Bioconversion of Oleic Acid by *Bacillus* Strain NRRL BD-447: Identification of 7-Hydroxy-17-oxo-9-*cis*-octadecenoic Acid

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ABSTRACT: Continuing efforts to isolate and assign structures to the products from the bioconversion of oleic acid with *Bacillus* strain NRRL-447 have resulted in identification of a positional isomer of the hydroxy-keto fatty acid previously reported. After high-performance liquid chromatographic isolation, the new compound was identified by gas chromatography–mass spectrometry and nuclear magnetic resonance analyses to be 7-hydroxy-17-oxo-9-*cis*-octadecenoic acid.

Paper no. J9103 in *JAOCS 76*, 1023–1026 (September 1999).

KEY WORDS: *Bacillus*, bioconversion, fatty acid, hydroxy, ketone, octadecenoic acid, oleic acid, oxo.

The United States imports 44,000 metric tons of castor oil annually, costing about $30 million. Ricinoleic acid (12-hydroxy-9-octadecenoic acid) makes up 90% of the fatty acids in castor oil and is the major hydroxy fatty acid used in industry. The hydroxyl group at C12 makes ricinoleic acid unique and permits novel derivatives and multiple industrial uses.

Domestic vegetable oils, particularly soybean oil, have been a commodity with annual surpluses of up to 1.2 billion pounds in the United States for many years. It would be economically beneficial to transform these surplus oils into replacement or new value-added products. To do this, the structure of soybean oil must be modified to change its physical and chemical properties. Therefore, discovery and development of new methods to convert existing surplus oils into unique fatty acids containing hydroxyl or oxo groups would be of great interest.

Industrially useful fatty chemicals, e.g., hydroxy and keto acids for use in surfactants, lubricants, detergents, cosmetics, and fragrances, are often manufactured from fats and oils by chemical reactions. These reactions are often run under severe conditions and produce random or mixed products. Direct synthesis of only one isomer is usually not possible with chemical synthesis, whereas microorganisms can perform reactions under milder conditions and are often specific in their syntheses. Enzymatic modification of surplus oils or their fatty acids may provide new products with greater industrial utility.

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Wallen *et al.* (1) first reported the microbial conversion of oleic acid to 10-hydroxystearic acid. Oleic acid can be converted to 10-hydroxystearic acid by several *Nocardia* strains at greater than 90% yield (2); to 10-hydroxy- and 10-ketostearic acid by *Flavobacterium* sp. DS5 (3); to 10-ketostearic acid by *Staphylococcus warneri* (4); and to 7,10-dihydroxy-8-(*E*)-octadecenoic acid by *Pseudomonas* sp. PR3 (5). The microbial production of hydroxy fatty acids from fatty acids was recently reviewed by Hou (6).

Previously we reported that two strains of *Bacillus pumilus* (NRRL BD-174 and NRRL BD-226) produced the 15-, 16-, and 17-hydroxyoctadecenoic acid isomers from oleic acid (7). More recently, we reported on the oxygenation of oleic acid by *Bacillus* strain NRRL-447 to the uniquely bifunctional compound, 7-hydroxy-16-oxo-9-*cis*-octadecenoic acid (8). We now report on the isolation and identification of an additional oxygenated product which is the positional isomer, 7-hydroxy-17-oxo-9-*cis*-octadecenoic acid.

MATERIALS AND METHODS

Bacillus strain NRRL BD-447 was obtained from the Agricultural Research Service Culture Collection and grown in TGY medium (8). Larger-scale bioconversions were done in 600 mL TGY media with 3 mL oleic acid (0.05%) added after 24 h growth of the organism. Oxygenation products were extracted with ether.

The products were separated and isolated by high-performance liquid chromatography (HPLC) using a Spectra Physics (San Jose, CA) 8800 solvent delivery system, a Dynamax 60A silica column (21.4 × 250 mm i.d.; Rainin Instrument Co., Inc., Woburn, MA), and the solvent system reported by Gérard *et al.* (9): solvent A—hexane/isopropanol/acetic acid (99.3:0.6:0.1, vol/vol/vol); and solvent B—hexane/isopropanol/acetic acid (79.8:20.18:0.1, vol/vol/vol). The gradient was modified as follows (min, solvent A/solvent B): 0, 99/1; 5, 85/15; 25, 60/40; 60, 60/40; 75, 40/60; 100, 40/60; 130, 0/100; 160, 0/100. Solvent flow was 4 mL/min with 10% directed to a Varex (Varex Corporation, Rockville, MD) ELSD II detector.

Product identification was done by gas chromatography–mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR). GC–MS was performed using a Hewlett-Packard (HP) 5989B mass spectrometer (Hewlett-Packard,

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Palo Alto, CA) equipped with an HP-5890 Series II Plus gas chromatograph and an HP-7673 autoinjector. Electron impact (EI), methane chemical ionization (CI), and isobutane CI mass spectra were obtained on the methyl ester, trimethylsilyl (TMS) derivatives, and $NaHB_4$-reduced compounds. Proton, one-dimensional and two-dimensional, ^{13}C NMR spectra including homonuclear and heteronuclear correlations were determined on the methyl ester dissolved in deuterated methanol with a Bruker ARX 400 spectrometer (Billerica, MA) operating at 400 and 100 MHz, respectively.

RESULTS AND DISCUSSION

A positional isomer of a previously identified bioconversion product containing both keto and hydroxy functionalities was isolated by HPLC in >97% purity and its structure elucidated with GC–MS and NMR. The EI spectrum of the methyl ester (Fig. 1, compound **II**) has a base peak at *m/z* 127 and a strong ion fragment at *m/z* 159 (Fig. 2A). No molecular ion (M) is seen; however, the fragment at *m/z* 308 would derive from M – H_2O. The fragment at *m/z* 127 probably results both from a loss of methanol from the *m/z* 159 fragment (159 – 32) and from cleavage adjacent to the C9 double bond toward the CH_3 terminal end of the molecule (C10,11 cleavage). The *m/z* 159 fragment results from bond cleavage between C7 and C8. This fragment shifts to *m/z* 231 after TMS derivatization (compound **III**), indicating that it contains one free hydroxyl group. This would account for the large fragment ion at *m/z* 127 seen for the methyl ester and the smaller 127 fragment after TMS derivatization of the methyl ester. These MS data are consistent with C7,8 bond cleavage of a fatty acid with hydroxy substitution on the C7 carbon and subsequent loss of methanol (10).

The TMS derivative of the fatty acid (compound **IV**) was prepared and subjected to analysis by both methane and isobutane CI–MS. Methane CI–MS of this TMS derivative (MW 456) provides largest ion fragments at *m/z* 367 $[MH - 90]^+$ and *m/z* 277 $[MH - 90 - 90]^+$ resulting from loss of one and two HOTMS groups, respectively (Fig. 2B). Small fragments at *m/z* 455 and 457 $[(M - H)^+$ and $(MH)^+]$ and at 485 [M + 29] and 497 [M + 41] are similar to the pattern seen previously. Similarly, isobutane CI of this derivative gives a molecular ion $(MH)^+$ at *m/z* 457, a base peak at *m/z* 367 (MH^+ – HOTMS), and a small fragment ion at *m/z* 277. These fragment ions from the various MS techniques indicate a molecular weight of 456, consistent with a hydroxy-keto-octadecenoic compound.

The position of the keto group was determined to be at C17 by comparison of the EI mass spectra of compound **II** before and after $NaBH_4$ reduction of the oxo functionality to hydroxyl (11) followed by TMS derivatization (compound **V**). The fragment ion at *m/z* 43 [CH_3–CO-] is replaced by a fragment ion at *m/z* 117 (CH_3-CH-OTMS) confirming the oxo substitution at C17.

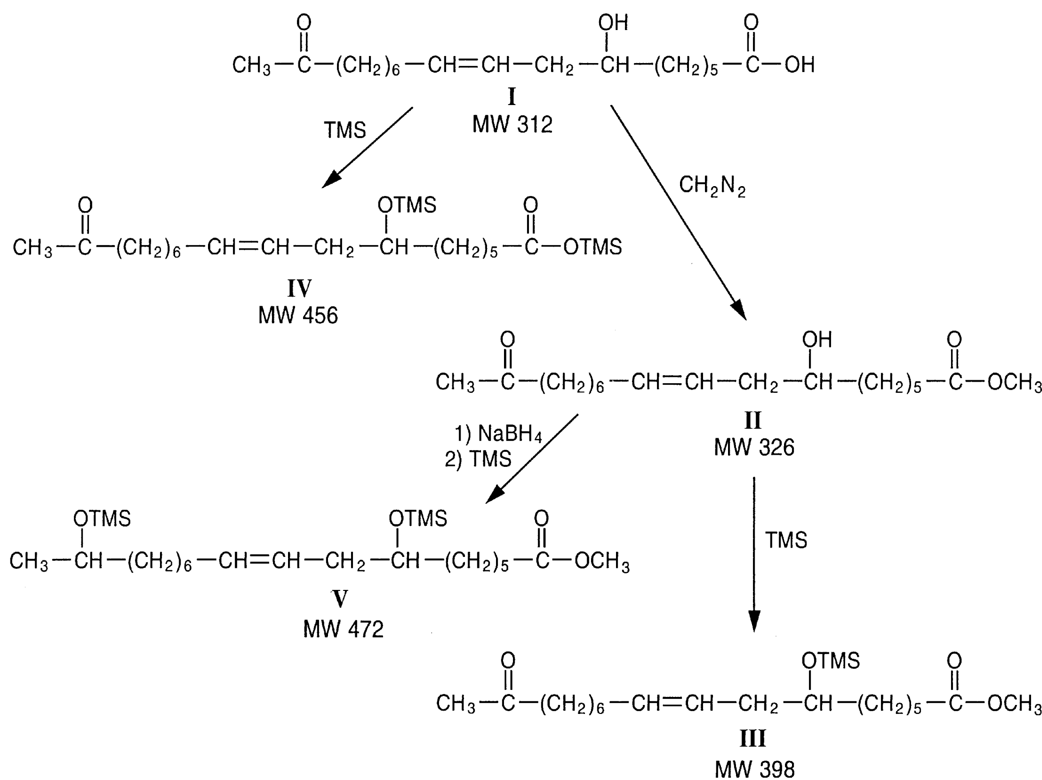

FIG. 1. Derivatization scheme used to identify the bioconversion product.

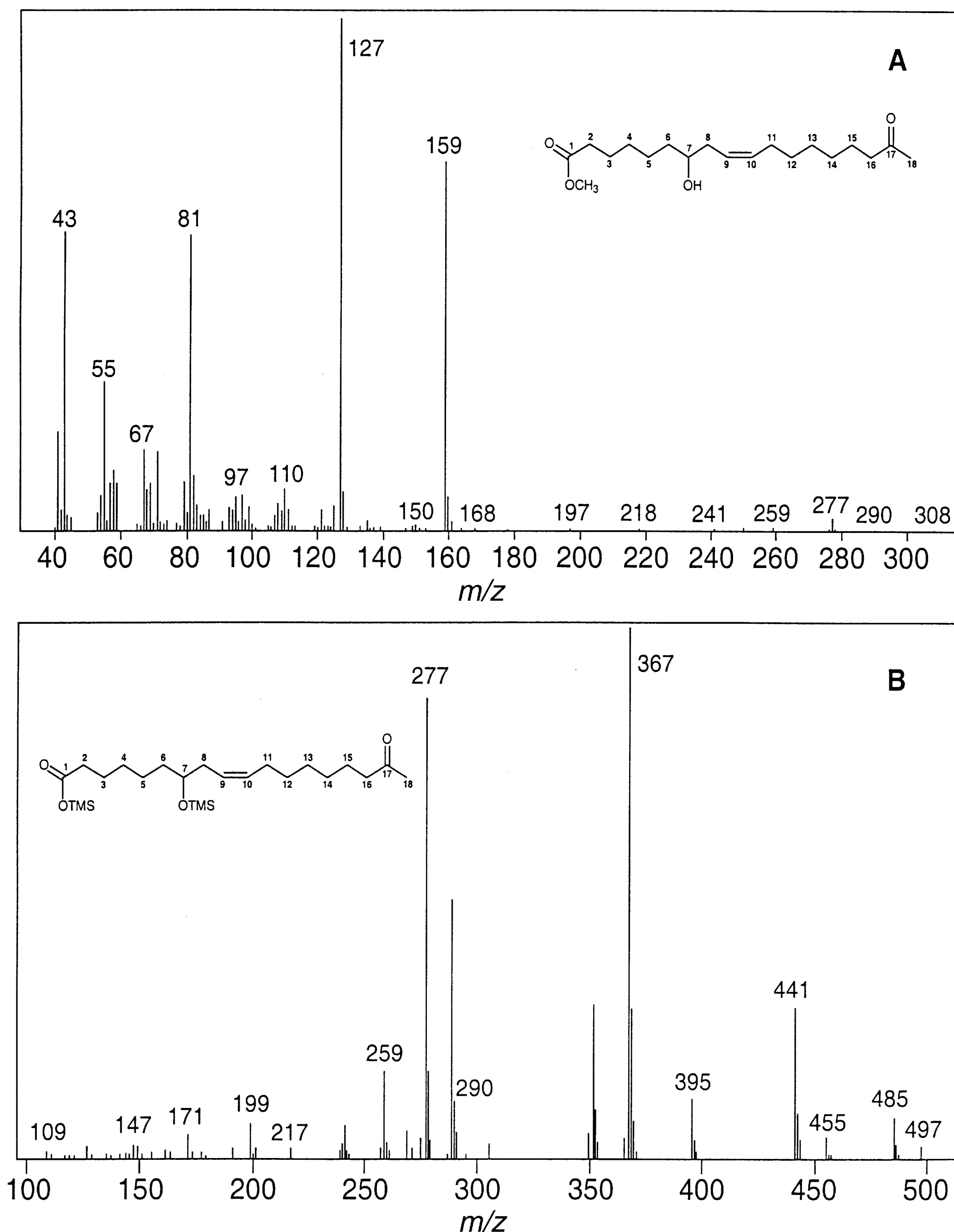

FIG. 2. (A) Electron impact mass spectrum of the methyl ester of 7-hydroxy-17-oxo-9-*cis*-octadecenoic acid. (B) Methane chemical ionization mass spectrometry of the trimethylsilyl derivative of 7-hydroxy-17-oxo-9-*cis*-octadecenoic acid.

Proton NMR analysis provided evidence for unsaturation, keto and hydroxy moieties, and for one methylene group between the hydroxy and double bond in compound **II**. The configuration of the double bond was determined to be *cis* from the observed coupling of 10.8 Hz between the 9,10 protons (Table 1). The carbonyl can be assigned to the C17 position because the chemical shift of the terminal methyl is 29.9 ppm in the ^{13}C spectrum and because there is a singlet methyl resonance at 2.12 ppm in the ^{1}H spectrum. The hydroxyl-bearing carbon is separated from the double bond by one methylene because the methylene protons observed at 2.19 ppm are coupled to a single proton at 3.59 ppm and an olefinic proton at 5.39 ppm. ^{13}C NMR spectra showed a single peak at 71.3 ppm, characteristic of having a hydroxyl attachment, and a distinctive peak at 209.3 ppm, characteristic of a ketone carbon. The following resonance signals also were present: two olefinic carbons at 125.1 and 133.4 ppm, methylene carbons from 23.7 to 43.7 ppm, and the terminal methyl carbon at 29.9

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

Carbon number	Proton resonance (ppm)	Hydrogens	Coupling constant, J(Hz)	^{13}C resonance (ppm)
1	—			174.2
2	2.30	2	7.5 (2,3)	34.0
3	1.63	2		24.9
4	1.32	2		29.1
5	1.32	2		25.4
6	1.46	2		36.6
7	3.59	1		71.3
8	2.19	2	7.4 (8,9)	35.4
9	5.39	1	10.8 (9,10)	125.1
10	5.54	1	6.9 (10,11)	133.4
11	2.02	2		27.3
12	1.29	2		29.4
13	1.29	2		29.0
14	1.29	2		29.0
15	1.55	2		23.7
16	2.40	2		43.7
17	—	—		209.3
18	2.12	3		29.9
OCH_3	3.65	3		51.5

ppm. Tulloch (12) tabulated the ^{13}C chemical shifts in spectra of methyl oxooctadecanoates. The signal for the terminal methyl carbon (C18) is found at 29.7 ppm when the oxo group is at the C17 position, which is consistent with our value. These data, together with the GC–MS data, suggest that this compound has 18 carbons, a hydroxy group at C7, a C9,10 double bond, and an oxo group at C17. Apparently, the enzyme system in NRRL BD-447 produces octadecenoic acids with positional isomerization of the oxo group similar to that found for hydroxyl groups in *B. pumilus* strains NRRL BD-174 and NRRL BD-226. This isomerization may impart unique properties to each compound, which may be exploited by industry.

ACKNOWLEDGMENTS

We thank David Weisleder for NMR analyses.

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[Received December 21, 1998; accepted April 23, 1999]