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BIOTRANSFORMATION OF DIGITOXIGENIN BY CELL SUSPENSION CULTURES OF *DIGITALIS PURPUREA**

MASAO HIROTANI and TSUTOMU FURUYA

School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108, Japan

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Key Word Index—*Digitalis purpurea*; Scrophulariaceae; tissue cultures; biotransformation; hydroxylation; glucosylation; steroid; digitoxigenin; digitoxigenone; epidigitoxigenin; periplogenin; digitoxigenin glucoside; epidigitoxigenin glucoside.

Abstract—Digitoxigenin was oxidized to digitoxigenone which was reduced to epidigitoxigenin and then glucosylated to epidigitoxigenin glucoside by cell cultures of *Digitalis purpurea*. The epidigitoxigenin glucoside, together with digitoxigenone and epidigitoxigenin, was isolated in considerable amounts, whereas digitoxigenin glucoside could only be detected in low concentration. Furthermore, it was confirmed by TLC and HPLC that digitoxigenin was hydroxylated to periplogenin.

INTRODUCTION

The first attempts of biotransformation of digitoxigenin were performed with plant cell suspension cultures by Stohs and Staba [1]. Subsequently, two studies on the biotransformation of digitoxigenin were performed with *Digitalis purpurea* and *D. lanata* cell cultures. Stohs and Rosenberg [2] reported that digitoxigenin was transformed by oxidation at C-3 to digitoxigenone, whereas Alfermann *et al.* [3] observed the formation of digoxigenin by 12β-hydroxylation. After simultaneous incubation of digitoxigenin and digitoxose, a compound was found which appeared to be digoxigenin monodigitoxoside. Recently, Jones *et al.* [4] reported the conversion of digitoxigenin to periplogenin in high yield by cell cultures of *Daucus carota*.

The present paper describes the studies on the biotransformation of digitoxigenin by cell suspension cultures of *D. purpurea*.

RESULTS

Digitoxigenin (**1**) (870 mg) was incubated with *Digitalis purpurea* cell suspension cultures (total fr. wt 1130 g) for 7 days. After harvest the callus and medium were extracted. The $CHCl_3$ and $CHCl_3$–MeOH(2:1) extracts of the callus and medium contained several biotransformation products and were chromatographed on Si gel several times (see Experimental). After acetylation and purification, compound **6** acetate was isolated as prisms, mp 221–223°, $C_{37}H_{52}O_{13}$, $[\alpha]_D^{20}+10.7°$. The IR spectrum of **6**-acetate showed α,β-unsaturated γ-lactone and ester absorption bands at 1750, 1735 and 1045 cm^{-1}. The main MS fragment peaks were observed at *m/e* 704[M^+], 357 [$M^+ - C_{14}H_{19}O_{10}$] and 331 [$C_{14}H_{19}O_9$]. The peak at *m/e* 357 suggested that the aglycone part was a digitoxigenin analogue. The peak at *m/e* 331 suggested that **6**-acetate was a tetraacetyl-D-glucopyranoside [5]. The ^1H NMR analysis of **6**-acetate showed an anomeric proton doublet at δ 4.6 (1H) with a coupling constant of 8 Hz, indicating that the sugar had the β-configuration [6] and a signal at δ 3.7, (1H) *s(br)* $W_{1/2}=20$ Hz, indicating that the proton of C-3 of the aglycone was in the β-configuration by comparison with the ^1H NMR data for synthetic 3β-hydroxy-5β-pregnan-20-one glucoside acetate and 3α-hydroxy-5β-pregnan-20-one glucoside acetate [7]. From these results, compound **6** was concluded to be epidigitoxigenin glucoside. At the same time, digitoxigenin glucoside (**5**)-acetate was detected in acetylated subfraction 2 on TLC.

In the next experiment, digitoxigenin was suspended with Tween 80 (in total 760 mg in 3.6 ml of Tween 80) and autoclaved and then added to 17-day-old suspension cultures. The callus was harvested after a further 5 days incubation. The $CHCl_3$ extracts from the callus (2.86 g) and medium (1.8 g) yielded eight Kedde-positive compounds on TLC. The $CHCl_3$ extract from the medium was chromatographed on Si gel (see Experimental). After purification of fraction 2 by HPLC, compound **2** was obtained as colourless prisms, mp 203–205°, $C_{23}H_{32}O_4$, $[\alpha]_D^{18}+30.5°$. The identity of **2** with digitoxigenone was confirmed by mp, $[\alpha]_D$, IR, MS, TLC and HPLC. Fraction 4 gave compound **3** as colourless prisms, mp 277–283°, $C_{23}H_{34}O_4$, $[\alpha]_D^{18}+25.2°$. The identity of **3** with epidigitoxigenin was confirmed by mp, $[\alpha]_D$, IR, MS and HPLC. From fraction 7, periplogenin was detected by TLC and HPLC. The other Kedde-positive compounds were not identical with authentic samples of 7β-, 12β- and 16β-hydroxydigitoxigenin by TLC and HPLC.

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DISCUSSION

As shown in Scheme 1, digitoxigenin (**1**) was oxidized to digitoxigenone (**2**) and reduced in high yield to the 3α-hydroxy compound epidigitoxigenin (**3**) and probably to a small extent back to the 3β-hydroxy compound digitoxigenin (**1**). Moreover, epidigitoxigen (**3**) was glucosylated to epidigitoxigenin glucoside (**6**), while unchanged digitoxigenin (**1**) was glucosylated in small yield to digitoxigenin glucoside (**5**). Stohs and Rosenberg [2] have reported that digitoxigenone was identified as one of the biotransformation products of digitoxigenin which is consistent with our results.

The glucosylation of digitoxin to purpurea glycoside A using cell cultures of *D. purpurea* and *D. lanata* has been investigated by Furuya *et al.* [8] and Reinhard *et al.* [9]. Furthermore, it was demonstrated that 5β-pregnane-3,20-dione was converted to 3β-hydroxy-5β-pregnan-20-one glucoside and 3α-hydroxy-5β-pregnan-20-one glucoside in almost equal amounts in cell cultures of *D. purpurea* [7]. However, the formation of epidigitoxigenin glucoside, which was first demonstrated in plant tissue cultures, was more extensive than production of digitoxigenin glucoside in this experiment. This fact is consistent with the results of enzymatic studies which showed that 3α-hydroxy-5β-pregnan-20-one has a higher affinity than 3β-hydroxy-5β-pregnan-20-one for sterol: UDPG glucosyltransferase and also that the 3β-hydroxy compound digitoxigenin has a very low affinity [10].

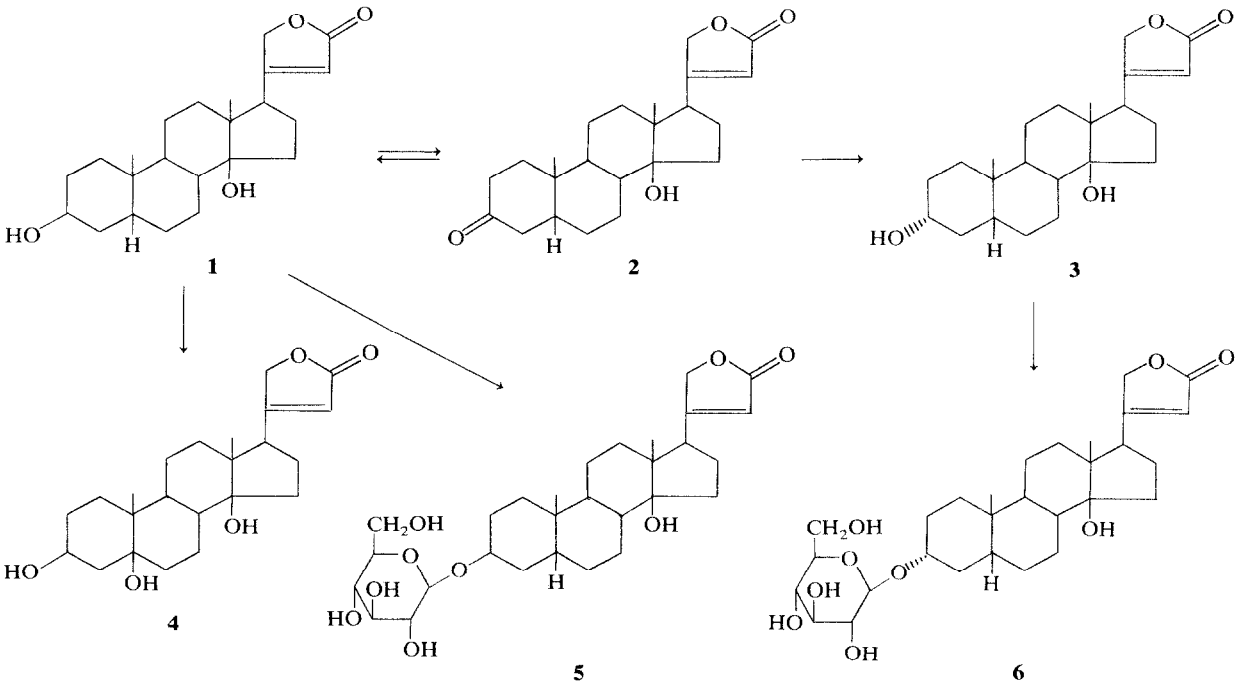
On the other hand, it was shown that digitoxigenin is hydroxylated at C-5 to peripolgenin (**4**), a conversion which was recently observed in high yield in the biotransformation of digitoxigenin using cell cultures of *Daucus carota* Ca 68 by Jones *et al.* [4]. The hydroxylation at C-12 and C-16 of digitoxigenin and related cardiac glycosides has been observed in cell cultures of *D. lanata* and *D. purpurea* by Alfermann *et al.* [3] and Furuya *et al.* [8]. In this experiment, we have detected eight Kedde-positive compounds in the culture medium. Three of them were isolated and identified as digitoxigenone, epidigitoxigenin and periplogenin, respectively. Two of the other compounds were presumed to be 7β- and 12β-hydroxydigitoxigenin in preliminary tests by TLC [11], but their identities have not been confirmed by HPLC until now.

Although 16β-hydroxylation of digitoxin to give gitoxin could be observed in *D. purpurea* callus, the 16β-hydroxy compound of digitoxigenin could not be found in this experiment. In using digitoxigenin instead of digitoxin as a substrate, it seems that the 3β-hydroxy group of digitoxigenin was rapidly converted to a 3α-hydroxy group by *D. purpurea* callus. Therefore, some unknown Kedde-positive compounds seem likely to be hydroxylation products of epidigitoxigenin.

EXPERIMENTAL

Mps were determined in a Büchi apparatus and a Kofler hot plate, and are uncorr. ^{1}H NMR spectra were determined in $CDCl_3$ using TMS as int. ref. MS were run using a direct insertion probe.

Tissue cultures and administration of digitoxigenin (**1**). The tissue cultures were derived from the seedlings of *Digitalis purpurea* and had been subcultured for 8 yr. Plant cell suspension cultures were grown on modified Murashige and Skoog's tobacco medium containing 0.5 ppm 2,4-D (2,4-dichlorophenoxyacetic acid), 0.05 ppm kinetin and 3% sucrose. The administration of digitoxigenin was carried out using two methods: (1) the medium (250 ml) was dispensed in a 1 l. flask containing 30 mg of crystalline digitoxigenin.



Scheme 1. Possible scheme for the biotransformation of digitoxigenin (**1**) by a cell suspension culture of *Digitalis purpurea*.

The callus (*ca* 30 g fr. wt per flask) from 3-week-old static cultures was incubated at 29° in a shaker for 7 days; (2) digitoxigenin (20 mg) suspended with Tween 80 was added under sterile conditions to each flask (250 ml medium/l. flask) at 17 days after inoculation (*ca* 10 g callus each flask) and cultured for a further 5 days.

Extraction procedure. The calluses were harvested with nylon cloth and homogenized with MeOH in a Waring blender and allowed to stand for 1 week at room temp. The homogenate was filtered and the residue was extracted 2× with fresh solvent. The filtrates were combined and the organic solvent was removed under red. pres. The residue was extracted with $CHCl_3$ and the aq. soln was extracted with $CHCl_3$–MeOH (2:1). The medium was extracted with the same method.

Isolation and identification of epidigitoxigenin glucoside (**6**) *acetate and detection of digitoxigenin glucoside* (**5**) *acetate.* Digitoxigenin (total 870 mg) was administered to *D. purpurea* callus (total fr. wt 1130 g). After shaking for 7 days, the callus and medium were extracted according to the method described above. The $CHCl_3$–MeOH (2:1) extracts from the callus were combined (2.998 g), chromatographed on Si gel (200 g) and eluted as follows: fraction I, 8 l. $CHCl_3$; fraction II, 0.7 l. 50% MeOH in $CHCl_3$. Fraction II was rechromatographed on Si gel (95 g) and eluted as follows: fraction A, 1 l. 3% MeOH in $CHCl_3$, 1 l. 5% MeOH in $CHCl_3$ and 0.9 l. 7% MeOH in $CHCl_3$; fraction B, 2 l. 7% MeOH in $CHCl_3$; fraction C, 1.1 l. 7% MeOH in $CHCl_3$; fraction D, 1.1 l. 7% MeOH in $CHCl_3$ and 1 l. 10% MeOH in $CHCl_3$. The colourless powder obtained from the fraction B was rechromatographed on Si gel (20 g), giving subfraction 1, 300 ml $CHCl_3$ and 500 ml 1% MeOH in $CHCl_3$; subfraction 2, 400 ml 3% MeOH in $CHCl_3$; subfraction 3, 100 ml 3% MeOH in $CHCl_3$; subfraction 4, 750 ml 5% MeOH in $CHCl_3$. Subfraction 2 was acetylated and purified by Si gel chromatography and recrystallized from dil EtOH to yield prisms of **6**-acetate, 76 mg, mp 221–223°; $[\alpha]_D^{20}+10.7°$ ($CHCl_3$; *c* 0.98). [Found: C, 62.77; H, 7.43. $C_{37}H_{52}O_{13}$ requires; C, 63.07; H, 7.39%]. UV λ_{max}^{MeOH} nm (log ε): 225 (4.31); IR ν_{max}^{KBr} cm^{-1}: 3530 (OH), 2950 (CH), 1750 (5-membered lactone), 1735 (COO), 1620 (C=C), 1230, 1045; MS (probe) 20 eV *m/e* (rel. int.): 704 [M^+] (2), 644 [M^+-HOAc] (1), 357 [$M^+-C_{14}H_{19}O_{10}$] (33), 339 (24), 331 [$C_{14}H_{19}O_9$] (74), 169 (100); ^1H NMR (100 MHz, $CDCl_3$): δ 0.84 (3H, *s*, C-18 or C-19), 0.86 (3H, *s*, C-19 or C-18), 2.01 (3H, *s*, Ac), 2.04 (3H, *s*, Ac), 2.05 (3H, *s*, Ac) 2.08 (3H, *s*, Ac), 3.7 (1H, *s(br)*, $W_{1/2}=20$ Hz, C-3), 4.6 (1H, *d*, $J=8$ Hz). Digitoxigenin glucoside (**5**) acetate was detected in acetylated subfraction 2 on TLC (R_f, 0.31, 0.41; C_6H_6–EtOAc, 5:6, C_6H_6–Me_2CO, 3:1, respectively) in comparison with synthetic digitoxigenin glucoside acetate. Thus subfraction 2 contained a small amount of digitoxigenin glucoside (**5**).

Isolation and detection of conversion products of digitoxigenin (**1**). Digitoxigenin (total 760 mg, suspended with Tween 80) was added to the 17 day-precultured *D. purpurea* callus. After 5 days culture, callus (fr. wt 2.5 kg) and medium (*ca* 6 l.) were separated with nylon cloth and extracted according to the procedure above. The $CHCl_3$ and $CHCl_3$–MeOH fractions from callus and medium were examined on TLC. The $CHCl_3$ fraction (1.8 g) from the medium gave 8 Keddepositive spots (R_f 0.55, 0.47, 0.42, 0.35, 0.26, 0.20, 0.10, 0.07; $CHCl_3$–EtOH, 10:1). The $CHCl_3$ extract was chromatographed on Si gel (180 g Merck Si gel, 230 mesh) and eluted with 3% MeOH in $CHCl_3$ and collected as follows: fraction 1, 645 ml; fraction 2, 90; fraction 3, 195; fraction 4, 75; fraction 5, 480; fraction 6, 165; fraction 7, 165; fraction 8, 315. HPLC (13.8 min) of fraction 2 on μ Bondapak C18 gave **2** as colourless prisms (Me_2CO–petrol), 7.3 mg, mp 203–205° (Kofler); $[\alpha]_D^{18}+30.5°$ ($CHCl_3$; *c* 0.38); $C_{23}H_{32}O_4$ (required 372.230; M^+, 372.227);"IR ν_{max}^{KBr} cm^{-1}: 3470 (OH), 2930, 2855 (CH), 1780, 1750 (lactone), 1715 (CO), 1625 (C=C), 1030; MS (probe) 75 eV *m/e* (rel. int.): 372 [M^+] (100), 284 (6), 219 (50), 201 (31), 178 (39), 149 (34), 126 (23), 111 (64), 109 (98). Fraction 4 gave **3** as colourless prisms (MeOH), 39.5 mg, mp 277–283°; $[\alpha]_D^{21}+25.2°$ (MeOH; *c* 0.22); $C_{23}H_{34}O_4$ (required 374.246; M^+, 374.245); IR ν_{max}^{KBr} cm^{-1}: 3500, 3415 (OH), 2930, 2855 (CH), 1790, 1740 (lactone), 1630 (C=C), 1040; MS (probe) 75 eV *m/e* (rel. int.): 374 [M^+] (6), 356 [M^+-H_2O] (67), 338 [M^+-128] (100), 231 (15), 204 (88), 189 (41); R_f 0.42, 0.16 in $CHCl_3$–EtOH (10:1) and EtOAc–*n*-hexane (3:1) respectively; R_t 19.9 min by HPLC on μ Bondapak C18. Periplogenin from fraction 7 was detected by TLC R_f, 0.26, 0.09 in $CHCl_3$–EtOH (10:1) and EtOAc–*n*-hexane (3:1) respectively and HPLC (R_t, 10.3 min on μ Bondapak C18).

Synthesis of digitoxigenin glucoside (**5**) *acetate* [12]. A mixture of 187 mg digitoxigenin, 500 mg acetobromglucose, 310 mg silver-4-hydroxyvalerate and 5 ml Et_2O–CH_2Cl_2 (3:1) was stirred at room temp. for 24 hr. The Ag salt was filtered off and the filtrate was concd under red. pres. The residue was acetylated by the usual method and purified by Si gel chromatography and recrystallized from dil EtOH (80 mg), mp 160–161°; $[\alpha]_D^{26}-2.3°$ ($CHCl_3$; *c* 1.53); UV λ_{max}^{MeOH} nm (log ε): 227 (4.31); IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 2940 (CH), 1760 (α,β-unsaturated CO), 1630 (C=C), 1230, 1045; ^1H NMR (100 MHz, $CDCl_3$): δ 0.89 (3H, *s*, C-18 or C-19), 0.92 (3H, *s*, C-19 or C-18), 2.01~2.09 (4×3H, *s*, Ac), 4.06 (1H, *s*, $W_{1/2}=7$ Hz, C-3), 4.58 (1H, *d*, $J=8$ Hz).

Conditions for HPLC and data of cardiac aglycones. HPLC analysis was run on a Waters liquid chromatograph 6000A instrument detected with a Waters absorbance detector model 440 and a differential refractometer. A column (30 cm×4 mm) packed with μ Bondapak C18 was operated under the following conditions. The solvent was MeOH–H_2O (1:1) with a flow rate 2.0 ml/min. R_t (min) of compounds were as follows: digitoxigenin 17.4, epidigitoxigenin 19.9, digitoxigenone 13.8, periplogenin 10.3, 7β-hydroxdigitoxigenin 13.1, 12β-hydroxydigitoxigenin 3.2, 16β-hydroxydigitoxigenin 8.2.

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