Hydration of Squalene and Oleic Acid by Corynebacterium sp. S-401

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Received February 9, 1981

We screened microorganisms from soil using squalene as carbon source in order to obtain microorganisms which have specific activities for aliphatic linear compounds such as fatty acids and terpenes. As a result, we isolated Arthrobacter sp. Y-11 which specifically cleaves the squalene molecule at its center into two geranylacetone molecules. This bacterium also catalyzed stereoselective scission of some squalene derivatives and oxidation of linear terpenoid alcohols. These previous works suggested that this screening method using squalene is useful for selecting lipophilic microorganisms which have promising reactivities on aliphatic hydrophobic compounds.

In this report we describe the hydration reaction of squalene and oleic acid with Corynebacterium sp. S-401 which was isolated from soil by the screening method mentioned above. The isolate hydrates the squalene molecule to form tertiary alcohols when it grows on a squalene medium supplemented with yeast extract. These reactions are interesting in view of the degradation pathways of naturally abundant linear terpenoids.

Resting cells of S-401 also stereospecifically hydrated oleic acid to form 10R-hydroxyoctadecanoic acid with relatively high yields being accompanied with a further oxidized product, 10-oxooctadecanoic acid.

MATERIALS AND METHODS

Culture conditions. A strain of Corynebacterium sp. S-401 isolated from soil was used throughout this study. Three types of culture medium were used in this study. Medium A: NH₄NO₃, 0.04%, MgSO₄*7H₂O, 0.01%, yeast extract, 0.02%, squalene, 0.03%. Medium B: Medium B was the same as A enriched with 0.1% yeast extract and 0.3% squalene. Medium C: Medium A without squalene was enriched with 0.3% yeast extract. Squalene was sterilized separately and mixed before inoculation.

Isolation of microorganisms from soil. Soil samples were incubated in medium A (3 ml in a test tube) on a reciprocal shaker for 3 days. After three successive transfers, the culture broths were extracted with dichloromethane and the reaction products were surveyed on TLC plates using an n-hexane-ether solvent system. Single colonies were isolated from the culture broths that accumulated degradation products by plate culture.

Hydration of squalene. Into medium B (100 ml) in a 500 ml conical flask seed culture of Corynebacterium sp. S-401 (1 ml) was inoculated. Cultivation was carried out on a rotary shaker at 30°C for 3 days. After the cultivation period, the pH of the broth was adjusted to 2~3 with 3N HCl solution and the broth was extracted 3 times each with 100 ml of dichloromethane. Dichloromethane extracts were combined and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pres-
sure at 40°C and the residual oil was purified by extraction with n-hexane and subsequent silica gel column chromatography using n-hexane-ether as the solvent system.

Preparation of resting cells of Corynebacterium sp. S-401. Corynebacterium sp. S-401 was cultured on medium C (200ml) in a 1 liter conical flask on a rotary shaker at 30°C for 2 days. Cells were collected by centrifugation at 15,000 x g for 10 min and washed 3 times with phosphate buffer (0.1 M, pH 7.0), and then suspended in 100 ml of the same phosphate buffer to a cell concentration of OD<sub>600</sub> = 10.

Hydration of oleic acid. To a cell suspension (100 ml) in a conical flask (500 ml), 5 g of oleic acid was added and the mixture was incubated on a rotary shaker at 30°C for 3 days. After adjustment of the pH of the reaction mixture to 3 with 3 N HCl, the reaction mixture was extracted with dichloromethane (100 ml) 3 times. Products were purified in the form of methyl esters.

Analytical method. Infrared adsorption spectra were recorded on a Hitachi Model 215 Grating IR Spectrometer. Mass spectra were obtained with a Hitachi RMU-6E and NMR spectra with a Hitachi R-24B (60 MHz) or JEOL Model PS 100 (100 MHz). Optical rotation was determined on a Hitachi Model DIP-181 Digital Polarimeter.

Chemicals. Squalene was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo) and used without further purification. Oleic acid was a product of Wako Chemical Ind. Ltd. Merck silica gel (type 60) was used for TLC and Merck silica gel 60 (70~230 mesh) was used for column chromatography.

RESULTS

Identification of Corynebacterium sp. S-401

The microbiological properties of strain S-401 are given in Table I. Morphological results were obtained from electronmicrographs using cells grown on bouillon broth and sampled at 24 hr intervals for one week. The cells showed neither a spherical shape nor chain forms. They did not form mycelia at any stage of their growth. DL-Diaminopimelic acid was detected on paper chromatography in cell wall hydrolysate of this bacterium.\(^3\)

The GC content was determined using the cells which were cultured in a medium containing 2% of glycine.\(^4\) The value of GC content was 69.8% which was calculated on T<sub>m</sub> 97.9°C with maximum hyperchromicity 1.29.

On the basis of these observations, this bacterium was identified as the genus Corynebacterium according to Bergey’s Manual, 7th and 8th editions.\(^5,6\)

Hydration products of squalene

Cultivation of Corynebacterium sp. S-401 with squalene (7.61 g) in 3 liters of medium B afforded 4.22 g of crude oil after dichloromethane extraction. The crude oil was dissolved in n-hexane and the resulting white precipitate was removed by centrifugation. After evaporation of the solvent, 3.94 g of an oily product was obtained. On silica gel TLC using n-hexane–ether (3:7), the oily product showed 6 spots. The most nonpolar spot was assigned as recovered squalene and the following five spots were tentatively named SqI through SqV according to their polarity from a higher Rf value.

The crude oily mixture (3.71 g) was placed on a silica gel column (200 g) and eluted stepwise with n-hexane–ether and ethyl acetate. Squalene (0.72 g, 12% recovery) was obtained by 2 liters n-hexane elution. Solvents for elution and yields of SqI–SqV were as follows: SqI, n-hexane–ether (95:5) 500 ml,
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0.96 g, 12% (conversion rate); SqII, n-hexane–ether (1:1) 250 ml, 0.73 g, 9%; SqIII, n-hexane–ether (1:1) 1.5 liters, 0.22 g, 3%; SqIV, ether 250 ml, 0.35 g, 4%; and SqV; ether 500 ml and then ethyl acetate 500 ml, 0.06 g, 0.7%.

Structure of SqI

The spectrometric properties and elemental analysis results of SqI were as follows. IR \( \nu_{\text{max}} \) cm\(^{-1} \): 3200–3500 (O–H). NMR (in CDCl\(_3\)) \( \delta \): 5.11 (5H, t, olefinic protons), 1.9–2.1 (19H, m, =CH–CH\(_2\)=), 1.59 (15H, s, (Z)-CH\(_3\)=C=), 1.41 (4H, m, CH\(_2\)), 1.19 (6H, s, CH\(_3\)=C–O). MS \( m/z \): 428 (M\(^+\)). Elemental analysis, found: C, 83.83; H, 12.19. Calcd. for C\(_{30}\)H\(_{52}\)O: C, 84.04; H, 12.23%.

The IR spectrum of SqI revealed that it has a hydroxy group as the sole functional group. Since NMR signals showed no \( \alpha \)-methine proton of the hydroxy group, this alcohol is assigned as tertiary which coincided with the result of acetylation reaction. Mild acetylation conditions such as acetic anhydride-pyridine at room temperature did not give acetate. The acetylation of SqI was performed with a 68% yield by heating it at 100°C with sodium acetate in acetic anhydride for 11 hr.

SqI acetate had the following spectrometric characteristics: IR \( \nu_{\text{max}} \) cm\(^{-1} \): 1735 (C=O), 1260. NMR (in CDCl\(_3\)) \( \delta \): 5.1 (4H, olefinic protons), 1.9–2.2 (16H, CH\(_2\)), 1.65 (1~2H, s, (E)-CH\(_3\)=C=), 1.60 (12H, s, (Z)-CH\(_3\)=C=), 1.3~1.5 (4H, m, CH\(_2\)), 1.2 (12H, s, CH\(_3\)=C–O). MS \( m/z \): 470 (M\(^+\)–H\(_2\)O).

The spectrum of SqII showed only a hydroxy group as a probable functional group. Comparison of NMR and mass spectra of SqII with those of SqI indicated that the structure of the main portion of SqII is 2,3,22,23-tetrahydrosqualene-2,23-diol and SqII contains some isomeric diols as minor components. All hydroxy groups are tertiary according to the NMR spectrum.

SqII was acetylated with acetylchloride in dichloromethane in the presence of pyridine at room temperature for 24 hr (94% yield). SqII acetate showed two spots on TLC (\( n \)-hexane–ether = 1:1, \( R_f \)=0.84 and 0.65). These two components were separated by column chromatography and the ratio of the major component (nonpolar) and the minor one (polar) was determined as 52:7. These two isomers showed almost the same MS pattern and the nonpolar major diacetate still possessed the signal of the (E)–CH\(_3\)= group (1/9 of olefinic methyl group) in its NMR spectrum.

In order to confirm the structure of the main
component of SqII (2,3,22,23-tetrahydro-squalene-2,23-diol), it was hydrogenated on Pd-C and the resulting saturated diol was dehydrated to give a diolefinic compound. The procedure was same as that for SqI (67% yield). The NMR spectrum of this diolefin showed two olefinic methyl groups in almost a 1:1 ratio; 1.69 (6H, s, (E)-CH$_3$-C=), and 1.59 (6H, s, (Z)-CH$_3$-C=). On the basis of these NMR data and the ratio of diacetate isomers, the content of the main terminal hydration product was estimated to be about 80% that of SqII.

Structure of SqIll, SqIV and SqV

Spectrometric properties of these three compounds were as follows. SqIll, IR $\nu_{max}$ cm$^{-1}$: 3450 (O-H). NMR (in CDCl$_3$+D$_2$O) $\delta$: 5.1 (3H, olefinic protons), 1.9~2.2 (12H, m, CH$_2$-C=), 1.69 (3H, s, (E)-CH$_3$-C=), 1.60 (9H, s, (Z)-CH$_3$-C=), 1.2 and 1.18 (about 12H, s, CH$_3$-C-O). MS $m/z$: 428 (M$^+$-2H$_2$O). $[\alpha]_D^{22} = +0.624$ (c=5.8, in EtOH). SqIV, IR $\nu_{max}$ cm$^{-1}$: 3450 (O-H). NMR (in CDCl$_3$+D$_2$O) $\delta$: 5.1 (2H, olefinic protons), 1.9~2.2 (8H, m, CH$_2$-C=), 1.69 and 1.60 (6H, s, (E)- and (Z)-CH$_3$-C=), 1.2 and 1.18 (about 12H, s, CH$_3$-C-O). MS $m/z$: 428 (M$^+$-3H$_2$O). $[\alpha]_D^{22} = +0.936$ (c=6.5 in EtOH). SqV, IR $\nu_{max}$ cm$^{-1}$: 3450 (O-H). NMR (in CDCl$_3$+CD$_3$COOD) $\delta$: 5.1 (1H, olefinic proton), 1.9~2.2 (4H, m, CH$_3$-C=), 1.59 (3H, s, (Z)-CH$_3$-C=). MS $m/z$: 428 (M$^+$-4H$_2$O). $[\alpha]_D^{22} = +0.197$ (c=7.3, in EtOH).

When these three sets of spectrometric data were compared, the number of olefinic protons, allylic methylene protons and olefinic methyl protons decreased and methylene protons and CH$_3$-C-OH protons increased parallel with the polarity on TLC plates. No $\alpha$-methylene proton of the hydroxy group was observed in all NMR spectra. All these alcohols are tertiary and the site of hydration seems to be random although terminal double bond hydrated products were rather predominant. The results are shown in Fig. 1.

Hydration of oleic acid

From the reaction mixture of 100 ml of cell suspension with 5 g of oleic acid 5.88 g of crude extract was obtained. The crude product was extracted with methanol to give 5.53 g of a crude oil which was esterified in 200 g of dry methanol-HCl (7%) at room temperature for 2 days. The resulting mixture of esters (6.4 g) was separated on a silica gel column (120 g) using dichloromethane and dichloromethane-methanol (20:1). The yields of the three main fractions were as follows: Methyl oleate, 3.92 g; nonpolar product (OL-I), 0.52 g; and polar product (OL-II), 1.25 g.

Structure of OL-I

OL-I formed crystals having a mp of 47°C. Spectrometric properties of OL-I were as follows. IR $\nu_{max}$ cm$^{-1}$: 1740 (CO-O), 1700 (C=O). NMR (in CDCl$_3$): 3.64 (3H, s, CH$_3$), 2.3 (6H, quartette, CH$_2$=C=O), 1.0~1.8 (24H, m, CH$_2$), 0.85 (3H, t; CH$_3$). MS $m/z$: 312 (M$^+$), 157, 156, 99. Elementary
analysis. Found: C, 72.85; H, 11.53. Calcd. for 
C_{19}H_{36}O_3: C, 73.03; H, 11.61%.

IR and NMR spectra indicated that OL-I is a keto-ester. The location of the carbonyl group was determined from the mass spectrum. The abundance of fragments 99, 156 and 157 suggested β-fission of the carbonyl group which was located at the C-10 position of the stearic acid skeleton. Thus the structure of OL-I was assigned as methyl 10-oxo-octadecanoate.

Structure of OL-II

OL-II formed crystals having a mp of 43°C. Spectrometric properties of OL-II were as follows. IR ν\text{max} cm⁻¹: 3450 (O–H), 1740 (CO–O). NMR (in CDCl₃) δ: 9.76 (1H, broad, O–H), 3.64 (3H, s, OCH₃), 3.6–3.65 (1H, m, H–C–O), 2.3 (2H, t, CH₂–C=O), 1.1–1.7 (28H, m, CH₂), 0.86 (3H, t, CH₃). MS m/z: 265 (M⁺–H₂O–OCH₃), 201. Elemental analysis, Found: C, 72.59; H, 12.23. Calcd. for C_{19}H_{38}O₃: C, 72.56; H, 12.18%. [α]_250 = -0.41 ± 0.02, [α]_365 = -0.58 (c = 4.11, MeOH).

Spectrometric data suggested that OL-II is a hydroxy fatty acid methyl ester and its mass spectrum was identical with that of methyl 10-hydroxyoctadecanoate. The values of optical rotation coincided with those of (-)-methyl 10R-hydroxyoctadecanoate.⁷,⁸

Optical purity of this ester is about ≥80% on the basis of the value [α]_236 = -0.51 and [α]_365 = -0.67 which were the highest values in Schroepfer’s publication.⁸¹

Consequently the cells of Corynebacterium sp. S-401 catalyzed stereospecific hydration of oleic acid to give (-)-10R-hydroxyoctadecanoic acid (IX) with a 22.4% yield and the subsequent oxidation product, 10-oxooctadecanoic acid (VIII), with a 9.1% yield, as shown in Fig. 2.

DISCUSSION

In a series of attempts to isolate soil microorganisms which have transforming activities for linear aliphatic compounds such as squalene and fatty acids, we obtained Corynebacterium sp. S-401 using squalene as carbon source in the screening process. Corynebacterium sp. S-401 hydrated squalene to form mono-, di-, tri-, tetra- and pentahydrated products when it was cultivated with squalene in a yeast extract rich medium. All hydroxy groups were tertiary and the main products were mono-(SqI) and dihydrated squalene (SqII). The site(s) of hydration in these two compounds are mainly at the terminal olefin group of the squalene molecule.

One of the most advantageous points of microbial transformation is the stereospecificity and another useful aspect is selective reaction of a special functional group among many in one molecule. So, these simple and selective hydration procedures suggest to us the potential of these lipophilic bacteria for organic synthesis and they are attractive also from the view point of degradation paths of branched olefinic hydrocarbons in nature.

When the resting cells of S-401 were supplied with oleic acid as an example of a naturally abundant olefinic compound, 10-oxooctadecanoic acid and (-)-10R-hydroxyoctadecanoic acid were obtained with 22.4 and 9.1% yields, respectively.

Wallen et al. reported the production of 10-hydroxyoctadecanoic acid from oleic acid with a 14% yield using Pseudomonas sp.⁹ The stereochemistry, substrate specificity and reaction mechanisms of this reaction were investigated by Schroepfer Jr. et al. using a crude enzyme preparation.⁷,⁸,¹⁰ The stereochemistry of the hydroxy acid produced by Corynebacterium sp. S-401 was the same as the Pseudomonas product. Although, Corynebacterium sp. and Pseudomonas sp. belong
to different Gram-stain groups, they cata-
lyze the same hydration reaction leading to
the same stereochemistry. This fact suggests
the probable common degradation path of
unsaturated fatty acids in nature which might
be initiated with hydration on a double bond.

The resting cells of *Corynebacterium* sp. S-
401 failed to catalyze hydration of oleic acid
amide, oleonitrile, oleyl alcohol, oleyl al-
dehyde or *cis*-9-octadecene. Accordingly the
carboxy group of oleic acid was seem to be
essential in this reaction. Cells of S-401 also
catalyzed a number of wax ester syntheses.
These various activities of S-401 indicate to us
a new aspect of lipophilic bacteria screened
with an unusual carbon source.

*Acknowledgment.* We wish to thank Dr. Tatsuji Seki,
Department of Fermentation Technology, Osaka
University, for kind suggestion about the GC content
determination.

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