ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 16 (2002) 275–281

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Biotransformation of phenolic compounds by the cultured cells of *Catharanthus roseus*

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Received 13 September 2001; received in revised form 12 October 2001; accepted 20 October 2001

Abstract

The cultured cells of *Catharanthus roseus* were able to convert 2-, 3-, and 4-hydroxybenzyl alcohols into their corresponding hydroxybenzyl-β-D-glucopyranosides or β-D-glucopyranosylbenzyl alcohols, and then convert 2- and 3-hydroxybenzyl-β-D-glucopyranosides into primeverosides and vicianosides. Further, the *C. roseus* cells were capable of hydroxylation of 2-hydroxybenzoic acid to afford 2,5-dihydroxybenzoic acid and then glucosylation of the newly introduced phenolic hydroxyl group.

Keywords: Phenolic compound; *Catharanthus roseus*; Biotransformation

1. Introduction

Glucosylations using cultured plant cells have been the subject of increasing attention [1–9], as one-step enzymatic glycosylation is useful for preparation of glycosides rather than chemical glycosylation which requires tedious steps including the protection and the deprotection of hydroxyl groups of sugar moieties. On the other hand, the ability of cultured plant cells to hydroxylate foreign substrates into potentially useful substances has been quite interesting [4,10–14]. These biocatalytic approaches would be superior to chemical synthesis, since a position-specificity is expected from an enzymatic reaction. There are several reports on the regioselective glucosylation of salicyl alcohol by cultured plant cells showing that only benzyl hydroxyl group was glucosylated to give isosalicin [15,16]. Recently, we reported that the cultured cells of *Catharanthus roseus* are able to convert exogenous 1-phenylethanol into its glucoside and disaccharides [17]. However, little attention has been paid to the glycosylation of phenolic hydroxyl group and the hydroxylation of phenolic compounds by cultured plant cells. On continuing the investigation of biotransformation by cultured plant cells, we now report the specific capabilities of *C. roseus* cells to glycosylate and hydroxylate phenolic compounds.

2. Experimental

2.1. Analysis

Analytical and preparative TLCs were carried out on glass sheets (0.25 and 0.5 mm) coated with silica gel (Merck silica gel 60; GF_{254}). GLC was carried out

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1381-1177/02/$ – see front matter
PII: S1381-1177(01)00073-X

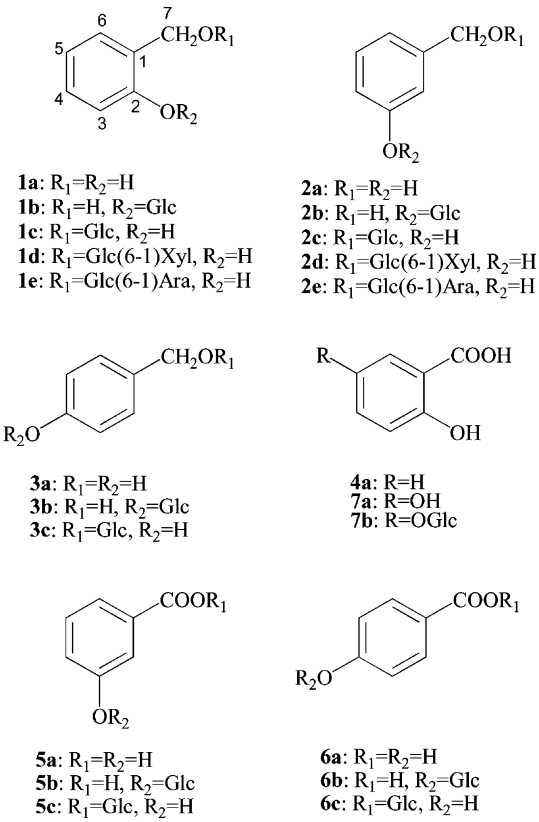
with FID and a capillary column (0.25 mm × 25 m) coated with 0.25 μm CP cyclodextrin β 236M-19 (WCOT) using N_2 as carrier gas (50 $cm^3\,min^{-1}$) at column temperature: 100 °C. NMR spectra were measured on a JEOL GSX500 [500 MHz (^1H) and 125.8 MHz (^{13}C)] NMR spectrometer. FABMS was taken on a JEOL SX102A mass spectrometer.

2.2. Plant materials

Callus tissues induced from the leaves of *C. roseus* [18] have been maintained for approximately 10 years under subculturing on SH agar medium [19] containing 3% of sucrose and 10 mM of 2,4-dichlorophenoxyacetic acid at 25 °C under illumination (4000 lx) for every 3–5 weeks. Prior to use in this study, the callus tissues were transferred to 300 ml conical flasks containing 100 ml of SH liquid medium containing 3% of sucrose and 10 mM of 2,4-dichlorophenoxyacetic acid and cultured at 25 °C for 3 weeks on a rotary shaker (75 rpm) under illumination (4000 lx).

2.3. Biotransformation of hydroxybenzyl alcohols (*1a–3a*) and hydroxybenzoic acids (*4a–6a*)

A substrate (each 150 mg), such as 2-, 3- and 4-hydroxybenzyl alcohols (**1a**–**3a**) and 2-, 3- and 4-hydroxybenzoic acids (**4a**–**6a**), in MeOH (1 ml) and glucose (7.8 g) were administered to the flask (1000 ml) containing the suspension cells (150 g) of *C. roseus* in 600 ml of SH medium, and the cultures were incubated at 25 °C for 7 days on a rotary shaker (75 rpm) under illumination (4000 lx). After the incubation, the cells and medium were separated by filtration with suction. The cells were extracted with MeOH and the extract was concentrated by evaporation in vacuo. The methanolic fraction was partitioned between H_2O and EtOAc and then the aqueous layer was further extracted with BuOH. The filtered medium was extracted with BuOH. The BuOH extracts from the cells and the culture medium were combined and then subjected to preparative TLC with MeOH:EtOAc (1:3) and HPLC with a Puresil C18 (Waters) column using MeOH:H_2O (1:3, v/v) for separation of hydroxybenzyl alcohol derivatives and acetonitrile:H_2O (1:4, v/v) for that of hydroxybenzoic acid derivatives to give corresponding glucoside, vicianoside, and primeveroside.



Scheme 1.

However, no further conversion products were observed in spite of careful HPLC analyses (Scheme 1).

Small amounts of the disaccharides were taken to the vial containing 4.0 M HCl (0.1 ml) and heated to 80 °C for 2 h and then cooled. After removal of the solvent in a stream of N_2, the residue was converted to a pentafluoropropionate with pentafluoropropionic anhydride (0.4 ml) in CH_2Cl_2 (0.4 ml) in a sealed tube at 120 °C for 2 h. Excess reagents were removed under a stream of N_2 and the derivatives were analyzed by chiral GC on CP cyclodextrin β 236M-19. Peaks of the derivatives from the disaccharides were assigned to those of L-arabinose and D-glucose for the vicianoside and those of D-xylose and D-glucose for the primeveroside.

The structures of the products were determined on the basis of their FABMS, ^1H, ^{13}C NMR, H–H COSY, C–H COSY, and HMBC spectra. ^{13}C NMR spectral data of glycosylation products were shown in Tables 1

Table 1
^{13}C Chemical shifts of the products (**1b**–**e**, **2b**–**e**, **3b** and **c**) in the biotransformation of hydroxybenzyl alcohol by the cultured cells of *C. roseus* (CD_3OD)

Position	**1b**	**1c**	**1d**	**1e**	**2b**	**2c**	**2d**	**2e**	**3b**	**3c**
C-1	124.8	125.9	125.9	126.0	145.2	141.5	141.1	141.6	120.2	120.2
C-2	158.3	157.3	157.4	157.4	117.0	117.0	119.0	117.0	130.2	131.9
C-3	118.2	117.1	117.2	117.1	160.0	159.5	159.1	159.4	118.5	116.8
C-4	131.0	130.9	131.0	130.9	117.4	121.3	121.0	121.3	159.3	159.1
C-5	124.8	121.3	121.4	121.4	131.2	131.3	130.9	131.3	118.5	116.8
C-6	131.0	131.8	132.0	131.9	122.6	116.6	116.7	116.5	130.2	131.9
C-7	63.7	68.6	68.8	67.5	65.8	72.5	72.5	72.7	65.6	72.5
C-1′	104.5	104.3	104.3	104.4	103.2	104.0	103.9	104.3	103.2	103.7
C-2′	76.2	75.9	75.8	75.9	75.4	76.0	75.7	76.1	75.7	75.9
C-3′	79.2	78.8	78.7	78.8	79.0	78.9	78.6	78.9	78.9	78.9
C-4′	72.5	72.4	72.3	72.5	72.2	72.5	72.2	72.8	72.2	72.5
C-5′	79.2	78.8	77.9	77.9	78.8	78.8	77.7	78.0	78.8	78.8
C-6′	62.1	63.5	70.6	70.3	63.4	63.6	70.4	70.5	63.3	63.6
C-1″			106.3	105.9			106.2	106.2		
C-2″			75.7	73.2			75.5	73.4		
C-3″			78.4	75.0			78.4	75.2		
C-4″			72.0	70.3			71.8	70.5		
C-5″			67.7	68.8			67.6	67.7		

and 2. Retention times for the products in the HPLC were as follows (min): **1b**, 7.9; **1c**, 11.5; **1d**, 13.1; **1e**, 12.1; **2b**, 7.7; **2c**, 11.3; **2d**, 12.9; **2e**, 11.9; **3b**, 3.9; **3c**, 11.8; **5b**, 2.2; **5c**, 2.8; **6b**, 1.9; **6c**, 2.5; **7a**, 3.7; **7b**, 1.9.

2-Hydroxymethylphenyl β-D-glucopyranoside (**1b**): 5 mg; FABMS *m/z* 309 $[M + Na]^+$; ^{1}H NMR (CD_3OD) δ 7.20 (dd, 1H, J = 7.3, 2.0 Hz, H-3), 7.25 (td, 1H, J = 7.3, 2.0 Hz, H-4), 7.02 (td, 1H, J = 7.3, 2.0 Hz, H-5), 7.32 (dd, 1H, J = 7.3, 2.0 Hz, H-6), 4.86 (d, 1H, J = 12.2 Hz, H-7a), 4.77 (d, 1H, J = 12.2 Hz, H-7b), 4.56 (d, 1H, J = 7.3 Hz, H-1′), 3.29–3.53 (m, 4H, H-2′,3′,4′,5′), 3.69 (dd, 1H, J = 12.1, 5.1 Hz, H-6a′), 3.87 (dd, 1H, J = 12.1, 1.6 Hz, H-6b′). The structure was confirmed by direct comparison of ^{1}H and ^{13}C NMR and FABMS spectral data with those of authentic sample (Sigma).

Table 2
^{13}C Chemical shifts of the products (**5b** and **c**, **6b** and **c**, and **7b**) in the biotransformation of hydroxybenzoic acid by the cultured cells of *C. roseus* (CD_3OD)

Position	**5b**	**5c**	**6b**	**6c**	**7b**
C-1	122.8	122.5	127.5	127.4	120.3
C-2	141.3	141.3	133.4	132.8	158.9
C-3	159.3	159.7	117.9	117.4	118.5
C-4	118.2	121.1	163.3	163.4	125.1
C-5	116.4	116.8	117.9	117.4	151.8
C-6	131.4	131.1	133.4	132.8	120.4
C-7	167.8	166.9	167.5	167.2	177.1
C-1′	104.0	104.0	102.5	102.5	104.5
C-2′	75.0	74.9	75.6	75.7	75.8
C-3′	79.7	79.7	79.1	79.0	78.8
C-4′	72.5	72.4	72.1	72.1	72.2
C-5′	78.9	78.8	78.8	78.8	78.8
C-6′	63.6	63.1	63.3	63.2	63.3

2-Hydroxybenzyl β-D-glucopyranoside (**1c**): 28 mg; FABMS *m/z* 309 $[M + Na]^+$; ^{1}H NMR (CD_3OD) δ 6.76 (d, 1H, J = 7.8 Hz, H-3), 7.10 (t, 1H, J = 7.6 Hz, H-4), 6.79 (t, 1H, J = 7.6 Hz, H-5), 7.31 (d, 1H, J = 7.3Hz, H-6), 4.91 (d, 1H, J = 12.2 Hz, H-7a), 4.71 (d, 1H, J = 12.2 Hz, H-7b), 4.38 (d, 1H, J = 7.3 Hz, H-1′), 3.23–3.38 (m, 4H, H-2′,3′,4′,5′), 3.67 (dd, 1H, J = 12.2, 5.1 Hz, H-6a′), 3.87 (dd, 1H, J = 11.9, 1.6 Hz, H-6b′). The structure was confirmed by direct comparison of ^{1}H and ^{13}C NMR and FABMS spectral data with those of authentic sample (Sigma).

2-Hydroxybenzyl 6-*O*-(β-D-xylopyranosyl)-β-D-glucopyranoside (**1d**): 8 mg; FABMS *m/z* 441 $[M + Na]^+$; ^{1}H NMR (CD_3OD) δ 6.74 (d, 1H, J = 7.4 Hz, H-3), 7.08 (t, 1H, J = 7.4 Hz, H-4), 6.77 (t, 1H, J = 7.4 Hz, H-5), 7.29 (d, 1H, J = 7.4 Hz, H-6), 4.86 (d, 1H, J = 12.0 Hz, H-7a),

4.69 (d, 1H, J = 12.0 Hz, H-7b), 4.36 (d, 1H, J = 7.5 Hz, H-1′), 3.18 (dd, 1H, J = 9.0, 7.5 Hz, H-2′), 3.30 (dd, 1H, J = 9.0, 8.5 Hz, H-3′), 3.31 (t, 1H, J = 9.0 Hz, H-4′), 3.41–3.45 (m, 1H, H-5′), 4.08 (dd, 1H, J = 11.0, 2.0 Hz, H-6a′), 3.72 (dd, 1H, J = 11.0, 5.5 Hz, H-6b′), 4.32 (d, 1H, J = 7.5 Hz, H-1″), 3.18 (dd, 1H, J = 9.0, 7.5 Hz, H-2″), 3.43 (t, 1H, J = 9.0 Hz, H-3″), 3.43–3.47 (m, 1H, H-4″), 3.82 (dd, 1H, J = 11.0, 5.5 Hz, H-5a′), 3.72 (t, 1H, J = 11.0 Hz, H-5b′).

2-Hydroxybenzyl 6-*O*-(α-L-arabinopyranosyl)-β-D-glucopyranoside (**1e**): 6 mg; FABMS *m/z* 441 $[M + Na]^+$; ^{1}H NMR (CD_3OD) δ 6.78 (dd, 1H, J = 7.8, 1.3 Hz, H-3), 7.11 (td, 1H, J = 7.8, 1.3 Hz, H-4), 6.81 (td, 1H, J = 7.8, 1.3 Hz, H-5), 7.33 (dd, 1H, J = 7.8, 1.3 Hz, H-6), 4.89 (d, 1H, J = 12.0 Hz, H-7a), 4.73 (d, 1H, J = 12.0 Hz, H-7b), 4.40 (d, 1H, J = 8.0 Hz, H-1′), 3.24 (dd, 1H, J = 9.0, 8.0 Hz, H-2′), 3.35 (t, 1H, J = 9.0 Hz, H-3′), 3.28–3.32 (m, 1H, H-4′), 3.46–3.49 (m, 1H, H-5′), 4.12 (dd, 1H, J = 11.5, 2.5 Hz, H-6a′), 3.75 (dd, 1H, J = 11.5, 6.0 Hz, H-6b′), 4.35 (d, 1H, J = 6.8 Hz, H-1″), 3.60 (dd, 1H, J = 8.8, 6.8 Hz, H-2″), 3.53 (dd, 1H, J = 8.8, 3.5 Hz, H-3″), 3.78–3.80 (m, 1H, H-4″), 3.86 (dd, 1H, J = 12.4, 3.3 Hz, H-5a′), 3.55 (t, 1H, J = 12.4, 2.0 Hz, H-5b′).

3-Hydroxymethylphenyl β-D-glucopyranoside (**2b**): 16 mg; FABMS *m/z* 309 $[M + Na]^+$; ^{1}H NMR (CD_3OD) δ 7.06 (t, 1H, J = 2.0 Hz, H-2), 6.94–6.97 (m, 1H, H-4), 7.20 (t, 1H, J = 7.8 Hz, H-5), 6.94–6.97 (m, 1H, H-6), 4.53 (s, 1H, H-7), 4.86 (d, 1H, J = 7.5 Hz, H-1′), 3.37 (dd, 1H, J = 9.0, 7.5 Hz, H-2′), 3.33–3.41 (m, 3H, H-3′,4′,5′), 3.86 (dd, 1H, J = 11.7, 2.0 Hz, H-6a′), 3.65 (dd, 1H, J = 11.7, 5.1 Hz, H-6b′).

3-Hydroxybenzyl β-D-glucopyranoside (**2c**): 35 mg; FABMS *m/z* 309 $[M + Na]^+$; ^{1}H NMR (CD_3OD) δ 6.81 (m, 1H, H-2), 6.83 (dd, 1H, J = 8.4, 1.4 Hz, H-4), 7.09 (t, 1H, J = 8.4 Hz, H-5), 6.65 (m, 1H, H-6), 4.81 (d, 1H, J = 11.5 Hz, H-7a), 4.55 (d, 1H, J = 11.5 Hz, H-7b), 4.30 (d, 1H, J = 7.7 Hz, H-1′), 3.19 (dd, 1H, J = 9.6, 7.7 Hz, H-2′), 3.23 (t, 1H, J = 9.6 Hz, H-3′), 3.25–3.31 (m, 2H, H-4′,5′), 3.85 (dd, 1H, J = 11.8, 2.0 Hz, H-6a′), 3.64 (dd, 1H, J = 11.8, 5.1 Hz, H-6b′).

3-Hydroxybenzyl 6-*O*-(β-D-xylopyranosyl)-β-D-glucopyranoside (**2d**): 10 mg; FABMS *m/z* 441 $[M + Na]^+$; ^{1}H NMR (CD_3OD) δ 6.82–6.84 (m, 2H, H-2,4), 7.09 (t, 1H, J = 7.8 Hz, H-5), 6.65 (dt, 1H, J = 7.8, 2.5 Hz, H-6), 4.79 (d, 1H, J = 11.5 Hz, H-7a), 4.54 (d, 1H, J = 11.5 Hz, H-7b), 4.31 (d, 1H, J = 7.5 Hz, H-1′), 3.20 (dd, 1H, J = 9.5, 7.5 Hz, H-2′), 3.26 (t, 1H, J = 9.5 Hz, H-3′), 3.28–3.47 (m, 2H, H-4′,5′), 4.07 (dd, 1H, J = 12.0, 2.0 Hz, H-6a′), 3.71 (dd, 1H, J = 12.0, 6.0 Hz, H-6b′), 4.30 (d, 1H, J = 8.0 Hz, H-1″), 3.17 (dd, 1H, J = 9.5, 8.0 Hz, H-2″), 3.42 (t, 1H, J = 9.5 Hz, H-3″), 3.48–3.51 (m, 1H, H-4″), 3.82 (dd, 1H, J = 11.5, 5.5 Hz, H-5a′), 3.15 (dd, 1H, J = 11.5, 10.8 Hz, H-5b′).

3-Hydroxybenzyl 6-*O*-(α-L-arabinopyranosyl)-β-D-glucopyranoside (**2e**): 7 mg; FABMS *m/z* 441 $[M + Na]^+$; ^{1}H NMR (CD_3OD) δ 6.82–6.83 (m, 2H, H-3,4), 7.09 (t, 1H, J = 8.0 Hz, H-5), 6.65 (dt, 1H, J = 8.0, 2.3 Hz, H-6), 4.79 (d, 1H, J = 12.0 Hz, H-7a), 4.55 (d, 1H, J = 12.0 Hz, H-7b), 4.30 (d, 1H, J = 7.5 Hz, H-1′), 3.20 (dd, 1H, J = 9.5, 7.5 Hz, H-2′), 3.29–3.40 (m, 3H, H-3′,4′,5′), 4.07 (dd, 1H, J = 11.5, 2.5 Hz, H-6a′), 3.71 (dd, 1H, J = 11.5, 6.0 Hz, H-6b′), 4.30 (d, 1H, J = 7.0 Hz, H-1″), 3.55 (dd, 1H, J = 9.0, 7.0 Hz, H-2″), 3.46 (dd, 1H, J = 8.8, 3.3 Hz, H-3″), 3.74–3.76 (m, 1H, H-4″), 3.82 (dd, 1H, J = 12.3, 3.3 Hz, H-5a′), 3.48–3.51 (m, 1H, H-5b′).

4-Hydroxymethylphenyl β-D-glucopyranoside (**3b**): 5 mg; FABMS *m/z* 309 $[M + Na]^+$; ^{1}H NMR (CD_3OD) δ 7.03 (d, 2H, J = 8.8 Hz, H-2,6), 7.23 (d, 2H, J = 8.8 Hz, H-3,5), 4.49 (s, 2H, H_2-7), 4.71 (d, 1H, J = 7.3 Hz, H-1′), 3.39 (dd, 1H, J = 8.6, 7.3 Hz, H-2′), 3.35–3.42 (m, 3H, H-3′,4′,5′), 3.84 (dd, 1H, J = 12.0, 1.8 Hz, H-6a′), 3.65 (dd, 1H, J = 12.0, 4.2 Hz, H-6b′).

4-Hydroxybenzyl β-D-glucopyranoside (**3c**): 13 mg; FABMS *m/z* 309 $[M + Na]^+$; ^{1}H NMR (CD_3OD) δ 6.70 (d, 2H, J = 8.6 Hz, H-2,6), 7.19 (d, 2H, J = 8.6 Hz, H-3,5), 4.77 (d, 1H, J = 11.2 Hz, H-7a), 4.51 (d, 1H, J = 11.2 Hz, H-7b), 4.27 (d, 1H, J = 7.6 Hz, H-1′), 3.16 (dd, 1H, J = 9.2, 7.6 Hz, H-2′), 3.23 (t, 1H, J = 9.2 Hz, H-3′), 3.24–3.30 (m, 2H, H-4′,5′), 3.85 (dd, 1H, J = 12.0, 1.8 Hz, H-6a′), 3.64 (dd, 1H, J = 12.0, 5.5 Hz, H-6b′).

3-Carboxyphenyl β-D-glucopyranoside (**5b**): 15 mg; FABMS *m/z* 299 $[M - H]^-$; ^{1}H NMR (CD_3OD) δ 6.88 (s, 1H, H-2), 7.03 (dd, 1H, J = 8.0, 2.0 Hz, H-4), 7.29 (t, 1H, J = 8.0 Hz, H-5), 7.55 (dd, 1H, J = 8.0, 2.0 Hz, H-6), 4.33 (d, 1H, J = 7.5 Hz, H-1′), 3.36–3.50 (m, 4H, H-2′,3′,4′,5′), 3.85 (dd, 1H,

J = 11.9, 2.2 Hz, H-6a′), 3.69 (dd, 1H, J = 11.9, 5.7 Hz, H-6b′).

3-Hydroxybenzoic acid β-D-glucopyranosyl ester (**5c**): 12 mg; FABMS *m/z* 299 $[M - H]^-$; ^{1}H NMR (CD_3OD) δ 6.85 (s, 1H, H-2), 6.69 (dd, 1H, J = 8.0, 2.0 Hz, H-4), 7.13 (t, 1H, J = 8.0 Hz, H-5), 7.47 (dd, 1H, J = 8.0, 2.0 Hz, H-6), 4.89 (d, 1H, J = 7.5 Hz, H-1′), 3.36–3.49 (m, 4H, H-2′,3′,4′,5′), 3.88 (dd, 1H, J = 12.0, 2.0 Hz, H-6a′), 3.69 (dd, 1H, J = 12.0, 4.5 Hz, H-6b′).

4-Carboxyphenyl β-D-glucopyranoside (**6b**): 7 mg; FABMS *m/z* 299 $[M-H]^-$; ^{1}H NMR (CD_3OD) δ 7.92 (d, 2H, J = 8.9 Hz, H-2,6), 7.08 (d, 2H, J = 8.9 Hz, H-3,5), 4.81 (d, 1H, J = 7.5 Hz, H-1′), 3.39–3.47 (m, 4H, H-2′,3′,4′,5′), 3.83 (dd, 1H, J = 11.9, 2.2 Hz, H-6a′), 3.63 (dd, 1H, J = 11.9, 5.7 Hz, H-6b′); ^{13}C NMR (CD_3OD) (see Table 2).

4-Hydroxybenzoic acid β-D-glucopyranosyl ester (**6c**): 5 mg; FABMS *m/z* 299 $[M - H]^-$; ^{1}H NMR (CD_3OD) δ 7.92 (d, 2H, J = 8.9 Hz, H-2,6), 7.08 (d, 2H, J = 8.9 Hz, H-3,5), 4.96 (d, 1H, J = 7.6 Hz, H-1′), 3.36–3.49 (m, 4H, H-2′,3′,4′,5′), 3.86 (dd, 1H, J = 12.0, 2.0 Hz, H-6a′), 3.67 (dd, 1H, J = 12.0, 5.1 Hz, H-6b′).

2,5-Dihydroxybenzoic acid (**7a**): 18 mg; FABMS *m/z* 153 $[M-H]^-$; ^{1}H NMR (CD_3OD) δ 6.62 (d, 1H, J = 8.4 Hz, H-3), 6.76 (dd, 1H, J = 8.4, 2.0 Hz, H-4), 7.26 (d, 1H, J = 2.4 Hz, H-6); ^{13}C NMR (CD_3OD) δ 122.7 (C-1), 156.5 (C-2), 118.4 (C-3), 122.7 (C-4), 150.7 (C-5), 117.8 (C-6), 178.2 (C-7).

5-*O*-(β-D-glucopyranosyl)-2,5-dihydroxybenzoic acid (**7b**): 70 mg; FABMS *m/z* 315 $[M - H]^-$; ^{1}H NMR (CD_3OD) δ 6.70 (d, 1H, J = 9.0 Hz, H-3), 7.08 (dd, 1H, J = 9.0, 3.5 Hz, H-4), 7.57 (d, 1H, J = 3.5 Hz, H-6), 4.72 (d, 1H, J = 7.5 Hz, H-1′), 3.38 (dd, 1H, J = 9.0, 7.5 Hz, H-2′), 3.39 (t, 1H, J = 9.0 Hz, H-3′), 3.30–3.41 (m, 2H, H-4′,5′), 3.84 (d, 1H, J = 12.0 Hz, H-6a′), 3.66 (dd, 1H, J = 12.0, 4.5 Hz, H-6b′).

*2.4. Time course experiments in the biotransformation of salicylic acid (**4a**)*

Each 50 g of the suspension cells of *C. roseus* was portioned to five flasks containing 100 ml of the SH medium. Salicylic acid (**4a**) (21 mg) and glucose (1.3 g) were administered to the flasks and the mixtures were incubated on a rotary shaker (75 rpm) at 25 °C. At a regular time interval, the cells and medium were separated by filtration, and then extracted with MeOH. The products were extracted and purified in the same procedure described above. The amounts of the products (**7a** and **7b**) were calculated on the basis of the peak area of their HPLC analyses with Puresil C18 column using MeOH:H_2O (1:3, v/v).

3. Results and discussion

3.1. Glycosylation of hydroxybenzyl alcohols

Callus tissues induced from the leaves of *C. roseus* were used in this study. After administration of 2-hydroxybenzyl alcohol (**1a**) to the cultured cells of *C. roseus*, products **1b–e** were isolated by a combination of preparative TLC and HPLC and the yields of the products are shown in Table 3. Products **1b** and **c** were identified to *o*-hydroxymethylphenyl β-D-glucopyranoside and *o*-hydroxybenzyl β-D-glucopyranoside, respectively. On the other hand, the structure of **1d** and **e** were determined to be disaccharides, of which the sugar moieties were attached to the benzylic hydroxyl group, *o*-hydroxybenzyl 6-*O*-(β-D-xylopyranosyl)-β-D-glucopyranoside (primeveroside) and

Table 3
Biotransformation of the phenolic compounds by the cultured cells of *C. roseus*

Substrate	Product	Conversion (%)
1a	**1b**	3
	1c	19
	1d	5
	1e	4
2a	**2b**	11
	2c	23
	2d	7
	2e	5
3a	**3b**	3
	3c	9
4a	**7a**	12
	7b	47
5a	**5b**	10
	5c	8
6a	**6b**	5
	6c	3

o-hydroxybenzyl 6-*O*-(α-L-arabinopyranosyl)-β-D-glucopyranoside (vicianoside), respectively. No disaccharides, which were linked the sugar moiety to the phenolic hydroxyl group, were obtained. Compared with the glucosylation of benzylic hydroxyl group and phenolic hydroxyl groups, that of phenolic hydroxyl group was lower.

On administration of 3-hydroxybenzyl alcohol (**2a**), *m*-hydroxymethylphenyl β-D-glucopyranoside (**2b**), *m*-hydroxybenzyl β-D-glucopyranoside (**2c**), *m*-hydroxybenzyl 6-*O*-(β-D-xylopyranosyl)-β-D-glucopyranoside (**2d**) and *m*-hydroxybenzyl 6-*O*-(α-L-arabinopyranosyl)-β-D-glucopyranoside (**2e**) were isolated. The glucosylation of phenolic hydroxyl group was under a half of that of benzylic hydroxyl group.

In case of 4-hydroxybenzyl alcohol (**3a**), *p*-hydroxymethylphenyl β-D-glucopyranoside (**3b**) and *p*-hydroxybenzyl β-D-glucopyranoside (**3c**) were obtained. No disacchrides such as primeveroside and vicianoside of **3a** were obtained.

Thus, the cultured cells of *C. roseus* were found to convert hydroxybenzyl alcohols into their glucosides, primeverosides and vicianosides. The conversion into primeveroside and vicianoside was regioselective; the sugar moieties of them were attached to only benzylic hydroxyl group. However, in the case of 4-hydroxybenzyl alcohol, no disaccharide formation was observed. The efficiency of glucosylation tended to decrease in the order of benzylic hydroxyl group and phenolic hydroxyl group. As regards the substrate specificity of glucosylation, the cultured cells of *C. roseus* glucosylated the 3-hydroxyl derivative more than the 2- and 4-hydroxyl derivatives.

3.2. Biotransformation of hydroxybenzoic acids

Administration of *o*-hydroxybenzoic acid (salicylic acid) (**4a**) to the *C. roseus* cells resulted in the formation of compounds **7a** and **b**. The negative FABMS spectrum of **7a** showed a peak at *m/*z 153 ($[M-H]^-$). The ^{1}H, ^{13}C and COSY NMR spectra of **7a** interpreted the compound **7a** to be 2,5-dihydroxybenzoic acid (gentisic acid). On the other hand, the negative FABMS spectrum of **7b** showed a peak at *m/z* 315 ($[M-H]^-$). The ^{1}H and ^{13}C NMR analyses of **7b** revealed the product **7b** to be a β-D-glucopyranoside of **7a**. The H-1′ signal at δ 4.72 showed an HMBC correlation to C-5 signal at δ 151.8, identifying **7b** as 5-*O*-(β-D-glucopyranosyl)-2,5-dihydroxybenzoic acid.

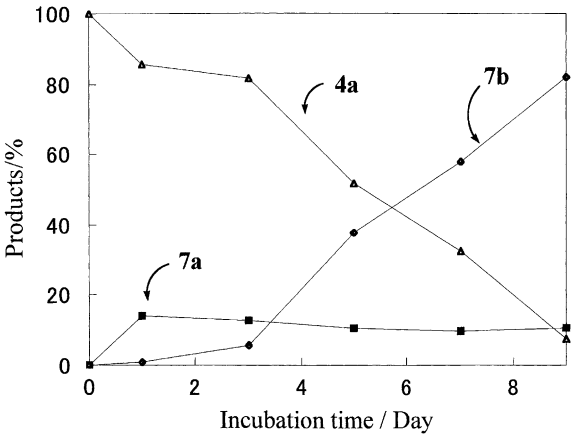


Fig. 1. Time course in the biotransformation of *o*-hydroxybenzoic acid (**4a**).

To investigate the biotransformation pathway of **7b**, the time course in the biotransformation of **4a** was followed. As shown in Fig. 1, **4a** was converted into **7a** at 1 day's incubation, whereas the glucoside **7b** was predominantly accumulated in the cells after 3 days' incubation with the decrease of the amount of **7a**. This result suggested that the product **7a** was first formed by hydroxylation at C-5 of **4a** and then glucosylation of the newly introduced hydroxyl group was occurred to give the glucoside **7b**.

On the other hand, incubation of 3-hydroxybenzoic acid (**5a**) afforded both of 3-carboxylphenyl β-D-glucopyranoside (**5b**) and 3-hydroxybenzoic acid β-D-glucopyranosyl ester (**5c**). On administration of 4-hydroxybenzoic acid (**6a**), 4-carboxylphenyl β-D-glucopyranoside (**6b**) and 4-hydroxybenzoic acid β-D-glucopyranosyl ester (**6c**) were obtained.

Thus, the cultured cells of *C. roseus* were found to be able to glucosylate both of hydroxyl and carboxyl groups of 3- and 4-hydroxybenzoic acids. On the other hand, the cultured cells hydroxylated the 5-position of 2-hydroxybenzoic acid (salicylic acid) to produce gentisic acid which is, in general, known to be biosynthesized from 3-hydroxybenzoic acid [20]. The observation that only salicylic acid, but no 3-hydroxybenzoic acid, was hydroxylated to afford gentisic acid may suggest the existence of biosynthetic

pathway of gentisic acid from salicylic acid in *C. roseus* cells. Characterization of enzymes which catalyze the hydroxylation and glucosylation of phenolic compounds is now in progress.

Acknowledgements

This work was in part supported by a Grant for Scientific Research from the Nagase and Technology Foundation of Japan.

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