Chapter 13

Production of Hydroxy Fatty Acids by Biocatalysis

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Introduction

Plant systems produce hydroxy fatty acids, which are important industrial materials. The hydroxy group gives a fatty acid special properties, such as higher viscosity and reactivity compared with other fatty acids. At present, imported castor oil and its derivatives are the only commercial source of these industrial hydroxy fatty acids.

Because of their special chemical attributes, hydroxy fatty acids are used in a wide range of products, including resins, waxes, nylons, plastics, corrosion inhibitors, cosmetics, and coatings. Furthermore, they are used in grease formulations for high-performance military and industrial equipment. Ricinoleic and sebacic acids, two of the castor oil derivatives, are classified by the Department of Defense as strategic and critical materials. 12-Hydroxystearates (esters with C-10 to C-12 alcohols) are used in leather coatings requiring oil resistance and water imperviousness and in roll leaf foils because of their alcohol solubility and excellent wetting and adhesion to metallic particles (1). Because of fluctuating supplies and prices for castor oil, some companies have sought alternative raw materials, primarily petroleum-based feedstocks. Like ricinoleic acid, the hydroxy fatty acids of lesquerella also have double bonds and a carboxyl group that provide sites at which chemical reactions can occur (Fig. 1).

We have been investigating the production of value-added products from soybean oil. A Japanese patent application by Soda *et al.* (2) claimed the production of ricinoleic acid from oleic acid by *Bacillus pumilus*. Our initial goal was to produce ricinoleic acid from oleic acid by biocatalysis and hence to reduce the dependency on imported castor oil. Although we could not demonstrate the production of ricinoleic acid from oleic acid as did other investigators, including Soda's own group (2), our efforts led to discoveries of many new hydroxy fatty acids. These new products have potential industrial applications. Microbial oxidation of unsaturated fatty acids was reviewed recently (3).

Monohydroxy Fatty Acid

Production of 10-Hydroxystearic Acid

Microbial hydration of unsaturated fatty acid was first reported by Wallen *et al.* (4) from our laboratories. They found that a *Pseudomonad* isolated from fatty material

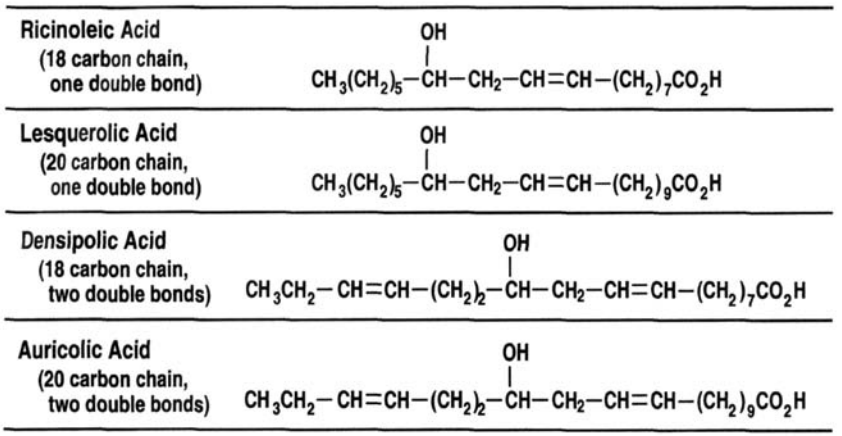

Fig. 1. Hydroxy fatty acids from castor oil and lesquerella oil.

hydrated oleic acid at the *cis*-9-double bond produced 10-hydroxystearic acid (10-HSA) with a 14% yield. The 10-HSA is optically active (5,6) and has the d-configuration (6). Incubation of this organism with oleic acid in a medium enriched with deuterium oxide yields 10-HSA containing one deuterium atom (6). Moreover, this deuterium was shown to be on carbon atom 9 and in the L-configuration. By using soluble cell-free extracts of a *Pseudomonad*, Niehaus and Schroepfer (7) were able to demonstrate the conversion of oleate to 10-HSA under anaerobic conditions and the reversibility of the reaction. These findings, coupled with the observed stereospecific uptake of one atom of solvent hydrogen into 10-HSA and the lack of conversion of either the *cis*- or *trans*- 9,10-epoxystearic acid to 10-HAS, are compatible with a mechanism involving hydration of the double bond of oleic acid and rule out an epoxide intermediate (8). The same enzyme preparation was later found to catalyze both the hydration of *cis*- and *trans*-9,10-epoxystearic acids to yield threo- and erythro-9,10-dihydroxystearic acid, respectively (9). Niehaus *et al.* (10) demonstrated the interconversion of oleic acid and 10-HSA by a soluble (105,000 × *g* supernatant) enzyme preparation from a *Pseudomonad*. This further ruled out the possible intermediate role of an epoxide in the overall conversion for two reasons. First, the enzymatic conversion of oleate to 10-HSA was observed to proceed under anaerobic conditions, a feature not characteristic of enzymatic epoxidations of olefins. Second, neither the DL-*cis*-9,10-epoxystearate nor the DL-*trans*-9,10-epoxystearate served as precursors of either oleate or 10-hydroxystearate under the conditions studied.

By using the squalene screening method (11), Seo *et al.* (12) isolated a culture, *Corynebacterium* sp. S-401, from soil, that hydrates the squalene molecule to form tertiary alcohols. They found that resting cells of strain S-401 also stereospecifically hydrated oleic acid to 10-ketostearic (10-KSA) and (-)-10*R*-hydroxystearic acids with 22.4 and 9.1% yield, respectively. Strain S-401 failed to catalyze hydration of

oleoamide, oleonitrile, oleyl alcohol, oleyl aldehyde, or *cis*-9-octadecene. Accordingly, the carboxy group of oleic acid seems to be essential in this reaction.

Cells of *Rhodococcus rhodochrous* also hydrated oleic acid to 10-HSA and 10-KSA at 55 and 12% yields, respectively (13). Hydration of oleic acid to 10-HSA was also demonstrated in resting cell suspensions of seven *Nocardia* species under anaerobic conditions (14). *Nocardia cholesterolicum* NRRL 5769 gave a yield >90% with optimum conditions at pH 6.5 and 40°C. A minor amount of 10-KSA was detected. The reaction proceeds *via* hydration of the double bond as shown by labeling experiments with deuterium oxide and ^{18}O-labeled water. The system was specific for fatty acids with *cis* unsaturation at the 9 position. Anaerobiosis favors bioconversion to 10-HSA (15) and higher pH favors bioconversion to 10-KSA (14).

Thus far, the microbial hydration of oleic acid was found in *Pseudomonas* (4), *Nocardia (Rhodococcus)* (13,14), *Corynebacterium* (12), *Sphingobacterium* (16), and *Micrococcus* (17). Works of El-Sharkawy *et al.* (18) considerably extended the genera of microorganisms known to hydrate oleic acid to include a range of eucaryotic organisms. Strains from several other genera including *Absida, Aspergillus, Candida, Mycobacterium*, and *Schizosaccharomyces* were also found capable of catalyzing the hydration of oleic acid. Resting cells of *Saccharomyces cerevisae* (baker's yeast, type II: Sigma, St. Louis, MO) converted oleic acid to 10-HSA with a 45% yield (18). Three other cultures, *Nocardia aurantia* ATCC 12674, *Nocardia* sp. NRRL 5646, and *Mycobacterium fortuitum* UI 53378, all converted oleic acid to 10-KSA with 65, 55, and 80% yields, respectively. Small amounts of 10-HSA were also produced by these cultures except strain NRRL 5646.

The stereospecificity of microbial hydrations of oleic acid to 10-HS A was investigated by Yang *et al.* (19) on the basis of the ^{1}H nuclear magnetic resonance (NMR) spectral analysis of diastereomeric *S*-(+)-*O*-acetylmandelate esters of hydroxystearates (18). They found that although *R. rhodochrous* ATCC 12674-mediated hydration of oleic acid gave mixtures of enantiomers 10(*R*)-hydroxystearic acid and 10(*S*)-hydroxystearic acid, *Pseudomonas* sp. NRRL B-3266 produced optically pure 10(*R*)-hydroxystearic acid. The remaining microorganisms investigated (16) stereoselectively hydrated oleic acid to 10(*R*)-hydroxystearic acid containing 2 and 18%, respectively, of the contaminating 10(*S*)-hydroxystearic acid.

Although hydration of oleic acid to 10-HSA was investigated at the cell-free enzyme level (7–10), attempts to purify hydratase were not successful. Very little was known about the physical and chemical properties of oleate hydratase. Purification and characterization of oleate hydratase from *Nocardia cholesterolicum* NRRL 5767 were investigated by Huang *et al.* (20). The cell-free extracts, which were obtained after French Press disintegration of the cells and centrifugation, were fractionated by ammonium sulfate. The enzyme activity was found in the fraction of 60–75% ammonium sulfate saturation. The enzyme fraction was further purified through a Mono-Q ion exchange and Superose (Pharmacia, Piscataway, NJ) gel filtration column chromatography. The purified enzyme fraction showed a single Protein band on acrylamide gel electrophoresis. The hydration proceeded linearly for 6 h. The optimum

pH for the enzyme reaction is between 6.5 and 7. The K_m value for the hydratase reaction at 30°C is 2.82×10^{-4} mol/L. The molecular weight estimated from Supherose HR 10/30 gel filtration column is ~120,000 Da and from SDS-PAGE is ~32,000 Da (21). Therefore, oleate hydratase is a tetramer, composed of four identical subunits. Lanser (22) reported the conversion of oleic acid to 10-ketostearic acid by a microorganism from other genera, *Staphylococcus* sp. The yield was >90% with <5% byproduct, 10-hydroxystearic acid.

Hou reported (23) that *Flavobacterium* sp. DS5 converted oleic acid to 10-KSA in 85% yield. Optimum time, pH, and temperature for the production of 10-KSA are as follows: 36 h, 7.5, and 30°C. A small amount of 10-HSA (~10% of the main product 10-KSA) is also produced during the bioconversion. 10-KSA is not further metabolized by strain DS5 and accumulates in the medium. In contrast to growing cells, a resting cell suspension of strain DS5 produces 10-HSA and 10-KSA in a ratio of 1:3. The cell-free crude extract obtained from ultrasonic disruption of the cells converts oleic acid mainly to 10-HSA (10-HSA: 10-KSA = 97:3). This result strongly suggested that oleic acid is converted to 10-KSA *via* 10-HSA. Stereochemistry of 10-HSA from strain DS5, determined by ^{1}H NMR of the mandelate esters, showed 66% enantiomeric excess in 10(*R*) form.

The *Flavobacterium* DS5 enzyme system also catalyzes the conversion of linoleic acid. In contrast to oleic acid substrate, which yields mainly the keto product, linoleic acid substrate yields mainly 10-hydroxy-12(*Z*)-octadecenoic acid (10-HOA) with 55% yield (24). The optimum conditions for the production of 10-HOA were pH 7.5, temperature 20–35°C, and 36 h of incubation. Two minor products produced were 10-methoxy-12-octadecenoic acid and 10-keto-12-octadecenoic acid (10-KOA).

Strain DS5 oxidized unsaturated but not saturated fatty acids. The relative activities were in the following order: oleic > palmitoleic > arachidonic > linoleic > linolenic > γ-linolenic > myristoleic acids.

With the resting cells suspension, the ratio of products, 10-HOA:10-KOA was 97:3. Less 10-KOA was produced in comparison with that of growing cells. The cells were disrupted with ultrasonic oscillation and centrifuged to obtain cell-free crude extract. The linoleic acid conversion enzyme(s) resided in the cell-free crude extract, and only 10-HOA was produced from linoleic acid.

Positional Specificity of Strain DS5 Hydratase

From substrate specificity studies (23,24), it seems that DS5 hydratase hydrates a specific carbon position of the unsaturated fatty acid substrates. To clarify this point and the effect of substrate carbon chain length on the strain DS5 hydratase activity, we studied the hydration of mono-, di-, and triunsaturated C-18 fatty acids as well as other carbon chain length monounsaturated fatty acids.

Strain DS5 converted α-linolenic acid to 10-hydroxy-12,15-octadecadienoic acid and a minor product 10-keto-12,15-octadecadienoic acid (24). Strain DS5 also con-

verted γ-linolenic acid to 10-hydroxy-6(*Z*),12(*Z*)-octadecadienoic acid. The enzyme hydrated 9-unsaturation but did not alter the original 6,12-unsaturations. Strain DS5 converted myristoleic acid to two products, 10-keto myristic and 10-hydroxymyristic acids. Palmitoleic acid also gave two bioconversion products, 10-ketopalmitic and 10-hydroxypalmitic acids.

Previously, the strain DS5 bioconversion products from oleic and linoleic acids were identified as 10-ketostearic (23) and 10-hydroxy-12(*Z*)-octadecenoic acid (24), respectively. It is interesting to find that all unsaturated fatty acids tested are hydrated at the 9,10 positions with the oxygen functionality at C-10 despite their varying degree of unsaturations. DS5 hydratase was not active on saturated fatty acids and other non-9(*Z*)-unsaturated fatty acids such as elaidic [9(*E*)-octadecenoic], arachidonic [5(*E*),8(*E*),1 l(*E*),14(*E*)-eicosatetraenoic], and erucic [13(*E*)-docosenoic] acids (25). From all of the data gathered, it is concluded that DS5 hydratase is indeed a C-10 positional-specific enzyme. The fact that elaidic acid was not hydrated indicates that the unsaturation must be in the *cis* configuration for DS5 hydratase activity.

The strain DS5 system produced more keto product from palmitoleic and oleic acids and more hydroxy product from myristoleic, linoleic, and α- and γ-linolenic acids. The reason for this preference is not clear. Among the 18-carbon unsaturated fatty acids, an additional double bond in either side of the C-10 position lowers the enzyme hydration activity. A literature search revealed that all microbial hydratases hydrate oleic and linoleic acids at the C-10 position (Fig. 2). Therefore, the positional specificity of microbial hydratases might be universal.

Hydration of Other Fatty Acids

Hydrations of unsaturated fatty acids other than oleic acid were also reported. Wallen *et al.* (26) prepared three new unsaturated 10-hydroxy fatty acids, all optically active,

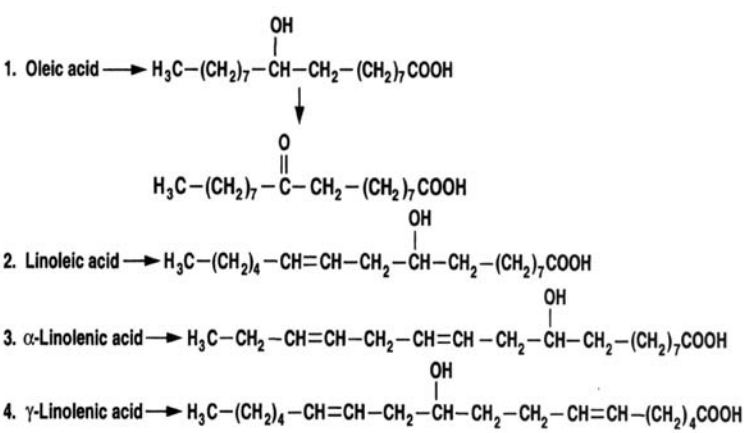

Fig. 2. Bioconversion products from unsaturated fatty acids by strain DS5 hydratase.

by the anaerobic microbial hydration of a *cis*-9-double bond. Substrates that formed these new hydroxy fatty acids are linoleic, linolenic, and ricinoleic acids. The yields were as follows: linoleic acid to 10-hydroxy-12(*Z*)-octadecenoic acid, 20 mole %; linolenic acid to 10-hydroxy-12(*Z*),15(*Z*)-octadecadienoic acid, 21 mol %; and ricinoleic acid to 10, 12-dihydroxystearic acid, 41 mol %. Giesel-Buhler *et al.* (27) reported the production of 10-hydroxy-12-octadecenoic acid from linoleic acid by resting cells of *Acetobacterium woodii* through hydration.

In a patent disclosure, Litchfield and Pierce (13) claimed that cells of *Rhodococcus rhodochrous* catalyzed the hydration of linoleic acid to 10-hydroxy-12-octadecenoic acid at 22% yield with 10-keto-12-octadecenoic acid as a co-product. The hydration enzyme is inducible by the presence of oleic acid at the early stage of cell growth.

More recently, Koritala and Bagby (28), using washed resting cells suspension of *Nocardia cholesterolicum* under anaerobic conditions, reported the hydration of linoleic and linolenic acids to 10-hydroxy-12(*Z*)-octadecenoic (yield 71%) and 10-hydroxy-12(*Z*),15(*Z*)-octadecadienoic acids (yield 77%), respectively. The production of 10-hydroxy fatty acids by hydratase from various microbes is summarized in Table 1.

Other than hydration, the hydroxylation of oleic acid was also reported. Lanser *et al.* (29) found that two strains of *Bacillus pumilus* (NRRL BD-174 and BD-226) produced 15-, 16-, and 17-hydroxy-9-*cis*-octadecenoic acids.

Dihydroxy Unsaturated Fatty Acid

In our continuing screening program for new industrial chemicals from vegetable oils and their component fatty acids, we isolated a new bacterial strain, PR3, which converted oleic acid to a new compound, 7,10-dihydroxy-8(*E*)-octadecenoic acid

TABLE 1
Hydratases from Various Microbes That Produced 10-Hydroxy Product

Microbe	Reference	
Pseudomonas	Wallen *et al.* 1962	(4)
Corynebacterium	Seo *et al.* 1981	(12)
Rhodococcus	Litchfield and Pierce 1986	(13)
Bacillus	Soda *et al.* 1987	(2)
Nocardia	Koritala *et al.* 1989	(14)
Micrococcus	Blank *et al.* 1991	(17)
Sarcina	Blank *et al.* 1991	(17)
	El-Sharkaway *et al.* 1992	(18)
Aspergillus	El-Sharkaway *et al.* 1992	(18)
Candida	El-Sharkaway *et al.* 1992	(18)
Mycobacteriu	El-Sharkaway *et al.* 1992	(18)
Schizosaccharomyces	El-Sharkaway *et al.* 1992	(18)
Staphylococcus	Lanser 1993	(22)
Flavobacterium	Hou 1994	(23)
Sphingobacterium	Kaneshiro *et al.* 1994	(16)

(DOD), involving both hydration and possibly hydroxylation (30,31). Strain PR3, isolated from a water sample at a pig farm in Morton, IL, formed a smooth, round, white colony on agar plate. The microorganisms were motile, short, rod-shaped bacteria. Flagella stain showed multiple polar flagellae. Strain PR3 grew aerobically and could not grow anaerobically. The oxidase activity of the cells was positive. Based on these observations, strain PR3 belongs to the genus *Pseudomonas* (30). Strain PR3 produced fluorescein on King's medium B as well as pyrocyanin on King's medium A, suggesting that the organism was a strain of *P. aeruginosa*. Further identification was conducted with DNA reassociation measurements (32).

Strain PR3 converted oleic acid to a new compound, 7,10-dihydroxy-8(*E*)-octadecenoic acid. The absolute configuration of DOD was determined with the aid of circular dichroism to be *R,R* (33). The production of DOD from oleic acid reached a maximum after 48 h of incubation with a yield of 63%. Further incubation reduced DOD content in the medium, thus strain PR3 metabolizes DOD.

The production of DOD from oleic acid is unique in that it involves an addition of two hydroxy groups at two positions and a rearrangement of the double bond of the substrate molecule. The reaction at the Δ9,10 position resembles hydration, and the reaction at the C-7 position seems like a hydroxylation. Subsequent investigation of reactions catalyzed by PR3 led to the isolation of another new compound, 10-hydroxy-8-octadecenoic acid (HOD) (34). 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. Kinetic studies (34) showed 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 postulated as follows (Fig. 3): a hydratase in strain PR3 attacks oleic acid at the C-10 position, introduces a hydroxy group, and at the same time shifts the double bond from C-9 to C-8. The resulting product (HOD) is then oxidized by a hydroxylase at the C-7 position to produce DOD.

Recently, we were able to produce DOD with a cell-free enzyme preparation at a higher yield. The yield of DOD production by strain PR3 was improved to >80%.

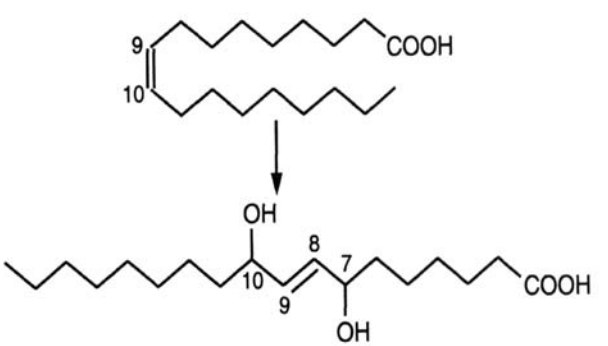

Fig. 3. 7,10-Dihydroxy-8(*E*)-octadecenoic acid produced from oleic acid by *Pseudomonas aeruginosa* PR3.

It was also found that strain PR3 converted ricinoleic acid to a new compound, 7,10,12-trihydroxy-8(*E*)-octadecenoic acid at 35% yield (35). The reaction mechanism is the same as that for the conversion of oleic acid to DOD. Physiologic activity tests of DOD revealed that DOD has some activity against *Bacillus subtilis* and a common pathogen, *Candida albican*.

A similar type of compound, dihydroxyoctadecenoic acid, was produced by *Pseudomonas* 42A2 (36). However, the positions for the double bond and hydroxy groups in that report were determined later and were shown to correspond to 7,10-dihydroxy-8(*E*)-octadecenoic acid (37,38).

Trihydroxy Unsaturated Fatty Acid

Recently, we discovered the production of a new compound, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid from linoleic acid by a new microbial isolate. Production of trihydroxy unsaturated fatty acids in nature is rare. The only compounds reported were all produced in trace amounts. 8,9,13-Trihydroxy docosaenoic acid was produced by yeast as an extracellular lipid (39). 9,10,13-Trihydroxy-11(*E*)- and 9,12,13-trihydroxy-10(*E*)-octadecenoic acids were detected in beer (40). It has been suggested that these trihydroxy fatty acids are formed from linoleic acid during the processes of malting and mashing of barley (41). Gardner *et al.* (42) reported the production of diastereomeric (*Z*)-11,12,13-trihydroxy-9-octadecenoic acids and four isomers of (*E*)-9,12,13(9,10,13)-trihydroxy-10(11)-octadecenoic acids by acidcatalyzed transformation of 13(*S*)-hydroperoxylinoleic acid. Kato *et al.*(43,44) reported that hydroxy and epoxy unsaturated fatty acids present in some rice cultivars acted as antifungal substances and were active against rice blast fungus. It was postulated that these fatty acids were derivatives of linoleic and linolenic acid hydroperoxides. Recently, mixed hydroxy fatty acids, isolated from the *Sasanishiki* variety of rice plant suffering from the rice blast disease, were shown to be active against the fungus (45). 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 (46,47). 9,12,13-Trihydroxy-10(*E*)-octadecenoic acid was also isolated from *Colocasia antiquorum* inoculated with *Cemtocystis fimbriata* and showed activity against black rot fungus (48).

Other than extraction from plant materials, our discovery is the first report on production of trihydroxy unsaturated fatty acids by microbial transformation. The microorganism that performs this unique reaction was isolated from a dry soil sample collected from McCalla, AL. Strain ALA2 is a gram-positive, nonmotile rod (0.5 μm × 2 μm). The strain was identified as *Clavibacter* sp. ALA2 (49).

The chemical structure of the new compound was determined by mass spectrometry (MS), Fourier transform infrared Spectroscopy (FTIR) and NMR. The chemical ionization mass spectrum of the methyl ester prepared with diazomethane gave a molecular ion of *mlz* 345. Fragments of 327 (M-18), and 309 (M-2 × 18) were also seen. The electron impact spectrum of the methylated product provided more frag-

ments for structural analysis. Large fragments corresponding to α-cleavage with ions *mlz* 227 (25%) and 129 (100%) place two hydroxy groups at the C-12 and C-13 positions and the third hydroxy group at a position higher than carbon 13. Proton and ^{13}C NMR analyses further confirmed the structure. Resonance signals (ppm) and corresponding molecular assignments, given in Table 2, located three hydroxy groups at C-12, C-13, and C-17 and further confirmed the identity of the bioconversion product as 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid. The coupling constant of 10.7 Hz at C-9,10 confirmed our infrared data that the unsaturation is in *cis* configuration (49) (Fig. 4). The structure of THOA resembles those of plant self-defense substances.

Other Reaction Products

Typical reaction products produced from linoleic acid by strain ALA2 analyzed by gas chromatography (GC) are shown in Figure 5. In addition to the main reaction product at retention time (Rt) 24 min, there were small amounts of products at Rt 17 and 10 min. Mass spectral analysis of fragments indicated that these were 12-[5-ethyl-2-tetrahydrofuranyl]-7,12-dihydroxy-9*Z*-dodecenoic for Rt 17 and 12-[5-ethyl-2-tetrahydrofuranyl]-12-hydroxy-9*Z*-dodecenoic acid for Rt 10. The yield of the main product (THOA) was 35% and the relative amounts of these products produced were THOA:Rt 17:Rt 10 = 9:1.3:1 (50).

TABLE 2
Proton and ^{13}C Nuclear Magnetic Resonance Signals and Molecular Assignments for Bioconversion Product

	Resonance Chemical shifts (ppm)/coupling (Hz)			
Carbon number	^{13}C	Proton		
1	174.4	—		
2	34.1	2.29	t	$J2,3 = 7.4^a$
3	24.9	1.60	m	
4	29.0	1.30	bs	
5	29.0	1.30	bs	
6	29.0	1.30	bs	
7	29.5	1.30	bs	
8	27.3	2.04	m	$J8,9 = 7.0$
9	133.8	5.55	m	$J9,10 = 10.7$
10	124.6	5.40	m	$J10,11 = 7.2$
11	31.7	2.29	m	
12[b]	73.7	3.48	m	
13[b]	73.8	3.48	m	
14	33.5	1.48	m	
15	21.7	1.30	bs	
16	39.1	1.45	m	
17	68.0	3.82	m	$J17,18 = 6.1$
18	23.5	1.18	d	
OCH_3	51.5	3.65	s	

[a]Coupling constant, *J*, is expressed in Hz.
[b]Shift may be interchanged.

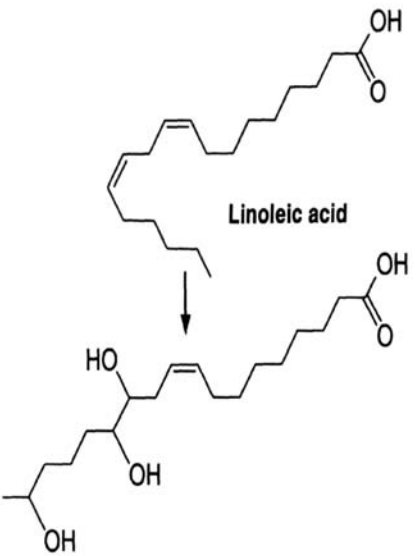

Fig. 4. 12,13,17-Trihydroxy-9(*Z*)-octadecenoic acid produced from linoleic acid by *Clavibacter* sp. ALA2.

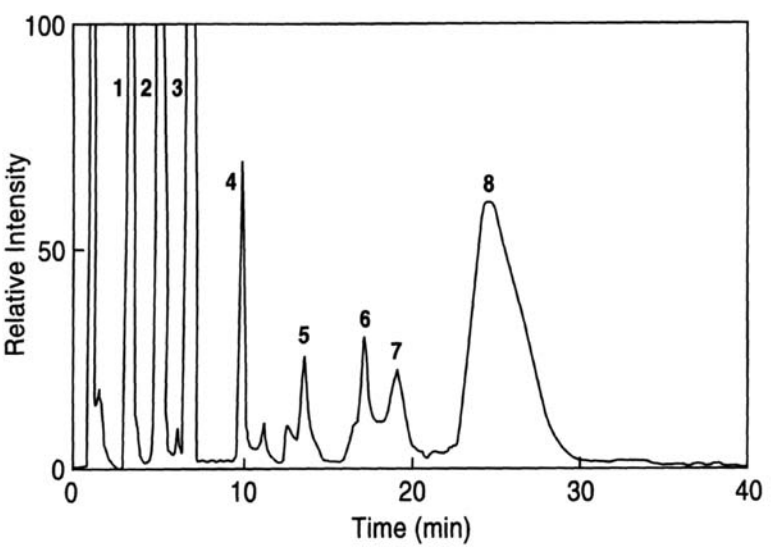

Fig. 5. A typical gas chromatography of strain ALA2 reaction products. 1: Internal standard, palmitic acid; 2: substrate, linoleic acid; 3–7: unknown; 8: product, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid.

The optimum conditions for the bioconversion of linoleic acid to THOA were pH 7.0 and temperature 30°C (51); the maximum production of THOA was found after 5–6 d of reaction. Further incubation did not reduce THOA content in the medium, indicating that strain ALA2 did not metabolize THOA.

The biological activity of THOA at 200 ppm concentration was tested against many plant pathogenic fungi (52,53). The results, expressed in percentage of growth inhibition, are as follows: *Erisyphe graminis* (common disease name, wheat powdery mildew) 77%; *Puccinia recondita* (wheat leaf rust) 86%; *Pseudocercosporella herpotrichoides* (wheat foot rot) 0%; *Septoria nodorum* (wheat glume blotch) 0%; *Pyricularia grisea* (rice blast) 0%; *Rhizoctonia solani* (rice sheath blight) 0%; *Phytophthora infestans* (potato late blight) 56%; and *Botrytis cinerea* (cucumber botrytis) 63%.

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