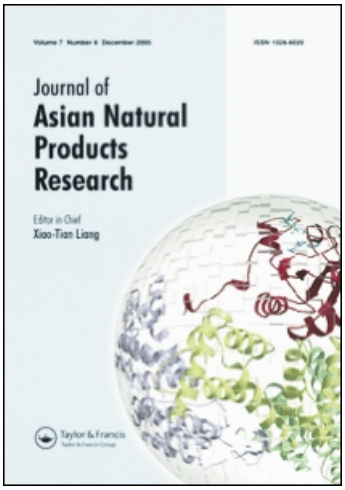

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C-27 AND C-3 GLUCOSYLATION OF DIOSGENIN BY CELL SUSPENSION CULTURES OF *COSTUS SPECIOSUS*

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3-O-[β-D-glucopyranosyl-(1″ → 2′)-β-D-glucopyranosyl], 27-O-β-D-glucopyranosyl-(25*R*)-spirost-5-ene-3β,27-diol was isolated from cell suspension cultures of *Costus speciosus*, following incubation with diosgenin, and its structure was elucidated using a combination of one- and two-dimensional ^{1}H and ^{13}C NMR spectral data, and positive and negative ion ESMS spectral data.

Keywords: *Costus speciosus*; Cell suspension cultures; Biotransformation; Glucosylation; Diosgenin; 3-O-[β-D-glucopyranosyl-(1″ → 2′)-β-D-glucopyranosyl]; 27-O-β-D-glucopyranosyl-(25*R*)-spirost-5-ene-3β,27-diol

INTRODUCTION

Various plant cell cultures are capable of glucosylating a variety of exogenously supplied substrates [1]. For example, the glucosylation of simple phenols by various cell suspension cultures has been reported by Umetami, Tanaka and Tabata [2]. Cell suspension cultures of *Salix matsudana* transform salicyl alcohol into salicin and isosalacin, while salicylic acid was converted into salicylic acid-2-O-β-D-glucopyranoside and

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salicylic acid-7-O-β-D-glucopyranoside [3]. In previous work, we reported the bioconversion 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* [4 6]. Paczkowski and Wojciechowski [7] reported the glucosylation of diosgenin and solasodine into its mono-glucoside by soluble glycosyltransferase(s) from *Solanum melongena* leaves, while Inoue *et al.* [8] have demonstrated the transformation of a furostanol glycoside (protogracillin) into a spirostanol glycoside (gracillin) by β-glucosidase in *Costus speciosus* rhizomes. These studies showed that diosgenin glycoside can be produced by both glycosyltransferase and glucosidase in plant cells. The fungus *Cunninghamella elegans* has been reported to transform diosgenin into its di-and tri-hydroxy derivatives [9].

The callus and cell suspension cultures of *C. speciosus* contain cholesterol, campesterol, stigmasterol and β-sitosterol, while diosgenin (**1**) and other steroidal sapogenins have not been detected. Diosgenin accumulates only in shoot cultures of *C. speciosus* [10].

It is well known that the accumulation of secondary metabolites depends on the relative rates of biosynthesis and catabolism; accumulation can occur when the rate of biosynthesis is higher than the rate of catabolism [11]. The absence of **1** in the callus and suspension cultures of *C. speciosus* may be due to its conversion, immediately after its biosynthesis. In order to determine whether **1** can be transformed to other substances, it was incubated in cell suspension cultures of *C. speciosus*. Another aim of the experiment was to determine whether diosgenin glycoside(s) can be formed by direct glycosylation of the aglycone **1**.

We now report the biotransformation of diosgenin (**1**) by cell suspension cultures of *C. speciosus* into a new C-27 glucoside, 3-O-[β-D-glucopyranosyl-(1″ → 2′)-β-D-glucopyranosyl], 27-O-β-D-glucopyranosyl-(25*R*)-spirost-5-ene-3β,27-diol (**2**), and its structure elucidation using a combination of one- and two-dimensional ^{1}H and ^{13}C NMR, and positive and negative ion ESMS spectral data.

RESULTS AND DISCUSSION

Incubation of cell suspension cultures of *Costus speciosus* with **1** (75 mg l^{-1}), followed by isolation, purification by column chromatography and

preparative TLC, afforded metabolite **2** (R_f 0.19). Spots of compounds **1** and **2** showed identical visible absorbance reflectance spectra after reaction with anisaldehyde-sulphuric acid reagent. Control experiments showed that in the absence of cells, substrate **1** (R_f 0.81) remained unchanged in the culture medium, and that metabolite **2** was only produced when cell suspension cultures of *C. speciosus* were present in the medium. Although the PCV (packed cell volume) and GI (growth index) were decreased (*ca.* 40%), cell suspension cultures of *C. speciosus* seemed to be resistant to inoculation with **1** (75 mg l^{-1}).

The positive ion electron spray mass spectrum (ESMS) of **2**, determined in a cation-assisted matrix, displayed pseudomolecular ions at m/z 917 $(M+H)^+$, 934 $(M+NH_4)^+$, 939 $(M+Na)^+$, and 955 $(M+K)^+$. The negative ion ESMS determined in a Cl^- assisted matrix, showed pseudomolecular ions at m/z 915 $(M-H)^-$, 951 $(M+^{35}Cl)^-$ and 953 $(M+^{37}Cl)^-$. These data indicated that metabolite **2** had a molecular weight of 916 daltons demonstrating **2** to be a trihexoside analogue of **1**.

The ^{13}C NMR spectrum of **2** exhibited 45 carbon signals, including a quaternary acetal-type resonance at δ 109.6 (C-22), two alkene resonances at δ 141.1 (C-5) and 121.6 (C-6), and three methyl group resonances at δ 16.4 (C-18), 19.5 (C-19), and 15.0 (C-21), all of which corresponded closely to those observed for diosgenin; however, a fourth methyl group resonance was not observed. The ^{13}C NMR spectrum also displayed three anomeric glucosyl signals at δ 101.5 (C-1′), 106.7 (C-1″) and 105.1 (C-1‴).

The ^1H NMR spectrum of **2** displayed signals attributable to two tertiary methyl groups at δ 0.82 (H-18) and 1.01 (H-19), while only a single secondary methyl group was observed at δ 1.10 ($d, J = 6.9$ Hz). Three anomeric β-D-glucopyranosyl proton resonances were also observed at δ 5.06 (H-1′, $d, J = 7.6$ Hz), 5.27 (H-1″, $d, J = 7.7$ Hz) and 4.77 (H-1‴, $d, J = 7.7$ Hz).

The HMBC spectrum of **2** displayed correlations between the anomeric glucosyl protons at δ 5.06 (H-1′) with C-3 (δ 79.3) and δ 5.27 (H-1″) with C-2′ (δ 84.8), while H-2′ (δ 4.14) exhibited correlations with C-1′ (δ 101.5), C-1″ (δ 106.7) and C-3′ (δ 77.9). These data indicated that the inner β-D-glucopyranosyl residue was attached to C-3, and that the pair of β-D-glucopyranosyl residues were mutually 1, 2-linked. Comparison with the ^{13}C NMR spectral assignments determined for the sugar moieties of diosgenin-3-O-β-D-glucopyranosyl-(1″ → 3′)-β-D-glucopyranoside (glucoside **3**) [12] showed that the C-2′ resonance of **2** exhibited a downfield shift of 10.4 ppm, indicative of α-glucosylation [13]. Correlations observed in the ROESY spectrum of **2** also established the presence of a 1, 2-glucosidic linkage. In particular, correlations were observed between H-1′ (δ 5.06) and H-3 (δ 3.84) and between H-1″ (δ 5.27) and H-2′ (δ 4.14).

One- and two-dimensional NMR spectral data (including COSY, TOCSY, ROESY, HMBC and HSQC spectra) showed that the third glucopyranosyl residue was attached to C-27. TOCSY and COSY correlations identified the resonances and connectivities of other protons associated with each of the glycosyl residues, while the HSQC spectrum identified the corresponding ^{13}C resonances. The HMBC spectrum of **2** indicated correlations between H-27$_A$ (δ 3.46) and C-1‴ (δ 105.1), C-26 (δ 63.7), C-25 (δ 36.7), C-24 (δ 24.0); H-27$_B$ (δ 3.93) and C-26, C-25, C-24; and H-1‴ (δ 4.77) and C-27 (δ 72.0). The attachment of the third glucopyranosyl residue to C-27 was also confirmed by the ROESY spectrum of **2**, which showed a correlation between H-1‴ and H-27$_A$.

Consequently the structure of **2** was determined to be 3-O-[β-D-glucopyranosyl-(1″ → 2′)-β-D-glucopyranosyl], 27-O-β-D-glucopyranosyl-(25*R*)-spirost-5-ene-3β,27-diol.

To our knowledge this is the first report of the C-27 glucosylation of diosgenin by cell suspension cultures of *C. speciosus*. This is also the first report of the isolation of 3-O-[β-D-glucopyranosyl-(1″ → 2′)-β-D-glucopyranosyl], 27-O-β-D-glucopyranosyl-(25*R*)-spirost-5-ene-3β,27-diol from a natural source. This work showed that *C. speciosus* cells can biosynthesize diosgenin glycoside by glucosylation of the aglycone, in addition to the bioconversion of the furostanol glycoside as previously reported [8].

It seems likely that cell suspension cultures of *C. speciosus* hydroxylate diosgenin at C-27 prior to glucosylation. We are investigating the possibility of isolating 27-hydroxydiosgenin, the presumed intermediate biotransformation product, and endeavour to characterize the enzymes that are responsible for the bioconversion.

EXPERIMENTAL SECTION

General Experimental Procedures

NMR spectra were recorded at 400.13 (^1H) and 100.62 MHz (^{13}C) using an inverse 5 mm probehead installed in a Bruker DRX 400 spectrometer. Gradient selection was utilized in HMBC and HSQC experiments. Chemical shifts (δ ppm) are reported relative to solvent peaks observed for pyridine-d_5 ($^1H = 8.70$ ppm, low field signal; $^{13}C = 123.5$ ppm, high field signal). Coupling constants are reported to a precision of ± 0.2 Hz. ^{13}C NMR signal multiplicities (*d*, *t* or *q*; *s* suppressed) were determined using the DEPT sequence with a 135° detection pulse. Two-dimensional COSY and HMBC

(80 msec mixing time) spectra were determined in absolute value mode, while TOCSY, ROESY (250 msec spin lock time) and HSQC spectra were determined in phase-sensitive mode.

Positive and negative ion electrospray mass spectra (ESMS) were obtained using a Fisons VG Platform II instrument. Samples were introduced into the spectrometer using $CH_3CN : H_2O$ (1 : 1) as solvent.

The visible absorbance reflectance spectra of the spots of substrate **1** and metabolite **2** were determined using a Shimadzu TLC Scanner CS 930. Stationary phase: silica gel F_{254} precoated plate (E. Merck); mobile phase: $CHCl_3 : MeOH : H_2O$ (40 : 16 : 3); detection was performed with anisaldehyde-sulphuric acid reagent (100°C, 5 min).

Cell Suspension Culture and Biotransformation Conditions

Cell suspension cultures were initiated from callus cultures of *C. speciosus* (code F8), as previously reported [10]. The calli were cultivated in 300 ml Erlenmeyer flasks containing 50 ml of modified Murashige and Skoog medium [14] supplemented with sucrose (30 g l^{-1}), kinetin (2 mg l^{-1}), 2,4-dichloro phenoxyacetic acid (0.5 mg l^{-1}) and casein hydrolysate (1 g l^{-1}) on a gyrotary shaker (120 rpm) at 25 ± 1° under continuous light (*ca.* 1500 lux). Biotransformation experiments were typically performed by inoculating cells (6–7 g fresh weight) into liquid medium (50 ml) containing **1** (75 mg l^{-1}) and incubated for 12 days. After 12 days cultures were harvested, followed by PCV (packed cell volume) and GI (growth index) determination [10], filtered, weighed, oven dried at 40° (until their water content was ca. 2%), and powdered.

Biotransformation of 1 and Isolation of 2

Incubation of diosgenin **1** (75 mg l^{-1}), and with the *C. speciosus* cell cultures as described above, produced powdered biomass (23.2 g), which was refluxed for 1 h with $CHCl_3$ (4 times); the residues were then refluxed (4 times) with MeOH for 2 h. Concentration of the combined MeOH extracts under reduced pressure using a rotatory evaporator afforded a semi-solid brown residue (1.93 g), which was submitted to column chromatography on silica gel 60 (E. Merck, 70–230 mesh) using $CHCl_3 : MeOH : H_2O$ (40 : 16 : 3) as eluent [12]. Purification of the metabolite fraction by preparative TLC (silica gel 60 F_{254} precoated plate, Merck; 0.25 mm layer) using $CHCl_3 : MeOH : H_2O$ (40 : 16 : 3) as developing solvent, yielded metabolite **2** (10 mg).

TABLE I ^{1}H and ^{13}C NMR chemical shifts for glucosides **2** and **3** (ppm in pyridine-d_5)[a]

Position	**2** ^{1}H	**2** ^{13}C	**3**[b] ^{13}C
1_A	0.93	37.5	37.4
1_B	1.68		
2_A	1.80	30.3	30.2
2_B	2.14		
3	3.84	79.3	78.8
4_A	2.65	39.3	39.3
4_B	2.82		
5		141.1	140.8
6	5.34	121.6	121.8
7_A	1.44	32.3	32.2
7_B	1.83		
8	1.51	31.7	31.6
9	0.84	50.3	50.2
10	–	37.1	37.0
11_A	1.32	21.2	21.1
11_B	1.45		
12_A	1.16	39.9	39.9
12_B	1.66		
13	–	40.5	40.5
14	1.01	56.7	56.6
15_A	1.98	32.2	31.8
15_B	1.40		
16	4.49	81.2	81.1
17	1.75	62.9	62.9
18	0.82	16.4	16.4
19	1.01	19.5	19.4
20	1.90	42.1	42.0
21	1.10 (d, $J = 6.9$)	15.0	15.0
22	–	109.6	109.3
23_A	1.62	31.3	31.8
23_B	1.66		
24_A	1.62	24.0	29.3
24_B	1.66		
25	2.05	36.7	30.6
26_A	3.72	63.7	66.9
26_B	4.03		
27_A	3.46	72.0	17.3
27_B	3.93		
1′	5.06 (d, $J = 7.6$)	101.5	102.1
2′	4.14	84.8	74.4
3′	4.35	77.9	88.9
4′	4.23	71.5	69.8
5′	3.88	78.2	78.1
$6_{A'}$	4.34	62.7	62.5
$6_{B'}$	4.50		
1″	5.27 (d, J 7.7)	106.7	106.1
2″	4.13	77.1	75.7
3″	4.24	78.1	78.3
4″	4.30	71.6	71.6
5″	3.98	78.6	78.3
$6_{A''}$	4.46	62.9	62.5
$6_{B''}$	4.57		

TABLE I (Continued)

Position	**2** ^1H	**2** ^{13}C	**3**[b] ^{13}C
$1'''$	4.77 ($d, J = 7.7$)	105.1	–
$2'''$	4.01	75.2	–
$3'''$	4.23	78.6	–
$4'''$	4.23	71.7	–
$5'''$	3.95	78.8	–
$6_{A'''}$	4.38	62.9	–
$6_{B'''}$	4.56		

[a]Signals were assigned by means of 2D NMR experiments. Coupling constants (J in Hz) are given in parentheses.
[b]Inoue *et al.*, 1995 [12].

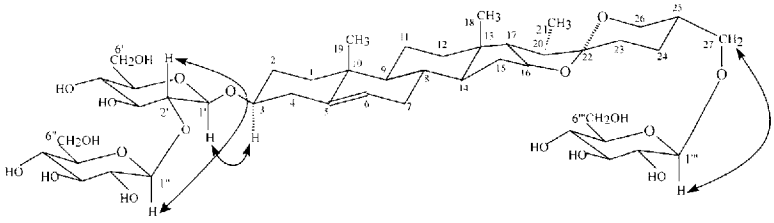

FIGURE 1 Chemical structure of metabolite **2** and selected ROESY correlations (↔).

Metabolite 2, white amorphous solid; *in situ* visible absorbance reflectance, λ_{max} nm (silica gel F_{254} precoated plate; anisaldehyde-sulphuric acid reagent): 428; positive ion ESMS ($+60$ V, Na^+, K^+ and NH_4^+ assisted matrix) m/z (% rel. int.): 917 ($[M+H]^+$, 100), 934 ($[M+NH_4]^+$, 40), 939 ($[M+Na]^+$, 70), 955 ($[M+K]^+$, 48); negative ion ESMS (-100 V, Cl^- assisted matrix), m/z (% rel. int.): 915 ($[M-H]$, 100), 951 ($[M+^{35}Cl]^-$, 23), 953 ($[M+^{37}Cl]^-$, 18); ^1H NMR and ^{13}C NMR spectral data see: Table I.

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