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Cyclization of 2,3-Oxidosqualene with Microsomal Fraction of *Cephalosporium caerulens*[1]

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It was demonstrated that the precipitate at 105000×*g* of cell-free extracts of *Cephalosporium caerulens* exhibited the activities for cyclizing 2,3-oxidosqualene into lanosterol and 3β-hydroxyprotosta-17(20) [16,21-*cis*], 24-diene. However, the cell-free preparations possessed no activity for cyclizing into 3β-hydroxyprotosta-13(17), 24-diene. Major part of these activities were located in the microsomal fraction which precipitates at 105000×*g* and deoxycholate treatment solubilized these cyclases.

The recent rapid progress in the studies on cyclization of 2,3-oxidosqualene (I) prompted us to investigate the biosynthesis of 3β-hydroxyprotosta-17(20)[16,21:*cis*],24-diene (II). This tetracyclic triterpene alcohol is an important intermediate in biosyntheses of antibiotics, fusidic acid (III),[3] helvolic acid (IV),[4] cephalospolin P_1 (V)[5] and viridominic acid C (VI),[6] and possesses a unique framework of prototype sterol.[7]

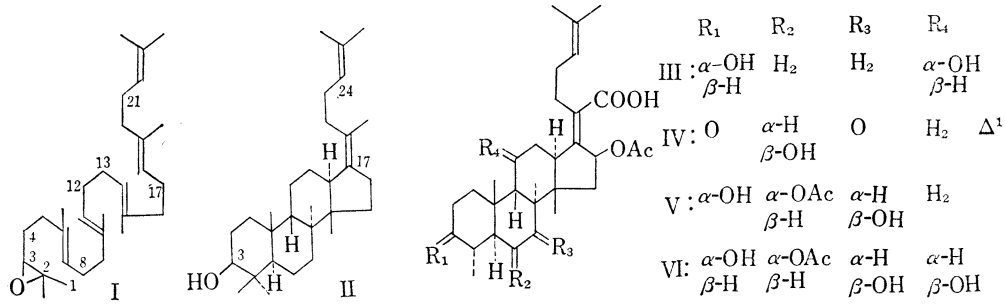

Chart 1

According to the present view, the cyclization of I in biosynthesis of sterol first leads to the cation (VIIa)[8] or its stabilized equivalent (VIIb)[9] which is subsequently converted into a final product, lanosterol (VIII) or cycloartenol (IX), by concerted 1,2-hydrogen and methyl migrations and terminating in the loss of a proton from C-9 or C-19. In contrast

1) Presented at the 43rd Annual Meeting of the Japanese Biochemical Society, Oct, 10, 1970, Tokyo.
2) Location: *1-1-1, Yayoi, Bunkyo-ku, Tokyo.*
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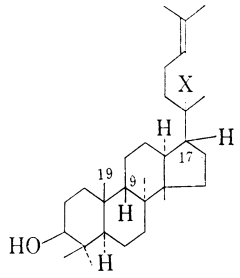
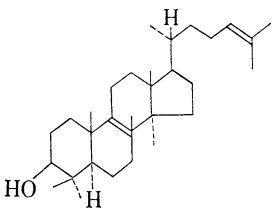
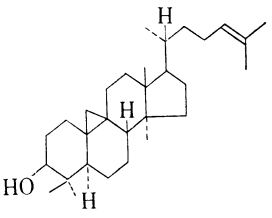


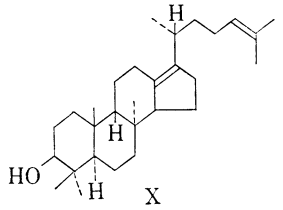
VIIa : X=+
VIIb : X=nucleophile



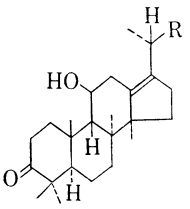
VIII

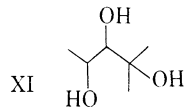


IX



X





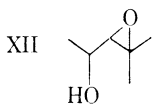


Chart 2

with this, the framework of II is considered to be formed by direct stabilization of the cation (VIIa) or its equivalent (VIIb), *viz.* by elimination of a proton from C-17 followed by formation of a double bond between C-17 and C-20.

Bloch and his co-workers recently purified 2,3-oxidosqualene-lanosterol cyclase from hog liver microsomes[10] and clarified the comparative properties of the corresponding cyclase from soluble fraction of yeast cell homogenates.[11] Furthermore, the substrate specificity of liver cyclase was extensively determined by two research groups of E.E. van Tamelen[12] and of E.J. Corey,[13] to give rise to some fruitful informations concerning cyclization mechanism and an involved intermediate such as VII.

On the other hand, van Tamelen, *et al.*[14] and Caspi, *et al.*[15] respectively investigated biosynthesis of fusidic acid (III) in growing culture of *Fusidium coccineum* utilizing (4,8,12,13, 17,21-^{3}H)-I and (3RS,4R)-(2-^{14}C,4-^{3}H)-mevalonic acid (MVA), in order to provide other direct evidence for the role of VII as an intermediate in cyclization. The data, thus obtained, of incorporation of labeled precursor into III and its chemical degradation were consistent with postulate that a prototype sterol (II) is formed by direct elimination of a proton from C-17 of VII. Previously we reported that cell-free extracts of *Emericellopsis* and *Cephalosporium* species converted (2-^{14}C)-MVA into VIII and II.[16] In this paper, transformation of I to VIII and II by microsomal fraction of *Cephalosporium caerulens* was described.

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Experimental

Materials——3RS-(2-^{14}C)-Mevalonolactone was obtained from the Radiochemical Center (Amersham, England). Squalene and lanosterol (VIII) were purchased from Tokyo Kasei Kogyo Co., Ltd. Squalene was purified before use by chromatography on silica gel column followed by thiourea clathrate formation[17] to give one peak on gas–liquid chromatography with a column of OV-17. Pure VIII (mp 140—141°) was obtained by purification of the acetate dibromide and debromination and saponification as described by Johnston, *et al.*[18] 24, 25-Dihydrolanosterol (mp 146—148°)[19] was prepared by catalytic hydrogenation of VIII. 3β-Hydroxyprotosta-17(20)[16,21: *cis*],24-diene (II, mp 117—117.5°)[6] and 3β-hydroxyprotosta-13(17),24-diene (X, mp 104—105°)[7] were isolated from a nonsaponifiable lipid fraction of mycelia of *C. caerulens* and purified by column chromatography on 10% $AgNO_3$–silica gel column followed by repeated recrystallizations from CH_3OH. (^{14}C)-Squalene was synthesized by an incubation of (^{14}C)-mevalonolactone with 15000×***g*** supernatant of yeast cell homogenates as described previously,[20] and purified by silica gel column chromatography. ^{14}C-Labeled I was chemically synthesized from (^{14}C)-squalene according to the procedure of Willett, *et al.*[21]

Gas–Liquid Chromatography (GLC)——GLC was carried out by means of Shimadzu GC-4A gas chromatograph with all glass column (1 m×4 mm inner diameter) and injection system, equipped with flame ionization detector. Column packed with 1.5% OV-17 on silanized Chromosorb W (60—80 mesh) was operated at 230° with N_2 pressure of 1.6 kg/cm^2 and inlet temperature of 250° and detector temperature of 250°.

Thin–Layer Chromatography (TLC)——TLC was carried out on Silica gel G plates (0.25 mm layer thickness, 10% calcium sulphate binder). The following solvent systems were used: *n*-hexane–ethyl acetate (3: 1) for isolation of triterpene alcohol fraction, or *n*-hexane–ethyl acetate (20: 1) for separation of squalene and I. Nonradioactive reference standards were detected by exposure to iodine vapor.

Radioactivity Measurements——Scanning of TLC plates was done with a Packard radiochromatogram scanner model 7201. Radioactivities of samples were measured in a Packard liquid scintillation spectrometer model 2003 in a sintillation fluid containing 2,5-diphenyloxazol (4 g) and 1,4-bis[2-(5-phenyloxazoyl)]-benzene per liter of toluene. The radioactivities of materials absorbed on TLC plate were determined by counting appropriate portion of scraped silica gel suspended in scintillation fluid.

Growth of *C. carulens* and Preparation of Cell-Free Extracts——Stock culture of *C. caerulens* was maintained on potato dextrose agar. Liquid growth medium (1 liter) consisted of glucose (50 g), corn steep liquor (30 g), KH_2PO_4 (2 g), KCl (0.5 g), $MgSO_4$ (0.2 g), $ZnSO_4$ (0.2 g), $CaCO_3$ (2 g), and pH was adjusted at 7.0 with KOH. Growth of this fungus was permitted to proceed at 37° for 120 hr with shaking (reciprocal shaker, 120 rpm). The mycelia were harvested by centrifugation and washed several times with 50 mM Tris-HCl buffer (pH 7.4) containing 0.25M sucrose and 1 mM of EDTA. The yield of mycelia was 6—7 g wet weight per 100 ml. All subsequent steps were carried out at 4° unless stated otherwise. The washed mycelia (6 g) were suspended in 6 ml of the buffer and the suspension was homogenized in a Braun homogenizer for 2 min with 0.5 mm glass beads. The homogenates thus obtained were centrifuged at 800×***g*** for 5 min to spin down the nuclei and cell debris, then at 15000×***g*** for 10 min to spin down mitocondria and finally at 105000×***g*** for 1 hr to sediment the remaining particulate matter. Protein concentration was determined by the method of Lowry, *et al.*[22]

Incubation of (1,5,9,16,20,24-^{14}C)-I——The reaction mixture contained the following components in a final volume of 1.0 ml: Tris-HCl buffer, pH 7.4, 100 μmole; Tween 80, 1.0 mg; ^{14}C-I, 1 μmole, 1.0×10^6 dpm; enzyme solution (5.0 mg protein). The reaction was carried out at 37° for 1 hr and terminated by addition of 10% KOH in CH_3OH (w/v). After standing at 70° for 30 min under an atmosphere of N_2, the reaction mixture was repeatedly extracted with ether. The ether extracts were combined, dried over Na_2SO_4 and evaporated to dryness under a stream of N_2. In order to separate ^{14}C-I and triterpene alcohol fraction, the residue was applied to silica gel column (2 g of Wakogel C-200, 8 cm×8 mm column). ^{14}C-I was eluted with 20 ml of *n*-hexane–benzene (1: 1) and triterpene alcohol fraction with 20 ml of benzene. The triterpene alcohol fraction was further chromatographed on thin-layer plate, if necessary, to obtain lanosterol fraction. Assay of this lanosterol fraction was carried out by co-crystallization with authentic specimens.

Incubation of Nonlabeled I——The reaction mixture contained the following components in a final volume of 5.0 ml: Tris-HCl buffer, pH 7.4, 500 μmole; Tween 80, 5 mg; I, 10 μmole; enzyme solution (25 mg protein). The isolation of triterpene alcohol fraction was the same procedure as described above. Assay of lanosterol fraction was made by GLC. Calibration curves for authentic samples, II, VIII, IX and 24,25-

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dihydrolanosterol, were made. An appropriate amount of 24,25-dihydrolanosterol was added to each sample of biosynthesized lanosterol fraction as an internal standard for calibration.

Co-Crystallization with Authentic Triterpene Alcohols——Triterpene alcohol fraction was divided into three equal portions to which each 10 mg of authentic triterpene alcohols were added. The mixture were dissolved in 0.2 ml of dry pyridine, added 0.1 ml of benzoylchloride and then allowed to stand over night at room temperature. To the reaction mixture ice-H_2O was added to decompose excess of benzoylchloride and precipitated benzoate was extracted with three 5 ml quantities of ether. The combined extracts were washed with 3% HCl, $NaHCO_3$ saturated solution and H_2O. After the extracts were dried over Na_2SO_4, the solvent was removed under a stream of N_2. The benzoates thus obtained were recrystallized to constant specific radioactivity from EtOH.

Treatment of Microsomal Fraction with Sodium Deoxycholate (DOC)——Microsomal fraction obtained above were suspended in 50 mM Tris-HCl buffer and the suspension was incubated for 10 min at 30° with 0.5 mg of DOC per mg of protein. The incubated sample was passed through a column of Sephadex G-25 and followed by centrifugation at $105000 \times g$ for 1 hr.

Result and Discussion

Previously, the biosyntheses of lanosterol (VIII) and 3β-hydroxyprotosta-17(20)[16,21-*cis*],24-diene (II) from MVA was demonstrated by the supernatant at $10000 \times g$ of *Emericellopsis salmosynnemata* Grosklags et Swift (IFO 8517). Based from this preliminary result, we first examined cyclization of 2,3-oxidosqualene (I) with the supernatant at $15000 \times g$ of this fungus. Contrary to our expectation, quantitative GLC analysis of sterol fraction of reaction mixture clarified that this supernatant possessed a negligible cyclization activity. Then *Emericellopsis synnematicola* Mathur et Thirumalachar (IFO 8519), *E. humicola* (Cain) Gilman (IFO 8518), *E. microspora* Bachus et Orpurt (NHL 2417) and *C. caerulens* were tested. The former three *Emericellopsis* species show the high conversion yields of MVA into nonsaponifiable lipid fraction[16] and the last one is known to produce helvolic acid (IV) in the highest yield.

Table I. Distribution of I-Cyclizing Activities in Subcellular Fractions

Fraction		VIII formed (mμmole)	II formed (mμmole)
$15000 \times g$	ppt	1	0
	sup	117	85
	heated sup	5	2
$105000 \times g$	ppt	148	127
	sup	7	2
	ppt+sup	190	106

Among the four microorganisms tested, only the supernatant at $15000 \times g$ of *C. caerulens* converted I into VIII and II in approximately 1.2% and 0.85% yields, respectively, which were calculated by quantitative GLC analysis of sterol fraction (*cf.* Table I). This supernatant was further centrifuged at $105000 \times g$ to spin down the particulate matter corresponding to a microsomal fraction of liver homogenates from which 2,3-oxidosqualene-lanosterol cyclase was successfully solubilized.[10] Cyclization of I was first examined both with the microsomal fraction and with the supernatant. Quantitative GLC assay of conversion products revealed that cyclization of I catalized by the microsomal fraction but not by the supernatant as shown in Fig. 1 and Table I.

To determine the structures of cyclization products and their conversion yields, incubation of ^{14}C-labeled I with the microsomal fraction was carried out and sterol fraction was isolated by column chromatography. As seen in Fig. 2, radioactivity was mainly located at the position corresponding to lanosterol fraction. This sterol fraction was assayed according to dilution method. Three recrystallizations of respective specimens afforded constant spe-

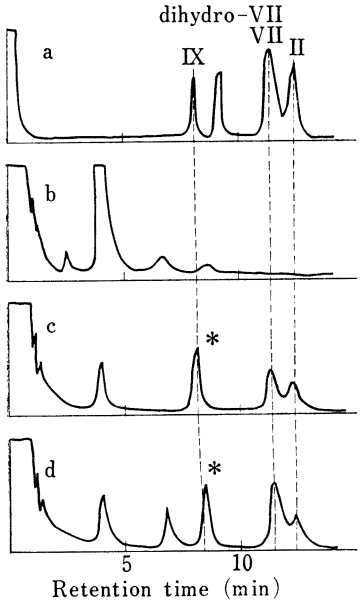

Fig. 1. Gas–Liquid Chromatogram of the Products synthesized from I

a) mixture of standard samples, II, VII, dihydro-VII and IX
b) sterol fraction synthesized from I with a supernatant at 105000×*g*
c) sterol fraction synthesized from I with a precipitate at 105000×*g*
d) sterol fraction synthesized from I with a superantant and precipitate at 105000×*g*

* t_R of this peak is quite similar to that of IX. However the incorporation experiment of ^{14}C-I clarified that cyclization of I to IX did not occur at all.

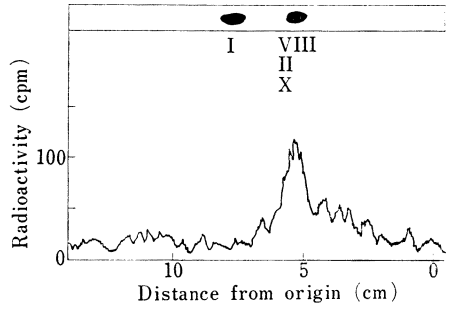

Fig. 2. Thin–Layer Chromatogram of Sterol Fraction Derived from ^{14}C-I

cific activities in the case of VIII-benzoate (505 dpm/mg) and II-benzoate (310 dpm/mg) and a negligible value in that of 3β-hydroxyprotosta-13(17),24-diene-(X)-benzoate as seen in Table II. Conversion yields into VIII and II were 3.0% and 1.9%, respectively.

X is isolated in the amount larger than that of II from a nonsaponifiable lipid fraction of mycelia of this fungus.[7] Despite of this fact the microsomal fraction could not show any ability for synthesizing X. The similar experiments mentioned above were carried out under addition of II or X to the reaction mixture, to determine whether or not cyclization path might be affected under these conditions. As seen from the results summarized in Table III and IV, any significant changes were not observed in comparison with the data from the experiment without addition of II or X.

The suspension of microsomal fraction was treated with DOC, passed through Sephadex G-25 and then again centrifuged at 105000×*g* to separate into precipitate and supernatant. As seen in Table V the supernatant thus obtained showed the activity almost the same as that of a precipitate untreated with DOC but the precipitate after DOC treatment lost a major part of the activity. This preliminary result shows that 2,3-oxidosqualene cyclases of *C. caerulens* might be solubilized from the microsomal fraction by DOC treatment.

The cyclase activity from this fungus was very weak as compared with those of liver and yeast homogenates and this caused great difficulty to carry out further purification of enzymes and separation of two cyclase activities. To solve this difficulty several attempts

Table II. Identification of ^{14}C-Labeled Cyclization Products by Recrystallization with Authentic Substance

Substance	Specific radioactivity after recrystallization (dpm/mg)		
	1	2	3
VIII-Benzoate	640	506	505
II-Benzoate	394	313	310
X-Benzoate	86	0	0

Table III. Determination of Specific Activities of ^{14}C-Labeled Cyclization Products, obtained under Addition of II,[a] by Recrystallization with Authentic Substance

Substance	Specific radioactivity after recrystallization (dpm/mg)		
	1	2	3
VIII-benzoate	709	620	618
II-benzoate	590	530	531
X-benzoate	56	0	0

a) 2 mg of II were dispersed in the reaction mixture before addition of enzyme preparation.

Table IV. Determination of Specific Activities of ^{14}C-Labeled Cyclization Products, Obtained under Addition of X,[a] by Recrystallization with Authentic Substance

Substance	Specific radioactivity after recrystallization (dpm/mg)		
	1	2	3
VIII-benzoate	741	625	630
II-benzoate	575	542	535
X-benzoate	81	10	0

a) 2 mg of X were dispersed in the reaction mixture before addition of enzyme preparation.

Table V. Distribution of I-Cyclases in Microsomal Subfractions Obtained by DOC Treatment[a]

Fraction	VIII formed (mμmole)	II formed (mμmole)
$105000 \times g$ ppt	150	132
Subfraction after DOC treatment clear sup	109	98
ppt	19	14

a) Cyclase activties were assayed by quantitative GLC.

such as utilization of mycelia at various growing stages, changes of destructing procedure of mycelia or screening of more suitable strain using techniques for mutation, failed to provide any improvement. Thus our first planning of isolation of 2,3-oxidosqualene-3β-hydroxy-protosta-17(20)[16,21:*cis*],24-diene cyclase was compelled to stop until more effective and stable enzyme system became available.

However the following facts observed in the present investigation are noteworthy.

1) Cell-free extracts for cyclization activity of I was krst prepared from mycelia of fungus. In the case of *C. caerulens*, the microsomal fraction possesses activities for cyclizing I into both VIII and II.

2) Liver lanosterol cyclase and phytoflagellate cycloartenol cyclase are located mainly in the microsomal fraction but in yeast, lanosterol cyclase is found in the soluble fraction. In *C. caerulens*, major part of cyclase activity are located in the microsomal fraction.

3) DOC treatment solubilized these cyclases of C. *caerulens*.

4) X, another prototype sterol possessing a framework of Alisol A (XI) and B (XII),[23] is isolated from mycelia of *C. caerulens* in the amount larger than that of II. Contrary to this fact, X is not synthesized in incubation of I with the cyclase preparations of this fungus.

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