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BIOSYNTHESIS OF PONASTERONE A AND ECDYSTERONE FROM CHOLESTEROL IN *PODOCARPUS MACROPHYLLUS**

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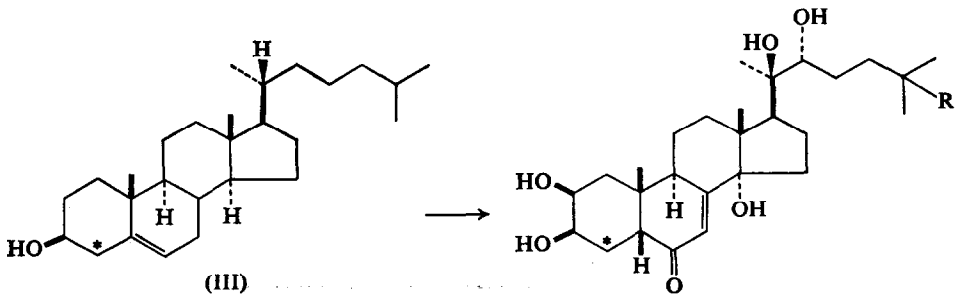
Abstract—Administration of cholesterol-4-^{14}C to *Podocarpus macrophyllus* seedlings resulted in the formation of the insect-metamorphosing substances ponasterone A and ecdysterone in radioactive form.

INTRODUCTION

AFTER the recent discovery of the steroids which control moulting and metamorphosis of arthropods from the animal kingdom and later from the plant kingdom, the biosynthesis of these substances has naturally become an interesting subject of investigation. Indeed, it has been shown that cholesterol is the precursor of the metamorphosing hormone ecdysone in an insect, *Calliphora erythrocephala*.[1] It seems possible that the phytoecdysones may be also synthesized in plants from cholesterol which is now an accepted plant constituent. In fact, the biosynthesis of another metamorphosing hormone, ecdysterone, from cholesterol in a plant, *Podocarpus elatus* R. Brown (Podocarpaceae), has already been demonstrated.[2]

We have also been interested in this subject and started our work in an investigation of the biosynthesis of ponasterone A, the first metamorphosing steroid discovered in the plant kingdom[3] which has been isolated from a variety of sources. For this purpose we have used the conifer *P. macrophyllus* Lambert (Podocarpaceae) which is known to contain both



* Part VI in the series on 'Biochemical Syntheses'; Part V, H. HIKINO, D. KUWANO and T. TAKEMOTO, *Yakugaku Zasshi*, **89**, 1149 (1969). This paper is also Part VIII in the series on Steroids; Part VII, H. HIKINO, S. ARIHARA and T. TAKEMOTO, *Tetrahedron* **25**, 3909 (1969).

[1] P. KARLSON and H. HOFFMEISTER, *Z. Physiol. Chem.* **331**, 298 (1963).
[2] E. HEFTMANN, H. H. SAUER and R. D. BENNETT, *Naturwissenschaften* **55**, 37 (1968); H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **7**, 2027 (1968).
[3] K. NAKANISHI, M. KOREEDA, S. SASAKI, M. L. CHANG and H. Y. HSU, *Chem. Comm.* **1966**, 915.

ponasterone A (I) and ecdysterone (II) as the major phytoecdysones,[4] and investigated their synthesis in a plant treated with radioactive cholesterol (III).*

RESULTS

Cholesterol-4-^{14}C (III) was administered twice a week for 1 month to intact *Podocarpus macrophyllus* leaves. 4 weeks after the last treatment the leaves were harvested and extracted with methanol. The less-polar fraction of the extract contained a radioactive substance which appeared to be unchanged cholesterol (III). The polar portion of the extract was extracted with *n*-butanol. The butanol extract was chromatographed over alumina to afford three fractions. The first contained a radioactive substance which was slightly less polar than ponasterone A and had a conjugated system. However, since the amount obtained was insufficient for further structural study, and since no reference compound having the same properties as this substance was available, it could not be identified. The second fraction was rechromatographed over silica gel to give a crystalline material having the same mobility on TLC as ponasterone A. After dilution with carrier ponasterone A, the substance was crystallized from methanol and ethyl acetate to afford radioactive ponasterone A (I) possessing constant specific activity (Table 1). TLC on silica gel plates, developed with two solvent systems, showed only a single spot, corresponding exactly with ponasterone A. To confirm the identity of the radioactive substance with ponasterone A, it was acetylated to furnish a product which showed only a single radioactive spot by TLC, coinciding with ponasterone A triacetate.

TABLE 1. RECRYSTALLIZATION OF PONASTERONE A

Solvent used for crystallization	cpm /μmole
MeOH (3 times)	1000
AcOEt (once)	970
MeOH (once)	1020

The third fraction was also rechromatographed over silica gel to yield crystalline material which corresponded chromatographically to ecdysterone. The substance was diluted with carrier ecdysterone and crystallized from methanol to furnish the radioactive ecdysterone (II) having constant specific activity (Table 2). The identity of the radioactive substance with ecdysterone was further evidenced by acetylation which gave a radioactive derivative corresponding chromatographically to ecdysterone triacetate.

TABLE 2. RECRYSTALLIZATION OF ECDYSTERONE

Solvent used for crystallization	cpm/μmole
MeOH (3 times)	122
MeOH (once)	125
MeOH (once)	122

* Part of the material contained herein formed a preliminary communication: H. HIKINO, T. KOHAMA and T. TAKEMOTO, *Chem. Pharm. Bull. (Tokyo)* **17**, 415 (1969).

[4] S. IMAI, S. FUJIOKA, K. NAKANISHI, M. KOREEDA and T. KUROKAWA, *Steroids* **10**, 557 (1967).

The plant, *P. macrophyllus*, is known to contain, besides ponasterone A and ecdysterone, the other metamorphosing steroids, makisterone A,[5] B, C and D.[6] However, since the content of these latter phytoecdysones are small, the biosynthesis of these substances could not be examined. We have nevertheless proved that cholesterol serves as a precursor of ponasterone A and ecdysterone in the plant. However, the pathway of biosynthesis from cholesterol to these metamorphosing substances remains to be elucidated.

EXPERIMENTAL*

Administration of Radioactive Cholesterol to Podocarpus macrophyllus

Cholesterol-4-^{14}C was applied in acetone soln. to the leaves of three *Podocarpus macrophyllus* seedlings grown in soil, about 70 cm tall, by the techniques reported earlier.[7] A total of ten such treatments were given. The leaves were treated once only. Total dose $1{\cdot}54 \times 10^7$ cpm.

Isolation of Radioactive Ponasterone A and Ecdysterone

Four weeks after the last treatment, the leaves were harvested (610 g), cut into pieces and extracted with MeOH. The MeOH extract was washed with Et_2O to give the Et_2O extract (6·5 g) and the residue. By preparative TLC of part of the Et_2O extract, the radioactive zone corresponding to cholesterol was removed and eluted (total $3{\cdot}60 \times 10^5$ cpm). The residue was diluted with water and extracted with *n*-butanol to give the butanol extract (11·8 g, $4{\cdot}75 \times 10^5$ cpm) which was chromatographed over neutral alumina (60 g).

Fractions eluted with AcOEt were combined (350 mg, $1{\cdot}88 \times 10^5$ cpm) and rechromatographed over silica gel (10 g). Elution with $CHCl_3$–MeOH (50:1) yielded a substance ($4{\cdot}44 \times 10^4$ cpm), TLC (AcOEt–MeOH = 10:1): R_f 0·49.

Fractions eluted with the same solvent were combined (626 mg, $1{\cdot}87 \times 10^5$ cpm) and rechromatographed over silica gel (15 g). Elution with $CHCl_3$–MeOH (10:1) afforded crystalline material (111 mg, $9{\cdot}30 \times 10^4$ cpm) which was diluted with ponasterone A (13 mg) and recrystallized 3 × from MeOH to furnish the radioactive ponasterone A having a specific activity of $10{\cdot}0 \times 10^2$ cpm/μmole as colourless needles, TLC (AcOEt–MeOH = 10:1): R_f 0·38 (ponasterone A 0·38), ($CHCl_3$–MeOH = 5:1): R_f 0·65 (ponasterone A 0·65). On further crystallization from AcOEt and from MeOH there was no change in specific activity (Table 1).

Fractions eluted with AcOEt–MeOH (10:1) were combined (400 mg, $1{\cdot}80 \times 10^5$ cpm) and rechromatographed over silica gel (50 g). Elution with $CHCl_3$–MeOH (10:1) yielded crystalline material (305 mg, $1{\cdot}45 \times 10^5$ cpm) which was diluted with ecdysterone (55 mg) and crystallized 3 × from MeOH to give radioactive ecdysterone possessing a specific activity of 122 cpm/μmole as colourless needles, TLC (AcOEt–MeOH = 10:1): R_f 0·10 (ecdysterone 0·10), ($CHCl_3$–MeOH = 5:1): R_f 0·40 (ecdysterone 0·40). On recrystallization from MeOH there was no change in specific activity (Table 2).

Acetylation of Radioactive Ponasterone A

Radioactive ponasterone A (0·5 mg) in pyridine (0·1 ml) was treated with Ac_2O (0·05 ml) at room temperature for 36 hr. Isolation in the usual manner gave the radioactive acetate, TLC (AcOEt): R_f 0·50 (ponasterone triacetate 0·50), ($CHCl_3$–MeOH = 10:1): R_f 0·75 (ponasterone A triacetate 0·75).

Acetylation of Radioactive Ecdysterone

The radioactive ecdysterone (0·5 mg) was acetylated in a similar manner to that described above to furnish the radioactive acetate, TLC (AcOEt): R_f 0·30 (ecdysterone triacetate 0·30), ($CHCl_3$–MeOH = 10:1): R_f 0·55 (ecdysterone triacetate 0·55).

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* TLC was carried out on silica gel G plates, developing solvents being shown in parentheses. Aliquots of radioactive samples were counted on planchets at infinite thinness under a 2π gas-flow detector.

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[6] S. Imai, S. Fujioka, E. Murata, Y. Sasakawa and K. Nakanishi, *Tetrahedron Letters* 3887 (1968).
[7] R. D. Bennett and E. Heftmann, *Phytochem.* 4, 577 (1965).