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BIOTRANSFORMATION OF 18β-GLYCYRRHETINIC ACID BY CELL SUSPENSION CULTURES OF *GLYCYRRHIZA GLABRA*

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Key Word Index—*Glycyrrhiza glabra*; Leguminosae; 18β-glycyrrhetinic acid; glycosylation; biotransformation; plant cell culture.

Abstract—Two biotransformation products formed from 18β-glycyrrhetinic acid by cell suspension cultures of *Glycyrrhiza glabra* were isolated and their structures determined by chemical and spectral data as 3-*O*-[α-L-arabinopyranosyl-(1→2)-β-D-Glucuronopyranosyl]-24-hydroxy-18β-glycyrrhetinic acid and 30-*O*-β-D-glycopyranosyl-18β-glycyrrhetinic acid. The formation of glycyrrhizin, the main triterpene glucuronide of the licorice root, was not detected among the biotransformation products. This is the first report of the glucuronylation of an exogenous triterpene in plant cell cultures.

INTRODUCTION

The stolon and root of licorice, *Glycyrrhiza glabra* L., contain glycyrrhizin (**1**), an oleanane-type triterpene glucuronide, which is used as a natural sweetener as well as an antiinflammatory drug for hepatitis. However, we have detected no glycyrrhizin in callus or cell suspension cultures of this plant, although they contained β-amyrin, a possible biosynthetic precursor of glycyrrhizin, in addition to betulinic acid, a lupane-type triterpene [1]. This suggested that the cultured cells were incapable of producing glycyrrhizin due to their inability to perform some (or all) of the specific oxidation and glycosylation reactions in the final part of biosynthetic pathway leading to glycyrrhizin. In this study, we have examined whether or not cultured cells of *G. glabra* can convert exogenously supplied 18β-glycyrrhetinic acid (**2a**) into its glucuronide, glycyrrhizin.

RESULTS AND DISCUSSION

18β-Glycyrrhetinic acid (**2a**) (600 mg) was administered to 15-day-old cell suspension cultures of *G. glabra* at the linear growth stage. Fifteen days later the cells (443 g fr. wt) were harvested and extracted with methanol. The methanol extract was suspended in water, adjusted to pH 4 with HCl, and extracted [EtOAc–MeOH (19:1)]. The EtOAc-soluble fraction was subjected to column chromatography (silica gel and LiChroprep RP-8) and prep. TLC, successively, to give two major metabolities, A (60 mg) and B (5 mg). The ^{1}H and ^{13}C NMR assignments of these two biotransformation products were performed by DEPT pulse sequence, two-dimensional H–H and H–C shift correlation data.

Metabolite A (**3a**), which tasted as sweet as **1**, was methylated with diazomethane. The methyl ester (**3b**) was methanolized with 5% HCl–methanol to give an agly-

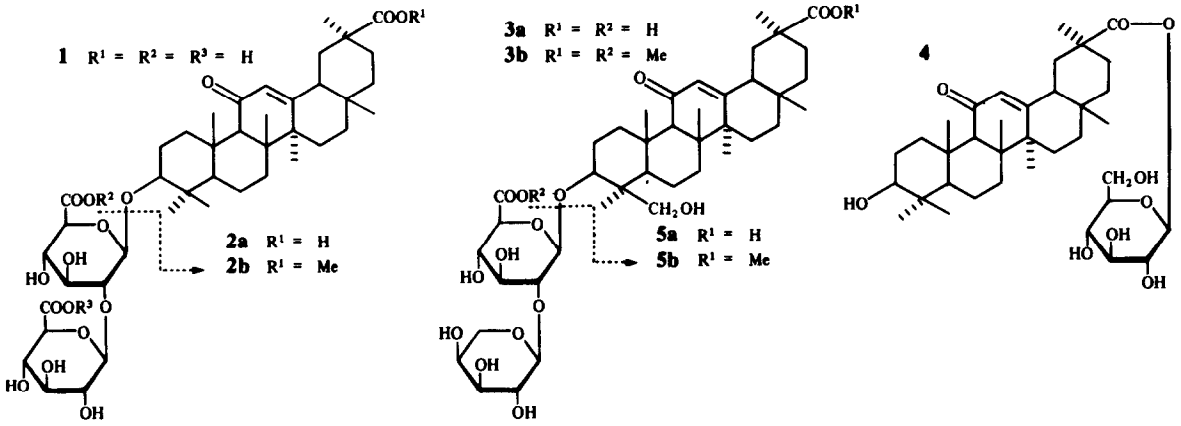

Table 1. ^{13}C NMR data for compounds **2a**, **3b** and **4**

	C	2a	3b	4
Sapogenol moiety	3	77.9	90.5	77.9
	11	199.5	199.2	199.7
	12	169.5	170.3	169.2
	24	16.5	64.5	16.6
	30	179.0	176.8	175.8
3-*O*-β-D-Glucuronopyranosyl moiety	1′		105.3	
	2′		78.9	
	3′		77.7	
	4′		73.0	
	5′		77.1	
	6′		169.1	
	OCH_3		51.7	
2′-*O*-α-L-Arabinopyranosyl moiety	1″		104.9	
	2″		73.5	
	3″		74.9	
	4″		70.2	
	5″		67.5	
30-*O*-β-D-Glucopyranosyl moiety	1‴			96.0
	2‴			74.4
	3‴			78.8
	4‴			71.2
	5‴			79.5
	6‴			62.3

cone and methyl glycosides. The GC analysis of the TMSi derivatives of the methyl glycosides indicated that the sugar moiety of metabolite A consisted of arabinose and glucuronic acid. This was supported by the ^{1}H and ^{13}C NMR spectra of **3b** (Table 1). The ^{1}H NMR spectrum of **3b** exhibited signals assignable to six tertiary methyl groups, two carboxyl methyl groups, and two anomeric protons of the sugar moiety. The chemical shifts (glucuronic acid: δ4.90, $J = 7.6$ Hz; arabinose: δ 5.43, $J = 7.4$ Hz) of the anomeric protons in the ^{1}H NMR spectrum and of the carbons assignable to the sugar moiety in the ^{13}C NMR spectrum indicated the presence of β-linked glucuronopyranose and α-linked L-arabinopyranose, respectively. In the ^{13}C NMR spectrum of **3b**, a new signal assignable to a tertiary carbinol group appeared at δ 63.2 in place of the C-24 methyl group signal of glycyrrhetinic acid, suggesting that the latter group was oxidized to a hydroxymethyl group. The aglycone of **3b** obtained by methanolysis was identified as 24-hydroxyglycyrrhetinic acid methyl ester (**5b**) by comparisons with an authentic sample isolated from licorice roots [2]. Glycosylation shifts in the ^{13}C NMR spectrum were observed for C-3 of the aglycone and C-2 of glucuronic acid. Therefore, the structure of metabolite A was determined as 3-*O*-[α-L-arabinopyranosyl-(1→2)-β-D-glucuronopyranosyl]-24-hydroxy-18β-glycyrrhetinic acid (**3a**).

Metabolite B (**4**), which was found to have no free carboxyl group, was methanolized with 5% HCl–methanol to give an aglycone (methyl ester) and a methyl sugar. The sugar moiety was identified as glucose by GC analysis of the TMSi derivative. The methylated aglycone was identified as 18β-glycyrrhetinic acid methyl ester by TLC analysis and ^{13}C NMR. The ^{1}H NMR signal at δ 6.40 together with its coupling constant ($J = 8.0$ Hz) was assignable to an anomeric proton of 4 and indicated that β-D-glucopyranose was attached to the C-30 carboxyl group of the aglycone. Thus, the structure of metabolite B was concluded to be 30-*O*-β-D-glucopyranosyl-18β-glycyrrhetinic acid (**4**).

The ability of three cell lines of *G. glabra* to glycosylate **18β**-glycyrrhetinic acid (**2a**) supplied to the liquid medium 8 (exponential growth stage) and 28 (stationary stage) days after inoculation is shown in Table 2. All the cell lines tested transformed glycyrrhetinic acid (**2a**) into **3a** and **4**, but the efficiency was markedly different

Table 2. Biotransformation of compound **2a** by three cell lines of *G. glabra*

Cell line	Growth stage	Per cent of **2a** transformed to 3a*	4*
RNS-1A	Exponential	4.9	1.0
	Stationary	—	1.3
RNS-1B	Exponential	1.8	2.6
	Stationary	0.3	1.1
RNS-1C	Exponential	13.0	1.5
	Stationary	—	0.2

*Most of **3a** and **4** (>95%) were found in the cells.

Compound **2a** (6 mg in 0.25 ml DMSO and 0.05 ml Tween 80) was added to the medium (30 ml) eight (exponential growth stage) or 28 (stationary stage) days after inoculation with cells (1 g). The cells were harvested for analysis after a further four days of incubation at 25° in the dark.

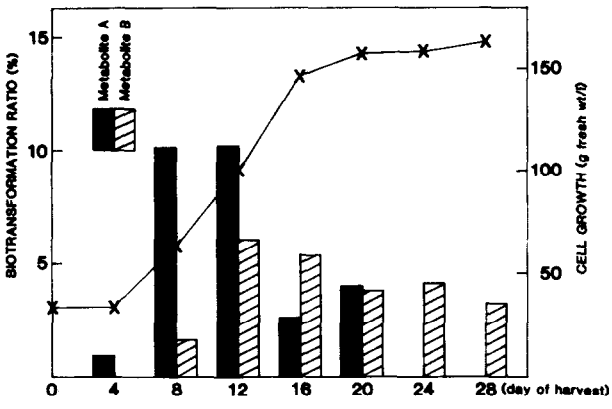

Fig. 1. Time course of cell growth and biotransformation of 18β-glycyrrhetinic acid (**2a**) to metabolite A (**3a**) and B (**4**) by *G. glabra* cell suspension cultures. Compound **2a** (6 mg) was added to separate cultures on different dates with intervals of four days and the cells were harvested after four days of incubation. The data represent the mean of three replicates.

between the cell lines as well as between the culture stages. Cell line RNS-1C converted 13% of **2a** administered at the exponential growth stage into **3a**, but none at the stationary stage. Furthermore, the ratio of **3a** to **4** changed during the period of cell growth (Fig. 1). Compound **3a** was formed mainly in the exponential and linear growth stages, while **4** tended to increase with the decline of cell growth from the late linear to the stationary stage. More than 95% of these metabolites accumulated in the cells. The formation of glycyrrhizin (**1**) from the substrate **2a** was undetectable in any cell lines tested. Although glucosylation of various exogenous compounds has been reported for plant cell cultures [3, 4], the occurrence of glucuronylation has never been reported to our knowledge. Although metabolite **3a** is a new saponin, its sugar moiety as well as its aglycone is known to exist independently as partial structures of different saponins isolated from licorice by other workers [2, 5, 6]. The 30-*O*-glucopyranosyl derivative of glycyrrhizin (**1**) has also been isolated from licorice [7]. It seems, therefore, that cultured cells of *G. glabra* partly retain the metabolic abilities specific to the licorice plant in transforming glycyrrhetinic acid (**2a**) to its glycoside, even though they are incapable of synthesizing glycyrrhizin (**1**).

EXPERIMENTAL

Mps: uncorr. ^{1}H and ^{13}C NMR spectra were measured in pyridine-d_5 at 600 and 125 MHz, respectively, and TMS was used as an int. standard. TLC: Merck silica gel 60 F254.

Chemicals. 18β-Glycyrrhetinic acid (**2a**) and glycyrrhizin (**1**) were supplied by Maruzen Kasei Co. Ltd, Japan. Prof. I. Kitagawa of Osaka University kindly gave us the authentic sample of 24-hydroxy-18β-glycyrrhetinic acid methyl ester (**5b**) and its NMR data.

Plant material and culture method. Cell suspension cultures (strains RNS-1A, -1B, and -1C) were established from the callus derived from a young root of *G. glabra* and maintained in Linsmaier-Skoog (LS) medium [8] containing 100 μM 1-naphthaleneacetic acid and 1 μM 6-benzyladenine for 3 years, as described elsewhere [1]. These cultures were agitated on a reciprocal shaker (100 strokes min^{-1}) in the dark at 25°, and subcultured at intervals of 24 days. For biotransformation experiments, cells (1 or 3 g) were inoculated in a flask (100 or 300 ml) containing the medium (30 or 100 ml) and incubated under the same conditions as above.

Substrate administration. For chemical analysis of biotransformation products, 0.6 g of 18β-glycyrrhetinic acid (**2a**) was dissolved in a mixture of 25 ml DMSO and 5 ml Tween 80, and an aliquot (1 ml) of the soln containing 20 mg of **2a** was added to each one of 30 flasks (100 ml medium/300 ml) 15 days after inoculation. The cultures were harvested after 15 days of incubation at 25° in the dark. For studying the efficiency of biotransformation for each cell line, 0.3 ml of a soln containing 6 mg of **2a** in 0.25 ml DMSO and 0.05 ml Tween 80 was added to the medium (30 ml) in a 100 ml flask 8 or 28 days after inoculation, and incubated for 4 days before harvest. For the time course experiment, 0.3 ml of the above soln was added to the medium every 4 days during a culture period of 28 days, and each culture with 3 replicates was incubated for 4 days before harvest. The cells were filtered off onto Miracloth and extracted with 60 ml MeOH, and the concd MeOH soln (5 ml) used for HPLC analysis. The filtered medium was adjusted to pH 4 with 1 M HCl, and extracted (× 3) with EtOAc–MeOH (19:1). The concd extract was dissolved in 5 ml MeOH and subjected to HPLC analysis.

Quantitative analysis of metabolites. The sample (10 μl) was analysed by reverse phase ion pair HPLC; column (4.6 × 150 mm) packed with TSK gel ODS 120A (Toyo Soda), solvent system: MeOH–H_2O (3:2) containing 15 mM tetra-*n*-butylammonium bromide (pH 6.0), flow rate: 1.5 ml min^{-1}, column temp.: 60°, detection: UV 254 nm. R_t: **3a**, 6.88 min; **4**, 20.01 min. The quantities of **3a** and **4** were calculated from the corresponding peak areas.

Isolation and identification of metabolites. The harvested cells (443 g fr. wt) were extracted with MeOH (11 × 3) and the extract concd *in vacuo*. The concd extract was suspended in 500 ml H_2O, adjusted to pH 4 with 1 M HCl, then extracted with EtOAc–MeOH (19:1). The concd extract (3.7 g) was subjected to silica gel CC (200 g Wako gel C-200) eluted successively with $CHCl_3$, EtOAc, EtOAc–MeOH (9:1), EtOAc–MeOH (4:1), EtOAc–MeOH (1:1) and MeOH.

Isolation of metabolite A (**3a**). A fraction (0.50 g) eluted with EtOAc–MeOH (4:1) was subjected to CC on a LiChroprep RP-8 column (size B); solvent system: MeCN–H_2O–HOAc (40:60:1), detection: UV 254 nm. The fraction corresponding to the peak at 19 min was subjected to prep. TLC using BuOH–HOAc–H_2O (7:1:2) to give **3a** (R_f 0.22). **3a** was methylated with CH_2N_2 and the methyl ester (**3b**) purified with LiChroprep RP-8 CC (MeCN–H_2O, 1:1, 5 ml min^{-1}) to give a powder (38 mg), mp 256–258°, $[\alpha]_D$ + 69° (MeOH; c 0.46), negative FABMS *m/z* 821 $[M-H]^-$, ^{1}H NMR; δ 0.76 (3H, *s*, Me), 1.04 (3H, *s*, Me), 1.15 (3H, *s*, Me), 1.18 (3H, *s*, Me), 1.34 (3H, *s*, Me), 1.38 (3H, *s*, Me), 2.42 (1H, *s*, H-9), 3.68 (3H, *s*, COOMe), 3.72 (3H, *s*, COOMe), 4.90 (1H, *d*, *J* = 7.6, GlcUA H-1), 5.43 (1H, *d*, *J* = 7.4, Ara H-1), 5.83 (1H, *s*, H-12); ^{13}C NMR: see Table 1.

Methanolysis of **3b**. A soln of **3b** (10 mg) in 5% HCl–dry MeOH (5 ml) was heated under reflux for 2 hr. The reaction mixture was neutralized with Ag_2CO_3 and the ppt. removed by centrifugation. The soln was evapd to dryness under red. pres. and the residue dissolved in H_2O was extracted with Et_2O. The Et_2O layer was subjected to prep. TLC ($CHCl_3$–MeOH 19:1) to give the aglycone of **3b** (R_f 0.5, 3 mg). It was identified as 24-hydroxy-18β-glycyrrhetinic acid methyl ester (**5b**) by comparison (MS, ^{1}H and ^{13}C NMR) with an authentic sample. The aq. layer was dried *in vacuo*, and the residue dissolved in 1 ml pyridine. An

aliquot of the pyridine soln (0.1 ml) was treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 0.2 ml) at 40° for 30 min. GC analysis of the reaction mixture revealed the presence of TMSi derivatives of methyl glucuronide and methyl arabinoside. The GC analysis was carried out by using a column (1 m × 3 mm) packed with 1% Silicone OV-17: isothermal 120°, N_2 at 30 ml/min, detector FID.

Isolation of metabolite B (**4**) A fraction (0.52 g) eluted with EtOAc–MeOH (9:1) was subjected to LiChroprep RP-8 CC (MeCN–H_2O 1:1, 5 ml min^{-1}, UV detector 254 nm), to give crude metabolite B (4, R_t 22 min). After CH_2N_2 treatment, **4**, which was not methylated, was isolated by LiChroprep RP-8 CC and recrystallized from MeOH – H_2O to yield needles (5 mg); mp 236–238°, $[\alpha]_D$ + 156° (MeOH; *c* 0.13), SIMS *m/z* 633 $[M+H]^+$, 655 $[M+Na]^+$; ^1H NMR: δ 0.83 (3H, *s*, Me), 1.10 (3H, *s*, Me), 1.11 (3H, *s*, Me), 1.28 (3H × 2, *s*, Me), 1.35 (3H × 2, *s*, Me), 2.51 (1H, *s*, H-9), 6.02 (1H, *s*, H-12), 6.40 (1H, *d*, J = 8.0, Glc H-1); ^{13}C NMR: see Table 1.

Methanolysis of **4**. **4** (1 mg) was methanolized in 5% HCl–dry MeOH under reflux for 2 hr. The reaction mixture gave the same GC peaks as did the TMSi derivative of the methyl glucoside. The aglycone obtained by methanolysis was treated with CH_2N_2 and identified as 18β-glycyrrhetinic acid methyl ester (**2b**) by TLC: $CHCl_3$–MeOH (19:1), R_f 0.55.

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