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Production of isomeric 9,10,13 (9,12,13)-trihydroxy-11*E* (10*E*)-octadecenoic acid from linoleic acid by *Pseudomonas aeruginosa* PR3[1]

H Kim, HW Gardner and CT Hou

Oil Chemical Research, National Center for Agricultural Utilization Research, ARS, USDA, Peoria, IL 61604, USA

Trihydroxy unsaturated fatty acids with 18 carbons have been reported as plant self-defense substances. Their production in nature is rare and is found mainly in plant systems. Previously, we reported that a new bacterial isolate, *Pseudomonas aeruginosa* PR3, converted oleic acid and ricinoleic acid to 7,10-dihydroxy-8(*E*)-octadecenoic acid and 7,10,12-trihydroxy-8(*E*)-octadecenoic acid, respectively. Here we report that strain PR3 converted linoleic acid to two compounds: 9,10,13-trihydroxy-11(*E*)-octadecenoic acid (9,10,13-THOD) and 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (9,12,13-THOD). Stereochemical analyses showed the presence of 16 different diastereomers — the maximum number possible. The optimum reaction temperature and pH for THOD production were 30°C and 7.0, respectively. The optimum linoleic acid concentration was 10 mg/ml. The most effective single carbon and nitrogen sources were glucose and sodium glutamate, respectively. However, when a mixture of yeast extract (0.05%), $(NH_4)_2HPO_4$ (0.2%), and NH_4NO_3 (0.1%) was used as the nitrogen source, THOD production was higher by 8.3% than when sodium glutamate was the nitrogen source. Maximum production of total THOD with 44% conversion of substrate was achieved at 72 h of incubation, after which THOD production plateaued up to 240 h. THOD production and cell growth increased in parallel with glucose concentration up to 0.3%, after which cell growth reached its maximum and THOD production did not increase. These results suggested that THODs were not metabolized by strain PR3. This is the first report of microbial production of 9,10,13- and 9,12,13-THOD from linoleic acid. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 109–115.

Keywords: bioconversion; trihydroxy octadecenoic acid; linoleic acid; *Pseudomonas aeruginosa* PR3

Introduction

Hydroxy fatty acids are important industrial materials because the hydroxyl group gives fatty acids special properties such as higher viscosity and reactivity compared with non-hydroxylated fatty acids [1]. Hydroxy fatty acids are used in a wide range of industrial products including resins, waxes, nylons, plastics, lubricants, cosmetics, and additives in coatings and paintings [34]. During past decades, much effort has been focused on the microbial production of hydroxy fatty acids from various fatty acid substrates. These hydroxy fatty acids produced in microbial systems are classified into three types: monohydroxy [15–18,28,29,31,38], dihydroxy [10,21,22,29] and trihydroxy fatty acids [19,20,23,24,30]. In addition to these multi-hydroxy fatty acids produced in microbial systems, there are numerous reports of the formation of 9,10,13-trihydroxy-11(*E*)-octadecenoic acid (9,10,13-THOD), 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (9,12,13-THOD), 9,10,11-trihydroxy-12-octadecenoic acid (9,10,11-THOD), and 11,12,13-trihydroxy-9-octadecenoic acid (11,12,13-THOD) in plants, starting with a report of their occurrence in wheat flour [9]. THODs in plants are formed sequentially from fatty acid hydroperoxides to epoxyhydroxy fatty acids, which then solvolyze. The enzymes catalyzing formation of epoxyhydroxy fatty acids in plants are epoxyalcohol synthase and peroxygenase [12,13]. Animal hydroxy-epoxides, the hepoxylins, solvolyze to form trihydroxy fatty acids [35]. Linoleic acid hydroperoxides are converted into THODs by homolytic and heterolytic mechanisms through epoxyhydroxy fatty acid intermediates [6], and these chemical systems are believed to contribute to the formation of THODs in biological systems. Of these trihydroxy fatty acids, 9,10,11-THOD, 9,10,13-THOD, and 9,12,13-THOD have gained special attention. Kato *et al.* [25] reported that the mixed hydroxy fatty acids isolated from the *Sasanishiki* variety of rice plant suffering from the rice blast disease exhibited a strong inhibition activity toward germination and elongation of the germ tube of the conidia of rice blast fungus. Their structures were identified as 9*S*,12*S*,13*S*-trihydroxy-10-octadecenoic acid and 9*S*,12*S*,13*S*-trihydroxy-10,15-octadecadienoic acid [26,27]. 9,12,13-THOD was also isolated from tubers of taro (*Colocasia antiquorum*) inoculated with *Ceratocystis fimbriata* and showed activity against black rot fungus [32]. Recently, Hamberg [12] reported the biosynthesis of antifungal trihydroxy unsaturated fatty acids in potato leaves.

Although the biological significance of these THODs was reported, their production in nature is restricted mainly to plant systems, and in trace amounts. No microbial system has been reported to produce trihydroxy unsaturated fatty acids. Previously, we reported that a bacterium, *Pseudomonas aeruginosa* PR3, was able to convert unsaturated fatty acids to dihydroxy fatty acids [21,22,28,29]. Here we report that the organism also converts

Correspondence: Dr CT Hou, Oil Chemical Research, ARS, USDA, 1815 N. University St., Peoria, IL 61604, USA

[1]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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linoleic acid to two compounds: 9,10-13-THOD and 9,12,13-THOD. The structures of these products were determined by gas chromatography/mass spectrometry (GC/MS). We also report the optimum conditions for the production of these two THODs from linoleic acid by *P. aeruginosa* PR3.

Materials and methods

Microorganisms

P. aeruginosa NRRL strain B-18602 (PR3), isolated from a water sample from a hog farm near Peoria, IL, was grown aerobically at 28°C in 125 ml Erlenmeyer flasks containing 50 ml of standard medium with shaking at 200 rpm. The standard medium contained per liter: 4 g dextrose, 2 g K_2HPO_4, 2 g $(NH_4)_2HPO_4$, 1 g NH_4NO_3, 0.5 g yeast extract, 0.014 g $ZnSO_4$, 0.01 g $FeSO_4{\cdot}7H_2O$ and 0.01 g $MnSO_4{\cdot}7H_2O$. For the studies on optimization of THOD production, the standard medium was modified as specified. The medium was adjusted to pH 7.0 with a 1:3 dilution of phosphoric acid. Cultures were maintained on agar slants with the standard medium with the addition of 1.5% agar.

Chemicals

Linoleic acid and the methyl ester of elaidic acid (99^{+}% purity by GC) were purchased from NU-Check-Prep Inc. (Elysian, MN, USA). A mixture of trimethylsilylimidazole (TMSI) and pyridine (1:4 v/v) was purchased from Supelco Inc. (Bellefonte, PA, USA). All other chemicals were reagent grade and were used without further purification. Other chemicals were purchased from Sigma (St. Louis, MO, USA), unless mentioned otherwise.

Bioconversion

Linoleic acid (0.5 g) as substrate was added to a 24-h-old culture in the standard or modified medium and then flasks were transferred to the adjusted condition followed by incubation for an additional 72 h. We used 1 N HCl and 1 N NaOH to adjust the pH of 24-h-old cultures before the addition of substrate to determine the effect of pH on THOD production. At the end of the cultivation, the culture broth was acidified to pH 2 with 6 N HCl followed by immediate extraction twice with an equal volume of ethyl acetate and diethyl ether. The solvent was evaporated from the combined extracts using rotary evaporator.

Analysis of products

Products in crude extracts were analyzed and identified by GC/MS. Crude samples were first methylated with diazomethane for 1 min at room temperature. After the solvent was evaporated under a stream of nitrogen, the methyl esters were derivatized with a mixture of TMSI and pyridine (1:4 v/v) for at least 20 min at room temperature. The TMSI-derivatized sample was analyzed using 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 capillary column with 30 m×0.25 mm i.d., 0.25 μm film thickness (Supelco Inc.). GC was run with a temperature gradient of 20°C/min from 70°C to 200°C, holding 1 min at 200°C, and then 0.7°C/min to 240°C, followed by holding for 15 min at 240°C (helium flow rate=0.67 ml/min). For quantitative analysis, the methyl ester of elaidic acid was added to the sample as an internal standard prior to TMSI derivatization. The internal standard method was confirmed by gravimetric measurement of the product. Chemical structures of the analyzed products were determined with the electron impact mass spectra obtained with GC/MS. The values presented in each experiment of this study are averages of duplicates. The error range was within 10% of average values.

Stereochemical analysis

Complete stereochemical analysis of 9,10,13-THOD and 9,12,13-THOD was accomplished by the method of Hamberg [11] with some modifications. The method involves thin layer chromatography (TLC) separation into classes of *cis*-1,4-diols and *trans*-1,4-diols followed by separation using boric acid-impregnated TLC into *erythro* and *threo* diols. This gives four classes of two GC-separable 9,10,13-THOD and 9,12,13-THOD peaks. Each of these two potentially contains mirror image stereoisomers, e.g. 9(*S*),10(*S*),13(*S*)-THOD and 9(*R*),10(*R*),13(*R*)-THOD, which can be determined by derivatizing them with (−)-menthylchloroformate followed by oxidative cleavage. The modification used in this study employed boric-acid-impregnated plates, instead of the Na arsenite plates used by Hamberg [11]. TLC plates (Merck, Darmstadt, Germany; silica gel 60 F_{254}, 20 m×20 cm×250 μm) were briefly submerged in an aqueous solution of 11 g boric acid/200 ml, positioned vertically to drain and dry, and then activated at 110°C in an oven for 1 h. The *erythro* (lower R_f) and *threo* separation (higher R_f) of either the *cis*-1,4-diols or the *trans*-1,4-diols fraction was accomplished by development with $CHCl_3$/MeOH (95:5, v/v). Detection was with aqueous 0.1% Na 8-anilino-1-napthalenesulfonate (ANS) spray followed by long-UV viewing. TLC scrapings were extracted with ethyl acetate/MeOH (4:1, v/v). The solvent was evaporated and then treated with 4 ml of a saturated solution of mannitol (ca. 7.5% mannitol) in MeOH/H_2O (1:1 v/v) to sequester boric acid from complexes with THOD. After 45 min of incubation, the THODs were extracted with 4 ml $CHCl_3$ and the $CHCl_3$ layer was washed twice with 2 ml H_2O. All other procedures were as described by Hamberg [11], except that the (−)-menthylcarbonyl (MC) derivatives were oxidatively cleaved with $KMnO_4$ in acetic acid [14] instead of by oxidative ozonolysis.

Results

Identification of products

P. aeruginosa PR3 converted linoleic acid to a mixture of six major products with GC retention times from 37.8 to 40.5 min (Figure 1). Mass spectra of the TMSI-derivatized methyl esters of 9,10,13-THOD and 9,12,13-THOD were previously reported by numerous investigators [3–5,11,32,36,37]. The mass spectra of TMSI-derivatized and methyl-esterified peaks A and D in Figure 1 were identical and afforded ions at *m/z* [ion structure] (percent relative intensity) 460 [rearrangement with elimination of $CH_3(CH_2)_4CHO$] (2), 439 $[M\text{-}TMSIOH\text{-}CH_3O]^+$ (0.3), 317 (3), 301 (3), 259 $[CHOTMSI(CH_2)_7COOCH_3]^+$ (100), 230 (4), 211 (6), 173 (7), 155 (22), 147 (12) 129 (8), 109 (8), 75 (8), 73 $[TMSI]^+$ (53). These mass spectra were in excellent agreement with the spectrum of a highly purified sample of 9,10,13-THOD (methyl ester, TMSI ether) reported by Hamberg [11]. Therefore, peaks A and D were considered to be two or more different stereo isomers of 9,10,13-THOD.

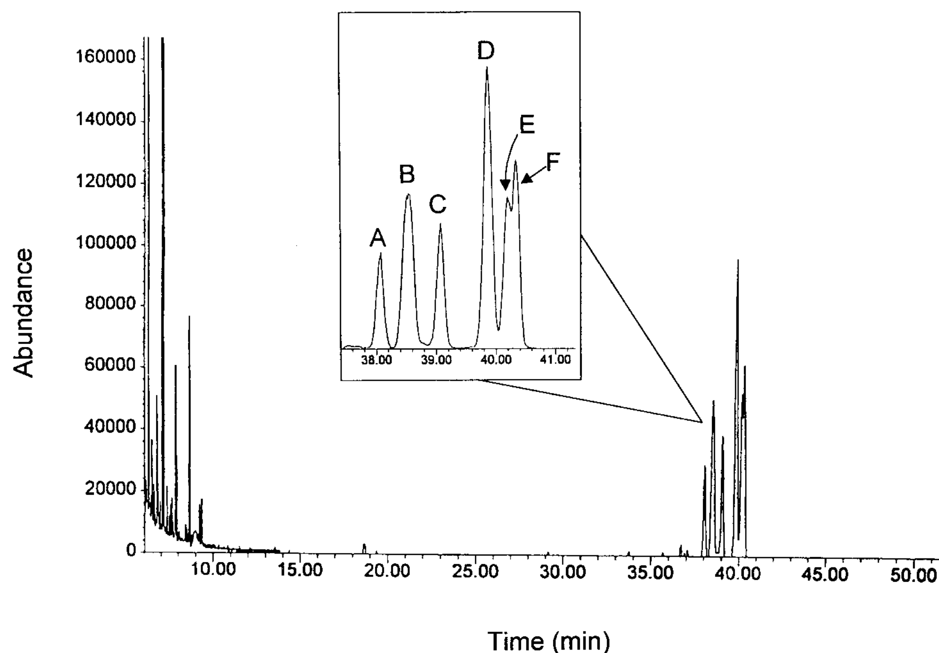

Figure 1 Gas chromatograms of the methylated, trimethylsilylated products from the bioconversion of linoleic acid for 72 h by *P. aeruginosa* PR3. Peaks A and D: 9,10,13-THOD; peaks C, E and F: 9,12,13-THOD; peak B: mixture of 9,10,13-THOD and 9,12,13-THOD. Other reaction conditions are in Materials and methods section.

GC peaks C, E, and F gave virtually identical mass spectra with ions at m/z [ion structure] (percent relative intensity) 460 [rearrangement with elimination of $CH_3(CH_2)_4CHO$] (7), 439 $[M\text{-}TMSIOH\text{-}CH_3O]^+$ (1), 387 (2), 317 (1), 297 (2), 259 (12), 230 (5), 173 $[CHOTMSI(CH_2)_4CH_3]^+$ (100), 155 (4), 147 (12), 129 (4), 103 (9), 75 (7), 73 $[TMSI]^+$ (51). These mass spectra are in good agreement with the spectrum of a highly purified sample of 9,12,13-THOD (methyl ester, TMSI ether)

Table 1 Total stereochemical analysis of 9,10,13-THOD and 9,12,13-THOD obtained from bioconversion of linoleic acid by *P. aeruginosa* PR3

	Percent by GC-FID[a]		Percent stereoisomer[a,b] % *R*		% *S*		Identity of triOH	Percent of total (mean, *n*=2)
	(1)	(2)	(1)	(2)	(1)	(2)		
cis-1,4-Diols								
threo 9,10,13	9.25	8.68	50.5	49.2	49.5	50.8	9(*R*),10(*R*),13(*S*)	4.53
							9(*S*),10(*S*),13(*R*)	4.47
threo 9,12,13	10.2	10.0	54.4	54.1	45.6	45.9	9(*R*),12(*S*),13(*S*)	5.48
							9(*S*),12(*R*),13(*R*)	4.62
erythro 9,10,13	13.4	12.2	52.8	53.4	47.2	46.6	9(*R*),10(*S*),13(*R*)	6.80
							9(*S*),10(*R*),13(*S*)	6.00
erythro 9,12,13	14.0	15.0	50.4	49.6	49.6	50.4	9(*R*,12(*S*),13(*R*)	7.25
							9(*S*),12(*R*),13(*S*)	7.25
trans-1,4-Diols								
threo 9,10,13	10.8	11.2	53.3	51.5	46.7	48.5	9(*R*),10(*R*),13(*R*)	5.75
							9(*S*),10(*S*),13(*S*)	5.22
threo 9,12,13	11.1	12.2	48.8	42.6	51.2	47.4	9(*R*),12(*R*),13(*R*)	5.92
							9(*S*),12(*S*),13(*S*)	5.76
erythro 9,10,13	14.8	14.3	49.7	46.8	50.3	53.2	9(*R*),10(*S*),13(*S*)	7.52
							9(*S*),10(*R*),13(*R*)	7.01
erythro 9,12,13	16.4	16.5	54.6	53.2	45.4	46.8	9(*R*),12(*R*),13(*S*)	8.86
							9(*S*),12(*S*),13(*R*)	7.58

[a]Values represent two separate analyses, (1) and (2).
[b]Percent (*R*) or (*S*) is derived from GC separation of methyl 2(*R*)- or 2(*S*)-menthylcarbonyl-heptanoate derivative obtained from 9,10,13-THOD or separation of dimethyl 2(*R*)- or 2(*S*)-menthylcarbonyl-decandioate from 9,12,13-THOD. The stereoconfiguration of the other carbons follows from the configuration of the 1,4-diol and 1,2-diol permitting analysis of all possible isomers (total of 16).

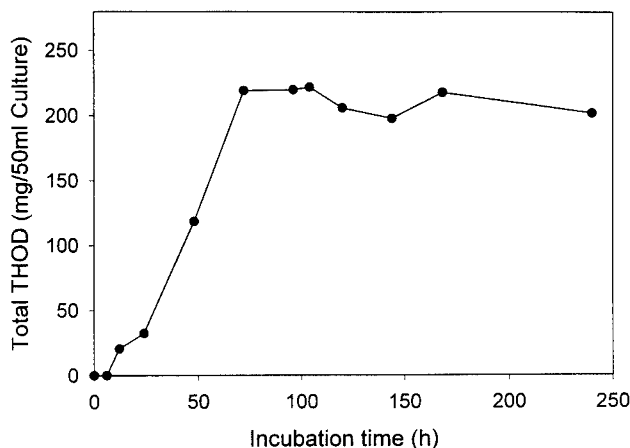

Figure 2 Time course of production of THOD from linoleic acid by *P. aeruginosa* PR3. Incubation time represents reaction time after linoleic acid (500 mg) was added to the 24-h-old culture. See Materials and methods section for other reaction conditions.

reported by Hamberg [11]. Accordingly, peaks C, E and F were different stereo isomers of 9,12,13-THOD. Because GC peak B (Figure 1) exhibited mass spectral ions characteristic of both 9,10,13-THOD and 9,12,13-THOD, this peak was obviously a mixture. Based on the results obtained above, it was concluded that the six major peaks of the GC chromatogram in Figure 1 represented different stereo isomers and positional isomers of THOD. Because GC alone is incapable of separating all the positional- and stereo isomers of THOD, the mixture was subjected to total stereochemical analysis as described below.

Stereoconfiguration determination

Sixteen stereo- and positional isomers could be separated by GC following a TLC procedure [11]. TLC was used to separate *cis*-1,4-diols from *trans*-1,4-diols, as shown in Figure 4 by group 1, and one containing eight isomers each. The second separation on boric-acid-impregnated TLC plates gives separation into *erythro* (low R_f) and *threo* (high R_f) diols indicated by *e* and *t* in Figure 4. Each of the four TLC fractions then afforded two GC peaks comprised of earlier-eluting 9,10,13-THOD followed by a slightly later-eluting 9,12,13-THOD, making a total of eight. Each of these two GC peaks was comprised of mirror image stereo isomers making a total of 16 positional- and stereo isomers; i.e. all possible combinations of isomers.

Table 2 Effect of nitrogen sources on THOD production

Nitrogen source	Total THOD production (mg/50 ml culture)
Control[a]	220
Yeast extract (0.1%)	113
Peptone (0.1%)	118
Tryptone (0.1%)	95
NH_4NO_3 (25 mM)	135
NH_4Cl (50 mM)	107
$NaNO_3$ (50 mM)	119
Urea (25 mM)	153
Sodium glutamate (50 mM)	203

[a]Standard medium was used as control medium.

Four TLC-separated fractions, each containing two GC-separable peaks of 9,10,13-THOD and 9,12,13-THOD, were each subjected to derivatization by (−)-methylchloroformate followed by oxidative cleavage of the double bond by $KMnO_4$ and methyl esterification. Each of the four fractions afforded a mixture of methyl 2(*R*)- and 2(*S*)-MC-heptanoates from 9,10,13-THOD and dimethyl 2(*R*)- and 2(*S*)-MC-decanedioate from 9,12,13-THOD, all of which could be separated by GC as diastereomers. This strategy gives the stereoconfiguration of either the 9-hydroxyl for 9,12,13-THOD or the 13-hydroxyl for 9,10,13-THOD. The stereoconfiguration of the other hydroxyls can then be deduced from the *erythro*/*threo* diol and *cis*-1,4-diol/*trans*-1,4-diol configuration determined by TLC. We found that all 16 possible isomers were present (Table 1).

In order to obtain large quantities of THOD, the following production conditions were studied. The total amount of THODs produced was calculated from GC using the internal standard method [28].

Time course of THOD production

The reaction was carried out with standard medium at 28°C for the time specified after addition of linoleic acid to the 24-h-old culture. Control studies with the autoclaved 1-day-grown culture media showed no product formation. The total amount of THODs produced in the culture increased with time up to 72 h (Figure 2). After maximum THOD production (220 mg/50 ml culture, 44% weight yield) at 72 h, the amount of THOD in the medium remained virtually unchanged up to 240 h, indicating that THODs were not further metabolized. This result was different from that reported for the production of 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) from oleic acid by the same strain [21] in which DOD was metabolized by *P. aeruginosa* PR3 after it peaked at 48 h. However, the production of 12,13,17-9(*Z*)-octadecenoic acid (THOA) from linoleic acid by *Clavibacter* sp. ALA2 [19,23] showed similar results in that THOD was not further metabolized. Therefore, THOD production was standardized at 72 h whenever other variables were evaluated.

Effect of substrate concentration on THOD production

Varied amounts of linoleic acid were added to 24-h-old cultures and THOD production was determined after a 72-h incubation under standard conditions. The productions of THOD at linoleic acid concentrations of 100, 200, 300, 500, 700, and 1000 mg were: 12.5, 26, 80, 190, 235, 207, and 208 mg/50 ml, respectively. THOD production increased with the substrate concentration and peaked at 500 mg substrate, after which production decreased slightly and remained at a plateau up to 1 g. Therefore, 500 mg was

Table 3 Effect of carbon sources on THOD production

Carbon source (0.4%)	Total THOD production (mg/50 ml culture)
Glucose (control)	215
Fructose	n.d.[a]
Sucrose	179
Glycerol	8.6
Starch	184
Maltose	202

[a]Not detected.

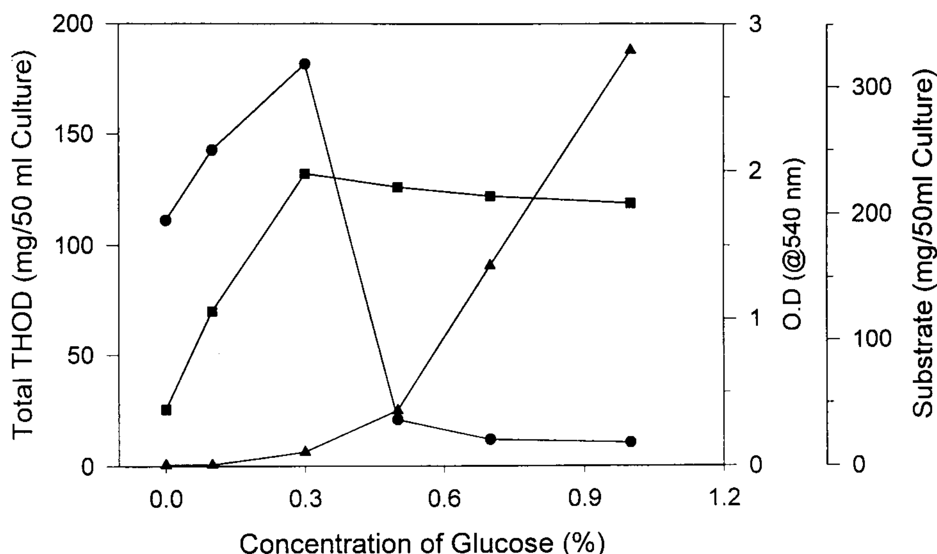

Figure 3 Effect of glucose concentration on THOD production from linoleic acid. Media with different concentrations of glucose were inoculated with *P. aeruginosa* PR3 and then incubated for 24 h under standard conditions followed by addition of substrate and additional incubation for 72 h. (●) Total THOD production. (■) o.d. (▲) Substrate.

selected as an optimal substrate concentration for the production of THODs at the standard culture conditions.

Effect of temperature on THOD production

The effect of reaction temperature on THOD production was studied between 15°C and 45°C. THOD production at temperatures (°C) of 15, 20, 25, 30, 35, 40, and 45 were 74, 130, 210, 260, 250, and 252 mg/50 ml, respectively. THOD production increased significantly in parallel with temperature up to 30°C, after which it reached a maximum at 40°C followed by a sharp decrease at 45°C. This result was different from that for DOD production by the same strain where DOD production was very sensitive to the reaction temperature [21].

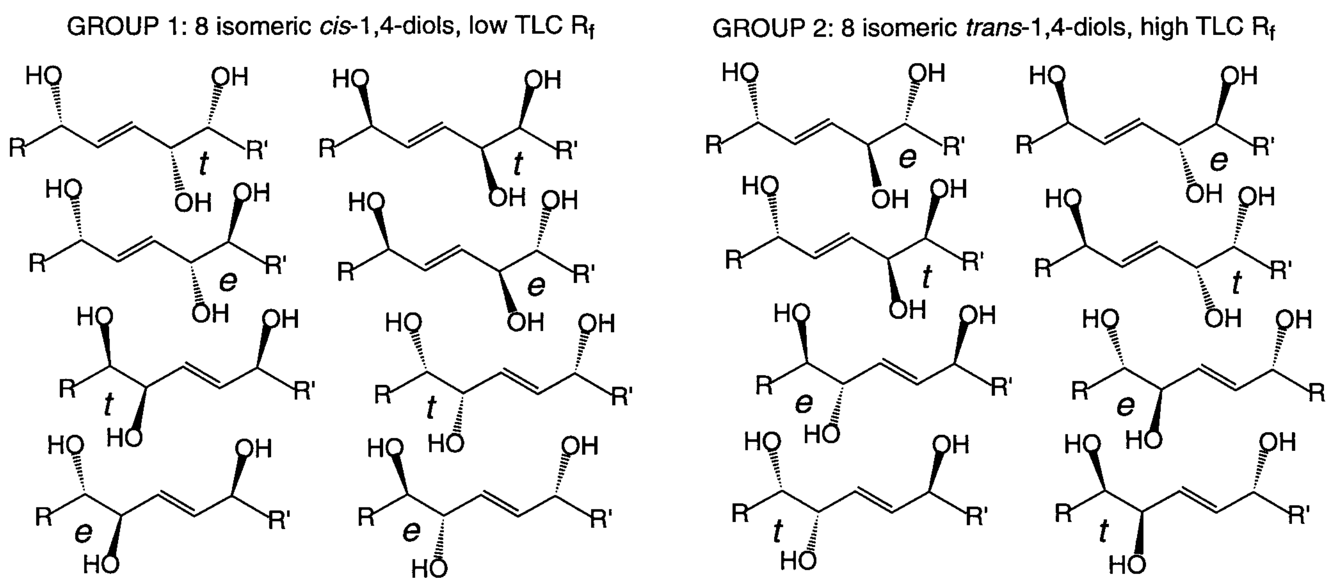

Figure 4 Stereochemical analysis of methyl 9,10,13(9,12,13)-trihydroxy-11(10)*E*-octadecenoic acid. R = $CH_3(CH_2)_4$–; R′ = –$(CH_2)_7COOCH_3$. The first separation, into "*cis*-1,4-diols" and "*trans*-1,4-diols", was accomplished by TLC as shown by Groups 1 and 2 containing eight isomers each. The second separation, on boric-acid-impregnated TLC plates, gave separation into *erythro* (low R_f) and *threo* (high R_f) diols indicated by *e* and *t*. These groups of four are then separated and quantified for their content of 9,10,13-trihydroxyls vs. 9,12,13-trihydroxyls by GLC (two separable peaks of TMSI derivatives). The mirror images 9,10,13-trihydroxyls and 9,12,13-trihydroxyls, i.e., group 1 *threo* isomers, were resolved by forming MC derivatives followed by oxidative cleavage of the double bond, methyl esterification and GLC analyses of dimethyl 2(*R*)- and 2(*S*)-hydroxy(MC)-decandioate (from 9,12,13-THOD) and methyl 2(*R*)- and 2(*S*)-heptanoate (from 9,10,13-THOD). This latter analysis gives the stereoconfiguration of the 9-hydroxyl and 13-hydroxyl, respectively, and the stereoconfiguration of the other hydroxyls follows from other structural features illustrated.

Effect of pH on THOD production

THOD production, pH 5, 5.5, 6, 6.5, 7, 7.5, 8, and 8.5 were: 4, 10, 70, 190, 235, 40, 20, and 4 mg/50 ml, respectively. THOD production was relatively sensitive to the reaction pH. Thus, the optimum pH for production of THODs was 7.0. This was in accordance with that for DOD production by the same strain [21].

Effect of nitrogen sources on THOD production

The combined nitrogen source containing yeast extract (0.05%), $(NH_4)_2HPO_4$ (0.2%), and NH_4NO_3 (0.1%) in the standard medium was replaced with various organic and inorganic nitrogen compounds for testing the production of THOD. THOD was produced with all the nitrogen sources tested with relative productions of 43–92.3% compared to the control (Table 2). As shown in the table, sodium glutamate was the most effective of these nitrogen sources tested for THOD production, but was less sufficient compared to the standard medium control.

Effect of carbon sources on THOD production

The effects of various carbon sources on THOD production are shown in Table 3. Among the carbon sources tested, maltose, starch, sucrose and glucose were effective for THOD production. THOD production relative to the control (glucose) ranged between 83.2% and 94%. However, with glycerol, THOD production was only 4% when compared to that with glucose and no THOD was detected with fructose.

Effect of glucose concentration on THOD production

As glucose was selected as the most effective carbon source, the effects of glucose concentration were studied on cell growth and THOD production. As shown in Figure 3, THOD production and cell growth increased in parallel with glucose concentration up to 0.3%. However, as glucose concentration was increased, cell growth reached a maximum, but THOD production was significantly lower, while the substrate remaining was higher. These results suggested that *P. aeruginosa* PR3 utilized linoleic acid as a substitute carbon source for their growth when the carbon source was not sufficient in the medium and THODs were produced as non-metabolizable byproducts in this microbial process.

Discussion

The distribution of 16 isomers of THOD found in this study (Table 1) was reminiscent of the THODs reported to arise from autooxidation of linoleic acid [11]. It is known from the literature that these THODs arise from 9- and 13-hydroperoxides of linoleic acid both chemically, via alkoxyl radicals or heterolytic rearrangement [6], or by plant enzymes, hydroperoxide-dependent peroxygenase [13] or epoxyalcohol synthase [12]. In the case of enzyme-produced THODs, there is usually more selectivity in the isomers produced [11–13]. In the present study, it appears that either a stereochemically non-specific dioxygenase is involved, like soybean lipoxygenase-3 [8], or *P. aeruginosa* may, in some way, cause autoxidation of linoleic acid. It should be noted that the control incubation did not produce THOD and the carbohydrate supplied, particularly glycerol and fructose (Table 3), had an effect which did not lead to the production of THOD. According to Brash [2], prokaryotes have not yet been found to contain lipoxygenase-like dioxygenases; however, it was reported that *Pseudomonas* 42A2 produced 10-hydroperoxy-8(*E*)-octadecenoic acid from oleic acid [9], which is the probable precursor of 10(*S*)-hydroxy-8(*E*)-octadecenoic acid and 7(*S*), 10(*S*)-dihydroxy-8(*E*)-octadecenoic acid found as products of oleic acid in *P. aeruginosa* [7,28]. Since *P. aeruginosa* did not oxidize the C-7, and -10 of linoleic acid, linoleic acid, surprisingly, is not a substrate for an oleic acid-type of conversion.

Iron in the medium may prove to be an excellent catalyst of autoxidation. Thus, *P. aeruginosa* may be involved in activating iron by complexing it with ligands or changing the redox balance. For example, the redox balance of Fe^{2+}/Fe^{3+} is important in maintaining autoxidation of phospholipid liposomes [33]. Recently, Spiteller and Spiteller [36] reported the involvement of Fe^{2+} and/or Fe^{3+} as an oxidant in the decomposition of linoleic acid hydroperoxide to the corresponding products including 9,12,13-THOD and 9,10,13-THOD. The intermediary compounds reported in the literature mentioned above were also detected in our reaction mixture with linoleic acid as substrate (data not shown).

Therefore, a microbial lipoxygenase may be involved in the production of THODs from linoleic acid in the *P. aeruginosa* PR3 system. It would be of interest to isolate the intermediates and determine their stereo configuration.

Acknowledgement

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