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ECDYSTERONE BIOSYNTHESIS IN *PODOCARPUS ELATA*

HORST H. SAUER, RAYMOND D. BENNETT and ERICH HEFTMANN

Division of Biology, California Institute of Technology, Pasadena, California and
Western Regional Research Laboratory,* Albany, California

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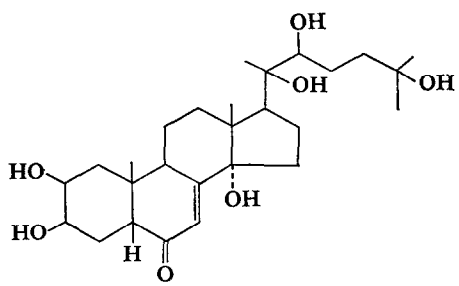
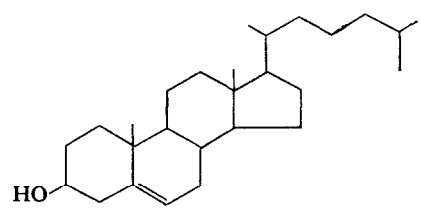
Abstract—The insect molting hormone ecdysterone was isolated in radioactive form after administration of cholesterol-4-^{14}C to *Podocarpus elata* seedlings. Cholestenone-4-^{14}C, however, was not significantly incorporated into ecdysterone by this plant. The biosynthetic implications of these results are discussed.

INTRODUCTION

THE STEROID hormones controlling molting in insects, ecdysterone (I) and ecdysone (20-desoxyecdysterone), have been known for several years, but only one study of their biosynthesis has been reported. Karlson and Hoffmeister found that *Calliphora erythrocephala* larvae converted tritiated cholesterol (II) to ecdysone.[1] Both ecdysterone and ecdysone, as well as several related compounds having molting activity, have now been found in plants[2–5] in much higher concentrations than in insects. For an investigation of the early stages in the biosynthesis of molting hormones, we have accordingly chosen a plant, *Podocarpus elata*, which contains ecdysterone.[6] A preliminary account of part of this work has appeared elsewhere.[7]



(I) Ecdysterone



(II) Cholesterol

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. Work conducted under a cooperative agreement with the California Institute of Technology. Requests for reprints should be addressed to Erich Heftmann.

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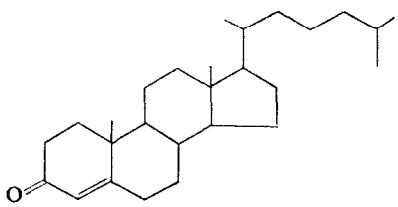
RESULTS

Since cholesterol was already known to be a precursor of ecdysone in an insect, we first administered cholesterol-4-^{14}C to *Podocarpus elata* seedlings, twice a week for 4 weeks. Subsequent extraction of the plants gave a polar fraction, which was examined by TLC. Most of the radioactivity appeared to be associated with cholesterol, but two more polar radioactive substances were present, one of which corresponded chromatographically to ecdysterone. The ecdysterone-like material was isolated by column chromatography on alumina and further purified by preparative TLC. To prove the identity of the radioactive material with ecdysterone, we then subjected both to the same reactions, acetylation with pyridine-acetic anhydride and oxidation with sodium periodate. The products were examined by TLC in two systems. In each case the radioactive products corresponded chromatographically to those of ecdysterone.



(III) Cholestenone

We then investigated the possibility that cholestenone (III) may be an intermediate between cholesterol and ecdysterone. Two groups of *P. elata* seedlings were treated, under the same conditions, with either cholesterol-4-^{14}C or cholestenone-4-^{14}C, respectively. The cholesterol-treated plants were worked up and, as before, a polar fraction was obtained which appeared to contain radioactive ecdysterone. After purification by column chromatography on silica gel and preparative TLC, a portion of this material was diluted with carrier ecdysterone. No change in specific activity was observed after crystallization from three solvents (Table 1).

TABLE 1. RECRYSTALLIZATION OF ECDYSTERONE*

Solvent used for crystallization	Counts/min/μmole†
—	519 ± 29
Benzene–acetone	519 ± 29
Benzene–methanol	513 ± 29
Ethyl acetate–methanol	521 ± 29

* Portions of 0·2 mg or less were plated from solution on ringed planchets over an area of 12·7 cm^2 and counted in duplicate on a Beckman Widebeta II instrument. Counter efficiency was 34 per cent and background was 2 counts/min.

† 90 per cent confidence level.

The cholestenone-treated plants were worked up in the same manner. TLC of the polar fraction showed no radioactivity corresponding to ecdysterone, although as little as 10 per cent of that found in the cholesterol-treated plants could have been detected.

DISCUSSION

The radioactive ecdysterone obtained from plants treated with cholesterol-4-^{14}C corresponded in mobility in two TLC systems to authentic ecdysterone. In addition, its acetylation and oxidation products were chromatographically identical to those of ecdysterone. Finally, its radiochemical purity was demonstrated by dilution with carrier ecdysterone and crystallization from three solvents. Thus, there can be little doubt that cholesterol is a precursor of ecdysterone in *Podocarpus elata.*

Turning our attention to the steps following cholesterol in the biosynthetic pathway, we first considered the change from Δ^5 to 5B-H. In animals such a transformation involves a Δ^4-3-ketone as an intermediate.[8] Recently the same requirement was observed in a plant, *Digitalis lanata,* where progesterone was found to be an obligatory intermediate between the Δ^5-precursor pregnenolone and the 5B-cardenolides.[9] When we administered cholestenone-4-^{14}C to *P. elata* plants, however, no significant incorporation of radioactivity into ecdysterone was observed, which suggests that a different biosynthetic mechanism may be operating in this case. It should be noted that ecdysterone, unlike the steroids saturated at the 5-position whose biosynthesis has been studied previously, contains a double bond in Ring B (Δ^7). Thus the biosynthetic pathway may involve introduction of this double bond while the Δ^5-unsaturation is still present, and a Δ^4-3-ketone may not be required for such a transformation. Insects are known to be capable of converting cholesterol to 7-dehydrocholesterol,[10] but the detailed mechanism has not been investigated. Robbins *et al.*[10] suggested that the latter compound may be a precursor of ecdysone in insects. Further investigations on this subject are now in progress.

EXPERIMENTAL

Methods

TLC techniques were as described previously.[11] All chromatograms were run on Silica Gel G plates purchased from Analtech, Inc., Wilmington, Delaware.* For column chromatography, neutral alumina (Woelm, Eschwege, Germany) and silica gel, particle size 0·05–0·2 mm (Brinkmann Instruments, Westbury, New York), were used. Aliquots of radioactive samples were counted on planchets at infinite thinness under a gas-flow detector (see Table 1, legend, for details).

Materials

Cholesterol-4-^{14}C (50 μc/μM) and cholestenone-4-^{14}C (20 μc/μM) were purchased from New England Nuclear Corporation. Ecdysterone was isolated from rhizomes of *Polypodium vulgare,*[12] supplied by Mr. E. C. Robbins, Gardens of the Blue Ridge, Ashford, North Carolina.† *Podocarpus elata* seedlings were obtained from Husbands' Rockcliffe Gardens, Yorba Linda, California.

Administration of Radioactive Steroids

In the first experiment five *P. elata* seedlings, about 15 cm tall, were each treated with 1 μc of cholesterol-4-^{14}C by the technique previously described.[13] A total of ten such treatments were given, twice a week. In the second experiment two groups of five plants each were treated with 1 μc-doses of cholesterol-4-^{14}C and 1 μc-doses of cholestenone-4-^{14}C, respectively, in the same manner as above.

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

† Dr. David B. Lellinger of the Smithsonian Institution has since informed us that the plant was probably the taxonomically similar *Polypodium virginianum* L. A specimen of this plant material has been filed in the U.S. National Herbarium.

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First Cholesterol-Treated Plants

The entire plants were harvested 3 days after the last treatment, frozen in liquid N_2, and lyophilized. The dried material (16 g) was homogenized with 500 ml of 95% ethanol. The homogenate was transferred to a flask with 500 ml of 95% ethanol and stirred for 24 hr at 25°. The solution was filtered and the filter cake extracted twice by stirring with 500 ml of 95% ethanol for 24 hr at 25°. The ethanolic filtrates were combined and evaporated. The residue was taken up in 500 ml of water and extracted with two 200-ml portions of hexane. The extracts were washed with 75 ml of water, combined, and evaporated, giving a nonpolar fraction (211 mg, $1{\cdot}07 \times 10^6$ counts/min).

The aqueous layer was then extracted with four 150-ml portions of butanol. The extracts were washed with the same 75 ml of H_2O as above, combined, and evaporated, giving a polar fraction (1·08 g, $2\ 22 \times 10^7$ counts/min). A portion of the polar fraction was subjected to TLC with $CHCl_3$–95% ethanol (6:4) and scanned for radioactivity. The major peak appeared to be associated with cholesterol, but two much smaller, more polar peaks were observed, one of which had the same mobility as a cochromatographed sample of ecdysterone.

The polar fraction was chromatographed on a 30-g column of alumina, Grade III. Fractions of 75 ml each were collected with the following eluents: 1, 5%; 2, 10%; 3, 20%; 4, 50%; and 5, 70% methanol in benzene. TLC of the fractions with $CHCl_3$–95% ethanol (7:3) indicated that Fraction 4 (6 5 mg, $3{\cdot}60 \times 10^5$ counts/min) contained radioactive ecdysterone, which was isolated by preparative TLC with CH_2Cl_2–95% ethanol (7:3), giving 4·2 mg, $1{\cdot}47 \times 10^5$ counts/min. For final purification, $\frac{1}{10}$ of this material was chromatographed on a $200 \times 50 \times 0{\cdot}25$ mm Silica Gel G layer in the same system. The plate was scanned, and the zone corresponding to the center of the radioactive peak was removed and eluted ($1{\cdot}01 \times 10^4$ counts/min).

A 0·2-mg sample of authentic ecdysterone was treated with 0·1 ml of pyridine and 0·09ml of acetic anhydride for 15 hr at 25° and then evaporated to dryness in vacuum. The products had the following R_f values in two TLC systems. CH_2Cl_2–95% ethanol (9:1): 0·61, 0·58, 0 41; ethyl acetate–cyclohexane (5:1): 0 39, 0·33, 0·17. A portion of the radioactive ecdysterone, after acetylation as above, showed, in each system, peaks corresponding only to these three products.

A 0·2-mg sample of authentic ecdysterone was dissolved in 0·2 ml of methanol and treated with a solution of 5 mg of $NaIO_4$ in 0·1 ml of methanol–water (1:1) for 3 hr at 25°. The mixture was then diluted with 1 ml of water and extracted with three 2-ml portions of $CHCl_3$–ethanol (3:2) The extracts were washed with two 1-ml portions of water, combined, and evaporated. This reaction also gave three products, with the following R_f values. CH_2Cl_2–95% ethanol (17:3): 0·72, 0·66, 0·57; CH_2Cl_2–acetone–formamide (160:40:1): 0 45, 0·12, 0·12. The radioactive ecdysterone was subjected to the same reaction, and again the radioactive products corresponded to those of authentic ecdysterone.

Second Cholesterol-Treated Plants

The plants were harvested, lyophilized, and extracted as above. The residue from evaporation of the alcohol extracts was taken up in 100 ml of water and extracted with three 50-ml portions of CH_2Cl_2. The extracts were washed with 25 ml of water, combined, and evaporated, giving a nonpolar fraction (228 mg, $1{\cdot}94 \times 10^7$ counts/min).

The aqueous layer was then extracted with three 50-ml portions of butanol. The extracts were washed with the same 25-ml portion of water as above, combined, and evaporated, giving a polar fraction (215 mg, $9\ 01 \times 10^6$ counts/min). TLC of the polar fraction with ethyl acetate–acetone–water (14:6:1), developed twice, showed a major peak corresponding to cholesterol and two minor, more polar peaks, one of which corresponded to ecdysterone.

The polar fraction was chromatographed on a 50-g column of silica gel, packed into a chromatographic tube of 35 mm dia. as a slurry in ethyl acetate–acetone–water (34:6:1). Six fractions of 250 ml each were eluted with the same solvent. Fractions 3–6, which contained the ecdysterone, were combined, giving 29 mg, $1{\cdot}99 \times 10^5$ counts/min. Purification by preparative TLC with CH_2Cl_2–95% ethanol (7:3) gave chromatographically homogeneous ecdysterone (2·7 mg, $1{\cdot}00 \times 10^5$ counts/min). About $\frac{1}{10}$ of this material was diluted with 9·2 mg of authentic ecdysterone and crystallized as shown in Table 1.

Cholestenone-Treated Plants

The workup was the same as for the second group of cholesterol-treated plants. Nonpolar fraction: 126 mg, $1{\cdot}78 \times 10^7$ counts/min. Polar fraction: 230 mg, $1{\cdot}62 \times 10^7$ counts/min. TLC of the polar fraction with ethyl acetate–acetone–water (14:6:1), developed twice, showed a major peak corresponding to cholestenone. No radioactive peaks were observed corresponding to ecdysterone or to the other polar radioactive metabolite of cholesterol.

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