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STUDIES ON INSECT MOULTING HORMONES: BIOSYNTHESIS OF ECDYSONE, ECDYSTERONE AND 5β-HYDROXYECDYSTERONE IN *POLYPODIUM VULGARE*

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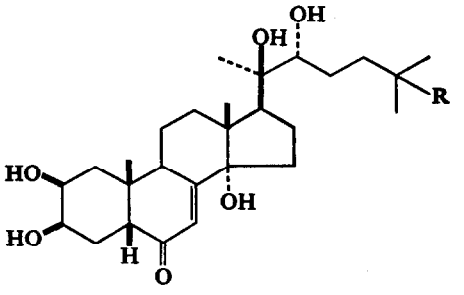
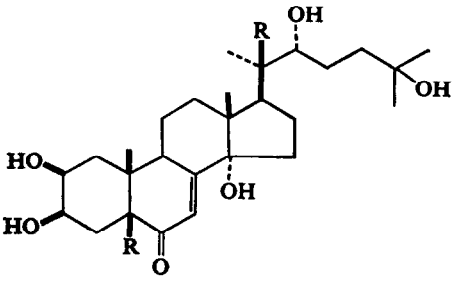
Abstract—The incorporation of [2-^{14}C]-mevalonic acid into ecdysterone, and of [4-^{14}C]-cholesterol into ecdysone, ecdysterone and 5β-hydroxyecdysterone in *Polypodium vulgare* has been demonstrated.

INTRODUCTION

ECDYSTERONE (I), a steroid with moulting hormone activity in several arthropods,[1] is widely distributed in the plant kingdom[2,3] and is present in high concentrations in the rhizomes of the fern *Polypodium vulgare* L. (0·07–1·0 per cent of dry weight).[4] The rhizomes also contain, in smaller amounts, ecdysone (II), the first moulting hormone isolated from an insect,[5] and 5β-hydroxyecdysterone (III)[6] (0·002 and 0·04 per cent respectively). The only other known plant source of ecdysone is bracken fern.[7] 5β-Hydroxyecdysterone has also been isolated from *Vitex megapotamica*.[8]



Ecdysterone (I) R = OH
Ponasterone A (IV) R = H



Ecdysone (II) R = H
5-β-hydroxyecdysterone (III) R = OH

These and several related phytoecdysones presumably arise via the isopentenoid pathway to the steroids. The conversion of [2-^{14}C]-mevalonic acid into ecdysterone (I) and ponasterone A (IV) in *Taxus baccata* has been recently demonstrated[2] and [4-^{14}C]-cholesterol has

[1] A. KRISHNAKUMARAN and H. A. SCHNEIDERMAN, *Nature* **220**, 601 (1968).
[2] For references see N. J. DE SOUZA, E. L. GHISALBERTI, H. H. REES and T. W. GOODWIN, *Biochem. J.* **114**, 895 (1969).
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[4] G. HEINRICH and H. HOFFMEISTER, *Experientia* **23**, 995 (1967). J. JIZBA, V. HEROUT and F. ŠORM, *Tetrahedron Letters* 1689 (1967). J. JIZBA and V. HEROUT, *Coll. Czech. Chem. Commun.* **32**, 2867 (1967).
[5] A. BUTENANDT and P. KARLSON, *Z. Naturforsch.* **9b**, 389 (1954).
[6] G. HEINRICH and H. HOFFMEISTER, *Tetrahedron Letters* 6063 (1968). J. JIZBA, V. HEROUT and F. ŠORM, *Tetrahedron Letters* 5139 (1967).
[7] J. N. KAPLANIS, M. J. THOMPSON, W. E. ROBBINS and B. M. BRYCE, *Science* **157**, 1436 (1967).
[8] H. RIMPLER, *Tetrahedron Letters* 329 (1969).

been converted into ecdysterone in *Podocarpus elata*[9] and into ponasterone A in *P. macrophyllus*.[10]

We now report the incorporation of [^{14}C]-mevalonic acid into ecdysterone and the biosynthesis of ecdysone, ecdysterone and 5β-hydroxyecdysterone from [4-^{14}C]-cholesterol by *Polypodium vulgare*.

RESULTS AND DISCUSSION

Table 1 provides a summary of the feeding techniques used in the experiments, the percentage levels of incorporations of the radioactive substrates into the phytoecdysones and the specific radioactivity of the phytoecdysones.

The results of Exp. 4 clearly show the biosynthesis of ecdysterone (I) from [2-^{14}C]-mevalonic acid in *Polypodium vulgare*. These findings are similar to those obtained previously[2] for *Taxus baccata*. It is interesting to note, however, that although *P. vulgare* is a richer source of ecdysterone than *T. baccata*, the level of incorporation of mevalonic acid for the former is only about one-tenth of that for *T. baccata*. To what extent the biosynthesis of the phytoecdysones is dependent on factors such as the age of the plant, seasonal differences, and the site of application of the substrate is not known. The results indicate that the rate of conversion of mevalonic acid into the phytoecdysones is slow. This would explain the very poor incorporation observed in Exps. 1–3 with sliced tissues for limited incubation periods. The results of the experiment with labelled cholesterol (Exp. 5) show that ecdysterone (I), ecdysone (II), and 5β-hydroxyecdysterone (III) in *P. vulgare* can arise from cholesterol. The radiochromatogram scan (Fig. 1) illustrates the separation between the triacetates of ecdysone and

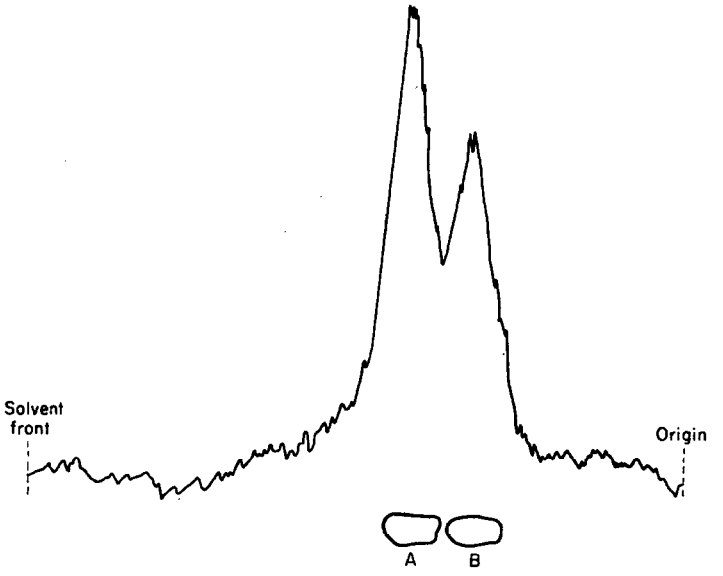

FIG. 1. RADIOSCAN OF A THIN-LAYER CHROMATOGRAM ON SILICA GEL G DEVELOPED WITH ETHYL ACETATE–HEXANE (4:1) OF A MIXTURE OF ECDYSONE AND ECDYSTERONE TRIACETATES.

Markers: A, ecdysone triacetate; B, ecdysterone triacetate.

[9] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* 7, 2027 (1968).
[10] H. HIKINO, T. KOHAMA and T. TAKEMOTO, *Chem. Pharm. Bull.* 17, 415 (1969).

TABLE 1. INCORPORATION OF LABELLED MEVALONATE (MVA) AND CHOLESTEROL INTO PHYTOECDYSONES BY *P. vulgare*

Expt. No.	Radioactive substrate	Month	Feeding technique	Duration	Incorporation (%)			Specific radioactivity (cpm/μmole)	
					Ecdysterone	Ecdysone	5β-Hydroxy Ecdysterone	Ecdysterone (triacetate)	Ecdysone (triacetate)
1	5 μc [2-^{14}C] MVA	January	Sliced rhizomes	24 hr	0·006*†	—	—	—	—
2	10 μc [2-^{14}C] MVA	February	Sliced leaves and rhizomes	24 hr	0·0001*†	—	—	—	—
3	10 μc [2-^{14}C] MVA	June	Sliced leaves and rhizomes	44 hr	0·001*†	—	—	—	—
4	10 μc [2-^{14}C] MVA	June	Leaves of whole plant	3 weeks	0·022*	0·002*†	0·001*†	36·5 (33·1)	—
5	10 μc [4-^{14}C] Cholesterol	June–July	Leaves of whole plant	5 weeks	0·14	0·055	0·003†	765 (854)	463 (577)

* Based on the utilization of one optical isomer.

† Maximum values; purification to constant specific activity was not possible.

ecdysterone when a portion of the ecdysone, incompletely separated from ecdysterone by preparative TLC, was acetylated and subjected to TLC. This is, to our knowledge, the first demonstration of the conversion of cholesterol into ecdysone in plants. The present results, together with those reported by other groups,[9,10] implicate cholesterol as a possible intermediate in the biosynthetic pathway to the phytoecdysones. Whether cholesterol is an obligatory *in vivo* precursor remains an open question. Cholesterol is widely distributed among plant families[11,12] and is the major component of the sterol fraction in chloroplasts of several plants.[13] In addition, the biosynthesis of cholesterol in plants fed with mevalonic acid has been demonstrated in *Solanum tuberosum*,[14] *Dioscorea spiculiflora* and *Digitalis purpurea*.[15] Among ferns, there is one report of the presence of cholesterol in *Polystichum filix mas*.[16] The major sterol of *Polypodium vulgare* is β-sitosterol,[17,18] accompanied by smaller amounts of another sterol which on GLC had the same retention time as campesterol.[18] The GLC data showed no peak with R_T corresponding to cholesterol at levels not higher than 1 per cent of the β-sitosterol content. In the hope of shedding some light on the possible intermediacy of cholesterol in the biosynthesis of the phytoecdysones, we isolated the sterols in the experiment in which [2-^{14}C]-mevalonic acid was incorporated into ecdysterone and separated them into individual sterols by reversed phase TLC. By both the spark chamber and scintillation counting techniques, no significant levels of radioactivity above background were found associated with the band having the same R_f as cholesterol. As can be seen from Table 2, only 0·24 per cent of the labelled cholesterol absorbed by the plant is accounted for

TABLE 2. FATE OF [4-^{14}C]-CHOLESTEROL IN *P. vulgare*

Fraction	Total activity (d.p.m.)
Fed to the leaves of the plant	$2{\cdot}2 \times 10^7$
In washings of leaves before processing the plant	$5{\cdot}14 \times 10^5$
In isolated sterol fraction	$3{\cdot}38 \times 10^2$
In phytoecdysones	$5{\cdot}20 \times 10^4$

in the phytoecdysone and sterol fraction. This would suggest either that cholesterol is be rapidly metabolized in the plant tissue or rapidly photooxidized, or both.

The chromatoplate developed by the reverse phase technique for the separation of sterols in Exp. 4 also showed that no significant radioactivity was associated with bands having the same mobility as those of campesterol and β-sitosterol, the dominant sterols of *P. vulgare*. The spark chamber photograph of the plate, however, revealed a faint radioactive

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[12] EL S. AMIN, O. AWAD, M. ABD EL SAMAD and M. N. ISKANDER, *Phytochem.* **8**, 295 (1969); C. E. COOK, M. E. TWINE, C. R. TALLENT, I. HARPER, G. HEUNISCH, J. B. LEWIS and M. E. WALL, *Phytochem.* **8**, 1025 (1969); A. M. GAWIENOSKI and C. C. GIBBS, *Steroids* **12**, 545 (1968); R. J. KEMP and E. I. MERCER, *Biochem. J.* **110**, 119 (1968); E. RICHARDSON, J. R. BAUR, R. S. HALLIWELL and R. LANGSTON, *Steroids* **11**, 231 (1968); N. J. DE SOUZA and W. R. NES, *Science* **162**, 363 (1968).
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[14] D. F. JOHNSON, R. D. BENNETT and E. HEFTMANN, *Science* **140**, 198 (1963).
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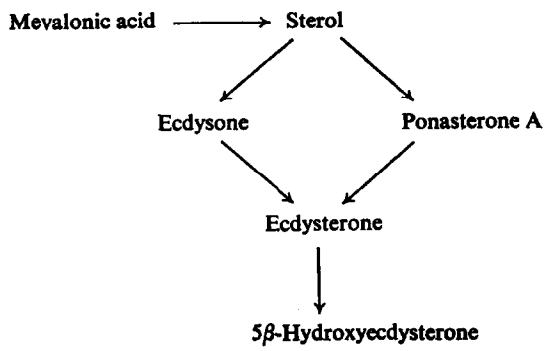
[17] G. BERTI and F. BOTTARI, in *Progress in Phytochemistry*, (edited by L. REINHOLD and Y. LIWSCHITZ), Vol. 1, p. 589, Interscience, New York, (1968).
[18] N. J. DE SOUZA, E. L. GHISABERTI, H. H. REES and T. W. GOODWIN (unpublished results).

band with an R_f corresponding to that of 7-dehydrocholesterol. When the radioactivity was diluted with authentic 7-dehydrocholesterol and recrystallized, a sample of constant specific radioactivity ($5{\cdot}4 \times 10^3$ cpm/mmole) was obtained. Since the total counts associated with this sample was very small, we hope later to confirm this finding by a more rigorous verification of the radioactive purity of the 7-dehydrocholesterol. The only reports[19] of $\Delta^{5,7}$-sterol systems in plants are those of ergosterol and 7-dehydrostigmasterol. However, a common structural feature of the phytoecdysones is the double bond at C-7, and the involvement of $\Delta^{5,7}$-sterol as an intermediate in their biosynthesis is possible. 7-Dehydrocholesterol has been suggested as a precursor of ecdysones in insects and by analogy with the known conversion by nsects of Δ^5- to $\Delta^{5,7}$-sterols,[20] Heftmann *et al.*[9] have suggested that a similar pathway may be operative in plants for the phytoecdysones. The combined results of this work and those we reported earlier[2] are in agreement with the possible operation of a sequence such as that shown in Scheme 1 for the biosynthesis of the phytoecdysones.



Ecdysone is converted into ecdysterone in insects and crustaceans,[21] and this precursor–product relationship has also been suggested[22] for the plant system.

EXPERIMENTAL

General

M.ps were determined on a Kofler block. Radioactivity measurements were made on a Beckman LS 200 B liquid scintillation counter. The samples were dissolved in 20–50 μl methanol before addition of 15 ml of a scintillator solution of toluene containing 5·0 g of 2,5-diphenyloxazole and 0·3 g of *p*-bis-(4-methyl-5-phenyl-oxazol-2-yl) benzene per 1 l. Chromatoplates were scanned for radioactivity on a model RTLS-IA scanner (Panax Equipment Ltd., Redhill, Surrey). The spark chamber technique[23] was also used to locate radioactive zones. We are grateful to Dr. F. W. Hemming for help in using this apparatus.

Materials

Ecdysone was kindly supplied by F. Hoffmann-La Roche and Co. Ltd., Basel, Switzerland. Ecdysterone was isolated by us from the rhizomes of *Polypodium vulgare* and was identical (m.p., u.v., i.r., NMR, and m.s.) with an authentic sample.

Plants, Incubation Conditions and Feeding Techniques

Wild *P. vulgare* plants were collected in the neighbourhood of Liverpool, potted in soil from their source and grown either in a greenhouse at Ness Botanical Gardens, Cheshire, or in our laboratories.

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[20] W. E. ROBBINS, M. J. THOMPSON, J. N. KAPLANIS and T. J. SHORTINO, *Steroids* **4**, 635 (1964).
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[22] T. TAKEMOTO, Y. HIKINO, S. ARIHARA and H. HIKINO, *Tetrahedron Letters* 2475 (1968).
[23] B. R. PULLAN, in *Quantitative Paper and Thin-layer Chromatography* (edited by E. J. SHELLARD), p. 123, Academic Press, New York (1968).

With sliced tissue, the incubation procedure of Goad and Goodwin[24] was followed. With whole plants, labelled mevalonate was administered to fresh tissue[2,25] over 20 days. [2-^{14}C]DL-Mevalonic acid lactone (1 μc) in aqueous Nonidet P 42 (0·2 ml of a 0·01 % (v/v) solution) was applied as microdrops to the leaves at the upper third portion of five newly emerged stems. The average length of the stems from the rhizomes to the tip of the stems was approx 25 cm. Similar applications were made every alternate day until 10 μc of mevalonic acid lactone had been deposited.

Labelled cholesterol was administered to whole plants by applying [4-^{14}C]-cholesterol (1 μc), in 0·2 ml of an 80 % ethanol solution containing 0·05 % (v/v) Tween 80, to the leaves as just described. When the droplets were no longer visible (*ca.* 15 min), the leaves were sprayed with a 10 % (v/v) solution of silicone fluid, MS 200, in light petroleum.[26] Similar applications were made twice a week until 10 μc substrate had been deposited on the leaves.

Chromatographic Procedures

a. Column chromatography. Crude extracts were adsorbed on celite and placed on top of a column of neutral alumina act. III (ratio 100:1). The column was eluted with benzene and benzene–methanol (3, 5, 10, 20, 50, 70 and 100 %); the volume used of each eluent was twice the hold-up volume of the column. The fractions were analysed by TLC. Ecdysterone was found mainly in the 50 % methanol–benzene fraction.

b. Thin-layer chromatography. (i) For the phytoecdysones and their acetates plates of silica gel G (Merck) (0·25 mm) were used. Plates were developed in EtOAc–EtOH (4:1) and/or $CHCl_3$–EtOH–Acetone (100:33:16·5) for the phytoecdysones and in EtOAc–hexane (4:1) for their acetates. The compounds were located by spraying with H_2SO_4, heating at 110° and visualized in u.v. light. For preparative purposes only the edge of the plates were sprayed. The compounds were eluted with warm MeOH. (ii) For the separation of non-saponifiable lipids, plates of silica gel G (0·25 mm, impregnated with Rhodamine 6G) were used. The plates were developed in CH_2Cl_2–acetone (9:1) and the compounds eluted with ether. (iii) For reversed phase TLC separation of sterols, Kieselguhr G impregnated with paraffin was used.[27] Plates were developed in acetone–water (4:1), and the compounds located by spraying with a 10 % solution of Rhodamine 6G in acetone. After elution of the compounds with ether, the paraffin was removed by chromatography on a neutral alumina column (act. II).

Isolation, Purification and Identification of Radioactive Phytoecdysones

At the end of the feeding period the experimental materials were processed essentially by the method of Heftmann *et al.*[9] In the experiment using cholesterol as substrate the leaf surfaces were washed with a spray of benzene before the plants were processed and the radioactivity in the washings determined. The phytoecdysones were isolated by column chromatography and separated from each other by preparative TLC. The radioactive material with the same R_f as authentic ecdysterone was repeatedly purified by preparative TLC. The ecdysterone content of the eluates was determined by u.v. spectroscopy after each purification and the specific activity calculated. When this was found not to drop significantly, the material was crystallized to constant m.p. and specific activity and compared (m.p., mass, u.v. and i.r. spectra) with authentic ecdysterone. When measurable levels of radioactivity were found associated with the metabolite having the same R_f as ecdysone, the metabolite was diluted with authentic ecdysone and crystallized to constant m.p. and specific activity. Ecdysterone and ecdysone were identified further by conversion to the corresponding 2,3,22-triacetates and crystallization of these derivatives to constant m.p. and specific activity. Radioactive 5β-hydroxyecdysterone was identified by comparison of its mobility on TLC with that of an authentic sample. Furthermore, after acetylation, the radioactivity was found associated with material which had the same R_f as the major product obtained from a similar reaction on authentic 5β-hydroxyecdysterone. Lack of carrier compound prevented recrystallization of the radioactive material to constant specific activity.

Isolation and Separation of Radioactive Sterols

The hexane extract obtained during the processing of the whole plants was combined with the residues eluted from the chromatographic column before elution of the phytoecdysones. The extract was saponified with 10 % ethanolic KOH, the unsaponifiable lipids separated in the usual manner and the sterols isolated by preparative TLC. When necessary, the sterols fraction was then separated into its components by preparative reversed phase TLC.

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