APPLIED MICROBIOLOGY, Mar. 1975, p. 400-404
Vol. 29, No. 3
Copyright © 1975 American Society for Microbiology
Printed in U.S.A.

Oxidative Degradation of Squalene by *Arthrobacter* Species

YASUHIRO YAMADA, HIROBUMI MOTOI, SHINICHI KINOSHITA, NOBUO TAKADA, AND HIROSUKE OKADA*

Department of Fermentation Technology, Faculty of Engineering, Osaka University, Yamada-kami, Suita-shi, Osaka 565, Japan

Received for publication 11 October 1974

An organism isolated from soil and identified as *Arthrobacter* sp. was studied for its squalene degradation. The degradation product from squalene, which accumulated in the culture broth, was isolated and identified as trans-geranylacetone by mass spectrometry, gas chromatography, infrared spectrometry, and nuclear magnetic resonance spectrometry. Addition of a high concentration of K_2HPO_4 to the culture medium resulted in accumulation of fairly large amounts of carboxylic acids in addition to geranylacetone. These carboxylic acids were identified as isovaleric, β,β'-dimethylacrylic, geranic, and (+)-(R)-citronellic acids. Among these acids, α,β-saturated carboxylic acids were found to be predominant in quantity.

Squalene is a naturally abundant acyclic triterpene, formation of which is thought to occur by the tail-to-tail condensation of two farnesylpyrophosphate (C_{15}) molecules via presqualenepyrophosphate (8, 10), and is an important precursor of steroids and triterpenes. The mechanism of its cyclization to lanosterol via 2,3-oxidosqualene in mammalian livers has been reported by several workers (5, 13). Zander et al. (14) have reported another nonoxidative cyclization of squalene in *Tetrahymena pyriformis*. By this organism, squalene is cyclized to form tetrahymanol by a hydration reaction. Recently, 12,13-dehydrosqualene formation by *Staphylococcus aureus* (11, 12) and also by *Halobacterium cutirubrum* (9) has been reported, in which the molecule of squalene is dehydrogenated at its center of symmetry. In spite of these findings, no information on microbial degradation of squalene is available.

We now wish to report the isolation and identification of the products of degradation of squalene by *Arthrobacter* sp. This organism is an isolate from soil capable of utilizing squalene and belongs to the genus *Arthrobacter*.

MATERIALS AND METHODS

Microorganisms and culture conditions. A strain of *Arthrobacter* sp. isolated from soil was used throughout this study. A detailed description of the bacterium will be presented elsewhere. The soil samples were inoculated into test tubes, each containing 3 ml of the selection medium consisting of 1.5% squalene, 0.2% yeast extract, and 0.4% NH_4NO_3, and incubated at 27 C for 3 days on a reciprocating shaker. After extraction of the culture broth with dichloromethane and evaporation of the solvent, the products were analyzed by thin-layer chromatography, using *n*-hexane-ether (95:5) as the solvent system. Single colonies were isolated from the culture broths that accumulated products of squalene degradation, as evidenced by thin-layer chromatography.

A 0.5-ml portion of fresh seed culture was inoculated into 500-ml Sakaguchi flasks (a globular flask with flattened shoulder) containing 50 ml of medium. Medium A contained 2% corn-steep liquor and a specified amount of squalene and was adjusted to pH 6.9 with NaOH; medium B consisted of 2% glucose, 0.5% KNO_3, 0.02% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract, 0.1% KH_2PO_4, and 0.5% squalene and was adjusted to pH 6.5. Flasks were incubated at 30 C on a reciprocating shaker.

Assay procedures. Growth was monitored by absorbance at 550 nm after the removal of oily materials from the culture broth by extraction with 3 volumes of a solvent mixture (ethanol-butanol-chloroform, 10:10:1), centrifugation of the cells at 12,000 × *g* for 10 min, and resuspension in water.

The quantities of squalene and its degradation products were estimated by gas chromatography. Samples (50 ml) of culture broth were extracted three times with 30-ml portions of dichloromethane. The combined organic layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. The oily residue was weighed and subjected to gas chromatography. The content of geranylacetone was estimated from the peak height by comparison with that of a known amount of an authentic sample.

Analytical methods. Gas chromatography was performed on a Hitachi gas chromatography 063 instrument equipped with a thermal conductivity detector. Separations were performed on a stainless-steel column (100 by 0.3 cm) containing SE-30. Helium served as carrier gas at a flow rate of 21 ml/min. Temperatures of the column injection port and detector will be specified below. Infrared spectra were recorded on a Hitachi model 215 grating infrared spectrometer. Oily samples were run on neat liquid films between NaCl plates. The nuclear magnetic resonance (NMR) spectra in $CDCl_3$ were recorded with a JEOL model PS 100 spectrophotometer, with tetramethylsilane as the internal standard. Mass spectra were obtained with a Hitachi RMU-6E mass spectrometer.

Chemicals. Squalene was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo) and used without further purification. Geranylacetone used as the authentic sample was a gift from Takasago Perfumery Co. Ltd. Isovaleric acid was prepared from β,β'-dimethylacrylic acid by catalytic hydrogenation. Other chemicals were obtained from commercial sources.

RESULTS

Biotransformation of squalene by Arthrobacter sp.: identification of geranylacetone. Geranylacetone was isolated from the culture broth as follows. After 38 h of culture in medium A supplemented with 242 mg of squalene per flask, the culture broths were combined (500 ml) and extracted three times with 300-ml portions of dichloromethane. The extracts were combined and dried over anhydrous sodium sulfate. After evaporation of the solvent, 10 ml of *n*-hexane was added to the residue, and the resulting precipitate was removed by filtration. The oily residue (1.740 g) obtained after evaporation of the solvent contained 55% geranylacetone and 35% unchanged squalene when analyzed gas chromatographically at the following temperatures: column, 310 C; injection port, 340 C; detector, 340 C. The yield, calculated on the basis of two molecules of geranylacetone from one molecule of squalene, was 55.87%. Pure geranylacetone (0.917 g) was obtained by distillation of the oily residue under reduced pressure.

The sample thus obtained had a bp of 90 to 95 C at 7 mm of Hg. Elemental analysis showed: C, 80.21%; H, 12.03%; the calculated values for $C_{13}H_{22}O$ were: C, 80.35%; H, 11.41%. The infrared spectrum of the sample was almost identical to that of authentic geranylacetone (cis-trans mixture) and had values of $\nu_{max}{}^{film}$ at 1,720, 1,440, 1,380, 1,360, and 1,155/cm. The 1,720/cm band is attributed to the $>C{=}O$ group of the molecule. This observation was confirmed by mass spectral analysis (70 eV) where the parent ion was observed at *m/e* 194. Other peaks were observed at *m/e* 151, 136, 125, 107, 69, 43, and 41; *m/e* 151 was assigned as $M^+ - C_2H_3O$.

The NMR spectra of the compound in deuterated chloroform showed singlet signals at δppm of 1.59, 6H (cis-C_6—CH_3 and cis-C_{10}—CH_3); 1.69, 3H (trans-C_{10}—CH_3); and 2.11, 3H (CO—CH_3) and a triplet band at 5.09 (olefinic protons at C_5 and C_9). Evidence for the trans geometric isomerism of the C_5—C_6 double bond was that the NMR signal of two methyl groups at C_6 and C_{10} appeared at the same δ value, 1.59 (2).

Preparation of geranylacetone semicarbazone. To confirm the molecular formula, the semicarbazone derivative was prepared. After recrystallization from an ether-cyclohexane mixture, the semicarbazone had an mp of 94 to 95 C. Elemental analysis of the compound showed the following percent composition: C, 66.48; H, 10.44; N, 16.65. The calculated values for $C_{14}H_{25}N_3O$ were: C, 66.89; H, 10.03; N, 16.72.

The infrared spectrum of the sample was identical to that of the semicarbazone derived from authentic geranylacetone (cis-trans mixture) and had absorption bands at $\nu_{max}{}^{nujol}$ values of 3,540, 3,300, 1,690, and 1,580/cm. The main mass spectral peaks observed had *m/e* values of 251 (parental ion), 182, and 69.

On the basis of these data obtained from the isolated compound and its semicarbazone, the carbonyl compound produced from squalene by *Arthrobacter* sp. was identified as trans-geranylacetone.

Time course of the production of geranylacetone. Figure 1 shows a typical time course of growth and geranylacetone formation in medium B. After a long lag period (12 to 20 h), cell growth and geranylacetone accumulation started. Geranylacetone formation was found to be parallel with the cell mass increase in the logarithmic growth phase and continued after the logarithmic growth phase at a higher rate than growth. The pH value of the culture broth remained constant during the culture period. The yield of geranylacetone obtained to squalene added was 16% (wt/wt), which was much smaller than that obtained in medium A (39%).

Isolation and identification of carboxylic acids produced by Arthrobacter sp. Preliminary experiments had shown that several spots other than squalene and geranylacetone were observed when cells were cultured in medium A supplemented with 1% K_2HPO_4. For the

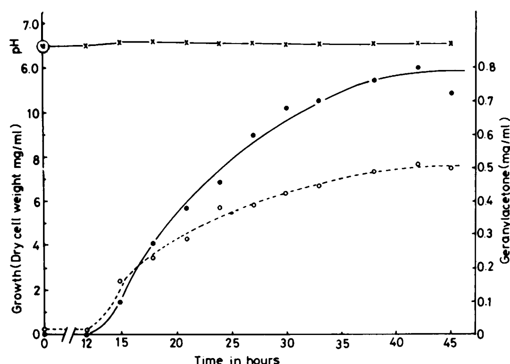

FIG. 1. *Time course of the production of geranylacetone by Arthrobacter sp. Symbols:* ×, *pH;* ●, *geranylacetone;* O, *growth.*

isolation and identification of these products, the following experiments were carried out.

Twelve flasks, each containing 75 ml of medium A supplemented with 1% K_2HPO_4 and 0.65 g of squalene, were inoculated and incubated for 65 h. After acidification of the combined broths to pH 2 with 3 N HCl, the oil fraction was prepared according to the procedure described previously. Yield was 5.21 g. The oil was dissolved in dichloromethane and extracted with 10% sodium carbonate solution. The alkaline solution was acidified with 3 N HCl and extracted with dichloromethane. After drying over anhydrous sodium sulfate, the solvent was evaporated to give carboxylic acids in a yield of 0.54 g. The yield of neutral compounds that were not extracted with sodium carbonate solution was 4.3 g. The gas chromatogram of this neutral moiety showed that it contained 44% geranylacetone and 50% unchanged squalene.

The carboxylic acids thus obtained were separated into five peaks by gas chromatography (temperatures: injection port, 250 C; column, 170 C; detector, 260 C). These peaks were designated A, B, C, D, and E in the order of retention time (Fig. 2). Fractions corresponding to peaks A plus B, C plus D, and peak E were collected at the detector outlet of the gas chromatograph instrument and used for identification experiments.

Identification of isovaleric and β,β'-dimethylacrylic acids. Infrared spectra of the mixture of acids A and B in film showed $\nu_{c=c}$ absorption at 1,640/cm. When the mixture was hydrogenated on palladium-charcoal (5%) in methanol under atmospheric pressure, a pure carboxylic acid, which was identical with peak A carboxylic acid by gas chromatography, was obtained. The infrared spectra of the carboxylic acid obtained by hydrogenation of the mixture coincided perfectly with that of isovaleric acid. This suggests that the carboxylic acid of peak B has a skeleton of isovaleric acid. Mass spectra of the A-B mixture showed two parental molecular ions at *m/e* of 102 and 100, which correspond to isovaleric acid (A) and carboxylic acid (B), respectively. The NMR spectra in $CDCl_3$ of the A-B mixture showed signals of $(CH_3)_2$—CH— methyl protons at δ 0.96 (doublet), CH_3—C= methyl protons at δ 1.90 and 2.15 (singlet), and an olefinic proton at δ 5.62 ppm. From these data carboxylic acid B was identified as β,β'-dimethylacrylic acid. The ratio of the quantities of isovaleric and β,β'-dimethylacrylic acids estimated from the NMR spectra was 3:1.

Identification of citronellic and geranic acids. The mass spectra of the C—D mixture showed two parental molecular ions at *m/e* 170 and 168. The analysis of other fragments indicated that these carboxylic acids were citronellic acid (C) (M^+, *m/e* 170) and geranic acid (D) (M^+, *m/e* 168). The NMR spectra of the C—D mixture in $CDCl_3$ showed signals at a δppm value of 1.61 (cis CH_3—C=, singlet), 1.69 (trans CH_3—C=, singlet), 5.05 (olefinic proton, triplet), 5.65 (olefinic proton, singlet), and 2.12 (>CH—CH_3, doublet). The infrared spectra of the mixture showed absorption bands at a ν_{max}^{film} value of 1,705 (C=O) and 1,640/cm

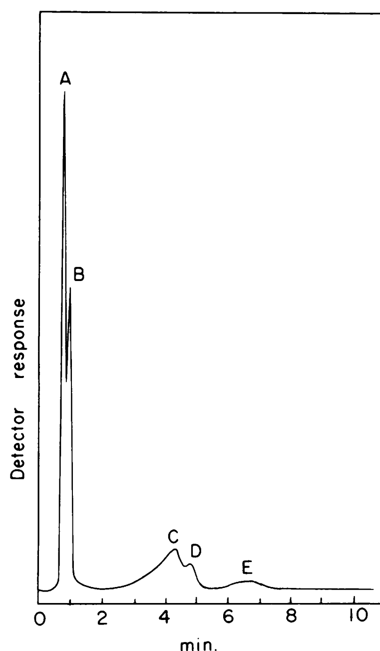

FIG. 2. *Gas chromatogram of carboxylic acids on SE-30 column (100 by 3 mm) column temperature, 170 C.*

(C=C). These data support the assignment stated above.

To confirm the molecular structures of acids C and D, isolation of these two acids in the form of their methyl esters was performed. A mixture of carboxylic acids (A, B, C, D, etc.) (320 mg) was methylated with diazomethane in ether. From the reaction mixture, 214 mg of methyl esters was obtained by the standard method. The methyl esters of acids C and D were separated by gas chromatography (temperatures: injection port, 220 C; column, 150 C; detector, 215 C) (Fig. 3). Peaks C′ and D′ correspond to carboxylic acids C and D, respectively. Peak E′ was a mixture of carboxylic acid esters which were not identified. Fractions corresponding to peaks C′ and D′ were collected separately and used for identification experiments.

Identification of methyl (+)-R-citronellate. The infrared absorption spectra of the methyl ester C′ in film showed an ester carbonyl group absorption band at 1,740/cm and no strong absorption in the region of 1,600 to 1,700/cm, which indicated that the ester C′ was an α,β-saturated carboxylic acid ester. The mass spectra of the ester C′ showed a peak of parental molecular ion M^+ at *m/e* 184 and of other fragments at 152 *m/e* 152 ($M^+ - CH_3OH$), 110, 95, 82, 74, 69, 55, and 41. The NMR spectra of the ester C′ in $CDCl_3$ showed signals at δppm values of 0.98, 3H (CH—CH_3, doublet), 1.60, 3H (cis-CH_3—C=, singlet), 1.69, 3H (trans-CH_3—C=, singlet), 5.07, 1H (=C—H, triplet), and 3.61 (CH_3—O—). From these data, the ester C′ was assigned as methyl citronellate.

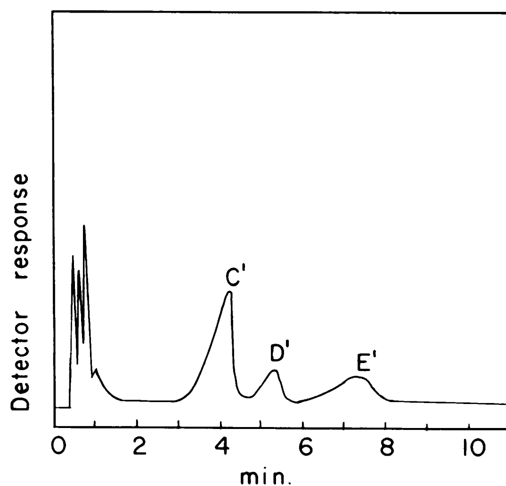

FIG. 3. *Gas chromatogram of methyl esters on SE-30 column (100 by 3 mm); column temperature, 150 C.*

This ester showed a plain optical rotatory dispersion curve in the region of 250 to 600 nm. The optical rotation values obtained were $[\alpha]_{300}^{25} = +204°$, $[\alpha]_{350}^{25} = +91°$, and $[\alpha]_{400}^{25} = +68°$ (C = 0.0044 in methanol). This suggested that the ester is methyl (+)—R—citronellate (6, 7).

Identification of methyl geranate. The infrared absorption spectra of the methyl ester D′ in film showed an ester carbonyl group at 1,720/cm and a strong absorption band of C=C conjugated with a carbonyl group at 1,645/cm. The mass spectra of the ester D′ showed a peak of parental molecular ion at *m/e* 182 and other fragments at 151 ($M^+ - CH_3O$), 123, 113, 83, 69, 55, and 41. These spectral data suggested that the ester D′ is methyl geranate.

DISCUSSION

Arthrobacter sp. which was isolated from soil can oxidatively decompose squalene (C_{30}) (I) into geranylacetone (C_{13}) (II). The yield of geranylacetone to squalene consumed was 56% on the basis that one molecule of squalene gives two molecules of geranylacetone. This fact suggests that squalene molecules in part were cleaved at the two sites shown by wavy lines in the structural formula in Fig. 4. In mammalian liver, squalene is first oxidized at its terminal double bond to give squalene-2,3-oxide (5, 8), which is then cyclized. In the case of *Arthrobacter* sp. the central part of squalene is attacked, and fission occurs symmetrically at $C_{10}=C_{11}$ and $C_{14}=C_{15}$, although the cleavage mechanism has not been elucidated. Another central attack of the squalene molecule to form 12,13-dehydrosqualene by *S. aureus* and by *H. cutirubrum* has been suggested (12, 14).

When medium A was supplemented with a high concentration of dipotassium phosphate, squalene was oxidized to carboxylic acids in addition to geranylacetone. The carboxylic acids comprised up to 10% of the total recovered

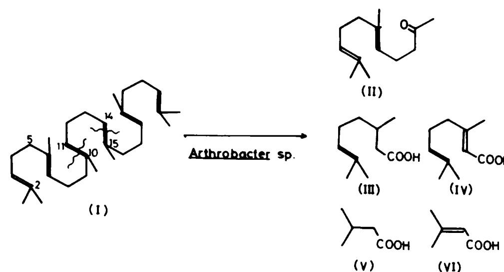

FIG. 4. *Main products of oxidative degradation of squalene by Arthrobacter sp.*

oil. The main carboxylic acids were identified as isovaleric acid (V), β,β'-dimethylacrylic acid (VI), geranic acid (IV), and (+)—(R)—citronellic acid (III). Though several efforts to oxidize geranylacetone using the intact cells were not successful, it is probable that these carboxylic acids are derived from squalene by a "central attack" mechanism which is common to geranylacetone formation. The fact that all acids accumulated have beta-substituted methyl groups might suggest that this configuration is resistant to biological oxidation by this organism. The α,β-saturated form was found to be predominant for both C_5 and C_{10} acids.

The oxidative cleavage of squalene by *Arthrobacter* sp. is a useful method of obtaining pure trans-geranylacetone. The supposed intermediate of the oxidative degradation of squalene by this organism, which retains a C_{30} skeleton, probably has functional groups such as hydroxyl groups in its central position. As has been suggested by others (1, 3, 4), flexible linear molecules, such as squalene, which have no specific groups could hardly be the object of selective chemical reactions, especially in central parts of the molecule. Thus, the application of this microorganism to introduce functional groups into central parts of the squalene molecule is interesting from the viewpoint of the synthesis of squalene derivatives.

ACKNOWLEDGMENTS

We thank Sumitomo Chemical Co. Ltd. for optical rotatory dispersion analysis and Takasago Perfumery Co. Ltd. for supplying authentic geranylacetone.

LITERATURE CITED

1. Baldwin, J. E., A. K. Bhatnagar, and R. W. Haper. 1970. Site-specific dehydrogenation of ring D in steroids. Chem. Commun. **1970**:659–661.
2. Bates, R. B., R. H. Caringham, R. P. Rakutis, and J. H. Schauble. 1962. Stereochemistry of terpenoid double bonds of type $—CH_2C(CH_3)=CHCH_2—X$. Chem. Ind. **1962**:1020–1021.
3. Breslow, R., and S. W. Baldwin. 1970. Conversion of chorestanol to 12-ketocholestanol and to Δ^{14}- and $\Delta^{8(14)}$-choresterol by remote oxidation. J. Am. Chem. Soc. **92**:732–734.
4. Breslow, R., and M. A. Winnik. 1969. Remote oxidation of unactivated methylene groups. J. Am. Chem. Soc. **91**:3083–3084.
5. Dean, P. D. G., P. R. Ortiz de Montellano, K. Bloch, and E. J. Corey. 1967. A soluble 2,3-oxidosqualene sterol cyclase. J. Biol. Chem. **242**:3014–3019.
6. Djerassi, C., and G. W. Krakower. 1959. Optical rotatory studies. XXI. Effect of ring size. J. Am. Chem. Soc. **81**:237–284.
7. Eisnebraun, E. J., and S. M. McElvain. 1955. The configurations of the 3-methylcyclopentanones and related compounds. J. Am. Chem. Soc. **77**:3383–3384.
8. Epstein, W. W., and H. C. Rilling. 1970. Studies on the mechanism of squalene biosynthesis. The structure of presqualene pyrophosphate. J. Biol. Chem. **245**:4597–4605.
9. Kushwaka, S. C., E. L. Pugh, J. K. G. Kramer, and M. Kates. 1972. Isolation and identification of dehydrosqualene and C_{40}-carotenoid pigments in *Harobacterium cutirubrum*. Biochim. Biophys. Acta **260**:492–506.
10. Rilling, H. C., and W. W. Epstein. 1969. Studies on the mechanism of squalene biosynthesis. Presqualene, a pyrophosphated precursor of squalene. J. Am. Chem. Soc. **91**:1041–1042.
11. Suzue, G., K. Tsukada, and S. Tanaka. 1967. A new triterpenoid from a mutant of *Staphylococcus aureus*. Biochim. Biophys. Acta **144**:186–188.
12. Suzue, G., K. Tsukada, and S. Tanaka. 1968. Occurrence of dehydrosqualene (C_{30} phytoene) in *Staphylococcus aureus*. Biochim. Biophys. Acta **164**:88–93.
13. Willet, J. D., K. B. Sharpless, K. E. Lord, E. E. van Tamelen, and P. B. Clayton. 1967. Squalene-2,3-oxide, an intermediate in the enzymatic conversion of squalene to lanosterol and cholesterol. J. Biol. Chem. **242**:4182–4191.
14. Zander, J. M., J. B. Greig, and E. Caspi. 1970. Tetrahymanol biosynthesis. Studies in vitro on squalene cyclization. J. Biol. Chem. **245**:1247–1254.