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INCORPORATION OF 4-^{14}C-22,23-^{3}H-SITOSTEROL INTO DIOSGENIN BY *DIOSCOREA DELTOIDEA* TISSUE SUSPENSION CULTURES

S. J. STOHS, J. J. SABATKA and H. ROSENBERG

Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Nebraska, Lincoln, NB 68508, U.S.A.

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Key Word Index—*Dioscorea deltoidea*; Dioscoreaceae; suspension cultures; diosgenin biosynthesis; sitosterol; C-26 oxygenation.

Abstract—Sitosterol-4-^{14}C-22,23-^{3}H with a ^{3}H/^{14}C ratio of 5·0 was incorporated into diosgenin such that the ^{3}H/^{14}C ratio in diosgenin was approx. 2·3. The per cent of ^{14}C incorporation was 0·92% and for ^{3}H was 0·42%. The results indicate that C-23 is not involved in the transformation of sitosterol into diosgenin. The first step in the cyclization of the sterol side-chain may either involve oxygenation at C-26 or direct hydroxylation at C-22 via a mixed function oxidase system. Other indirect evidence suggests that the C-26 oxygenation mechanism is operative.

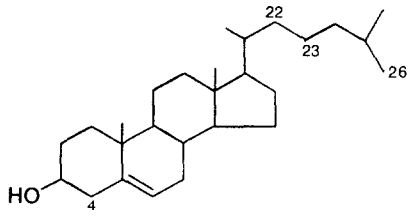
INTRODUCTION

A NUMBER of radioactive precursors have been shown to be incorporated into diosgenin (**2**) by *Dioscorea* plants, tuber homogenates, and tissue cultures. Such precursors include: 2-^{14}C-acetate,[1] 2-^{14}C-mevalonate,[2] 4-^{14}C-cholesterol,[3] 4-^{14}C- and 26-^{14}C-cholesterol (**1**),[4] 4-^{14}C, 25-^{3}H-cholesterol,[5] and 26-^{14}C-26-hydroxycholesterol.[6] The sequence in which oxygen is introduced at positions 16, 22 and 26 constitute major unanswered questions regarding the biosynthesis of diosgenin. The incorporation of cholesterol-4-^{14}C-25-^{3}H-cholesterol indicates that stereospecific oxygenation at C-26 is not dependent upon the presence of a Δ^{24}-double bond.[5] Furthermore, the incorporation of 26-hydroxycholesterol-26-^{14}C suggests that oxygenation at C-26 may be the first step in diosgenin biosynthesis from cholesterol.[6] *Digitalis lanata* plants do not incorporate either 22-keto- or 22-hydroxy-cholesterol-23-^{14}C into sapogenins,[7] providing indirect support for the theory that oxygenation occurs first at C-26.

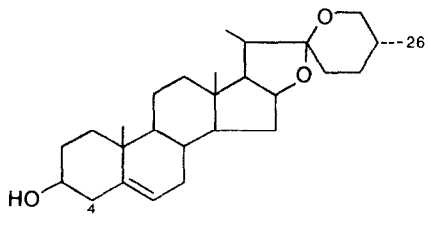
The most common sterol among higher plants is sitosterol (**3**), a C_{29} sterol. No previous studies have been reported on the incorporation of sitosterol into diosgenin, a C_{27} sapogenin. We have previously observed sitosterol as one of the major sterols in suspension cultures of *Dioscorea deltoidea* (unpublished observations). We have incubated suspension cultures of *D. deltoidea* with 4-^{14}C-22,23-^{3}H-sitosterol in an attempt to answer two questions: (1) can sitosterol serve as a precursor of diosgenin, and (2) if sitosterol is incorpor-

[1] HEFTMANN, E., BENNETT, R. D. and BONNER, J. (1961) *Arch. Biochem. Biophys.* **92**, 13.
[2] BENNETT, R. D., HEFTMANN, E., PRESTON, W. H. and HAUN, J. R. (1963) *Arch. Biochem. Biophys.* **103**, 74.
[3] BENNETT, R. D. and HEFTMANN, E. (1965) *Phytochemistry* **4**, 577.
[4] STOHS, S. J., KAUL, B. and STABA, E. J. (1969) *Phytochemistry* **8**, 1679.
[5] JOLY, R. A., BONNER, J., BENNETT, R. D. and HEFTMANN, E. (1969) *Phytochemistry* **8**, 1709.
[6] BENNETT, R. D., HEFTMANN, E. and JOLY, R. A. (1970) *Phytochemistry* **9**, 349.
[7] TSCHESCHE, R., HULPKE, H. and FRITZ, R. (1968) *Phytochemistry* **7**, 202.

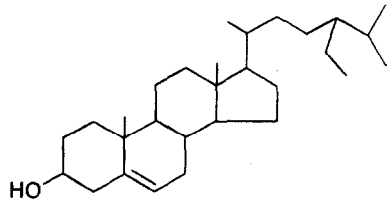
ated into diosgenin, what is the ratio of the $^{3}H/^{14}C$ that is incorporated and does oxygenation occur first at C-22 or C-26?



(1) Cholesterol



(2) Diosgenin



(3) Sitosterol

RESULTS AND DISCUSSION

Following incubation of 4-^{14}C-22,23-^{3}H-sitosterol in *D. deltoidea* suspension cultures for 30 days, the diosgenin fraction was isolated following acid hydrolysis, and contained both ^{14}C and ^{3}H when co-chromatographed with nonradioactive diosgenin in two solvent systems. The distribution of radioactivity in the various extraction fractions as well as the $^{3}H/^{14}C$ ratios is given in Table 1. The diosgenin was acetylated and the radioactivity was shown to co-chromatograph with standard diosgenin acetate in three TLC systems. The radioactive diosgenin acetate was subsequently co-crystallized to constant specific activity, thus verifying the incorporation of the sitosterol into diosgenin (Table 2).

TABLE 1. DISTRIBUTION OF RECOVERED RADIOACTIVITY FOLLOWING INCUBATION OF *Dioscorea deltoidea* SUSPENSION CULTURES WITH 4-^{14}C, 22,23-^{3}H-SITOSTEROL

Fraction	% Recovered ^{3}H	% Recovered ^{14}C	Ratio dpm $^{3}H/^{14}C$
Pre-hydrolysis tissue extract	53·6%	59·1%	4·7
Post-hydrolysis tissue extract	43·6	36·1	5·2
Hydrolysis filtrate	<0·1	<0·1	5·0
Culture medium	2·4	3·9	4·2
Diosgenin	0·41	0·92	2·3

Following the incubation of *D. deltoidea* suspension cultures with 4-^{14}C-22,23-^{3}H-sitosterol for 30 days, the tissue, medium, acid hydrolyzed tissue and hydrolysis filtrate were extracted as described under Experimental. The per cent total extractable radioactivity and the ratio of the dpm's $^{3}H/^{14}C$ in each fraction is given in the table.

The $^3H/^{14}C$ ratio of the administered sitosterol was approx. 5·0, while the $^3H/^{14}C$ ratio of the isolated diosgenin was approx. 2·3 (Table 1), indicating that some radioactivity from the starting material was lost. This loss of radioactivity is most probably due to loss of ^3H at the C-22 position. If oxygenation occurred at C-26 or if hydroxylation at C-22 via a mixed function oxidase followed by cyclization, both protons would be lost from C-22 and therefore a loss of one-half of the ^3H would be expected. However, if oxygenation occurred at C-22 and involved a $\Delta^{22,23}$ intermediate, all of the ^3H at C-22 and one-half of the ^3H at C-23 would be lost. As such, beginning with a $^3H/^{14}C$ ratio of 5·0 for sitosterol, with a C-26 oxygenation or a direct hydroxylation at C-22, one would expect a theoretical $^3H/^{14}C$ ratio of 2·5, while if oxygenation occurred at C-22 with a $\Delta^{22,23}$ intermediate a $^3H/^{14}C$ ratio of 1·25 would result. Our results indicate that oxygenation at C-26 or direct hydroxylation at C-22 occurs since we have obtained a $^3H/^{14}C$ ratio of 2·3 for the incorporated radioactivity in diosgenin.

TABLE 2. CO-CRYSTALLIZATION OF ^{14}C, ^3H-DIOSGENIN ACETATE TO CONSTANT SPECIFIC ACTIVITY

Solvent system	Specific activity (dpm/mg) ^{14}C	^3H
Me_2CO–$CHCl_3$	338 ± 12	751 ± 19
$CHCl_3$–MeOH	317 ± 13	740 ± 22
Me_2CO–$CHCl_3$	326 ± 15	762 ± 13

The tissue of *D. deltoidea* suspension cultures incubated for 30 days with 4-^{14}C-22,23-^3H-sitosterol was dried, powdered, $CHCl_3$ extracted, acid hydrolyzed and re-extracted with $CHCl_3$. Carrier diosgenin and sitosterol were added to the post-acid hydrolyzed $CHCl_3$ extract which was then acetylated, and the diosgenin acetate was isolated by column chromatography as described under Experimental. The isolated diosgenin acetate was recrystallized from the solvents listed above. Each value represents the mean of 3–5 aliquots ± the s.d.

Under identical conditions, we have obtained approximately 7·3% incorporation of both 4-^{14}C- and 26-^{14}C-cholesterol into diosgenin as compared to the present 0·9% incorporation of the 4-^{14}C- from sitosterol. This decreased efficiency of incorporation is not unexpected since two carbons must be removed from the side-chain of sitosterol while cholesterol, a C_{27} sterol can be incorporated directly into diosgenin. In addition, the amounts of sitosterol are much larger in *Dioscorea* than the amounts of cholesterol and other C_{27} sterols (unreported observations), thus diluting out our labeled substrate. The results clearly demonstrate that sitosterol can serve as a precursor for diosgenin, with the loss of two carbons from the side-chain. Our results are in agreement with the previous indirect evidence which supports the theory that in sapogenin biosynthesis oxygenation occurs first at C-26 followed by cyclization.[6,7]

EXPERIMENTAL

Culture incubations. Suspension cultures of *D. deltoidea* were grown and maintained as previously reported.[4,8,9] To each of 20–500 ml flasks containing 125 ml of 10-day-old culture was added one μc 4-^{14}C-sitosterol (Amer-

[8] KAUL, B., STOHS, S. J. and STABA, E. J. (1969) *Lloydia* **32**, 347.
[9] KAUL, B. and STABA, E. J. (1968) *Lloydia* **31**, 171.

sham/Searle, 61 mc/mM) + 5 μc 22,23-^{3}H-sitosterol (New England Nuclear, 30 c/mM). The tissues were harvested by filtration after 30 days, pooled, and freeze dried, yielding 30·8 g of dried tissue. The tissue was powdered and extracted with $CHCl_3$ in a Soxhlet for 20 hr. The remaining tissue (29·7 g) was air dried, powdered, and acid hydrolyzed using 30 ml 10% HCl per 2 g powdered tissue, reflexing for 2 hr. The hydrolyzed tissue was filtered, washed with distilled water, freeze dried, powdered, and extracted with $CHCl_3$ in a Soxhlet for 30 hr. The medium in which the tissue had been grown was freeze dried, powdered and Soxhlet extracted for 24 hr with $CHCl_3$. The filtrate and washings from the acid hydrolysis were adjusted to pH 5 and $CHCl_3$ extracted. The distribution of recovered radioactivity is given in Table 1.

Chromatography. Co-chromatography of the post-acid hydrolysis extract on silica gel H plates, developed in $CHCl_3$ (× 4) or C_6H_6–EtOAc (3:1, developed × 2), indicated that approx. 1% of the total recovered ^{14}C and 0·4% of the ^{3}H was present in diosgenin. To the post-hydrolysis extract was added 50 mg of diosgenin and 50 mg of sitosterol as carriers. This extract was then chromatographed on a 100 g silica gel 60 (Brinkmann) column which was eluted sequentially with the following solvents: 1000 ml $CHCl_3$; and 500 ml of 0·5, 1, 1·5, 2, and 4% MeOH in $CHCl_3$. A flow rate of approx. 1 ml/min was maintained, collecting 20 ml fractions. Fractions 33–37 contained a mixture of the diosgenin and sitosterol. These fractions were pooled, and evaporated to dryness. The pooled fractions were acetylated by adding 20 ml Ac_2O and 2 ml pyridine, and reflexing for 2 hr. The mixture was evaporated to approximately one-half original volume, 30 ml of methanol was added, and the mixture evaporated down to approximately one-fourth volume. The methanol additions and subsequent evaporations were repeated a total of four times. Distilled water (20 ml) which contained 0·8 ml of H_2SO_4 was added, and the mixture extracted (× 3) with 30 ml $CHCl_3$. The $CHCl_3$ extracts were pooled, dried, and concentrated. TLC on silica gel H plates developed with hexane-ether (9:1, developed 2 ×) or $CHCl_3$-ether (10:1) and on silica gel H plates containing 10% $AgNO_3$ developed 2 × with petrol-benzene (3:2), indicated that approx. 2·5% of the ^{14}C and 0·96% of the ^{3}H present in the acetylated post-acid hydrolysis fraction co-chromatographed with diosgenin acetate. The acetylated mixture was loaded on a 35 g silica gel 60 column, eluted with hexane–ether (9:1), collecting 10 ml fractions. Fractions 30–40 contained the diosgenin acetate. These tubes were combined and dried. The diosgenin acetate was recrystallized to constant specific activity and the results are given in Table 2.

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