Phytochemistry, 1971, Vol. 10, pp. 2987 to 2990. Pergamon Press. Printed in England.

SHORT COMMUNICATION

Δ^4-β-SITOSTEN-3-ONE FROM β-SITOSTEROL BY LEAF HOMOGENATES

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(*Received* 1 *January* 1971)

Abstract—β-Sitosterol-4-^{14}C is metabolized to Δ^4-β-sitosten-3-one by *Cheiranthus cheiri* leaf homogenates. Greater than 60% conversion occurs within 2 hr. Under identical conditions, leaf homogenates of *Strophanthus kombé* fail to metabolize β-sitosterol, while *Digitalis purpurea* leaf homogenates yield only very small amounts of the metabolite.

INTRODUCTION

ALTHOUGH β-sitosterol is one of the predominant sterols of higher plants, until recently there was no evidence that it was further metabolized to other steroids in developing plants. Bennett *et al.*[1] have no shown that β-sitosterol-3-^{14}C can serve as the starting material for progesterone, digitoxigenin, digoxigenin and gitoxigenin in the leaves of *Digitalis lanata*. Cholesterol, the most prevalent sterol in animal systems, has repeatedly been shown to function as a precursor in the biosynthesis of a variety of plant steroids,[2,3] although it has been isolated from only a relatively small number of plants. We have investigated the metabolism of β-sitosterol-4-^{14}C by leaf homogenates of *Cheiranthus cheiri*, *Digitalis purpurea* and *Strophanthus kombé*.

RESULTS

Leaf homogenates of *Cheiranthus cheiri*, *Digitalis purpurea* and *Strophanthus kombé* were incubated with β-sitosterol-4-^{14}C in buffer and then extracted. The extracts were

TABLE 1. Δ^4-SITOSTEN-3-ONE FROM β-SITOSTEROL-4-^{14}C

Leaf homogenate source	Extracted radioactivity as Δ^4-β-sitosten-3-one (%)
Control	1·48
Cheiranthus cheiri	61·59
Cheiranthus cheiri, heated	0·45
Digitalis purpurea	2·89
Strophantus kombé	0·40

The homogenate from one g of plant leaf was incubated with 0·10 μc β-sitosterol-4-^{14}C in the presence of an NADPH generating system for 2 hr. The reaction mixtures were extracted, concentrated and chromatographed on TLC plates. The results are expressed as the percentage of the total extractable ^{14}C present as Δ^4-β-sitosten-3-one. Each value represents the average of 3–6 experiments.

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[2] E. HEFTMANN, *Lloydia* **31**, 293 (1968).
[3] H. SINGH, V. K. KAPOOR and A. S. CHAWLA, *J. Sci. Ind. Res.* **28**, 339 (1969).

concentrated and examined by TLC. Initial studies with *C. cheiri* indicated that β-sitosterol (I) was converted to a less polar metabolite, with maximum conversion occurring in approximately 2 hr. This metabolite was co-chromatographed in three TLC systems with Δ^4-β-sitosten-3-one (II), giving identical R_fs.

The abilities of leaf homogenates from *C. cheiri*, *D. purpurea*, and *S. kombé* to metabolize β-sitosterol are given in Table 1. In 2 hr, 61% of the extracted radioactivity was present as Δ^4-β-sitosten-3-one following incubation with leaf homogenates of *C. cheiri*. Heating the *C. cheiri* homogenates for 10 min at 100° completely destroyed the metabolic activity. Homogenates from young leaves (4–6 months old) of *S. kombé* were unable to metabolize β-sitosterol following incubation times up to 6 hr. *D. purpurea* leaf homogenates yielded only very small amounts of Δ^4-β-sitosten-3-one as compared to flasks devoid of leaf homogenate.

Further evidence that the metabolite was Δ^4-β-sitosten-3-one was obtained by isolating the metabolite from *C. cheiri* by preparative TLC and co-crystallizing it to constant specific activity with nonradioactive Δ^4-β-sitosten-3-one (Table 2).

TABLE 2. CO-CRYSTALLIZATION OF Δ^4-β-SITOSTEN-3-ONE TO CONSTANT SPECIFIC ACTIVITY

Solvent	Counts/min/mg
95% Ethanol	767 ± 17
Acetone–90% methanol	725 ± 20
Petroleum–methanol	761 ± 41
Acetone–90% methanol	721 ± 34

Δ^4-β-Sitosten-3-one-4-^{14}C which had been prepared from β-sitosterol-4-^{14}C by incubation with *C. cheiri* leaf homogenates was isolated by preparative TLC. Non radioactive-Δ^4-β-sitosten-3-one was synthesized from β-sitosterol by Oppenauer oxidation. The Δ^4-β-sitosten-3-one-4-^{14}C was co-crystallized to constant specific activity with the non-radioactive material. Quadruplicate aliquots were counted in a Beckman LS-100 liquid scintillation counter. The results are expressed as the average counts per minute per milligram with the standard deviation.

DISCUSSION

The results indicate that β-sitosterol can be metabolized by plant enzymes. The metabolite Δ^4-β-sitosten-3-one may be a necessary intermediate in the metabolism of β-sitosterol to other plant steroids. For example, Tschesche *et al.*[4] have shown that the biosynthesis of spirostanols as tigogenin and gitogenin from cholesterol must pass through Δ^4-cholesten-3-one.

β-Sitosterol has been isolated from numerous sources and is frequently found as the glycoside or ester. In the wood of several plants, Δ^4-β-sitosten-3-one has been found in conjunction with β-sitosterol,[5,6] and may have escaped detection in many cases. 3β-Sitostanol[6,7] and sitostene[8] have also been isolated in association with β-sitosterol. In

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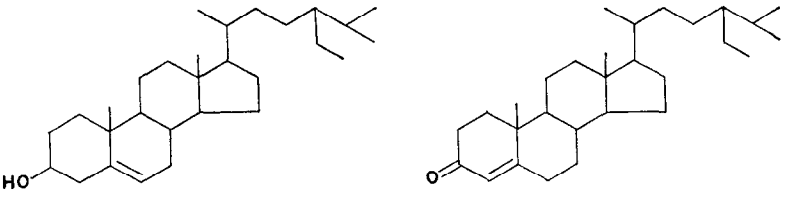

(I) β-Sitosterol (II) Δ^4-β-Sitostene-3-one

potato leaves, Δ^4-cholesten-3-one has been identified as an intermediate in the conversion of cholesterol to 3β-cholestanol.[9] Whether β-sitosterol can directly serve as a precursor for many types of plant steroids, as has been shown for cholesterol, remains to be determined. It is apparent that the conversion of β-sitosterol to Δ^4-β-sitosten-3-one under our experimental conditions does not occur readily in all plant species that contain steroids. The reason for this difference is not known, but may be related to the relative significance of this reaction in the subsequent metabolism of β-sitosterol to other steroids.

EXPERIMENTAL

Homogenates. Leaves from 4–6 month old plants of *Cheiranthus cheiri*, *Digitalis purpurea* or *Strophanthus kombé* were homogenized in a buffer medium containing 0·25 M sucrose, 0·05 M tris chloride pH 7·4, 0·005 M $MgCl_2$, 0·003 M L-cysteine HCl, 0·045 M mercaptoethanol, and 1 mg/ml bovine serum albumin (Sigma Chemical Co.). Homogenization was performed in an ice bath using a Sorvall Omni-Mixer Homogenizer operated at maximum speed for two 20-sec intervals. Thirty per cent homogenates were prepared. Homogenates were filtered through one layer of muslin under vacuum and the filtrate used as the enzyme source.

Incubation. β-Sitosterol-4-^{14}C having a specific activity of 61 mg/mM was obtained from Amersham-Searle Corp. and was shown by TLC to have a radiochemical purity of greater than 99 per cent. The homogenate from one gram of plant leaves was incubated with 0·10 μC β-sitosterol-4-^{14}C, to which was also added 7·0 mg glucose-6-phosphate, 1·5 mg $NADP^+$, 2·5 units glucose-6-phosphate dehydrogenase (Sigma Chemical Corp.), and sufficient homogenization buffer medium to give a volume of 5·0 ml. The β-sitosterol-4-^{14}C was added to the reaction flask in 0·10 ml of 70% EtOH. All incubations were performed at 30° on a water bath shaker, with the flasks being aerated with 95% O_2–5% CO_2. Results for 2-hr incubations are reported in Table 1.

The reaction mixtures were extracted with 40 ml EtOAc–HOAc (100:1) for 1 hr on a shaker and the resulting emulsion broken by centrifugation. The organic phase was removed, dried over Na_2SO_4 and evaporated to dryness *in vacuo*. The extraction efficiency was 84 ± 9%.

Δ^4-β-Sitosten-3-one identification. Aliquots of the extracts were co-chromatographed with the reference standards β-sitosterol and Δ^4-sitosten-3-one on silica gel H (Brinkman) TLC plates divided into 2-cm wide columns, developing three successive times with diisopropyl ether–petroleum–HOAc (70:30:1), or once with either CH_2Cl_2–MeOH (97:3) or benzene–EtOAc (4:1). The reference standards were located by exposing the developed TLC plates to I_2 vapors. The I_2 was allowed to evaporate and the areas corresponding to the standards as well as the remainder of each column were transferred to counting vials with the aid of a razor blade. Toluene counting solution was added and the samples were counted in a Beckman LS-100 liquid scintillation counter. Samples were normally counted for 20 min, while samples containing less than 1000 counts/min above background were counted for longer periods of time. A background count of 10–15 counts/min was routinely obtained for ^{14}C.

Δ^4-β-Sitosten-3-one synthesis. Δ^4-β-Sitosten-3-one was synthesized by the Oppenauer oxidation process.[10] 2 g of β-sitosterol were dissolved in a dry mixture of 15 ml acetone and 30 ml benzene with 8·0 g aluminum tert.-butoxide, and the mixture was refluxed for 8 hr. Upon cooling, 4 ml water was added, following by 10 ml 10% H_2SO_4 with vigorous shaking, and then 30 ml water. The organic phase was removed, and the aqueous phase was shaken out several times with benzene. The pooled organic phase was then dried,

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and concentrated *in vacuo*. TLC of the resulting oil on Adsorbosil-5, developing with hexane–ether (5:2), revealed that the reaction was not complete. Crystallization failed to improve the purity. The mixture was acetylated by refluxing for 1 hr in 5 ml Ac_2O, 10 ml benzene and 0·5 ml pyridine. The mixture was cooled and excess Ac_2O was decomposed with methanol. The mixture was concentrated at 40° under vacuum, 5 ml water added, and extracted three times with benzene. The pooled benzene extracts were washed with water and evaporated to dryness. TLC of the product revealed only the presence of Δ^4-β-sitosten-3-one and β-sitosterol acetate.

Column chromatography. The acetylation mixture was chromatographed on a 100 g Adsorbosil-5 column, collecting 20 ml fractions. The column was eluted sequentially with 350 ml hexane–ether (9:1), 500 ml hexane–ether (5:1), and finally with hexane–ether (5·2). β-Sitosterol acetate was eluted in fractions 23–25 while pure Δ^4-β-sitosten-3-one was obtained in fractions 27–32. The Δ^4-β-sitosten-3-one was recrystallized from acetone–methanol, yielding 368 mg of crystalline product melting at 84–86°, which agrees with that previously reported.[7,11]

Acknowledgements—The authors gratefully acknowledge the technical assistance of Miss Jorja Sturek. These investigations were supported in part by research grant GB-13232 of the National Science Foundation, Washington, D.C., and U.S.P.H.S. grant AM-13803.

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