Malaysian Journal of Pharmaceutical Sciences,
Vol. 3, No. 1, 35—44 (2005)

PENERBIT USM

GLUCOSYLATION OF *p*- AND *m*-HYDROXYPHENOLS BY PLANT CELL SUSPENSION CULTURES OF *SOLANUM MAMMOSUM*

ACHMAD SYAHRANI[1], TINI PALUPI[1], UNTARI FAJAR TRIYANTI[1], FAIQATUL HIMMAH[1], RAINER EBEL[2] AND GUNAWAN INDRAYANTO[1]*

[1] Plant Biotechnology Research Group, Faculty of Pharmacy, Airlangga University, Jl. Dharmawangsa Dalam, Surabaya 60286, Indonesia

[2] Institute for Pharmaceutical Biology, University of Duesseldorf, Universitaetstrasse 1, Geb. 26.23, D-40225 Duesseldorf, Germany

Cell suspension cultures of Solanum mammosum were able to transform exogenously supplied resorcinol and hydroquinone into resorcinol-O-β-D-glucopyranoside and arbutin, respectively. The highest level of arbutin in biomass was formed within 3 days after inoculation with hydroquinone.

Keywords: *Solanum mammosum, resorcinol, resorcinol-O-β-D-glucopyranoside, hydroquinone, arbutin*

INTRODUCTION

Various plant cell cultures are capable of glucosylating a variety of exogenously supplied substrates (Suga and Hirata 1990). The glucosylation of some phenol derivatives by cell suspension cultures of *Catharanthus roseus* was reported by Shimoda *et al.* (2002). Mizukami *et al.* (1987) reported glucosylation of phenol, catechol and resorcinol by cultured cells of *Gardenia jasminoides*. High yield formation of arbutin from hydroquinone was reported by using cell suspension cultures of *C. roseus* (Inomata *et al.* 1991) and *Rauwolfia serpentina* (Lutterbach and Stöckigt 1992).

The bioconversions of salicyl alcohol into salicin, salicylamide into salicylamide-2-O-β-D-glucopyranoside, *p*-aminobenzoic acid into *p*-aminobenzoic acid-7-O-β-D-glucopyranosyl ester, and *o*-aminobenzoic acid into *o*-aminobenzoic acid-7-O-β-D-glucopyranosyl ester and *o*-aminobenzoic acid-7-O-β-D-(β-D-1,6-O-glucopyranosyl)-glucopyranosyl ester by cell suspension cultures of *Solanum mammosum* (local name: *terung susu*) were previously reported by our group (Syahrani *et al.* 1997a, 1997b, 1999). Recently, Hartanti *et al.* (2002) reported high yield formation

* Corresponding author: Gunawan Indrayanto, e-mail: indrayanto@hotmail.com

of *o*-aminobenzoic acid 7-O-β-D-(β-1,6-O-D-glucopyranosyl)-glucopyranosyl ester (*ca.* 20–30% dry weight) from *o*-aminobenzoic acid by suspension cultures of *S. mammosum*.

Glucosylation by plant cell cultures is very interesting due to the difficulty to perform this reaction by chemical synthesis or by microbiological bioconversion (Yokoyama and Inomata 1998). By glucosylation, water insoluble compounds are transformed to water soluble compounds, so their formulation will be easier.

The glycosides of *o*- and *m*-hydroxyphenol can be used as preventing agents for aging-related graying hair and as enhancers of melanin formation in hair (Japanese Patent), while arbutin is used as a potent suppressor of melanin synthesis in human skin (Inomata *et al.* 1991).

In this report, we describe the conversion of resorcinol **(1)** to resorcinol-O-β-D-glucopyranoside **(2)** (Fig. 1), and hydroquinone **(3)** to arbutin **(4)** (Fig. 2) by cell suspension cultures of *S. mammosum*.

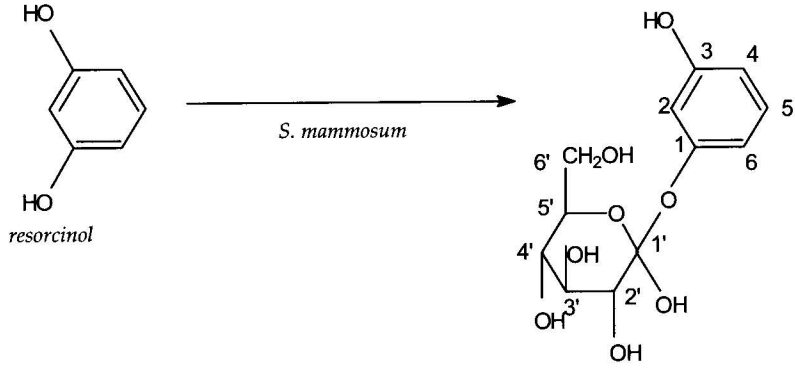

Fig. 1: Conversion of resorcinol to resorcinol-O-β-D-glucopyranoside by cell suspension cultures of *S. mammosum*.

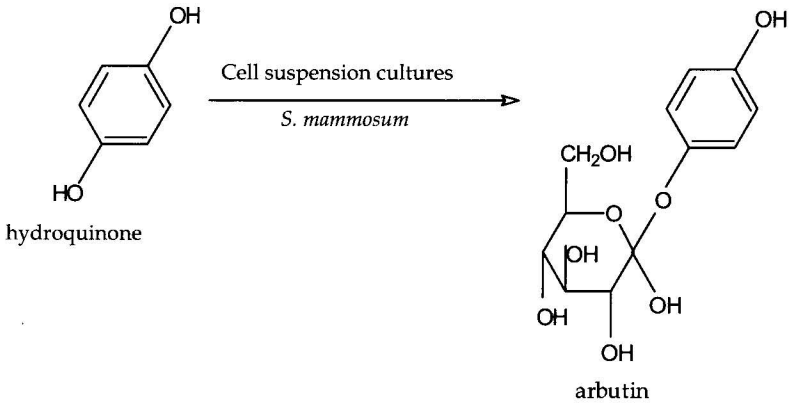

Fig. 2: Conversion of hydroquinone to arbutin by cell suspension cultures of *S. mammosum*.

MATERIALS AND METHODS

General

Nuclear Magentic Resonance (NMR) spectra were determined at 500 MHz (^{1}H) and 125.8 MHz (^{13}C) on a Bruker DRX 500 spectrometer (for **2**), and a Hitachi FT-NMR spectrometer at 22.5 MHz (^{13}C for **3** and **4**). Tetramethylsilane (TMS) was used as internal standard.

The positive and negative ion electrospray mass spectra (ESI/MS) were determined using a Finnigan LCQ/Deca coupled to an Agilent 1100 Diode Array Detector (DAD) - high performance liquid chromatography (HPLC) system. Flow rate was at 0.4 mL/min. Solvent systems were as follows: **A**, water (+ 0.1% v/v formic acid, Merck), **B**, 100% acetonitrile (Merck); gradient: 0 min, 10% **B**; 2-35 min, linear gradient from 10% to 100% B. The injection volume was 5 µL, capillary temperature was set to 260°C and the spray voltage was adjusted to 4.5 kV.

The *in situ* ultra violet (UV) absorbance reflectance spectra of the thin layer chromatography (TLC) spots of substrates **1** and **3**, and metabolites **2** and **4** were determined using a Shimadzu TLC Scanner CS 930. The stationary phase was silica gel F254 (precoated plates, Merck) and the mobile phase was EtOAc:MeOH:H_2O (Merck) 77:13:10.

Optical rotations were measured on a Perkin Elmer 341 LC polarimeter (optical path length, 100 mm).

Cell suspension cultures

Cell suspension cultures of *S. mammosum* were cultivated in 40 Erlenmeyer flasks (300 mL) containing 50 mL of modified MS (Murashige and Skoog 1962) medium, supplemented with 3% sucrose (Merck), 2 mg/L kinetin (Merck), 1 mg/L 1-napthylacetic acid (Merck) and 1 g/L casein hydrolysate (Merck) on a gyrotary shaker (100 rpm) at 25 ± 1°C under continuous light (*ca.* 1500 lux, Philips TL 54/40w) as previously reported (Syahrani *et al.* 1999), or incubated in totally dark environment.

Toxicity and biotransformation experiments

Cells (10 g fresh weight) were inoculated into liquid medium (50 mL) containing various concentrations (200–1500 mg/L) of **1** (Sigma) and **3** (Sigma), and without **1** and **3** (control cultures). After 7 days of incubation, the cultures were harvested, filtered and weighed. Packed cell volume (PCV) was determined according to the method previously described (Seitz *et al.* 1985). The growth index was measured as the ratio of the masses of the harvested and the inoculated cells (fresh weights, respectively).

Biotransformation experiments were typically performed by inoculating cells (10 g fresh weight) into the liquid medium (50 mL) containing **1** (750 mg/L) or **3** (400 mg/L) and incubating for 7 days. After 7 days, the cultures were harvested, filtered, weighed, oven dried at 40°C over night (until their water content was *ca.* 2%) and powdered.

Isolation of 2

The oven dried (40°C) powdered biomass (11.6 g) collected from 40 Erlenmeyer flasks was refluxed (2 hr) in 300 mL MeOH. The MeOH extract was concentrated under reduced pressure to afford a dark brown semi solid residue (3.31 g), which was subjected to column chromatography on silica gel 40 (70–230 mesh ASTM, Merck) using EtOAc/MeOH/H_2O 77:13:10 as eluent. Fractions containing **2** were further purified by preparative TLC (precoated silica gel 60 F254;

E. Merck) using EtOAc:MeOH:H_2O 77:13:10 as developing solvent, yielding **2** (37.5 mg).

Optical rotation (measured following hydrolysis with 2N HCl in MeOH): $[\alpha]^{20}_D$ = + 59.9° (*c* = 1.05, MeOH); *in situ* UV absorbance reflectance, λ_{max} nm (silica gel): 273; positive ionisation mode ESI/MS: *m/z* (% rel.int): 273.3 ($[M+H]^+$); negative ionisation mode: *m/z* 271.4 ($[M-H]^-$, 5.7), 317.4 ($[M-H+HCOOH]^-$, 100.0), 543.4 ($[2M-H]^-$, 84.0), 589.0 ($[2M-H+HCOOH]^-$, 19.3) and 814.8 ($[3M-H]^-$, 90.2); ^1H and ^{13}C NMR data, see Table 1.

Table 1: ^1H- and ^{13}C-NMR Spectral Data (in CD_3OD, Merck) of **2**[a]

Position	δ_H	δ_C	COSY	HMQC[d]	HMBC[d]
1		160.8 *s*			
2	6.55 (br *t*, *J* = 2.2)	105.6 *d*	6	2	3, 6
3		160.0 *s*			
4	6.44 (*ddd*, *J* = 8.1, 2.2, 0.6)	110.9 *d*	5	4	2, 3, 6
5	7.05 (br *t*, *J* = 8.1)	131.3 *d*	4, 6	5	1, 2, 3, 6
6	6.56 (*ddd*, *J* = 8.1, 2.2, 0.6)	109.3 *d*	5, 2	6	1, 2, 4
1'	4.85[b] (*J* ≈ 7.5[c])	102.7 *d*	2'	1'	1, 3', 5'
2'	3.43 *m*[c]	75.4 *d*	1'[e]	2'	[e]
3'	3.45 *m*[c]	78.5 *d*	[e]	3'	[e]
4'	3.40 *m*[c]	71.8 *d*	[e]	4'	[e]
5'	3.40 *m*[c]	78.6 *d*	6'A, 6'B[e]	5'	[e]
6'	A: 3.88 (*dd*, *J* = 12.1, 1.7)	63.0 *t*	A: 5',	6'	A: 4', 5'
	B: 3.70 (*dd*, *J* = 12.1, 5.0)		6'B		B: 4', 5'
			B: 5',		
			6'A		

[a] δ in parts per million (ppm) and *J* (parentheses) in Hz

[b] partially obscured by residual water signal

[c] assignments and chemical shifts based on correlated spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC) spectra

[d] from H to C

[e] cross peaks (in part) not detected due to overlapping signals

J (coupling constant), s (singlet), dd (double doublet), ddd (doublet of double doublets), br *t* (broad triplet), m (complex multiplet)

Isolation of 4

The oven dried (40°C) powdered biomass (10.1 g) collected from 40 Erlenmeyer flasks was refluxed (2 hr) in 300 mL MeOH. The MeOH extract was concentrated under reduced pressure to afford a dark brown semi solid residue (2.1 g), which was subjected to column chromatography on silica gel 40 (70-230 mesh ASTM, Merck) using EtOAc:MeOH:H_2O 77:13:10 as eluent, yielding pure **4** (207 mg). *In situ* UV

absorbance reflectance, λ_{max} nm (silica gel): 285; ^{13}C-NMR (DMSO-d_6): 150.3 (C-1), 117.8 (C-2, C-6), 115.5 (C-3, C-5), 152.1 (C-4), 101.8 (C-1'), 73.4 (C-2'), 76.7 (C-3'), 69.9 (C-4'), 77.0 (C-5') and 60.9 (C-6).

Analysis of 3 and 4

The contents of **3** and **4** in the medium and the cell biomass were determined densitometrically using a Shimadzu CS 930 TLC Scanner and precoated silica gel F254 plates eluted with EtOAc:MeOH:H_2O 77:13:5. Quantitation was performed by measuring the absorbance reflectance of the analyte spots at 285 nm (R_f values of **3** and **4** were 0.63 and 0.32, respectively). The concentrations of **3** and **4** were determined from calibration graphs on each TLC plate using authentic standards of hydroquinone (Sigma) and arbutin (Sigma). With this method, linearity of response were achieved for loading of 0.5–6.9 μg/spot (for hydroquinone) with line equation: Y = 325 + 8434 X, relative process standard deviation, V_{xo} (Funk *et al.* 1992) = 5.3%, r = 0.9983, n = 5; and 0.5–10 μg/spot (for arbutin) with line equation: Y = 154 + 4416 X, V_{xo} = 7.0%, r = 0.9971, n = 5, respectively. The results of recovery studies (mean ± SD, n = 4) using standard addition method to biomass were 101.6 ± 8.0% (for hydroquinone) and 101.2 ± 8.0% (for arbutin). Bioconversion capacity was calculated as percentage (%) of the total conversion of the substrate to product in each Erlenmeyer flask.

RESULTS AND DISCUSSION

The toxicity of substrates **1** or **3** towards the cell suspension cultures of *S. mammosum* was tested in the range of 200–1500 mg/L. Cell death was observed at concentrations above 800 mg/L for **1** and 400 mg/L for **3**. At concentrations of 750 mg/L (for **1**) and 400 mg/L (for **3**), respectively, cells survived and their colours remained green, but their growth indices (1.42 for **1**; 1.25 for **3**) and PCV (46% for **1**; 40% for **3**) were reduced compared to cultures without administration of the substrates. Thus, these substrate concentrations were selected in further experiments. Apparently, the *para* position of the dihydroxy groups in **3** resulted in more pronounced toxicity to this cell suspension culture compared to the *meta* arrangement present in **1**.

Incubation of cell suspension cultures of *S. mammosum* with **1** (750 mg/L), followed by isolation, purification by column chromatography and preparative TLC, afforded metabolite **2**. After administration of substrate **3** (400 mg/L) to the same cell suspension culture of *S. mammosum*, metabolite **4** was isolated. TLC analysis showed that the spots of substrates **1** (R_f = 0.74) or **3** (R_f = 0.63), and their metabolites **2** (R_f = 0.46) or **4** (R_f = 0.32) exhibited very similar *in situ* UV absorbance reflectance spectra. Control experiments showed that in the absence of cells, substrates **1** or **3** remained unchanged in the culture medium, and that metabolites **2** or **4** were only produced in the presence of cell suspension cultures of *S. mammosum*.

The positive ion electrospray mass spectrum (ESI/MS) of **2** yielded the pseudomolecular ion at *m/z* 273.3 $(M+H)^+$, while prominent ions in the negative mode included *m/z* 271.4 $(M-H)^-$ (5.7%), 317.4 $(M-H+HCOOH)^-$ (100.0%), 543.4 $(2M-H)^-$ (84.0%), 589.0 $(2M-H+HCOOH)^-$ (19.3%) and 814.8 $(3M-H)^-$ (90.2%). These data indicated that metabolite **2** had a molecular weight of 272 and that it was a glucoside of **1**. The ^{13}C-NMR spectrum of **2** comprised 1 methylene, 9 methine and 2 quaternary carbon resonances (see Table 1). The ^{1}H-NMR spectrum of **2** included a signal at δ 4.85 (H-1′) attributable to the anomeric glucopyranosyl proton which was partially obscured by the residual water signal, preventing direct observation of its coupling constant. However, from the COSY spectrum a large diaxial coupling ($J \approx 7.5$ Hz) between H-1′ and H-2′ was clearly discernible, thus confirming the β configuration of H-1′. Four aryl proton signals were also observed in the ^{1}H-NMR spectrum of **2** (see Table 1). The HMBC spectrum of **2** included correlations between the anomeric proton at δ 4.85 (H-1′) with C-1 (δ 160.8), C-3′ (δ 78.5) and C-5′ (δ 78.6). Upon hydrolysis of **2** with 2 N HCl in MeOH, an optical rotation ($[\alpha]^{20} = +59.9°$, $c = 1.05$) was obtained which was comparable to that of authentic β-D-glucose measured under similar conditions. These data indicated that **2** was resorcinol-O-β-D-glucopyranoside.

DAD - HPLC analysis of metabolite **4** and authentic standard arbutin (Sigma) showed identical retention times and online UV spectra. Furthermore, ^{13}C NMR spectra obtained for **4** and authentic arbutin were virtually superimposable. Thus, metabolite **4** was unequivocally determined to be identical to arbutin.

To our knowledge, this is the first report of the glucosylation of hydroquinone and resorcinol by cell suspension cultures of *S. mammosum*.

The time course of the biotransformation of **3** to **4** by cell suspension cultures of *S. mammosum* is shown in Figure 3. Quantitative analysis showed that the maximum level of **4** in cells was achieved on the 3rd day after administration of the substrate in both incubation conditions (22.9 mg/g and 23.3 mg/g dry weight, respectively). Substrate **3** was not detected any more in the medium one day after substrate administration. Incubation of the cultures in the dark could not stimulate the formation of arbutin, while Inomata *et al.* (1991) observed a much higher yield of the formation of **4** from **3** by cell suspension cultures of *C. roseus* that were incubated in the dark. This present work showed that incubation in the dark could seemed stimulate the degradation of the product between the 5th and 7th day. The maximum biotransformation capacity of substrate **3** by cell suspension cultures of *S. mammosum* reported here (27% on the 4th day of incubation) was significantly lower than the glucosylation rate of salicyl alcohol by the same cultures as reported previously (52%) (Syahrani *et al.* 1997a). Obviously, upon replacing the -CH_2OH moiety by an -OH group in the substrate, the glucosylation capability of the suspension cultures of *S. mammosum* was reduced significantly.

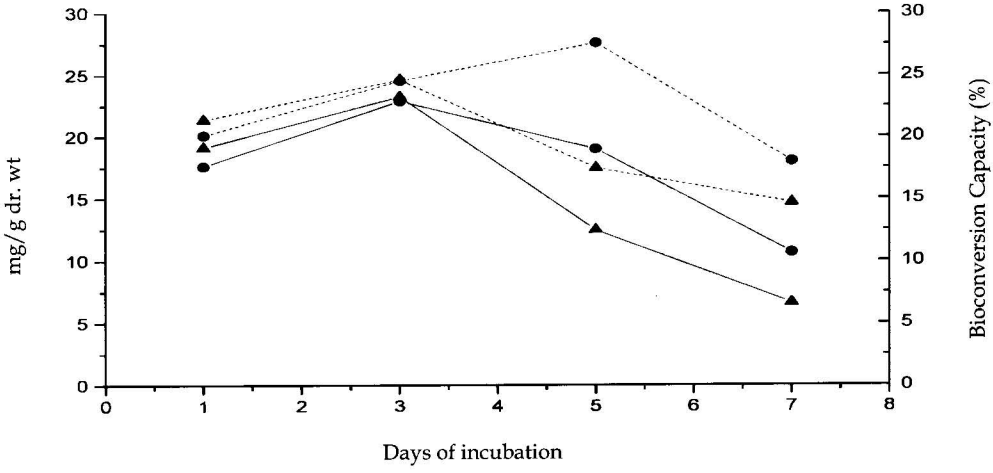

Fig. 3: Time course of formation of **4** in cell suspension cultures of *S. mammosum* after administration of **3** and its bioconversion rate.
— : mg/g dry weight of **4** in cells ... : bioconversion rate (in %)
● : incubation with light ▲ : incubation in the dark

CONCLUSION

The present work showed that the suspension cultures of *S. mammosum* have the capability to convert resorcinol and hydroquinone into resorcinol-O-β-D-glucopyranoside and arbutin, respectively. The maximum bioconversion capacity of hydroquinone to arbutin was 27%. Incubation in the dark could not stimulate the biotransformation capacity of the hydroquinone.

REFERENCES

FUNK, W., DAMMAN, V. & DONNERVERT, G. (1992) *Qualitätssicherung in der Analytischen Chemie,* pp. 12 -180 (New York, Basel, Cambridge: VCH, Weinheim).

HARTANTI, L., WIDJAJA, I., SYAHRANI, A. & INDRAYANTO, G. (2002) High yield formation of o-aminobenzoic acid diglucoside in cell suspension cultures of *Solanum mammosum, Journal of Asian Natural Product Research,* 4: 61–65.

INOMATA, S., YOKOYAMA, M., SETO, S. & YANAGI, M. (1991) High level production of arbutin from hydroquinone in suspension cultures of *Catharanthus roseus* plant cells, *Applied Microbiology Biotechnology,* 36: 315–319.

JAPANESE PATENT NUMBER 05221834 and 3120087.

LUTTERBACH, R. & STÖCKIGT, J. (1992) High yield formation of arbutin from hydroquinone by cell suspension cultures of *Raulwolvia sepentina, Helvetica Chimica Acta,* 75: 2009–2011.

MIZUKAMI, H., HIRANO, A. & OHASHI, H. (1987) Effect of subtituents groups on the glucosyl conyugation of xenobiotic phenol by cultured cells of *Gardenia jasminoides, Plant Science,* 48: 11–15.

MURASHIGE, T. & SKOOG, F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures, *Physiologia Plantarum,* 15: 473–479.

SEITZ, H. U., SEITZ, U. & ALFERMANN, W. (1985) *Pflanzliche Gewebekultur,* pp. 78–79 (Stuttgart: Gustav Fischer Verlag).

SHIMODA, K., YAMANE, S., HIRAKAWA, H., OHTA, S. & HIRATA, T. (2002) Biotransformation of phenolic compounds by cultured cells of *Catharanthus roseus, Journal of Molecular Catalyst B: Enzymatic,* 16: 275–281.

SUGA, T. & HIRATA, T. (1990) Biotransformation of exogenous substrates by plant cell cultures, *Phytochemistry,* 29: 2393–2406.

SYAHRANI, A., INDRAYANTO, G., WILKINS, A. & SUTARJADI (1997a). Glucosylation of salicyl alcohol by cell suspension cultures of *Solanum mammosum, Natural Product Sciences,* 3: 71–74.

SYAHRANI, A., INDRAYANTO, G., SUTARJADI & WILKINS, A. (1997b) Bioconversion of salicylamide by cell suspension cultures of *Solanum mammosum, Chemical Pharmaceutical Bulletin,* 45: 555–557.

SYAHRANI, A., RATNASARI, E., INDRAYANTO, G. & WILKINS, A.L. (1999) Biotransformation of o- and p- amino benzoic acids and N-acetyl-aminobenzoic acid by cell suspension culture of *Solanum mammosum, Phytochemistry,* 51: 615–620.

YOKOYAMA, M. & INOMATA, S. (1998) *Catharanthus roseus* (Periwinkle): *In vitro* culture, and high level production of arbutin by biotransformation, IN: BAJAJ, Y.P.S. (Ed.). *Biotechnology in Agriculture and Forestry,* 41: 67–79 (Heidelberg: Springer).