Bioconversion of Salicylamide by Cell Suspension Cultures of *Solanum mammosum*

Achmad SYAHRANI,[a] Gunawan INDRAYANTO,*,[a] SUTARJADI,[b] and Alistair WILKINS[c]

Laboratory of Pharmaceutical Biotechnology, Faculty of Pharmacy, Airlangga University,[a] Jl. Dharmawangsa Dalam, Surabaya 60286, Indonesia, Laboratory of Pharmacognosy, Faculty of Pharmacy, Airlangga University,[b] Jl. Dharmawangsa Dalam, Surabaya 60286, Indonesia, and Department of Chemistry, School of Science and Technology, University of Waikato,[c] Hamilton-New Zealand. Received August 26, 1996; accepted November 20, 1996

A new conversion product, salicylamide 2-*O*-β-D-glucopyranoside was isolated from cell suspension cultures of *Solanum mammosum* following the administration of salicylamide. The time-course of the bioconversion was monitored in these suspension cultures.

Key words *Solanum mammosum*; Solanaceae; cell suspension culture; glycosylation; bioconversion; salicylamide

Various plant cell cultures are capable of glycosylating a variety of exogenously supplied compounds.[1] The glycosylation of coumarins, flavonoids, anthraquinones and simple phenols by various cell suspension cultures has been published by Umetami *et al.*[2] Mizukami *et al.*[3] reported that some cell suspension cultures (*e.g.*, *Lithospermum erythrorhizon*, *Gardenia jasminoides*) were able to transform salicylate derivates such as salicyl alcohol into salicin and isosalicin. Dombrowski and Alferman[4,5] reported the transformation of salicyl alcohol into salicin and isosalicin, salicylic acid into salicylic acid 2-*O*-β-D-glucoside and salicylic acid 1-*O*-β-D-glucosylester in the suspension cultures of *Salix matsudana*. The formation of glucosyl conjugates of exogenous compounds is considered to be a detoxification process as described earlier.[6]

Investigation in our laboratory showed that cell suspension cultures of *Solanum mammosum* (code sm.) could convert salicyl alcohol to salicin. No report of biotransformation of salicylamide (**1**), a derivate of salicylate, to its glycosides by plant cell suspension cultures has appeared in the literature. In this paper, we report the formation of salicylamide glucoside (**2**) from **1** exogenously introduced into suspension cultures of *Solanum mammosum*.

The toxicity of **1** toward *Solanum mammosum* cell suspension cultures was investigated for concentrations in the range 200—1000 mg/l. Cell death was observed at the highest administered concentration (1000 mg/l). At the lower concentration of 800 mg/l, cells survived, but did not grow (see Fig. 2). In all of our experiments 800 mg/l of **1** was administered. This concentration is greater than that at which salicylic acid (172.5 mg/l) and salicyl alcohol (310, 620 mg/l) were administered by previous authors.[3-5]

TLC analysis showed the presence of metabolite **2**. Two control experiments showed that in the absence of cells, **1** remained unchanged in the control medium, and that **2** was only produced when **1** was added to the suspension cultures. The differential scanning colorimeter (DSC) thermogram of **2** indicated that the product was reasonably pure. The negative ion electrospray mass spectrum (MS) of **2**, determined in the presence of NH_4COOH, showed peaks at *m/z* 344 $[M+COOH]^-$, 334 $[M-H+2H_2O]^-$, 298 $[M-H]^-$ and 136 $[C_7H_6NO_2]^-$, suggesting that **2** has a molecule weight of 299 daltons and is a glucosyl analogue of salicylamide. NMR spectral data were consistent with the identification of **2** as salicylamide 2-*O*-β-D-glucopyranoside. In particular, the coupling constant of the H-1′ glucosyl proton (J=7.6 Hz) established the configuration at this center to be equatorial (β),[7] while irradiation of the glucosyl H-1′ resonance (5.02 ppm) in a nuclear Overhauser enhancement (NOE) difference experiment enhanced H-3′ (3.40 ppm, signal also coincident with H-2′), H-5′ (3.49 ppm) and H-3 (7.40 ppm) thereby confirming **2** to be a 2-*O*-β-glucoside.

The time course of formation of **2** from **1** in cell suspension cultures of *Solanum mammosum* is depicted in Fig. 3. Quantitative analysis showed that the greatest level of **2** (71.5 mg/flask or equivalent to 140.2 mg/g dry weight) was formed in the cells 5 d after cell inoculation of 40 mg of **1** in flasks containing 50 ml of medium each (*i.e.*, an inoculation level of 800 mg/l). Glucoside **2** was not detected in medium during the 6 d inoculation period, while salicylamide was detected in both cells and the medium

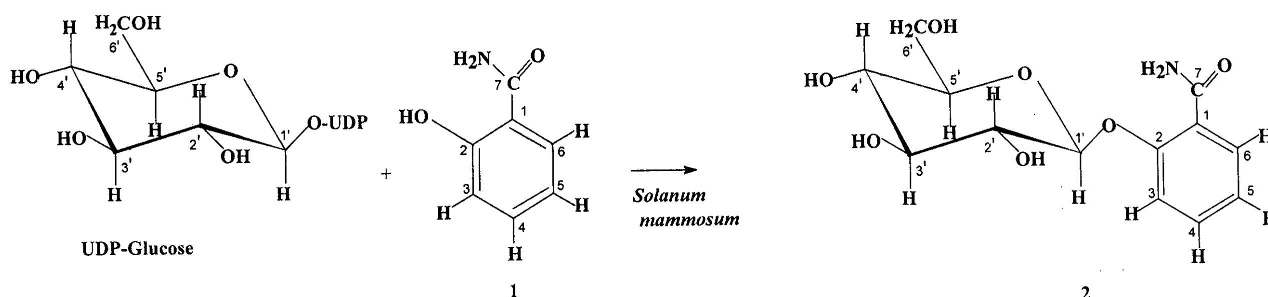

Fig. 1. Bioconversion of **1** to **2**

* To whom correspondence should be addressed.

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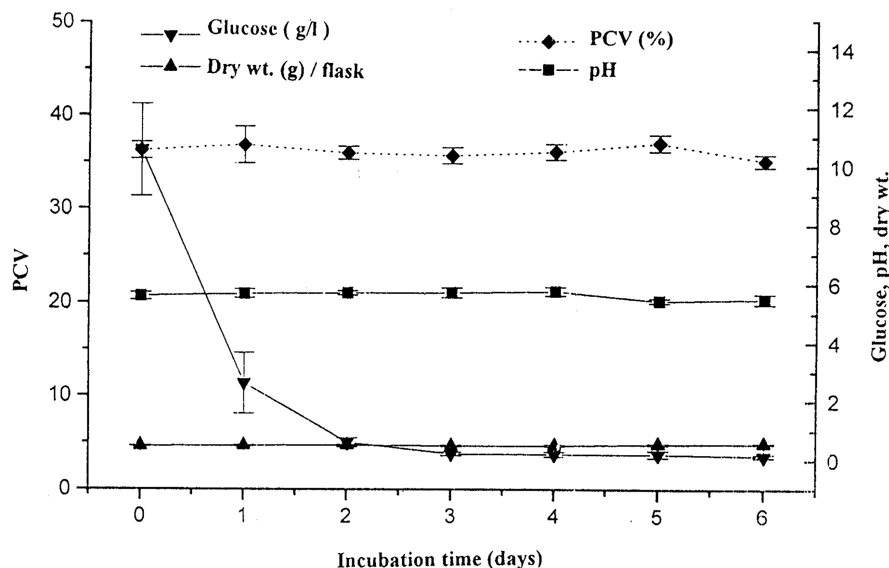

Fig. 2. Effect of **1** (800 mg/l) in the Medium on Growth [Dry wt./flask; Packed Cell Volume (PCV)] of Cell Suspension Cultures of *Solanum mammosum*

Values represent mean ± S.D. ($n=6$).

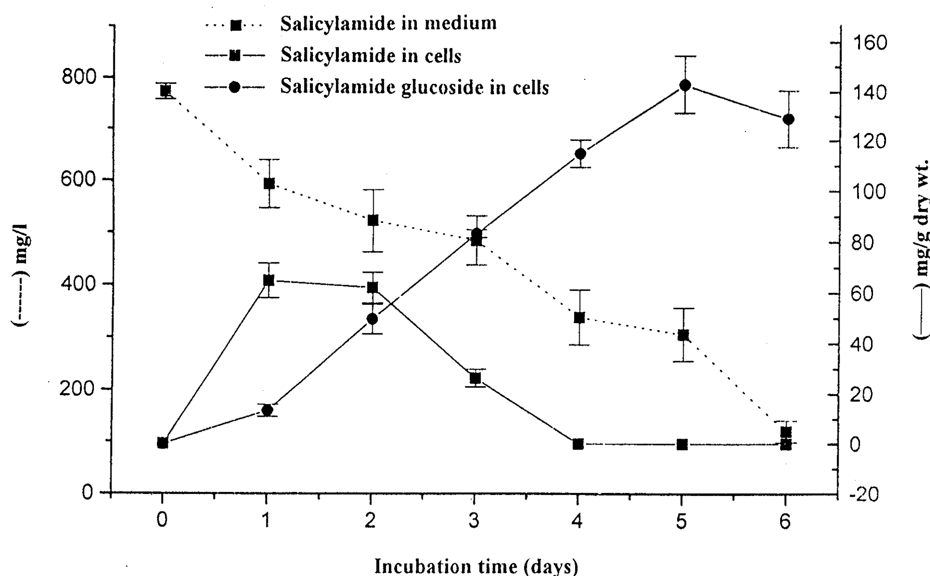

Fig. 3. The Time Course of the Bioconversion of **1** to **2** in Cell Suspension Cultures of *Solanum Mammosum*

Values represent mean ± S.D. ($n=6$).

during the incubation period.

The glucosylation capability of cell suspension cultures of *Solanum mammosum* reported here, namely an 81.9% conversion of **1** to **2**, is higher than that reported previously for *Gardenia jasminoides* (30% for salicyl alcohol)[3] and *Salix matsudana* (48% for salicyl alcohol).[5] To our knowledge this is the first report of the bioconversion of salicylamide into its glucoside **2** by cell suspension cultures of *Solanum mammosum*. Also, glucoside **2** does not appear to have been previously isolated from natural sources.

Experimental

General Procedure ^{1}H (300.13 MHz) and ^{13}C (75.47 MHz) NMR spectra were determined at 300 K in dimethylsulfoxide (DMSO-d_6) using a Bruker AC-300 spectrometer fitted with a 5 mm probe head. Chemical shifts (δ ppm) are reported relative to the solvent peaks DMSO-d_6 (δ ^{1}H = 2.60; ^{13}C = 39.5 ppm). ^{13}C-NMR signal multiplicity (s, d, t or q) was determined using the distortionless enhancement by polarization transfer (DEPT) sequence with a 135° detection pulse. NMR signal assignments of **2** were substantiated in NOE-difference and two-dimensional correlated spectroscopy (COSY), heteronuclear correlated spectroscopy (HETCORR) and heteronuclear multiple bond correlation (HMBC) experiments. The negative ion electrospray MS of **2** was determined using a VG Platform instrument. The concentration of glucose in the medium was determined colorimetrically (Hewlett Packard spectrophotometer HP 8452) using enzymatic colorimeter test Peridochrom reagent (Boehringer Mannheim). The DSC thermogram of glucoside **2** was determined using a Shimadzu DT 30 thermal analyzer.

Cell Suspension Cultures Clone sm. of the cell suspension cultures used in these studies was initiated from the callus cultures of *Solanum mammosum* (code sm.) established previously.[8] The calli were cultivated in a 300 ml Erlenmeyer flask containing 50 ml of modified Murashige and Skoog medium[9] supplemented with sucrose (30 g/l), kinetin (2 mg/l), naphthalene acetic acid (1 mg/l) and caseinhydrolysate (1 g/l) on a gyrotary shaker (120 rounds per min) at 25 ± 1 °C under continuous light (*ca.* 2000 lux).

Toxicity and Bioconversion Experiments Fresh weight cells (10 g) were

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inoculated into liquid medium (50 ml) without **1** (control cultures) and into liquid medium containing various concentrations of **1** (200, 400, 600, 800 and 1000 mg/l). After 6 d incubation the cultures were harvested, collected, filtered, weighed, dried and powdered. The standard protocol for the bioconversion experiment was as follows: fresh weight cells (10 g) were inoculated into a 300 ml Erlenmeyer flask containing 50 ml medium to which **1** (800 mg/l) had been added. Inoculated solutions were cultured for 1 to 6 d.

Extraction, Isolation and Purification of 2 The oven dried (40 °C) powdered biomass (5.65 g) was refluxed (2 h) in MeOH. The MeOH extract was concentrated under reduced pressure and submitted to column chromatography (CC) (1.84 g) on Silicagel 40 (Merck, 70—230 mesh), developed with EtOAc–MeOH–H_2O (77 : 13 : 10). The glucoside fraction was purified by preparative TLC (Silicagel 60 GF 254, Merck; 0.25 mm layer; developed with the EtOAc–MeOH–H_2O (77 : 13 : 10); detection: UV 254 nm). Glucoside **2** (224 mg) was obtained after removal of MeOH (used as the elution solvent for TLC bands). A sharp endothermic peak of DSC thermogram was observed at 163.4 °C.

Compound 2 Electrospray MS (cone voltage −40 V, NH_4COOH assisted matrix) *m/z* [negative ions] (% rel. int.): 361.1 $[M+62]^-$ (21.6), 344.3 $[M+COOH]^-$ (100.0), 334.2 $[M-H+2H_2O]^-$ (21.6), 298.2 $[M-H]^-$ (7.6), 136.2 $[C_7H_6NO_2]^-$ (59.7) and 118.3 $[136-H_2O]^-$ (26.3). ^{1}H-NMR (DMSO-d_6, 300.13 MHz) δ: 7.40 (1H, br d, J=8.3 Hz, H-3), 7.55 (1H, br td, J=7.9, 1.8 Hz, H-4), 7.20 (1H, br t, J=7.7 Hz, H-5), 7.90 (1H, dd, J=7.7, 1.8 Hz, H-6), 5.02 (1H, d, J=7.6 Hz, H-1′), 3.40 (1H, m, H-2′), 5.69 (1H, br s, OH at C-2′), 3.40 (1H, m, H-3′), 5.19 (1H, br s, OH at C-3′), 3.27 (1H, m, H-4′), 5.25 (1H, br s, OH at C-4′), 3.49 (1H, m, H-5′), 3.58, 3.83 (2H, m, H-6′, H-6″), 4.74 (1H, br s, OH at C-6′), 7.65 (1H, br s, NH′), 7.91 (1H, br s, NH″). ^{13}C-NMR (DMSO-d_6, 75.47 MHz) δ: 123.4 (C-1), 155.6 (C-2), 116.4 (C-3), 132.6 (C-4), 122.3 (C-5), 130.7 (C-6), 166.2 (C-7), 101.9 (C-1′), 73.4 (C-2′), 76.3 (C-3′), 69.7 (C-4′), 77.3 (C-5′), 60.8 (C-6′).

Quantitative Analysis of 1 and 2 The contents of **1** (Rf=0.81) and **2** (Rf=0.31) in the medium and the biomass were determined densitometrically using a Shimadzu CS 930 TLC scanner on Silicagel 60 GF 254 (Merck), following elution with EtOAc–MeOH–H_2O (77 : 13 : 10). Quantitation was performed by measuring the absorbance reflectant (at λ_{max}) of the analyst spots (304 nm for **1** and 294 nm for **2**). The determinations of **1** and **2** were made by calculation with a calibration graph obtained using salicylamide (Sigma) and the isolated glucoside as external standards on the same plate. The method of validation was made according to Funk *et al.*[10] The linearity of **1** and **2** was achieved from 0.8 to 20 μg/spot (relative process standard deviation, V_{xO}=1.74% for **1** and 3.19% for **2**, n=6). The accuracy (by standard addition) was 93.84±1.65% (**1**) and 91.21±2.76% (**2**). The confidence range data (p<0.05) of the intercept (V_{Baf}) was 0.121±0.327 (**1**) and 0.124±0.588 (**2**), whilst for slope (V_{Bbf}) was 1.127±0.223 (**1**) and 1.100±0.147 (**2**).

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