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# GLUCOSYLATION OF PHENOLIC COMPOUNDS BY PLANT CELL CULTURES

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**Key Word Index**—Glucosylation; biotransformation; plant cell culture; simple phenol; coumarin; flavonoid; anthraquinone.

**Abstract**—Ten culture strains derived from seven species of plants were examined for their ability to glucosylate exogenous phenolic compounds including four simple phenols, four coumarins, three flavonoids and three anthraquinones. Suspension cultures of *Datura*, *Lithospermum*, *Perilla* and *Catharanthus* were capable of glucosylating *m*-hydroxybenzoic acid, umbelliferone, esculetin, daphnetin, and liquiritigenin. In particular, *Datura* cultures glucosylated *ca* 90% of umbelliferone within 24 hr after administration. Only *Perilla* cultures were capable of glucosylating rhein and emodin. The ability of glucosylating isomeric hydroxybenzoic acids varied widely with culture strains. A culture strain of *Mallotus* transformed *p*-hydroxybenzoic acid to *p*-*O*-*β*-D-glucosylbenzoic acid and *p*-hydroxybenzoic acid glucose ester, whereas only *o*-*O*-*β*-D-glucosylbenzoic acid was formed from *o*-hydroxybenzoic acid.

## INTRODUCTION

Tissues of intact plants are able to convert exogenous phenolic compounds into their corresponding glucosides [1–8]. Similarly, several workers have reported that plant cell cultures are also capable of glucosylating such substrates as phenols [9–14], steroids [15–17], terpenoids [15], and cardenolides [15, 18, 19]. Thus, cultured cells may be utilized for obtaining glucosides, since a position-specific glucosylation would be expected from an enzymatic reaction. In addition, the one-step glucosylation by biotransformation may be more efficient than a two-step chemical synthesis consisting of glucosylation and deacetylation. However, the successful glucosylation of an exogenous substrate by the former requires that the recipient cells possess a strong glucosyltransferase activity capable of introducing a glucose molecule to a specific position of the substrate without giving any by-products. Although such a function may vary with culture strains as well as chemical structures of substrates, no detailed studies have been made concerning these basic problems.

This paper now reports the results of comparative studies on specific capabilities of plant cell cultures to glucosylate phenolic compounds.

## RESULTS

Chemical examination confirmed that culture strains used in the present study lacked the compounds used for biotransformation experiments and their glucosides, except for the cultured cells of *Lithospermum erythrorhizon* (LE), *Datura innoxia* (DI) and *Mallotus japonicus* (MJ-W) which accumulated small amounts of *p*-hydroxybenzoic acid and its *O*-glucoside [20]. Each test compound (5 μmol/tube) was administered aseptically to various cell cultures at the early stationary growth stage.

### *Glucosylation of coumarins*

Four hydroxycoumarin derivatives, i.e. umbelliferone (7-hydroxycoumarin), esculetin (6,7-dihydroxycoumarin), daphnetin (7,8-dihydroxycoumarin) and fraxetin (7,8-dihydroxy-6-methoxycoumarin), were used as substrates. Umbelliferone was most efficiently converted into its glucoside by DI (90% in 24 hr). *Perilla frutescens* (PF-R, PF-O), *Catharanthus roseus* (CR) and LE also gave a good yield (46 to 75%) of the glucoside (Table 1). As regards esculetin, DI and CR yielded esculin (7-hydroxycoumarin-6-*O*-glucoside) and cichoriin (6-hydroxycoumarin-7-*O*-glucoside), whereas LE gave only the former. DI and CR were also superior to the other cultures in glucosylating daphnetin (37 and 32%, respectively). Fraxetin was found to be glucosylated more efficiently by DI and *Gardenia jasminoides* (GJ-B) (30 and 24%, respectively).

In general, culture strains capable of glucosylating umbelliferone also glucosylated esculetin and daphnetin. The conversion rate for the coumarins tended to decrease with an increasing number of substituted groups in chemical structures in cell lines DI, PF-R, CR, and LE. Exceptionally, however, a few cultures such as GJ-B and MJ-R (*Mallotus japonicus*), which hardly glucosylated umbelliferone, could glucosylate fraxetin at a relatively high rate, suggesting that the substrate specificity varies with cell lines.

### *Glucosylation of flavonoids*

Three flavonoids, liquiritigenin (7, 4′-dihydroxyflavanone), naringenin (5,7,4′-trihydroxyflavanone) and baicalein (5,6,7-trihydroxyflavone) were used as substrates, of which liquiritigenin was more readily glucosylated by 5 strains (DI, PF-R, PF-O, CR, and LE) with

Table 1. Efficiency of glucosylation (%) of coumarins by various cell suspension cultures (see Experimental for culture conditions)

| Culture strain* | Umbelliferone | | Esculetin | | Daphnetin | | Fraxetin | |
|---|---|---|---|---|---|---|---|---|
| DI | 90.0 ± 5.0† | (2.3)‡ | 65.0 ± 1.3 | (4.7) | 36.6 ± 1.7 | (0) | 30.1 ± 0.8 | (0) |
| PF-R | 74.9 ± 1.9 | (0) | 28.8 ± 1.2 | (0) | 13.4 ± 1.2 | (6.4) | 9.8 ± 1.2 | (0) |
| PF-O | 63.0 ± 3.1 | (2.6) | 10.4 ± 0.9 | (1.2) | 11.3 ± 1.6 | (15.5) | 6.9 ± 2.5 | (0) |
| CR | 55.9 ± 2.4 | (2.9) | 43.2 ± 1.4 | (19.6) | 31.6 ± 0.4 | (2.1) | 0 | (0) |
| LE | 46.3 ± 1.1 | (24.2) | 37.3 ± 2.2 | (7.2) | 16.9 ± 1.2 | (0) | n.t.§ | |
| BF | 10.1 ± 1.2 | (46.8) | 3.7 ± 1.2 | (19.4) | 4.6 ± 0.5 | (11.9) | 15.4 ± 1.6 | (22.6) |
| GJ-A | 1.3 ± 0.1 | (46.5) | 15.7 ± 1.5 | (5.9) | 1.5 ± 0.3 | (1.8) | 0 | (15.4) |
| GJ-B | 0.9 ± 0.3 | (4.9) | 9.1 ± 1.5 | (8.0) | 3.7 ± 0.5 | (0) | 23.7 ± 1.3 | (2.8) |
| MJ-R | 0 | (1.6) | 0 | (9.4) | 0 | (36.4) | 12.3 ± 1.6 | (2.8) |
| MJ-W | 0 | (39.0) | 0 | (0.3) | 0 | (10.5) | n.t. | |

*DI = *Datura innoxia*, PF-R and PF-O = *Perilla frutescens* var. *crispa*, CR = *Catharanthus roseus*, LE = *Lithospermum erythrorhizon*, BF = *Bupleurum falcatum*, GJ-A and GJ-B = *Gardenia jasminoides* forma *grandiflora*, MJ-R and MJ-W = *Mallotus japonicus*.

†Mean ± s.e. (7 replicates).

‡Values in parentheses indicate the percentages of total residual aglycones in medium and cells.

§Not tested.

Table 2. Efficiency of glucosylation (%) of flavonoids

| Culture strain | Liquiritigenin | | Naringenin | | Baicalein | |
|---|---|---|---|---|---|---|
| DI | 23.5 ± 2.7* | (4.5)† | 0 | (24.9) | 0 | (0) |
| PF-R | 17.0 ± 2.1 | (4.6) | 6.0 ± 0.9 | (0) | 0 | (0) |
| PF-O | 23.0 ± 1.4 | (1.0) | 3.2 ± 1.2 | (0.8) | 0 | (0.3) |
| CR | 34.1 ± 2.1 | (3.8) | 0 | (21.9) | 0 | (0) |
| LE | 24.9 ± 1.4 | (26.9) | 0 | (0) | n.t.‡ | |
| BF | 2.0 ± 0.4 | (n.t.) | 1.3 ± 0.1 | (0.2) | 0 | (0) |
| GJ-A | 0 | (14.7) | 0 | (14.9) | 0 | (0) |
| GJ-B | 5.8 ± 0.3 | (14.7) | 0 | (10.7) | 0 | (0) |
| MJ-R | 0 | (0.7) | 0 | (10.0) | 0 | (0) |
| MJ-W | 0 | (n.t.) | 0 | (13.5) | 0 | (0) |

*Mean ± s.e. (7 replicates).

†Values in parentheses indicate the percentages of total residual aglycones in medium and cells.

‡Not tested.

yields of 17 to 34% (Table 2). Naringenin with an extra hydroxy group at C-5 was glucosylated only by PF-R, PF-O and *Bupleurum falcatum* (BF), though the yields were low (1.3–6.0%). Furthermore, baicalein with three neighboring hydroxy groups in the A ring was never glucosylated by any culture strain, probably owing to stereochemical hindrance.

*Glucosylation of anthraquinones*

Three anthraquinones, alizarin (1,2-dihydroxyanthraquinone), rhein (1,8-dihydroxyanthraquinone-3-carboxylic acid) and emodin (1,3,8-trihydroxy-6-methylanthraquinone) were administered to various cultures. As shown in Table 3, five strains (PF-R, PF-O, CR, LE, and GJ-B) glucosylated alizarin, though the conversion rates were generally low (2–12%). Only PF-R was capable of glucosylating all the three anthraquinones, whereas the rest of strains failed to glucosylate both rhein and emodin. The glucosylated rhein isolated from the PF-R cultures behaved similarly to authentic rhein-8-*O*-glucoside on both TLC and HPLC, and it yielded rhein on both acidic hydrolysis and enzymic hydrolysis with almond β-glucosidase.

*Glucosylation of simple phenols*

Salicyl alcohol (*o*-hydroxybenzylalcohol) and three isomers of hydroxybenzoic acids (HBA) were used as substrates. As shown in Table 4, salicyl alcohol was glucosylated only by DI and PF-R. On the other hand, both *m*- and *p*-HBA were glucosylated by all the culture strains tested except for BF that failed to glucosylate *m*-HBA. The glucosylation ratio of *m*-HBA to *p*-HBA was nearly 1 : 1 in 5 out of 9 culture strains, while PF-R, CR and GJ-A glucosylated more *m*-HBA than *p*-HBA. Glucosylation of *o*-HBA was observed only in three cultures, PF-R, CR, and MJ-W (7–61%). It is of special interest that MJ-W, though incapable of glucosylating any coumarins, flavonoids or anthraquinones, glucosylated *o*-, *m*-, and *p*-HBA at a conversion rate of 61, 30, and 25% in 24 hr, respectively. Analyses have shown that MJ-W trans-

Table 3. Efficiency of glucosylation (%) of anthraquinones

| Culture strain | Alizarin | | Rhein | | Emodin | |
|---|---|---|---|---|---|---|
| DI | 0 | (53.3) | 0 | (1.4)* | 0 | (0.9) |
| PF-R | 19.0±1.9 | (37.9) | 11.6±0.9† | (0) | 5.7±1.1 | (9.6) |
| PF-O | 0 | (35.8) | 4.8±0.4 | (0.3) | 0 | (6.3) |
| CR | 0 | (70.0) | 5.2±0.2 | (2.7) | 0 | (5.1) |
| LE | 0 | (70.4) | 11.6±1.1 | (4.4) | n.t.‡ | |
| BF | 0 | (56.3) | 0 | (0.9) | 0 | (5.3) |
| GJ-A | 0 | (90.4) | 0 | (6.4) | 0 | (3.2) |
| GJ-B | 0 | (48.3) | 2.2±0.2 | (4.6) | 0 | (2.0) |
| MJ-R | 0 | (50.0) | 0 | (0) | 0 | (1.4) |
| MJ-W | 0 | (51.3) | 0 | (0) | 0 | (1.7) |

*Values in parentheses indicate the percentages of total residual aglycones in medium and cells.

†Mean ± s.e. (7 replicates).

‡Not tested.

formed *p*-HBA into both *p*-*O*-*β*-D-glucosylbenzoic acid and *p*-HBA glucose ester, whereas *o*-HBA was converted exclusively into *o*-*O*-*β*-D-glucosylbenzoic acid.

## DISCUSSION

Pridham [8] was first to demonstrate that hydroquinone and resorcinol administered to the leaves and seedlings of various higher plants were rapidly converted to the corresponding glucosides and that this activity was absent or very weak in Bryophytes and Thallophytes. It has been reported thereafter that cultured cells of higher plants, such as *Datura innoxia* [9], *D. ferox* [10], *Agrostemma githago* [10], *Digitalis purpurea* [10] and *Gardenia jasminoides* [21], also converted hydroquinone to the monoglucoside arbutin. The present experiments have shown that plant cell cultures can glucosylate various foreign phenolics which are not present originally in the cultured cells although the efficiency of glucosylation varies with the culture strain depending on the chemical structure.

The efficiency of glucosylation tended to decrease in the order of coumarins, simple phenols, flavonoids, and anthraquinones. As regards hydroxybenzoic acid, most of the culture strains glucosylated *m*- and *p*-isomers more readily than the *o*-isomer. These results suggest that phenolic compounds can be glucosylated efficiently by cultured cells when the molecules are relatively small in size, and have few substituents adjacent to the hydroxy group. However, there are a few exceptions to this rule as observed in the glucosylation of fraxetin and *o*-HBA by GJ-B and MJ-W, respectively.

The position of glucosylation in the substrate molecules seems to be specific to culture strains; e.g. strains DI and CR converted esculetin into both esculin and its isomer cichoriin, while strain LE formed only esculin [14]. In this connection it has been reported that DI glucosylated salicyl alcohol to form isosalicin [9], whereas *Gardenia* and *Lithospermum* cultures converted it largely into salicin, an isomer of isosalicin [12]. Apparently, the position specificity of glucosylation varies with plant species, but it is not known whether the isomeric glucosides are synthesized by two different position-specific glucosyltransferases [22, 23] or transglucosylases [24] or by the same enzymes.

## EXPERIMENTAL

*General.* Mps: uncorr. $^1H$ and $^{13}C$ NMR were measured at 200 and 50.10 MHz, respectively, and TMS was used as int. standard.

*Plant materials and culture method.* Ten culture strains used in the present experiments were derived from the following plant materials in respective years indicated: DI (*Datura innoxia* Mill, root, 1969), PF-R and PF-O (*Perilla frutescens* Britton var. *crispa* Decne, leaf, 1976), CR (*Catharanthus roseus* G. Don, stem, 1979), LE (*Lithospermum erythrorhizon* Sieb. et Zucc., seedling, 1971), BF (*Bupleurum falcatum* L., leaf, 1979), GJ-A and GJ-B (*Gardenia jasminoides* Ellis forma *grandiflora* Makino, floral bud, 1974 and 1976, respectively), and MJ-R and MJ-W (*Mallotus japonicus* Muell. Arg., seedling, 1970). MJ-R is a variant culture strain isolated from MJ-W and produces anthocyanins in light. PF-R and PF-O originated from different varieties of *P. frutescens* var. *crispa*.

All the cell suspension cultures were agitated in 100 ml conical flasks containing 30 ml of Linsmaier–Skoog medium [25] supplemented with 1 μM 2,4-dichlorophenoxyacetic acid on a reciprocal shaker at a speed of 100 strokes/min at 25° in the dark, and subcultured at intervals of 2 weeks. In glucosylation experiments, cultured cells (0.2–0.5 g) were inoculated into a test tube (18 × 200 mm) containing 7 ml of the above medium and incubated under the same conditions as mentioned above.

*Substrate administration and quantitative analysis.* At the early stationary growth stage (10–14 days after inoculation depending on the culture strain), a substrate (5 μmol/tube) dissolved in 70% EtOH (0.2 ml) was aseptically added to the medium. Twenty-four hr after administration the cells and medium were harvested, homogenized with a Teflon homogenizer, then centrifuged at 1000 × *g* for 15 min. The supernatant was extracted with a double vol. of $Et_2O$, twice. After the $Et_2O$ layer was concd, the residue was subjected to TLC to separate the aglycone. The amount of the aglycone was measured by a TLC-chromatoscanner (CS-900, Shimadzu) or HPLC (Nihon Bunko). The glucoside in the aq. layer (2 ml) was hydrolysed with β-glucosidase (2 mg) for 15 hr at 37°, and the aglycone extracted with $Et_2O$ (4 ml × 2) was quantitatively determined by TLC-chromatoscanner or HPLC to estimate the amount of the glucoside formed. The efficiency of glucosylation was expressed as the percentage of the substrate glucosylated in 24 hr of incubation.

*Chromatographic conditions.* Solvent systems used for TLC (Si gel 60 F-254, Merck) were as follows: toluene–$HCO_2Et$–$HCO_2H$ (5:4:1) for umbelliferone, esculetin, daphnetin, fraxetin, liqui-

Table 4. Efficiency of glucosylation (%) of phenol derivatives

| Culture strain | Salicyl alcohol | | *o*-Hydroxybenzoic acid | | *m*-Hydroxybenzoic acid | | *p*-Hydroxybenzoic acid | |
|---|---|---|---|---|---|---|---|---|
| DI | 19.0±0.3* | (0)† | 0 | (70.4) | 47.7±2.9 | (0) | 43.7±4.5 | (0) |
| PF-R | 8.1±0.3 | (0) | 19.5±2.6 | (43.2) | 32.3±1.8 | (0) | 3.3±0.2 | (0) |
| PF-O | 0 | (0) | 0 | (50.8) | 23.8±3.1 | (0) | 27.1±1.4 | (0) |
| CR | 0 | (10.5) | 6.7±2.4 | (41.1) | 52.3±1.4 | (0) | 4.3±0.2 | (0) |
| LE | n.t.‡ | | 0 | (74.3) | 23.4±1.3 | (0) | n.t. | |
| BF | 0 | (21.1) | 0 | (62.7) | 0 | (0) | 33.9±2.8 | (0) |
| GJ-A | 0 | (9.9) | 0 | (68.2) | 35.0±2.4 | (0) | 18.4±0.9 | (20.5) |
| GJ-B | 0 | (8.1) | 0 | (56.5) | 27.5±2.5 | (0) | 27.3±2.2 | (0.8) |
| MJ-R | 0 | (n.t.) | 0 | (76.5) | 19.7±1.3 | (0) | 18.8±0.9 | (3.8) |
| MJ-W | 0 | (n.t.) | 60.9±9.4 | (30.2) | 30.2±2.0 | (7.7) | 24.5±1.1 | (2.5) |

*Mean±s.e. (7 replicates).
†Values in parentheses indicate the percentages of total residual aglycones in medium and cells.
‡Not tested.

ritigenin, naringenin, alizarin, emodin, *m*- and *p*-hydroxybenzoic acid; $C_6H_6$–$HCO_2Et$–$HCO_2H$ (74:24:1) for rhein; and EtOAc–MeOH–$H_2O$ (100:16.5:13.5) for rhein-8-*O*-glucoside. Each compound was located under UV light (254 nm) by comparison with the standard sample.

Conditions for reversed-phase HPLC were as follows; column: TSK GEL ODS 120A, 10 μm (Toyo Soda), 150 × 4.6 mm, flow rate: 1.0 ml/min, pressure: 70 kg/cm², detection: absorption at 270 nm for rhein-8-*O*-glucoside, 275 nm for baicalein, 285 nm for salicyl alcohol, and 300 nm for *o*-hydroxybenzoic acid, solvent system: $H_2O$–MeOH (30:100) for baicalein, $H_2O$–MeOH–HOAc (150:100:5) for rhein-8-*O*-glucoside and salicyl alcohol, and $H_2O$–MeOH–HOAc (200:50:5) for *o*-hydroxybenzoic acid. Retention time: baicalein, 4.29 min; rhein-8-*O*-glucoside, 13.4 min; salicyl alcohol, 4.26 min; *o*-hydroxybenzoic acid, 28 min.

*Isolation and identification of glucosides.* Glucosylated products of esculetin were identified according to the method described elsewhere [14]. To identify the metabolites of *o*-hydroxybenzoic acid, cultured cells of *M. japonicus* (MJ-W, 743 g fr. wt) were supplied with *o*-hydroxybenzoic acid (2.2 mmol) and extracted with MeOH (3.7 l × 2) 24 hr after administration. The extract was evaporated to dryness *in vacuo* and the residue (15 g) was extracted with MeOH (100 ml × 2). The MeOH-soluble fraction (4.9 g) was chromatographed on a column (Si gel, 1 × 44 cm) fitted with a lab pump using EtOAc (1.5 l) as solvent. The eluate (1.4 g) was subjected to prep. TLC on Si gel GF-254 with the solvent system BuOH–HOAc–$H_2O$ (4:1:5) to isolate *o*-*O*-β-D-glucosylbenzoic acid (**1**) as amorphous powder (32 mg), mp 141–142°. This compound was confirmed to be identical with a synthetic specimen of **1** (mmp, comparisons of UV, IR, $^1$H NMR, $^{13}$C NMR spectra, and TLC). $^1$H NMR (DMSO-$d_6$): δ3.1–3.8 (*m*, 6H, glucosyl), 4.89 (*d*, 1H, anomeric proton, $J$ = 7.5 Hz), 7.10 (*t*, 1H, $J$ = 8 Hz), 7.30 (*d*, 1H, $J$ = 8 Hz), 7.52 (*dt*, 1H, $J_{ortho}$ = 8 Hz, $J_{meta}$ = 1.6 Hz), 7.67 (*dd*, 1H, $J$ = 8 and 1.6 Hz). $^{13}$C NMR (DMSO-$d_6$): δ60.7 (C-6'), 69.7 (C-4'), 73.4 (C-2'), 76.2 (C-3'), 77.3 (C-5'), 101.9 (C-1'), 116.9 (C-3), 122.0 (C-1, C-5), 130.5 (C-6), 133.1 (C-4), 156.4 (C-2), 167.2 (–COO–).

*p*-Hydroxybenzoic acid (1.56 mmol × 4) was added to cell cultures of *M. japonicus* (MJ-W) at intervals of 12 hr to obtain glucosylated products. Twenty-four hr after the final administration, harvested cells (611 g fr. wt) were extracted with MeOH (3.1 l × 2). After evapn, the residue (10 g) was extracted with MeOH (100 ml × 2). The MeOH-soluble fraction (3.4 g) was applied to a Si gel column (1 × 44 cm) fitted with a lab pump and eluted with EtOAc (1.5 l). The eluate (1.2 g) was recrystallized from MeOH to yield *p*-hydroxybenzoic acid glucose ester (**2a**) as colourless prisms (247 mg), mp 228°. This compound was identical in all respects with a synthetic sample of **2a**. $^1$H NMR (DMSO-$d_6$): δ3.1–3.8 (*m*, 6H), 5.54 (*d*, 1H, anomeric proton, $J$ = 7.5 Hz), 6.87 (*d*, 2H, $J$ = 9 Hz), 7.87 (*d*, 2H, $J$ = 9 Hz). $^{13}$C NMR (DMSO-$d_6$): δ60.5 (C-6'), 69.5 (C-4'), 72.5 (C-2'), 76.4 (C-3'), 77.8 (C-5'), 94.5 (C-1'), 115.3 (C-3, C-5), 119.7 (C-1), 131.8 (C-2, C-6), 162.2 (C-4), 164.3 (–COO–).

The combined mother liquid was subjected to prep. TLC on Si gel GF-254 with the solvent system EtOAc–MeOH–$H_2O$ (100:16.5:13.5) for development and eluted with MeOH. This afforded *p*-*O*-β-D-glucosylbenzoic acid (**2b**) as amorphous powder (34 mg), mp 209–210°, which was identified by direct comparison with a synthetic sample of **2b**. $^1$H NMR (DMSO-$d_6$): δ3.1–3.8 (*m*, 6H), 4.99 (*d*, 1H, anomeric proton, $J$ = 7.5 Hz), 7.10 (*d*, 2H, $J$ = 9 Hz), 7.89 (*d*, 2H, $J$ = 9 Hz). $^{13}$C NMR (DMSO-$d_6$): δ60.6 (C-6'), 69.6 (C-4'), 73.1 (C-2'), 76.5 (C-3'), 77.0 (C-5'), 99.8 (C-1'), 115.8 (C-3, C-5), 124.1 (C-1), 131.0 (C-2, C-6), 160.8 (C-4), 166.8 (–COO–).

*Synthesis of* **1**, **2a** *and* **2b**. Compounds **1** and **2b** were prepared using *o*- and *p*-hydroxybenzoic acid methyl esters, respectively, as starting materials by the method of ref. [26].

For the synthesis of **2a**, 1-*O*-acyl-β-D-glucose tetraacetate was prepared by the condensation of 3,4,6-tri-*O*-acetyl-α-D-glucopyranose 1,2-(*t*-butyl orthoacetate) [27] with *p*-hydroxybenzoic acid according to the method of ref. [28]. The acetate obtained was dissolved in MeOH, cooled in icy water, treated with dry $NH_3$ gas for 15 hr for the *O*-deacetylation of sugar, and deionized with Dowex 50W-X4 to give **2a**.

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