Fatty Acid Bioconversions by *Pseudomonas aeruginosa* PR3

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ABSTRACT: The bioconversions of oleic acid, linoleic acid, and ricinoleic acid by *Pseudomonas aeruginosa* PR3 were investigated. The conversion of oleic acid to 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) was improved to better than 80% yields by modifying the culture medium and reaction parameters. The microbial cultures were stable and retained the same level of DOD production capacity for up to 6 mon as examined herewith. Strain PR3 did not bioconvert linoleic acid. However, strain PR3 converted ricinoleic acid to a novel compound, 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD), with a yield of about 35%. The product was further metabolized by strain PR3. TOD was purified by thin-layer chromatography, and its structure was determined by ^{1}H and ^{13}C nuclear magnetic resonance and mass spectrometry. This is the first report on the production of 7,10,12-trihydroxy unsaturated fatty acid by microbial transformation.

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KEY WORDS: Bioconversions, dihydroxy unsaturated fatty acid, oleic acid, *Pseudomonas aeruginosa,* ricinoleic acid, 7,10,12-trihydroxy-8(*E*)-unsaturated fatty acid.

Microbial conversions of unsaturated fatty acids have been widely exploited to produce new, value-added products. Hou (1) recently reviewed the work on some biological oxidation systems. Wallen *et al.* (2) reported the first bioconversion of oleic acid to 10-hydroxystearic acid by a Pseudomonad. In our laboratories, the bioconversion of fatty acids to produce mono-, di-, and tri-hydroxy unsaturated fatty acids has also been found (3–6). The production of a novel compound, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD), from oleic acid by strain PR3 has been described most extensively. Under optimal conditions, the yield of bioconversion is greater than 60% (5). PR3 is a strain of *Pseudomonas aeruginosa*, and its DOD production is inversely correlated with the accumulation of phenazine 1-carboxylic acid (PCA)(7). The production of DOD and PCA by strain PR3, however, was not consistent, and studies were conducted to stabilize and maximize cultures for the bioconversion of oleic acid.

Oxygenated metabolites of unsaturated fatty acids play a variety of important roles in biological systems. Enzymatic conversion of lipid hydroperoxides to trihydroxy fatty acids has been reported in many higher plants (8). 8,9,13-Trihydroxy docosaenoic acid is an extracellular lipid component in yeast (9). 9,10,13-Trihydroxy-11(*E*)- and 9,12,13-trihydroxy-10(*E*)-octadecenoic acids were detected in beer (10) and presumably resulted from converting linoleic acid during the barley malting process (11). Trihydroxy unsaturated fatty acids, 9*S*,12*S*,13*S*-trihydroxy-10-octadecenoic acid and 11,12,13-trihydroxy-9(*Z*),15(*Z*)-octadecadienoic acid, isolated from rice plants with blast disease, exhibited antifungal activity (12–15). 9,12,13-Trihydroxy-10(*E*)-octadecenoic acid was also isolated from *Colocasia antiguorum* inoculated with *Ceratocystis fimbriata* and was shown to possess antiblack rot fungal activity (16). Recently, Hou (6) reported the first production of a trihydroxy unsaturated fatty acid, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid by microbial transformation of linoleic acid with *Clavibacter* sp. ALA2.

This paper describes the stability of and improved DOD production by strain PR3. In addition, it describes the finding of a novel trihydroxy unsaturated fatty acid, 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD), which is produced from ricinoleic acid by strain PR3.

EXPERIMENTAL PROCEDURES

Microorganisms. Pseudomonas sp. PR3 was isolated from a water run-off of a pig farm located in Morton, Illinois (4,5). The culture used in this study was previously maintained on TGY (17) agar medium. It was subsequently transferred monthly for 6 mon onto fresh TGY and screening agar media. The screening medium (SM) contained (per liter) 4 g dextrose, 0.5 g yeast extract, 10 g $(NH_4)_2HPO_4$, 2 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.014 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.008 g $MnSO_4 \cdot H_2O$, and 0.1 g nicotinic acid (5). The medium was adjusted to pH 7.0 with diluted phosphoric acid.

Chemicals. Oleic acid, linoleic acid, and ricinoleic acid (all of 99+% purity) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). All other chemicals were reagent-grade and used without further purification. Thin-layer precoated Silica Gel 60 plates were obtained from EM Separations Technology (Gibbstown, NJ).

Bioconversion reactions. Bioconversions were carried out in either SM or modified Wallen fermentation (WF) medium. The WF medium contained (per liter) 4 g dextrose, 5 g yeast extract, 4 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.0075 g $FeSO_4 \cdot 7H_2O$, and its pH was adjusted to 7.3 with 3N H_2SO_4 (18). The production of DOD from oleic acid by *Pseudomonas*

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sp. PR3 in different cultural media was carried out according to the basic procedures described previously (5). Oleic acid (0.3 mL, 1%) was added to 18- or 48-h-old cultures and then shaken for an additional 2 d at 28°C and 200 rpm. For the bioconversion of ricinoleic acid by strain PR3, an aliquot of the fatty acid (0.5–1%) was added to an 18-h-old culture in 30 mL WF medium, and the bioconversion was allowed to proceed for 2–3 d under the same conditions. At the end of the conversion, lipids were recovered from the acidified broth by extracting twice with an equal volume of methanol/ethyl acetate (1:9, vol/vol) (19). The solvent was then removed from the combined extracts with a rotary evaporator. The concentrated lipid extracts were transferred to 1-dram vials and dried under a nitrogen stream for further analysis.

Analysis of products. Bioconversion was monitored by gas chromatography (GC), thin-layer chromatography (TLC), and mass spectrometry (MS). Lipid extracts were esterified with diazomethane. The methyl esters were injected into an HP (Hewlett-Packard; Palo Alto, CA) model 5890 Series II gas chromatograph, equipped with a Supelco (Bellefonte, PA) SPB-1 capillary column (15 m × 0.32 mm, 0.25 µm film thickness), a flame-ionization detector and an HP 7673 autosampler, and HP ChemStation software was used for data acquisition and integration. The temperature of the injector and the detector was set at 240 and 250°C, respectively, and helium was used as carrier gas at 1 mL/min. The oven temperature was programmed as follows: 190 to 204°C at 2°C per min, 204 to 230°C at 5°C per min, and holding at 230°C for an additional 12 min. For quantitative analysis, palmitic acid was added as an internal standard prior to solvent extraction. DOD in samples assayed by GC was quantitated in reference to its own standard curve (mass/peak area linear relationship) and methyl palmitate internal standard. The linear relationship between mass and peak area, however, has not been determined for TOD. Therefore, the quantitation of TOD was determined by total recovery weights.

TLC analyses were carried out on Silica Gel 60 (0.25 mm thickness) plates (EM Science, Gibbstown, NJ) developed in chloroform/methanol/acetic acid (9:1:0.1, by vol). The chromatograms were visualized first with sulfuric acid spray, followed by charring with a heat gun, and then with vanillin/sulfuric acid spray, followed by brief heating.

Isolation and identification of new hydroxy fatty acid. Bioconversion of ricinoleic acid in 100 mL of WF culture medium and harvest of the products were carried out according to the procedures described above. The products were separated in a mini-column (7 cm × 5 mm i.d.) of Silica Gel 60 (230–400 mesh) with a gradient of hexane and ethyl acetate. The column was washed with two bed volumes of hexane/ethyl acetate (20:80, vol/vol). A portion of the products (55 mg) in a minimal volume of the column wash solvent was then applied onto the column. Subsequent column elution was carried out by the following sequential steps: four bed-volumes wash solvent, one bed-volume 100% ethyl acetate, two bed-volumes 100% ethyl acetate, and one bed-volume ethyl acetate/methanol (50:50, vol/vol). The isolated material was further analyzed by GC–mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR).

The sample was first methylated, and trimethylsilyl (TMS) derivatives were subsequently prepared by using Sylon BTZ (Supelco, Bellefonte, PA) according to the manufacturer. Electron-impact GC–MS was obtained with an HP model 5890 gas chromatograph, coupled to an HP model 5972 mass selective detector. Separations of components were achieved in an HP-5 (30 m × 0.25 mm i.d., 0.25 µm film thickness) column with a temperature gradient programmed to start at 70°C, increasing at 20°C/min to 170°C with 1 min hold at this temperature, increasing at 5°C/min to 250°C and holding for 15 min. The underivatized sample was analyzed by proton and ^{13}C NMR as described previously (5).

RESULTS AND DISCUSSION

Stability of strain PR3 and improved production of DOD. Pseudomonas aeruginosa strain PR3 was discovered and characterized for its ability to convert oleic acid to DOD (4,5,7). Under optimal conditions (pH 7.0, 2 d at 30°C) in SM, product yield was greater than 60% (5). However, the microbe showed inconsistent DOD production during subsequent transfer and maintenance of the culture. This problem suggested a need to reexamine the culture medium, especially the high concentration (10 g/L) of $(NH_4)_2HPO_4$ of SM. Although NH_4^+ can serve as a nitrogen source for microbial growth, high concentration of NH_4^+ in the presence of excessive amounts of phosphate ion could lead to the formation of insoluble complexes with certain metal ions, such as Mg^{+2}, Zn^{+2}, or Mn^{+2}. Preliminary results showed that the minerals of SM altered the capacity of strain PR3 to produce DOD; the production was in the order of SM without $(NH_4)_2HPO_4$ > SM without K_2HPO_4 > SM with only 2 g/L $(NH_4)_2HPO_4$ > SM. Studies on different cultural media showed that WF, which contained no added $(NH_4)_2HPO_4$, was consistently 3–10 times better than SM for DOD production. With WF for the bioconversion of oleic acid with PR3 cultures that were maintained on TGY agar slants for 1–6 mon, the yields of DOD production were 65–75% at 28°C and 200 rpm shaker speed for 48 h. Thus, strain PR3 is stable and can be readily maintained for 6 mon without losing conversion capacity. Further modification of SM affected the ability of PR3 to produce DOD (Table 1). Replacing 10 g/L $(NH_4)_2HPO_4$ in SM with 2 g/L $(NH_4)_2HPO_4$, 1 g/L NH_4NO_3, and excluding $MgSO_4$ (SM4), the cultural conversion increased greatly from a 7 to 89% yield. On the other hand, when the same replacements for 10 g/L $(NH_4)_2HPO_4$ were made but excluding $MnSO_4$ from SM (SM5), the yield remained at about 7%. The reason for this large variation of DOD production by metal ions is not known. However, it is known that manganese is required for the catalysis of certain enzymes, such as cytochrome P-450 class (20), and perhaps that enzyme is involved in the formation of DOD.

Production and isolation of product. Strain PR3 did not produce any appreciable amounts of new conversion products

TABLE 1
Comparison of Different Culture Media in the Production of DOD by Strain PR3[a]

Culture medium[b]	DOD production (mg) (I)	(II)	Yield (%)
SM	23.5	17.8	7.0
SM4	273.2	259.9	89.0
SM5	22.2	18.9	7.0
WF	203.6	188.9	65.3

[a]Oleic acid (1%) was added to 48-h-old PR3 cultures, and the bioconversion was allowed for 48 h at 28°C and 200 rpm prior to lipid extraction. I and II are separate experiments; each figure of DOD production is the average of duplicate runs.
[b]The composition of SM and WF is described in the Experimental Procedures section. SM4 medium is SM that contained no $MgSO_4$ and had 10 g/L $(NH_4)_2HPO_4$ replaced by 2 g/L $(NH_4)_2HPO_4$ and 1 g/L NH_4NO_3, whereas SM5 medium is SM that contained no $MnSO_4$ and had the same replacement for 10 g/L $(NH_4)_2HPO_4$. Abbreviations: DOD, 7,10-dihydroxy-8(*E*)-octadecenoic acid; SM, screening medium; WF, Wallen fermentation.

when incubated with linoleic acid instead of oleic acid. GC analysis indicated that most of the linoleic acid substrate was also consumed by the microorganism (data not shown). On the contrary, strain PR3 converted ricinoleic acid to produce a new compound, resembling a trihydroxy fatty acid when compared to DOD by GC retention times (RT) (Fig. 1). The new compound had a characteristic RT of 14.17 min, whereas DOD had an RT of 10.89 min and the internal standard, palmitic acid, had an RT of 2.86 min. Production of the new compound, however, was highly sensitive to time. By extending the conversion time from 48 to 72 h prior to lipid extraction, the yield of new compound, relative to the GC peak area of methyl palmitate, had greatly decreased from 20.0 to 2.3%. This indicated that the new compound formed in the cultural medium could be further metabolized by strain PR3. Subsequently, a 48-h conversion time after the addition of substrate was applied to produce the compound. The yield of this bioconversion reaction was 35.2%, based on the total weights of compound recovered from a TLC separation. The new compound was isolated by silica gel column chromatography, which provided an effective method for obtaining small quantities of pure material for further structural analysis. After the crude lipid extracts were loaded onto a mini-column of Silica Gel 60, contaminants were removed by eluting the column with four bed-volumes of the column wash solvent, followed

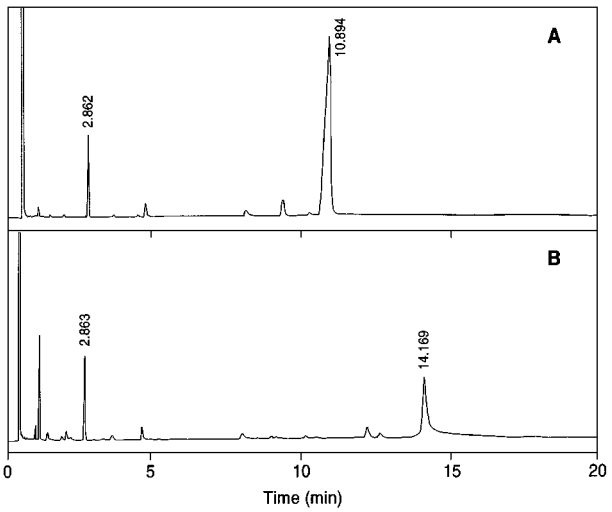

FIG. 1. Gas chromatograms (GC) of methyl esters recovered after bioconversion by strain PR3 of substrate (A) oleic acid and (B) ricinoleic acid. GC peaks are palmitic acid internal standard [retention time (RT) = 2.86 min], DOD (RT = 10.89 min), and the newly identified 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) (RT = 14.17 min).

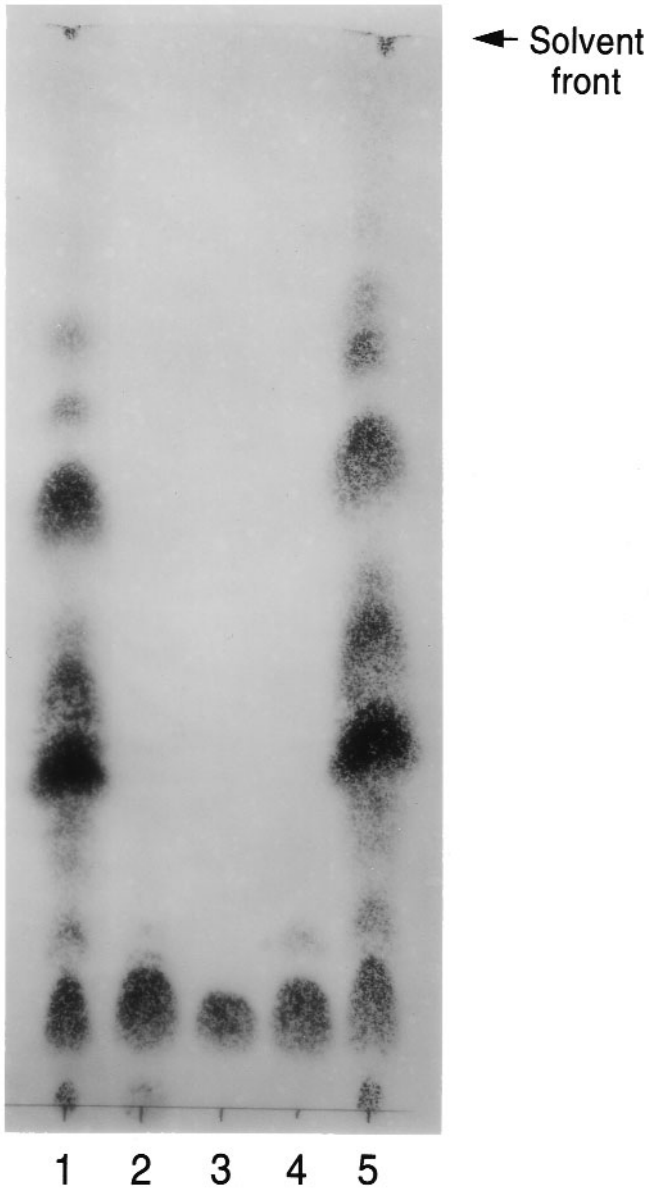

FIG. 2. Thin-layer chromatogram of the bioconversion products of strain PR3 on ricinoleic acid. Lanes 1 and 5 are crude lipid extracts, whereas lanes 2, 3, and 4 are fractions eluted from a silica gel column with one bed-volume 100% ethyl acetate right after four bed-volumes hexane/ethyl acetate (20:80, vol/vol), two bed-volumes 100% ethyl acetate, and one bed-volume ethyl acetate/methanol (50:50, vol/vol), respectively.

by one bed-volume of 100% ethyl acetate. Subsequent elution with two bed-volumes 100% ethyl acetate yielded a homogeneous fraction as shown on TLC (Fig. 2).

Structure determination of TOD: NMR analysis. Proton NMR showed the following resonance signals: olefinic protons -CH=CH- at 5.65 ppm; three tertiary protons -CH-O- at 4.30, 4.01, and 3.79 ppm, with the first two being adjacent to a double bond; $-CH_2-COOH$ at 2.27 ppm; methylene groups ranged from 1.30 to 1.62 ppm; and a terminal $-CH_3$ at 0.90 ppm. The coupling constant was 15 Hz for the olefinic products, indicating a possible *trans*-configuration across the double bond. The ^{13}C NMR spectra showed three carbon peaks at 69.03, 69.78, and 73.00 ppm, characteristic of a hydroxyl attachment. There was a distinct methylene carbon at 45.69 ppm and two olefinic carbons at 133.87 and 135.02 ppm. The remaining carbon peaks were at 14.44, 23.70, 26.11, 26.28, 26.71, 30.17, 30.50, 33.05, 35.02, 38.23, 38.96, and 177.80 ppm. The NMR data indicate that this compound is a trihydroxy octadecenoic acid with a *trans*-configuration and that a methylene group interrupts two of the three carbons each bearing a hydroxyl group.

GC–MS analysis. The electron impact mass spectrum of TMS derivatives of the methylated sample is shown in Figure 3. The spectrum showed that seven *m/z* peaks with their corresponding relative intensities were important to the structure determination: 187(100%), 231(19%), 359(11%), 431(1.8%), 239(46%), 269(11%), and 341(5%). As shown in the postulated structure (Fig. 3), *m/z* 187 was the fragment from the terminal methyl end of the molecule with a hydroxyl group at the C-12 position. The fragment from the carboxyl end with a second hydroxyl group attached to the C-7 position corresponded to ion *m/z* 231. The assignment of a hydroxyl group at the C-7 position was consistent with the presence of a large fragment at *m/z* 431. The release of a TMS group from *m/z* 431 produced fragment ion *m/z* 341. The third hydroxyl group was present at the C-10 position as shown in a fragment of *m/z* 359. Releasing of TMS from C-10 produced the ion of *m/z* 269. Fragment ion *m/z* 329 had been converted to *m/z* 239 upon the release of TMS due to rearrangement of the double bond at the C-8 position. These fragments located the hydroxyl groups at C-7, C-10, and C-12 and the double bond at C-8 of the molecule. Based on both NMR and MS data, we concluded that the

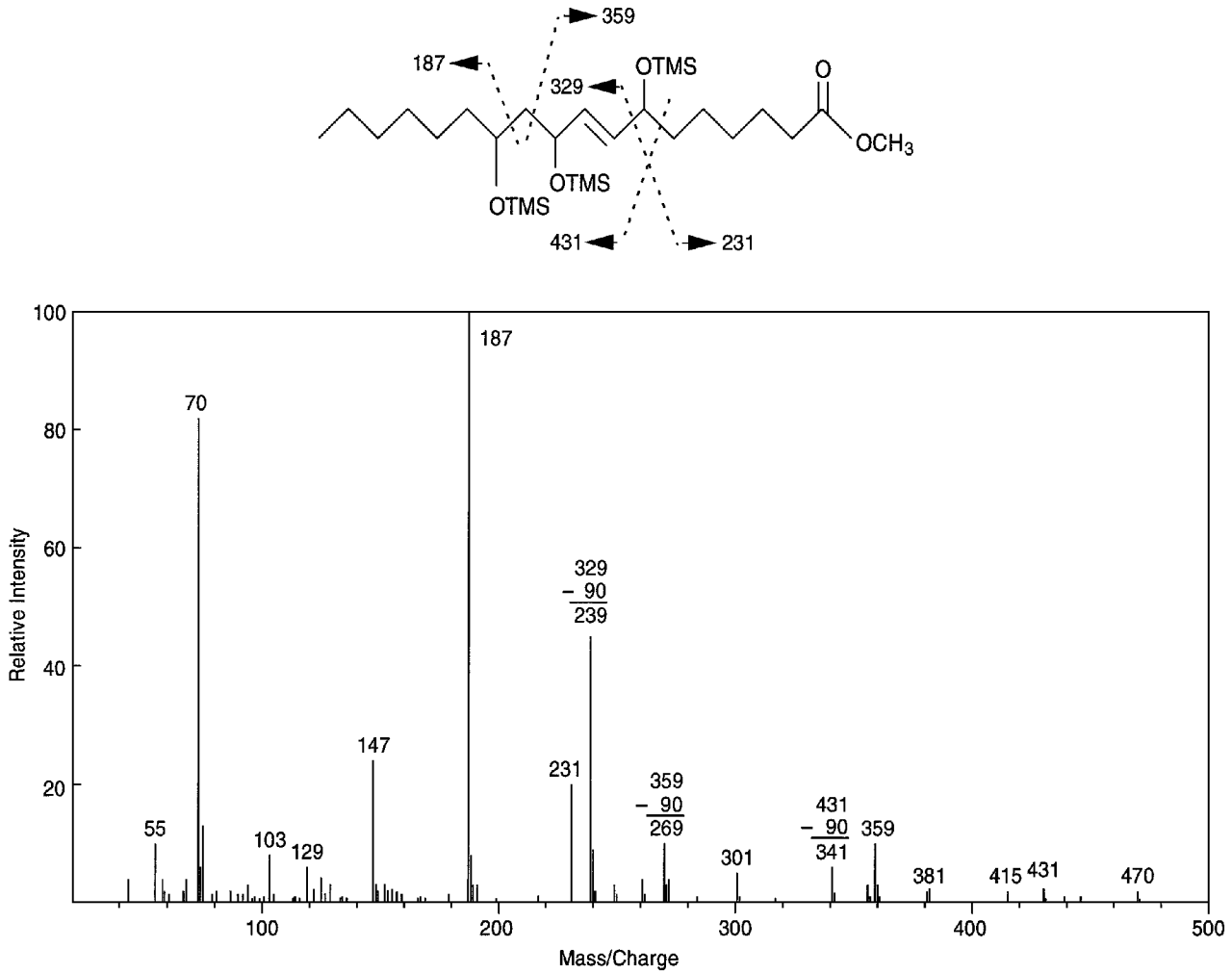

FIG. 3. Electron impact mass spectrum of trimethylsilyl (TMS) derivative of methylated TOD. See Figure 1 for other abbreviation.

new compound as produced by transformation of ricinoleic acid with strain PR3 is TOD. Therefore, the mechanism of TOD production can be identical to that of DOD, which involves hydroxylation at two positions and a rearrangement of the double bond of the substrate molecule (4,5). The formation of DOD was shown to involve an intermediate 10-hydroxy-8(*Z*)-octadecenoic acid (21). We are examining the bioconversion reaction to see if the formation of TOD also involves an intermediate step. Biological activity of TOD is also under investigation.

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