Production of Hydroxy Fatty Acids from Unsaturated Fatty Acids by *Flavobacterium* sp. DS5 Hydratase, a C-10 Positional- and *cis* Unsaturation-Specific Enzyme

Ching T. Hou*

Oil Chemical Research, NCAUR, ARS, USDA, Peoria, Illinois 61604

ABSTRACT: A new microbial isolate, *Flavobacterium* sp. DS5, converted oleic and linoleic acids to their corresponding 10-keto- and 10-hydroxy fatty acids. The hydration enzyme seems to be specific to the C-10 position. Conversion products from α- and γ-linolenic acids were identified by gas chromatography/mass spectrometry, Fourier transform infrared, and nuclear magnetic resonance as 10-hydroxy-12(*Z*),15(*Z*)-octadecadienoic and 10-hydroxy-6(*Z*),12(*Z*)-octadecadienoic acids, respectively. Products from other 9(*Z*)-unsaturated fatty acids also were identified as their corresponding 10-hydroxy- and 10-keto-fatty acids. *Trans* unsaturated fatty acid was not converted. From these results, it is concluded that strain DS5 hydratase is indeed a C-10 positional-specific and *cis*-specific enzyme. DS5 hydratase prefers an 18-carbon monounsaturated fatty acid. Among the C_{18} unsaturated fatty acids, an additional double bond at either side of the 9,10-position lowers the enzyme hydration activity. Because hydratases from other microbes also convert 9(*Z*)-unsaturated fatty acids to 10-hydroxy fatty acids, the C-10 positional specificity of microbial hydratases may be universal.

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KEY WORDS: Bioconversion, *Flavobacterium*, hydratase, hydration, hydroxy fatty acids, unsaturated fatty acids.

Microbial hydration of unsaturated fatty acids was first reported by Wallen *et al.* (1) from our laboratories. They found that a *Pseudomonas* isolated from fatty material hydrated oleic acid at the unsaturated 9,10-position to produce 10-hydroxystearic acid with 14% yield. The 10-hydroxystearic acid is optically active (2,3) and has the D-configuration (3). Seo *et al.* (4) found that resting cells of *Corynebacterium* sp. S-401 stereospecifically hydrated oleic acid to (–)-10(*R*)-hydroxystearic acids with 9.1% yield. 10-Oxostearic acid also was formed in 22.4% yield. Cells of *Rhodococcus rhodochrous* also hydrated oleic acid to 10-hydroxystearic acid (10-HSA) and 10-ketostearic (10-KSA) acid at 55 and 12% yields, respectively (5). Hydration of oleic acid to 10-HSA was also demonstrated in resting cell suspensions of seven *Nocardia* species under anaerobic conditions (6). *Nocardia cholesterolicum* NRRL 5769 gave a yield that exceeded 90% with optimum conditions at pH 6.5 and 40°C. A minor by-product, 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.

Micrococcus also hydrates oleic acid (7). Three *Nocardia* strains were able to hydrate linoleic and linolenic acids to their corresponding 10-hydroxy products (8). The works of El-Sharkawy *et al.* (9) considerably extended the groups of microorganisms known to hydrate oleic acid to include a range of eucaryotic organisms. Strains from several genera, including *Absida, Aspergillus, Candida, Mycobacterium*, and *Schizosaccharomyces*, were 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-hydroxystearic acid with a 45% yield (10). Three other cultures—*N. aurantia* ATCC 12674, *Nocardia* sp. NRRL 5646, and *M. fortuitum* UI 53378—converted oleic acid to 10-oxo-octadecanoic acid with 65, 55, and 80% yields, respectively. Small amounts of 10-HSA also were produced by these cultures, except for strain NRRL 5646. Lanser (11) reported the conversion of oleic acid to 10-KSA by *Staphylococcus* sp. The yield was greater than 90% with less than 5% of by-product 10-HSA. Kaneshiro *et al.* (12) found that *Sphingobacterium* sp. NRRL B-14797 converted oleic acid exclusively to 10(*R*)-hydroxystearic acid with 70% yield.

Although hydration of oleic acid to 10-HSA was investigated at cell-free enzyme levels (13–16), attempts to purify hydratase were not successful. Little was known about the physical and chemical properties of oleate hydratase. Oleate hydratase from *N. cholesterolicum* NRRL 5767 was purified by Huang *et al.* (17) by using Mono-Q ion-exchange and Superose gel-filtration column chromatography. 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} M. The molecular weight estimated from a Superose HR 10/30 gel-filtration column is about 120,000 daltons, and from denatured sodium dodecyl sulfate–polyacrylamide gel electrophores's (SDS–PAGE) it is about 32,000 daltons (18). Therefore, oleate hydratase is a tetramer composed of four identical subunits.

In our continuing screening of microorganisms capable of converting fatty acids to value-added products, a new microbial culture, strain DS5, isolated from a dry soil sample col-

*Address correspondence at Oil Chemical Research, NCAUR, ARS, USDA, 1815 N. University St., Peoria, IL 61604.

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lected in Peoria, Illinois, was found to convert oleic acid to 10-KSA with 85% yield. Strain DS5 also converted linoleic acid to 10-hydroxy-12(*Z*)-octadecenoic acid (10-HOA) with 64% yield. Hydroxy fatty acids are useful in lubricants, surfactants, plasticizers, detergents, coatings, paints, and antifungal agents. 10-HOA is a positional isomer of ricinoleic acid, an important material used in the production of sebacic acid. The latter is used commercially in the synthesis of esters and resins.

It was interesting to find that DS5 hydratase oxidizes both oleic and linoleic acids at the 9,10 double bond. To determine positional specificity and the effect of substrate carbon chainlengths on strain DS5 hydratase activity, we studied the hydration of mono-, di-, and triunsaturated C_{18} fatty acids and other monounsaturated fatty acids with different carbon chainlengths, as well as *trans*-unsaturated fatty acid. In this paper, we also investigated the nature of DS5 hydratase, identified the bioconversion products from α- and γ-linolenic acids, and products from other unsaturated fatty acids. We found that strain DS5 hydratase is indeed a C-10, positional-specific, and *cis*-specific enzyme and prefers an 18-carbon monounsaturated fatty acid.

MATERIALS AND METHODS

Microorganisms. Microorganisms from soil and water samples were constantly screened for their ability to modify oleic acid. Growth medium and conditions have been reported previously (19–21). Microbial isolates were identified by the Biolog automated bacteria and yeast identification system (Microstation, Hayward, CA).

Chemicals. Linoleic and oleic acids [purity >99% by gas chromatography (GC)] were purchased from Nu-Chek-Prep Inc. (Elysian, MN). Other unsaturated fatty acids (99% purity) were purchased from Sigma. 10-HSA was as shown in our previous work (6). All solvents used were ACS-grade and were obtained from commercial sources. Kieselgel 60 and thin-layer precoated Kieselgel $60F_{254}$ plates were obtained from EM Science (Cherry Hill, NJ).

Bioconversion. Bioconversions were carried out by adding 0.3 mL (0.26 g) substrate unsaturated fatty acid to a 24-hour-old culture, and the flasks were shaken again at 200 rpm at 30°C for two days. For studies with resting cell suspensions and cell-free crude extracts, strain DS5 was grown for one day and centrifuged at 8,000 rpm for 20 m into separate cells and supernatant. The cell pellet was washed twice with a 0.05 M potassium phosphate buffer of pH 7.5 and resuspended in a small amount of the same buffer to $OD_{650\,nm} = 4$. Substrate unsaturated fatty acid was added to the cell suspension or the cell-free crude extract, and the mixture was incubated at 30°C for 18 h to study bioconversion. At the end of this time, the reaction mixture was acidified to pH 2 with 6 N hydrochloric acid, and then extracted twice with an equal volume of diethyl ether. The solvent was removed from the combined extracts with a rotary evaporator.

Isolation of products. Crude extracts containing reaction products were subjected to high-performance liquid chromatography (HPLC) to isolate pure material for further identification. A Dynamax-60A silica column (25 cm × 21.4 mm i.d.; Rainin Instruments, Woburn, MA) and methylene chloride/methanol (97:3, vol/vol) as solvent were used with a DuPont Instruments (Wilmington, DE) chromatographic pump equipped with a Waters (Milford, MA) Model 403 reflective index detector and an ISCO Inc. (Lincoln, NE) V^4 variable-wavelength detector. Purity of fractions was analyzed with thin-layer chromatography (TLC) and GC.

Analyses of products. The reaction products were analyzed by TLC and GC as described previously (19,20). Toluene/dioxane/acetic acid (79:14:7, vol/vol/vol) was the TLC solvent system. For GC, the samples were methylated with diazomethane. GC of these methyl esters were run isothermally at 200°C. For quantitative analysis, palmitic acid was added as internal standard prior to solvent extraction. A linear relationship was established on the peak area ratios of product vs. methyl palmitate.

Chemical structure of the product was identified through 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) 5890 GC coupled to a Hewlett-Packard 5970 Series Mass Selective Detector. The column outlet was connected directly to the ion source. Separations were effected in a methylsilicone column (15 m × 0.25 mm) with a temperature gradient of 8°C per min from 160 to 250°C after an initial hold at 160°C for 3 min. Proton and ^{13}C NMR spectra were determined in deuterated chloroform with a Bruker WM-300 spectrometer (Rheins, Tepten, Germany), operated at a frequency of 300 and 75.5 MHz, respectively. FTIR analyses of the free acid products were run as films on KBr on a Perkin Elmer infrared Fourier transform Model 1750 spectrometer (Perkin Elmer, Inc., Oakbrook, IL).

RESULTS AND DISCUSSION

Strain DS5 is a Gram-negative, nonmotile rod (0.5 × 2 μm) that produces yellowish-brown pigment. Comparison with known strains through the Biolog GN microstation established that strain DS5 belongs to genus *Flavobacterium* and has a 50% similarity to the closest species of *F. gleum*. Therefore, strain DS5 is assigned as *Flavobacterium* sp. DS5 (22).

Hydration of oleic acid by strain DS5. Strain DS5 converted oleic acid to 10-KSA in 85% yield. The product purified from HPLC is a white, solid material. It formed crystals in ether at low temperature. The plate-like crystals were washed with hexane and gave a single spot ($R_f = 0.45$) on silica-gel TLC and 99.8% purity upon GC analysis. The melting point is 79.2°C. Structure of 10-KSA was determined by mass spectroscopy, proton, and ^{13}C NMR and FTIR (22).

Optimum time, pH, and temperature for the production of 10-KSA are 36 h, 7.5, and 30°C, respectively. About 10% of 10-HSA also is 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 at a ratio of 25:75, and the product ratio for cell-free crude extract, obtained from ultrasonic disruption of the cells, yielded mainly 10-HSA (Table 1). These results strongly suggest that DS5 hydratase is (i) associated with the cell and not an extracellular enzyme, and (ii) soluble in nature and not in particulate form. In addition, they strongly suggested that oleic acid is converted to 10-KSA *via* 10-HSA. The second step, the conversion of 10-HSA to 10-KSA, is catalyzed by a secondary alcohol dehydrogenase, which requires a cofactor, NAD. In the cell-free crude extract, the cofactor NAD is less available to the enzyme to carry out the reaction. The stereochemistry of product 10-HSA from strain DS5, determined by ^{1}H NMR of the mandelate esters of methyl-10-hydroxystearate (10) obtained from DS5, showed 66% enantiomeric excess in the 10(*R*) form. Enantiomeric purity of 10-HSA from other microbes (10) was [% e.e. in 10(*R*) isomer]: *N. restrictus* ATCC 14887 [65.8%], *Pseudomonas* sp. NRRL 3266 [100%], *Pseudomonas* sp. NRRL 2994 [95.8%], *R. rhodochrous* ATTC 12674 [11.7%], *M. fortuitum* UI 53378 [76.4%], Baker's yeast [64.1%], and *Sphingobacterium* sp. (12)NRRL B-14797 [100%].

Hydration of linoleic acid. The *Flavobacterium* DS5 enzyme system also catalyzes the conversion of linoleic acid. In contrast to the oleic acid substrate, from which the main product is 10-keto acid, DS5 enzyme produces mainly 10-hydroxy acid from linoleic acid. DS5 enzyme converted linoleic acid to 10-HOA with 55% yield (23). GC of the methylated products shows a retention time at 13.7 min. The specific optical rotation of 10-HOA is $[\alpha]_D^{24} = -5.58$ (methanol).

The main reaction product purified from HPLC (conditions described previously) is a colorless, oily liquid. It showed a single spot ($R_f = 0.38$) on TLC and 98.9% purity upon GC analysis. The main product identified by GC/MS, FTIR, and NMR was 10-HOA (22). A minor product with GC retention time (RT) 12.6 min was identified as 10-keto-12-octadecenoic acid. The optimum conditions for the production of 10-HOA were pH, 7.5; temperature, 20–35°C; and 36 h of incubation.

Hydration of α- and γ-linolenic acids. From the results described above, DS5 hydratase converts oleic and linoleic acids to their corresponding 10-hydroxy and 10-keto fatty acids. It seems that DS5 hydratase is a C-10 positional-specific enzyme. To clear this point and the effect of substrate carbon chainlength on the DS5 hydratase activity, we studied the hydration of triunsaturated C_{18} fatty acids, *trans*-unsaturated fatty acid, as well as other carbon chainlength monounsaturated fatty acids.

TABLE 1
Biotransformation of Oleic Acid by Strain DS5

System	Product ratio (10-HSA/10-KSA)[a]
Growing cells	10:90
Resting cell suspension	25:75
Cell-free crude extract	97:3

[a]10-HSA, 10-hydroxystearic acid; 10-KSA, Co-ketostearic acid.

Product from α-linolenic acid. The purified major product has a GC RT of 12.7 min and purity of 98.5%. It is a liquid at 4°C and a solid at freezer temperature (–20°C). The electron-impact mass spectrum of the free acid product gave a heaviest ion of *m/z* 278 (M – 18). Large fragments, corresponding to α-cleavage with ions *m/z* 187 and 169 (relative intensity 22 and 100%, respectively), place the hydroxy group at the C-10 position (Fig. 1A). This was further confirmed by GC/MS of the methyl ester, prepared with diazomethane, which gave the largest fragments at *m/z* 201 and 169. Therefore, the product is likely 10-hydroxy-12,15-octadecadienoic acid.

FTIR of the free acid (Fig. 2A) showed absorption of the acid hydroxy group around 2800–3200 cm^{-1} and of the alkyl hydroxy group at 3392 cm^{-1}. The carbonyl group was seen at 1710 cm^{-1}. No keto carbonyl was detected. In the absence of a significant absorbance at 970 cm^{-1}, which would be evidence of *trans* double bonds, the unsaturation seen at 3010 cm^{-1} is *cis*.

The reaction product was also subjected to proton and ^{13}C NMR analyses. Resonance signals (ppm) and corresponding molecular assignments, given in Table 2, further confirmed the identity of the bioconversion product as 10-hydroxy-12(*Z*),15(*Z*)-octadecadienoic acid. In comparison with NMR data of a previous paper (9), there are slight revisions and fewer ambiguities in current assignments. The olefinic coupling constant at $C_{12,13}$ of 10.7 Hz and $C_{15,16}$ of 10.6 Hz confirmed our infrared data that the unsaturations are in the *cis* configuration. A minor product, with a GC RT of 11.97 min, was identified by GC/MS as 10-keto-12,15-octadecadienoic acid (24).

Product from γ-linolenic acid. The purified product has a GC RT of 11.4 min and purity of 98.5%. It is a liquid at 4°C

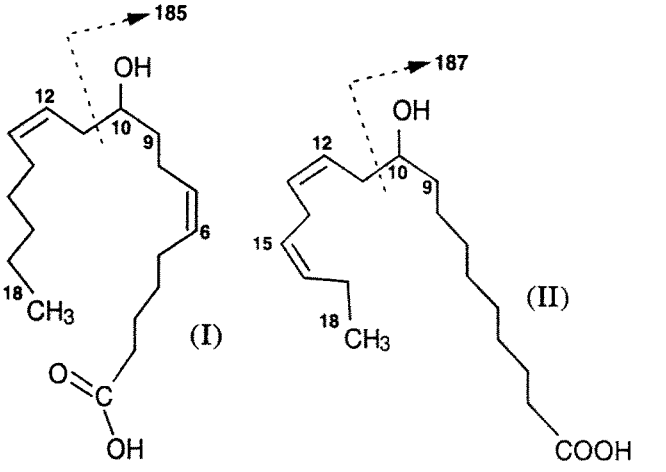

FIG. 1. Conversion products from α- and γ-linolenic acids by DS5 hydratase. (I), 10-hydroxy-6(*Z*),12(*Z*)-octadecadienoic acid; (II), 10-hydroxy-12(*Z*), 15(*Z*)-octadecadienoic acid.

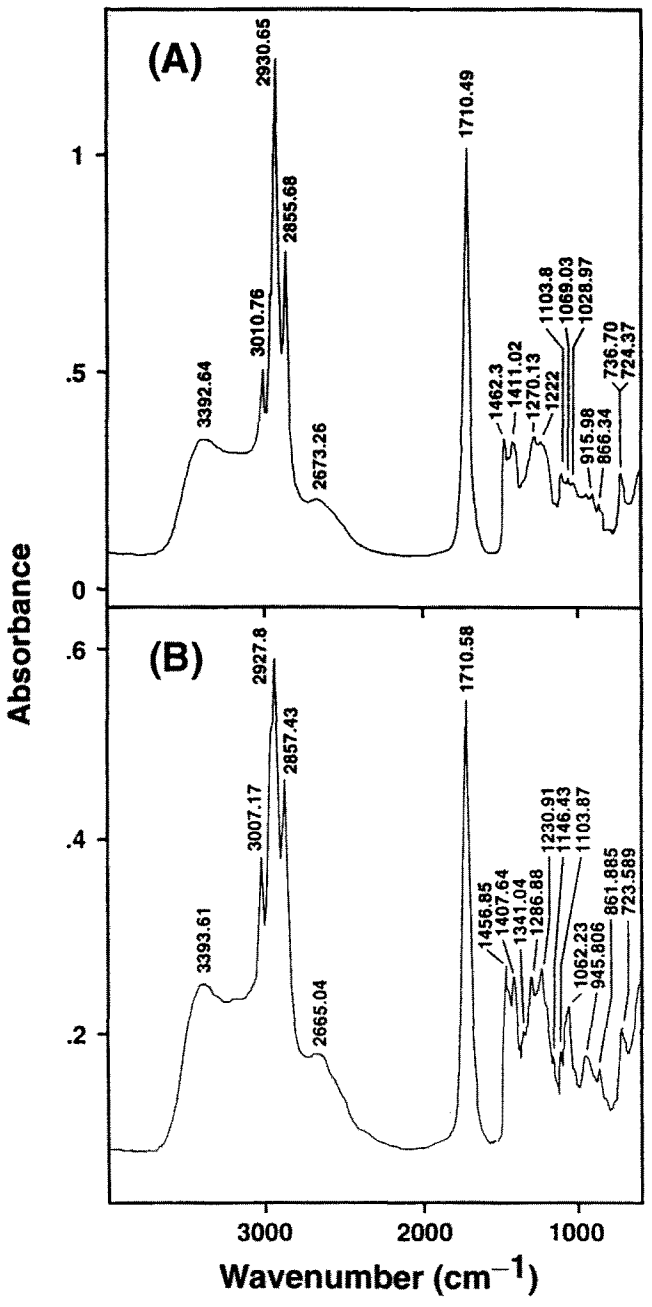

FIG. 2. Fourier transform infrared of conversion products from α- and γ-linolenic acids.

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

From α-linolenic acid		
	Resonance signals (ppm)	
Carbon number	Proton/J (Hz)	^{13}C
1		179.5
2	2.30 *t* (2,3 = 7.5)	34.0
3	1.59 *m*	24.6
4	1.27 *m*	
5	1.27 *m*	
6	1.27 *m*	28.9–29.5
7	1.27 *m*	
8	1.27 *m*	25.6
9	1.44 *m* (9,10 = 7.5)	36.6
10	3.61 *m* (10,11 = 7.3)	71.5
11	2.22 *dd* (11,12 = 7.3)	35.1
12	5.38 *m* (12,13 = 10.7)	125.3
13	5.49 *m* (13,14 = 7.3)	131.3
14	2.77 *m* (14,15 = 7.1)	25.6
15	5.28 *m* (15,16 = 10.6)	126.8
16	5.35 *m* (16,17 = 7.0)	132.0
17	2.04 *m* (17,18 = 7.5)	20.5
18	0.94 *t*	14.2
From γ-linolenic acid		
1		179.4
2	2.33 *t* (2,3 = 7.5)	33.9
3	1.63 *m* (3,4 = 7.5)	24.2
4	1.39 *m*	29.0
5	2.04 *m*	26.7
6	5.36 *m* (6,7 = 10.9)	129.7
7	5.36 *m*	129.7
8	2.14 *m*	23.6
9	1.52 *m*	36.4
10	3.63 *m* (10,11 = 7.0)	71.1
11	2.21 *m*	35.3
12	5.36 *m* (12,13 = 10.9)	124.8
13	5.54 *m* (13,14 = 6.9)	133.5
14	2.04 *m*	27.3
15	1.33 *m*	29.3
16	1.27 *m*	31.5
17	1.27 *m* (17,18 = 6.6)	
18	0.87 *t*	14.0

and a solid at freezer temperature (–20°C). The electron-impact spectrum of the free acid of the product gave a heaviest ion at 278 (M – 18). Large fragments, corresponding to α-cleavage with ions *m/z* 185 and 167 (relative intensity 55 and 78%, respectively), place the hydroxy group at the C-10 position (Fig. 1B). This was further confirmed by GC/MS of the methyl ester of the product, which showed the largest fragments at *m/z* 199 and 167. Therefore, the product is likely 10-hydroxy-6,12-octadecadienoic acid.

FTIR of the free acid (Fig. 2B) showed absorption of the acid hydroxy group around 2800–3200 cm^{-1} and the alkyl hydroxy group at 3393 cm^{-1}. The carbonyl group was seen at 1710 cm^{-1}. No keto carbonyl was detected. In the absence of a significant absorbance at 970 cm^{-1}, which would be evidence of *trans* double bonds, the unsaturation seen at 3007 cm^{-1} is *cis*.

The reaction product also was subjected to proton and ^{13}C NMR analyses. Resonance signals (ppm) and corresponding molecular assignments, given in Table 2, further confirmed the identity of the bioconversion product as 10-hydroxy-6(*Z*),12(*Z*)-octadecadienoic acid. The olefinic coupling constant at $C_{6,7}$ of 10.9 Hz and $C_{12,13}$ of 10.9 Hz confirmed our infrared data that the unsaturations are in the *cis* configuration. The enzyme hydrated the C_9 double bond and did not alter the original olefinic configurations at C_6 and C_{12}.

TABLE 3
Substrate Specificity of Unsaturated Fatty Acids Conversion Enzymes in Strain DS5

Acids	Retention time (min) Substrate	Products[a]	Relative activity %
Myristoleic	2.70	3.80 k	48
		4.08 h	
Palmitoleic	4.21	5.85 k	161.8
		7.31 h	
Oleic	7.25	13.30 k	289.5
		14.30 h	
Petroselinic	7.24	12.50	0.9
Linoleic	6.92	12.60 k	100
		13.70 h	
α-Linolenic	6.99	11.84 k	97.2
		12.61 h	
γ-Linolenic	6.58	11.29 k	89.9

[a]k, 10-keto product; h, 10-hydroxy product.

TABLE 4
Hydratases from Various Microbes Which Produced 10-Hydroxy Product

Microbes	Substrates	Reference
Pseudomonas sp. NRRL 3266	Oleic	1
Corynebacterium sp.	Oleic	4
Rhodococcus rhodochrous	Oleic	5
	Linoleic	5
Acetobacterium woodii	Linoleic	25
Micrococcus sp.	Oleic	7
Saccharomyces cerevisae	Oleic	8
Mycobacterium fortuitum	Oleic	8
Nocardia cholesterolicum	Oleic	6
	Linoleic	9
	Linolenic	9
Staphylococcus sp.	Oleic	10
Flavobacterium sp.	Oleic	21
	Linoleic	22
	Linolenic	24
Sphingobacterium sp.	Oleic	11

Products from other unsaturated fatty acids. Strain DS5 converted myristoleic acid to two products (GC RT 3.8 and 4.08 min). The electron-impact spectrum of the methyl ester of RT 3.8 min gave a molecular ion of *m/z* 256. The large fragment, corresponding to α-cleavage with *m/z* 199, places the keto group at the C-10 position. The methyl ester of product RT 4.08 min gave a molecular ion of *m/z* 258. The large fragments corresponding to α-cleavage with *m/z* 201 and 169 (relative intensity 15 and 70%, respectively) also place the hydroxy group at the C-10 position. Therefore, products RT 3.8 and 4.08 min are likely 10-keto myristic and 10-hydroxymyristic acids, respectively.

Palmitoleic acid also gave two bioconversion products (RT 6.9 min and RT 7.27 min). The electron-impact spectrum of the methyl ester of RT 6.9 min gave a molecular ion of *m/z* 284. The large fragment, corresponding to α-cleavage with *m/z* 199, places the keto group at the C-10 position. The methyl ester of product RT 7.27 min gave a molecular ion of 286. The large fragments, corresponding to α-cleavage with *m/z* 201 and 169 (relative intensity 20 and 60%, respectively), place the hydroxy group at the C-10 position. Therefore, products RT 6.9 and 7.27 min are likely 10-ketopalmitic and 10-hydroxypalmitic acids, respectively.

It is interesting to find that all the unsaturated fatty acids tested with DS5 hydratase are hydrated at the C-10 position despite their varying degrees of unsaturation. 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(Z),8(Z),11(Z),14(Z)-eicosatetraenoic], and erucic [13(Z)-docosenoic] acids (24). From all 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 indicated that the unsaturation must be in the *cis* configuration for DS5 hydratase activity.

The strain DS5 system produced relatively more keto product from palmitoleic and oleic acids and more hydroxy product from myristoleic, linoleic, and α- and γ-linolenic acids (Table 3). The reason for the product preference is not clear. Among the 18-carbon unsaturated fatty acids, additional double bonds at either side of the C-10 position lower the enzyme hydration activity. Searching through the literature (Table 4), we found that all microbial hydratases hydrate oleic and linoleic acids at the C-10 position. Therefore, the positional specificity of microbial hydratases may be universal.

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