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Stereospecificity of Microbial Hydrations of Oleic Acid to 10-Hydroxystearic Acid

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We recently described a simple method for ascertaining the stereochemical purities of hydroxy fatty acids (S. H. El-Sharkawy, W. Yang, L. Dostal, and J. P. N. Rosazza, Appl. Environ. Microbiol. 58:2116–2122, 1992) based on the ^{1}H-nuclear magnetic resonance spectral analysis of diastereomeric *S*-(+)-*O*-acetylmandelate esters of hydroxystearates. This report describes the stereochemistries of microbial hydrations of oleic acid to 10-hydroxystearic acid by *Nocardia aurantia* (also known as *Rhodococcus rhodochrous*) ATCC 12674, *Nocardia restrictus* ATCC 14887, *Mycobacterium fortuitum* UI-53387, *Pseudomonas* species strain NRRL-2994, *Pseudomonas* species strain NRRL B-3266, and baker's yeast. 10(*R*)-hydroxystearic acid isolated from *Pseudomonas* species strain NRRL-2994 was the standard for use in the ^{1}H-nuclear magnetic resonance spectral technique to permit simple assignments of the absolute configurations of 10-hydroxystearic acid produced by different microorganisms. While the *R. rhodochrous* ATCC 12674-mediated hydration of oleic acid gave mixtures of enantiomers 10(*R*)-hydroxystearic acid and 10(*S*)-hydroxystearic acid, *Pseudomonas* species strain NRRL-B-3266 produced optically pure 10(*R*)-hydroxystearic acid. The remaining microorganisms stereoselectively hydrated oleic acid to 10(*R*)-hydroxystearic acid containing between 2 and 18% of the contaminating 10(*S*)-hydroxystearic acid.

Since the first report of the microbiological production of 10-hydroxystearic acid from oleic acid (compound 1 [see Fig. 1]) by Wallen and coworkers (18, 19), the hydration of oleic acid to 10-hydroxystearic acid has been reported with *Pseudomonas* sp. strain NRRL-2994 (17), *Pseudomonas* sp. strain NRRL-B-3266 (16), and *Rhodococcus rhodochrous* ATCC 12674 (10). We recently reported that oleic acid was efficiently converted to 10-hydroxystearic acid by resting cells of *Saccharomyces cerevisiae* (baker's yeast, type II; Sigma Chemical Co.) and other yeasts, fungi, and bacteria (5).

Baker and Gunstone (2) reported the chemical synthesis of 9-hydroxystearic acid. While it was impossible to measure the optical rotation of this hydroxy fatty acid, the D configuration was assigned on the basis of mixture melting point data. Schroepfer and Bloch (17) demonstrated that the methyl ester of Gunstone's synthetic acid was slightly levorotatory ($[\alpha]^{23}$, 546° = −0.16). 10-Hydroxystearic acid was prepared by incubating oleic acid with *Pseudomonas* sp. strain NRRL-2994 under the conditions described by Wallen et al. (19). Wallen et al. (19) suspected that the hydroxy acid was optically active, but it was not possible to measure its optical rotation. Wallen et al. (18) later reported the specific rotation of methyl-10-hydroxystearic acid to be $[\alpha]^{26}$, 550° = −0.03. Schroepfer and Bloch (17) reported that the methyl ester of the 10-hydroxyoctadecanoic acid produced from oleic acid by *Pseudomonas* sp. strain NRRL-2994 was levorotatory and assigned the stereochemistry of the hydroxylated position as D, or *R* as in 10(*R*)-hydroxystearic acid (compound 2 [see Fig. 1]). These investigators later reported that during the course of the 9,10-hydration reaction solvent hydrogen was stereospecifically introduced at the 9 position in the corresponding L or *S* configuration (15).

Most investigators (4, 6, 7, 9, 10, 19) who have studied the microbial hydration of oleic acid either have not recorded the stereochemical purities of hydroxyoctadecanoic acid products or have relied upon extremely small specific rotation measurements to infer isomeric compositions. We recently (5) introduced a simple method for ascertaining the stereochemical purities of hydroxy fatty acids based on the ^{1}H-nuclear magnetic resonance (NMR) analysis of diastereomeric *S*-(+)-*O*-acetylmandelate esters. This report describes the bioconversion of oleic acid (compound 1 [see Fig. 1]) into 10-hydroxystearic acid (*R* and/or *S* configuration [compounds 2 and/or 3]) by six microorganisms known to accomplish this reaction and the use of ^{1}H-NMR spectral analysis of diastereomeric *S*-(+)-*O*-acetylmandelate esters of the isolated 10(*R*, *S*)-hydroxystearates (compounds 4 and 5 [see Fig. 1]) to determine the stereochemical purities of the hydrated oleic acid product.

MATERIALS AND METHODS

Chemicals. Oleic acid, 4-(*N*,*N*-dimethylamino)-pyridine, dicyclohexylcarbodiimide, and *S*-(+)-*O*-acetylmandelic acid were obtained from Sigma. Diazald (*N*-methyl-*N*-nitrosotoluene-*p*-sulfonamide) was purchased from Aldrich Chemical Co.

Instrumentation. ^{1}H-NMR spectra were obtained on a Bruker AMX–600-MHz high-field spectrometer. ^{1}H-NMR spectra were recorded at 600.136 MHz, using $CDCl_3$ as a solvent and tetramethylsilane as an internal standard. Chemical shift values are reported in parts per million, and coupling constants (*J* values) are given in hertz. Abbreviations for ^{1}H-NMR signals are as follows: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; and m, multiplet.

Microorganisms and fermentation. Microorganisms used in this work are contained in the University of Iowa College of Pharmacy culture collection and were maintained on Sabouraud-maltose agar (2% agar) or sporulation agar (ATCC #5 medium) (1). Our medium #5 slants consisted of the

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following: yeast extract, 0.1%; beef extract, 0.1%; tryptose, 0.2%, $FeSO_4$, 1 mg/liter; glucose, 1%; and agar, 2%. The medium was adjusted to pH 7.2 before autoclaving. Dry baker's yeast was purchased from Sigma (YSC-2, type II, *S. cerevisiae*), and Red Star brand yeast was purchased from a local grocery store. Other cultures examined in this work included *Nocardia aurantia* ATCC 12674 (also known as *R. rhodochrous*), *Nocardia restrictus* ATCC 14887, *Mycobacterium fortuitum* UI-53387, *Pseudomonas* sp. strain NRRL-2994, and *Pseudomonas* sp. strain NRRL-B-3266.

Cultures were grown by using our standard two-stage fermentation protocol (3, 5) in 50 ml of sterile yeast-malt extract broth held in stainless-steel-capped, 125-ml DeLong culture flasks. The composition of the medium is 0.4% glucose, 0.4% yeast extract, and 1% malt extract in distilled water; the medium was adjusted to pH 5.8 with 6 N HCl before autoclaving at 121°C for 15 min. Cultures were generally incubated with shaking at 150 rpm at 25 to 27°C on New Brunswick Scientific G25 Gyrotory shakers. A 10% inoculum derived from 48-h-old stage I cultures was used to initiate stage II cultures, which were incubated for 24 h before receiving oleic acid (22.6 μl/ml; 20.2 mg/ml) as a substrate. Cultures were sampled at various time intervals by removing 0.5 ml of the entire culture. Samples were adjusted to pH 2 with 6 N HCl and extracted with an equal volume of ethyl acetate-propanol (9:1, vol/vol), and the organic and aqueous layers were separated by centrifugation for 1 min in a desktop centrifuge. The organic solvent layer was removed, evaporated to dryness, and reconstituted in 50 μl of methanol, and samples were spotted onto thin-layer chromatography (TLC) plates for analyses. Preparative-scale incubations were conducted as described in detail before (5), as were methods for the extraction, chromatographic purification, and identification of hydroxy fatty acid products.

Preparation of (*S*)-(+)-*O*-acetylmandelate esters of methyl-10-hydroxystearate. 10-Hydroxystearic acid isolated from *R. rhodocus* ATCC 12674 was methylated with diazomethane and reacted with (*S*)-(+)-*O*-acetylmandelic acid as described before (5), and the diastereomeric derivative mixture of compounds 4 and 5 [(*S*)-(+)-*O*-acetylmandelate esters] were isolated by preparative TLC. In a typical derivatization reaction, (*S*)-(+)-*O*-acetylmandelic acid (9.2 mg; 47 μmol) and 1 mg (8.6 μmol) of 4-(*N*,*N*-dimethylamino)-pyridine were dissolved in 200 μl of dichloromethane (dried over Na_2SO_4); the mixture was stirred and cooled in an ice bath. A solution of 10 mg (32 μmol) of methyl-10-hydroxystearate in 300 μl of CH_2Cl_2 was added along with 9.7 mg (47 μmol) of dicyclohexylcarbodiimide, which was dissolved in 50 μl of CH_2Cl_2. The reaction was quantitative within 2 h (TLC [5]), and the reaction mixture was filtered to remove a white precipitate. The filtrate was evaporated to dryness and reconstituted with CH_2Cl_2 for preparative TLC (hexane-ethyl acetate, 3:1). The major UV_{254} quenching band at R_f = 0.5 was scraped and eluted with ethyl acetate to give the analytical sample of 5 mg of methyl-10-(*S*)-(+)-*O*-acetylmandeloylstearates 4 and 5 after solvent evaporation. The 600-MHz ^{1}H-NMR spectrum gave the following data: 0.87 (3H, t, J = 7.2, H-18), 0.88 (3H, t, J = 7.2, H-18), 1.01 to 1.31 [18H, m, $(CH_2)_9$], 1.35 to 1.39 (2H, m, H-9 or H-11), 1.56 to 1.64 (2H, m, H-3 or H-4), 2.19 (3H, s, CH_3CO), 2.29 (2H, dd, J = 7.5, H-2), 2.30 (2H, dd, J = 7.5, H-2), 3.6658 (3H, s, OCH_3), 3.6708 (3H, s, OCH_3), 4.85 to 4.89 (1H, m, H-10), 5.8708 (1H, d, J = 2.2, H-2′), 5.8744 (1H, d, J = 2.2, H-2′), 7.35 to 7.39 (3H, m, H-3″, H-4″, H-5″), 7.47 (2H, dd, J = 6, H-2″, H-6″).

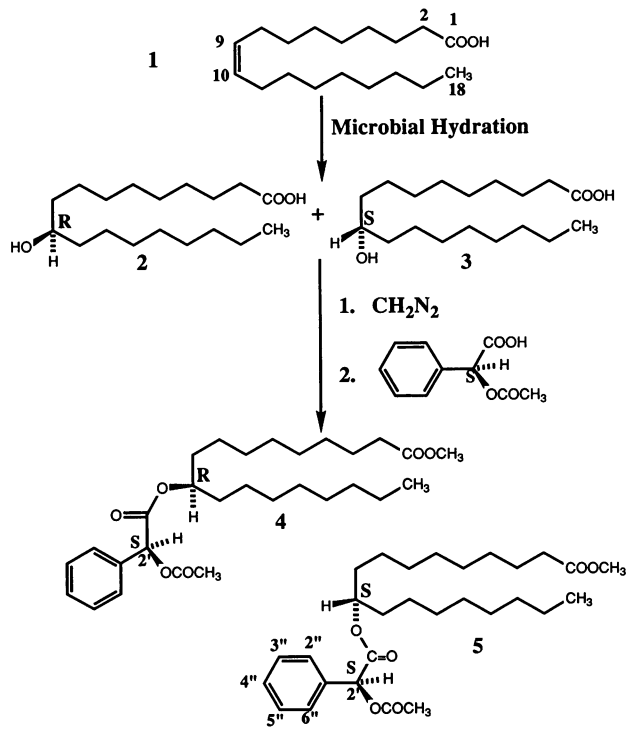

FIG. 1. Microbial transformation of oleic acid (compound 1) to 10(*R*)-hydroxystearic acid (compound 2) and 10(*S*)-hydroxystearic acid (compound 3) and chemical derivatization of these hydroxy fatty acids to their corresponding methyl-10(*R*)-[*S*-(+)-*O*-acetylmandeloyl] (compound 4) and methyl-10(*S*)-[*S*-(+)-*O*-acetylmandeloyl] (compound 5) stearates.

The ^{1}H-NMR spectra of mandelate esters of methyl-10-hydroxystearate (compounds 4 and/or 5) from the other microorganisms gave identical proton NMR spectra, except for the relative intensities of the pairs of diastereoisomeric protons for H-2, H-18, —OCH_3, and H-2′. Figure 2 shows the signals for the —OCH_3 methyl ester functional group for compounds 4 and/or 5 obtained from each microorganism. The results in Fig. 2 are expressed in terms of the percent enantiomeric purity (enantiomeric excess, or %ee). Enantiomeric purities are determined by the equation: % ee = $[(R - S)/(R + S)] \times 100$, where R represents the NMR signal peak area of the R isomer (compound 4) and S represents the NMR signal peak area of the S isomer (compound 5).

RESULTS AND DISCUSSION

Hydroxy fatty acids are valuable as lubricants, surfactants, plasticizers, and components in detergent, coating, and paint industries and in the synthesis of resins (5). Since these bifunctional monomers may be useful in the preparation of chiral polymers, it is important to have available a simple method for determining the stereochemical purities of 10-hydroxystearic acids. It is theoretically possible to obtain either 10(*R*)-hydroxystearic acid (compound 2), 10(*S*)-hydroxystearic acid (compound 3), or mixtures of these two enantiomers (compounds 2 and 3) by microbial hydration of oleic acid (Fig. 1). The assignment of stereochemistry to 10-hydroxyoctadecanoic acids has been based largely on the elegant and detailed work of Schroepfer and coworkers (16, 17). However, the routine adaptation of such chemical

methodologies for ascertaining hydroxyl group stereochemistry is laborious, and the use of measured rotations of much less than 1° renders this process difficult. The use of *S*-(+)-*O*-acetylmandelates in determining the stereochemical purities of secondary alcohols is well established (5, 11). The derivatization reaction is simple to perform, quantitative in yield, and not subject to racemization during reaction workup. Furthermore, we previously demonstrated that ^{1}H-NMR spectra of compounds 4 and/or 5 exhibited four sets of signals which could be useful in distinguishing between stereoisomers. These were signals for protons at H-2 and H-18, the methyl ester, and the methine proton of the mandelic acid moiety. With the resolution achieved by using ^{1}H-NMR at 600 MHz, it is also possible to distinguish as little as 1% of a contaminating isomer (11).

The microorganisms selected for this work all have been well documented for their abilities to convert oleic acid to 10-hydroxystearic acid. For these experiments, the microbial hydration of oleic acid was carried out (5), and the resulting 10-hydroxystearic acid products were isolated and purified for derivatization and ^{1}H-NMR analysis. Each of the 10-hydroxystearates isolated from the six different microorganisms was quantitatively methylated with diazomethane before being converted to the *S*-(+)-*O*-acetylmandelate ester.

Since *Pseudomonas* sp. strain NRRL 2994 produces 10(*R*)-hydroxystearic acid (compound 2) (13, 14, 17), the ^{1}H-NMR spectral properties of compound 4 obtained from this microorganism were used to assign the absolute configurations of the *S*-(+)-*O*-acetylmandelate derivatives of hydroxystearates produced by the other organisms. With the standard compound, signals for H-2 and H-18 occurred as triplets at 2.29 and 0.898 ppm, respectively, and both the methyl ester signal at 3.67 ppm and the mandelic acid methine signal at 5.87 ppm occurred as singlet signals in the proton NMR spectrum. In racemic compounds (compounds 4 and 5), all of the signals mentioned above become twinned (5), with the relative peak areas providing a measure of the respective amounts of the isomers.

The proton NMR signals for the methoxyl groups of the methyl esters of compounds 4 and 5, centered at 3.67 ppm, are the best resolved of all signals mentioned. Thus, the relative intensities of these peaks provide a measure of the stereochemical purities (percent enantiomeric excess) of compounds 2 and 3 formed by oleic acid hydration. The results are shown in Fig. 2.

Pseudomonas sp. strains NRRL B-3266 and NRRL 2944 gave essentially pure compound 4 with measured values of 100 and 95% enantiomeric excess, respectively. These results indicate that the stereochemistry of the 10-hydroxystearic acid formed by these pseudomonads is largely of the *R*-absolute configuration. On the other hand, *R. rhodochrous* ATCC 12674 gave a mixture of compounds 4 and 5 with a measured enantiomeric excess of 11.7%, or a composition consisting of 56% 10(*R*) (compound 4) and 44% 10(*S*) (compound 5). All other cultures gave the 10*R* isomer (compound 4) as the major metabolite, with measured enantiomeric excess values ranging between 64.1 and 76.4%. The results observed and calculated through the methyl ester functional group were also confirmed by observations of the other three proton NMR signals. For example, with the *R. rhodochrous*-derived 10(*R*)-mandelate derivative, signals for H-2 and H-18 each occurred as two sets of overlapping triplets at 2.29 and 0.88 ppm, respectively, and the methyl ester signal at 3.67 ppm and the methine proton of the mandelic acid residue (Ar-CH-CO—) at 5.87 ppm each appeared as two singlets of similar relative intensities.

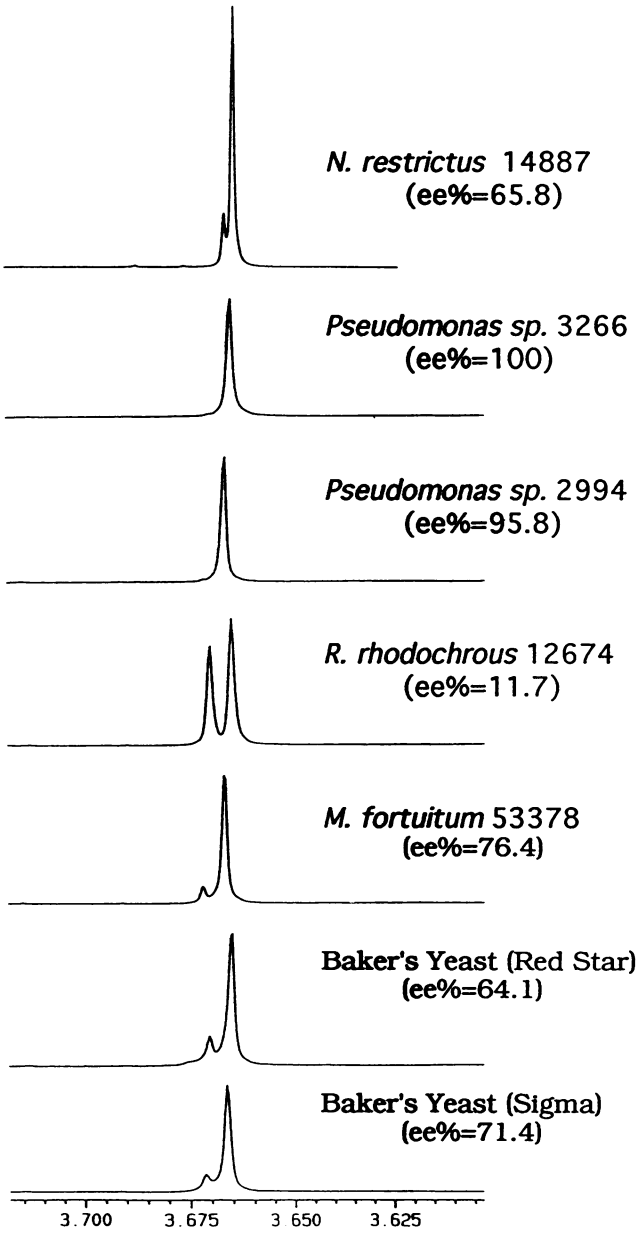

FIG. 2. ^{1}H-NMR spectral signals for the methyl ester functional groups of compounds 4 and 5 obtained from various microorganisms. Optical purities are expressed as percent enantiomeric excess (ee%).

Our results indicate that, with the organisms examined, microbial hydrations of oleic acid are largely 10*R* specific and the stereochemistry of hydration is mixed, depending on the organism used. The highest enantiomeric purities were realized with the two *Pseudomonas* strains examined; the most relaxed hydration stereospecificity occurred with *R. rhodochrous*. This work demonstrates the power and simplicity in the derivatization and NMR spectral method for determining the absolute configurations and the stereochemical purities of 10-hydroxystearic acids.

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