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THE MODE OF C-16 HYDROXYLATION IN THE BIOSYNTHESIS OF GITOXIGENIN IN *DIGITALIS PURPUREA*[1,2]

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Abstract—(16α-^{3}H); (4-^{14}C)-Pregnenolone (^{3}H:^{14}C ratio 3·15) was administered to two *Digitalis purpurea* plants and the biosynthesized digitoxigenin (^{3}H:^{14}C ratio 3·17) and gitoxigenin (^{3}H:^{14}C ratio 3·1) were isolated. The results indicate a direct replacement of the 16β-hydrogen of pregnenolone by a hydroxyl group in the biosynthesis of gitoxigenin.

INTRODUCTION

THE MODE of hydroxylation of steroids at non-activated secondary carbons by animal tissues and micro-organisms is relatively well understood.[3–6] Ample evidence is now available indicating that the incoming hydroxyl assumes the stereochemistry of the displaced hydrogen.[3–7] Rather surprisingly, little work was carried out on this topic in higher plants. In this paper we concern ourselves with the gross stereochemical aspects of hydroxylation of steroids in higher plants. It will be shown that the introduction of the 16β hydroxyl in the biosynthesis of gitoxigenin from pregnenolone in *Digitalis purpurea* proceeds by the direct displacement of the 16β proton.

RESULTS AND DISCUSSION

16α-^{3}H-Pregnenolone was prepared by the catalytic tritiation of 16-dehydropregnenolone acetate.[8] The product obtained was equilibrated with base and purified by paper and thin-layer chromatography. The homogeneous 16α-^{3}H-pregnenolone was mixed with 4-^{14}C-pregnenolone and an aliquot of this material (Ia) was diluted with nonradioactive material. The dilute (16α-^{3}H); (4-^{14}C)-pregnenolone (Ia) was crystallized several times and the ^{14}C specific activity and the ^{3}H:^{14}C ratio remained constant (Table 1). Equilibration of the

[1] Part X in the series "The Biosynthesis of Plant Sterols"; for part IX, see K. R. VARMA, J. A. F. WICKRAMASINGHE and E. CASPI, *J. Biol. Chem.* **244**, 3951 (1969).

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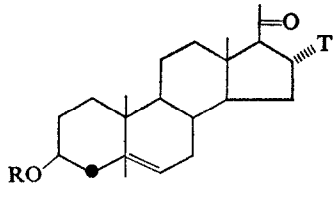
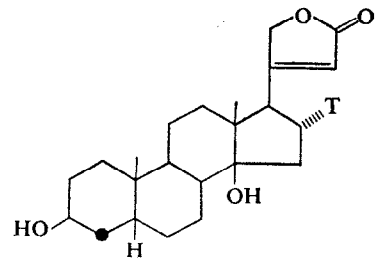
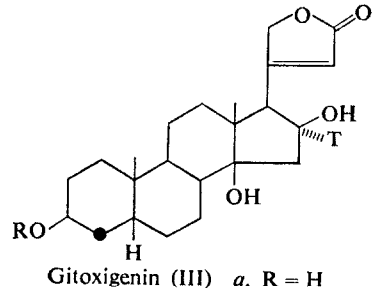


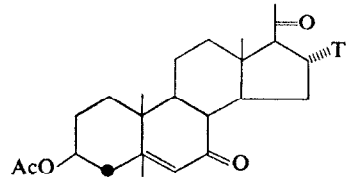
Pregnenolone (I) *a*. R = H
b. R = Ac



Digitoxigenin (II)



Gitoxigenin (III) *a*. R = H
b. R = Ac



(IV)

TABLE 1. THE ^{3}H:^{14}C RATIOS AND THE ^{14}C SPECIFIC ACTIVITIES OF DILUTED RADIOACTIVE PREGNENOLONE (1a) AND 7-KETO PREGNENOLONE ACETATE (IV)

	^{3}H:^{14}C ratio	dpm/mg of ^{14}C
Pregnenolone		
Crude	3·4	527
1st crystallization	3·1	550
2nd crystallization	3·1	551
3rd crystallization	3·2	539
4th crystallization	3·15	543
Pregnenolone (equilibrated)		
1st crystallization	3·1	563
2nd crystallization	3·1	550
7-Keto-pregnenolone acetate		
1st crystallization	3·1	500
2nd crystallization	3·1	530
3rd crystallization	3·05	538

mixed sample with base did not change the ^{14}C specific activity and $^{3}H:^{14}C$ ratio. To test whether isotopic hydrogen is present at the allylic carbon 7, the acetate (Ib) was converted to the 7-ketone[9] (IV). The oxidation proceeded without loss of isotopic hydrogen (Table 1).

The (16α-^{3}H); (4-^{14}C)-pregnenolone (13·8 μc ^{14}C) was administered to two *Digitalis purpurea* plants as previously described.[10] After 33 days, the plants were harvested and processed.[10,11] The distribution of radioactivity in the various extracts is given in Table 2. The residue of the chloroform extract, containing the bulk of the cardenolide-glycosides, was hydrolyzed.[10–12] The digitoxigenin (II) and gitoxigenin (IIIa) were isolated by a combination of chromatographic procedures described previously.[10,11,13] The identity and homogeneity of the two cardenolides were confirmed by co-crystallization of the free alcohols (II and IIIa) with cold materials to constant specific activity and constant $^{3}H:^{14}C$ ratios. The purity of the gitoxigenin was further corroborated by conversion to the acetate IIIb (Table 3).

It is evident that the $^{3}H:^{14}C$ ratios of the administered pregnenolone (Ia) ($^{3}H:^{14}C$ ratio 3·15), the digitoxigenin (II) ($^{3}H:^{14}C$ ratios 3·15) and the gitoxigenin (IIIa) ($^{3}H:^{14}C$ ratio 3·1) are essentially identical. Oxidation of the gitoxigenin ($^{3}H:^{14}C$ ratio 3·1) to gitoxigenone[14]

TABLE 2. DISTRIBUTION OF TRACERS IN EXTRACTS OF *D. purpurea* LEAVES AFTER THE ADMINISTRATION OF (16α-^{3}H)-(4-^{14}C)-PREGNENOLONE (Ia) (13·8 μc; 30·5 × 10^{6} dpm ^{14}C; $^{3}H:^{14}C$ ratio 3·15)

Extract	Radioactivity × 10^{-6} dpm	$^{3}H:^{14}C$ ratio
Leaf washings	4·5	3·3
Ligroin	0·08	3·1
Chloroform	6·07	2·7
Chloroform–ethanol (2:1)	0·89	2·3
Chloroform–ethanol (3:2)	2·13	2·5

Percent recovery of radioactivity (corrected for the unabsorbed activity): 35·42% (^{14}C) and 29·86% (^{3}H).

($3H:^{14}C$ ratio 0·6) proceeded with the loss of *ca.* 81 per cent of tritium (Table 3). Equilibration of the gitoxigenone with base removed the residual amount of the isotopic hydrogen which was located at C-15. The presence of tritium at C-15 is not surprising in view of the previous reports on the distribution of isotopic hydrogens in catalytically hydrogenated olefins.[15,16]

The results indicate that in the biosynthesis of gitoxigenin in *D. purpurea* the incoming 16β-hydroxyl assumes the stereochemistry of the displaced 16β-hydrogen. The gross aspects of the mechanism of hydroxylation of steroids at secondary carbons in *D. purpurea* are thus similar to those operating in animal tissues and microorganisms.

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TABLE 3. THE ^{3}H:^{14}C RATIOS AND ^{14}C SPECIFIC ACTIVITIES OF THE BIOSYNTHESIZED DIGITOXIGENIN (2), GITOXIGENINS (IIIa) AND THEIR DERIVATIVE

	^{3}H:^{14}C ratio	^{14}C dpm/mg
Digitoxigenin		
1st crystallization	3·15	1073
2nd crystallization	3·1	815
3rd crystallization	3·2	887
4th crystallization	3·2	867
5th crystallization	3·2	898
Gitoxigenin		
Crude	3·1	—
1st crystallization	3·0	725
2nd crystallization	3·0	631
3rd crystallization	3·1	547
4th crystallization	3·1	528
5th crystallization	3·0	525
Gitoxigenin acetate		
1st crystallization	3·1	592
2nd crystallization	3·2	463
3rd crystallization	3·3	445
4th crystallization	3·2	438
Gitoxigenone		
Purified by TLC	0·60, 0·6	
After base treatment	0·08	

EXPERIMENTAL

Chromatography

Silica gel (Merck $HF_{254+366}$) was used for TLC in the indicated solvents. Strips of Whatman No. 1 paper (40 cm long) were used for paper chromatography. In the case of Bush systems, chromatographic papers were pretreated prior to development.[13,17] A Vanguard Automatic Chromatogram Scanner, Model 880, was used for scanning the plates for radioactivity. The chromatographically purified precursors and products were further checked for homogeneity (and identity) by co-crystallization to constant specific activities (^{14}C) and ^{3}H:^{14}C ratios.

Counting[18]

Counting was carried out in a Packard Tri-carb automatic liquid scintillation spectrometer, series 314E. The samples were dissolved in 15 ml of a scintillator solution of toluene containing 4 g of 2,5-diphenyloxazole and 100 mg of *p*-bis-[2-(5-phenyloxazolyl)]-benzene per 1000 ml.

16α-*^{3}H-pregnenolone*[8] was obtained from Drs. Shlomo Burstein and Marcel Gut of this Foundation. The sample was purified by paper chromatography and TLC (benzene–EtOAc, 3:1) until homogeneous.

4-*^{14}C-Pregnenolone* was purchased from New England Nuclear Corporation, Boston, Massachusetts. The homogeneity of the compound was established by TLC in benzene–EtOAc (3:1).

Gitoxigenin and *digitoxigenin* were purchased from Fluka Company, Basel, Switzerland.

Administration of a Mixture of (16α-^{3}H);(4-^{14}C)-pregnenolone to Digitalis purpurea *Plants*

The purified 16α-^{3}H-pregnenolone (40 μc) and 4-^{14}C-pregnenolone (13·9 μc) were mixed and an aliquot (27,000 dpm of ^{14}C) was diluted with nonradioactive pregnenolone (50 mg). The sample was crystallized from EtOAc and the ^{14}C specific activity and ^{3}H:^{14}C ratio were constant in four crystallizations (Table 1). The remaining solids and mother liquors were pooled, dissolved in 80% aq. ethanol containing 5% potassium

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hydroxide (10 ml) and the mixture was refluxed for 3 hr. The recovered pregnenolone had an unchanged ^3H:^{14}C ratio (Table 1).

The undiluted (16α-^{3}H)-(4-^{14}C)-pregnenolone (13·8 μc; 30·5 × 10^6 dpm ^{14}C) (^{3}H:^{14}C ratio 3.15) was administered to two young *D. purpurea* plants as previously described.[10] After 33 days, the plant was cut above the soil and processed.[10,11,18] The distribution of the radioactivity in the various extracts is given in Table 2.

Hydrolysis of the Chloroform Extract[10–12]

An aliquot of the extract (2·02 × 10^6 dpm) was dissolved in methanol (4 ml), then H_2SO_4 (0·1 N; 4 ml) was added and the mixture was refluxed for 20 min. Most of the methanol was removed under reduced pressure and the remaining aqueous phase was warmed for 20 min at 70°. To the cooled solution a saturated solution of NaCl (10 ml) was added and the mixture was extracted with EtOAc (4 × 30 ml). The combined extract was washed sequentially with dilute saline, dilute aq. NaOH, again with saline, and then dried and reduced to a residue (2·02 × 10^6 dpm^{14}C).

Isolation of (16α-^{3}*H*)-(4-^{14}*C*)-*digitoxigenin* (*II*)

The above residue was fractionated on TLC (EtOAc–benzene, 4:1) and the products in the zones corresponding to II and IIIa were recovered (EtOAc, soxhlet).

The digitoxigenin fraction (2·9 × 10^5 dpm ^{14}C) was diluted with cold material (5 mg) and purified by TLC as previously described[10,11] to yield a homogeneous product (1·7 × 10^5 dpm ^{14}C). A portion of the radiochemically pure residue (4·6 × 10^4 dpm ^{14}C) was further diluted with cold material (38 mg) and crystallized several times from ethyl acetate. The results are summarized in Table 3.

Isolation of (16α-^{3}*H*)-(4-^{14}*C*)-*gitoxigenin* (*IIIa*). The residue of the crude gitoxigenin (IIIa) zone was fractionated by a combination of paper and thin-layer chromatography until it was homogeneous[10,11] (2·3 × 10^4 dpm ^{14}C). The residue was diluted with cold gitoxigenin and crystallized several times from EtOAc (Table 3).

(16α-^{3}*H*)-(4-^{14}*C*)-*gitoxigenin*-3-*monoacetate* (*IIIb*). Crystalline radioactive gitoxigenin (IIIa) (15 mg) was acetylated in the conventional manner (pyridine–Ac_2O at room temp for 16 hr). The product obtained (IIIb) was homogeneous by TLC (EtOAc–benzene, 1:1) and was crystallized from methanol (Table 3).

Oxidation of gitoxigenin.[14] Gitoxigenin (40 mg) was suspended in HOAc and then a mixture of CrO_3 (30 mg), water (0·2 ml) and HOAc (2 ml) was added and the mixture was stirred for 2 hr at ambient temperature. Within a few minutes the steroid dissolved. The reaction mixture was diluted with water and the product was extracted with $CHCl_3$ (3 × 75 ml). The combined extract was washed with water (4 × 25 ml), saline, dried and reduced to a residue (45 mg). The crude solid was fractionated by TLC (benzene–EtOAc, 1:1) and the product in the major zone was recovered (25 mg). The homogeneous residue resisted crystallization and an amorphous powder was obtained: ν_{max} ($CHCl_3$) 3460, 1780, 1745, 1705, 1630 cm^{-1}; λ_{max} (MeOH) 221 nm (ε, 7000); mass spectrum: *m/e* 368 (M-18; <1%), 325 (M-61; 27%), 324 (M-62; 100%), 310 (M-76, 8%), 309 (M-77, 33%).

To a solution of (16α-^{3}H)-(4-^{14}C)-gitoxigenin (IIIa) (15 mg) in pyridine (1·5 ml) a mixture of CrO_3 (50 mg) water (0·1 ml) and pyridine (1·5 ml) was added and the solution was stored for 16 hr at room remperature. The product was recovered in the conventional manner to yield, after purification by TLC (EtOAc–benzene, 1:1), gitoxigenone (9 mg). The product gave an identical i.r. and mass spectrum as that obtained in the cold run. The counting results are given in Table 3.

(16α-^{3}*H*)-(4-^{14}*C*)-7-*Keto-pregnenolone*-3β-*acetate* (*IV*)

A dilute sample of the radioactive ^{3}H:^{14}C pregnenolone (30 mg; 1·6 × 10^4 dpm ^{14}C) was acetylated in the usual manner. The homogeneous acetate (TLC, EtOAc–benzene, 1:3) was treated with a mixture of HOAc, Ac_2O and chromic acid by the method of Marshall *et al.*[9] The recovered product was submitted to TLC (EtOAc–benzene, 1:4) and then crystallized from EtOAc–Et_2O to yield the 7-ketone (IV) (21 mg); m.p. 154–155°; ν_{max} (KBr) 3390 (w), 1725, 1705, 1665, 1625, 1240 cm^{-1}; λ_{max} (MeOH) 235 nm (ε, 16,800) (reported:[9] m.p. 152/153°; λ_{max} (MeOH) 236 nm (ε, 13,200)). The ketone (IV) was crystallized to constant specific activity (^{14}C) and constant ^{3}H:^{14}C ratio (Table 1).

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