Journal of Industrial Microbiology & Biotechnology (2000) 24, 167–172

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Production of 10,12-dihydroxy-8(*E*)-octadecenoic acid, an intermediate in the conversion of ricinoleic acid to 7,10,12-trihydroxy-8(*E*)-octadecenoic acid by *Pseudomonas aeruginosa* PR3

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A bacterial isolate, *Pseudomonas aeruginosa* (PR3), has been reported to produce a new compound, 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD), from ricinoleic acid (Kuo TM, LK Manthey and CT Hou. 1998. J Am Oil Chem Soc 75: 875–879). The reaction is unique in that it involves an introduction of two additional hydroxyl groups at carbon 7 and 10 and a rearrangement of the double bond from carbon 9–10 (*cis*) to 8–9 (*trans*). In an effort to elucidate the metabolic pathway involved in the formation of TOD from ricinoleic acid by PR3, we have isolated another compound from the reaction mixture using HPLC. The structure of the new compound was determined to be 10, 12-dihydroxy-8(*E*)-octadecenoic acid (DHOD) by GC/MS, FTIR, and NMR. The structural similarity between DHOD and TOD and the results from the time course study of the above two compounds strongly suggested that DHOD was an intermediate in the bioconversion of ricinoleic acid to TOD by PR3. The optimum pH and temperature for the production of DHOD from ricinoleic acid by PR3 was 6.5 and 25°C, respectively. This is the first report on the production of 10,12-dihydroxy-8(*E*)-octadecenoic acid from ricinoleic acid by PR3. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 167–172.

Keywords: biotransformation; dihydroxy octadecenoic acid; trihydroxy octadecenoic acid; *Pseudomonas aeruginosa*; ricinoleic acid; hydroxylation

Introduction

Plant systems are known to produce hydroxy fatty acids. Hydroxy fatty acids are important industrial materials because the hydroxyl group on fatty acid gives a fatty acid special properties such as higher viscosity and reactivity compared with other non-hydroxylated fatty acids [1]. Ricinoleic and sebacic acids, two of the castor oil derivatives, are used in the synthesis of high-performance polymers for military equipments and classified by the Department of Defense as strategic and critical materials. Presently, imported castor oil and its derivatives are the only commercial sources of these industrial hydroxy fatty acids.

Microbial enzyme systems convert unsaturated fatty acids to three types of hydroxy fatty acid products, namely: monohydroxy, dihydroxy and trihydoxy fatty acids. Microbial production of 10-hydroxystearic acid from oleic acid was reported [8,15,17,19]. The production of dihydroxy unsaturated fatty acid from oleic acid by *Pseudomonas aeruginosa* PR3 was also reported [4,5]. It involved both hydroxylation and possibly isomerization. The absolute configuration of dihydroxy fatty acid product was determined to be 7(*S*),10(*S*)-dihydroxy-8(*E*)-octadecenoic acid (DOD) [2]. 10-Hydroxy-8(*E*)-octadecenoic acid (HOD) was identified as an intermediate in the bioconversion of oleic acid to DOD by *Pseudomonas aeruginosa* PR3 and *Pseudomonas* sp 42A2 [3,6]. The absolute configuration of HOD was determined to be 10(*S*)-hydroxy-8(*E*)-octadecenoic acid with a minor isomer of *R* form (about 3%) [2]. Linoleic acid was converted to 10-hydroxy-12(*Z*)-octadecenoic acid by *Flavobacterium* sp DS5 [9]. Strain DS5 also converted α- and γ-linolenic acid to 10-hydroxy-6(*Z*),12(*Z*)-octadecadienoic and 10-hydroxy-12(*Z*),15(*Z*)-octadecadienoic acids, respectively [11,12]. Microbial production of trihydroxy fatty acid, 12, 13, 17-trihydroxy-9(*Z*)-octadecenoic acid from linoleic acid was also reported [13]. Trihydroxy unsaturated fatty acids are known to inhibit the growth of plant pathogenic fungi [14]. Microbial oxidation of unsaturated fatty acids was thoroughly reviewed [10].

Recently, Kuo *et al* [16] found that strain PR3 converted ricinoleic acid to 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD). The reaction mechanism involved in TOD production from ricinoleic acid looked similar to that of DOD production from oleic acid in that both the substrates (oleic acid and ricinoleic acid) contained C18 fatty acid backbone with a *cis*-double bond at carbon 9 except for a hydroxyl group pre-introduced at C12 of ricinoleic acid. In an effort to elucidate the metabolic pathway leading to TOD from ricinoleic acid, we isolated a light yellowish, crystal-like compound from the reaction mixture. The structure of the new compound was determined by GC/MS, FTIR, and NMR to be 10,12-dihydroxy-8(*E*)-octadecenoic acid (DHOD). In this paper, we described the isolation and structure determination of this new compound. Optimum conditions for the production of this new compound were also reported. Evidence obtained strongly suggested that DHOD was a plausible intermediate in the bioconversion of ricinoleic acid to TOD.

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Received 28 July 1999; accepted 18 November 1999

Materials and methods

Microorganisms

Pseudomonas aeruginosa NRRL strain B-18602 (PR3) was originally isolated from a water sample in Morton, Illinois [7]. The culture was grown at 28°C aerobically in a 125-ml Erlenmeyer flask containing 50 ml of medium with shaking at 200 rpm. The medium used hereafter contained per liter: 4 g dextrose, 4 g K_2HPO_4 1 g $(NH_4)_2HPO_4$, 1 g yeast extract, 0.1 g $MgSO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 0.01 g $MnSO_4 \cdot 7H_2O$. The medium was adjusted to pH 7.0 with diluted phosphoric acid. Cultures were maintained on agar slant with the medium mentioned above except for the addition of 1.5% agar.

Chemicals

Ricinoleic acid with 99$^+$% purity by gas chromatography (GC) was purchased from Sigma Chemical Co (St Louis, MO, USA). Mixture of trimethylsilylimidazole (TMSI) and pyridine (1:4 v/v) was purchased from Supelco Inc (Bellefonte, PA, USA). Methyl ester of elaidic acid was purchased from NU-Check-Prep Inc (Elysian, MN, USA). All other chemicals were reagent grade and were used without further purification. Kieselgel 60 and thin-layer precoated Kieselgel 60 F_{254} plates were obtained from EM Science (Cherry Hill, NJ, USA). Other chemicals were purchased from Sigma, unless mentioned otherwise.

Bioconversion

Ricinoleic acid (0.2 g) as substrate was added to a 24-h-old culture in the above medium and the flasks were transferred to the adjusted condition followed by continued incubation for an additional 30 h. 1 N HCl and 1 N NaOH were used to adjust the medium pH to study the effect of pH on the production of DHOD. At the end of the cultivation, the culture broth was acidified to pH 2 with 6 N HCl and was extracted twice with an equal volume of ethyl acetate followed by diethyl ether. The solvent was evaporated from the combined extracts with a rotary evaporator.

Analysis of products

Crude extract was subjected to high performance liquid chromatography (HPLC) to purify DHOD. HPLC was conducted with a Hewlett Packard Series 1100 equipped with a Hewlett Packard 1100 series diode array detector and a Microsorb silica column (41.4 mm ID $\times$ 25 cm) from Rainin Instrument Co (Woburn, MA, USA). Purification was started by injecting the crude extract dissolved in methylene chloride/methanol (2:1 v/v) into the HPLC column pre-equilibrated with hexane/ethyl acetate (80:20 v/v). Separation was achieved by eluting the column with a linear gradient from hexane/ethyl acetate (80:20 v/v) after initial holding for 20 min to hexane/ethyl acetate (0:100 v/v) for a total running time of 180 min with a flow rate of 7 ml min^{-1}. Fractions were collected according to the peaks monitored at 240 nm wave length. The collected fractions were analyzed by GC and thin-layer chromatography (TLC). For GC analysis, the samples were first methylated with diazomethane for 1 min at room temperature. The methyl esters then reacted with the mixture of TMSI and pyridine (1:4 v/v) for at least 20 min at room temperature. The TMS derivatized sample was analyzed with a Hewlett Packard model 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA). The GC was equipped with a flame ionization detector, a Supelco SPB-1™ capillary column with 15 m $\times$ 0.32 mm ID, 0.25 μm thickness (Supelco Inc), and a Hewlett Packard 3392A integrator. GC was run isothermally at 205°C for 30 min. For quantitative analysis, methyl ester of elaidic acid was added to the sample as an internal standard prior to TMS derivatization. The TLC was developed with a solvent system consisting of toluene : dioxane : acetic acid (79 : 14 : 7 v/v/v). The spots were visualized first by iodine vapor and then spraying the plate with 50% sulfuric acid followed by heating in a 100°C oven for 10 min.

Chemical structure of the HPLC-purified sample was determined by GC/mass spectrometry (GC/MS), nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectroscopies. Electron-impact mass spectra were obtained with a Hewlett Packard (Avondale, PA, USA) 5890 GC coupled to a Hewlett Packard 5972 Series Mass Selective Detector. The column outlet was connected directly to the ion source. Separations were carried out in a methylsilicone column (30 m $\times$ 0.25 mm ID, 0.25 μm film thickness) with a temperature gradient of 20°C min^{-1} from 70°C to 170°C, holding 1 min at 170°C, and then 5°C min^{-1} to 250°C, followed by holding for 15 min at 250°C (helium flow rate = 0.67 ml min^{-1}). For GC/MS, TMS derivatives of the methyl ester of the purified compound were prepared as mentioned above. Proton and ^{13}C NMR spectra were determined in deuterated chloroform with a Bruker ARS-400 spectrometer (Billerica, MA, USA), operated at a frequency of 400 and 100 MHz, respectively. FTIR analysis of the purified free acid was run as films on KBr on a Perkin Elmer infrared Fourier transform Model 1750 spectrometer (Perkin Elmer, Oakbrook, IL, USA).

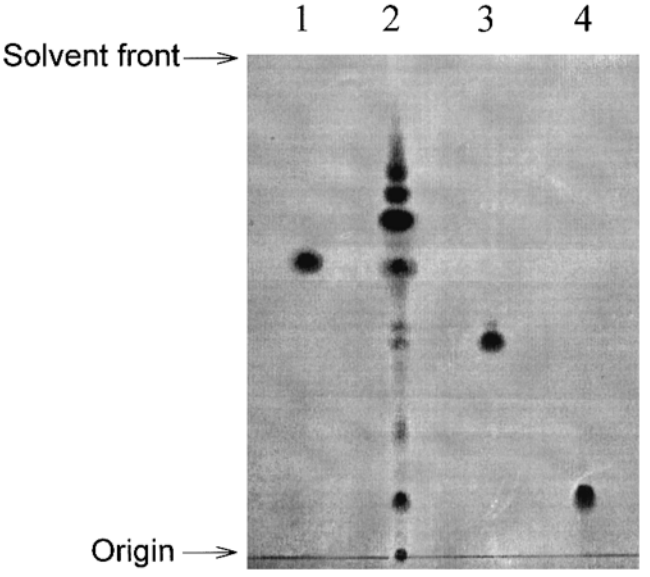

Figure 1 Thin-layer chromatography of DHOD. The solvent system consisted of toluene/dioxane/acetic acid (79:14:7, v/v/v). Lane 1: ricinoleic acid; lane 2: crude reaction products; lane 3: purified DHOD; lane 4: purified TOD. Spots were visualized with iodine vapor.

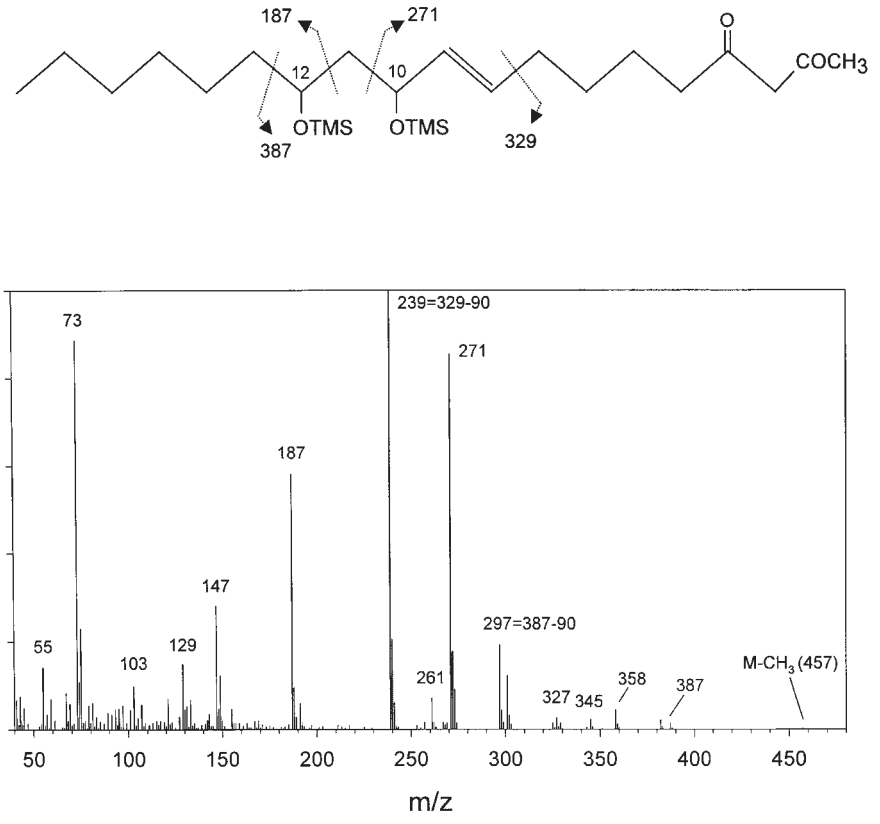

Figure 2 EI mass spectrum of TMS derivatives of methylated DHOD.

Results and discussion

Purification of DHOD

Two hundred milligrams of the crude extract (see Materials and methods) dissolved in 2 ml solvent mixture of methylene chloride/methanol (2:1 v/v) was injected into the HPLC column and separated by eluting the column with a gradient solvent system described in the Materials and methods section. The collected fractions were analyzed by TLC and GC. Most of the targeted product was collected around 115–135 min after injection. Fractions containing the targeted product were combined, concentrated, and subjected to HPLC again under the same condition. After the second HPLC, 15 mg of the pure targeted product (>95% by GC) was obtained. TLC of the purified sample revealed one major spot (Figure 1).

Structure determination

The electron impact GC/MS data of the TMS derivative of the methylated sample are given in Figure 2. This was consistent with the TMS derivative of a methylated C18 dihydroxy monoenoic fatty acid with a molecular mass of 472. The locations of the hydroxyl group were apparent from the fragments observed in the EI spectrum of the TMS derivative of the methylated fatty acid. The intense fragments arising from alpha cleavage to the derivatized hydroxyl groups to give fragments containing the TMS and both the TMS and a double bond were observed at m/z 187 and m/z 271, respectively. These fragments along with those observed at m/z 387 and m/z 329 allocated the hydroxyl groups at C10 and C12 and a double bond at C8-9.

The purified free fatty acid was subjected to IR analysis. The presence of the hydroxyl group was indicated by the broad strong IR absorption at 3600 cm^{-1} region. An absorption observed at 1702 cm^{-1} represented the presence of carbonyl group and the absorption at 966 cm^{-1} indicated that the unsaturation was *trans* [18].

The purified free acid was subjected to proton and ^{13}C NMR analysis to further confirm the elucidated structure of the purified sample. Resonance signals (ppm) and corresponding molecular assignments are given in Table 1. Resonance signal of the olefinic protons (—CH═CH—) obtained from proton NMR was observed at 5.62 ppm with coupling constant of 15.4 Hz strongly indicating *trans*-configuration across the double bond. Two protons (—CH—O—) were observed at 3.75 and 4.23 ppm. ^{13}C NMR confirmed the presence of the following carbons: carbonyl carbon at around 177.4 ppm (C1), a double bond between 131.7 (C8) and 134.8 ppm (C9), and two —CHOH— carbons at 70.3 (C10) and 69.1 ppm (C12). Other protons and carbons are as shown in the table. The data obtained from GC/MS, FTIR, and proton and ^{13}C NMR confirmed that the purified compound was 10,12-dihydroxy-8(*E*)-octadecenoic acid (DHOD).

Optimum conditions for the production of DHOD

The time course study of DHOD production was carried out at 28°C for the time specified after ricinoleic acid was

Table 1 Proton and ^{13}C nuclear magnetic resonance signals and molecular assignments for DHOD

Carbon number	Resonance signal (ppm)	
	Proton	^{13}C
1		177.4
2	2.22	36.2
3	1.58	26.7
4	1.42	29.9
5	1.42	30.2
6	1.42	30.5
7	2.04	33.2
8	5.62 ($J_{8,9}$ = 15.4 Hz)[a]	131.7
9	5.45	134.8
10	4.23	70.3
11	1.58	45.8
12	3.75	69.1
13	1.58	38.9
14	1.25	26.5
15	1.25	30.3
16	1.25	33.0
17	1.25	23.7
18	0.88	14.4

[a]*J*: coupling constant.

added to the culture. The amount of the produced DHOD and TOD and the amount of the remaining substrate are shown in Figure 3. Maximum amount of the produced DHOD and TOD were reached at 30 h and 37 h, respectively. After peaking at 30 h, the production of DHOD declined sharply. However, the production of TOD increased beyond 30 h and reached a maximum at 37 h after which it remained on a plateau up to 84 h. This indicated that DHOD might be an intermediate of TOD production from ricinoleic acid. Further incubation reduced the TOD content in the medium indicating that PR3 metabolized TOD. The fact that the substrate ricinoleic acid disappeared within 24 h of reaction indicated that ricinoleic acid was also converted to compounds other than DHOD and TOD. Based on these results, the production of DHOD was standardized at 30 h wherever other variables were evaluated.

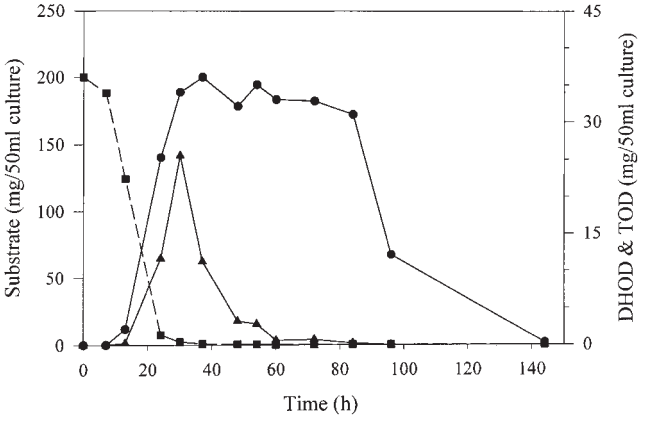

Figure 3 Time course production of DHOD and TOD from ricinoleic acid. Substrate (200 mg) was added to a 24-h-old culture in a shake flask followed by an additional 30-h incubation. ●: TOD (mg per 50 ml culture), ▲: DHOD (mg per 50 ml culture), ■: ricinoleic acid remained in the culture (mg per 50 ml culture) (see Materials and methods for details).

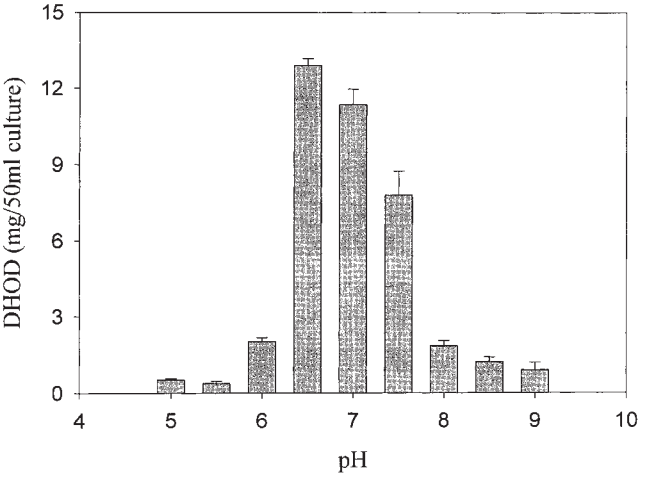

Figure 4 Effect of pH on the production of DHOD from ricinoleic acid. Substrate (200 mg) was added to a 24-h-old culture in a shake flask followed by an additional 30-h incubation. pH was adjusted with 1 N HCl or 1 N NaOH prior to the addition of substrate.

The effect of pH on the production of DHOD was studied. Substrate was added to the 24-h-old culture of which pH was adjusted to the desired value ranging from 5.0 to 9.0 and then incubation continued for an additional 30 h under the standard condition. As shown in Figure 4, the optimum pH for the production of DHOD appeared to be 6.5 which was the same as that reported for the production of 10-hydroxy-8(*E*)-octadecenoic acid (HOD), an intermediate of 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) production from oleic acid by the same strain [6].

The effect of reaction temperature on the production of DHOD from ricinoleic acid by PR3 was also studied with the range from 10°C to 40°C. Incubation continued for an additional 30 h under the adjusted temperature after the addition of substrate. The DHOD production increased significantly as the temperature increased from 10°C (Figure 5) and reached a maximum at 25°C after which a sharp decrease in DHOD production was observed at 30°C and

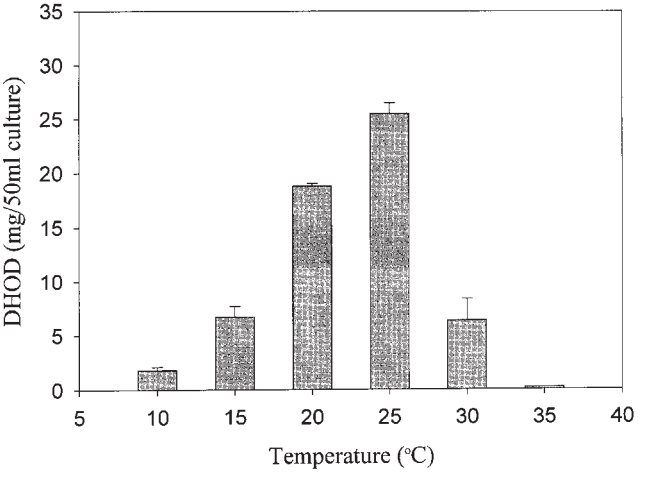

Figure 5 Effect of incubation temperature on the production of DHOD from ricinoleic acid. Cells were grown at 28°C for 24 h in pH 7.0 dextrose medium. After addition of ricinoleic acid (200 mg), incubation continued at the temperature specified for an additional 30 h.

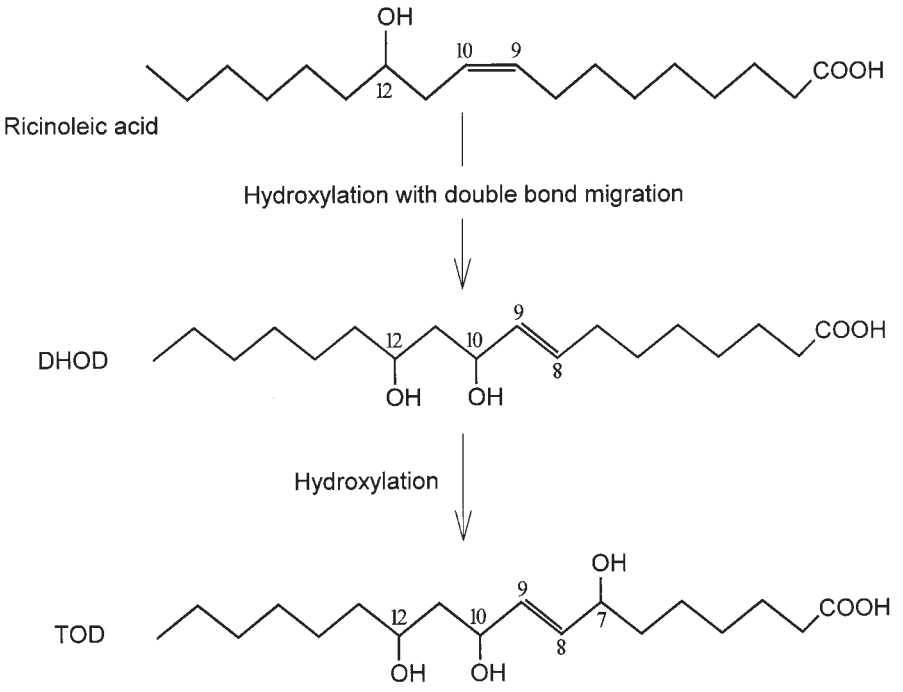

Figure 6 Postulated bioconversion pathway of ricinoleic acid leading to TOD.

over. This result was different from those reported for the production of HOD [6] and DOD [5] by the same strain in which 30°C was the optimum temperature for their production.

Based on the structural similarity between DHOD and TOD and the results from this study, the overall bioconversion pathway of ricinoleic acid leading to TOD by strain PR3 was postulated as shown in Figure 6. Substrate (ricinoleic acid) is first converted to DHOD during which one hydroxyl group is introduced at C10 and the double bond is shifted from *cis* C9-10 to *trans* C8-9. The resulting plausible intermediate DHOD is then subject to another hydroxylation at C7 by possibly the same enzyme(s) involved in the first hydroxylation reaction resulting in TOD formation. This reaction pathway resembles that involved in the production of DOD from oleic acid by the same strain [6] in that it involves an introduction of two new hydroxyl groups at carbon 7 and 10 and a rearrangement of the double bond from carbon 9–10 (*cis*) to 8–9 (*trans*). This suggested that the same enzyme system in *Pseudomonas aeruginosa* PR3 was possibly used for the production of DOD and TOD from oleic acid and ricinoleic acid, respectively. Further work should be done to investigate the specific enzyme(s) involved in this reaction.

Acknowledgements

We gratefully acknowledge the excellent technical assistance of AL Ashbury. We thank Dr D Weisleder for NMR, Mr A Lanser for FTIR analysis.

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