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BIOSYNTHESIS OF DIOSGENIN FROM 26-HYDROXYCHOLESTEROL IN *DIOSCOREA FLORIBUNDA*

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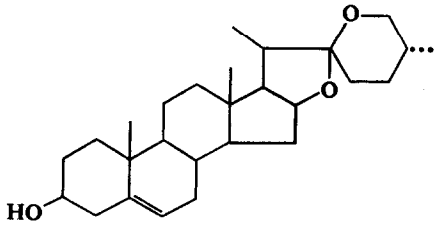
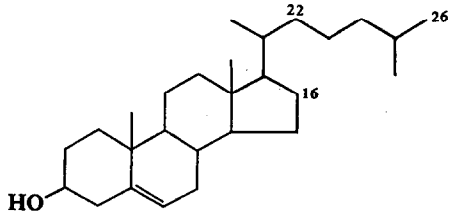
Abstract—26-Hydroxycholesterol-26-^{14}C was converted by a *Dioscorea floribunda* plant to diosgenin. This result suggests that oxygenation at C-26 may be the first step in the biosynthesis of diosgenin from cholesterol. No diosgenin was formed from kryptogenin-26-^{14}C by *D. composita*, nor was diosgenin-26-^{14}C converted to its C-25 epimer, yamogenin, by the latter plant.

INTRODUCTION

STEROIDAL sapogenins (spirostanols), e.g. diosgenin (I), are known to be made from cholesterol (II) in plants,[1] but the intermediate biosynthetic steps have not yet been elucidated. The incorporation of cholesterol-4-^{14}C-25-^{3}H into diosgenin[2] has demonstrated that the stereospecific oxygenation at C-26[3] is not dependent upon the presence of a Δ^{24}-double bond. However, the main questions involve the sequence in which introduction of oxygen at positions 16, 22, and 26 occurs. The evidence on this subject has thus far been indirect. Tschesche *et al.*[4] reported that neither 22-keto- nor 22-hydroxycholesterol-23-^{14}C were converted to sapogenins by *Digitalis lanata* plants. We have recently found that a furostanol, in which the F-ring was held open by glucosidation of the C-26 hydroxyl, was an intermediate in the conversion of cholesterol to diosgenin in *Dioscorea floribunda*.[5, 6] This suggests that



(I) Diosgenin



(II) Cholesterol

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[1] E. HEFTMANN, *Lloydia* **30**, 209 (1967).
[2] R. A. JOLY, J. BONNER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **8**, 1709 (1969).
[3] R. JOLY and C. TAMM, *Tetrahedron Letters* 3535 (1967).
[4] R. TSCHESCHE, H. HULPKE and R. FRITZ, *Phytochem.* **7**, 2021 (1968).
[5] R. A. JOLY, J. BONNER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **8**, 857 (1969).
[6] R. A. JOLY, J. BONNER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **8**, 1445 (1969).

C-26 is probably not the last of the three carbons to be oxygenated, since otherwise spontaneous ring closure to a spirostanol should occur before the glucoside unit could be attached. Thus, any oxygenation sequence beginning with C-22 or ending with C-26 seems unlikely. We have now studied this problem further by administering 26-hydroxycholesterol-26-^{14}C to a *D. floribunda* plant. This compound was prepared from kryptogenin-26-^{14}C, which we have recently synthesized.[7] Kryptogenin (Δ^5-cholestene-3β,26-diol-16,22-dione) is frequently found as a companion of diosgenin and has been postulated as a possible precursor of the latter.[8] We have, therefore, also treated a *D. composita* plant with kryptogenin-26-^{14}C. A second *D. composita* plant was treated with diosgenin-26-^{14}C,[7] in an effort to study its metabolism.

RESULTS AND DISCUSSION

No significant incorporation of radioactivity from kryptogenin into diosgenin or its C-25 epimer, yamogenin, was observed. Several radioactive components were detected in the extract by TLC, but under the hydrolytic conditions used in the workup, kryptogenin is largely destroyed. Since it would be difficult to differentiate actual metabolites of the latter from decomposition products, this was not attempted.

In contrast to Tschesche *et al.*,[4] who administered labeled tigogenin to two *Digitalis* species and recovered 80–95 per cent of it unchanged, we found that diosgenin was metabolized to a considerable extent by *Dioscorea composita*. However, the major metabolites of diosgenin could not be identified. About 1 per cent of the administered diosgenin was converted to the glycoside dioscin (diosgenin 2′-*O*,4′-*O*-bis-α-L-rhamnopyranosyl-β-D-glucopyranoside). No significant radioactivity was found to be associated with yamogenin.

26-Hydroxycholesterol-26-^{14}C was prepared by Clemmensen reduction of kryptogenin-26-^{14}C, according to the method of Scheer *et al.*,[9] and administered to a rather large *D. floribunda* plant. In order to avoid dilution of any diosgenin formed from the radioactive substrate by the large amount of diosgenin already present in the plant, the application of 26-hydroxycholesterol-26-^{14}C was restricted to four leaves of the plant, and only these leaves were worked up. After isolation of diosgenin acetate and yamogenin acetate by preparative TLC, each was diluted with carrier material and crystallized (Table 1). The diosgenin acetate, after an initial drop, attained constant molar specific activity, which was not changed upon conversion to diosgenin and further crystallization. The specific activity of the yamogenin acetate, however, decreased rapidly to a point where further crystallization was not feasible. The radioactivity associated with diosgenin represents 0·28 per cent of that originally administered to the plant. While the possibility that radioactive yamogenin was formed in very low yield (at most, 0·002 per cent) cannot be excluded, it seems most likely that the latter was, as the data indicate, unlabeled. The kryptogenin-26-^{14}C had previously been shown[7] to be a single isomer having the same configuration at C-25 as diosgenin, and therefore the 26-hydroxycholesterol-26-^{14}C must also have had this configuration, racemization at C-25 being unlikely under the conditions of the Clemmensen reduction. The results of this experiment suggest that the stereochemistry at C-25 of the sapogenins is fixed when cholesterol is oxygenated at one of the terminal methyl groups, and no interconversion of the isomers is possible thereafter. Our failure to find yamogenin as a metabolite of diosgenin-26-^{14}C supports this conclusion.

[7] R. D. BENNETT, H. H. SAUER and E. HEFTMANN, *J. Labeled Compounds* **5**, 160 (1969).
[8] E. HEFTMANN, *Lloydia* **31**, 293 (1968).
[9] I. SCHEER, M. J. THOMPSON and E. MOSETTIG, *J. Am. Chem. Soc.* **78**, 4733 (1956).

TABLE 1. RECRYSTALLIZATION OF RADIOACTIVE STEROIDS*

Compound	Solvent used for crystallization	Cpm/μmole†
A. Diosgenin acetate		23·2 ± 1·5
	MeOH	13·7 ± 1·0
	MeOH	12·3 ± 1·0
	EtOH	10·7 ± 1·0
	EtOH	10·9 ± 1·0
	Hexane	11·2 ± 1·0
Diosgenin	Acetone	11·7 ± 1·0
	Hexane–CH_2Cl_2	11·6 ± 1·0
B. Yamogenin acetate		14·4 ± 0·90
	MeOH	3·04 ± 0·66
	MeOH	0·64 ± 0·66

* 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% and background was 4 cpm.

† 90% confidence level.

The yield of diosgenin from 26-hydroxycholesterol in this experiment was somewhat lower than that obtained previously from cholesterol-4-^{14}C (0·50 per cent) in *D. floribunda*.[2] However, the plants used in the earlier experiment, being smaller and younger, were, presumably, more active biosynthetically. Furthermore, in the older plant used for the present work, yamogenin was the predominant sapogenin isolated, whereas the younger plants used earlier contained mostly diosgenin. It seems likely, therefore, that the conditions for observing the biosynthesis of diosgenin were less favorable when 26-hydroxycholesterol was used as a precursor. Our findings are consistent with the hypothesis that oxidation at one of the terminal methyl groups is the first step in the conversion of the saturated side-chain of cholesterol into the spiroketal moiety of the sapogenins. Depending on which one of the methyl groups is oxygenated, diosgenin or yamogenin is formed.

EXPERIMENTAL

Methods and Materials

TLC techniques were as described previously.[10] All chromatograms were run on silica gel G plates purchased from Analtech, Inc., Wilmington, Delaware.* Aliquots of radioactive samples were counted on planchets at infinite thinness under a gas-flow detector having an efficiency of 34% (see Table 1, legend, for details).

26-Hydroxycholesterol-26-^{14}C. The synthesis of kryptogenin-26-^{14}C described previously[7] was repeated, with two modifications which substantially improved the yield. In the preparation of the diazoketone, a glass tube was used instead of metal tubing for distillation of CH_2N_2, which previously appears to have undergone partial decomposition by contact with the metal edges. The rearrangement of the diazoketone was carried out by the method of Newman and Beal,[11] using silver benzoate as catalyst, except that the reaction mixture was refluxed for 5 min in 80% dioxane. This solvent gave a better yield than either MeOH or *t*-butyl alcohol and also produced the desired free acid directly. From 15·0 mg (0·25 mc) of *N*-methyl-^{14}C-*N*-nitroso-*p*-toluenesulfonamide† 0·4 mg (2·72 × 10^6 cpm) of kryptogenin-26-^{14}C was obtained.

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

† New England Nuclear Corporation.

[10] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **5**, 747 (1966).

[11] M. S. NEWMAN and P. F. BEAL, *J. Am. Chem. Soc.* **72**, 5163 (1950).

Zinc amalgam was prepared by shaking 75 mg of mossy zinc with 7·5 mg of $HgCl_2$, 125 μl of H_2O, and 3·5 μl of conc. HCl for 5 min. The aqueous solution was removed, and the zinc amalgam was washed with 100 μl of H_2O. A portion of the kryptogenin from above ($2{\cdot}17 \times 10^6$ counts/min) was dissolved in 100 μl of EtOH and added to the zinc amalgam, followed by 2μl of conc. HCl. The mixture was refluxed for 2 hr while a total of 20 μl of conc. HCl was added in 2 μl-portions. The EtOH was then removed under a stream of N_2, 200 μl of H_2O added, and the mixture extracted with three 400-μl portions of EtOAc. The extracts were washed with 200 μl of 5% $KHCO_3$ and 200 μl of H_2O, combined, and evaporated. The residue was subjected to preparative TLC with cyclohexane–EtOAc (2:3). Elution of the zone corresponding to 26-hydroxycholesterol gave $2{\cdot}00 \times 10^5$ cpm. This material, after further purification by preparative TLC with CH_2Cl_2-MeOH (47:3), gave $1{\cdot}17 \times 10^5$ cpm of 26-hydroxycholesterol-26-^{14}C, radiochromatographically homogeneous by TLC with CH_2Cl_2–acetone (4:1).

Administration of radioactive steroids. The ^{14}C-labeled compounds were each administered to the leaves of a *Dioscorea* plant in nine equal doses, twice weekly. Plant No. 1, a *D. composita* about 3 months old, was treated with a total of $3{\cdot}06 \times 10^5$ cpm of kryptogenin-26-^{14}C,[7] applied to the leaf surface as a solution in EtOH–dimethylsulfoxide (9:1). Plant No. 2, also a *D. composita* about 3 months old, was treated with a total of $2{\cdot}43 \times 10^5$ cpm of diosgenin-26-^{14}C,[7] by the technique previously described.[12] Plant No. 3, a *D. floribunda*, was about 1 yr old and was much larger than the other two. A total of $1{\cdot}04 \times 10^5$ cpm of 26-hydroxycholesterol-26-^{14}C was applied to four leaves of this plant by the technique previously described.[12]

Plant No. 1. Four days after the last treatment, the shoot portion of the plant was frozen in liquid nitrogen and lyophilized. The dry material (6·8 g) was homogenized in a blender with 90 ml of H_2O. The homogenate was then treated with 45 ml of conc. HCl and refluxed for 2 hr. The mixture was extracted with six 75-ml portions of CH_2Cl_2–Et_2O (3:1), and the extracts were washed successively with 50 ml of H_2O, 50 ml of 10% $KHCO_3$, and 50 ml of H_2O, combined, and evaporated, to give 563 mg ($1{\cdot}32 \times 10^5$ cpm). TLC of an aliquot of this material with CH_2Cl_2–acetone (9:1) showed two major radioactive peaks, one at the origin and one near the solvent front, and several smaller peaks, one of which corresponded to diosgenin, in between. The diosgenin was isolated and purified by preparative TLC in the same system to give 30 mg ($6{\cdot}96 \times 10^3$ cpm). This material was acetylated, and diosgenin acetate, together with yamogenin acetate, was isolated by preparative TLC in the same system as above, yielding 13 mg ($1{\cdot}80 \times 10^3$ cpm). The mixture was then purified by repeated preparative TLC with CH_2Cl_2–Et_2O (97:3) and isolation of the diosgenin acetate–yamogenin acetate zone. The radioactivity dropped continuously, and the experiment was abandoned when only 45 cpm remained associated with the sapogenin acetates.

Plant No. 2. Four days after the last treatment, the shoot system was frozen in liquid N_2 and lyophilized. The dried material (3·8 g) was worked up by the method described previously[5] to give a benzene extract (189 mg, $2{\cdot}38 \times 10^4$ cpm) and a butanol extract (308 mg, $6{\cdot}88 \times 10^3$ cpm). TLC of an aliquot of the benzene extract with CH_2Cl_2–acetone (9:1) showed three radioactive peaks of approximately equal intensity, one corresponding to diosgenin and the other two being much more polar. The latter two were not identical with any of the reference sapogenins available to us. The diosgenin was isolated by preparative TLC in the same system (0·3 mg, $8{\cdot}01 \times 10^3$ cpm), and a portion of this material ($3{\cdot}98 \times 10^3$ cpm) was acetlyated. TLC of the acetate with CH_2Cl_2–Et_2O (97:3) showed only one peak, corresponding in mobility to diosgenin acetate. No significant radioactivity was observed corresponding to yamogenin acetate.

An aliquot of the butanol extract, examined by TLC with CH_2Cl_2–MeOH–H_2O (28:12:3), showed a peak corresponding in mobility to dioscin. This material was isolated by preparative TLC in the same system to give 59 mg ($3{\cdot}08 \times 10^3$ cpm).

Plant No. 3. Four days after the last treatment, the four leaves to which 26-hydroxycholesterol-26-^{14}C had been applied were removed, frozen in liquid N_2, and lyophilized. The dry material (0·8 g) was homogenized in a tissue grinder with 10 ml. of H_2O. The homogenate was centrifuged and the supernatant liquid removed. The residue was then extracted by refluxing for 5 min, in succession, with 5 ml of 50%, 5 ml of 75%, and two 5-ml portions of 100% EtOH. These extracts were combined with the supernatant from above and concentrated to 1·0 ml. Then 0·5 ml of conc. HCl and 1·5 ml of EtOH were added to the concentrate, and the mixture was refluxed for 2 hr. The EtOH was removed under a stream of N_2, 1 ml of H_2O was added, and the mixture was extracted with two 3-ml portions of EtOAc. The extracts were washed with 1 ml of 10% $KHCO_3$ and 1 ml of H_2O, combined, and evaporated, to give 82 mg ($2{\cdot}89 \times 10^4$ cpm). This material was chromatographed on a 4-g column of Grade III alumina.* Fractions of 10 ml each were collected with the following eluents: 1–2, hexane; 3–4, 10%; 5–6, 25%; 7–8, 50% benzene in hexane; 9–12, benzene; 13–14, 10%; and 15–16, 50 % Et_2O in benzene. These fractions were assayed by TLC with CH_2Cl_2–MeOH (97:3). Fractions 10 and 11 (10 mg, $1{\cdot}80 \times 10^3$ cpm) contained diosgenin, which was isolated by preparative TLC in the same system to give 3·5 mg ($1{\cdot}10 \times 10^3$ cpm). This material was further purified by preparative TLC with cyclohexane–EtOAc (1:1), and yielded 2·7 mg (820 cpm) of diosgenin, which was acetylated. Preparative TLC by continuous development[13] with CH_2Cl_2 for 2 hr then separated diosgenin acetate (0·6 mg, 599 cpm) from

* Woelm, Eschwege, Germany.

[12] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 475 (1965).

[13] R. D. BENNETT and E. HEFTMANN, *J. Chromatog.* **12**, 245 (1963).

yamogenin acetate (1·8 mg, 270 cpm). Each of the two sapogenin acetates was diluted with carrier material and crystallized, as shown in Table 1. The radiochemically pure diosgenin acetate was then converted to diosgenin with $LiAlH_4$.[14]

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[14] R. D. BENNETT, E. HEFTMANN, W. H. PRESTON, JR., and J. R. HAUN, *Arch. Biochem. Biophys.* **103**, 74 (1963).