_2O), 272 (25%, MH^+ + NH_3 − CO_2), 200 (47%, MH^+ − C_7H_{15}), 156 (100%, M^+ − $C_9H_{16}O_2$), and 141 (74%, M^+ − $C_9H_{17}O_2$).

Esterification of 3 to methyl-10-oxostearate (3a). 10-Oxostearate (3) (300 mg, 1 mmol) from *N. aurantia* (ATCC 12674) was suspended in 5 ml of anhydrous ether, 15 ml of diazomethane ether solution (0.15 g of diazomethane, prepared from Diazald [Aldrich]) was added to the suspension, and the reaction solution was set at room temperature for 1 h. The solvent was then removed under a stream of nitrogen to produce 311 mg of white crystal of methyl-10-oxostearate (3a). The methyl ester prepared as described above had the following properties: high-resolution electron impact mass spectrum, *m/z* 312.2663 for $C_{19}H_{36}O_3$ (calculated, 312.2904), 281 (M^+ − $-OCH_3$), 199 [CH_3OCO-$(CH_2)_8$-C=O], 214 [CH_3OCO-$(CH_2)_8$-CO-CH_3], and 184 [$CH_3OCO(CH_2)_7$-CH=CH_2].

Reduction of 3a to racemic 2a with $NaBH_4$. A total of 250 mg (0.8 mmol) of 3a was dissolved in 5 ml of anhydrous methanol, 250 mg (6.6 mmol) of $NaBH_4$ powder was added to the solution, and the mixture was stirred at room temperature for 1 h. The reaction was stopped by adding 1 ml of 6 N HCl, the solvent was removed under reduced pressure, and 5 ml of water was added to the white residue. Ethyl acetate extraction of the aqueous solution followed by concentration gave 240 mg of racemic methyl-10-hydroxystearate (2a), which was spectrally identical to 2a obtained by chemical methylation of 2.

Baeyer-Villiger oxidation of methyl-10-oxo-octadecanoate. A 50 mg of sample 3a was suspended in 1 ml of benzene and mixed with 100 mg of *m*-chloroperoxybenzoic acid in 2 ml of benzene; the mixture was stirred at room temperature for 4 days, cooled in ice-water, filtered to remove the white precipitate, and washed with benzene. After evaporation of the organic solvent, the residue was dissolved in 8 ml of 10% aqueous NaOH solution, refluxed for 2 h, and stirred at room temperature overnight. The reaction solution was extracted with ether, and the residue obtained upon removal of the solvent was redissolved in ethyl acetate for TLC. The fraction at R_f 0.28 (petroleum ether-ether-formic acid, 60:40: 0.75, vol/vol/vol) was isolated by preparative TLC before analysis by electron impact gas chromatography-mass spectrometry. Both standard 1-octanol (R_f of 0.28 in the same solvent system) and the fraction isolated from the first ether extraction gave identical mass (electron impact) fragmentation patterns with major peaks at *m/z* (percent relative abundance) 112 (M^+ [$C_8H_{18}O$] − H_2O, 5%), 84 (C_5H_8O, 47%), 70 (C_4H_6O, 65%), and 56 (C_4H_8, 100%).

Preparation of (*S*)-(+)-*O*-acetylmandelate esters (2b) of racemic and microbiologically generated methyl-10-hydroxystearate (2a). (*S*)-(+)-*O*-Acetylmandelic acid (92 mg, 0.47 mmol) and 10.5 mg (0.086 mmol) of 4-(*N*,*N*-dimethylamino)-pyridine were dissolved in 2 ml of dichloromethane (dried over Na_2SO_4), stirred, and cooled in an ice bath. A solution of 100 mg of racemic methyl-10-hydroxystearate (2a) in 3 ml of CH_2Cl_2 was added along with 97 mg (0.47 mmol) of dicyclohexylcarbodiimide dissolved in 0.5 ml of CH_2Cl_2. The reaction was complete within 2 h (TLC), and the reaction mixture was filtered to remove a white precipitate. The filtrate was evaporated under reduced pressure to dryness and then reconstituted with CH_2Cl_2 for preparative TLC (hexane-ethyl acetate, 3:1). The major UV (254 nm) quenching band at R_f 0.5 was scraped and eluted with ethyl acetate to give 90 mg of methyl-10-(*S*)-(+)-*O*-acetylmandeloylstearate 2b after evaporation. The physical properties of 2b were as follows: high-resolution mass spectrometry (fast atom bombardment; matrix, 3-nitrobenzylalcohol) for $C_{29}H_{47}O_6$ $(M+H)^+$ (calculated value, 491.3373; found value, 491.3357) and for $C_{29}H_{46}O_6Na$ $(M+Na)^+$ (calculated value, 513.3192; found value, 513.3199); CI *m/z* (percent relative abundance) 508 (M + 1 + NH_3, 100%), 491 (M + 1, 0.93%), 450, 314, 297, 265, 212, 168, 152, 124, 106; ^1H NMR ($CDCl_3$), *d*, 0.88 (3H, t, *J* = 7.2, H-18), 0.89 (3H, t, *J* = 7.2, H-18), 1.03 to 1.31 [18H, m, $(CH_2)_9$], 1.37 (2H, dd, *J* = 7, H-9 or H-11), 1.56 to 1.62 (2H, m, H-3 or H-4), 2.91 (3H, s, CH_3CO), 2.29 (2H, dd, 7.5, H-2), 2.30 (2H, 33, 7.5, H-2), 3.67 (3H, d, *J* = 3, OCH_3), 4.85 to 4.89 (1H, m, H-10), 5.87 (1H, d, *J* = 2.2, H-2′), 7.35 to 7.38 (3H, m, H-3″, H-4″, H-5″), 7.47 (2H, dd, *J* = 6, H-2″, H-6″). The proton-proton correlation spectroscopy (COSY) spectrum of this compound

TABLE 1. Cultures that form hydroxyoctadecanoic acid (2) or oxo-octadecanoic acid (3) products from oleic acid

Species	Strain[a] or description	Products observed in culture extracts by TLC		
		2	3	1
Absidia coerulia (−)	UI 27B	−	+	−
Aspergillus terreus	UI 58	−	+	−
A. terreus	ATCC 11156	−	+	+
Candida intermedia	UI 5159	+	−	−
C. intermedia	ATCC 12089	+	−	−
C. lipolytica	UI 5699	−	+	−
C. lipolytica	UI MrCAN	+	−	−
C. lipolytica	ATCC 8661	+	−	−
C. lipolytica	ATCC 9773	+	+	−
C. parasilosis var. *quercus*	ATCC 56466	+	+	−
Corynebacterium mediolarum	ATCC 14004	+	+	+
Mycobacterium fortuitum	UI 53378	+	+	+
Nocardia aurantia	ATCC 12674	+	+	−
Nocardia butanica	ATCC 21197	+	−	+
Nocardia sp.	NRRL 5646	−	+	−
Pseudomonas sp.	NRRL B2994	+	+	−
Pseudomonas sp.	NRRL B3266	+	+	−
Rhodotorula rubra	ATCC 20129	+	+	−
R. rubra	IFO 889	+	−	+
Saccharomyces cerevisiae	Sigma type II (baker's yeast)	+	+	−
S. cerevisiae	NRRL Y2034	+	−	−
Saccharomyces sp.	UI Sacch	−	+	−
Schizosaccharomyces octosporus	NRRL Y854	−	+	−

[a] UI, University of Iowa, College of Pharmacy, culture collection; NRRL, Northern Regional Research Laboratories, Peoria, Ill.; ATCC, American Type Culture Collection, Rockville, Md.

shows cross-peaks corresponding to the coupling of H-18 to H-17, H-17 to H-16, and H-2 to H-3.

In similar fashion, a solution of 10 mg (0.032 mmol) of metabolite methyl-10-hydroxystearate (2a) and 10 mg (0.47 mmol) of dicyclohexylcarbodiimide in 2 ml of CH_2Cl_2 was added to a 2-ml CH_2Cl_2 solution of *O*-acetylmandelic acid (9 mg, 0.047 mmol) and 4-(*N*,*N*-dimethylamino)-pyridine (1 mg). The mixture was stirred over an ice bath for 5 h and then filtered to remove a white precipitate. Removal of the reaction solvent under nitrogen gave a crude residue containing mainly (*S*)-(+)-*O*-acetylmandelate ester of metabolite methyl 10-hydroxystearate 2b. TLC of this residue gave a major spot at R_f 0.5 (hexane-ethyl acetate, 3:1, vol/vol). The 600-MHz ^{1}H NMR spectrum gave the following results: 0.87 (3H, t, J = 7.2, H-18), 1.03 to 1.31 [18H, m, $(CH_2)_9$], 1.35 to 1.39 (2H, m, H-9 or H-11), 1.85 to 1.63 (2H, m, H-3 or H-4), 2.19 (3H, s, CH_3CO), 2.30 (2H, dd, J = 7.5, H-2), 3.67 (3H, s, OCH_3), 4.85 to 4.89 (1H, m, H-10), 5.87 (1H, s, H-2′), 7.36 to 7.38 (3H, m, H-3″, H-4″, H-5″), 7.47 (2H, dd, J = 6, H-2″, H-6″).

DISCUSSION

Methods commonly used in our laboratory for the screening of microorganisms (4) were employed to identify cultures capable of biotransforming oleic acid into oxygenated products. Screening experiments were conducted with TLC as the method for analysis, and no attempts were made to optimize oleic acid metabolite yields. A total of 78 microorganisms were examined, and TLC analyses revealed that 23 of these, representing several genera, were capable of forming either 10-hydroxyoctadecanoic acid (2), or 10-oxo-octadecanoic acid (3) (Table 1). Before this work, only *Pseudomonas* and *Nocardia* (*Rhodococcus*) species had been reported as forming hydrated oleic acid derivatives (8, 12, 13, 17, 18, 20, 32–34, 39). We found that two of the four pseudomonads examined formed oxygenated oleic acid metabolites. Wallen et al. (39) found no oleic acid metabolites from *Candida*, *Penicillium*, *Phycomyces*, *Calcarisporium arbuscula*, and *Lachnocladium* isolates. Noteworthy in our results are the preponderance of yeast species that produce oxygenated oleic acid derivatives with good yields. Under our biotransformation conditions, 7 of 10 *Candida* strains performed the oxidation reaction, as did three of the four *Saccharomyces* species, three of the four *Nocardia* species, and each of the *Corynebacterium*, *Rhodotorula*, and *Schizosaccharomyces* species examined. In addition, only 2 of 16 *Aspergillus* species formed oleic acid products, as did the only *Absidia* and *Mycobacterium* strains. This work considerably extends the groups of microorganisms now known to microbiologically transform oleic acid to include a range of eucaryotic organisms. Furthermore, the conditions used in our experiments were demonstrated to be useful when applied to cultures well known to achieve hydration of oleic acid. Cultures that reproducibly appeared to produce relatively large amounts of metabolites were selected for preparative scale reactions. In general, preparative-scale fermentations gave reaction profiles and relative yields of metabolites similar to those observed during screening. However, preparative-scale reactions were allowed to proceed until substrates were consumed before culture harvest and extraction and isolation of metabolites.

Resting cell suspensions of *S. cerevisiae* were used to produce 10-hydroxyoctadecanoic acid (10-hydroxystearic acid) (2) on a preparative scale with a 45% yield. The biocatalyst was obtained as an active resting cell suspension in 0.1 M phosphate buffer (pH 6.5) containing 1% glucose as an energy source. The metabolite was isolated by solvent extraction and purified by column chromatography, and the structure was confirmed by ^{1}H NMR, ^{13}C NMR, and mass spectral analyses and by comparison with an authentic standard (18). The metabolite exhibited no olefinic signals at 5.32 and 5.38 ppm in the proton NMR spectrum or at 129.74 and 130.02 ppm in the carbon NMR spectrum, indicating that the metabolite had saturated the double bond of oleic acid. New signals in ^{1}H and ^{13}C NMR spectra of the metabolite resonating at 4.60 (1H, m) and 69.54 ppm suggested the presence of a hydroxyl group in the metabolite at either the C-9 or C-10 position. The presence of a hydroxyl group in the metabolite structure was confirmed by an IR absorption band at 3,420 cm^{-1}. The low-resolution mass spectrum gave *m/z* 300, which also indicated that the double bond of oleic acid had been saturated and that oxygen had been added to the structure. Gas chromatography-mass spectrometry of the metabolite methyl ester indicated a molecular ion at *m/z* 314.4687 for empirical formula $C_{19}H_{38}O_3$ plus other indicator fragments at *m/z* 201 and 172, which fix the location of the hydroxyl group at position 10. This is the first report of a yeast species forming 10-hydroxy-octadecanoic acid.

Oleic acid was transformed to 10-oxo-octadecanoic acid (3) on a preparative scale by cultures of *M. fortuitum*, *Nocardia* sp. (NRRL 5646), and *N. aurantia* (ATCC 12674) with yields of 80, 55, and 65%, respectively. The metabolite from each organism was isolated by solvent extraction, purified chromatographically, and subjected to spectral and chemical analyses. Absent in the proton and carbon NMR

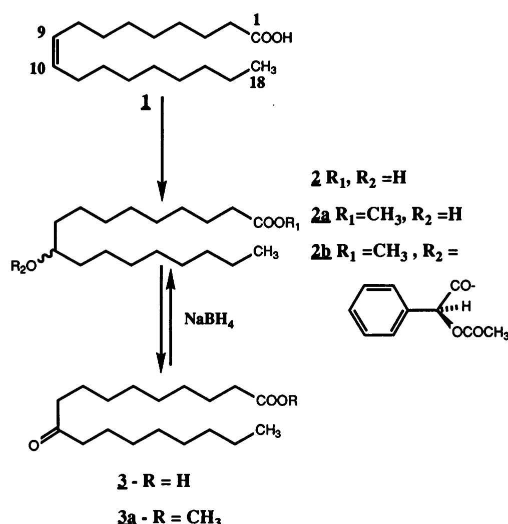

FIG. 1. Microbial transformations of oleic acid (1) to 10-hydroxyoctadecanoic acid (2), 10-oxo-octadecanoic acid (3), and chemical derivatives.

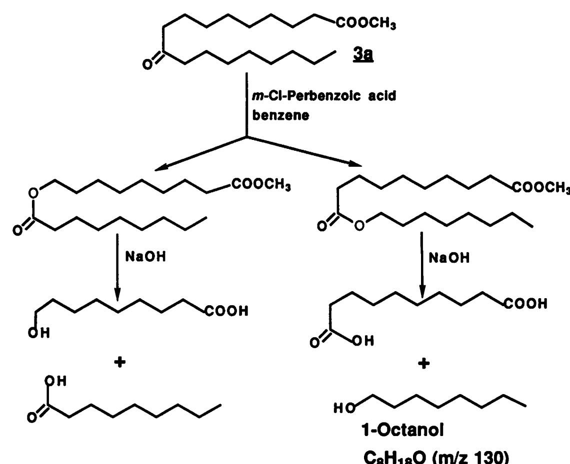

FIG. 2. Expected products from the Baeyer-Villiger oxidation of 3a.

spectra were signals for the olefinic protons present in the substrate oleic acid. These were replaced by a new signal at 211.35 ppm, indicative of a ketone. The presence of a ketone functional group in the metabolite structure was confirmed by IR spectral analysis by an intense absorption band at 1,720 cm^{-1}. The low-resolution ammonia chemical ionization mass spectrum gave *m/z* 316, which is consistent for a molecular ion of a metabolite containing an oxygen atom plus one double bond. The high-resolution mass spectrum of the metabolite methyl ester (3a) gave *m/z* 312.2663 for $C_{19}H_{36}O_3$, and major fragments at *m/z* 199 and 214, which fix the location of the ketone at position 10.

The pathway for formation of oxidized oleic acid derivatives likely follows that outlined in Fig. 1. Initial hydration of the olefinic center of oleic acid would afford 10-hydroxystearic acid (2), and enzymatic dehydrogenation by alcohol dehydrogenases would afford 10-oxostearic acid (3). The nature of oleic acid metabolites formed during the microbial transformation reaction was highly dependent upon biotransformation conditions. Under the conditions used in this work, three cultures produced 3 as the major metabolite. When shaker speeds were reduced to 150 rpm and volumes of medium were reduced to 1/10 the volume of DeLong culture flasks, greater proportions of 2 were formed (data not shown).

Based upon all spectral measurements and on comparisons with values in the literature, the structures of the oxo-octadecanoic acid and hydroxyoctadecanoic acid metabolites are reasonable. Mass spectral analysis of oxygenated fatty acids obviously represents a powerful tool in the assignment of metabolite structure (37, 38). However, fragment ions expected from oxygenated fatty acid positional isomers are predictably quite similar with low-resolution mass spectral analyses, and structure identification by mass spectrometry alone requires standards for direct spectral comparison with the instruments and conditions used in acquiring spectra for isolated metabolites (37, 38). The use of derivatives of oxygenated fatty acids provides a means of tagging fragments and thus confirming structures. Others have employed chemical methods for localizing the positions of fatty acid double bonds (1).

We explored the possibility of using Baeyer-Villiger chemical oxidation of ketones as a means of providing confirmation of the position of the ketone in the structure of the 10-oxo-octadecanoic acid (3) (12, 22). Baeyer-Villiger oxidation of the metabolite 3 would lead to a mixture of monocarboxylic and dicarboxylic acids and one hydroxy acid product, depending upon which side of the ketone functional group underwent the oxidation reaction (Fig. 2). Although complex mixtures of acid products would be expected, only one neutral alcohol product can be obtained by ketone oxidation. The expected oxidation reaction would occur between positions 10 and 11, giving an 8-carbon alcohol, 1-octanol. The fraction isolated from Baeyer-Villiger oxidation of 3a by preparative TLC gave mass spectral fragmentations identical to those of standard 1-octanol, thus confirming its identity, and the location of the oxygen atom of the ketone (3a).

The assignment of stereochemistry and assessment of stereochemical purities of 10-hydroxyoctadecanoic acids has been largely based on the elegant and detailed work conducted by Bloch and Schroepfer (32–34). Other workers in the field (8, 12, 13, 17, 18, 20, 39) who have studied microbial transformations of oleic acid either have not reported on the stereochemical purities of hydroxyoctadecanoic acid products or have relied upon extremely small specific rotation measurements or indirect methods to infer isomeric composition. The routine adaptation of chemical methodologies described by Schroepfer and Bloch for ascertaining hydroxyl group stereochemistry is laborious, and the use of measured rotations of much less than 1^0 renders this process difficult. We have explored the use of a simple derivatization procedure coupled with high-field ^1H NMR spectral analysis of a diastereomeric hydroxy-fatty acid derivative for this purpose. Parker (25) and Liu et al. (21) have described the use of *S*-(+)-*O*-acetylmandelate esters of alcohols followed by proton NMR spectral analysis of the diastereomeric esters to define stereochemical purities of other types of isomeric alcohols. The strategy was to prepare a racemic alcohol mixture (2a) (Fig. 1) by $NaBH_4$ reduction of methyl-10-oxo-octadecanoate (3a) and to compare the spectral properties of the resulting *R*,*S*-10-*S*-(+)-*O*-acetylmandeloyl-methyloctadecanoate (2b) with those of 2b from a fermentation mixture. The derivative is easily prepared (21, 25) and was completely characterized by proton NMR and mass spectral analysis. A

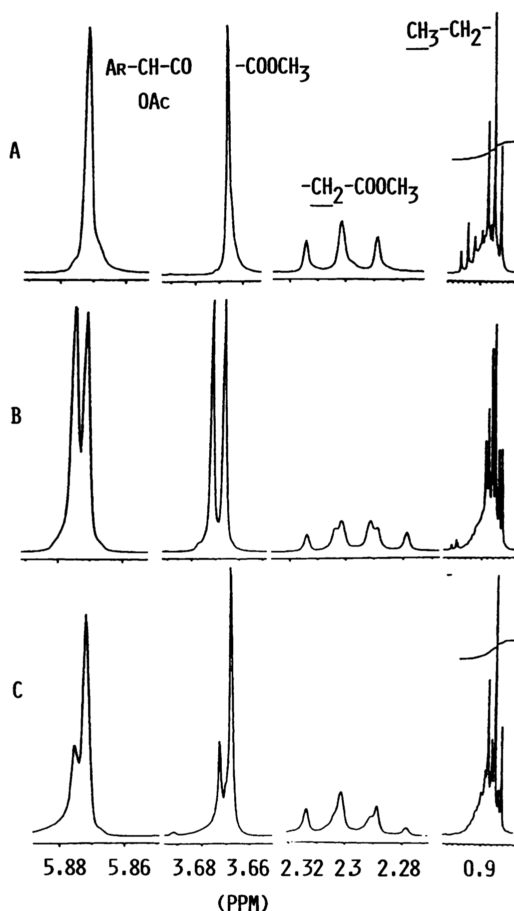

FIG. 3. Selected ^{1}H NMR spectral signals of 2b for the methine proton of the mandelic acid moiety (5.88 ppm), the methyl ester functional group (3.67 ppm), the methylene group protons for position 2 (2.30 ppm), and the methyl group protons for position 18. (A) 2b from microbiological source; (B) 2b from racemic 2a by $NaBH_4$ reduction of ketone (3); (C) equal mixture of A and B.

proton-proton correlation spectrum was used to confirm interactions of the various protons of the mandelate ester. For racemic 2b, signals for protons at H-2 and H-18 each occurred as doublets of overlapping triplets centered at 2.29 and 0.88 ppm, respectively (Fig. 3B); the methyl ester signal at 3.67 ppm and the signal for the methine proton of the mandelic acid residue (ArCHCO) at 5.87 ppm each appeared as two singlets of equivalent intensity (Fig. 3B). All other ^{1}H NMR signals at 600 MHz appeared to be normal. The results indicate that, in racemic 2b, four different sets of signals can be used to distinguish 10-hydroxyoctadecanoic acid enantiomers. For the natural product (Fig. 3A), the corresponding signals were simplified as a triplet each for H-2 and H-18 and a singlet for the methyl ester and methine signals. The results indicate that the 2b obtained from fermentation is enantiomerically pure. To test the method further, a mixture of the racemic and natural 2a was examined by NMR with the expected results (Fig. 3C). These results indicate that the derivatization and NMR technique represents a powerful and simple means of ascertaining stereochemical purities of hydroxy-fatty acids. With the resolution achieved with ^{1}H NMR at 600 MHz, it is possible to distinguish as little as 1% of a contaminating isomer (21). Our work is complemented by that of Cardillo et al. (6), who used a similar approach employing Mosher ester derivatives of hydroxy-alkanes to determine stereochemical purities. The present work does not define the absolute stereochemistry of 2b. However, this will be the subject of another more detailed communication, in which the absolute stereochemistries of hydroxystearate derivatives from a variety of microorganisms are elaborated.

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