*Phytochemistry*, Vol 27, No 11, pp 3475-3479, 1988
Printed in Great Britain

0031-9422/88 $3.00+0.00


# BIOTRANSFORMATION OF DIGITOXIGENIN BY CELL SUSPENSION CULTURES OF *STROPHANTHUS AMBOENSIS**

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(*Received* 8 *February* 1988)

**Key Word Index**—*Strophanthus amboensis*, Apocynaceae, cell cultures; biotransformation, 5β-hydroxylation, glucosylation, cardenolides, digitoxigenin, 3-epiperiplogenin, periplogenin β-D-glucoside

**Abstract**—Six biotransformation products of digitoxigenin by cell suspension cultures of *Strophanthus amboensis* are described.

## INTRODUCTION

In our previous paper, we investigated the biotransformation of digitoxigenin (**1**) by cell suspension cultures of *Strophanthus gratus*, and reported on 17βH-digitoxigenin, 3-*epi*-17βH-digitoxigenin, 17βH-acovenosigenin-A, periplogenin, 17βH-periplogenin, 3-*epi*-17βH-periplogenin and 3-*epi*-4β-hydroxy-17βH-digitoxigenin as products [1]. The biotransformation of **1**, a precursor of cardiac glycosides, by plant cell cultures has been carried out with *Digitalis lanata* [2, 3], *D. purpurea* [2, 4], *Thevetia neriifolia* [5] and *Daucus carota* [6] in the hope of obtaining new cardenolides. *Strophanthus amboensis* is one of the Apocynaceous plants containing a number of cardiac glycosides [7, 8]. Dohnal *et al.* [9] have reported on the growth and phytochemical analysis of *S. amboensis* callus tissue culture and identified phytosterols and ursolic acid but cardenolides could not be detected. This paper deals with the biotransformation of **1** by a cell suspension culture of this plant.

## RESULTS AND DISCUSSION

The cell strain used for this work was derived from the leaves of *Strophanthus amboensis* [See Experimental]. However, no cardenolides were detected by TLC analysis of extracts of the cells. After digitoxigenin (**1**) (600 mg) was incubated with the cells (1.0 kg fr. wt) for 18 days, the cells and the medium were extracted with chloroform and chloroform–methanol (2 1), respectively. Seven Kedde-positive spots were detected on TLC of the chloroform extracts and four Kedde-positive spots were found in the chloroform–methanol extracts. These spots were distinct from the biotransformation products of **1** found in the cell cultures of *Strophanthus gratus* [1]. After these extracts were combined (6.1 g) and separated, the products **2**–**4**, **5**-tetraacetate and **7** were isolated as crystalline compounds and their chemical structures were elucidated. Furthermore, product **6**-tetraacetate was identical with an authentic sample by HPLC and TLC.

Product **2** was isolated as colourless prisms (25.0 mg, yield 4.2%), and had the composition $C_{23}H_{34}O_4$ on the basis of high-resolution mass spectroscopy. In the $^1$H NMR spectrum of product **2** (Table 1), the H-3 signal appeared as a multiplet ($W_{1/2}=26$ Hz) at $\delta$3.59, thus showing that H-3 had the β-configuration. The other proton signals were similar to those of **1**. These results indicate that **2** is 3α,14-dihydroxy-5β,14β-card-20(22)-enolide (3-epidigitoxigenin) Compound **2** has been identified previously as a product of the biotransformation of **1** in a *D. purpurea* cell culture [4].

Product **3** (33.0 mg; yield 5.3%), the main product in this experiment, had molecular formula $C_{23}H_{34}O_5$ (high-resolution mass spectrometry), and its structure was determined as 3β,5,14-trihydroxy-5β,14β-card-20(22)-enolide (periplogenin) through the $^1$H and $^{13}$C NMR spectral data (Tables 1 and 2). 5β-Hydroxylation of **1** by plant cell cultures has been previously observed with *D. purpurea* [4], *D. carota* [6] and *S gratus* [1].

Product **4** (9.0 mg; yield 1.4%) had molecular formula $C_{23}H_{34}O_5$ (high-resolution mass spectrometry), and the $^1$H NMR spectral data of products **3** and **4** were 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-3α) and 4.05 (1H, *m*, $W_{1/2}=22$ Hz, H-3β). In the $^{13}$C NMR spectra, the data for C-1 to C-10 of products **3** and **4** showed moderate differences From the data, the structure of product **4** was established to be 3α,5,14-trihydroxy-5β,14β-card-20(22)-enolide (3-epiperiplogenin). The formation of 3-epiperiplogenin (**4**), which has been isolated from leaves of *Adonis vernalis* [10], is now demonstrated in plant cell cultures.

Product **5**-tetraacetate (7.5 mg, yield 0.7%) was isolated after acetylation, had molecular formula $C_{37}H_{52}O_{13}$ (high-resolution mass spectrometry). The main mass spectral fragment peaks were observed at *m/z* 704 $[M]^+$, 357 $[C_{23}H_{33}O_3]^+$ and 331 $[C_{14}H_{19}O_9]^+$. The peak at *m/z* 357 suggested that the aglycone part was a digitoxigenin analogue, and the peak *m/z* 331 suggested that product **5**-tetraacetate was a tetraacetyl D-glucopyr-

*Part 57 in the series 'Studies on Plant Tissue Cultures'. For Part 56 see Furuya, T., Orihara, Y., Takagi, S and Yoshida, T (1988) *Plant Tiss Cult Letters* (in press)

Table 1 $^1$H NMR spectral data for biotransformation products **2**–**4**, **5**-tetraacetate and **7** (400 MHz, $CDCl_3$)

| Protons | **2** | **3*** | **4**† | **5**-tetraacetate | **7***† |
|---|---|---|---|---|---|
| H-3 | 3 59 *m* | 4 12 *br s* | 4 05 *m* | 4 01 *br s* | 4 22 *br s* |
| | (26) | (7) | (22) | (7 5) | (7) |
| H-17 | 2 71 *dd* | 2 84 *dd* | 2 79 *dd* | 2 77 *dd* | 2 83 *dd* |
| | (9, 6) | (9, 6) | (9, 6) | (9, 5 5) | (9, 6) |
| 3H-18 | 0 80 *s* | 0 88 *s* | 0 88 *s* | 0 87 *s* | 0 89 *s* |
| 3H-19 | 0 85 *s* | 0 93 *s* | 0 89 *s* | 0 90 *s* | 0 93 *s* |
| H-21a | 4 53 *dd* | 4 91 *dd* | 4 81 *dd* | 4 80 *dd* | 4 91 *dd* |
| | (18, 2) | (18, 2) | (18, 2) | (18, 1 8) | (18, 2) |
| H-21b | 4 92 *dd* | 5 03 *dd* | 4.99 *dd* | 4 98 *dd* | 5 03 *dd* |
| | (18, 2) | (18, 2) | (18, 2) | (18, 1 8) | (18, 2) |
| H-22 | 5 80 *dd* | 5 89 *dd* | 5 89 *dd* | 5 88 *dd* | 5 90 *dd* |
| | (2, 2) | (2, 2) | (2, 2) | (1 8, 1 8) | (2, 2) |
| H-1′ | | | | 4 55 *d* | 4 41 *d* |
| | | | | (8) | (9) |
| H-2′ | | | | 5 00 *dd* | 3 17 *dd* |
| | | | | (9 5, 8) | (9, 9) |
| H-3′ | | | | 5 22 *dd* | — |
| | | | | (9 5, 9.5) | |
| H-4′ | | | | 5 08 *dd* | — |
| | | | | (9 5, 9 5) | |
| H-5′ | | | | 3.67 *ddd* | — |
| | | | | (9 5, 5, 2 5) | |
| H-6′ | | | | 4 12 *dd* | 3 65 *dd* |
| | | | | (12, 2 5) | (12, 2 5) |
| H-6″ | | | | 4 25 *dd* | 3 85 *dd* |
| | | | | (12, 5) | (12, 5) |
| MeCOO | | | | 2 01 *s*, 2 02 *s*, 2 02 *s*, 2 08 *s* | |

*Measured in $CD_3OD$

†Measured at 300 MHz

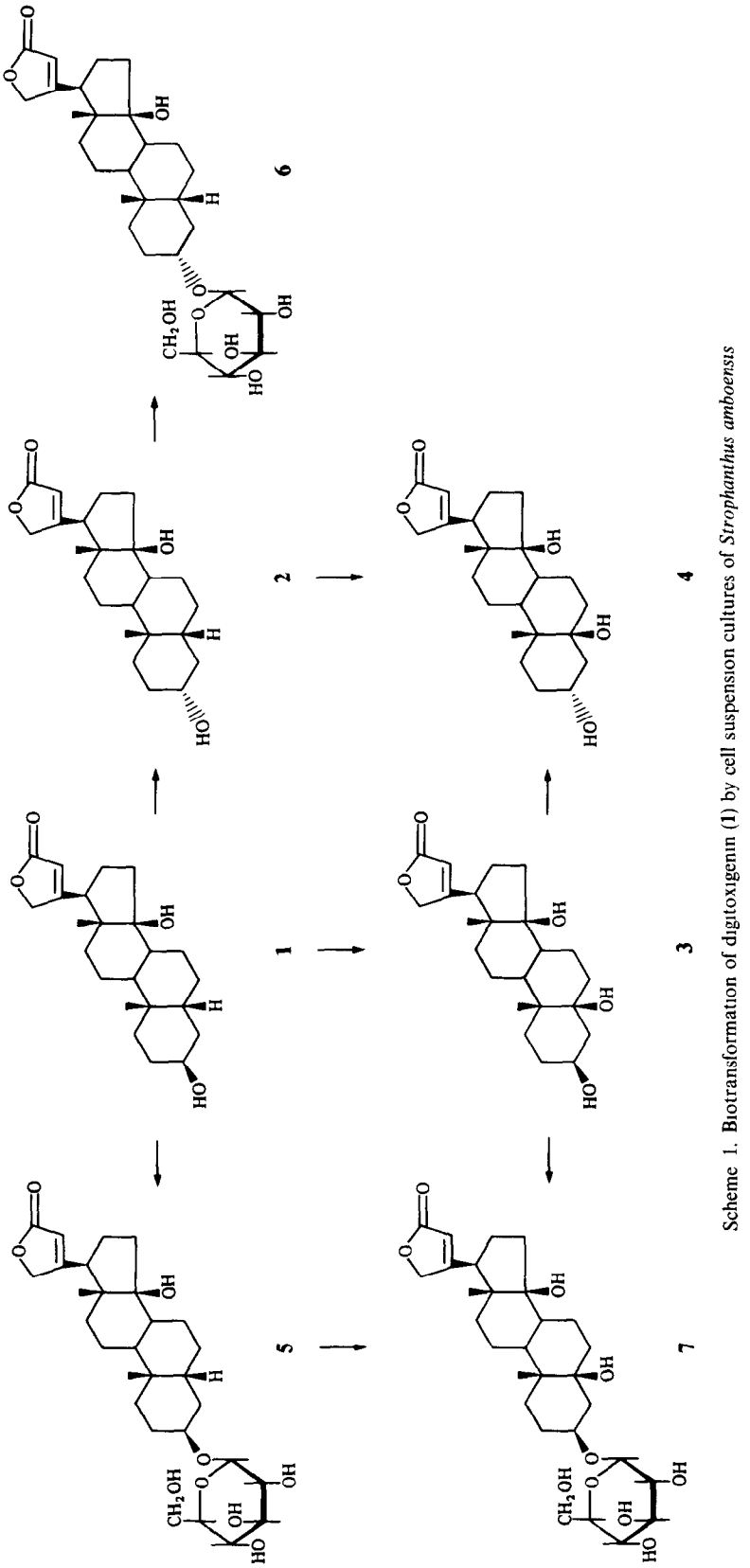
—Indicates signals were unresolved for signals of solvent.

The figures in parentheses are coupling constants in Hz except for $W_{1/2}$ values for *br s* and *m*

anoside [11]. In the $^1$H NMR spectrum, the signal for H-3 was observed at $\delta$4.01 (1H, *br s*, $W_{1/2}$=7.5 Hz, H-3α), and the signal for an anomeric proton was observed at $\delta$4.55 (1H, *d*, $J$=8 Hz), indicating that the sugar had the β-configuration [12] Moreover, consideration of the $^{13}$C NMR spectral data of product **5**-tetraacetate showed that the compound was digitoxigenin β-D-glucoside tetraacetate At the same time, product **6**-tetraacetate was identified as 3-epidigitoxigenin β-D-glucoside tetraacetate by comparison with the authentic compound (HPLC and TLC) The formation of digitoxigenin β-D-glucoside (**5**) and 3-epidigitoxigenin β-D-glucoside (**6**) has been demonstrated using cell cultures of *D purpurea*, with production of compound **6** predominating over compound **5** [4]. This fact is consistent with the results of enzymatic studies which showed that digitoxigenin (**1**) has a very low affinity for sterol. UDPG glucosyltransferase purified from the cells and plant [13]. From these results, it is interesting to note the different extents of glycosylation, the axial 3β-hydroxy group (**5**) was glucosylated preferentially compared to the equatorial 3α-hydroxy group (**6**), as was observed with the cell cultures of *S amboensis*

The main mass spectral fragment peaks of product **7** (26 5 mg, yield 3.0%) were observed at *m/z* 552 $[M]^+$, 390 $[C_{23}H_{34}O_5]^+$ and 318 $[C_{19}H_{26}O_4]^+$ The peaks at *m/z* 390 and 318 suggested that the aglycone part was a 5-hydroxydigitoxigenin analogue [14] In the $^1$H NMR spectrum, the signal for H-3 was observed at $\delta$4.22 (1H, *br s*, $W_{1/2}$=7 Hz, H-3α), and the signal for an anomeric proton was observed at 4 41 (1H, *d*, $J$=9 Hz), indicating the β-configuration On comparison of the $^{13}$C NMR chemical shift values of product **7** and periplogenin (**3**), the data for the aglycone part were similar to each other except for the C-2, C-3 and C-4 signals The data for the sugar part, C-1′ to C-6′, corresponded to the data for the sugar part of digitoxigenin β-D-glucoside [15]. From these data, the structure of product **7** was elucidated as periplogenin β-D-glucoside, which was isolated from seeds of *Strophanthus vanderijstii* [16], and is a new biotransformation product of **1** produced by plant cell cultures.

The possible biotransformation pathway of **1** by cell suspension cultures of *S. amboensis* is shown in Scheme 1 The epimerization of 3β-OH to 3α-OH and 5β-hydroxylation performed by the cell cultures of *S. gratus* have been observed with the cell cultures of *S. amboensis*, but 1β- and 4β-hydroxylation and isomerization of the 17β-butenolide ring were not demonstrated in *S amboensis*. On the other hand, the glycosylation ability exhibited by



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

*D purpurea*, was also found in *S amboensis* but not in *S gratus* The new biotransformation products, 3-epiperiplogenin (**4**) and periplogenin β-D-glucoside (**7**), are being evaluated for cardiotonic activity *in vitro* The various biotransformation abilities of plant cell cultures from the different origins will contribute possibly to the provision of novel cardiac glycosides, some of which may prove useful in the pharmaceutical industry [17]

## EXPERIMENTAL

Mps uncorr NMR 300 and 400 MHz ($CDCl_3$ or $CD_3OD$) FDMS was taken with a JEOL JMS D-300 instruments equipped with a direct inlet system HPLC of the biotransformation products was performed using a Nucleosil 5C18 (10 × 300 mm) column, coupled to a UV detector and a differential refractometer

*Culture methods* The leaves of *Strophanthus amboensis* were sterilized by 70% EtOH and a saturated soln of bleaching powder and then rinsed with sterile $H_2O$ and cut into *ca* 5 mm segments These segments were placed on modified Murashige and Skoog's tobacco medium containing 1 0 ppm 2,4-dichlorophenoxyacetic acid, 0 1 ppm kinetin and 3% sucrose in Jan, 1983 The calli were subcultured at 30 in the dark every 4 weeks In the biotransformation experiments, the calli were transferred to a liquid medium containing digitoxigenin (**1**) suspended with Tween 80, and incubated in a shaker (90 spm) for 18 days

*Detection and separation of biotransformation products* Digitoxigenin (**1**) (600 mg) was added to the calli (1 0 kg fr wt) from 4-week-old static cultures, and after 18 days, the $CHCl_3$ and the $CHCl_3$–MeOH (2 1) extracts from the calli and the medium were obtained according to the method described in a previous paper [1] The $CHCl_3$ extracts from the calli and the medium were examined on TLC with Kedde's reagent and 10% $H_2SO_4$, seven Kedde-positive spots ($R_f$ 0 41, 0 26, 0 18, 0 15, 0 10, 0 04, 0 01, $CHCl_3$–EtOH 10 1) were detected Four Kedde-positive spots ($R_f$ 0 34, 0 29, 0 24, 0 14; $CH_2Cl_2$–MeOH–$H_2O$ 84 15 1) were detected similarly in the $CHCl_3$–MeOH (2 1) extracts These extracts were combined (6 1 g), chromatographed on a silica gel column (250 g Wako gel C-200) and eluted as follows fraction A, $CHCl_3$ (4 0 l), fraction B, $CHCl_3$–MeOH (19 1, 1 8 l), fraction C, $CHCl_3$–MeOH (9 1, 2 4 l) and fraction D, $CHCl_3$–MeOH (4 1, 0 8 l)

*Isolation of 3-epidigitoxigenin* (**2**) Fraction A yielded the crude product **2** Further purification of product **2** was achieved by repeated HPLC (Nucleosil 5C18, 70% MeOH in $H_2O$, flow rate 3 ml/min) and product **2** was isolated from the fraction containing the peak at 15 4 min Product **2** was recrystallized from MeOH to give colourless prisms (25 0 mg), mp 268–271°, $C_{23}H_{34}O_4$ (required 374 2457, $[M]^+$ at *m/z* 374 2468), IR $\nu_{max}^{KBr}$ $cm^{-1}$ 3430, 1745, 1635 For $^1H$ and $^{13}C$ NMR spectral data of product **2** see Tables 1 and 2 EIMS *m/z* (rel int) 374 $[M]^+$ (6), 356 $[M-H_2O]^+$ (25), 338 $[M-2\times H_2O]^+$ (4), 246 $[C_{17}H_{26}O]^+$ (34), 203 $[C_{15}H_{23}]^+$ (100), 162 $[C_{12}H_{18}]^+$ (20), 147 $[C_{11}H_{15}]^+$ (14)

*Isolation of periplogenin* (**3**) Product **3** (33 0 mg) was isolated from fraction B after purification by HPLC ($R_t$ 8 1 min solvent 80% MeOH in $H_2O$) Product **3**, mp 136–140° (from MeOH), $C_{23}H_{34}O_5$ (required 390 2405, $[M]^+$ at *m/z* 390 2398), IR $\nu_{max}^{KBr}$ $cm^{-1}$ 3320, 1775, 1740, 1620 $^1H$ and $^{13}C$ NMR see Tables 1 and 2 EIMS *m/z* (rel int) 390 $[M]^+$ (1), 372 $[M-H_2O]^+$ (16), 354 $[M-2\times H_2O]^+$ (22), 318 $[C_{19}H_{26}O_4]^+$ (100), 300 $[C_{19}H_{24}O_3]^+$ (7), 262 $[C_{17}H_{26}O_2]^+$ (9), 219 $[C_{15}H_{23}O]^+$ (40), 201 $[C_{15}H_{21}]^+$ (59), 145 $[C_{11}H_{13}]^+$ (18)

Table 2 $^{13}C$ NMR chemical shifts of biotransformation products **2–4**, **5** tetraacetate and **7** (100 MHz, $CDCl_3$)

| C | 2 | 3* | 4† | 5-tetraacetate | 7*† |
|---|---|---|---|---|---|
| 1 | 33 1 | 26 5 | 29 4 | 29 6 | 26 6 |
| 2 | 30 5 | 28 8 | 29 8 | 26 9 | 27 0 |
| 3 | 71 5 | 69 4 | 67 6 | 73 4 | 75 9 |
| 4 | 36 1 | 36 2 | 41 6 | 33 1 | 34 6 |
| 5 | 41 4 | 76 5 | 74 8 | 36 3 | 75 1 |
| 6 | 26 8 | 38 1 | 36 5 | 26 4 | 36 2 |
| 7 | 21 4 | 25 1 | 23 7 | 21 4 | 25 0 |
| 8 | 41 9 | 42 2 | 41 0 | 41 9 | 41 6 |
| 9 | 36 1 | 40 4 | 38 9 | 35 7 | 40 1 |
| 10 | 34 9 | 42 0 | 39 7 | 35 1 | 41 8 |
| 11 | 20 9 | 23 1 | 21 0 | 21 2 | 22 6 |
| 12 | 39 9 | 41 3 | 39 8 | 40 0 | 40 9 |
| 13 | 49 4 | 51 2 | 49 3 | 49 6 | 50 9 |
| 14 | 85 5 | 86 6 | 85 3 | 85 6 | 86 4 |
| 15 | 34 7 | 33 6 | 33 0 | 33 1 | 33 4 |
| 16 | 26 9 | 28 3 | 26 7 | 26 9 | 28 0 |
| 17 | 50 8 | 52 3 | 50 5 | 50 9 | 52 0 |
| 18 | 15 6 | 16 7 | 15 9 | 15 8 | 16 4 |
| 19 | 23.1 | 17 6 | 15 8 | 23 7 | 17 3 |
| 20 | 174 3 | 177 5 | 174 3 | 174 4 | 177 3 |
| 21 | 73 3 | 75 7 | 73 6 | 74 1 | 75 4 |
| 22 | 117 6 | 118 1 | 117 6 | 117 7 | 117 9 |
| 23 | 174 4 | 178 7 | 174 3 | 174 4 | 178 4 |
| 1′ | | | | 98 7 | 101 9 |
| 2′ | | | | 71 5 | 75 1 |
| 3′ | | | | 72 8 | 78 2 |
| 4′ | | | | 68 6 | 71 6 |
| 5′ | | | | 71 7 | 78 3 |
| 6′ | | | | 62 1 | 62 8 |
| MeCOO | | | | 20 6 | |
| | | | | 20 6 | |
| | | | | 20 7 | |
| | | | | 20 8 | |
| MeCOO | | | | 169 1 | |
| | | | | 169 4 | |
| | | | | 170 4 | |
| | | | | 170 7 | |

*Measured in $CD_3OD$
†Measured at 75 2 MHz

*Isolation of 3-epiperiplogenin* (**4**) Product **4** (9 0 mg) was isolated from fraction B after purification by rechromatography on silica gel (Wako gel C-300) Product **4**, mp 232–235° (from MeOH–$H_2O$), $C_{23}H_{34}O_5$ (required 390 2405, $[M]^+$ at *m/z* 390 2383), IR $\nu_{max}^{KBr}$ $cm^{-1}$ 3400, 1750, 1630 $^1H$ and $^{13}C$ NMR see Tables 1 and 2 EIMS *m/z* (rel int) 390 $[M]^+$ (4), 372 $[M-H_2O]^+$ (19), 354 $[M-2\times H_2O]^+$ (20), 336 $[M-3\times H_2O]^+$ (4), 318 $[C_{19}H_{26}O_4]^+$ (4), 262 $[C_{17}H_{26}O_2]^+$ (7), 247 $[C_{16}H_{23}O_2]^+$ (10), 219 $[C_{15}H_{23}O]^+$ (18), 201 $[C_{15}H_{21}]^+$ (100), 160 $[C_{12}H_{16}]^+$ (12), 145 $[C_{11}H_{13}]^+$ (12)

*Isolation of digitoxigenin β-D-glucoside* (**5**) *tetraacetate* After acetylation of fractions B and C with pyridine–$Ac_2O$ at room temp and purification by HPLC ($R_t$ 26 3 min solvent 70% MeOH in $H_2O$), product **5**-tetraacetate was recrystallized from MeOH–$H_2O$ (7 5 mg), mp 164–170°, $C_{37}H_{52}O_{13}$ (required 704 3407, $[M]^+$ at *m/z* 704 3411) $^1H$ and $^{13}C$ NMR see Tables 1

and 2. EIMS *m/z* (rel. int.): 704 $[M]^+$ (2), 357 $[C_{23}H_{33}O_3]^+$ (100), 331 $[C_{14}H_{19}O_9]^+$ (66), 246 $[C_{17}H_{26}O]^+$ (27), 203 $[C_{15}H_{23}]^+$ (50), 169 (61)

*Identification of* 3-*epidigitoxigenin* β-D-*glucoside* (**6**) *tetraacetate* During **5**-tetraacetate purification by HPLC, the minor fraction, with a peak at 31.5 min, was detected Because of the small amount of sample, the product was not isolated but instead identified with authentic 3-epidigitoxigenin β-D-glucoside tetraacetate [4] by HPLC and TLC [$R_f$ 0 56 the first development with $CHCl_3$–EtOH (7 1); the second development with $C_6H_6$–$Me_2CO$ (3 1)]

*Isolation of periplogenin* β-D-*glucoside* (**7**) Product **7** (26 5 mg), mp 215–218° (from EtOH), was isolated from fraction D after purification by DCCC [DCCC, $CHCl_3$–MeOH–$H_2O$ (5 6 4); ascending method, using a column of i d 2 mm]. $^1H$ and $^{13}C$ NMR see Tables 1 and 2 EIMS *m/z* (rel. int) 552 $[M]^+$ (0 4), 534 $[M-H_2O]^+$ (1), 516 $[M-2\times H_2O]^+$ (1), 390 $[C_{23}H_{34}O_5]^+$ (20), 372 $[C_{23}H_{32}O_4]^+$ (20), 354 $[C_{23}H_{30}O_3]^+$ (45), 336 $[C_{23}H_{28}O_2]^+$ (25), 318 $[C_{19}H_{26}O_4]^+$ (100), 299 $[C_{19}H_{23}O_3]^+$ (18), 273 (10), 219 $[C_{15}H_{23}O]^+$ (18), 201 $[C_{15}H_{21}]^+$ (34), 145 $[C_{11}H_{13}]^+$ (32). FDMS *m/z* (rel int) 552 $[M]^+$ (100), 373 (15), 163 (32)

*Acknowledgements*—We would like to thank Dr T Kishi (Head) and Mr T Takahashi of Kyoto Takeda Herbal Garden for *Strophanthus amboensis* plant We would also like to express our appreciation to Mr K Kushida (Varian Instrument Ltd) for measurement of 300 MHz NMR spectra and the members of the Analytical Centre of this University for 400 MHz NMR spectra and mass spectra This work was supported by a Grant-in-Aid for Scientific Research (Project-1) from School of Pharmaceutical Sciences, Kitasato University

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