10(*S*)-Hydroxy-8(*E*)-octadecenoic Acid, an Intermediate in the Conversion of Oleic Acid to 7,10-Dihydroxy-8(*E*)-octadecenoic Acid

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ABSTRACT: The new microbial isolate *Pseudomonas aeruginosa* (PR3) has been reported to produce from oleic acid a new compound, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD), with 10-hydroxy-8-octadecenoic acid (HOD) being a probable intermediate. The production of DOD involves the introduction of two hydroxyl groups at carbon numbers 7 and 10, and a rearrangement of the double bond from carbons 9-10 to 8-9. It has been shown that the 8-9 unsaturation of HOD was possibly in the *cis* configuration. Now we report that the rearranged double bond of HOD is *trans* rather than *cis*, as determined by spectral data. Also, it was found that the 10-hydroxyl was in the *S*-configuration as determined by gas chromatographic separation of *R*- and *S*-isomers after preparation of the (–)-menthoxycarbonyl derivative of the hydroxyl group followed by oxidative cleavage of the double bond and methyl esterification. This latter result coincides with our recent finding that the main final product, DOD, is in the 7(*S*),10(*S*)-dihydroxy configuration. In addition, a minor isomer of HOD (about 3 %) with the 10(*R*)-hydroxyl configuration was also detected. From the data obtained herein, we concluded that 10(*S*)-hydroxy-8(*E*)-octadecenoic acid is the probable intermediate in the bioconversion of oleic acid to 7(*S*),10(*S*)-dihydroxy-8(*E*)-octadecenoic acid by PR3.

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KEY WORDS: Bioconversion, dihydroxy octadecenoic acid, monohydroxy octadecenoic acid, oleic acid, *Pseudomonas aeruginosa*, stereo configuration.

Hydroxy fatty acids are known to be produced in nature mainly by plant systems. The hydroxyl group is known to give a fatty acid special properties, such as higher viscosity and reactivity compared with other nonhydroxylated fatty acids (1). As a result, hydroxy fatty acids are used in a wide range of industrial products, such as resins, waxes, nylons, plastics, lubricants, cosmetics, and additives in coatings and paintings. At present, imported castor oil and its derivatives are the major commercial source of hydroxy fatty acids. However, the fluctuating supplies and prices for castor oil cause users to seek alternative raw materials.

Since Wallen *et al*. (2) reported the first bioconversion of oleic acid to 10-hydroxystearic acid by a pseudomonad, microbial conversion of unsaturated fatty acids has been widely exploited to produce new, value-added hydroxy fatty acids. Microbial oxidation of unsaturated fatty acids was recently reviewed (3). Much research has been done in this and other laboratories to investigate the microbial conversion of vegetable oils and their component fatty acids to value-added products. For example, Hou *et al.* (4) reported the discovery of a new compound, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD), which was produced from oleic acid in >60% yield by a bacterial isolate *Pseudomonas* sp. PR3 (5). Strain PR3 was identified subsequently as *Pseudomonas aeruginosa* (6). Recently the yield of DOD by strain PR3 was improved to >80% yield by modifying the culture medium and reaction parameters (7). The formation of a similar compound by *Pseudomonas* 42A2 also was reported and studied (8,9).

The bioconversion of oleic acid into DOD is unique in that it involves the introduction of hydroxyl groups at carbon atoms 7 and 10 and a rearrangement of the double bond from carbons 9-10 to 8-9. Based on the complexity of these reactions, DOD formation seems to involve more than one transformation. To elucidate the metabolic pathway leading to DOD from oleic acid, Hou and Bagby (10) reported isolation of two additional products from the reaction mixture, of which one was identified as phenazine-1-carboxylic acid and the other as 10-hydroxy-8(*Z*)-octadecenoic acid (HOD). HOD was thought to be an intermediate in the bioconversion of oleic acid to DOD. Recently, another group reported the conversion of oleic acid by *Pseudomonas* sp. 42A2 into HOD and DOD, both with an 8,9-*trans* double bond (11).

Here we report that the double bond of HOD is *trans* rather than *cis* by nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) data and that the 10-hydroxyl group is mainly in the *S*-configuration with a minor percentage in the *R*-configuration. Some aspects of the pathway involved in this bioconversion are discussed as well.

EXPERIMENTAL PROCEDURES

Microorganisms. Pseudomonas aeruginosa NRRL strain B-18602 (PR3) was originally isolated from a water sample in Morton, IL. The culture was aerobically grown at 28°C in a 125-mL Erlenmeyer flask containing 50 mL of medium at 200 rpm. The medium used contained 4 g dextrose, 2 g

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K_2HPO_4, 2 g $(NH_4)_2HPO_4$, 0.5 g yeast extract, 1 g NH_4NO_3, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.008 g $MnSO_4 \cdot 7H_2O$, 0.014 g $ZnSO_4$, and 0.01 g nicotinic acid per liter. The medium was adjusted to pH 7.0 with dilute phosphoric acid. Cultures were maintained on an agar slant with the above medium except for the addition of 1.5% agar.

Chemicals. Dimethyl octanedioate and oleic acid with 99+% purity by gas chromatography (GC) were purchased from Sigma Chemical Co. (St. Louis, MO). Racemic methyl 2-hydroxydecanoate was obtained from Matreya Inc. (Pleasant Gap, PA) and (–)-menthoxycarbonylchloride [(–)-menthyl chloroformate] was from Aldrich (Milwaukee, WI). Kieselgel 60 and thin-layer precoated Kieselgel $60F_{254}$ plates were obtained from EM Science (Cherry Hill, NJ). Other reagent-grade chemicals were purchased from Sigma and used without further purification.

Bioconversion. Oleic acid (0.3 g) was added to a 24-h-old culture in the above medium and the flasks were incubated under the same conditions for an additional 48 h. At the end of the cultivation, the culture broth was acidified to pH 2 with 6 N hydrochloric acid. The culture broth was extracted once with an equal volume of ethyl acetate followed by extraction with an equal volume of ether. The solvent was evaporated from the combined extracts with a rotary evaporator.

Analysis of products. The crude extract (200 mg) was subjected to high-performance liquid chromatography (HPLC) for analysis and isolation of HOD. HPLC was conducted with a Hewlett-Packard (Palo Alto, CA) series 1100 equipped with a Hewlett-Packard 1100 series diode array detector (DAD) and a Microsorb silica column (41.4 mm i.d. × 25 cm) from Rainin Instrument Co. (Woburn, MA). Purification was carried out by injecting crude extracts (200 mg) dissolved in methylene chloride/methanol (2:1 vol/vol) onto the column equilibrated with hexane/ethyl acetate (95:5 vol/vol). Separation was achieved by eluting the column with a linear gradient from hexane/ethyl acetate (95:5 vol/vol), after an initial hold of 20 min, to hexane/ethyl acetate (50:50 vol/vol) for a total running time of 200 min at a flow rate of 7 mL/min. Fractions were collected with the peaks being monitored at 240 nm wavelength. Although the 240 nm wavelength was not satisfactory for detecting HOD directly, a characteristic pattern of other eluting compounds detected at this wavelength was used to locate the HOD peak reliably. The collected fractions were analyzed by GC and thin-layer chromatography (TLC). For GC analysis, the samples were first methylated with ethereal diazomethane for 2 min at room temperature. The methyl esters, from which the solvent was evaporated under a nitrogen stream, were dissolved in methylene chloride/methanol (95:5 vol/vol) and injected into a Hewlett-Packard model 5890 GC (Hewlett-Packard, Avondale, CA). The GC was equipped with a flame-ionization detector (FID), a Supelco SPB-1™ capillary column (15 m × 0.32 mm i.d., 0.25-µm thickness; Supelco Inc., Bellefonte, PA), and a Hewlett-Packard 3392A integrator. Compounds were eluted isothermally at 200°C. The retention time of HOD was 9.3 min. TLC plates were developed with toluene/dioxane/acetic acid (79:14:7 vol/vol/vol). The spots were visualized first by iodine vapor and then spraying the plate with 50% sulfuric acid and heating in a 100°C oven for 10 min.

The HPLC-purified HOD was characterized by GC/mass spectrometry (GC/MS), nuclear magnetic resonance (NMR), and Fourier transform infrared (FTIR) spectrometry. Electron-impact mass spectra were obtained with a Hewlett-Packard (Avondale, PA) 5890 GC coupled to a Hewlett-Packard 5972 series mass selective detector. The column outlet was connected directly to the ion source. Compounds were separated on a methylsilicone column (30 m × 0.25 mm i.d., 0.25-µm film thickness) using a temperature gradient of 20°C/min from 70 to 170°C, holding 1 min at 170°C, and then 5°C/min to 250°C, followed by holding for 15 min at 250°C (helium at 0.67 mL/min). For GC/MS trimethylsilyloxy (TMSiO) derivatives were prepared by treatment of methyl esters with pyridine/hexamethyldisilazane/trimethylchlorosilane (2:2:3, vol/vol/vol) for 15 min at room temperature. ^1H and ^{13}C NMR spectra were determined in deuterated chloroform with a Bruker ARS-400 spectrometer (Billerica, MA), operated at a frequency of 400 and 100 MHz, respectively. FTIR analysis of the free acid products were run as films on KBr with a PerkinElmer FTIR model 1750 spectrometer (PerkinElmer Inc., Oakbrook, IL).

Determination of stereochemistry. For chiral analysis, 1 mg of HOD methylated with diazomethane was reacted with (–)-menthoxycarbonylchloride, and then the menthoxycarbonyl (MCO) derivative was isolated by TLC using hexane/ethyl acetate (185:15 vol/vol) developed as previously described (12). This method was modified to oxidatively cleave the MCO derivative with 12 mg $KMnO_4$ in 0.3 mL acetic acid for 1 h at 37°C (13). After oxidative cleavage, the acetic acid was evaporated with a stream of nitrogen, and the products were methyl esterified with diazomethane. The methyl 2-MCO-decanoate and dimethyl octanedioate obtained by this procedure were dissolved in hexane and analyzed by GC/MS. Methyl 2(*R*,*S*)-MCO-decanoate was prepared from racemic methyl 2-hydroxydecanoate by the method described above (12). GC/MS for this experiment was completed with a Hewlett-Packard model 5890 (Palo Alto, CA) gas chromatograph equipped with a Hewlett-Packard 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, 30 m × 0.25 mm, film thickness 0.25 m. The oxidative cleavage products methyl 2-MCO-decanoate and dimethyl octanedioate were separated by temperature programming from 65 to 260°C at a rate of 10°C/min (helium at 0.67 mL/min). In order to separate diastereomeric methyl 2(*S*)- and 2(*R*)-MCO-decanoates for determination of stereo configuration, the temperature program had to be changed from 100 to 260°C at a rate of 2%C/min (helium at 0.67 mL/min).

RESULTS AND DISCUSSION

Purification of HOD. After incubating *P. aeruginosa* PR3 in the presence of oleic acid, a crude organic extract was obtained

from the culture. A sample of the extract was injected onto the HPLC column and separated by eluting the column with the gradient system described in the Experimental Procedures section. The collected fractions were analyzed by TLC and GC revealing that most of the HOD eluted between 165–185 min after injection. Fractions containing HOD were combined, concentrated, and reanalyzed by HPLC. After the second HPLC separation, 5 mg of purified HOD (>95% by GC) was obtained. TLC of the purified sample showed one spot.

Structure determination. The location of the hydroxyl group and double bond was deduced from the fragment ions observed in the electron-impact GC/MS data of the silylated methyl ester of HOD (Fig. 1). The intense fragment (*m/z* 271) arising from alpha cleavage to the silylated hydroxyl group gave a fragment ion containing both the silyl group and the double bond. This fragment ion, together with the *m/z* 241 ion, located the hydroxyl group at either C10 or C8, but the greater intensity of the *m/z* 271 ion suggested that the hydroxyl group was localized at C10 as shown for a similar silylated methyl ester, methyl 13-hydroxy-11(*E*)-octadecenoate (14). This result was also supported by the fact that the MCO derivative of HOD subjected to oxidative cleavage of the double bond by $KMnO_4$ followed by methylation generated methyl 2-MCO-decanoate and dimethyl octanedioate (see the experiments described below). These data are consistent with the TMSiO derivative of a methylated 10-hydroxy-8-octadecenoic acid with a molecular mass of 384.

FTIR analysis. Purified HOD (as the free fatty acid) was analyzed by FTIR. The presence of hydroxyl group(s) was indicated by the IR absorption at 3605 cm^{-1}. An absorption at 1706 cm^{-1} showed the presence of a carbonyl group, and the absorption at 965 cm^{-1} indicated *trans* unsaturation (15).

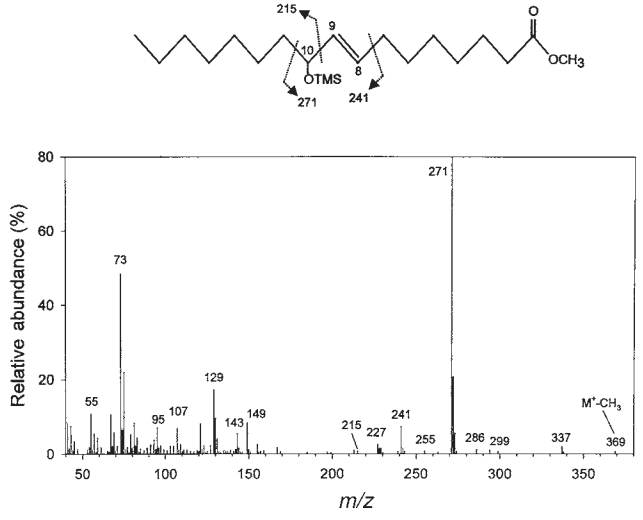

FIG. 1. Electron-impact mass spectrum of trimethylsilyl derivatives of purified and methylated 10-hydroxy-8-octadecenoic acid (HOD) obtained with a Hewlett-Packard 5890 (Avondale, PA) gas chromatograph coupled to an HP 5972 series mass selective detector. Compounds were separated using a temperature gradient of 20°C/min from 70 to 170°C, holding 1 min at 170°C, and then raised 5°C/min to 250°C, followed by holding at 250°C for 15 min (helium at 0.67 mL/min). OTMS, trimethylsilyloxy.

NMR analysis. Purified HOD was also subjected to ^1H and ^{13}C NMR analyses to confirm the structure of HOD. The proton and carbon resonance signals (ppm) and corresponding molecular assignments are given in Table 1. ^1H NMR absorption for the C8 and C9 olefinic protons (–CH=CH–) were observed at 5.61 and 5.42 ppm, respectively, with a coupling constant of 15.4 Hz strongly suggesting a *trans* double bond. One proton (–CH–O–) was observed at 4.02 ppm. Because of the downfield shift, compared to alcohols adjacent to saturated carbons, and the doublet of triplet multiplicity, the hydroxyl-bearing carbon was suggested to be vicinal to the double bond. ^{13}C NMR showed the presence of the following carbon atoms: carbonyl carbon at 174 ppm (C1), a double bond at 131.97 ppm (C8) and 133.14 ppm (C9), and a –CHOH– carbon at 73.27 ppm (C10). The chemical shifts of the remaining proton and carbon are listed in Table 1. The data obtained from GC/MS, FTIR, NMR, and oxidative cleavage confirmed that the purified compound was 10-hydroxy-8(*E*)-octadecenoic acid.

Determination of stereochemistry. Purified HOD was subjected to chiral analysis by the MCO method (see Experimental Procedures section). Oxidative cleavage of the MCO derivative of methyl HOD followed by methyl esterification gave two major peaks by GC at retention times of 10.65 and 20.2 min using the appropriate temperature program (see Experimental Procedures section). The first eluting peak was determined to be dimethyl octanedioate by comparison with the standard, and the second peak was determined to be methyl 2-MCO-decanoate by comparison with standard racemic methyl 2(*R,S*)-MCO-decanoate.

Although the above temperature programming was useful in detecting the two cleavage fragments, dimethyl octanedioate and methyl 2-MCO-decanoate, the program had to be changed in order to separate diastereomeric methyl 2(*S*)- and

TABLE 1
Proton and ^{13}C Nuclear Magnetic Resonance Signals and Molecular Assignments for HOD[a]

Carbon number	Resonance signal (ppm)	
	^1H	^{13}C
1	174.2	
2	2.3	34.01
3	1.62	24.62
4	1.25	28.57
5	1.25	28.78
6	1.25	29.26
7	2.01	32.01
8	5.61 ($J_{8,9}$ = 15.4 Hz)	131.97
9	5.42	133.14
10	4.02	73.27
11	1.46	37.28
12	1.52	25.48
13	1.25	28.81
14	1.25	29.55
15	1.25	29.55
16	1.25	31.86
17	1.25	22.66
18	0.86	14.1

[a]HOD, 10-hydroxy-8-octadecenoic acid.

2(*R*)-MCO-decanoates for determination of stereo configuration (see Experimental Procedures section). With this program, methyl 2-MCO-decanoate separated into a major peak at 60.15 min with a minor peak at 60.37 min (about 3%) (Fig. 2A). It is known from previous work that methyl 2(*S*)-MCO-alkanoates elute earlier than methyl 2(*R*)-MCO-alkanoates (12,13,17), specifically including methyl 2-MCO-decanoate

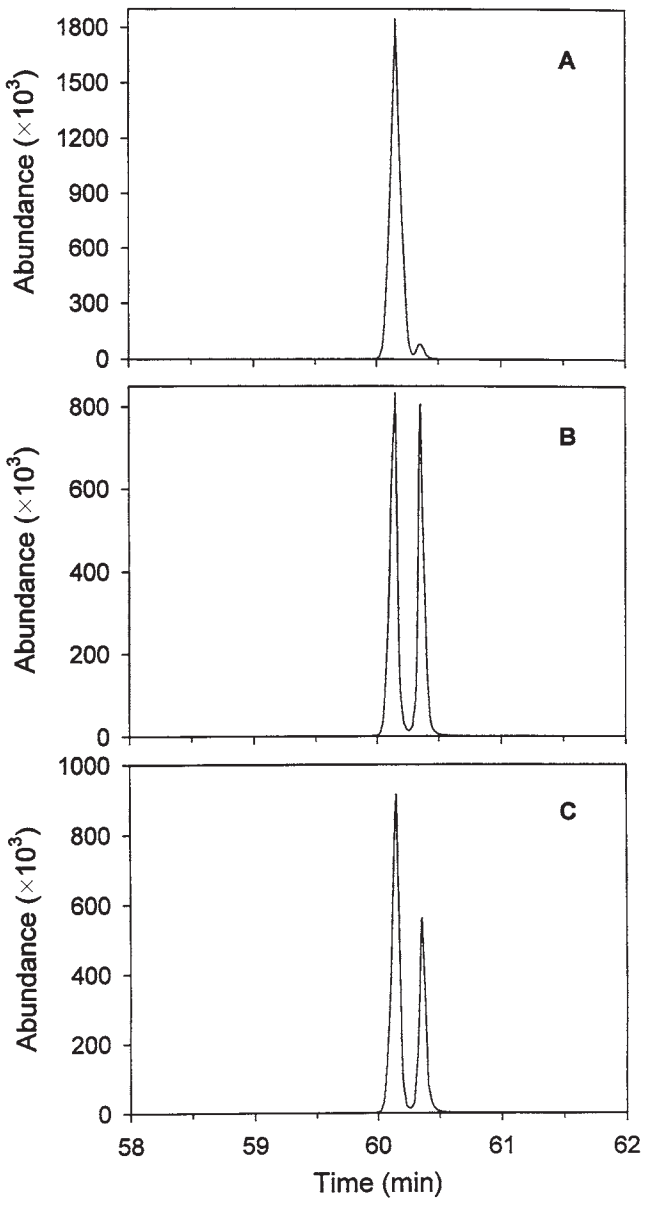

FIG. 2. (A) Total ion chromatogram of methyl 2-menthoxycarbonyl (MCO)-decanoate derived from the purified HOD by oxidative cleavage after MCO derivatization followed by methylation. First peak is centered at 60.15 min and the second peak is centered at 60.37 min. (B) Total ion chromatogram of methyl 2(*S,R*)-MCO-decanoate standard prepared by MCO derivatization of the racemic methyl 2(*S,R*)-hydroxydecanoate followed by methylation. First peak is the 2(*S*)-isomer (centered at 60.17 min) and the second peak is the 2(*R*)-isomer (centered at 60.37 min). (C) Total ion chromatogram of the coinjected MCO derivatives derived from the purified HOD (see A) and the standard racemic methyl 2(*S,R*)-hydroxydecanoate. First peak is centered at 60.16 min and the second peak is centered at 60.37 min. See Figure 1 for abbreviation.

(17). The injection of standard racemic methyl 2(*R,S*)-MCO-decanoate separated into two peaks, one [methyl 2(*S*)-MCO-decanoate] at 60.17 min and the other [methyl 2(*R*)-MCO-decanoate] at 60.38 min (Fig. 2B). Coinjection of the racemate with the MCO derivative from HOD demonstrated that the main stereoisomer from HOD augmented the methyl 2(*S*)-MCO-decanoate peak from the racemic standard (Fig. 2C). As expected, the GC-separated diastereomeric methyl 2(*S*)- and 2(*R*)-MCO-decanoates afforded virtually identical mass spectra essentially as reported previously (18), regardless of their origin from HOD or the racemic methyl 2-hydroxydecanoate. In analogy with the elution order determined previously of these diastereomeric methyl 2(*S*)- and 2(*R*)-MCO-decanoates (17,18), it was clear that the first major peak of the MCO derivative from HOD was the methyl 2(*S*)-MCO-decanoate and the second minor peak, the methyl 2(*R*)-MCO-decanoate, suggesting that the hydroxyl group at C10 of HOD was mainly in the *S*-configuration with a minor isomer of *R*-configuration (about 3%). These results coincided with our recent findings (18) that the main final product, DOD, was in the 7(*S*),10(*S*)-dihydroxy stereoconfiguration. A minor DOD isomer (about 3%) with a similar mass spectrum was separated from the main DOD isomer by HPLC (Kim, H., and C.T. Hou, unpublished data). It follows from the findings of 3% 10(*R*) HOD that the minor DOD isomer may have the 7(*S*),10(*R*)-dihydroxy configuration.

Based on the results of this study and previous work (10), the overall bioconversion pathway of oleic acid to DOD by strain PR3 is postulated as shown in Figure 3. Oleic acid is first converted to HOD by PR3, during which one hydroxyl group is introduced at C10(*S*) and a double bond is shifted from C9,10 *cis* to C8,9 *trans*. Although Hou (19,20) reported a hydratase-catalyzed C10-hydroxylation without retention of the C9,10-double bond by *Flavobacterium* sp. DS5, it is unlikely that a hydratase is involved in the PR3 bioconversion inasmuch as the double bond at C9,10 is not lost, but it is shifted to C8,9 as the *trans* isomer. The hydration of oleic, linoleic, and linolenic acid to their corresponding 10-hydroxy

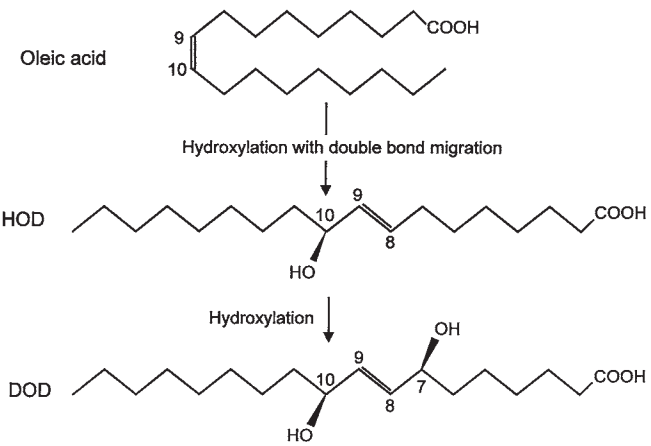

FIG. 3. Postulated bioconversion pathways of oleic acid leading to 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) by *Pseudomonas aeruginosa* (strain PR3). For other abbreviation see Figure 1.

products have been reported from various microbes as reviewed by Hou (20). Another group reported that a lipoxygenase-type enzyme located in the periplasm of *Pseudomonas* sp. 42A4 was involved in the formation of (*E*)-10-hydroperoxy-8-octadecenoic and (*E*)-10-hydroxy-8-octadecenoic acids from oleic acid (11). Although there is little evidence for oleic acid-specific lipoxygenases in the literature, a free radical mechanism of lipoxygenase would explain oxidation at C10 and the double bond shift. Allylic hydroxylations with double bond migration also are known to occur as a result of cytochrome P450 oxidation of fatty acids (21). The resulting plausible intermediate, HOD, is then subject to another hydroxylation at C7(*S*) by possibly the same enzyme(s) involved in the first hydroxylation reaction resulting in DOD formation. Further work should be done to investigate the bacterial enzyme(s) involved in hydroxylation.

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