# Identification of (*E*)-11-Hydroxy-9-octadecenoic Acid and (*E*)-9-Hydroxy-10-octadecenoic Acid by Biotransformation of Oleic Acid by *Pseudomonas* sp. 32T3

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**ABSTRACT:** *Pseudomonas* sp. 32T3, a newly identified strain originally isolated from a vegetable oil-contaminated soil, produces three monohydroxy acids—(*E*)-11-hydroxy-9-octadecenoic acid, (*E*)-10-hydroxy-8-octadecenoic acid, and (*E*)-9-hydroxy-10-octadecenoic acid—as bioconversion products of oleic acid. The bacterial cells were grown in a mineral medium containing oleic acid as the main carbon substrate. The compounds were identified as the corresponding methyl esters on the basis of their chromatographic and spectroscopic ($^1$H and $^{13}$C nuclear magnetic resonance and gas chromatography–mass spectrometry) features.

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**KEY WORDS:** Biotransformation, hydroxy fatty acids, oleic acid, *Pseudomonas.*

Hydroxy fatty acids are an important family of compounds with a wide range of applications, such as resins, surfactants, lubricant additives, plastics, coatings, cosmetics, nylons, and materials for the textile industry. Moreover, they protect plants against microbial infection, although the mechanism of these antimicrobial effects is poorly understood (1).

Bioconversion of oleic acid by a variety of microorganisms has been reported. The process may involve saturation of the alkyl chain, oxidation of the double bond to the ketoacid by *Staphylococcus* (2), *Nocardia, Mycobacterium* (3), or *Sphingobacterium* (4), oxidation to hydroxystearic acid by *Saccharomyces* (3) or *Rhodococcus* (5) or to several hydroxyoctadecenoic acids by *Nocardia, Selenomonas,* and *Enterococcus* (6,7) or *Alcaligenes* sp. (8). Hydroxy ketoacids have also been produced by a *Bacillus* strain (9). We reported that *Pseudomonas* sp. 42A2 produces (*E*)-10-hydroxy-8-octadecenoic acid and (*E*)-7,10-dihydroxy-8-octadecenoic acid and postulated that the formation of the monohydroxy derivative may occur through the corresponding hydroperoxide precursor (10–12). The diol derivative was also identified by Hou *et al.* (13) from cultures of *Pseudomonas aeruginosa*, and later the absolute configuration (14) of the monohydroxy derivative was also determined to be *S* (15).

In our current interest in the microbiological reutilization of waste oils, we report a new strain *Pseudomonas* sp. 32T3, originally isolated from a vegetable oil-contaminated soil. This strain produces the hydroxy derivatives (*E*)-11-hydroxy-9-octadecenoic acid, (*E*)-10-hydroxy-8-octadecenoic acid, and (*E*)-9-hydroxy-10-octadecenoic acid when grown in a mineral medium with oleic acid as the main carbon substrate. In this work we present the detection and chemical structural determination of hydroxy fatty acids obtained from cultures of *Pseudomonas* sp. 32T3 when incubated in salts mineral medium with crude oleic acid.

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## MATERIALS AND METHODS

*Microorganism and culture medium. Pseudomonas* sp. 32T3 was isolated from a vegetable oil-contaminated soil sample. The medium composition was as follows (g/L): $K_2HPO_4$, 2; $KH_2PO_4$, 1; KCl, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.012; $NaNO_3$, 7; and trace minerals, 0.05 mL. The medium was sterilized for 20 min at 121°C, and technical-grade oleic acid, previously sterilized in the same conditions, was added to 2% (vol/vol) as the carbon source. The pH was adjusted to 6.8.

*Chemicals.* All solvents and chemicals were analytically pure and obtained from commercial sources (Fluka, Steinheim, Switzerland; Panreac, Barcelona, Spain; and Aldrich, Madrid, Spain). The substrate for biotransformation, technical-grade oleic acid (*ca.* 80% purity by gas chromatography), was kindly supplied by Clarian (Barcelona, Spain).

*Bioconversion and product extraction*. The above culture medium was inoculated with 2% (vol/vol) of a *Pseudomonas* sp. 32T3 saline suspension (optical density at 540 nm of 2) from an overnight culture. The culture was aerobically grown at 30ºC in 250 mL of medium in a 1-L Erlenmeyer flask shaken at 150 rpm in a reciprocal shaker (Sanyo, Braun Biotech, Barcelona, Spain). Cultures were incubated for 120 h and samples were taken at 24, 48, 72, 96, and 120 h. The organic extracts were analyzed by thin-layer chromatography (TLC), and the amount of bioconversion products was highest after 72 h of incubation. At the end of the incubation, the culture was centrifuged, and the cell pellet was discarded. The supernatant was acidified to pH 2 with 6 N HCl and extracted twice with chloroform/methanol (2:1 vol/vol). The combined extracts were dried over anhydrous sodium sulfate and filtered, and the solvent was removed with a rotary evaporator.

*Purification of products*. The crude lipid extract was esterified with diazomethane (16) and purified by column chromatography on a 50 × 3 cm i.d. column packed with 60 g of silica gel 60 A C.C. Chromagel (SDS, Peypin, France). The column was eluted with mixtures of hexane/diethyl ether from 100:15 to 100:100 (all ratios vol/vol), increasing the amount of ether by 10% in each retention volume. A total of 60 fractions was eluted and analyzed by TLC, and those fractions containing compounds with $R_f$ similar to those of the expected monohydroxy octadecenoates were combined. These corresponded to fractions eluted with mixtures of hexane/diethyl ether 100:60, 100:75, and 100:90. The pooled material was repurified by column chromatography, eluting with mixtures of hexane/diethyl ether 100:5 to 100:100 to collect only the eluate with hexane/diethyl ether 100:40. This fraction was analyzed by gas chromatography–mass spectroscopy (GC–MS) and $^1$H and $^{13}$C nuclear magnetic resonance (NMR).

*Analysis of products*. TLC analyses were performed on 20 × 20 cm silica gel 60 $F_{254}$ (0.25 mm thickness) analytical plates (Panreac) and 20 × 20 cm silica gel 60 $F_{254}$ (0.50 mm thickness) preparative plates with a 20 × 4 cm concentration zone (Merck, Darmstadt, Germany). Plates were eluted with hexane/diethyl ether/acetic acid (80:50:1, vol/vol/vol), and spots were visualized by spraying with a solution of phosphomolybdic acid in absolute ethanol, followed by charring with a heat gun. GC analyses were carried out by injecting the samples into a Shimadzu GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a Supelco SPB-1 fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness, from Tecknokroma, St. Cugat, Spain). The integration of peaks was carried out on a Shimadzu C-R4A integrator-recorder. Helium was used as the carrier gas at a flow rate of 0.98 mL/min. Compounds were isothermally eluted at 200ºC.

For identification purposes, the compounds were transformed into their corresponding trimethylsilyl (TMS) derivatives as follows. In a 25-mL Erlenmeyer flask were placed 1–5 mg of the sample, 1 mL of anhydrous pyridine, 0.2 mL of hexamethyldisilazane, and 0.1 mL of trimethylchlorosilane. The reaction mixture was stirred for 12 h at room temperature; then 5 mL of hexane was added, quenched with 5 mL of water, and decanted. The aqueous layer was extracted with several 5-mL portions of hexane, and the organic phases were combined and dried on anhydrous sodium sulfate. After filtering, the solvent was stripped off and the residue was taken up in hexane and analyzed by GC–MS.

GC–MS analyses were run on a Hewlett-Packard 5989 A mass spectrometer coupled to a Hewlett-Packard 5890 series II-gas chromatograph (Hewlett-Packard, Palo Alto, CA) in electron impact (EI) and chemical ionization (CI) mode. CI used methane as the ionizing gas. Derivatized compounds as TMS derivatives were separated on a methyl silicone fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The temperature program was injection at 60ºC, increased at 15ºC/min up to 250ºC, and held at this temperature for 20 min more. Helium was used as the carrier gas at a flow rate of 0.60 mL/min.

$^1$H and $^{13}$C NMR spectra were obtained in $CDCl_3$ on a Varian Gemini-300 spectrometer (Varian Associates, Palo Alto, CA) operating at 300 MHz for $^1$H and 75.4 MHz for $^{13}$C. The values are expressed in δ scale relative to the chloroform signal (δ 7.26 ppm) of the nondeuterated material.

## RESULTS AND DISCUSSION

*Identification of the microorganism.* The original isolate was obtained from a sample taken from an oil-contaminated soil after an enrichment culture with kerosene and plated on Trypticase Soy Agar (TSA, Pronadisa, Barcelona, Spain). The isolate was a Gram-negative rod with respiration metabolism. It was catalase, oxidase, and nitrate-reduction positive and capable of growing on citrate. It was Voges-Proskauer, indole, and gelatin-hydrolysis negative. The isolated strain 32T3 was motile. Based on these results, the isolate was assigned to the genus *Pseudomonas*.

*Biotransformation of oleic acid by* Pseudomonas *sp. 32T3.* In previous studies we reported that *Pseudomonas* sp. 42A2 converted oleic acid into (*E*)-10-hydroxy-8-octadecenoic acid, (*E*)-10-hydroperoxy-8-octadecenoic acid, and (*E*)-7,10-dihydroxy-8-octadecenoic acid (11). In our efforts to identify new hydroxy fatty acid derivatives from oleic acid, the strain *Pseudomonas* sp. 32T3 was cultivated in mineral medium with technical-grade oleic acid as substrate for growth and biotransformation. Detection of the biotransformation products was carried out by comparison of their TLC behavior with that of the products of biotransformation of oleic acid by *Pseudomonas* sp. 42A2 (11). However, since the aim of this work was the characterization of monohydroxy derivatives from a new bacterial strain, we have not quantitatively estimated the yield of the biotransformation products.

*Identification of the conversion products.* Preliminary structure determination was performed by GC analysis of the methylated crude extract and revealed the presence, among others, of two peaks with retention times of 13.723 and 14.983 min on a SPB-1 fused-silica capillary column (Fig. 1A). One peak had a retention time similar to that of the methyl ester of (*E*)-10-hydroxy-8-octadecenoic acid (14.3 min) (12), suggesting that these products were monohydroxy fatty acids. TMS ether derivatives of alcohols have been widely used for identification purposes because of their high volatility, which makes them particularly useful for GC separation of nonvolatile products, and the characteristic α-cleavage products of high diagnostic value they induce (17). Derivatization, as the TMS ethers, showed the presence of a third component by GC–MS (Fig. 1B), probably an isomer of the other two esters according to their very similar chromatographic features. An improved resolution of a mixture of dihydroxyoctadecanoic acids through derivatization as the TMS derivatives was also noticed by Huang *et al*. (18). EI mass spectra of the TMS derivatives showed that the first-eluting compound (GC $R_t$ = 13.723 min) presented a high-

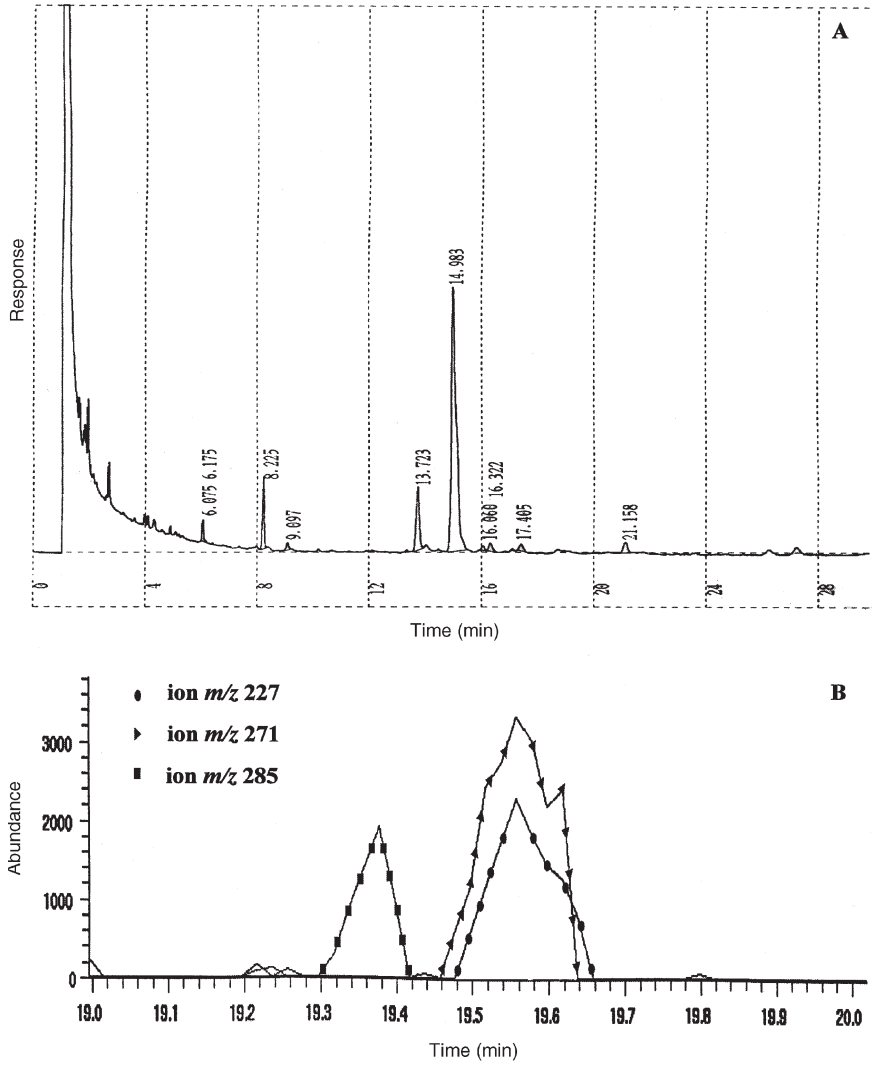


**FIG. 1**. (A) Gas chromatogram of the methyl esters of monohydroxy octadecenoic acids **1** (eluting at a retention time of 13.723 min) and **2–3** (coeluting at a retention time of 14.983 min) obtained by oxidation of oleic acid with *Pseudomonas* sp. 32T3. (B) Selected ion monitoring mass spectra of ions of *m/z* 227, 271, and 285 of the corresponding trimethylsilyl derivatives.

intensity fragment at *m/z* 285, which was attributed to the ion $[Me_3SiOCHCH{=}CH(CH_2)_7COOCH_3]^+$, indicating that the hydroxyl group was at position 11 (Fig. 2A). The spectra of the other two compounds, which coeluted at a GC $R_t$ of 14.983 min, showed the two largest fragment ions of *m/z* 271 $[Me_3SiOCHCH{=}CH(CH_2)_6COOCH_3]^+$ and *m/z* 227 $[Me_3SiOCHCH{=}CH(CH_2)_6CH_3]^+$, arising from α cleavage of the silylated hydroxyl group (Fig. 2B). No molecular ion was apparent from the spectra. The fragment ion of *m/z* 271 suggested the presence of a hydroxyl group at C10 while that of *m/z* 227 is consistent with a C8–C9 bond cleavage of a fatty acid with the hydroxyl group localized at C9. The other expected ions of *m/z* 201, 215 and 259, corresponding to α cleavage of the TMS ether, were not apparent from the spectra, most likely because they represent the unlikely breakage of a vinylic bond (Fig. 2). According to these data, the compounds were postulated to be (*E*)-11-hydroxy-9-octadecenoic acid, (*E*)-10-hydroxy-8-octadecenoic acid, and (*E*)-9-hydroxy-10-octadecenoic. Location of the double bond in each compound was determined on the basis of its NMR spectrum (see below).

The purified extract was subjected to CI–MS analysis. The spectra of the TMS derivatives provided evidence for the presence of monohydroxy octadecenoate derivatives of molecular weight 384 with a base peak of *m/z* 295 $[M - OTMS]^+$. Other important diagnostic ions were the fragments of *m/z* 369 $[M - CH_3]^+$, 263 $[M - OTMS - CH_3OH]^+$, 383 $[M - H]^+$, 385 $[M + H^+]$, as well as the α cleavage fragments of *m/z* 285, 271, and 201 (see above).

The $^1H$ NMR spectrum of the hydroxyester fraction showed in the olefinic region the presence of a doublet of triplets at δ 5.621 ($J$ = 15.0 Hz, $J'$ = 6.6 Hz) corresponding to $H_b$ of the $HOCHCH_a{=}CH_b$ group and a doublet of doublet of triplets centered at δ 5.444 ($J$ = 15.3 Hz, $J'$ = 7.0 Hz, $J''$ = 1.1 Hz) corresponding to $H_a$. The vinylic coupling constant

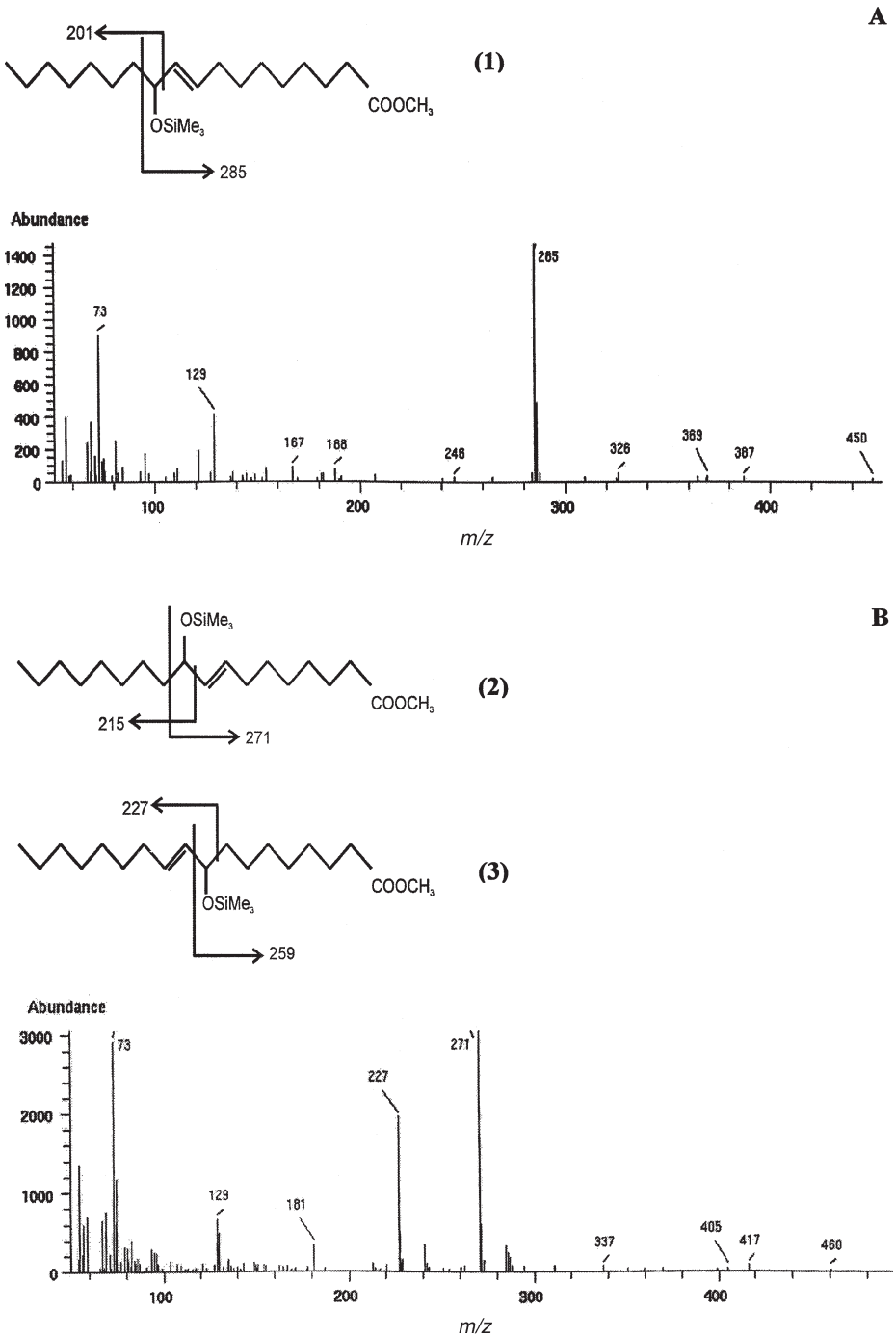


**FIG. 2**. (A) Electron impact (EI) mass spectrum and origin of ion of *m/z* 285 of the trimethylsilyl derivative of the methyl ester of (*E*)-11-hydroxy-9-octadecenoic acid (**1**). (B) EI mass spectrum and origin of ions of *m/z* 271 and 227 of the trimethylsilyl derivatives of the methyl esters of (*E*)-10-hydroxy-8-octadecenoic acid (**2**) and (*E*)-9-hydroxy-10-octadecenoic acid (**3**).

$J_{Ha\text{-}Hb}$ of 15.0–15.3 Hz indicated that the stereochemistry of the double bond was *trans*. Other key signals of the spectrum were a doublet of triplets at δ 4.03 with coupling constants $J$ = 6.6 Hz, $J'$ = 6.3 Hz, corresponding to the methine proton of an allylic hydroxyl group (HOCHC=C), a triplet at δ 2.30 ($J$ = 7.4 Hz) of a methylene group in α position to the carbonyl, and a multiplet at δ 2.02 of the allylic methylene(s). In the $^{13}C$ NMR spectrum the following diagnostic signals were apparent: 174.31 ppm (CO), 131.9–133.2 ppm (cluster of olefinic carbons), 77.29 and 73.24 (CHOH), and 51.4 ($OCH_3$). These data point to the presence of (*E*)-11-hydroxy-9-octadecenoic acid (**1**), (*E*)-10-hydroxy-8-octadecenoic acid (**2**), and (*E*)-9-hydroxy-10-octadecenoic acid (**3**), the first and third compounds being chemicals arising from bioconversion

of edible waste oil. Compound **1** may result from direct allylic oxidation of oleic acid at C-11, and in fact it was obtained by Knothe *et al.* (19) by chemical oxidation of oleic acid with $SeO_2$/*tert*-butylhydroperoxide. Compound **2** is a fungitoxic chemical isolated from the timothy plant *Epichloe typhina* (20) and was identified in the products of the action of *Pseudomonas* sp. 42A2 on oleic acid (11), and by Kim *et al*. (15) in the products of the action of *P. aeruginosa* on oleic acid. Compound **3**, reported by Koshino *et al.* (20), may well proceed from the oxidation of oleic acid at C-9 and migration of the double bond to C-11. Allylic hydroxylations with concomitant migration of double bonds are known to occur in the cytochrome P-450 oxidation of fatty acids (21).

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