CARBON-CARBON BOND CLEAVAGE OF FUCOSTEROL-24,28-OXIDE BY CELL-FREE EXTRACTS OF SILKWORM BOMBYX MORI*

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SUMMARY The 1500 X g supernatant of the silkworm Bombyx mori gut homogenate catalyzed the conversion of 24,28-epoxystigmast-5-en-3β-ol(III) to cholesta-5,24-dien-3β-ol(IV) which is a key step of stigmast-5-en-3β-ol(I) dealkylation in the insects. A structural analog 24,28-imino-stigmast-5-en-3β-ol(VI) was a potent inhibitor of this conversion.

Phytophagous insects which are uncapable of de novo biosynthesis of sterol, obtain its necessary cholesterol(V) through dealkylation of phytosterol derived from exogenous source. The major route of dealkylation of sitosterol(I) has been proposed (1) as : sitosterol(I)→fucosterol-24,28-oxide(III)→desmosterol(IV)→cholesterol(V). A key step of this sequence is carbon-carbon bond cleavage of the epoxide(III) and various evidences have been accumulated to support the intermediency of III during dealkylation of sitosterol(I) (1 - 5). A probable mechanism of this reaction involving migration of C-25-hydrogen of the epoxide(III) to C-24 during its conversion to desmosterol(IV) has been presented in the previous papers (4,5). In order to elucidate a more precise mechanism of the dealkylation, we have developed a cell-free system catalyzing transformation of the epoxide(III) into desmosterol(IV), which will be described in this report.

Experimental Procedure

Synthesis of [3α-^{3}H]-fucosterol-24,28-oxide(III) Fucosterol-24,28-oxide

*This is Part 22 in the series of "Studies on Steroids". For Part 21 see ref. 10.

Abbreviations : sitosterol = stigmast-5-en-3β-ol; fucosterol = stigmasta-5,(E)-24(28)-dien-3β-ol; fucosterol-24,28-oxide = 24,28-epoxystigmast-5-en-3β-ol; desmosterol = cholesta-5,24-dien-3β-ol.

(III)(3 mg) (6)† was oxidized with chromic oxide(5 mg)-pyridine(8 µl) complex in methylene dichloride(0.3 ml) (7), at room temperature for 15 min. Although thin layer chromatography(Merck, precoated plate, 0.25 mm thickness) of the product showed the presence of remaining starting material, this was used as such for the next step. Ultraviolet analysis assured the absence of 3-oxo-4-ene compound which would be otherwise formed by a facile isomerization of 3-oxo-5-ene. The crude products was dissolved in isopropanol(50 µl) and added to a solution of [^{3}H]-sodium borohydride(ca. 8 mCi)(New England Nuclear, 7 Ci/mM) in isopropanol(30 µl). After allowing to stand at room temperature for 1 hr, a cold sodium borohydride (1 mg) was added to complete the reaction. To remove 3α-hydroxyl isomer, the product(2.4 mg) was chromatographed on a column of silica gel(Wako gel C-200) affording [3α-^{3}H]-III(1.1 mg, 101 Ci/mole) by elution with benzene.

Preparation of cell-free extracts and incubation Fifth instar larvae of the silkworm Bombyx mori were gift of Mr. S.Sakurai, University of Tokyo. The gut was ligated and removed in a chilled 0.6 M KCl solution. Five guts were ground with sea sands(50-80 mesh)(10 g) in a motar containing 15 ml of Bucher's medium (8) at 5°. The homogenate was centrifuged at 1500 X g, for 10 min at 3° and the resulting supernatant was diluted with the same medium up to 20 ml. [3α-^{3}H]-III(2.24 uCi, 10 µg) was dissolved in dimethylformamide (50 µl) and added to the 1500 X g sup. Incubation was carried out at 30° for 2 hr with a gentle shaking in air. The enzyme reaction was terminated by the addition of methanol(20 ml).

Separation of sterols Lipids were extracted with ether(3 X 50 ml) and then saponified with 5 % KOH/methanol(10 ml) under reflux for 1 hr. The non-saponifiable fraction was added with carrier(each of 1 mg) of IV and V. This mixture was kept with benzoyl chloride(50 µl) in pyridine(1 ml) at room temperature overnight. By the usual work-up a mixture of benzoates was obtained, which was added with a carrier(3 mg) of III-benzoate. Column chromatography on silica gel with elution solvent of n-hexane-benzene(2 : 1) afforded "sterol" fraction which contained IV- and V-benzoates. A further elution with n-hexane-benzene(1 : 3) gave "epoxide" fraction corresponding to III-benzoate. The more polar fraction eluted with benzene-ethyl acetate(4 : 1) was designated as "polar"(Table 1). Resolution of IV- and V-benzoates was performed with 5 % $AgNO_3$-impregnated thin layer chromatography developed twice with n-hexane-benzene(4 : 1) (Rf : IV-benzoate, 0.24; V-benzoate, 0.58) or high pressure liquid chromatography(Fig. 1). The isolated IV-benzoate was recrystallized with carrier(150 mg) from acetone-methanol.

Radioactivity was measured on a Packard 3320 liquid scintillation counter using a solution of toluene containing 0.4 % DPO and 0.03 % di-Me-POPOP.

Results and Discussions

It is apparent from Table 1 that the 1500 X g supernatant of insect gut has the activity for carbon-carbon bond cleavage of the epoxide(III)(Exp. 1). When "Sterol" fraction was chromatographed on 5 % $AgNO_3$-impregnated thin layer plate which resolves effectively desmosterol(IV) and cholesterol(V)-benzoates, 99.4 % of the radioactivity were located on the band of desmosterol

† This compound was prepared by oxidation of II with m-chloroperbenzoic acid and was found to be almost 1 : 1 stereoisomeric mixtures of [24R, 28R]- and [24S, 28S]-epoxides (cf. ref. 11).

Table 1. Incorporation of [3α-^{3}H]-Fucosterol-24,28-oxide(III) into "Sterol", "Epoxide" and "Polar" Fractions.

Exp.	Enzyme Source	Recovery in Nonsaponif. fraction	Relative Radioactivity Incorporated into Fractions of "Sterol"	"Epoxide"	"Polar"
1	1500 X g sup of gut	93 %	21 %	49 %	28 %
2	" (Boiled)	92	0.1	78	21
3	" + Imine(VI)[a]	-	1	85	14
4	" + Allene(VII)[b]	-	24	41	35
5	25000 X g sup of gut	98	23	47	30
6	1500 X g sup of non-gut[c]	44	18	17	66
7	1500 X g sup of whole body	89	15	56	29

a. The same concentration as substrate.
b. Ten times concentration of substrate.
c. The remaining part after removing of gut.

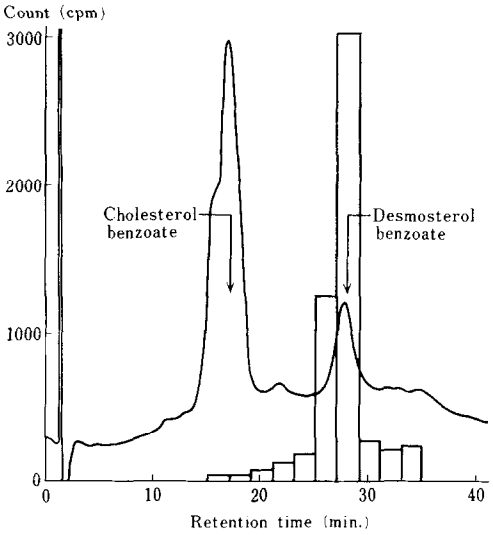
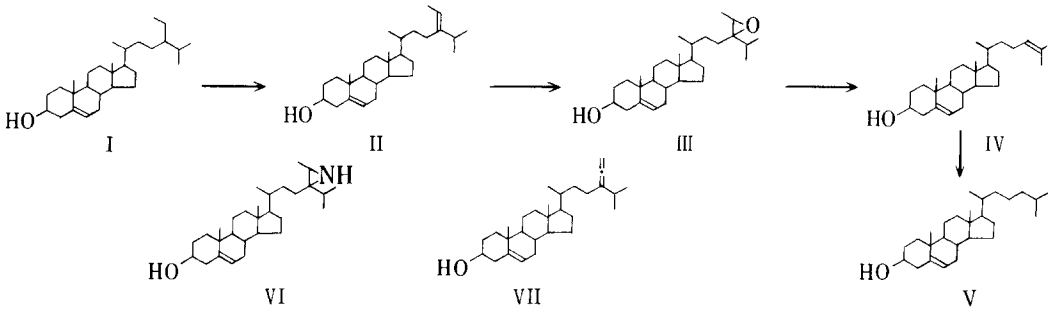


Fig. 1. High pressure liquid chromatography of "sterol" fraction by a Shimadzu-DuPont 830 Liquid Chromatograph. Column, Zorbax SiL (25 cm X 2.1 mm i.d.) ; mobile phase, 2 % CH_2Cl_2 in n-hexane ; pressure, 80 kg/cm^2 ; flow rate, 0.4 ml/min ; detector, UV photometer. Radioactivity eluted in every 2 min periods was measured by a liquid scintillation counter.

(IV)-benzoate. The result was confirmed with analysis by high pressure liquid chromatography(Fig. 1). A further corroboration was came from the constant



specific activity obtained by recrystallization with carrier : 661, 641, 636 and 639 cpm/mg for the first, second, third and fourth crystallizate, respectively. The incorporation ratio based on this value was estimated as ca. 15 %. It is remarkable that no cholesterol(V) which had been the major product of *in vivo* experiments (1), could be detected in the incubation product. The enzyme system appeared therefore, to lack in the activity of sterol Δ^{24} reductase, although this might be merely caused by depletion of the necessary cofactor, e.g., NADPH. When the same cell-free extracts was incubated with [^{3}H]-sitosterol(I) or [^{3}H]-fucosterol(II), most of the substrates have been remained unmetabolized‡. Thus, the presently developed cell-free preparations appear to catalyze rather specifically the conversion of epoxide(III) to desmosterol(IV) in the whole sequence of dealkylative metabolism of sitosterol(I) in *B. mori*. Although a detail of subcellular distribution of the enzyme activity must await future investigation, it was preliminarily observed that the 25,000 X g supernatant performed the conversion as effectively as the 1500 X g supernatant did (Exp. 5). It should be also noted that the insect gut seemed to be not the obligatory site of the dealkylation (Exp. 6 and 7).

Included also in Table 1 were inhibitory effects of 24,28-iminofucosterol (VI) and stigmasta-5,24,28-trien-3β-ol(VII) on the enzyme activity (Exp. 3 and 4). Previous observations *in vivo* suggested that these compounds exert its inhibitory effects on some step of dealkylation of sitosterol(I) in the

‡ N. Awata, unpublished.

insects (9, 10). The present experiments in vitro have established that the imine(VI) at the same level with substrate, completely blocked the conversion of epoxide(III) to desmosterol(IV), whereas addition of the allene(VII) even at 10 times concentrations of substrate induced almost no effect. Those results were in a good agreement with the expectation that the imine(VI) , a structural analog of the epoxide(III),would affect the step of III → IV, while the step involving fucosterol(II), i.e., I → II and /or II → III, may be the likely target for the other inhibitor allene(VII) with a close structural similarity with II.

By the use of the cell-free preparations it has recently revealed that [24S, 28S]-fucosterol-24,28-oxide, but not [24R, 28R]-isomer, is the exclusive precursor of desmosterol(IV) (11).

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