

## BIOTRANSFORMATION OF DIGITOXIGENIN BY CELL SUSPENSION CULTURES OF *STROPHANTHUS GRATUS*\*

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**Key Word Index**—*Strophanthus gratus*; Apocynaceae; cell cultures; biotransformation; 1 $\beta$ - and 4 $\beta$ -hydroxylation; isomerization of 17 $\beta$ -butenolide ring; cardenolides; digitoxigenin; 3-*epi*-17 $\beta$ H-periplogenin; 3-*epi*-4 $\beta$ -hydroxy-17 $\beta$ H-digitoxigenin.

**Abstract**—Biotransformation products of digitoxigenin by cell suspension cultures of *Strophanthus gratus* were isolated and their structures established as 17 $\beta$ H-digitoxigenin, 3-*epi*-17 $\beta$ H-digitoxigenin, 17 $\beta$ H-acovenosigenin-A, 17 $\beta$ H-periplogenin, 3-*epi*-17 $\beta$ H-periplogenin and 3-*epi*-4 $\beta$ -hydroxy-17 $\beta$ H-digitoxigenin, respectively. Furthermore, periplogenin was identified by TLC and HPLC. Biotransformations involving 1 $\beta$ - and 4 $\beta$ -hydroxylation and isomerization of the 17 $\beta$ -butenolide ring of digitoxigenin have been demonstrated for the first time in the cell cultures.

### INTRODUCTION

Several groups have reported on cardenolide production by cell cultures [1], regenerated embryos [2–4] and shoots [5–7] of *Digitalis lanata* and other *Digitalis* species. On the other hand, biotransformations of cardenolides with cell cultures of plants containing cardenolides, as well as with those of plants containing no cardenolides, have been investigated very intensively [8, 9]. Recently, the biotransformations of digitoxigenin [10] and gitoxigenin [11] by 5 $\beta$ -hydroxylation, and of  $\beta$ -methyl digitoxin [12] by 12 $\beta$ -hydroxylation have been utilized in the field of plant biotechnology.

G-Strophanthin (ouabain), obtained from the seeds of *Strophanthus gratus*, is widely used as an immediately effective cardiotonic. However, there has been no report on the use of tissue cultures of this plant until now. In order to produce cardenolides, especially G-strophanthin, three cell strains derived from the different parts (the leaves, the stems and the flower buds) of *S. gratus* have been obtained. In this paper we report the biotransformation of digitoxigenin (1), a precursor of cardiac glycosides, by cell suspension cultures of *S. gratus*.  $^1\text{H}$  and  $^{13}\text{C}$  NMR assignments of the biotransformation products have been performed using the DEPT pulse sequence, two-dimensional proton–proton and proton–carbon shift correlation data. The biotransformation percentage yield was calculated from the ratio of the amount of the isolated product to the starting amount of 1, taking into consideration their  $M_s$ .

### RESULTS AND DISCUSSION

The three cell strains, Sg-1, Sg-2 and Sg-3, were derived from the different parts of *Strophanthus gratus* (See Ex-

perimental). Kedde-positive spots were not detected on TLC in the chemical components of these cell strains. Digitoxigenin (1) (total 960 mg) was incubated with the three cell strains (total fr. wt 1.5 kg) for 16 days. After harvest the callus and medium were extracted with chloroform and chloroform–methanol (2:1). Eight Kedde-positive spots were detected on TLC in the extracts from the medium of the three cell strains. Products 2–4, 6 and 7 were isolated as crystals from these extracts using DCCC ( $\text{CHCl}_3$ – $\text{MeOH}$ – $\text{H}_2\text{O}$  5:6:4; descending method), silica gel column chromatography and HPLC. Product 8 diacetate was isolated by HPLC after acetylation. Furthermore, product 5 was identified with an authentic sample by TLC and HPLC. The products from the cells were detected on TLC, but not isolated as crystals.

Product 2 was isolated as colourless needles (45.0 mg, yield 4.7%, mp 160–163°) and it had the composition  $\text{C}_{23}\text{H}_{34}\text{O}_4$  on the basis of high-resolution mass spectroscopy. The IR spectrum showed peaks at 3400 (–OH), 1775 (sh), 1720 ( $\beta$ -substituted,  $\alpha,\beta$ -unsaturated five-membered lactone) and 1615 ( $\text{C}=\text{O}$ ). In the  $^1\text{H}$  NMR spectrum of product 2 (Table 1), the methyl proton signal of C-18 was shifted downfield to  $\delta$  1.03 (3H, s) and the methine proton signal of H-17, coupling with the methylene protons of C-16, was also shifted downfield to  $\delta$  3.17 (1H, dd,  $J=9.5, 9.5$  Hz). In the  $^{13}\text{C}$  NMR spectrum (Table 2), the C-12 carbon signal was shifted upfield to  $\delta$  32.10(t). Thereby the configuration of H-17 was revealed to be  $\beta$  [13]. The H-3 signal appeared as a broad singlet ( $W_{1/2}=7$  Hz) at  $\delta$  4.04, revealing its  $\alpha$ -configuration. On comparison of the  $^{13}\text{C}$  NMR chemical shifts of 2 and 1, the data for C-1 to C-10 were similar to each other. From all these data, product 2 was identified 3 $\beta$ ,14-dihydroxy-5 $\beta$ ,14 $\beta$ ,17 $\alpha$ -card-20(22)-enolide (17 $\beta$ H-digitoxigenin).

Product 3 (64.5 mg; yield 6.7%) had the same molecular formula,  $\text{C}_{23}\text{H}_{34}\text{O}_4$  (high-resolution mass spectrometry) as determined for product 2. The chemical shift values of H-17 and H<sub>3</sub>-18 in the  $^1\text{H}$  NMR spectrum and

\* Part 55 in the series 'Studies on Plant Tissue Cultures'. For Part 54 see Ayabe, S., Udagawa, A. and Furuya, T. (1988) *Arch. Biochem. Biophys.* (in press).

Table 1.  $^1\text{H}$  NMR spectral data for the biotransformation products **2-4**, **6**, **7** and **8**-diacetate (400 MHz)

H	2	3	4	6	7	8-diacetate*
1			3.76 <i>br s</i> (5.5)			
3	4.04 <i>br s</i> (7)	3.66 <i>m</i> (24)	4.12 <i>br s</i> (7.5)	4.12 <i>br s</i> (7)	3.92 <i>m</i> (23)	4.70 <i>m</i> (27)
4						5.23 <i>dd</i> (11.5, 9.5)
17	3.17 <i>dd</i> (9.5, 9.5)	3.19 <i>dd</i> (9.5, 9.5)	3.17 <i>dd</i> (9.5, 9.5)	3.17 <i>dd</i> (9.5, 9.5)	3.18 <i>dd</i> (9.5, 9.5)	3.09 <i>dd</i> (9.5, 9.5)
3H-18	1.03 <i>s</i>	1.03 <i>s</i>	1.03 <i>s</i>	1.03 <i>s</i>	1.03 <i>s</i>	0.94 <i>s</i>
3H-19	0.97 <i>s</i>	0.92 <i>s</i>	1.09 <i>s</i>	0.94 <i>s</i>	0.89 <i>s</i>	0.89 <i>s</i>
21a	4.84 <i>ddd</i> (18, 1.5, 1)	4.72 <i>ddd</i> (18, 1.5, 1)	4.83 <i>ddd</i> (18, 1.5, 1)	4.84 <i>ddd</i> (18, 1.5, 1)	4.84 <i>ddd</i> (18, 1.5, 1)	4.61 <i>ddd</i> (17.5, 1.5, 1)
21b	4.96 <i>dd</i> (18, 1.5)	4.82 <i>dd</i> (18, 1.5)	4.99 <i>dd</i> (18, 1.5)	4.95 <i>dd</i> (18, 1.5)	4.95 <i>dd</i> (18, 1.5)	4.72 <i>dd</i> (17.5, 1.5)
22	5.94 <i>dd</i> (3.5, 1.5)	5.88 <i>dd</i> (3.5, 1.5)	5.93 <i>dd</i> (3.5, 1.5)	5.94 <i>dd</i> (3.5, 1.5)	5.95 <i>dd</i> (3.5, 1.5)	5.79 <i>dd</i> (3.5, 1.5)
MeCOO						1.91 <i>s</i> , 1.93 <i>s</i>

\* Measured in  $\text{CDCl}_3$ ; the rest in  $\text{CD}_3\text{OD}$ . The figures in parentheses are coupling constants in Hz except for  $W_{1/2}$  values in *br s* and *m*.

Table 2.  $^{13}\text{C}$  NMR chemical shifts of the biotransformation products **2-4**, **6**, **7** and **8**-diacetate (100 MHz)

C	2	3	4	6	7	8-diacetate*
1	31.08	34.97	74.97	26.42	30.95	33.74
2	28.84	30.59	33.34	28.78	31.14	29.69
3	67.98	71.60	69.69	69.35	68.74	75.67
4	34.52	36.25	34.68	38.10	42.78	70.98
5	37.77	41.55	32.22	76.47	75.99	46.44
6	28.08	26.88	27.60	36.15	37.35	24.37
7	22.41	21.08	21.66	24.63	24.91	20.84
8	42.61	41.92	42.68	41.62	41.88	41.40
9	37.12	36.48	39.02	40.56	40.59	37.97
10	36.73	34.75	41.44	42.15	41.15	37.10
11	21.69	19.95	22.19	22.11	21.74	19.13
12	32.10†	30.93†	32.04†	32.03†	32.13†	30.76†
13	50.56	48.81	50.38	50.38	50.38	48.70
14	87.52	86.08	87.24	87.41	87.38	85.81
15	32.32†	31.54†	32.22†	32.32†	32.29†	31.79†
16	25.70	24.40	25.74	25.66	25.63	24.93
17	50.16	48.34	50.10	50.06	50.06	48.30
18	19.24	18.23	19.26	19.14	19.10	18.17
19	24.65	23.24	19.76	17.63	17.00	23.27
20	175.47	171.20	175.10	175.38	175.33	171.00
21	76.00	73.80	75.93	75.99	76.12	73.74
22	117.21	116.80	117.08	117.23	117.26	116.84
23	177.12	173.98	176.83	177.09	177.10	173.88
AcCO						20.84
						21.11
AcCO						170.50
						170.71

\* Measured in  $\text{CDCl}_3$ ; the rest in  $\text{CD}_3\text{OD}$ .

† Values in any vertical column may be interchanged.

of C-12 in the  $^{13}\text{C}$  NMR spectrum were very similar to those of product 2. The H-3 signal was observed as a multiplet ( $W_{1/2} = 24$  Hz) at  $\delta$  3.66 showing that it had the  $\beta$ -configuration. From these spectral data the structure of product 3 was elucidated as  $3\alpha,14$ -dihydroxy- $5\beta,14\beta,17\alpha$ -card-20(22)-enolide(3-*epi*-17 $\beta$ H-digitoxigenin).

Product 4 (3.7 mg; yield 0.4%, mp.  $253^\circ$ ) had molecular formula  $\text{C}_{23}\text{H}_{34}\text{O}_5$  (high-resolution mass spectrometry). In the mass spectrum of product 4, the base peak appeared at  $m/z$  201 [ $\text{C}_{15}\text{H}_{21}$ ] $^+$  which indicated that a hydroxyl group was introduced into the A or B ring of 1 [14]. In the  $^1\text{H}$  NMR spectrum, two equatorial protons were observed at  $\delta$  3.76 (1H, *br s*,  $W_{1/2} = 5.5$  Hz, H-1) and  $\delta$  4.12 (1H, *br s*,  $W_{1/2} = 7.5$  Hz, H-3 $\alpha$ ). Moreover, the cross-peak corresponding to the coupling between these protons (long-range coupling) was observed in the two-dimensional proton-proton shift correlation diagram which revealed that H-1 had the  $\alpha$ -configuration. In the  $^{13}\text{C}$  NMR spectrum, the signal of C-19 ( $\delta$  19.76, *q*) was shifted upfield by the  $\gamma$ -effect of the 1 $\beta$ -hydroxyl and the C-1 signal was observed at  $\delta$  74.97 (*d*). The other spectral data were similar to those of products 2 and 3. Therefore, the structure of product 4 was elucidated as  $1\beta,3\beta,14$ -trihydroxy- $5\beta,14\beta,17\alpha$ -card-20(22)-enolide (17 $\beta$ H-acovenosigenin-A). Sato and Nozaki [15] had reported this compound (mp  $253$ – $257^\circ$ ) as a microbial transformation product of 17 $\alpha$ -digitoxigenin (17 $\beta$ H-digitoxigenin) by *Absidia orchidis*.

Product 5 was detected as the minor fraction by HPLC (*R*<sub>f</sub> 8.1 min; solvent 80% MeOH in  $\text{H}_2\text{O}$ ) and identified with authentic periplogenin [16] by HPLC and TLC (*R*<sub>f</sub> 0.28;  $\text{CHCl}_3$ –EtOH 10:1).

Products 6 (28.0 mg; yield 2.8%) and 7 (23.5 mg; yield 2.3%) had the same molecular formula  $\text{C}_{23}\text{H}_{34}\text{O}_5$  (high-resolution mass spectrometry). The  $^1\text{H}$  NMR spectra of products 6 and 7 were very similar to each other except for the proton signal of H-3, at  $\delta$  4.12 (1H, *br s*,  $W_{1/2} = 7$  Hz, H-3 $\alpha$ ) and  $\delta$  3.92 (1H, *m*,  $W_{1/2} = 23$  Hz, H-3 $\beta$ ), respectively. On comparison of the  $^{13}\text{C}$  NMR chemical shift values of product 6 and periplogenin [14], the data for C-1 to C-10 were similar to each other, but for C-14, C-16, C-17, C-18, C-21 and C-23 moderate differences were observed and a very large difference was noted for C-12 (*ca* 8 ppm). From these data, the structures of products 6 and 7 were established to be  $3\beta,5,14$ -trihydroxy- $5\beta,14\beta,17\alpha$ -card-20(22)-enolide (17 $\beta$ H-periplogenin) and  $3\alpha,5,14$ -trihydroxy- $5\beta,14\beta,17\alpha$ -card-20(22)-enolide (3-*epi*-17 $\beta$ H-periplogenin), respectively. Product 6 has been reported before as alloperiplogenin, the aglycone of alloperiplocyarin [17], and a microbial transformation product of 17 $\beta$ H-digitoxigenin [15]. On the other hand, product 7 is a new compound in nature.

The molecular formula of product 8 ( $\text{C}_{23}\text{H}_{34}\text{O}_5$ , high-resolution mass spectrometry) showed the introduction of a hydroxyl group into 1, but it was impure as judged by the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra. Acetylation of product 8 gave a diacetate (3.0 mg; yield 0.2%;  $\text{C}_{27}\text{H}_{38}\text{O}_7$ , HRMS. In the mass spectrum of product 8 diacetate, the base peak appeared at  $m/z$  201 [ $\text{C}_{15}\text{H}_{21}$ ] $^+$  the same as for product 4. The signals for two acetoxy methyls were observed at  $\delta$  1.91 (3H, *s*) and  $\delta$  1.93 (3H, *s*) in the  $^1\text{H}$  NMR spectrum. The cross-peaks corresponding to the coupling between two protons at  $\delta$  4.70 (1H, *m*,  $W_{1/2} = 27$  Hz, H-3 $\beta$ ) and  $\delta$  5.23 (1H, *dd*,  $J = 11.5, 9.5$  Hz), and to the latter proton ( $\delta$  5.23) and the C-5 carbon ( $\delta$  64.44, *d*) were observed in the two-dimensional proton-proton

and the proton-carbon shift correlation diagrams, respectively. Moreover, on comparison of the  $^{13}\text{C}$  NMR chemical shift values of  $4\beta$ -acetoxy- $3\alpha,4\beta,14$ -trihydroxy- $5\beta,14\beta,17\beta$ -card-20(22)-enolide [18], the structure of product 8 diacetate was elucidated as  $3\alpha,4\beta$ -diacetoxy- $3\alpha,4\beta,14$ -trihydroxy- $5\beta,14\beta,17\alpha$ -card-20(22)-enolide (3-*epi*-4 $\beta$ -hydroxy-17 $\beta$ H-digitoxigenin diacetate), and it is a new compound. After digitoxigenin (1) (60 mg) was incubated for six days with the cultured Sg-3 cells (130 g fr. wt), products 2–5 and 6 were detected on TLC, but 7 and 8 were not observed. Therefore the biotransformation pathway of 1 by cell suspension cultures of *S. gratus* was presumed as shown in Scheme 1.

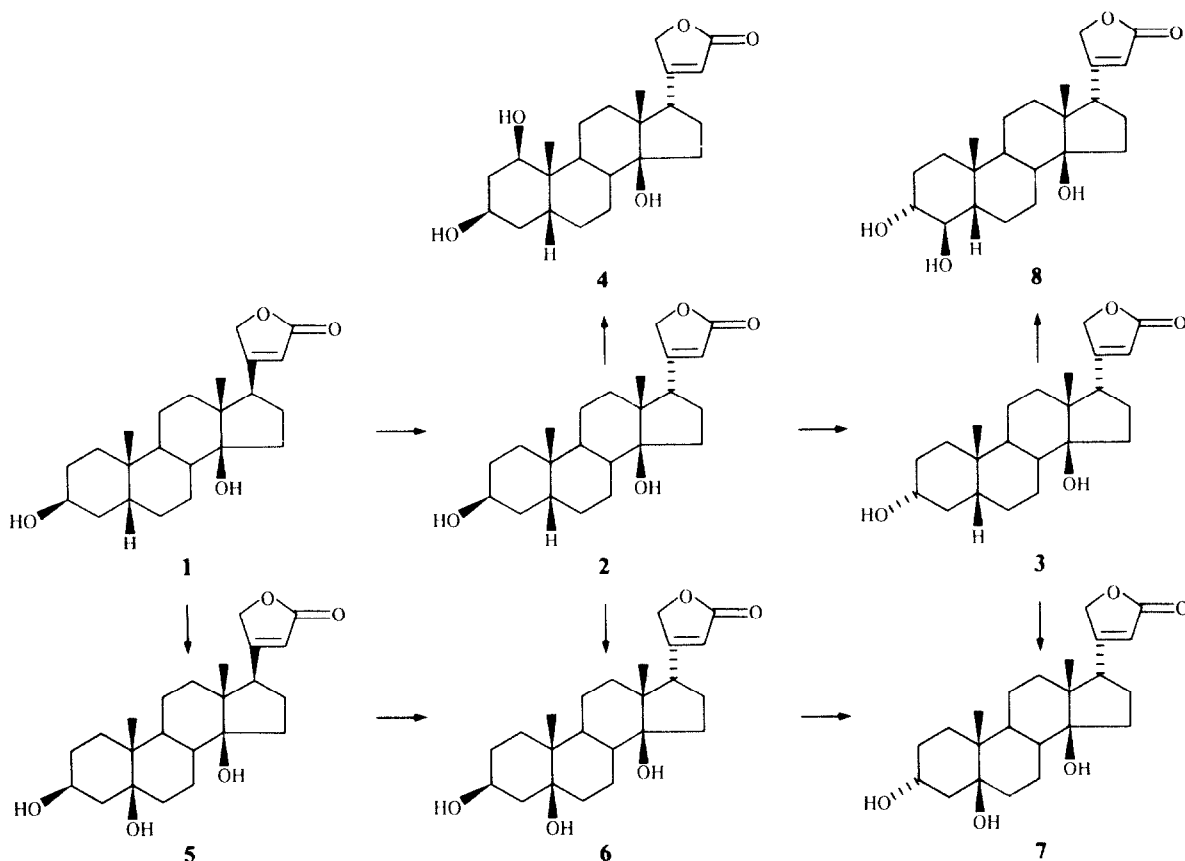
The biotransformation by isomerization of the 17 $\beta$ -butenolide ring (1 to 2 and 5 to 6) has been demonstrated for the first time by a plant cell suspension culture. However, the reaction did not occur with 10 cardenolides when incubated with tissue slices from the livers of 10 animal species including man [19], and in microbiological conversions of cardenolides. Since several 17 $\beta$ H-cardenolides have been found from natural sources, some plants are believed to possess an isomerization enzyme system for conversion of 17 $\alpha$ H- to 17 $\beta$ H-cardenolides [20, 21]. Thus, it is very interesting that the high isomerization ability has been demonstrated in the cell suspension cultures of *S. gratus*. Also, it is known that the reaction occurs chemically with sodium tosylate and sodium acetate in DMF by heating [22]. The epimerization of 3 $\beta$ -OH to 3 $\alpha$ -OH (2 to 3 and 6 to 7, probably via 3-keto) and 5 $\beta$ -hydroxylation (1 to 5, 2 to 6 and 3 to 7) performed by the cultured cells of *S. gratus* have been previously observed with cell cultures of other plants [16], though glycosylation has not been found in the cultured cells of *S. gratus*. The 1 $\beta$ - and 4 $\beta$ -hydroxylations in the cultured cells of *S. gratus* are notable, especially the 4 $\beta$ -hydroxylation which was not previously known even in microbial transformations of cardenolides. As 1 $\beta$ - and 5 $\beta$ -hydroxylations are indispensable reactions for the production of G-strophanthin, it is noteworthy that the reactions have been achieved not only with the whole plant but also with the cultured cells. Moreover, it is advantageous from the biotechnological aspect that the biotransformation products are mostly released into the medium, though some products are detected in the cells.

## EXPERIMENTAL

Mps: uncorr. NMR spectra were taken in  $\text{CDCl}_3$  or  $\text{CD}_3\text{OD}$ ;  $^{13}\text{C}$  NMR at 100 MHz and  $^1\text{H}$  NMR at 400 MHz. MS were run using a direct insertion probe at 20 eV. HPLC of biotransformation products was performed using a Nucleosil 5C18 (10  $\times$  300 mm) column, coupled to a UV detector and a differential refractometer.

**Callus induction and culture conditions.** The leaves, the volatile stems and the flower buds of *Strophanthus gratus* were sterilized with 70% EtOH and a saturated soln of bleaching powder and then rinsed with sterile  $\text{H}_2\text{O}$  and cut into *ca* 5 mm segments. These segments were placed on modified Murashige and Skoog's tobacco medium containing 1 ppm 2,4-D (2,4-dichlorophenoxyacetic acid), 0.1 ppm kinetin and 3% sucrose in Jan. 1983. The Sg-1 callus (obtained from the leaves), the Sg-2 callus (from the volatile stems) and the Sg-3 callus (from the flower buds) were subcultured at  $30^\circ$  in the dark every 4 weeks.

**Administration of digitoxigenin (1) and extraction procedure.** Digitoxigenin (1) (30 mg) suspended with Tween 80 was added



Scheme 1. Biotransformation of digitoxigenin (**1**) by cell suspension cultures of *Strophanthus gratus*

to each flask (250 ml medium/1 flask) and then the callus (*ca* 30 g fr. wt per flask) from 4-week-old static cultures was incubated at 30° in a shaker (90 rpm) for 6 or 16 days. The calli 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 twice with fresh solvent. The filtrates were combined and the organic solvent was removed under red. pres. The residue was extracted with  $\text{CHCl}_3$  and the aq. soln was extracted with  $\text{CHCl}_3$ -MeOH (2:1). The medium was extracted with the same method.

**Detection and separation of biotransformation products.** Digitoxigenin (**1**) (total 960 mg; 240, 360, and 360 mg) suspended with Tween 80 was added to the Sg-1, the Sg-2 and the Sg-3 callus (total fr. wt 1.5 kg; 400, 500 and 600 g), respectively. After incubation for 16 days, the  $\text{CHCl}_3$  and the  $\text{CHCl}_3$ -MeOH extracts from the callus and the medium were examined on TLC with Kedde's reagent and 10%  $\text{H}_2\text{SO}_4$ . 8 Kedde-positive spots ( $R_f$  0.43, 0.37, 0.33, 0.26, 0.23, 0.17, 0.13 and 0.08;  $\text{CHCl}_3$ -EtOH 10:1) were detected in both the  $\text{CHCl}_3$  and the  $\text{CHCl}_3$ -MeOH extracts from the medium of the three strains. After **1** (60 mg) was incubated for 6-days with the Sg-3 callus (130 g), 6 Kedde-positive spots ( $R_f$  0.43, 0.37, 0.33, 0.26, 0.23 and 0.17;  $\text{CHCl}_3$ -EtOH 10:1) were detected in the same extracts. These extracts (4.2 g) were separated into fraction A (Nos 23-33) and fraction B (Nos 34-42) by DCCC [droplet counter-current chromatography,  $\text{CHCl}_3$ -MeOH- $\text{H}_2\text{O}$  (5:6:4); descending method, using column of i.d. 2 mm, each fraction 10 g].

**Isolation of 17 $\beta$ H-digitoxigenin (2).** Fraction A was rechromatographed on a silica gel column (250 g Wako gel C-300) and eluted with a  $\text{CHCl}_3$ -MeOH solvent system and separated as follows; fraction A-1,  $\text{CHCl}_3$  (4.1 l); fraction A-2,  $\text{CHCl}_3$ -MeOH (97:3, 2.7 l) and fraction A-3,  $\text{CHCl}_3$ -MeOH (93:7, 2.9 l). Fraction A-1 yielded the crude product **2**. Further purification of product **2** was achieved by repeated HPLC (Nucleosil 5C18, solvent 70% MeOH in  $\text{H}_2\text{O}$ , flow rate: 3 ml/min) and product **2** was isolated from the fractions containing the peak at 11.8 min. Product **2** was recrystallized from MeOH- $\text{H}_2\text{O}$  to give colourless needles (45.0 mg), mp 160-163°,  $\text{C}_{23}\text{H}_{34}\text{O}_4$  (required 374.2457,  $[\text{M}]^+$  at  $m/z$  374.2453); IR  $\nu_{\text{max}}^{\text{KBr}}$   $\text{cm}^{-1}$ : 3400, 1775 (sh), 1720, 1615. For  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectral data of product **2** see Tables 1 and 2. EIMS  $m/z$  (rel. int.): 374  $[\text{M}]^+$  (7), 356  $[\text{M}-\text{H}_2\text{O}]^+$  (26), 338  $[\text{M}-2 \times \text{H}_2\text{O}]^+$  (4), 246  $[\text{C}_{17}\text{H}_{26}\text{O}]^+$  (22), 203  $[\text{C}_{15}\text{H}_{23}]^+$  (100), 162  $[\text{C}_{12}\text{H}_{18}]^+$  (18), 147  $[\text{C}_{11}\text{H}_{15}]^+$  (18).

**Isolation of 3-epi-17 $\beta$ H-digitoxigenin (3).** Product **3** (64.5 mg) was isolated from fraction A-2 by means of the same methods described above for product **2**. Product **3** ( $R_f$  14.0 min: HPLC), mp 159-162°,  $\text{C}_{23}\text{H}_{34}\text{O}_4$  (required 374.2457,  $[\text{M}]^+$  at  $m/z$  374.2453); IR  $\nu_{\text{max}}^{\text{KBr}}$   $\text{cm}^{-1}$ : 3400, 1780 (sh), 1720, 1615.  $^1\text{H}$  and  $^{13}\text{C}$  NMR: see Tables 1 and 2. EIMS  $m/z$  (rel. int.): 374  $[\text{M}]^+$  (5), 356  $[\text{M}-\text{H}_2\text{O}]^+$  (21), 341 (5), 246  $[\text{C}_{17}\text{H}_{26}\text{O}]^+$  (33), 203  $[\text{C}_{15}\text{H}_{23}]^+$  (100), 189  $[\text{C}_{14}\text{H}_{21}]^+$  (12), 174 (7), 162  $[\text{C}_{12}\text{H}_{18}]^+$  (21).

**Isolation of 17 $\beta$ H-acovenosigenin-A (4).** Product **4** (3.7 mg) was isolated from fraction A-2 by the same methods described

above for product 2. Product 4 ( $R_f$  8.4 min: HPLC), mp 253°,  $C_{23}H_{34}O_5$  (required 390.2406,  $[M]^+$  at  $m/z$  390.2403).  $^1H$  and  $^{13}C$  NMR: see Tables 1 and 2. EIMS  $m/z$  (rel. int.): 390  $[M]^+$  (5), 372  $[M-H_2O]^+$  (35), 354  $[M-2 \times H_2O]^+$  (46), 318  $[C_{19}H_{26}O_4]^+$  (24), 302  $[C_{19}H_{26}O_3]^+$  (28), 262  $[C_{17}H_{26}O_2]^+$  (14), 234  $[C_{15}H_{22}O_2]^+$  (13), 219  $[C_{15}H_{23}O]^+$  (49), 201  $[C_{15}H_{21}]^+$  (100), 195 (33), 178 (39), 160  $[C_{12}H_{16}]^+$  (33), 149 (49), 145  $[C_{11}H_{13}]^+$  (19).

**Isolation of 17 $\beta$ H-periplogenin (6).** After purification of fraction A-2 by HPLC ( $R_f$  7.3 min: solvent 80% MeOH in  $H_2O$ ), product 6 was recrystallized from MeOH- $H_2O$  (28.0 mg), mp 246°,  $C_{23}H_{34}O_5$  (required 390.2406,  $[M]^+$  at  $m/z$  390.2409); IR  $\nu_{max}^{KBr}$   $cm^{-1}$ : 3420, 1780, 1755, 1635.  $^1H$  and  $^{13}C$  NMR: see Tables 1 and 2. EIMS  $m/z$  (rel. int.): 390  $[M]^+$  (2), 372  $[M-H_2O]^+$  (21), 354  $[M-2 \times H_2O]^+$  (30), 336  $[M-3 \times H_2O]^+$  (17), 318  $[C_{19}H_{26}O_4]^+$  (100), 300  $[C_{19}H_{24}O_3]^+$  (30), 231 (7), 219  $[C_{15}H_{23}O]^+$  (28), 201  $[C_{15}H_{21}]^+$  (42), 145  $[C_{11}H_{13}]^+$  (11).

**Identification of periplogenin (5).** When product 6 was purified by HPLC, the minor fraction contained in the peak at 8.1 min was detected. The product was not isolated but identified with authentic periplogenin (5) by TLC ( $R_f$  0.28;  $CHCl_3$ -EtOH 10:1) and HPLC.

**Isolation of 3-epi-17 $\beta$ H-periplogenin (7).** Product 7 (23.5 mg) was isolated from fraction A-3 and fraction B after purification by HPLC  $R_f$  7.8 min: solvent 70% MeOH in  $H_2O$ ). Product 7, mp 199–201°,  $C_{23}H_{34}O_5$  (required 390.2406,  $[M]^+$  at  $m/z$  390.2403); IR  $\nu_{max}^{KBr}$   $cm^{-1}$ : 3500, 1780 (sh), 1720, 1620.  $^1H$  and  $^{13}C$  NMR: see Tables 1 and 2. EIMS  $m/z$  (rel. int.): 390  $[M]^+$  (4), 372  $[M-H_2O]^+$  (31), 354  $[M-2 \times H_2O]^+$  (43), 336  $[M-3 \times H_2O]^+$  (18), 318  $[C_{19}H_{26}O_4]^+$  (11), 219  $[C_{15}H_{23}O]^+$  (21), 201  $[C_{15}H_{21}]^+$  (100), 160  $[C_{12}H_{16}]^+$  (14), 145  $[C_{11}H_{13}]^+$  (13).

**Isolation of 3-epi-4 $\beta$ -hydroxy-17 $\beta$ H-digitoxigenin (8) diacetate.** From fraction A-3 product 8 was purified by HPLC ( $R_f$  9.4 min: solvent 70% MeOH in  $H_2O$ ) and repeatedly recrystallized from MeOH- $H_2O$  to give colourless needles (5.0 mg). These crystals,  $C_{23}H_{34}O_5$  (required 390.2406,  $[M]^+$  at  $m/z$  390.2406), were still impure as judged by  $^1H$  and  $^{13}C$  NMR. After acetylation with pyridine- $Ac_2O$  at room temp. and purification through repeated prep. TLC ( $R_f$  0.37;  $CHCl_3$ -EtOH 19:1), product 8 diacetate was recrystallized from MeOH- $H_2O$  (3.0 mg), mp 148–150°,  $C_{27}H_{38}O_7$  (required 474.2617,  $[M]^+$  at  $m/z$  474.2617).  $^1H$  and  $^{13}C$  NMR: see Tables 1 and 2. EIMS  $m/z$  (rel. int.): 474  $[M]^+$  (11), 432  $[M-C_2H_5O]^+$  (6), 372  $[M-HOAc-C_2H_5O]^+$  (92), 354  $[M-2 \times HOAc]^+$  (13), 229  $[C_{16}H_{21}O]^+$  (19), 219  $[C_{15}H_{23}O]^+$  (25), 201  $[C_{15}H_{21}]^+$  (100), 160  $[C_{12}H_{16}]^+$  (19), 145  $[C_{11}H_{13}]^+$  (31).

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