Pergamon

PII: S0031-9422(97)00748-6

*Phytochemistry*, Vol. 47, No. 7, pp. 1261–1265, 1998

Printed in Great Britain
0031 9422/98 $19.00+0.00

# BIOTRANSFORMATION OF DIGITOXIGENIN BY CULTURED *STROPHANTHUS* HYBRID CELLS

KIICHIRO KAWAGUCHI,* SACHIKO KOIKE, MASAO HIROTANI,† MICHIO FUJIHARA,‡ TSUTOMU FURUYA,§ RIKO IWATA¶ and KAZUO MORIMOTO¶

Medicinal Plant Garden, School of Pharmaceutical Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228, Japan; † School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Sirokane, Minato-ku, Tokyo 108, Japan; ‡ School of Allied Health Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228, Japan; § Faculty of Science, Okayama University of Science, 1-1 Ridai-cho, Okayama 700, Japan; ¶ Tsukuba Research Laboratory, Hitachi Chemical Co., Ltd., 48 Wadai, Tsukuba, Ibaraki 300-42, Japan

(*Received in revised form* 15 *July* 1997)

**Key Word Index**—*Strophanthus gratus*, *S. amboensis*; Apocynaceae; hybrid cells; electrofusion; biotransformation; digitoxigenin; 17$\beta H$-periplogenin $\beta$-D-glucoside; isomerization of 17$\beta$-lactone ring; glucosylation.

**Abstract**—Hybrid cells between *Strophanthus gratus* and *S. amboensis* were obtained by electrofusion and confirmed to be hybrids through isozyme and RFLP analyses. Because a new and hybrid compound, 17$\beta H$-periplogenin $\beta$-D-glucoside, was isolated as a biotransformation product of digitoxigenin by the hybrid cells, isomerization of 17$\beta$-lactone ring on *S. gratus* and glucosylation on *S. amboensis* were demonstrated simultaneously as the biotransformation abilities in the hybrid cells. Moreover, the productivity of the hybrid compound was increased by raising the sucrose concentration. © 1998 Elsevier Science Ltd. All rights reserved

## INTRODUCTION

Although *Strophanthus* plants produce cardiac glycosides, their cultured cells do not produce them *de novo*. We have investigated the species specific biological transformations of digitoxigenin (**1**), a precursor of cardiac glycosides, by suspension cultures of some *Strophanthus* species [1–4], and shown, for example, 1$\beta$-, 4$\beta$-hydroxylation and isomerization of the 17$\beta$-lactone ring by *S. gratus* cells, glucosylation by *S. amboensis* cells and 16$\beta$-hydroxylation by *S. intermedius*.

Cell fusion as a means of producing new heredity resources and of obtaining useful metabolites [5, 6] has recently made rapid progress. The hybrid cells obtained from parents having different biotransformation capabilities might be expected to produce new biotransformation products. In this paper, we have produced hybrid cells between *S. gratus* and *S. amboensis* by electrofusion and studied the biotransformation of **1** by fused cells.

## RESULTS AND DISCUSSION

### *Culture of fused protoplasts*

White cell lines of *S. gratus* (Sg) and yellowish green cell lines of *S. amboensis* (Sa) grown in the light were used as the parent lines for the production of heterokaryons. The difference between the two cell lines is that the cells of the green line contains chloroplasts. The diameters of the Sg protoplasts were about 50 $\mu$m and those of the Sa protoplasts were about 20 $\mu$m. Sg protoplasts were more susceptible to damage by electrofusion than Sa protoplasts. We, therefore, attempted to identify the fusion conditions that would not damage Sg protoplasts but that would at the same time yield high heterokaryon fusion rates. The optimum condition for electrofusion was found to be 1.0 kV cm$^{-1}$ DC pulse of 30 $\mu$s duration. The frequencies of protoplast fusion were counted by microscopic observation. The frequency of heterokaryon formation was estimated to be 5.5% of total protoplasts. The frequencies of homokaryon formation were 8.7% (Sg) and 2.2% (Sa). Hence, a method of selecting the heterokaryons from the homokaryons and infused protoplasts was needed. We first tried to cultivate Sg and Sa protoplasts in a variety of media based on Murashige and Skoog's (MS) [7] and 8p medium [8]. The highest frequency of cells undergoing division was obtained for Sg protoplasts on 1/2MS (twice diluted) medium (Fig. 1). No dividing cells were obtained from Sa protoplasts on this mode. We, therefore, selected 1/2MS medium for culturing protoplasts after fusion treatment. As the colour of Sg cells was white, while Sa cells were yellowish green when grown in the light,

* Author to whom correspondence should be addressed.

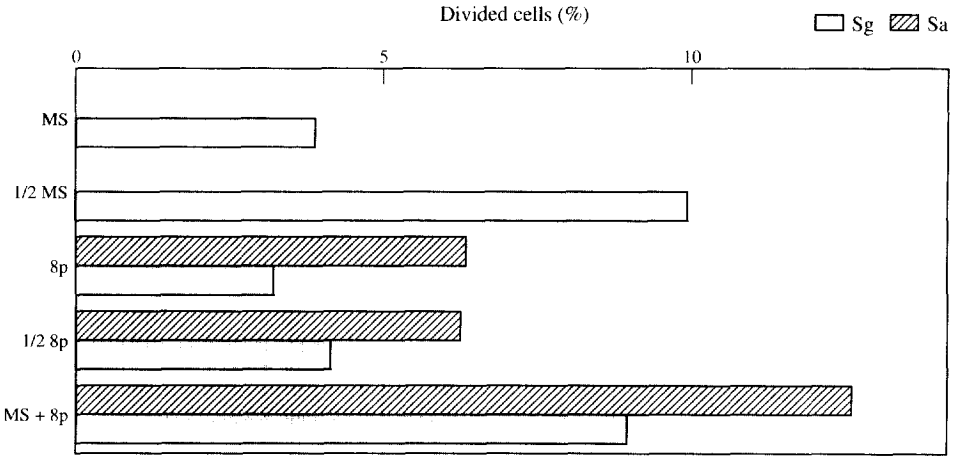



Fig. 1. Division frequency in a week old culture of *S. gratus* (Sg) and *S. amboensis* (Sa) protoplasts.

any yellowish green cells growing from the fusion-treated protoplasts in 1/2MS medium would be putative somatic hybrids. After 7–9 weeks, small colonies from fusion-treated protoplasts were observed in 1/2MS medium, and they grew faster than untreated protoplasts. On transferring to MS solid medium under continuous illumination, many cell lines between white and green were obtained 1–2 months later.

*Isozyme analysis*

Some of the cell lines obtained from fusion-treated protoplasts were homogenized and isozyme analyses were performed. The electrophoresis bands of acid phosphatase and leucine aminopeptidase in most of the cell lines examined were combinations of both parents' bands, and these cell lines were designated as hybrids. However, the intensity of bands differed cell by cell (data not shown). Because of the lack of distinction of the bands, some cell lines could not be assigned. Therefore, we used RFLP analysis to determine whether these cell lines were hybrids or not.

*RFLP analysis*

We randomly selected some yellowish green cell lines (B2-18, C14, 18, E5 and F8) from the many cell lines obtained from fusion-treated protoplasts. When the DNA extracted from the cell was digested with NheI, all of the cell lines had a combination of parents' DNA fragments hybridized with the probe (Fig. 2). The results indicate that they are all hybrids. When the DNA was digested with EcoRI, BamHI, XbaI, HindIII, EcoRV, XhoI, SpeI, BlnI, CpoI, NotI and Sse8387I, there were no particular fragments due to parents' DNA (data not shown).

*Biotransformation of digitoxigenin* (**I**) *by hybrid cells*

Experiment 1: after incubation of digitoxigenin (**1**) (120 mg) with F8 cells (262 g fr. wt) for 15 days, periplogenin β-D-glucoside (**7**) (65 mg; yield 37%, the main product) and compound **8** (7 mg; yield 4%) were isolated as the biotransformation products. At the same time, 17β*H*-periplogenin (**3**), 3-epi-17β*H*-periplogenin (**4**), 3-epiperiplogenin (**5**) and digitoxigenin β-D-glucoside (**6**) were identified with authentic samples by TLC and HPLC. The HR-FAB-mass spectrum of **8** exhibited a peak due to $[M + Na]^+$ at *m/z* 575.2880 ($C_{29}H_{44}O_{10}Na$). In the $^1H$ NMR spectrum of **8**, signals at δ 4.43 (1H, *br s*, $W_{1/2}$ = 8 Hz, H-3α), 3.30 (1H, *dd*, *J* = 9.5, 9.5 Hz, H-17β) and 1.06 (3H, *s*, $H_3$-18) were observed for the aglycone part and signals at δ 4.96 (1H, *d*, *J* = 8 Hz, H-1′), 3.93 (1H, *dd*, *J* = 8, 8 Hz, H-2′), 4.28 (1H, *dd*, *J* = 12, 5 Hz, H-6a′) and 4.47 (1H, *dd*, *J* = 12, 2 Hz, H-6b′) for the sugar part. In the $^{13}C$ NMR spectrum, the data for the aglycone part were similar to those of 17β*H*-periplogenin (**3**) [1], except for C-2 to C-4, and the data for the sugar part corresponded to the data for sugar part of periplogenin β-D-glucoside (**7**) [9]. From these spectral data, the structure of **8** was elucidated as 17β*H*-periplogenin β-D-glucoside. It is a new compound which is unique to the hybrid cells.

Experiment 2: after incubation of **1** with hybrid cell lines (F8, B18 and C14), the parent cells (Sg and Sa, separately) and a mixed culture of Sg and Sa cells, the chloroform and chloroform–isopropyl alcohol (3:2) fractions were analysed by HPLC (Table 1). Both **7** and **8** were detected in three hybrid cell lines (**7** > **8**: by comparison of peak area on HPLC) but only **7** was detected in Sa cells and the mixed culture of Sg and Sa cells. On the other hand, in agreement with previous studies [1, 2], neither **7** nor **8** was detected in Sg cells.

Therefore, the isomerization of the 17β-lactone ring brought about by Sg cells and the glucosylation by Sa

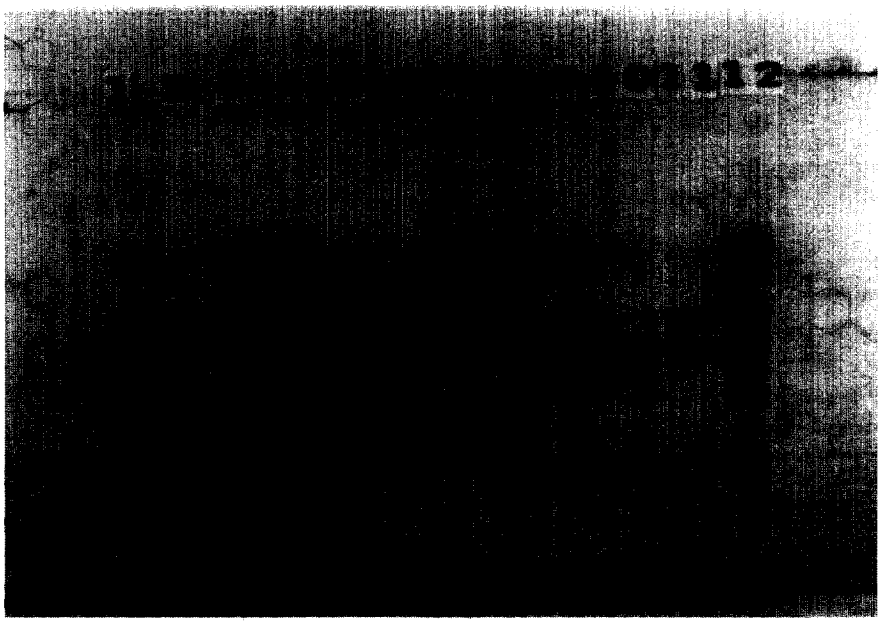


Fig. 2. Hybridization of labelled pRR217 to Nhe I digests of DNA from *S. gratus* (Sg), *S. amboensis* (Sa) and the cells developed from fusion treated protoplasts between Sg and Sa,

Table 1. Biotransformation products of digitoxigenin (**1**) by *S. gratus* (Sg), *S. amboensis* (Sa) and their hybrid cells (F8, B18 and C14 cell lines)

| Products | Sg | Sa | Sg+Sa | F8 | B18 | C14 |
|---|---|---|---|---|---|---|
| **3** | * | | * | * | * | |
| **4** | * | | * | | | |
| **5** | | * | * | * | * | |
| **6** | | * | * | * | * | * |
| **7** | | ** | * | ** | ** | ** |
| **8** | | | | * | * | * |

*,** Identified with authentic samples (*<**: peak area on HPLC).

cells occurred simultaneously in the hybrid cells. The possible biotransformation pathway of **1** by the hybrid cells is shown in Scheme 1. Although **3**–**5** were not detected in the C14 cell line, it seems that this cell line has higher activity of glucosylation (**1** to **6**, **2** to **7** and **3** to **8**) than of epimerization of 3$\beta$-hydroxyl to 3$\alpha$-hydroxyl (**2** to **5** and **3** to **4**) compared to F8 and B18 cell lines.

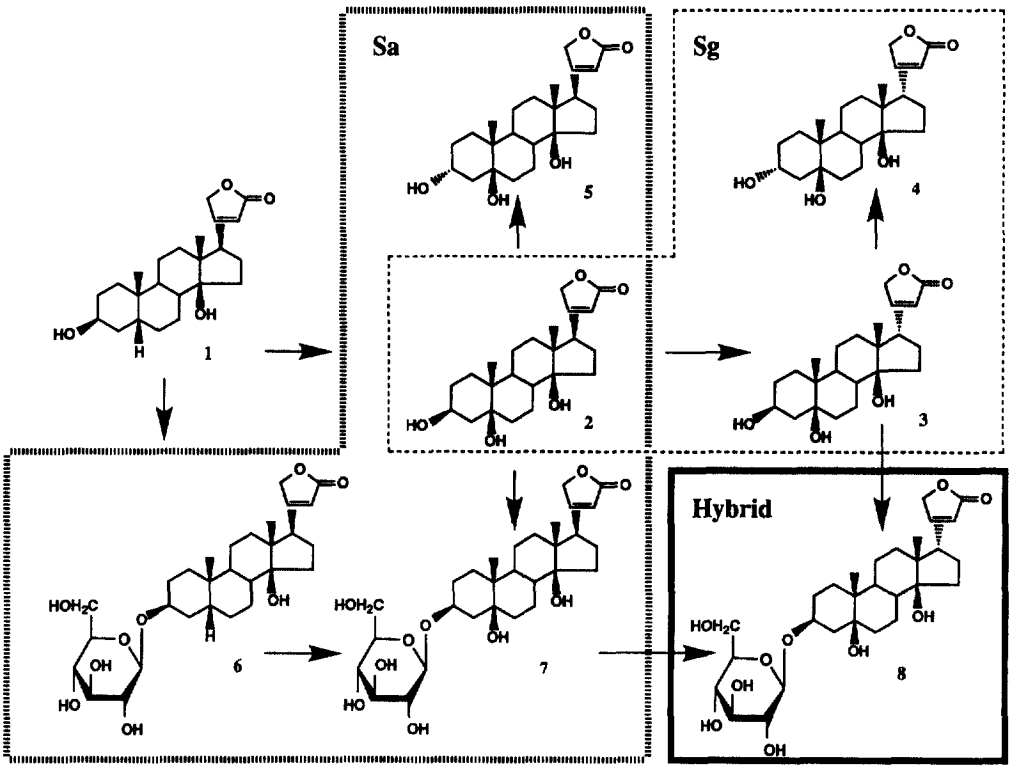
Experiment 3: after incubation of **1** (410 mg) with F8 cells (586 g fr. wt) for 18 or 19 days in a medium containing a higher concentration of sucrose (60 g $l^{-1}$), **7** (50 mg; yield 8.3%) and **8** (98 mg; yield 16.2%) were isolated as crystals. Periplogenin (**2**) and **3**–**6** were identified with authentic samples by HPLC. The greater production of **8** relative to **7** observed in this culture is due to unknown causes.

## EXPERIMENTAL

*General.* $^1$H and $^{13}$C NMR: Varian XL-400; EI-MS and positive FAB-MS: Jeol JMS-DX300; HR-FAB-MS: Jeol JMS-AX505.

*Protoplast isolation.* Callus cells induced from leaves of *Strophanthus gratus* [1] and *S. amboensis* [2] were used as parents. In order to select hybrid cells, the white cell line of *S. gratus* (Sg) and the yellowish green cell line of *S. amboensis* (Sa) grown in the light were used. Protoplasts were prepd from cell suspensions of Sg and Sa at 4–9 days after inoculation into MS medium containing sucrose 30 g $l^{-1}$, 1 mg $l^{-1}$, 2, 4-D and 0.1 mg $l^{-1}$ kinetin (DK medium). After incubating the cells in enzyme soln containing 0.6 M mannitol, 1% Cellulase Onozuka RS (Yakult Honsya Co., LTD), 0.1% Pectolyase Y23 (Seishinseiyaku Co., LTD) for 4 hr at 30°, the protoplasts were isolated by centrifugation in 20% sucrose.

*Protoplast fusion and culture.* The freshly isolated species of protoplasts (Sa and Sg) were mixed ($5 \times 10^4$ protoplasts $ml^{-1}$ each) and fused in a fusion chamber with 1.0 kV $cm^{-1}$ DC pulses of 30 $\mu$s duration using a somatic hybridizer (Shimazu Co., Ltd.). After 10 min, the treated cells were collected by centrifugation and cultured at $10^4$ cells $ml^{-1}$ in 1/2MS medium containing 0.6 M mannitol, 10 g $l^{-1}$ sucrose, 1 mg $l^{-1}$ 2, 4-D and 0.1 mg $l^{-1}$ kinetin in the dark at 30°. When the cell colonies grew to *ca* 0.5–1 mm in diameter (7–




Scheme 1. Biotransformation of digitoxigenin (1) by *S. gratus* (Sg), *S. amboensis* (sa) and their hybrid cells.

9 weeks after electrofusion), they were transferred to DK solid medium containing gellan gum 2 g $l^{-1}$. The cultures were grown under continuous illumination (10 000 lux) using a fluorescent lamp at 30°.

*Iosenzyme analysis.* Cells were homogenized with 0.1 M Na-Pi buffer (pH 7.0) at 4°. After centrifugation, isoenzymes in the supernatant were analysed by native PAGE (1 mm thick gel, 7.5% acrylamide). The gels were stained to detect acid phosphatase [10] and leucine aminopeptidase [11].

*RFLP analysis.* DNA was extracted from the cells according to the method of ref. [12]. The DNA was digested with restriction enzyme. Restriction fragments were sepd by electrophoresis in agarose gels. Southern blotting to nylon membrane (Hybond-N, Amersham) was carried out using the VacuGene vacuum blotting system (Pharmacia LKB). After drying, the membrane efficiently held DNA by crosslinking with a UV transilluminator. The labelling of DNA, hybridization and detection were performed using the DNA Labelling and Detection Kit Non-radioactive from Boehringer. Plasmid pRR217, containing ribosomal DNA from rice in the EcoRI cloning site of pBR325, was used as a probe [13]. The plasmid pRR217 was generously provided by Dr K. Oono.

*Biotransformation of digitoxigenin* (**1**) *by hybrid cells.* Digitoxigenin (**1**) (30–50 mg) suspended with Tween 80 (5%) was added to each flask (250 ml DK liquid medium 1 $l^{-1}$ flask) and then the cells (*ca* 30–70 g fr. wt per flask) from 4-week-old static cultures were incubated at 25° under light in a shaker (90 strokes $min^{-1}$) for 15–19 days. After incubation, $CHCl_3$ and $CHCl_3$–*iso*PrOH (3:2) extracts of the cells and the medium were prepared and examined on TLC with visualization using Kedde's reagent and 10% $H_2SO_4$. After purification using silica gel CC and pre. HPLC (Senshu Pak. PEGASIL ODS, solvent 60% MeOH in $H_2O$, flow-rate: 3 ml $min^{-1}$), the biotransformation products were isolated or identified with authentic samples by TLC and HPLC.

Experiment 1: **1** (120 mg: 30 mg × 4), F8 cells (262 g fresh wt), and 15 days incubation. Compounds **7** (65 mg; yield 37%) and **8** (7 mg; yield 4%) were isolated and **3**–**6** were identified.

Experiment 2: **1** (90 mg: 30 mg × 3) and F8 cells (173 g fresh wt); **1** (60 mg: 30 mg × 2) and B16 cells (106 g fresh wt); **1** (60 mg: 30 mg × 2) and C14 cells (87 g fresh wt); **1** (90 mg: 30 mg × 3) and Sg cells (178 g fresh wt); **1** (90 mg: 30 mg × 3) and Sa cells (133 g fresh wt); **1** (90 mg: 30 mg × 3) and Sg cells (87 g fresh wt) + Sa cells (81 g fresh wt), 17 days incubation in each case. These $CHCl_3$–*iso*PrOH (3:2) frs were analysed by HPLC, especially **7** and **8** ($R_t$ 10.2 min and 8.4 min: solvent 60% MeOH in $H_2O$). Both **7** and **8** (**7** > **8**) were detected in three hybrid cell lines, only **7** was detected in Sa cells and the mixed culture of Sg and Sa cells, neither **7** nor **8** was detected in Sg cells. Other products were shown in Table 2.

Table 2. Biotransformation products of digitoxigenin (**1**) formed by *S. gratus* (Sg), *S. amboensis* (Sa) and their hybrid cells (F8, B18 and C14 cell lines)

| Products | Sg | Sa | Sg + Sa[+] | F8 | B18 | C14 |
|---|---|---|---|---|---|---|
| **2** | * | | * | * | * | * |
| **3** | * | | * | * | * | |
| **4** | * | | * | | | |
| **5** | | * | * | * | * | |
| **6** | | * | * | * | * | * |
| **7** | | ** | * | ** | ** | ** |
| **8** | | | | * | * | * |

[+] Co-culture of Sg and Sa in same flask.

*,** Identified with authentic samples (* < **: peak area on HPLC).

Experiment 3: **1** (410 mg: 40 mg × 4, 50 mg × 2, 50 mg × 3) and F8 cells (586 g fresh wt: 255, 142 and 189 g) were incubated for either 18 or 19 days in DK liquid medium containing sucrose (60 g $l^{-1}$). Compound **7** (50 mg; yield 8.3%) and **8** (98 mg; yield 16.2%) were isolated and **2**–**6** were identified by HPLC (17.9, 16, 9.9, 11.6 and 13.2 min).

*17βH-Periplogenin β-D-glucoside* (**8**). Amorphous solid. HR-FAB-MS: $C_{29}H_{44}O_{10}+Na$ required 575.2832, $[M+Na]^+$ at *m/z* 575.2880; EI-MS *m/z* (rel. int.): 552 $[M]^+$ (5), 354 (69), 336 (52), 318 (100), 300 (40), 201 (43); $^1H$ NMR (400 MHz, $C_5D_5N$): δ 1.00 (3H, *s*, $H_3$-19), 1.06 (3H, *s*, $H_3$-18), 3.30 (1H, *dd*, *J* = 9.5, 9.5 Hz, H-17β), 3.93 (1H, *dd*, *J* = 8, 8 Hz, H-2′), 4.28 (1H, *dd*, *J* = 12, 5 Hz, H-6a′), 4.43 (1H, *br s*, $W_{1/2}$ = 8 Hz, H-3α), 4.47 (1H, *dd*, *J* = 12, 2 Hz, H-6b′), 4.69 (1H, *br d*, *J* = 18 Hz, H-21a), 4.85 (1H, *br d*, *J* = 18 Hz, H-21b), 4.96 (1H, *br d*, *J* = 8 Hz, H-1′), 5.99 (1H, *br s*, H-22); $^{13}C$ NMR (100 MHz, $C_5D_5N$): δ 26.3 (*t*, C-1), 26.9 (*t*, C-2), 74.1 (*d*, C-3), 34.1 (*t*, C-4), 73.4 (*s*, C-5), 36.5 (*t*, C-6), 24.4 (*t*, C-7), 40.9 (*d*, C-8), 39.5 (*d*, C-9), 41.3 (*s*, C-10), 21.2 (*t*, C-11), 31.2 (*t*, C-12), 49.4 (*s*, C-13), 85.4 (*s*, C-14), 31.8 (*t*, C-15), 25.1 (*t*, C-16), 49.0 (*d*, C-17), 18.7 (*q*, C-18), 17.5 (*q*, C-19), 173.0 (*s*, C-20), 74.3 (*t*, C-21), 116.8 (*d*, C-22), 174.4 (*s*, C-23), 101.6 (*d*, C-1′), 75.4 (*d*, C-2′), 79.0 (*d*, C-3′), 71.9 (*d*, C-4′), 79.1 (*d*, C-5′), 62.9 (*t*, C-6′).

*Acknowledgements*—We are indebted to Dr K. Oono, National Institute of Agrobiological Resources, Japan for supplying pRR217 and the members of the Analytical Centre of Kitasato University for the NMR and MS measurements. The authors are grateful to the Ministry of Education, Scientific Research (Grant No. 03453158), for financial support. This work was supported by a Grant-in-Aid for Scientific Research (Project 7) from the School of Pharmaceutical Sciences, Kitasato University.

## REFERENCES

1. Furuya, T., Kawaguchi, K. and Hirotani, M., *Phytochemistry*, 1988, **27**, 2129.
2. Kawaguchi, K., Hirotani, M. and Furuya, T., *Phytochemistry*, 1988, **27**, 3475.
3. Kawaguchi, K., Hirotani, M. and Furuya, T., *Phytochemistry*, 1989, **28**, 1093.
4. Kawaguchi, K., Hirotani, M. and Furuya, T., *Phytochemistry*, 1991, **30**, 1503.
5. Endo, T., Komiya, T., Masumitsu, Y., Morikawa, H. and Yamada, Y., *Plant Physiology*, 1987, **129**, 453.
6. Fujii, Y., Shimizu, K. and Takahashi, W., *Plant Tissue Culture Letters*, 1991, **8**, 31.
7. Murashige, T. and Skoog, F., *Plant Physiology*, 1962, **15**, 473.
8. Kao, K. and Michayluk, M., *Planta*, 1975. **126**, 105.
9. Kawaguchi, K., Hirotani, M., Yoshikawa, T. and Furuya, T., *Phytochemistry*, 1990, **29**, 837.
10. Arus, P. and Orton, T., *Journal of Hereditary*, 1983, **74**, 405.
11. Arus, P., Tanksley, S., Orton, T. and Jones, R., *Euphytica*, 1982, **31**, 417.
12. Honda, H. and Hirai, A., *Japanese Journal of Breeding*, 1990, **40**, 339.
13. Takaiwa, F., Oono, K. and Sugiura, M., *Plant Molecular Biology*, 1985, **4**, 355.