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INVESTIGATION OF INTERMEDIATE STEPS IN THE BIOSYNTHESIS OF ECDYSTERONE FROM CHOLESTEROL IN *PODOCARPUS ELATA*

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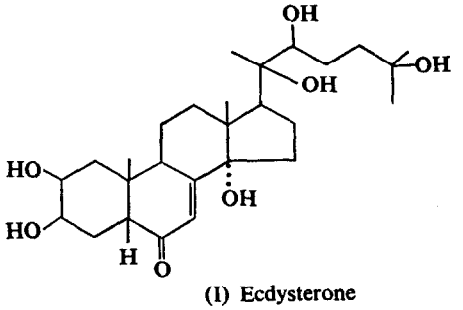
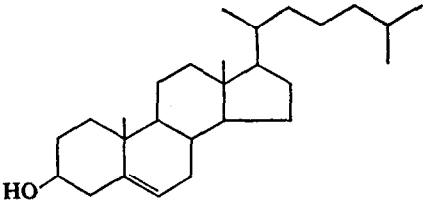
Abstract—Both 4-^{14}C- and 26-^{14}C-cholesterol were incorporated into ecdysterone by *Podocarpus elata*. This demonstrates that no degradation of the side chain is involved in the biosynthetic pathway. However, three possible intermediates in ecdysterone biosynthesis, 25-hydroxycholesterol-26-^{14}C, cholesterol 5α,6α-epoxide-4-^{14}C, and cholesterol 5β,6β-epoxide-4-^{14}C were not converted to ecdysterone. The significance of these results in the biosynthesis of ecdysterone is discussed.

INTRODUCTION

IN OUR first investigations into the biosynthesis of the insect molting hormone ecdysterone (I) in *Podocarpus elata*, we found that cholesterol (II) was a precursor of ecdysterone but Δ^4-cholesten-3-one was not.[1] We now describe further experiments aimed at clarifying the early stages in the biosynthesis of ecdysterone.



(I) Ecdysterone



(II) Cholesterol

RESULTS AND DISCUSSION

The conversion of cholesterol into ecdysterone involves changes at nine different positions in the molecule, and at this time there is no evidence to suggest the sequence in which these

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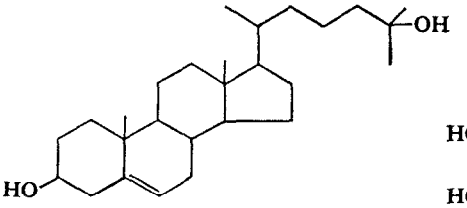
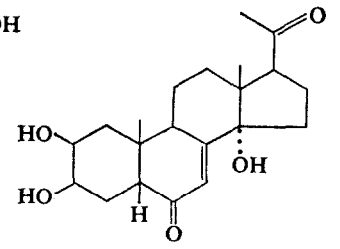
[1] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **7**, 2027 (1968).

transformations may occur. Accordingly, we have first investigated possible intermediates which can easily be obtained in radioactive form.

25-Hydroxycholesterol-26-^{14}C (III), prepared by a Grignard reaction between methyl iodide-^{14}C and 25-keto-26-norcholesterol, was administered to a *Podocarpus elata* seedling, twice a week, for 5 weeks. After workup of the plant, no significant radioactivity was found to be associated with ecdysterone. This indicates that hydroxylation at C-25 apparently follows some other transformation(s) in the molecule. Whatever the first step in the biosynthetic pathway might be, the enzymes controlling it seem to be specific enough that they cannot use 25-hydroxycholesterol as a substrate in place of cholesterol.



(III) 25-Hydroxycholesterol



(IV) Δ^7-Pregnene-6,20-dione-2β,3β,14α-triol

If the biosynthesis of ecdysterone were to involve a partial degradation of the cholesterol side-chain, this would also explain the failure of 25-hydroxycholesterol-26-^{14}C to be incorporated into ecdysterone. This possibility was investigated by the following experiment. One *P. elata* seedling was treated with cholesterol-26-^{14}C, while a second one was treated with a mixture of cholesterol-4-^{14}C and cholesterol-26-^{14}C. If labelled ecdysterone was obtained from the second plant but not from the first, a degradation of the side chain would be indicated. In fact, however, radioactive ecdysterone was isolated from both plants. As further confirmation that labelling at the 26-position had occurred, both samples of ecdysterone were degraded to Δ^7-pregnene-6,20-dione-2β,3β,14α-triol (IV). In the case of the plant treated with cholesterol-26-^{14}C, the IV obtained was nonradioactive, while the other ecdysterone sample, as expected, lost about half of its radioactivity in being converted to IV. These results demonstrate that the cholesterol side-chain is incorporated into ecdysterone intact.

Several possible mechanisms can be envisaged for the transformation of the 5,6-double bond of cholesterol into the 5β-H, 6-ketone system of ecdysterone. One of these, oxidation to Δ^4-cholesten-3-one and subsequent reduction, has been eliminated in a previous investigation.[1] Another mechanism involves the epoxidation of the double bond, followed by rearrangement to the ketone. In theory, either the 5α,6α- or the 5β,6β-epoxide of cholesterol could serve as an intermediate, since a 5α-H,6-ketone can be isomerized to the 5β-isomer by enolization. The radioactive α- and β-epoxides were prepared by oxidation of cholesterol-4-^{14}C with *p*-nitroperbenzoic acid and administered to a *P. elata* plant. Neither the 5α,6α- nor the 5β,6β-epoxide of cholesterol was incorporated into ecdysterone. Although these results seem to eliminate the epoxides of cholesterol as possible precursors, the epoxide → ketone mechanism could still be operating at a later stage of the biosynthetic pathway, e.g. after introduction of the Δ^7-double bond. Obviously, many alternatives for the early steps in ecdysterone biosynthesis remain to be investigated, and the elucidation of the entire biosynthetic pathway may be a formidable task.

EXPERIMENTAL

Methods and Materials

TLC techniques were as described previously.[2] All chromatograms were run on Silica Gel G plates, purchased from Analtech, Inc., Wilmington, Delaware. Aliquots of radioactive samples were counted on planchets at infinite thinness under a gas-flow detector having an efficiency of 34 per cent. All m.p.s. were determined on a Kofler block and are corrected. Methyl iodide-^{14}C, cholesterol-4-^{14}C, and cholesterol-26-^{14}C were purchased from New England Nuclear Corporation.

25-*Hydroxycholesterol*-26-^{14}*C*

To a Grignard reagent, freshly prepared from 18·0 mg (0·127 mmole) of $^{14}CH_3I$ (8·27 μc/μmole) and 20 mg of Mg turnings (carefully washed, dried, and cut into small pieces) in 1·5 ml of dry ether, was added 10·2 mg (0·0265 m-mole) of 25-keto-26-norcholesterol. The mixture was refluxed under N_2 for 5 hr, then poured onto ice, acidified with 2 N H_2SO_4, and worked up as usual. The crude product was twice purified by preparative TLC with CH_2Cl_2–acetone (9:1) and then recrystallized from ether–hexane to yield 1·95 mg of pure material, m.p. 176–178°; lit., m.p. 181·5–182·5°,[3] 177–179°.[4] No depression was observed when a mixed m.p. was taken. When subjected to TLC in CH_2Cl_2–acetone (9:1) and benzene–MeOH (9:1), and scanned, no radioactive impurities were detected and the compound was identical in mobility to authentic 25-hydroxycholesterol. The synthetic 25-hydroxycholesterol-26-^{14}C had a specific activity of $1{\cdot}65 \times 10^5$ counts/min/μmole. A portion of this material was diluted with carrier material and recrystallized from ether–hexane; no change in specific activity was observed.

Cholesterol-4-^{14}*C* 5α,6α- *and* 5β,6β-*epoxides*

Cholesterol-4-^{14}C (about 30 μc) was epoxidized with *p*-nitroperbenzoic acid.[5] The resulting mixture of the α- and β-epoxides was converted to the trimethylsilyl ethers by treating with 5 μl of N,O-bistrimethyl silyl-acetamide in 200 μl of CH_2Cl_2 for 30 min. The two isomers were then separated by preparative TLC with benzene and further purified by preparative TLC with ether until both were radiochemically homogeneous by TLC. The trimethylsilyl ethers were then hydrolyzed by treatment with i-PrOH–0·1 m-mole HCl (12:1) for 20 min.

Administration of Radioactive Steroids

The ^{14}C-labelled materials were each administered to the leaves of a *Podocarpus elata* seedling in ten equal doses, twice weekly for 5 weeks, by the technique previously described.[6] The total amounts administered to each plant were as follows: plant number 1, $1{\cdot}64 \times 10^5$ counts/min of 25-hydroxycholesterol-26-^{14}C; plant number 2, $9{\cdot}59 \times 10^6$ counts/min of cholesterol-26-^{14}C (46 μc/μmole); plant number 3, $1{\cdot}12 \times 10^7$ counts/min of cholesterol-26-^{14}C and $7{\cdot}65 \times 10^6$ counts/min of cholesterol-4-^{14}C (51 μc/μmole); plant number 4, $3{\cdot}1 \times 10^6$ counts/min of cholesterol α-epoxide-4-^{14}C; plant number 5, $2{\cdot}05 \times 10^5$ counts/min of cholesterol β-epoxide-4-^{14}C.

Plant Number 1

The plant was harvested and worked up as described previously[1] to give 265 mg of a butanol extract ($1{\cdot}20 \times 10^5$ counts/min). TLC with EtOAc–acetone–H_2O (14:6:1) showed that most of the radioactivity corresponded to 25-hydroxycholesterol. The more polar material of the extract was isolated by preparative TLC with the same system, giving 60 mg ($5{\cdot}25 \times 10^3$ counts/min). TLC of this material in the same system showed no radioactivity corresponding to ecdysterone, but other peaks due to both more and less polar material were present.

Plant Number

The butanol extract (536 mg, $2{\cdot}60 \times 10^6$ counts/min) was chromatographed on a 150-g column of silica gel (particle size 0·05–0·2 mm; Brinkmann Instruments, Westbury, New York), packed as a slurry in EtOAc. Fractions of 500 ml were collected with the following eluents: 1, EtOAc; 2–5, EtOAc–acetone–H_2O (14:6:1); and 6–7, EtOAc–MeOH (4:1). TLC of fractions 2–5 (167 mg, $7{\cdot}98 \times 10^5$ counts/min) indicated the presence of radioactive ecdysterone. They were subjected to preparative TLC with EtOAc–acetone–H_2O (14:6:1) and then with CH_2Cl_2–MeOH (17:3) and gave 3·6 mg of radiochemically pure ecdysterone ($3{\cdot}79 \times 10^4$ counts/min).

[2] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **5**, 747 (1966).
[3] A. L. WILDS and A. L. MEADER, *J. Org. Chem.* **13**, 763 (1948).
[4] A. I. RYER, W. H. GEBERT and N. M. MURRILL, *J. Am. Chem. Soc.* **72**, 4247 (1950).
[5] R. D. BENNETT, H. H. SAUER and E. HEFTMANN, *Phytochem.* **7**, 41 (1968).
[6] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 475 (1965).

A portion ($3{\cdot}79 \times 10^3$ counts/min) of this material was converted to the 2-monoacetate,[7] which was isolated by preparative TLC with EtOAc–acetone–H_2O (14:6:1). This material ($2{\cdot}05 \times 10^3$ counts/min) was oxidized with 200 μl of a 5% aqueous $NaIO_4$ solution in 200 μl of EtOH for 3 hr at 25°. After workup as usual, the oxidation product was hydrolyzed with 0·02 N K_2CO_3 in MeOH for 2 hr at 25°,[8] and the product, compound IV, was isolated by preparative TLC with CH_2Cl_2–MeOH (17:3). This material, which was identical in mobility to an authentic sample of IV, was devoid of radioactivity.

Plant Number 3

The butanol extract (270 mg, $3{\cdot}76 \times 10^6$ counts/min) was subjected to column chromatography and preparative TLC (see plant number 2) to give 2·5 mg of radiochemically homogeneous ecdysterone ($3{\cdot}63 \times 10^4$ counts/min). A portion ($7{\cdot}25 \times 10^3$ counts/min) was acetylated as above, and the purified 2-monoacetate ($4{\cdot}29 \times 10^3$ counts/min) was oxidized and hydrolyzed as above, yielding $1{\cdot}69 \times 10^3$ counts/min of compound IV. The product showed a single radioactive peak corresponding to authentic IV when subjected to TLC with CH_2Cl_2–MeOH (17:3) and scanned.

Plant Number 4

The butanol extract (599 mg, $1{\cdot}18 \times 10^6$ counts/min) was subjected to column chromatography (see plant number 2) and the ecdysterone-containing fractions were rechromatographed on a 40-g column of silica gel, eluted first with 150 ml of EtOAc–acetone–H_2O (17:3:1) and then with 400 ml of EtOAc–acetone–H_2O (14:6:1). TLC of the second fraction in the latter system showed a radioactive peak corresponding to ecdysterone. This material was isolated by preparative TLC with the same solvent to give 12·7 mg ($1{\cdot}36 \times 10^4$ counts/min). Preparative TLC with CH_2Cl_2–MeOH (17:3) then gave 1·0 mg of ecdysterone (582 counts/min). However, TLC with the same system showed that this radioactivity had a mobility slightly greater than that of ecdysterone.

Plant Number 5

The butanol extract (178 mg, $7{\cdot}20 \times 10^4$ counts/min), when subjected to column chromatography (see plant number 2), gave 71 mg ($2{\cdot}50 \times 10^4$ counts/min) of the ecdysterone-containing fractions. However, TLC with EtOAc–acetone–H_2O (14:6:1) showed no significant peaks corresponding to ecdysterone. The latter was then isolated by preparative TLC with the same system, yielding 3·3 mg (429 counts/min). TLC with CH_2Cl_2–MeOH (17:3) showed that none of this radioactivity was associated with ecdysterone.

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[7] H. MORIYAMA and K. NAKANISHI, *Tetrahedron Letters* 1111 (1968).
[8] B. N. GALBRAITH, D. H. S. HORN and E. J. MIDDLETON, *Chem. Commun.* 468 (1968).