A Novel Compound, 7,10-Dihydroxy-8(E)-Octadecenoic Acid from Oleic Acid by Bioconversion

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Sixty-two cultures from the Agricultural Research Service (ARS) Culture Collection and 10 cultures isolated from soil and water samples in Illinois were screened for their ability to convert agricultural oils to value-added industrial chemicals. A new compound, 7,10-dihydroxy-8(E)-octadecenoic acid (DOD), was produced from oleic acid at a yield of greater than 60% by bacterial strain PR3 which was isolated from a water sample in Morton, IL. To our knowledge, DOD has not been previously reported. The optimum time, pH and temperature for the production of DOD were 2 days, 7.0, and 30°C, respectively. The production of DOD is unique in that it involves hydroxylation at two positions and rearrangement of the double bond of the substrate molecule.

KEY WORDS: Chemiluminescence, fish meats, lipid peroxidation, mackerel, oxidative rancidity, sardine, sensory evaluation, shelf-life dating, tuna.

Agricultural oils, particularly soybean oil are often in great surplus. It is important to convert these surplus oils into new, value-added products (1,2). It is known that both agricultural oils and hydrocarbons are oxidized by microorganisms through similar catalytic processes or bioconversion pathways using enzymes such as oxygenases, epoxidases, dehydrogenases, hydratases or other enzymes that catalyze stereo and regiospecific reactions. Hou reviewed microbial transformation of important industrial hydrophobic compounds including aliphatic and aromatic hydrocarbons (3). Several microbial/enzymic approaches for modifying oils or fats have been reported in recent years, including the commercial application of lipases for fat splitting (4). *Nocardia corallina*, *Pseudomonas* sp., and *Puccinia graminis* are known to biosynthesize epoxy groups (5-7). Oleate serves as substrate for *Pseudomonas* and *P. graminis* to form 9,10-epoxy stearate (6,7). The cytochrome p-450 system from *Bacillus megatherium* appears to serve as a common enzyme for both epoxidation and hydroxylation of a variety of monounsaturated fatty acids (8). Marsh and James (9) showed that yeast biosynthesizes hydroxy stearic acid from stearic acid. Microbial conversion of oleic acid to 10-hydroxystearic acid was first reported by Wallen *et al.* in our laboratories (10). They found that a Pseudomonad isolated from a fatty material hydrated oleic acid at the double bond with a 14% yield. In a patent disclosure, *Rhodococcus rhodochrous* converts oleic acid to 10-hydroxystearic acid and minor amounts of 10-ketostearic acid (11). Recently, a strain of *Pseudomonas* was found to convert olive oil to a new surfactant, an unsaturated hydroxy fatty acid (12). We have identified several microorganisms that hydrate oleic acid to 10-hydroxystearic acid at greater than 90% yield (13).

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In our continuing screening program for new industrial chemicals from agricultural oils, we have discovered that a new bacterial strain, PR3, isolated from a water sample taken at a pig farm in Morton, IL, converts oleic acid to a new compound, 7,10-dihydroxy-8(E)-octadecenoic acid (DOD). This paper describes the production, purification and structure determination of this new compound.

EXPERIMENTAL

Microorganisms. Sixty-two microbial cultures (23 bacteria, 12 fungi, 14 Actinomycetes and 13 yeasts) from the ARS Culture Collection and 10 cultures from soil and water samples in Illinois were screened for their ability to modify oleic acid. The culture was grown aerobically at 30°C in a 125-mL shake flask (shake at 150 rpm) containing 30 mL of medium with the following composition (per liter): Dextrose, 4 g; $(NH_4)_2HPO_4$, 10 g; K_2HPO_4, 2 g; yeast extract, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; $MnSO_4.H_2O$, 0.008 g; $ZnSO_4.7H_2O$, 0.014 g; and nicotinic acid, 0.1 g. The medium was adjusted to pH 7.0 with 20% phosphoric acid.

Chemicals. Oleic acid (99+% purity) was purchased from Nu Check Prep, Inc. (Elysian, MN). All chemicals were reagent grade and were used without further purification. Thin-layer precoated Kieselgel 60 F_{254} plates were obtained from EM Science (Cherry Hill, NJ).

Bioconversions reaction. An aliquot of 0.4 mL oleic acid (0.35 g) was added to a 36- to 48-hr-old culture and the flasks were shaken at 150 rpm at 30°C for 2 days. At the end of this time, 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. The aqueous phase was again extracted with an equal volume of diethyl ether. The solvent was removed from the combined extracts with a rotary evaporator.

For larger scale, the bioconversion of oleic acid (0.8 g) was carried out as described above, but in 3.8 liter Fernbach flask containing 1 liter of medium. The reaction products were extracted with solvents as described previously. Then they were purified with solvent partitioning and silica gel column chromatography. Solvent was evaporated *in vacuo* from the combined extracts, and the residue was partitioned between hexane/acetonitrile (30 mL:30 mL). White material which appeared at the interface of these solvents was carefully collected and then washed twice with hexane. The nitrogen stream dried white material (95 mg) showed one spot by thin-layer chromatography (TLC) (Rf 0.34). Gas-liquid chromatography (GC) gave a major peak (97% of the total area) at 19.93 min. The acetonitrile fraction containing major product was washed once with fresh hexane and then dried with a rotary evaporator. The solid residue was dissolved in diethyl ether and separated on a silica gel G column (35 cm × 2.2 cm id), which was pre-equilibrated with diethyl ether. The column was washed with 200 mL ether and then eluted with ether methanol (70:30, v/v). Five-milliliter portions were collected and assayed with

TLC. Fraction numbers 45 to 55 contained the major product. These fractions were combined and evaporated to dryness. The solid residue was washed with hexane and then dried under nitrogen stream to obtain 250 mg white powder. TLC and GC analyses of this white powder showed that they were identical to those materials obtained previously with solvent partitioning. The overall recovery of this product was 71%. The melting point of this white powder is 64°C.

Analysis of products. The isolated reaction products were analyzed by TLC and GC. The TLC was developed with a solvent system consisting of toluene/dioxane/acetic acid (79:14:7, v/v). The chromatograms were visualized first by iodine vapor and then by spraying the plate with 50% sulfuric acid and heating in a 100°C oven for 10 min. For GC, the samples were methylated with diazomethane. The methyl esters, dissolved in diethyl ether, were injected into a Hewlett Packard model 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA) which was equipped with flame ionization detector, a Supelco SPB-1 capillary column 15 m, id 0.32 mm, 0.25 μm thickness (Supelco, Inc., Bellefonte, PA) and a Hewlett Packard 3392A integrator. GC was run isothermally at 200°C. For quantitative analysis, palmitic acid was added as an internal standard prior to the solvent extraction. A linear relationship was established on the peak area ratios of product vs methyl palmitate.

Infrared (IR) spectra of the free acid were obtained on KBr discs with a Mattson Polaris IR Spectrometer (Mattson Instruments, Inc., Madison, WI). A Mattson Galaxy 6020 IR spectrometer was used to obtain a spectrum of the methyl ester (thin film, KBr plate). Trimethylsilyl (TMS) derivatives of DOD were prepared with a derivatizing agent, Trisil-TBT by standard techniques. Electron impact mass spectra were obtained with a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970 Series Mass Selective Detector. The column outlet was connected directly to the ion source. Separations were effected in a methyl silicone column (15 m $\times$ 0.25 mm) with a temperature gradient of 8°C per min from 160°C to 250°C after initially holding at 160°C for 3 min. Chemical ionization mass spectra were obtained on a Finnigan TSQ mass spectrometer (Finnigan Corp., Sunnyvale, CA). Isobutane was the reagent gas (0.3 torr). Proton and ^{13}C nuclear magnetic resonance (NMR) spectra were determined in deuterated chloroform with a Bruker WM-300 spectrometer (Rheins, Tepten, Germany) operating at a frequency of 300 MHz and 75.5 MHz, respectively.

RESULTS AND DISCUSSION

Of the many cultures tested, only a bacterial strain PR3 isolated from a water sample at a pig farm in Morton, IL, was found to convert oleic acid to more polar products. Therefore, we studied the optimum conditions for the bioconversion and determined the structure of the reaction product. Strain PR3 is a Pseudomonad and has been deposited in the ARS Culture Collection. Detailed identification of strain PR3 will be published elsewhere.

Optimum conditions for the production of DOD. Oleic acid (0.4 mL) was added to two-day-old cultures (30 mL) in a 125-mL shake flask to start the reaction. The reaction was carried out at 30°C for the time specified. The amount of product DOD in the culture media increased with time: 0 hr, 0 mg; 12 hr, 60 mg; 24 hr, 130 mg; 36 hr, 180 mg; 48 hr, 250 mg; and 60 hr, 140 mg. A maximum (60% yield) was reached after 48 hr of reaction. Further incubation resulted in a decline in DOD content in the medium, indicating metabolism of DOD by strain PR3.

To determine the effect of pH on production of DOD, strain PR3 was grown on a pH 7.0 medium for 2 days. Then, the culture medium was adjusted to the desired pH (from 5.5 to 8.5) with either 2 N NaOH or 3 N HCl, and oleic acid substrate was added. The reactions proceeded for 2 days before harvest. The maximum yield of DOD occurred at pH 7.0 (pH 5.5, 15 mg; pH 6.0, 58 mg; pH 6.5, 160 mg; pH 7.0, 235 mg; pH 7.5, 120 mg; pH 8.0, 20 mg; and pH 8.5, 5 mg). At harvest time, each culture medium had become 0.3 pH units more acidic than their initial pH value.

The temperature optimum for the production of DOD by cells of strain PR3 was about 30°C. The amounts of DOD produced at 25°C and 35°C were 50% and 57%, respectively, of the amount produced at 30°C.

Structure determination: GC-MS analysis. The white powder (3 mg) was methylated with diazomethane and then analyzed by GC-MS (mass spectrometry) (Table 1). The mass spectrum of the major component had an apparent molecular weight of 328, corresponding to a dihydroxy C18 monoene ester. The dihydroxy structure was confirmed by GC/MS of the trimethylsilyl (TMS) derivative of the methylated compound, which incorporated two TMS groups for an apparent molecular weight of 472 and by CI GC/MS of the TMS derivative of the unmethylated sample which incorporated three TMS groups for an apparent molecular weight of 530. The locations of the hydroxy groups and the double bond were apparent from the fragments observed in the electron impact spectrum of the TMS derivative of the methylated product. Intense fragments arising from cleavage α to the derivatized hydroxyl groups to give a fragment containing both TMS groups and the double bond were observed at *m/z* 343 and *m/z* 359. These fragments located the hydroxyl groups at C7 and C10 and the double bond between the two hydroxyl groups at C8. Based on these MS data, the reaction product is 7,10-dihydroxy-8-octadecenoic acid.

NMR analysis. DOD was also subjected to proton and ^{13}C NMR analyses. Proton NMR of DOD showed the presence of the following resonance signals: -CH=CH- group at 5.63 ppm ($J_{AA'}$=14.3 Hz); two tertiary protons -C<u>H</u>-O- at 4.08 ppm; -C<u>H$_2$</u>-COOH at 2.32 ppm; eleven methylene groups from 1.2 to 1.6 ppm and a -C<u>H$_3$</u> group at

TABLE 1

MS Data for DOD and Derivatives *m/e* (Intensity)

Methylated DOD	
EI	41(90),43(100),55(87),57(88),59(22),69(46),71(37),81(35), 83(23),85(21),87(56)(,97(22),111(16),119(18),125(28), 130(21),137(17),141(28),157(18),165(19),183(16),197(9), 199(9),279(1),310(M-18$^+$;0.5)
TMS derivative methylated DOD	
EI	73(100),119(15),147(18),155(14),165(12),179(12),215(13), 231(18),253(53),269(63),330(5),343(42),359(28),441(2), 457(M-15$^+$;2)
TMS derivative of DOD	
CI	351(30),369(12),441(MH-90$^+$;100),515(M-15$^+$;2),531(MH$^+$;1)

0.86 ppm. The coupling constant for the olefinic protons indicates *trans* unsaturation. ^{13}C NMR confirmed the presence of the following groups: carbonyl at around 178 ppm; a C_8 C_9 double bond at 133.88 and 133.60 ppm; C_7 and C_{10} hydroxyl carbons at 72.3 and 72.4 ppm; $-CH_2-$ carbons cover the range from 22.6 to 37.1 ppm; and the terminal methyl carbon at 14.1 ppm.

Infrared (IR) analysis. The presence of hydroxy groups were indicated by the strong, broad IR absorption at the 3400 cm^{-1} region. Carbonyl absorption at 1712 cm^{-1} indicated free carboxyl. The absorption at 975 cm^{-1} confirmed that DOD contained *trans* unsaturation. Methyl ester of DOD was then analyzed as a thin film and showed absorption for ester carbonyl at 1740 cm^{-1} and *trans* unsaturation at 972 cm^{-1}. Thus, the structure is 7,10-dihydroxy-8(E)-octadecenoic acid.

DOD is insoluble in hexane, slightly soluble in toluene and benzene, and soluble in diethyl ether, chloroform, ethyl acetate, acetone, methanol and acetonitrile. The industrial uses of DOD are currently under investigation.

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