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10-Hydroxy-8(Z)-octadecenoic acid, an intermediate in the bioconversion of oleic acid to 7,10-dihydroxy-8(E)-octadecenoic acid

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SUMMARY

Previously, we reported the discovery of a new compound, 7,10-dihydroxy-8(E)-octadecenoic acid (DOD) which was produced from oleic acid by a new bacterial isolate PR3 [6,7]. The reaction is unique in that it involves a hydroxylation at two positions and a rearrangement of the double bond of the substrate molecule. Now, we have isolated another compound from the reaction mixture determined by GC/MS to be 10-hydroxy-8-octadecenoic acid (HOD). NMR and IR data indicate that the unsaturation is probably *cis*. The optimum pH and temperature for the production of HOD by strain PR3 were 6.5 and 30 °C, about the same as those for DOD. However, the amount of HOD detected remained small throughout an 48-h reaction period during which the amount of DOD increased sharply. At 48 h of reaction, the ratio between HOD : DOD was 1 : 10. HOD may be an intermediate in the biosynthesis of DOD from oleic acid.

INTRODUCTION

In our continuing screening effort for new industrial products from agricultural oils through biotechnology, we reported the discovery of a new compound, 7,10-dihydroxy-8(E)-octadecenoic acid (DOD) [6] which was produced from oleic acid at a greater than 60% yield by a new bacterial isolate *Pseudomonas* sp. PR3 [7]. This compound is optically active, $[\alpha]_D^{26} = 14\,^\circ C$ in methanol [10]. The reaction is unique in that it involves a hydroxylation at two positions and a rearrangement of the double bond of the substrate molecule. The known bio-oxidative reactions on the aliphatic double bond are the epoxide formation such as the epoxidation of propylene [4,5] and liquid alkenes [1,2,12] and fatty acids [2,3,13] and the production of hydroxy products, for example, the hydration of oleic acid to 10-hydroxystearic acid by oleate hydratase [11,14]. DOD formation involves more than one step and is different from previously known bio-oxidations.

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Since our first report, we have isolated two additional compounds from the reaction mixture. One, isolated as fine yellowish crystalline needles, was identified as phenazine-1-carboxylic acid [8]. The other product was a colorless oily material identified here to be likely 10-hydroxy-8(Z)-octadecenoic acid (HOD). The latter appears to be a plausible intermediate in the bioconversion of oleic acid to DOD.

MATERIALS AND METHODS

Microorganisms

Pseudomonas sp. PR3 was isolated from a water sample in Morton, Illinois. The culture was grown at 30 °C aerobically in a 125-ml Erlenmeyer flask (shaker at 150 rpm) containing 50 ml of medium as described in [6,7]. Oleic acid, 0.4 ml (0.35 g), was added to a 36- to 48-h-old culture of the above medium and the flasks were shaken at 150 rpm at 30 °C for an additional 2 days. At the end of the incubation, the culture broth was acidified to pH 2 with 6 N hydrochloric acid. Then the culture broth was extracted once with an equal volume of ethyl acetate and then with an equal volume of diethyl ether. The solvent was evaporated from the combined extracts with a rotary evaporator.

Chemicals

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

Analysis of products

HPLC was conducted with a Waters Delta Prep 3000 chromatograph (Millipore Corp., Milford, MA) equipped with a Waters Model 403 refractive index detector and a Isco Inc. (Lincoln, NE) V^4 variable wavelength detector. A Dynamax-60A silica column (25 cm × 21.4 mm id) was used. The column was eluted with either a mixture of methylene chloride : methanol : acetic acid (96 : 3 : 1 v/v) or a mixture of methylene chloride : methanol : acetic acid (98 : 1 : 1 v/v). The flow rate was 6 ml/min. The isolated reaction products were analyzed by thin-layer chromatography (TLC) and gas-liquid chromatography (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 a 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. HOD had a retention time of 15.8 min. For quantitative analysis, palmitic acid was added as an internal standard prior to solvent extraction. A linear relationship was established between the peak area ratios of products and methyl palmitate.

Infrared (IR) spectra of the free acid (thin film, KBr plate) were obtained with a Mattson Galaxy 6020 IR spectrometer (Mattson Instruments, Inc., Madison, WI). TMS derivatives of the unknown compound were prepared with derivatizing agent, Trisil-TBT by standard techniques. Electron impact GC-MS were obtained with a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970 Series Mass Selective Detector. Separations were effected in a methyl silicone column (15 m × 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. Proton and C-13 nuclear magnetic resonance spectra of the HOD were determined in deuterated chloroform with a Bruker WM-300 spectrometer (Rheins, Tepten, F.R.G.) operating at a frequency of 300 MHz and 75.5 MHz, respectively.

Isolation of HOD

The combined solid reaction products (4.2 g) collected from three 1-l flasks were dissolved in a small amount of methylene chloride. The sample was chromatographed on a silica gel 60 column (35 × 2.2 cm id) which was pre-equilibrated with methylene chloride. The column was washed with 320 ml methylene chloride and then was eluted with: 1) a 300-ml mixture of methylene chloride : methanol (98 : 2 v/v); 2) a 550-ml mixture of methylene chloride : methanol (95 : 5 v/v); and 3) a 400-ml mixture of methylene chloride : methanol (90 : 10 v/v). A fraction collecter, started after the column washing, collected 6-ml portions that were analyzed by TLC. Tube numbers 8–22 contained mainly the yellowish compound and unreacted oleic acid. Tube numbers 38–60 (200 mg) contained mainly HOD, and tube numbers 61–180 contained DOD (1.1 g). The combined HOD fraction was subjected to

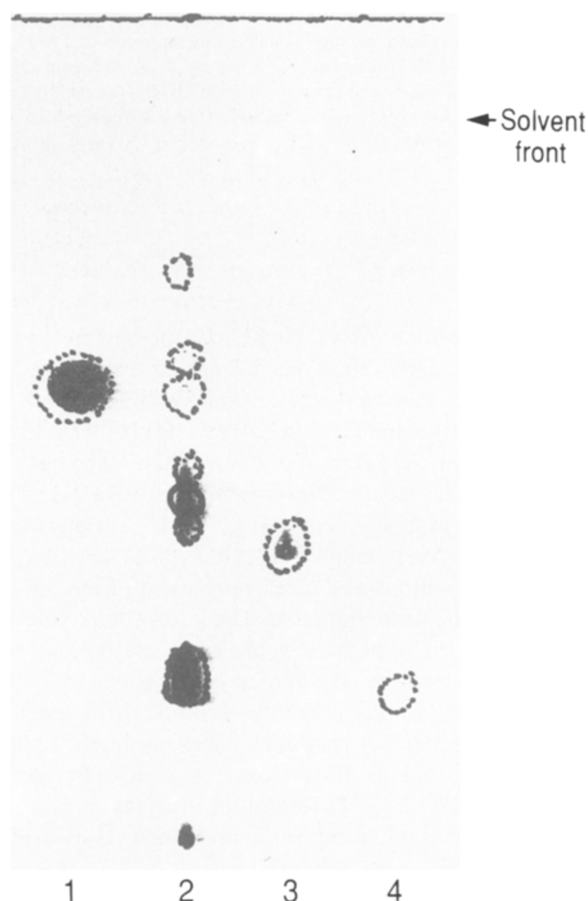

Fig. 1. Thin-layer chromatography of HOD. Solvent system = toluene : dioxane : acetic acid = 79 : 14 : 7 (v/v). Spots with dotted line were visualized with iodine vaper. 1. Oleic acid. 2. 48-h reaction products. 3. Purified HOD. 4. DOD.

HPLC with the methylene chloride : methanol : acetic acid (96 : 3 : 1 v/v) elution solvent system. Again, 6-ml portions were collected and analyzed with TLC. Fraction number 8 (160 mg, mainly HOD) was rechromatographed with methylene chloride : methanol : acetic acid (98 : 1 : 1 v/v). Fractions 10, 11 and 12 showed one single spot (Fig. 1, Rf = 0.41) on TLC analysis. These three fractions were combined, and the solvent was removed. A 100-mg sample of colorless oily material was obtained. Gas chromatography of the methylated materials showed essentially one peak (96% purity) at a retention time of 15.8 min.

RESULTS AND DISCUSSION

Structure determination

GM/MS analysis

The electron impact GC/MS data of the TMS derivative of the unmethylated sample were, m/e (intensity): 73(100), 75(49.7), 129(35.3), 149(9.6), 241(13.2), 287(12.7), 329(30.7), 355(0.6), 427($M-15^+$, 2.5) and 442(MH^+, 0.2). This is consistent with the di-TMS derivative of a C18 hydroxy monoenoic fatty acid with a relative molecular mass of 442. The location of the hydroxy group was apparent from the fragments observed in the electron impact spectrum of the TMS derivative of the free acid (Fig. 2). Intense fragment arising from cleavage alpha to the derivatized hydroxy group to give a fragment containing both the TMS and the double bond was observed at m/e 329. This fragment, together with the m/e 241 fragment, located the hydroxy group at either C10 or C8 and established that the hydroxyl group is alpha to a double bond [9]. The absence of the characteristic double alpha cleavage for an isolated hydroxyl (TMS derivative) group and the presence of the ion at m/e 241 provide unequivocal evidence for the location of both the double bond and the hydroxy group: either hydroxy group at C10 and double bond at C8 or hydroxy group at C8 and double bond at C9 [9]. Based on these MS data, the unknown product isolated is either 10-hydroxy-8-octadecenoic acid or 8-hydroxy-9-octadecenoic acid. The location of the hydroxyl group at position 8 and the double bond at position 9 would give strong m/z 343 fragment in the mass spectrum. Since this is absent, this unknown compound is likely the former. This agrees well with the fact that PR3 produces a large amount of 7,10-dihydroxy-8-octadecenoic acid [6,7].

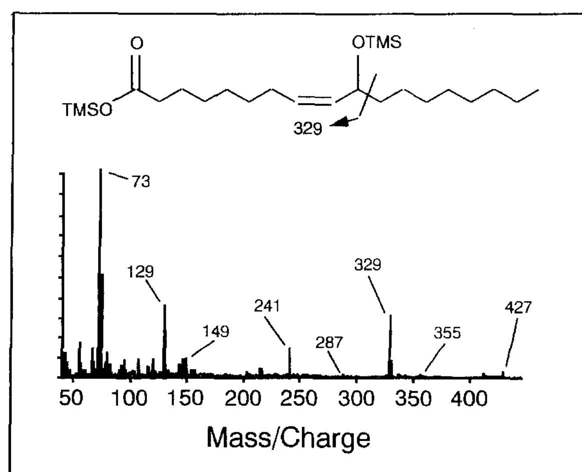

Fig. 2. EI mass spectrum of TMS derivative of HOD.

NMR analysis

Proton NMR of HOD showed the following resonance signals: olefinic protons $-CH{=}CH-$ at 5.63 ppm ($J_{AB'}$ = 11 Hz); one tertiary proton $-CH-O-$ at 4.08 ppm; $-CH_2-$CHOH$-$ at 2.55 ppm; $-CH_2-$COOH at 2.32 ppm; $-CH_2-$C= at 2.04 ppm; methylene groups cover from 1.2 to 1.6 ppm; and a terminal $-CH_3$ protons at 0.86 ppm. The coupling constant for the olefinic products indicates possible *cis* unsaturation. C-13 NMR confirmed the presence of the following carbons: carbonyl carbon at around 178 ppm; a C8, C9 double bond at 133.93 and 133.69 ppm; $-C{=}C-C-$ at 123.75 ppm; a $-C$H$-$OH carbon at 67.87 ppm; methylene carbons cover the range from 22.6 to 40.52 ppm; and the terminal methyl carbon at 14.11 ppm. Thus, the structure is likely 10-hydroxy-8(Z)-octadecenoic acid.

IR analysis

The presence of hydroxy group was indicated by the strong, broad IR absorption at 3400 cm^{-1} region. Other IR absorptions observed were: 1711 cm^{-1} for carbonyl

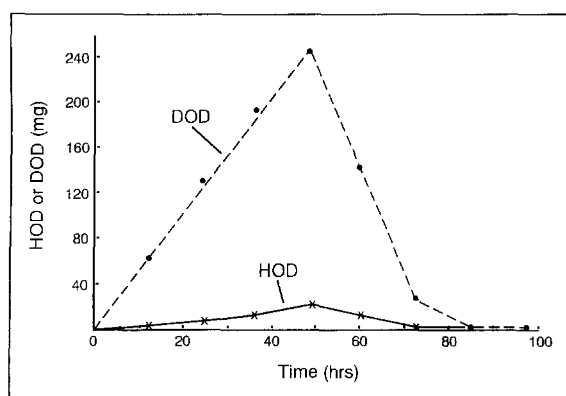

Fig. 3. Time course for the production of HOD by strain PR3. Reaction conditions: substrate oleic acid (0.35 g) was added to a 36-h-old culture in a shake flask at 30 °C. The production of HOD and DOD was assayed at 12-h interval of incubation.

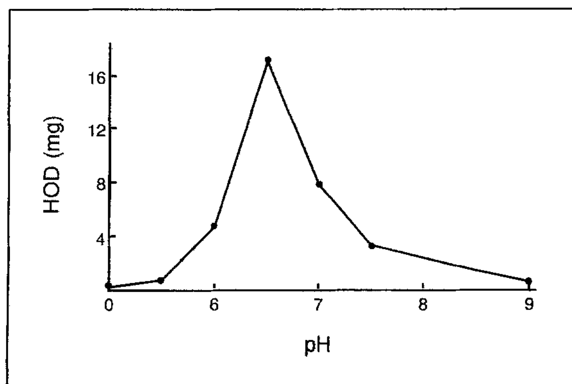

Fig. 4. Effect of pH on the production of HOD by strain PR3. Reaction conditions: Two-day-old cultures grown on pH 7.0 media were used. Prior to the addition of substrate oleic acid (0.35 g), the pH of the culture media was adjusted to desired by the addition of 3 N HCl or 2 N NaOH. The reaction was carried out at 30 °C for 2 days.

group; and 1409 cm^{-1} for *cis* unsaturation; no absorption near 965 cm^{-1} supported that the unsaturation was not *trans*.

Optimum conditions for the production of HOD

Time course results for the production of both HOD and DOD are shown in Fig. 3. The amount of HOD detected throughout the 48-h reaction period remained small while the amount of DOD increased sharply. At 48 h of reaction, the ratio of HOD to DOD was about 1 : 10. The optimum pH and temperature for the production of HOD were 6.5 (Fig. 4) and 30 °C (Fig. 5), about the same as those reported for DOD [6]. From the structure similarity between HOD and DOD, it is likely that HOD is an intermediate in the formation of DOD from oleic acid by strain PR3 and that the conversion from HOD to DOD is not a rate-limiting step. The bioconversion pathway for the production of DOD from oleic acid is, therefore, postulated as follows (Fig. 6): an enzyme in strain PR3 attacks oleic acid at C10 position, introducing a hydroxy group and at the same time shifting the double bond from C9 to C8 while retaining the *cis* unsaturation. The resulting product (HOD) is then oxidized by another (or the same) enzyme at C7 to produce DOD. This step also involves a rearrangement of HOD from *cis* to *trans* geometry which would make carbon-7 more accessible to the enzyme. To confirm this notion, radioactive HOD will be prepared and tested for its conversion to DOD. The industrial applications of both DOD and HOD are currently under investigation.

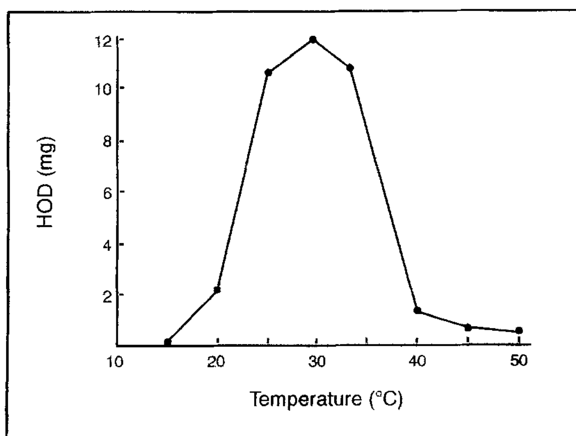

Fig. 5. Effect of temperature on the production of HOD by strain PR3. Cells were grown at 30 °C in pH 7.0 dextrose media for 2 days. After the addition of substrate oleic acid (0.18 g), the flasks were incubated at the temperature indicated for an additional 2 days. The production of HOD was determined by GC analysis.

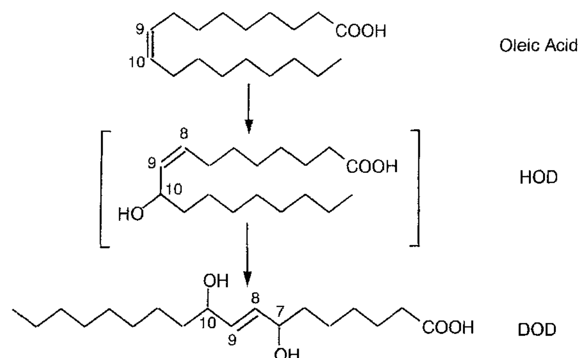

Fig. 6. Posturated bioconversion pathway of oleic acid by strain PR3.

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