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METABOLISM OF 5β-PREGNANE-3,20-DIONE AND 3β-HYDROXY-5β-PREGNAN-20-ONE BY *DIGITALIS* SUSPENSION CULTURES*

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Key Word Index—*Digitalis purpurea*, Scrophulariaceae; tissue cultures; metabolism, glucosylation, 5β-pregnane-3,20-dione, 3β-hydroxy-5β-pregnan-20-one, 3α-hydroxy-5β-pregnan-20-one, 5β-pregnane-3β,20α-diol, 5β-pregnane-3β,20β-diol, 5β-pregnane-3α,20α-diol, steroid glucosides

Abstract—*Digitalis purpurea* normal callus suspension culture is capable of metabolizing 5β-pregnane-3,20-dione (**1**) to 3β-hydroxy-5β-pregnan-20-one (**2**), 3α-hydroxy-5β-pregnan-20-one (**3**), 3β-hydroxy-5β-pregnan-20-one glucoside (**7**) and 3α-hydroxy-5β-pregnan-20-one glucoside (**8**). *Digitalis purpurea* habituated callus suspension culture is also capable of metabolizing **1** to **2**, **3**, 5β-pregnane-3β,20β-diol (**5**), (**7**), (**8**), 5β-pregnane-3β,20α-diol monoglucoside (**9**) and 5β-pregnane-3α,20α-diol monoglucoside (**11**). Furthermore, it was observed that 3β-hydroxy-5β-pregnan-20-one (**2**) is converted to **7**, **9** and **11** by both suspension cultures. At the same time, **1**, **3**, **5** and **8** were detected in normal callus, while 5β-pregnane-3β,20α-diol (**4**) and 5β-pregnane-3β,20β-diol monoglucoside (**10**) were present in the habituated callus culture.

INTRODUCTION

In recent years the biosynthesis of cardenolides has been investigated in several laboratories [1–3]. In *Digitalis lanata* plants pregnenolone was metabolized to 5β-pregnane-3,20-dione as well as cardenolides [4,5]. *Digitalis lanata* [6] and *Strophanthus kombe* [7] plants converted progesterone to 5β-pregnane-3,20-dione (**1**), 3β-hydroxy-5β-pregnan-20-one (**2**) and other cardenolide metabolites. Both **1** and **2** were incorporated into cardenolides [8]. Therefore, the following biosynthetic pathway for cardenolide production from pregnenolone has been proposed: pregnenolone → progesterone → 5β-pregnane-3,20-dione (**1**) → 3β-hydroxy-5β-pregnan-20-one (**2**) → 5β-pregnane-3β,14β-diol [9] → 3β,14β,21-trihydroxy-5β-pregnan-20-one [10] → digitoxigenin → digitoxin. The metabolism of progesterone by leaf homogenates [11], plant tissue culture [12–14] and microsomes from plant tissue cultures [15] has been reported but only 5α-metabolites were found without any 5β-metabolites In our earlier paper [16] we suggested the $\Delta^4 \rightarrow$ A/B *cis* stereospecific reduction to be an enzymatic control point. In order to investigate cardenolide production by plant tissue cultures, we have now examined the metabolism of 5β-pregnane-3,20-dione (**1**) and 3β-hydroxy-5β-pregnan-20-one (**2**) as key intermediates using two strains of *Digitalis purpurea* calluses. One strain, called normal, required auxins and cytokinins and the other, called habituated, did not require either auxins or cytokinins.

RESULTS

5β-Pregnane-3,20-dione (**1**) (total 810 mg) was incubated with *Digitalis purpurea* normal suspension callus cultures (total fr. wt 1220 g) for 5 days After harvest the callus and medium were extracted and 3 metabolic products were detected by TLC. These were isolated by silica gel column

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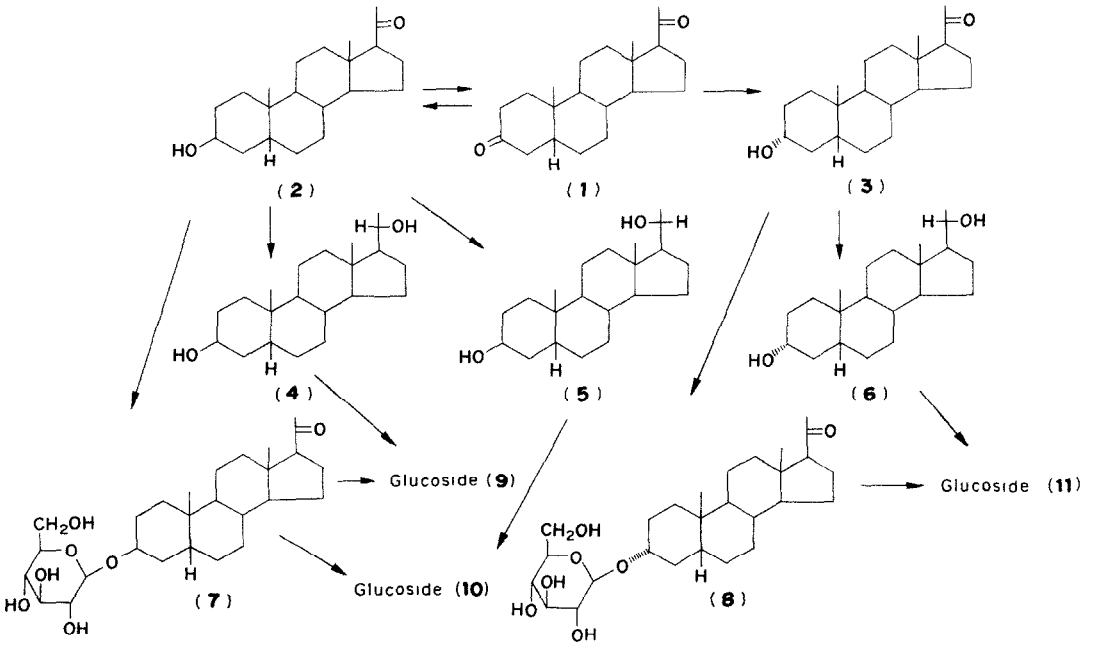

Scheme 1 Possible scheme for the metabolism of 5β-pregnane-3,20-dione and 3β-hydroxy-5β-pregnan-20-one by *Digitalis purpurea* normal and habituated calluses

chromatography and PLC on silica gel as described in the Experimental section Unchanged substrate (**1**) was recovered as needles (278 6 mg) Compound **2** was next isolated as colourless needles, mp 139–140°, and its formula ($C_{21}H_{34}O_2$) determined by high resolution MS Compound **2** was identical by mmp, IR, MS, TLC and GLC with authentic 3β-hydroxy-5β-pregnan-20-one Compound **3** was isolated as needles, mp 137 8°, $C_{21}H_{34}O_2$, and it was identical by mmp, IR, MS, TLC and GLC with authentic 3α-hydroxy-5β-pregnan-20-one.

Finally a mixture containing **7** and **8** was obtained as a powder. Hydrolysis of the mixture by treatment with apricot β-glucosidase gave 3β-hydroxy-5β-pregnan-20-one (**2**) and a trace amount of 3α-hydroxy-5β-pregnan-20-one (**3**) as the aglycones and glucose as the sugar Acid hydrolysis of the mixture gave the same amounts of **2**, **3** and glucose. After acetylation, the mixture of **7**-acetate and **8**-acetate gave colourless needles and their identities as steroidal monoglucose tetraacetates was indicated by elemental microanalysis ($C_{35}H_{52}O_{11}$) Their structures were confirmed by GC-MS comparison with synthetic 3β-hydroxy-5β-pregnan-20-one glucoside tetraacetate and 3α-hydroxy-5β-pregnan-20-one glucoside tetraacetate respectively In the MS of **7**-acetate the fragmentation peaks diagnostic of a hexopyranoside tetraacetate [17] were observed at *m/e* 331 and 301 (aglycone). Similarly, the MS of **8**-acetate gave peaks at *m/e* 331 and 301.

5β-Pregnane-3,20-dione (**1**) was also converted into 3β-hydroxy-5β-pregnan-20-one (**2**), its glucoside (**7**), 5β-pregnane-3β,20β-diol (**5**), 3α-hydroxy-5β-pregnan-20-one (**3**) and its glucoside (**8**), 5β-pregnane -3β,20α-diol monoglucoside (**9**) and 5β-pregnane-3α,20α-diol monoglucoside (**11**) by the habituated callus of *D purpurea* These products were detected by TLC. GLC and GC-MS (Table 1)

3β-Hydroxy-5β-pregnan-20-one (**2**) (total 270 mg) was administered to the suspension cultures of habituated callus of *D. purpurea* (total fr wt 1485 g) and after incubation for 7 days, the callus and medium were extracted with $CHCl_3$ and $CHCl_3$–MeOH. Three metabolic products were detected and isolated by silica gel column chromatography as described in the Experimental Compound **4** was identified by comparison with authentic 5β-pregnane-3β,20α-diol by TLC and GLC Compound **7** was isolated, acetylated

Table 1 Metabolites of 5β-pregnane-3,20-dione and 3β-hydroxy-5β-pregnan-20-one produced by normal and habituated calluses of *Digitalis purpurea*

Callus	Substrate	Metabolic product (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
Normal callus	5β-pregnane-3,20-dione (1)		+	+				+	+	(−)	(−)	(−)
Habituated callus	5β-pregnane-3,20-dione (1)		+	+		+		+	+	+		+
Normal callus	3β-hydroxy-5β-pregnan-20-one (2)	+		+		+		+	+	+		+
Habituated callus	3β-hydroxy-5β-pregnan-one (2)	(−)		(−)	+			+	(−)	+	+	+

+ Indicated the presence of metabolic products (−) Not examined in these experiments

and purified as described in the Experimental to give **7**-acetate, mp 206·5–207°. The IR spectrum of **7**-acetate had absorption bands at 1751, 1731 (COO) and 1688 (CO) cm^{-1}. The main MS fragmentation peaks were observed at *m/e* 648 (M^+) 331 ($C_{14}H_{19}O_9$) and 301 (M^+-$C_{14}H_{19}O_{10}$). The peak at *m/e* 331 suggested that **7**-acetate was a tetraacetyl-β-D-glucopyranoside. The NMR analysis of **7**-acetete showed an anomeric proton doublet at δ 4·54 (1H) with a coupling constant of 7·4 Hz, indicating that the sugar had the β-configuration [18]. Therefore **7**-acetate was 3β-hydroxy-5β-pregnan-20-one glucoside tetraacetate and this identification was confirmed by mp and spectral comparison with synthetic 3β-hydroxy-5β-pregnan-20-one-β-D-glucoside tetraacetate.

Fraction Z obtained from the initial silicagel chromatography was rechromatographed several times on silica gel to yield material which was acetylated and recrystallized from MeOH to give needles, mp 119·5–120°. The main MS peaks of the mixture were observed at *m/e* 345 ($C_{23}H_{37}O_2$; 19%), 331 (7) and 285 (100). Acid hydrolysis of the acetylated mixture gave 5β-pregnane-3β,20α-diol (**4**), 5β-pregnane-3β,20β-diol (**5**), 5β-pregnane-3α,20α-diol (**6**) and glucose. Fraction Z was therefore presumed to be a mixture of the monoglucosides of 5β-pregnane-3β,20α-diol, 5β-pregnane-3β,20β-diol and 5β-pregnane-3α,20α-diol.

3β-Hydroxy-5β-pregnan-20-one (**2**) was also metabolized to **1**, **3**, **5**, **7**, **8**, **9** and **11** by *D. purpurea* normal callus culture. These products were detected by TLC, GLC and GC–MS (Table 1).

DISCUSSION

In our earlier paper we suggested that *Digitalis* callus was unable to biosynthesize the cardenolides which are produced by the original plant. The fact that progesterone or pregnenolone was reduced stereospecifically to the 5α-compounds is probably due to regulation of gene expression in the undifferentiated cells. As shown in Scheme 1 the 3-ketone (**1**) was reduced to an axial alcohol, 3β-hydroxy-5β-pregnan-20-one (**2**) and an equatorial alcohol, 3α-hydroxy-5β-pregnan-20-one (**3**) and then **2** and **3** were glucosylated to give **7** and **8** respectively by both strains of suspension callus culture of *D. purpurea*. The almost equal amounts of **2** and **3** after acid hydrolysis was qualitatively detected by TLC and GLC. Reduction of 5α-pregnane-3,20-dione by plant suspension cultures previously [16] gave only the equatorial alcohol, 3β-hydroxy-5α-pregnan-20-one. In the present study, however, 5β-pregnane-3,20-dione (**1**) was reduced equally to both the axial and the equatorial alcohols by the cultured cells

The cultured cells may have retained the same reduction step, (**1**)→(**2**), as required for cardenolide biosynthesis in intact plants. Similarly we have already reported [19] the conversion of digitoxin into purpurea glycosides A and B and gitoxin by the suspension cultures of *D. purpurea* normal callus just as found in intact plants.

The glucoside-synthesizing systems of potato convert **3** to its β-glucopyranoside but not **2** [20]. Glucosides of 3β-hydroxy-5β-pregnan-20-one (**2**) and 3α-hydroxy-5β-pregnan-20-one (**3**) seem to be formed by the same biosynthetic pathway as glucosides from metabolites of progesterone [16], digitoxin [19] and testosterone [21] by suspension cultures of *D. purpurea* and *Nicotiana tabacum*. From the experimental results on the metabolic products found in the free fraction and hydrolyzates of the glucoside fraction, it seems likely

that glucosides of 5β-pregnane-3β,20α-diol (**4**), 5β-pregnane-3β,20β-diol (**5**) and 5β-pregnane-3α,20α-diol (**6**) are formed by the reduction of **7** and **8**.

The reaction (**1**) ⇄ (**2**) appears to be carried out by the same dehydrogenase as the reversible reaction of testosterone and androst-4-ene-3,17-dione described earlier [21].

In these tissue cultures, the metabolic transformations of 5β-pregnane-3,20-dione (**1**) and 3β-hydroxy-5β-pregnan-20-one (**2**) generally did not proceed to their possible completion but stopped after only a few steps although they were accompanied by the formation of glucosidic conjugates. Thus, many steps on the biosynthetic pathway to cardenolides which normally occur in *Digitalis* plants were inhibited in the undifferentiated *Digitalis* cells.

The present results show no differences in the metabolic pathway for cardenolide biosynthesis in normal and habituated *Digitalis* calluses. Therefore cardenolide biosynthesis does not appear to be regulated by applied exogenous hormones.

EXPERIMENTAL

Mp's are uncorrected. IR spectra were taken in KBr. NMR spectra were determined in $CDCl_3$ using tetramethylsilane as internal reference. MS were obtained using a direct insertion probe.

Tissue culture and administration of 5β-pregnane-3,20-dione (**1**) *and 3β-hydroxy-5β-pregnan-20-one* (**2**). Two normal and habituated strains of *D. purpurea* cultured cells were used for this expt. Normal callus was grown on the modified Murashige and Skoog's tobacco medium containing 0·5 ppm 2,4-D as auxin and 0·1 ppm kinetin as cytokinin. The other habituated callus, induced according to the method of K. Syōno and T. Furuya [22], was grown on the same medium but without auxin and cytokinin. The former was derived from a seedling of *D. purpurea* and sub-cultured for about 6 yr. The latter was derived from the former and sub-cultured for about 3 yr. The medium (250 ml) was dispensed in 1 l. flasks containing 20 or 30 mg 5β-pregnane-3,20-dione or 3β-hydroxy-5β-pregnan-20-one. The transplanted callus (25–35 g or 5–10 g), from a 3 week static culture, was incubated at 29° in a shaker.

Extraction procedure. Extractions were carried out according to the procedure described previously [16].

Isolation and identification of the metabolites of 5β-pregnane-3,20-dione (**1**) *in the normal callus.* After harvest the callus (1220·4 g fr. wt) and medium were separated and the medium was extracted with $CHCl_3$ (Fraction A) and $CHCl_3$–MeOH (2:1) (Fraction B). The callus was extracted by boiling under reflux with MeOH and filtered. The residue was extracted 3× with MeOH. Filtrates were combined, evaporated under red pres and extracted 3× each with $CHCl_3$ (Fraction C) and $CHCl_3$–MeOH (2:1) (Fraction D). Fractions A, B, C and D were compared by TLC with authentic compounds and then combined (3·58 g) and chromatographed on Si gel (50 g) eluted as follows: Fraction 1, 8 l. C_6H_6; Fraction 2, 2·7 l. 10% Et_2O in C_6H_6; Fraction 3, 1 l. $CHCl_3$; Fraction 4, 3·8 l. 5% MeOH in $CHCl_3$; Fraction 5, 3·8 l. total 10, 25, 30 and 50% MeOH in $CHCl_3$; Fraction 6, 1 l. MeOH. From Fraction 2 was recovered 278·6 mg 5β-pregnane-3,20-dione (**1**) mp 119–120°. PLC of Fraction 3 on Si gel G ($CHCl_3$–EtOAc, 4:1) gave (**2**) as colourless needles (from MeOH–H_2O) 29·1 mg, mp 139–140°, $C_{21}H_{34}O_2$, (required: 318·255, M^+ = 318·253), ν_{max} cm^{-1}: 3350 (OH), 2920, 2860 (CH), 1701 (CO) and 1033 (C–OH). MS (probe) 75 eV, *m/e* (rel. int.): 318 (M^+; 24%), 300 (M^+–H_2O; 39), 285 (M^+–H_2O–Me; 11), 84 (46), and 43 (100). The acetate was obtained from **2** in Ac_2O–Py overnight as colourless needles, mp 114° and R_f 0·32 on TLC (C_6H_6–EtOAc, 14:1). **2** Was identical by IR, MS, mmp, TLC (R_f 0·39; $CHCl_3$–EtOAc, 4:1) and GLC (R_t 15·6 min on QF-1) with authentic 3β-hydroxy-5β-pregnan-20-one. By the same method **3** was obtained as colourless needles (from MeOH–H_2O), 8·5 mg, mp 137·8°, $C_{21}H_{34}O_2$, (required: 318·255, M^+ = 318·253, ν_{max} cm^{-1}: 3380 (OH), 2920, 2830 (CH), 1700 (CO) and 1038 (C···OH). MS (probe) 75 eV, *m/e* (rel. int.): 318 (M^+; 43%), 300 (M^+–H_2O; 45), 285 (M^+–H_2O–Me; 13), 84 (52) and 43 (100). The acetate of **3** was obtained as colourless needles (from MeOH–H_2O), mp 89–90° and R_f 0·41 on TLC (C_6H_6–EtOAc, 14:1). **3** was identified by IR, MS, mmp TLC (R_f 0·33; $CHCl_3$–EtOAc, 4:1) and GLC (R_t 17·7 min on QF-1). From Fraction 4 a mixture of **7** and **8** was obtained partly as a colourless powder, 63·5 mg, R_f 0·37 on TLC ($CHCl_3$–MeOH, 7:1) and partly as colouless needles (from MeOH) of the acetates, 113·6 mg (Found: C, 64·65; H, 8·11. $C_{35}H_{52}O_{11}$ requires: C, 64·78; H, 8·09%). R_f 0·52 on TLC ($CHCl_3$–EtOAc, 4:1). MS of acetate *m/e* (rel. int.): 331 ($C_{14}H_{19}O_6$; 12%), 301 ($C_{21}H_{33}O$; 100), 242 (19), 200 (12), 169 (34), 157 (17), 140 (14), 115 (11), 109 (31), 98 (18), 73 (3), 43 (97). Their structures were confirmed using GC–MS (System 1) by comparison with synthetic 3β-hydroxy-5β-pregnan-20-one glucoside tetraacetate and 3α-hydroxy-5β-pregnan-20-one glucoside tetraacetate. In the MS of **7**-acetate (R_t 17·7 min) the fragmentation peaks diagnostic of a hexopyranoside tetraacetate were observed at *m/e* 331 and 301 ($C_{21}H_{33}O$). Similarly, the MS of **8**-acetate (R_t 16·0 min) gave peaks at *m/e* 331 and 301 ($C_{21}H_{33}O$).

Identification of the metabolites derived from 5β-pregnane-3,20-dione (**1**) *by the habituated callus.* 5β-Pregnane-3,20-dione (120 mg) was administered to the *D. purpurea* habituated callus. The callus (fr. wt 35 g) and medium (1·5 l.) were extracted with $CHCl_3$ and $CHCl_3$–MeOH (2:1) and the fractions were combined after comparison by TLC. This extract (210 mg) was chromatographed on Si gel (50 g) and eluted as follows: Fraction a, 200 ml $CHCl_3$ and 90 ml 1% MeOH in $CHCl_3$; Fraction b, 80 ml 1% MeOH in $CHCl_3$; Fraction c, 30 ml 1% MeOH in $CHCl_3$ and 45 ml 3% MeOH in $CHCl_3$; Fraction d, 155 ml 3% MeOH in $CHCl_3$ and 235 ml 10% MeOH in $CHCl_3$ and Fraction e, 55 ml 10% MeOH in $CHCl_3$ and 170 ml 20% MeOH in $CHCl_3$. Fraction c was evaporated to dryness and 3β-hydroxy-5β-pregnan-20-one (**2**), 3α-hydroxy-5β-pregnan-20-one (**3**) and 5β-pregnane-3β,20β-diol (**5**) were identified by TLC and GLC. R_f ($CHCl_3$–EtOAc, 4:1; $CHCl_3$–MeOH, 40:1) and R_t (min on QF-1) values were as follows: **2** 0·29, 0·52, 15·6; **3** 0·33, 0·43, 17·7; **5** 0·25, 0·29, 10·3. Fraction e (10 mg) was acetylated by Ac_2O–Py and **7**-acetate and **8**-acetate were identical by GC–MS (System 2) with synthetic 3β-hydroxy-5β-pregnan-20-one glucoside tetraacetate and 3α-hydroxy-5β-pregnan-20-one glucoside tetraacetate respectively. In the MS of 7-acetate (R_t 13·8 min) major peaks were observed at *m/e* 331 ($C_{14}H_{19}O$; 6%), 301 ($C_{21}H_{33}O$; 100) and 284 (64), similarly, the MS of **8**-acetate (R_t 12·2 min) gave peaks at *m/e* 331 ($C_{19}H_{14}O$; 26%), 301 ($C_{21}H_{33}O$; 100) and 285

(70) Moreover, the compound of R_t 19·6 min gave peaks at *m/e* 345 ($C_{23}H_{37}O_2$, 12%), 331 (16), 301 (25) and 285 (100) After acid hydrolysis of the acetylated glucoside fraction, 3β-hydroxy-5β-pregnan-20-one (**2**), 3α-hydroxy-5β-pregnan-20-one (**3**), 5β-pregnane-3β,20α-diol (**4**) and 5β-pregnane-3α,20α-diol (**6**) were detected by GLC and TLC R_t (min on QF-1) and R_f ($CHCl_3$–EtOAc, 4·1) were as follows **2** 15·6, 0·39, **3** 17·7, 0·33, **4** 11·5, 0·25, **6** 13·1, 0·15

Identification of the metabolites of 3β-hydroxy-5β-pregnan-20-one (**2**) *produced by the habituated callus* 3β-Hydroxy-5β-pregnan-20-one (270 mg) was administered to *D purpurea* habituated callus (total fr wt 1485 g) After shaking for 7 days, the callus and medium were extracted with $CHCl_3$ and $CHCl_3$–MeOH (2·1) Three metabolic products were detected by TLC R_f 0·25 ($CHCl_3$–EtOAc, 4·1), R_f 0·56 and 0·44 ($CHCl_3$–MeOH, 5·1) The $CHCl_3$ and $CHCl_3$–MeOH (2·1) extracts were combined (3·84 g) and chromatographed on Si gel (500 g) and eluted as follows Fraction V, 16·8 l $CHCl_3$, Fraction W 5·6 l 2% MeOH in $CHCl_3$, Fraction X, 1·4 l 5% MeOH in $CHCl_3$, Fraction Y, 1·6 l 10% MeOH in $CHCl_3$ and Fraction Z, 0·8 l 20% MeOH in $CHCl_3$ Fraction V was rechromatographed on Si gel (20 g) to give a small amount of **4** which was identical with authentic 5β-pregnane-3β,20α-diol by TLC (R_f 0·25 $CHCl_3$–EtOAc, 4·1) and GLC (R_t 11·4 min on QF-1) Fraction Y was also rechromatographed on Si gel (50 g) and eluted as follows. fraction O, 0·3 l C_6H_6, Fraction P, 0·4 l $CHCl_3$, Fraction Q, 1·2 l 2% MeOH in $CHCl_3$, Fraction R, 2·3 l 5% MeOH in $CHCl_3$ Fraction R (19·2 mg) was acetylated and purified by Si gel chromatography and recrystallized from MeOH to yield needles of **7**-acetate (6·36 mg), mp 206·5–207° ν_{max} cm^{-1} 2940, 2920 (CH), 1751, 1731 (COO), 1688 (CO), 1230, 1040 (COO), NMR 100 MHz, $CDCl_3$), δ 0·60 (3H, *s*, C-18), 0·92 (3H, *s*, C-19), 2·01, 2·02, 2·04, 2·07, 2·11 (3H, *s*, C-21 and 4 × 3H, *s*, MeCO), 4·54 (1H, *d*, *J* 7·4 Hz, 1′-H) MS (probe) 70 eV, *m/e* 648 M^+, 588 (M^+-MeCOOH), 331 ($C_{14}H_{19}O_9$), 301 (M^+-$C_{14}H_{19}O_{10}$) GLC R_t 18·15 min on OV-1 **7**-Acetate was identical by IR, NMR, GLC and mp with synthetic material Fraction Z was rechromatographed 3× on Si gel and then acetylated and recrystallized from MeOH to yield needles (3·6 l mg), mp 119·5–120°, ν_{max} cm^{-1} 2944 (CH), 1754, 1738 (COO), MS (probe) 70 eV, *m/e* (rel int) 345 ($C_{23}H_{37}O_2$, 19%), 331 ($C_{14}H_{19}O_9$, 7), 301 ($C_{21}H_{33}O$), 285 ($C_{23}H_{37}O_2$–MeCOOH, 100), NMR (100 MHz, $CDCl_3$) δ 0·63 (3H, *s*, C-18), 0·89 (3H, *s*, C-19), 1·97, 1·98, 1·99, 2·01, 2·04 (5× 3H, *s*, MeCOH), 4·56 (1H, *d*, *J* 7·0 Hz, 1′-H) After hydrolysis the product was identified as a mixture of 5β-pregnane-3β,20α-diol (**4**), 5β-pregnane-3β,20β-diol (**5**) and 5β-pregnane-3α,20α-diol (**6**) by TLC (R_f 0·30, 0·29, 0·18, $CHCl_3$–MeOH, 40·1) and GLC (R_t 11·5, 10·3, 13·1 min on QF-1) These data suggest that Fraction Z contained a mixture of the monoglucosides of 5β-pregnane-3β,20α-diol, 5β-pregnane-3β,20β-diol and 5β-pregnane-3α,20α-diol

Identification of the metabolites of 3β-hydroxy-5β-pregnan-20-one (**2**) *produced by the normal callus* $CHCl_3$ (56·8 mg) and $CHCl_3$–MeOH (99·0 mg) extracts were obtained after the administration of 3β-hydroxy-5β-pregnan-20-one (100 mg) to *D purpurea* normal callus (fr wt 32 g) The combined material was chromatographed on Si acid (40 g) eluted with $CHCl_3$ and $CHCl_3$–MeOH. The esters and aglycones were eluted in the first fraction. 5β-pregnane-3,20-dione (**1**) and 5β-pregnane-3β,20β-diol (**5**) were detected by TLC (R_f 0·52, 0·25, $CHCl_3$–EtOAc, 4·1) and GLC (R_t 35·1 min 10·3 min on QF-1) After acetylation of the glucoside fraction 3β-hydroxy-5β-pregnan-20-one glucoside tetraacetate (**7**-acetate), 3α-hydroxy-5β-pregnan-20-one glucoside tetraacetate (**8**-acetate) and 5β-pregnane-3ξ,20ξ-diol monoglucoside pentaacetate were detected with GC–MS (System 2). In the MS of **7**-acetate (R_t 13·8 min) peaks were observed at *m/e* 331 (tetraacetyl glucose oxonium ion, 4·3%), 301 ($C_{21}H_{33}O$, 81) and 284 (40) Similarly, the MS of **8**-acetate (R_t 12·2 min) gave peaks at *m/e* 331 (23%), 301 (68) and 284 (38) Moreover, the compound of R_t 19·6 min gave peaks at *m/e* 345 ($C_{23}H_{37}O_2$, 19·7%), 331 (8), 301 (13) and 285 (100) After acid hydrolysis of this acetylated glucoside fraction. 3β-Hydroxy-5β-pregnan-20-one (**2**), 3α-hydroxy-5β-pregnan-20-one (**3**) 5β-pregnane-3β,20α-diol (**4**) and 5β-pregnane-3α,20α-diol (**6**) were detected by GLC and TLC; R_t (min on QF-1) and R_f $CHCl_3$–MeOH (40·1) were as follows (**2**) 15·6, 0·52, (**3**) 17·7, 0·43, (**4**) 11·5, 0·30, (**6**) 13·1, 0·18

Hydrolysis of the mixture containing 3β-hydroxy-5β-pregnan-20-one glucoside (**7**) *and 3α-hydroxy-5β-pregnan-20-one glucoside* (**8**) The mixture of **7** and **8** (10 mg) obtained from *D purpurea* normal callus was refluxed with 5 ml alcoholic HCl for 1 hr After diluting with 30 ml H_2O, EtOH was removed under red pres The soln was extracted with $CHCl_3$ which washed with H_2O until neutral and evaporated to give a crystalline mixture identified by GLC and TLC as 3β-hydroxy-5β-pregnan-20-one (**2**) and 3α-hydroxy-5β-pregnan-20-one (**3**) The acidic soln, after removing **2** and **3**, was neutralized with dil KOH and conc under red pres Hydrolysate was identical to the hydrolysate obtained from β-D-glucose pentaacetate under the same hydrolytic conditions (TLC R_f 0·47, 0·34, BuOH–AcOH–H_2O, 5·1·1 anisaldhyde–H_2SO_4 reagent) The acetate mixture (23·2 mg) refluxed with 10% alcoholic HCl for 1 hr gave **2**, **3** and the same hydrolysate of β-D-glucose pentaacetate The mixture (0·7 mg) of **7** and **8** was incubated with apricot β-glucosidase in 1·5 ml 0·2 M acetate buffer, pH 4·5 for 24 hr at 31° 3β-Hydroxy-5β-pregnan-20-one (**2**), a trace amount of 3α-hydroxy-5β-pregnan-20-one (**3**) and glucose were detected by TLC (R_f 0·39, 0·33, $CHCl_3$–EtOAc, 4·1, R_f 0·34, BuOH–AcOH–H_2O, 5·1·1)

Synthesis of 3β-hydroxy-5β-pregnan-20-one glucoside tetraacetate (**7**-*acetate*) *and 3α-hydroxy-5β-pregnan-20-one-glucoside tetraacetate* (**8**-*acetate*) 3β-Hydroxy-5β-pregnan-20-one was treated with acetobromoglucose in the usual way [23] and the product was obtained as needles from MeOH, mp 206·5–208° $[\alpha]_D^{26}$ + 31·2° ($CHCl_3$, *c* 1·02) The IR, NMR, MS and GLC were identical with those of the metabolic product, (**7**-acetate) from *D purpurea* habituated callus administered 3β-hydroxy-5β-pregnan-20-one (**2**) 3α-Hydroxy-5β-pregnan-20-one glucoside tetraacetate was synthesized by the same method to give needles from MeOH, mp 144–145° $[\alpha]_D^{26}$ + 44·1° ($CHCl_3$, *c* 0·834) ν_{max} cm^{-1} 2940, 2870 (CH), 1750 (COO), 1703 (CO) MS (probe) 70 eV, *m/e* 648 (M^+) 588 (M^+-MeCOOH), 331, 301 ($C_{21}H_{33}O$) NMR (100 MHz, $CDCl_3$), δ 0·59 (3H, *s*, C-18), 0·91 (3H, *s*, C-19), 2·01, 2·02, 2·05, 2·07, 2·11 (3H, MeCO-, *s*, C-21 and 4 × 3H, *s*, MeCO), 4·59 (1H, *d*, *J* 7·4 Hz, 1′-H)

Glc operating conditions (a) 2 m × 3 mm column of 1·5% QF-1, oven 210°, detector block 230° N_2 carrier gas 44 ml/min (b) 2 m × 3 mm column of 1% OV-1, oven 280°, detector block 300°, N_2 carrier gas 40 ml/min

Operating conditions for GC–MS analysis System 1 1 m × 4 mm column of 1% OV-1 on chromosorb W at 280°, ion source 290°, ionizing energy 70 eV System 2 1 m × 2 mm column of 1% OV-1 on shimalite W at 285° ion source 290° ionizing energy 25 eV

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