Glycosides Having Chromophores as Substrates for Sensitive Enzyme Analysis. I. Synthesis of Phenolindophenyl-β-D-glucopyranosides as Substrates for β-Glucosidase

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Five new glucopyranosides, phenolindophenyl- (2a), phenolindo-3′-chlorophenyl-(2b), phenolindo-3′,5′-dichlorophenyl- (2c), 2,5-dimethylphenolindophenyl- (2d), and phenolindo-3′,5′-dibromophenyl-β-D-glucopyranoside (2e), were synthesized through two routes. Compounds 2c and 2e were synthesized by direct glycosidation (route A) of the Na salt of dihalogenophenolindophenol (4c and 4e). The direction of glycosidation was controlled by the choice of free phenolindophenols or the Na salts. Compounds 2a—d were synthesized *via* the condensation reaction (route B) of 4-aminophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosides (9a—c) with quinones (10 and 11). When 1,4-naphthoquinone (12) was used in this reaction, *N*-(2-naphthoquinonyl)-4-aminophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (13) was the major product. These glucopyranosides (2a—d) were hydrolyzed by β-glucosidase to give a blue product having high absorbance and they were concluded to be potential substrates for the assay of β-glucosidase.

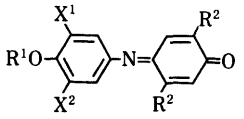
Keywords glucosidase activity; colorimetric assay; amylase; phenolindophenyl-β-D-glucopyranoside; enzyme analysis; 4-[(4-hydroxyphenyl)imino]-2,5-cyclohexadien-1-one

Glycosides bearing chromophores have been very important as substrates for sensitive enzyme analysis of glycosyl hydrolase activities, because the colorimetric assay of hydrolyzed chromophores has the advantages of simplicity of the procedure and excellent reproducibility of the data. In addition, these substrates are also applicable to kinetic studies of the hydrolysis and should be adaptable to automated measuring equipment.

Among the chromophores, 4-nitrophenols have been preferred, since they have considerable absorbance (ε: 16000—17000) and undergo large bathochromic shifts from undissociated (glycosidic) form to dissociated (hydrolyzed) form.[1] Therefore, 4-nitrophenyl glycosides have usually been used in analyses of enzymes such as glucosidase,[2] amylase,[3] *N*-acetyl-glucosaminidase,[4] and sialidase.[5] However, the analyses of the enzymes are subject to interference from colored biological substances (λ_{max}: 400—500 nm, yellow-red) such as bilirubin and hemoglobin.[6] Hence there is a requirement for substrates whose chromophore has absorbance in the longer wavelength region.[7]

In the course of studies on convenient and useful substrates for α-amylase (1,4-α-D-glucan 4-glucanohydrase, EC 3.2.1.1) assay,[8] we synthesized five new β-D-glucopyranosides (**2a—e**) of phenolindophenol (4-[(4-hydroxyphenyl)-imino]-2,5-cyclohexadien-1-one)derivatives (**1a—e**) (Chart 1), which have higher absorbance (ε: 35000—45000)[9] than 4-nitrophenols in the longer wavelength region (λ_{max}: 580—660 nm), through two routes (A and B) as follows.

Route A (Direct Method) Our first attempt at the synthesis of the glucosides (**2**) was the direct introduction of



1 : R^1=H 2 : R^1=β-D-glucopyranosyl

a : X^1=H, X^2=H, R^2=H
b : X^1=Cl, X^2=H, R^2=H
c : X^1=Cl, X^2=Cl, R^2=H
d : X^1=H, X^2=H, R^2=Me
e : X^1=Br, X^2=Br, R^2=H

Chart 1

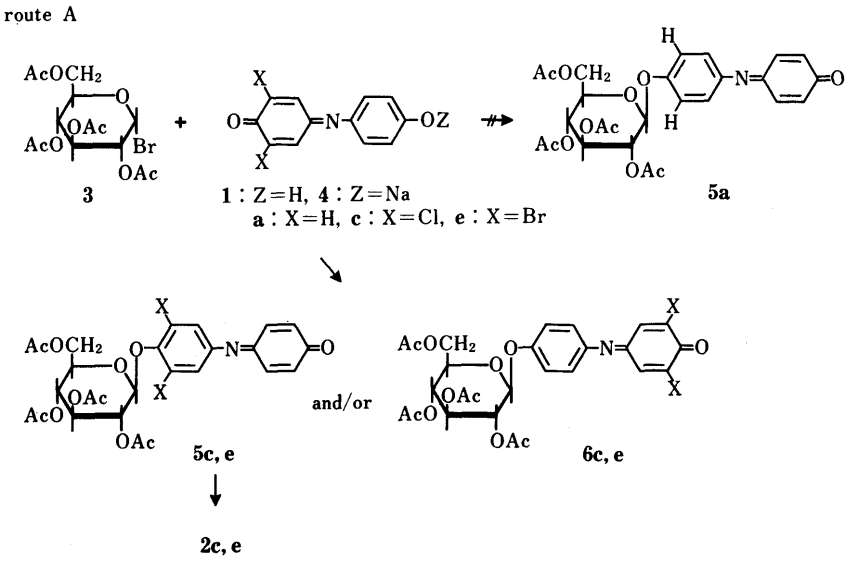


Chart 2

phenolindophenyl groups onto a glycosyl donor. When 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**3**)[10] was added to dihalogenophenolindophenols (**1c**, **1e**, **4c**, and **4e**), dihalogenophenolindophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosides (**5c**, **5e**, **6c**, and **6e**) were obtained. The deacetylation of 3′,5′-dihalogeno derivatives (**5c** and **5e**) gave dihalogenophenolindophenyl-β-D-glucopyranosides (**2c** and **2e**) (Chart 2). However, the reaction of 2,6-dihalogeno derivatives (**6c** and **6e**) gave a complex mixture of products which were difficult to purify. It was assumed that under the reaction conditions used (K_2CO_3 in MeOH), the unstable coplanar α,α′-disubstituted ketone structure in the 2,6-dihalogenophenolindophenyl group failed to give deacetylated compounds. Additionally, we could not obtain non-substituted phenolindophenyl glucosides (**5a**) by the reaction of **1a** and **4a** with **3**.

An interesting direction of glycosidation was found as follows. When a bromosugar (**3**) was allowed to react with the sodium (Na) salt of dihalogenophenolindophenols[11] (**4c** and **4e**), the C-1 position of **3** was attacked by the carbonyl group between the halogens of **4** to give exclusively **5c** and **5e**. On the other hand, when **1c** and **1e** having a free phenol group were used as acceptors in the presence of Ag_2O,[12] the attack occurred from the opposite side (the phenol function) and 2,6-dihalogeno derivatives (**6c** and **6e**) were mainly formed.

In studies of the acylation of phenolindophenols, Gamson *et al.*[13] found a similar direction of attack with or without pyridine as a catalyst. They proposed the following rationalization. Since the acetylation employed the Na salt of phenolindophenol as the starting material, the acetyl pyridinium ion would associate with the oxygen bearing the highest electron density to form an ion pair. The formation of the ion pair resulted in an orientation of the dihalogenophenolindophenolate ion which prevented attack on the more nucleophilic oxygen and promoted the acetylation on the less nucleophilic oxygen for steric and energetic reasons, giving 3′,5′-dihalogeno derivatives. On the other hand, in the absence of pyridine the course of the reaction proceeded as expected with the attack of the more nucleophilic oxygen of phenolindophenolate ion directly on the acetic anhydride, yielding 2,6-dihalogeno derivatives.

The above rationalization may be extended to explain our findings. In the glycosidation the Na cation may act similarly to the acetyl pyridinium ion in the above acetylation and two reaction courses may be available. The structures of four acetyl glucosides (**5c**, **5e**, **6c** and **6e**) were established by elemental analyses and spectral data. The proton nuclear magnetic resonance (^{1}H-NMR) and the carbon-13 nuclear magnetic resonance (^{13}C-NMR) data are given in Table I. The ^{1}H-NMR spectra of **5c** and **5e** in $CDCl_3$ showed four double doublet signals (each 1 proton) at δ 6.59—7.25 due to the quinone ring hydrogens and one singlet signal (2 protons) at δ 7.06 due to the benzene ring hydrogens indicating that **5c** and **5e** have a phenolindo-3′,5′-dihalogenophenyl group. In the case of **6c** and **6e**, there were two doublet signals (each 1 proton) at δ 7.35—7.79 due to the quinone ring hydrogens and two broad

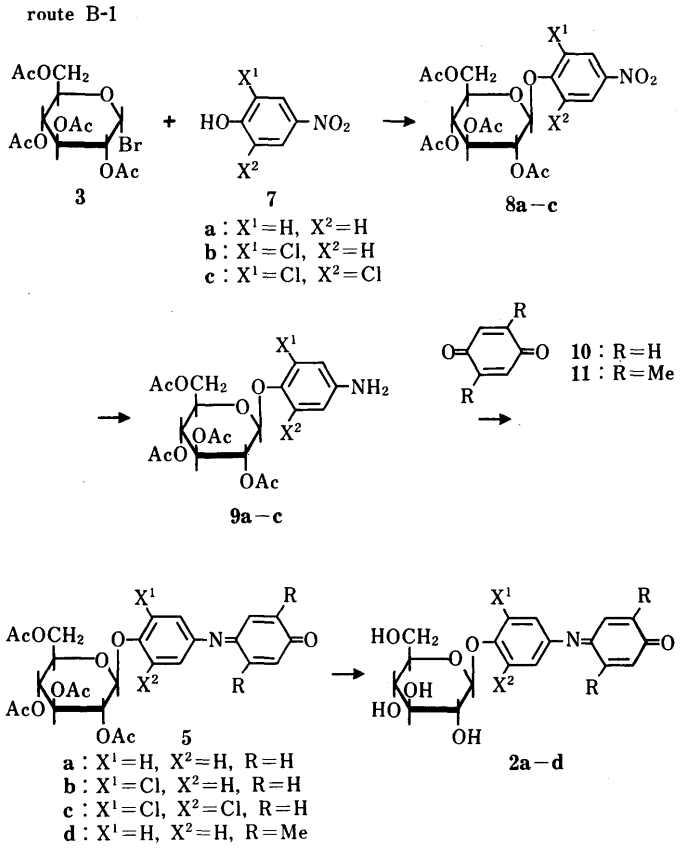


Chart 3

TABLE I. ^{1}H-NMR Data (199.5 MHz) for the Phenolindophenyl Group and ^{13}C-NMR Data (50.1 MHz) for Anomeric Carbon of **5c**, **5e**, **6c**, and **6e**[a]

Compd. No.	Phenolindophenyl group						Anomeric carbon
	H-2	H-6	H-3	H-5	H-2′, H-6′	H-3′, H-5′	
5c	6.60	6.71	7.05	7.25	7.06		101.0
	1H, dd	1H, dd	1H, dd	1H, dd	2H, s	—	(165)
	(10.4, 2.2)	(10.0, 2.2)	(10.4, 2.6)	(10.0, 2.6)			
5e	6.59	6.70	7.04	7.24	7.06		100.5
	1H, dd	1H, dd	1H, dd	1H, dd	2H, s	—	(167)
	(10.3, 2.2)	(10.0, 2.2)	(10.3, 2.7)	(10.0, 2.7)			
6c	—		7.35	7.52	6.90	7.09	98.8
			1H, d	1H, d	2H, brd	2H. brd	(162)
			(2.4)	(2.4)	(8.9)	(8.9)	
6e	—		7.61	7.79	6.91	7.07	98.8
			1H, d	1H, d	2H, brd	2H, brd	(161)
			(2.4)	(2.4)	(8.8)	(8.8)	

a) All spectra were taken in $CDCl_3$. Chemical shifts are in δ units. Coupling constants (in Hz) are given in parentheses.

doublet signals (each 2 protons) at δ 6.90—7.09 due to the benzene ring hydrogens, indicating that **6c** and **6e** have a 2,6-dihalogenophenolindophenyl group. ^{1}H-NMR signals were assigned by reference to data for phenolindophenyl esters obtained in an analogous study.[14] The ^{13}C-NMR spectra of the four acetyl glucosides in $CDCl_3$ showed a signal at δ 98.8—101.0 assigned to an anomeric carbon with a large coupling constant (J_{C-H} = 161—167 Hz), suggesting a β-glucosidic bond.[15] In addition, the structure of **5c** was confirmed by synthesis *via* another route (route B).

Route B-1 (Stepwise Method 1) In order to obtain various kinds of glucosides our second attempt was a stepwise synthesis of **2**, which involved the preparation of substituted 4-aminophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosides (**9**), as shown in Chart 3. The first step was the preparation of 4-nitrophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosides (**8**) by Koenigs–Knorr reaction of 4-nitrophenols (**7**) with **3**. The second step was the catalytic reduction of the nitro group to an amino group to yield **9**. The third step was the condensation of **9** with quinones (**10** and **11**) in the presence of trifluoroacetic acid (TFA) to yield the desired phenolindophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosides (**5**). The last step was the deacetylation. Four phenolindophenyl-β-D-glucopyranosides (**2a—d**) were obtained *via* this route.

When *p*-benzoquinone (**10**) and 2,5-dimethylbenzoquinone (**11**) were used in the above third step, the desired condensation reaction proceeded. However, in the case of 1,4-naphthoquinone (**12**), the 1,4-addition reaction of α,β-unsaturated ketone proceeded preferentially, followed by oxidation with excess quinones to give *N*-(2-naphthoquinonyl)-4-aminophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**13**). Only 0.9% of the products was 1-naphtholindophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**14**) (Chart 4). This may be due to the increase of electron density of the quinone ring and the decrease of electrophilicity at the carbonyl carbon in the fused aromatic ring.

Route B-2 (Stepwise Method 2) The deacetylation of **9a** could be carried out prior to the condensation. 4-Aminophenyl-β-D-glucopyranoside (**15**) thus obtained was condensed with quinones (**10** and **11**) in the presence of TFA to yield phenolindophenyl-β-D-glucopyranoside (**2a**) and 2,5-dimethylphenolindophenyl-β-D-glucopyranoside (**2d**) (Chart 5).

The structure of **2** was established by elemental analyses and spectral data (Tables II and III). ^{1}H-NMR spectra of **2** showed similar signal patterns due to phenolindophenol hydrogens to those of **5c** and **5e**. They also showed a signal at δ 4.88—5.14 assigned to an anomeric proton having a large coupling constant ($J_{1,2}$ = 6.8—7.6 Hz). This suggested the presence of a β-glucosidic bond in **2**,[16] which was also recognized by β-glucosidase hydrolysis as follows.

Since **2e** decomposed rapidly in water, the other four glucosides (**2a—d**) were examined for their suitability for the colorimetric assay of β-glucosidase activity. The assay was carried out in 5 mM phosphate buffer (pH 6.8) at 37 °C (the optimum conditions for hydrolysis by α-amylase).[17] All four substrates released phenolindophenols (**1a—d**) and the absorption of the blue color was measured at regular intervals under alkaline conditions (pH 11) to ensure sufficient dissociation. The K_m values and maximum velocities (V_{max}) of the four glucosides were obtained through Lineweaver–Burk plots and are summarized in Table IV.

It was found that **2b** and **2c** had larger affinity for the

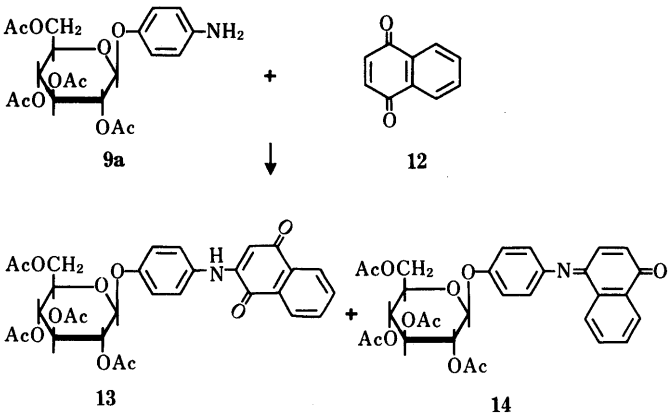


Chart 4

route B-2

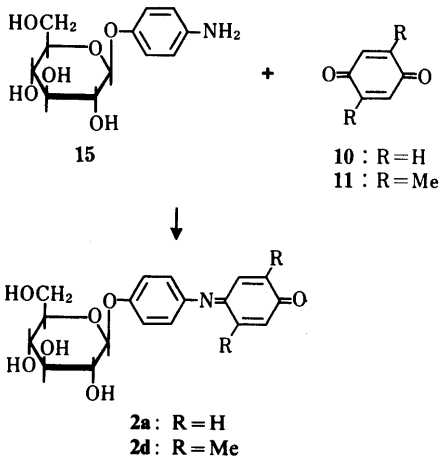


Chart 5

TABLE II. Melting Points and Elemental Analyses for **2**

Compd. No.	mp (°C)	Fromula	Elemental analysis (%) Calcd			Found		
			C	H	N	C	H	N
2a	149.0—151.0	$C_{18}H_{19}NO_7 \cdot 1/4H_2O$	59.09	5.37	3.83	59.22	5.31	3.87
2b	173.0—175.5	$C_{18}H_{18}ClNO_7$	54.62	4.58	3.54	54.61	4.56	3.54
2c	136.0—138.0	$C_{18}H_{17}Cl_2NO_7 \cdot 1/4H_2O$	49.73	4.06	3.22	49.49	4.05	3.18
2d	109.5—111.0	$C_{20}H_{23}NO_7 \cdot 1/2H_2O$	60.29	6.07	3.52	60.41	6.09	3.55
2e	130.0—134.0	$C_{18}H_{17}Br_2NO_7 \cdot 1/4H_2O$	41.28	3.37	2.67	41.44	3.44	2.62

TABLE III. ^{1}H-NMR Data (199.5 MHz) for the Phenolindophenyl Group and Anomeric Carbon of **2**[a]

Compd. No.	Phenolindophenyl group						Anomeric proton
	H-2	H-6	H-3	H-5	H-2′ H-6′	H-3′ H-5′	
2a	6.59 1H, dd (10.3, 2.2)	6.69 1H, dd (10.0, 2.2)	7.20 1H, dd (10.3, 2.6)	7.34 1H, dd (10.0, 2.6)	6.95 2H, br d (8.6)	7.16 2H, br d (8.6)	4.90 1H, d (7.1)
2b	6.62 1H, dd (10.5, 2.2)	6.71 1H, dd (10.0, 2.2)	7.16 1H, dd (10.5, 2.7)	7.34 1H, dd (10.0, 2.7)	7.10 6.91 1H, d 1H, dd (2.4) (8.8, 2.4)	— 7.35 1H, d (8.8)	4.97 1H, d (7.6)
2c	6.64 1H, dd (10.3, 2.2)	6.74 1H, dd (10.0, 2.2)	7.11 1H, dd (10.3, 2.7)	7.33 1H, dd (10.0, 2.7)	7.05 2H, s	—	5.08 1H, d (7.1)
2d	—	6.58 1H, br s	6.90 1H, br s	—	6.87 2H, d (8.7)	7.11 2H, d (8.7)	4.88 1H, d (6.8)
2e	6.63 1H, dd (10.2, 2.0)	6.72 1H, dd (10.0, 2.0)	7.09 1H, dd (10.2, 2.6)	7.33 1H, dd (10.0, 2.6)	7.24 2H, s	—	5.14 1H, d (7.6)

a) All spectra were taken in DMSO-d_6. Chemical shifts are in δ units. Coupling constants (in Hz) are given in parentheses.

TABLE IV. Properties of Phenolindophenyl-β-D-glucopyranosides (**2**) as β-Glucosidase Substrates

Compd. No.	K_m (M)	V_{max} (M/min)
2a	2.2×10^{-2}	2.4×10^{-6}
2b	5.3×10^{-3}	4.7×10^{-6}
2c	3.3×10^{-3}	1.3×10^{-6}
2d	1.1×10^{-2}	5.3×10^{-7}

enzyme than the others, and **2b** had the largest V_{max} value. In addition, released **1b** and **1c** showed a blue color over pH 8. Based on the above results, **2b** was considered to be the most suitable substrate among the four glucosides for the assay of β-glucosidase.

As the hydrolysis of the β-glucosides was the last step in the coupled enzymic assay system of α-amylase activity,[17] our findings that phenolindophenyl-β-D-glucopyranosides were good substrates for the β-glucosidase assay were also very important for the α-amylase assay. Moreover, these stepwise methods could be widely applied to the synthesis of other sugar glycosides. Preparation of phenolindophenyl maltooligosides and their clinical application are under study.

Experimental

Reagents and Materials All chemicals were of reagent grade unless otherwise noted. β-Glucosidase (from Sweet almond, 13.0 U/mg) was obtained from Toyobo Co., Ltd., Japan. Phenolindophenols were obtained from Tokyo Kasei Kogyo Co., Ltd., Japan. Water was deionized and distilled.

Apparatus All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. ^{1}H-NMR spectra were taken at 199.5 MHz and ^{13}C-NMR spectra were taken at 50.10 MHz with a JEOL JNM-FX200 NMR spectrometer with tetramethylsilane as an internal standard. The following abbreviations are used for the signal patterns: s = singlet, d = doublet, t = triplet, m = multiplet, and br = broad. Infrared (IR) spectra were taken with a JASCO A-202 spectrometer. Ultraviolet (UV) spectra were recorded with a Hitachi 557 spectrometer. Mass spectra (MS) were determined with a Hitachi M-80 spectrometer. High performance liquid chromatography (HPLC) was performed on a COSMOSIL 5 C18 column (4.6 mm i.d. × 250 mm) with a flow rate of 1.0 ml/min using a JASCO pump (BIP-1) and a refractive index (RI) detector (Shodex RI SE-51). The detector signals were processed and recorded using a reporting integrator (Chromatocorder 11, SIC). Analytical thin layer chromatography (TLC) was performed on Merck Kiesel gel 60F_{254} (type 60) plates. Column chromatography was performed on Merck Kiesel gel 60 (SiO_2, 230—400 mesh) and YMC-GEL ODS (250—350 mesh, from Yamamura Chemical Laboratories Co., Ltd.).

2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl Bromide (3) Phosphorus tribromide (69.4 g, 0.256 mol) and H_2O (10.0 g, 0.556 mol) were added dropwise to a solution of β-D-glucopyranose pentaacetate (100 g, 0.256 mol) in CH_2Cl_2 (100 ml) at room temperature. The reaction mixture was stirred at 30 °C for 10 h and the mixture was neutralized with K_2CO_3 at 30 °C, then filtered through a glass filter. The filtrate was evaporated under reduced pressure at 50 °C to give a hard crystalline mass. Recrystallization from Et_2O afforded pure **3** (98.4 g, yield 93.5%), mp 88—90 °C (lit.,[10] mp 88—89 °C).

General Procedure for Preparation of Phenolindo-3′,5′-dihalogenophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosides (5c and 5e) The Na salt of 2,6-dihalogenophenolindophenol (**4**, 121 mmol) and Ag_2O (28.3 g, 122 mmol) were added to a stirred solution of **3** (10.0 g, 24.4 mmol) in CH_3CN (200 ml), and the mixture was kept at 40 °C for 10—20 h until TLC (AcOEt–toluene, 1 : 1, v/v) indicated that the reaction was complete. The dark-colored mixture was cooled and diluted with CH_2Cl_2 (120 ml), then filtered through a pad of Celite. The insoluble material was thoroughly washed with CH_2Cl_2. The filtrate and washings were evaporated under reduced pressure at 50 °C. The syrupy residue was then chromatographed on silica gel with AcOEt–toluene (1 : 1, v/v) to give **5**. Recrystallization from EtOH afforded analytically pure **5**.

Phenolindo-3′,5′-dichlorophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**5c**): Yield, 5.98 g (44.1%). mp 151.0—152.0 °C. *Anal.* Calcd for $C_{26}H_{25}Cl_2NO_{11}$: C, 52.19; H, 4.21; N, 2.34. Found: C, 52.12; H, 4.20; N, 2.30. IR (KBr): 1750, 1648, 1452, 1382, 1242, 1216, 1092, 1040, 910, 876 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.03 (3H, s), 2.04 (6H, s), 2.11 (3H, s), 3.60—3.74 (1H, m), 4.14 (1H, dd, J = 12.0, 2.5 Hz), 4.24 (1H, dd, J = 12.0, 4.9 Hz), 5.19—5.43 (4H, m), and other signals as given in Table I. ^{13}C-NMR: as given in Table I. UV λ_{max}^{MeOH} nm (log ε): 264 (4.41), 438 (3.52).

Phenolindo-3′,5′-dibromophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**5e**): Yield, 7.66 g (45.7%). mp 145.5—147.0 °C. *Anal.* Calcd for $C_{26}H_{25}Br_2NO_{11}$: C, 45.44; H, 3.67; N, 2.04. Found: C, 45.44; H, 3.67; N, 2.07. IR (KBr): 1736, 1642, 1580, 1532, 1442, 1376, 1234, 1216, 1086, 1060, 1024, 872, 740 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.03 (6H, s), 2.04 (3H, s), 2.12 (3H, s), 3.63—3.72 (1H, m), 4.12 (1H, dd, J = 12.2, 2.9 Hz), 4.22 (1H, dd, J = 12.2, 4.5 Hz), 5.19—5.43 (4H, m), and other signals as given in Table I. ^{13}C-NMR: as given in Table I. UV λ_{max}^{MeOH} nm (log ε): 265 (4.43), 436 (3.52).

Reaction of 3 and 1c A stirred solution of **3** (1.70 g, 4.14 mmol) in benzene (30 ml) was added dropwise to a solution of **1c** (3.64 g, 13.6 mmol) and Ag_2O (3.16 g, 13.6 mmol) in benzene (130 ml), under reflux for 15 min. The reaction mixture was refluxed with stirring for an additional 20 h. The dark-colored mixture was filtered through a pad of Celite and insoluble material was thoroughly washed with CH_2Cl_2. The filtrate and washings were evaporated under reduced pressure at 50 °C. To separate **5c** and **6c** the syrupy residue was then chromatographed on silica gel with AcOEt–

toluene (2 : 3, v/v). Recrystallization from EtOH afforded analytically pure **5c** and **6c**, respectively.

5c: Yield, 267 mg (10.8%).

2,6-Dichlorophenolindophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**6c**): Yield, 963 mg (38.8%). mp 145.5—147.5 °C. *Anal.* Calcd for $C_{26}H_{25}Cl_2NO_{11}$: C, 52.19; H, 4.21; N, 2.34. Found: C, 52.18; H, 4.21; N, 2.36. IR (KBr): 1754, 1670, 1594, 1566, 1496, 1368, 1228, 1040, 908, 856, 798 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.04 (3H, s), 2.06 (3H, s), 2.08 (6H, s), 3.86—3.95 (1H, m), 4.19 (1H, dd, *J* = 12.2, 2.7 Hz), 4.31 (1H, dd, *J* = 12.2, 5.2 Hz), 5.13—5.38 (4H, m), and other signals as given in Table I. ^{13}C-NMR: as given in Table I. UV λ_{max}^{MeOH} nm (log ε): 274 (4.11), 312 (4.08), 494 (3.85).

Reaction of 3 and 1e Reaction of **3** (1.70 g, 4.14 mmol) and **1e** (4.86 g, 13.6 mmol) was carried out as described for the reaction of **3** and **1c**.

5e: Yield, 250 mg (8.8%).

2,6-Dibromophenolindophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**6e**): Yield, 722 mg (25.3%). mp 126.0—127.5 °C. *Anal.* Calcd for $C_{26}H_{25}Br_2NO_{11}$: C, 45.44; H, 3.67; N, 2.04. Found: C, 45.42; H, 3.67; N, 2.08. IR (KBr): 1742, 1662, 1494, 1372, 1226, 1166, 1068, 1032, 1008, 892, 836 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.04 (3H, s), 2.06 (3H, s), 2.08 (6H, s), 3.88—3.95 (1H, m), 4.19 (1H, dd, *J* = 12.2, 2.5 Hz), 4.31 (1H, dd, *J* = 12.2, 5.4 Hz), 5.14—5.33 (4H, m), and other signals as given in Table I. ^{13}C-NMR: as given in Table I. UV λ_{max}^{MeOH} nm (log ε): 318 (4.17), 496 (3.91).

General Procedure for Preparation of 4-Nitrophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosides (8) A 4-nitrophenol (**7**, 24.3 mmol) and Ag_2O (2.26 g, 9.74 mmol) were added to a stirred solution of **3** (2.00 g, 4.87 mmol) in CH_3CN (40 ml), and the mixture was kept at 40 °C for 10—20 h until TLC (AcOEt–toluene, 1 : 1, v/v) indicated that the reaction was complete. The dark-colored mixture was cooled and diluted with CH_2Cl_2 (120 ml), then filtered through a pad of Celite. Insoluble material was thoroughly washed with CH_2Cl_2. The filtrate and washings were evaporated under reduced pressure at 50 °C. The syrupy residue was then chromatographed on silica gel with AcOEt–toluene (1 : 1, v/v) to give **8**. Recrystallization from MeOH afforded analytically pure **8**.

4-Nitrophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**8a**): Yield, 1.40 g (61.3%). mp 122.0—123.0 °C. *Anal.* Calcd for $C_{20}H_{23}NO_{12}$: C, 51.28; H, 4.94; N, 2.98. Found: C, 51.25; H, 4.92; N, 2.99. IR (KBr): 1754, 1732, 1606, 1594, 1524, 1498, 1382, 1346, 1240, 1114, 1086, 1060, 914, 866 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.05 (3H, s), 2.07 (6H, s), 2.08 (3H, s), 3.90—3.99 (1H, m), 4.18 (1H, dd, *J* = 12.2, 2.5 Hz), 4.30 (1H, dd, *J* = 12.2, 5.4 Hz), 5.14—5.36 (4H, m), 7.08 (1H, dd, *J* = 9.9, 2.8 Hz), 7.09 (1H, dd, *J* = 9.9, 2.8 Hz), 8.20 (1H, dd, *J* = 9.9, 2.8 Hz), 8.22 (1H, dd, *J* = 9.9, 2.8 Hz). UV λ_{max}^{MeOH} nm (log ε): 218 (3.88), 290 (4.02).

2-Chloro-4-nitrophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**8b**): Yield, 2.06 g (84.0%). mp 147.0—148.0 °C. *Anal.* Calcd for $C_{20}H_{22}ClNO_{12}$: C,47.68; H, 4.40; N, 2.78. Found: C, 47.60; H, 4.38; N, 2.78. IR (KBr): 1756, 1586, 1530, 1486, 1358, 1236, 1084, 1040, 898, 834, 740, 600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.06 (3H, s), 2.07 (3H, s), 2.09 (3H, s), 2.10 (3H, s), 3.92—4.01 (1H, m), 4.22 (1H, dd, *J* = 12.5, 2.8 Hz), 4.31 (1H, dd, *J* = 12.5, 5.1 Hz), 5.16—5.45 (4H, m), 7.27 (1H, d, *J* = 9.1 Hz), 8.14 (1H, dd, *J* = 9.1, 2.7 Hz), 8.30 (1H, d, *J* = 2.7 Hz). UV λ_{max}^{MeOH} nm (log ε): 280 (3.95).

2,6-Dichloro-4-nitrophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**8c**): Yield, 2.46 g (93.9%). mp 181.0—182.0 °C. *Anal.* Calcd for $C_{20}H_{21}Cl_2NO_{12}$: C, 44.63; H, 3.93; N, 2.60. Found: C, 44.60; H, 3.91; N, 2.65. IR (KBr): 1752, 1538, 1350, 1240, 1064, 1034 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.04 (6H, s), 2.05 (3H, s), 2.11 (3H, s), 3.65—3.72 (1H, m), 4.09 (1H, dd, *J* = 12.2, 2.8 Hz), 4.20 (1H, dd, *J* = 12.2, 4.5 Hz), 5.17—5.42 (4H, m), 8.22 (2H, s). UV λ_{max}^{MeOH} nm (log ε): 272 (3.92).

General Procedure for Preparation of 4-Aminophenyl 2, 3, 4, 6-Tetra-*O*-acetyl-β-D-glucopyranosides (9) A stirred solution of **8** (2.00 g) in 1,4-dioxane (40 ml) was hydrogenated immediately after the addition of 200 mg of Pd/C (10%), at ordinary pressure at 35 °C for 15—40 h until TLC (AcOEt–toluene, 1 : 1, v/v) indicated that the reaction was complete. The reaction mixture was filtered through a pad of Celite. Insoluble material was thoroughly washed with CH_2Cl_2. The filtrate and washings were evaporated under reduced pressure at 50 °C. The syrupy residue was then chromatographed on silica gel with AcOEt–toluene (1 : 1, v/v) to give **9**. Recrystallization from MeOH afforded analytically pure **9**.

4-Aminophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**9a**): Yield, 1.84 g (98.3%). mp 130.5—131.5 °C. *Anal.* Calcd for $C_{20}H_{25}NO_{10}$: C, 54.67; H, 5.73; N, 3.19. Found: C, 54.60; H, 5.77; N, 3.14. IR (KBr): 3470, 3390, 1756, 1514, 1228, 1042 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.02 (3H, s), 2.03 (3H, s), 2.06 (6H, s), 3.18 (2H, br s), 3.73—3.82 (1H, m), 4.14 (1H, dd, *J* = 12.2, 2.4 Hz), 4.28 (1H, dd, *J* = 12.2, 5.0 Hz), 4.90 (1H, d, *J* = 7.3 Hz), 5.08—5.31 (3H, m), 6.58 (1H, dd, *J* = 8.7, 2.7 Hz), 6.60 (1H, dd, *J* = 8.7, 2.7 Hz), 6.82 (1H, dd, *J* = 8.7, 2.8 Hz), 6.83 (1H, dd, *J* = 8.7, 2.8 Hz). UV λ_{max}^{MeOH} nm (log ε): 235 (4.01), 295 (3.25).

2-Chloro-4-aminophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**9b**): Yield, 1.80 g (95.7%). mp 190.0—193.0 °C. *Anal.* Calcd for $C_{20}H_{24}ClNO_{10}$: C, 50.69; H, 5.09; N, 2.96. Found: C, 50.57; H, 5.11; N, 2.92. IR (KBr): 3440, 3350, 1740, 1610, 1492, 1358, 1214, 1060, 1028, 896 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.02 (3H, s), 2.03 (3H, s), 2.08 (3H, s), 2.09 (3H, s), 3.36 (2H, br s), 3.70—3.79 (1H, m), 4.10—4.32 (2H, m), 4.80—4.88 (1H, m), 5.10—5.32 (3H, m), 6.48 (1H, dd, *J* = 8.8, 2.7 Hz), 6.69 (1H, d, *J* = 2.7 Hz), 7.02 (1H, d, *J* = 8.8 Hz). UV λ_{max}^{MeOH} nm (log ε): 240 (4.03), 300 (3.36).

2,6-Dichloro-4-aminophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**9c**): Yield, 1.79 g (94.8%). mp 147.0—148.0 °C. *Anal.* Calcd for $C_{20}H_{23}Cl_2NO_{10}$: C, 47.26; H, 4.56; N, 2.76. Found: C, 47.17; H, 4.57; N, 2.70. IR (KBr): 3480, 3390, 1750, 1720, 1620, 1592, 1558, 1472, 1424, 1372, 1208, 1036, 900, 800 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.02 (3H, s), 2.03 (6H, s), 2.08 (3H, s), 3.71 (2H, br s), 3.60—3.74 (1H, m), 4.12 (1H, dd, *J* = 12.2, 2.4 Hz), 4.22 (1H, dd, *J* = 12.2, 7.6 Hz), 5.12—5.32 (4H, m), 6.58 (2H, s). UV λ_{max}^{MeOH} nm (log ε): 210 (4.51), 244 (4.02), 306 (3.41).

General Procedure for Preparation of Phenolindophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosides (5 and 14) and *N*-(2-Naphthoquinonyl)-4-aminophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (13) TFA (2.5 ml), molecular sieves (20.0 g), and **9** (9.09 mmol) were added to a stirred solution of quinones (**10**—**12**) (90.9 mmol) in 1,4-dioxane (120 ml), and the mixture was kept at room temperature for 1—10 h until TLC (AcOEt–toluene, 1 : 1, v/v) indicated that the reaction was complete. The dark-colored mixture was cooled and neutralized with K_2CO_3, then filtered through a glass filter. Insoluble material was thoroughly washed with CH_2Cl_2. The filtrate and washings were evaporated under reduced pressure at 50 °C. The syrupy residue was then chromatographed on silica gel with AcOEt–toluene (1 : 1, v/v) and on ODS gel with CH_3CN–H_2O (3 : 2, v/v) to give **5**, **14** and **13**. Recrystallization from EtOH afforded analytically pure **5**, **14** and **13**, respectively.

Phenolindophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**5a**): Yield, 2.54 g (52.8%). mp 133.5—134.5 °C. *Anal.* Calcd for $C_{26}H_{27}NO_{11}$: C, 58.98; H, 5.14; N, 2.65. Found: C, 58.51; H, 5.17; N, 2.54. IR (KBr): 1740, 1638, 1496, 1364, 1220, 1066, 1038, 904, 866, 838 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.04 (3H, s), 2.05 (3H, s), 2.08 (3H, s), 2.11 (3H, s), 3.86—3.91 (1H, m), 4.18 (1H, dd, *J* = 12.1, 2.4 Hz), 4.30 (1H, dd, *J* = 12.1, 5.0 Hz), 5.10—5.41 (4H, m), 6.54 (1H, dd, *J* = 10.4, 2.3 Hz), 6.68 (1H, dd, *J* = 10.0, 2.3 Hz), 6.87 (2H, br d, *J* = 9.0 Hz), 7.05 (2H, br d, *J* = 9.0 Hz), 7.13 (1H, dd, *J* = 10.4, 2.5 Hz), 7.29 (1H, dd, *J* = 10.0, 2.5 Hz). UV λ_{max}^{MeOH} nm (log ε): 260 (4.27), 470 (3.72).

Phenolindo-3′-chlorophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**5b**): Yield, 2.77 g (54.1%). mp 140.0—141.0 °C. *Anal.* Calcd for $C_{26}H_{26}ClNO_{11}$: C, 55.37; H, 4.65; N, 2.48. Found: C, 55.26; H, 4.65; N, 2.43. IR (KBr): 1748, 1644, 1484, 1366, 1236, 1080, 1036, 904, 868 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.04 (3H, s), 2.05 (3H, s), 2.09 (3H, s), 2.10 (3H, s), 3.83—3.91 (1H, m), 4.20 (1H, dd, *J* = 12.0, 2.7 Hz), 4.30 (1H, dd, *J* = 12.0, 4.9 Hz), 5.02—5.40 (4H, m), 6.57 (1H, dd, *J* = 10.4, 2.3 Hz), 6.69 (1H, dd, *J* = 10.1, 2.3 Hz), 6.75 (1H, dd, *J* = 8.6, 2.4 Hz), 6.97 (1H, d, *J* = 2.4 Hz), 7.08 (1H, dd, *J* = 10.4, 2.7 Hz), 7.24 (1H, d, *J* = 8.6 Hz), 7.26 (1H, dd, *J* = 10.1, 2.7 Hz). UV λ_{max}^{MeOH} nm (log ε): 260 (4.34), 462 (3.67).

5c: Yield, 3.30 g (60.7%)

2,5-Dimethylphenolindophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (**5d**): Yield, 1.15 g (22.7%). mp 118.0—120.0 °C. *Anal.* Calcd for $C_{28}H_{31}NO_{11}$: C, 60.32; H, 5.58; N, 2.53. Found: C, 60.33; H, 5.63; N, 2.48. IR (KBr): 2950, 1756, 1628, 1598, 1496, 1368, 1222, 1032, 902, 846 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.94 (3H, d, *J* = 1.3 Hz), 2.04 (3H, s), 2.06 (3H, s), 2.08 (6H, s), 2.26 (3H, d, *J* = 1.3 Hz), 3.87—3.94 (1H, m), 4.20 (1H, dd, *J* = 12.3, 2.6 Hz), 4.31 (1H, dd, *J* = 12.3, 5.2 Hz), 5.10—5.33 (4H, m), 6.53 (1H, d, *J* = 1.2 Hz), 6.79 (2H, br d, *J* = 8.7 Hz), 6.81 (1H, d, *J* = 1.2 Hz), 7.05 (2H, br d, *J* = 8.7 Hz). UV λ_{max}^{MeOH} nm (log ε): 267 (4.31), 477 (3.63).

1-Naphtholindophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside: (**14**): Yield, 4.7 mg (0.9%). mp 175.0—178.0 °C. High-resolution MS *m/z*: 579.1741 (Calcd for $C_{30}H_{29}NO_{11}$: 579.1739). IR (KBr): 1752, 1658, 1590, 1498, 1364, 1224, 1040, 902, 824 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.06 (3H, s), 2.07 (3H, s), 2.09 (3H, s), 2.10 (3H, s), 3.87—3.95 (1H, m), 4.20 (1H, dd, *J* = 12.3, 2.4 Hz), 4.33 (1H, dd, *J* = 12.3, 4.9 Hz), 5.10—5.37 (4H, m), 6.69 (1H, d, *J* = 10.5 Hz), 6.88 (2H, d, *J* = 8.7 Hz), 7.07 (2H, d, *J* = 8.7 Hz), 7.27 (1H, d, *J* = 10.5 Hz), 7.66 (1H, ddd, *J* = 7.3, 7.3, 1.7 Hz), 7.73 (1H, ddd, *J* = 7.3, 7.3, 1.7 Hz), 8.15 (1H, dd, *J* = 7.3, 1.7 Hz), 8.45 (1H, dd, *J* = 7.3, 1.7 Hz). UV λ_{max}^{MeOH} nm (log ε): 226 (4.28), 259 (4.40), 465 (3.73).

N-(2-Naphthoquinonyl)-4-aminophenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-

glucopyranoside (**13**): Yield, 1.36 g (25.1%). Oange oil. High-resolution MS *m/z*: 595.1679 (Calcd for $C_{30}H_{29}NO_{12}$: 595.1687). IR (KBr): 3460, 3320, 2950, 1750, 1672, 1600, 1572, 1514, 1366, 1348, 1294, 1220, 1118, 1036, 988, 904, 830, 780, 714 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.04 (3H, s), 2.05 (3H, s), 2.09 (6H, s), 3.84—3.93 (1H, m), 4.19 (1H, dd, J=12.2, 2.7 Hz), 4.31 (1H, dd, J=12.2, 5.0 Hz), 5.08—5.33 (4H, m), 6.24 (1H, s), 7.05 (2H, br d, J=9.0 Hz), 7.43 (2H, br d, J=9.0 Hz), 7.48 (1H, br s), 7.66 (1H, ddd, J=7.3, 7.3, 1.2 Hz), 7.75 (1H, ddd, J=7.3, 7.3, 1.2 Hz), 8.08 (1H, dd, J=7.3, 1.2 Hz), 8.10 (1H, dd, J=7.3, 1.2 Hz). UV λ_{max}^{MeOH} nm (log ε): 270 (4.45), 468 (3.68).

General Procedure for Preparation of Phenolindophenyl-β-D-glucopyranosides (2) (Deacetylation of 5) Anhydrous K_2CO_3 (0.50 mmol) was added to a suspension of **5** (2.00 mmol) in absolute MeOH (120 ml) with stirring, and the reaction mixture was kept at room temperature for 10—60 min until TLC (CH_2Cl_2–MeOH, 5:1, v/v) indicated that the reaction was complete. Then the solution was neutralized with Amberlite IRC-50 resin (H^+ form). The resin was filtered through a glass filter and was thoroughly washed with MeOH. The filtrate and washings were evaporated under reduced pressure at 50 °C. The syrupy residue was then chromatographed on silica gel with CH_2Cl_2–MeOH (4:1, v/v) to give **2**. Recrystallization from MeOH–H_2O afforded analytically pure **2**.

Phenolindophenyl-β-D-glucopyranoside (**2a**): Yield, 422 mg (58.4%). Melting point, elemental analysis: as given in Table II. IR (KBr): 1640, 1616, 1500, 1238, 1084, 1016, 870, 842 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.10—3.90 (m), 4.51 (1H, br s), 4.98 (1H, br s), 5.26 (1H, br s), and other signals as given in Table III. UV λ_{max}^{MeOH} nm (log ε): 260 (4.24), 304 (4.01), 478 (3.77).

Phenolindo-3′-chlorophenyl-β-D-glucopyranoside (**2b**): Yield, 493 mg (62.3%). Melting point, elemental analysis: as given in Table II. IR (KBr): 1642, 1612, 1492, 1256, 1088, 1044, 996, 872 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.10—3.80 (m), 4.50 (1H, br dd, J=5.6, 5.6 Hz), 5.00 (2H, m), 5.22 (1H, br d, J=4.0 Hz), and other signals as given in Table III. UV λ_{max}^{MeOH} nm (log ε): 262 (4.34), 468 (3.75).

Phenolindo-3′,5′-dichlorophenyl-β-D-glucopyranoside (**2c**): Yield, 445 mg (51.7%). Melting point, elemental analysis: as given in Table II. IR (KBr): 1650, 1620, 1452, 1256, 1068, 868, 810 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.03—3.75 (m), 4.17 (1H, br dd, J=5.5, 5.5 Hz), 4.92 (1H, br s), 4.99 (1H, br s), 5.25 (1H, br d, J=1.6 Hz), and other signals as given in Table III. UV λ_{max}^{MeOH} nm (log ε): 208 (4.46), 262 (4.35), 442 (3.52).

2,5-Dimethylphenolindophenyl-β-D-glucopyranoside (**2d**): Yield, 478 mg (61.4%). Melting point, elemental analysis: as given in Table II. IR (KBr): 2940, 1626, 1496, 1236, 1074, 902, 846 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.87 (3H, br s), 2.21 (3H, br s), 3.18—3.77 (m), 4.52 (1H, dd, J=5.6, 5.6 Hz), 4.97 (1H, d, J=4.9 Hz), 5.02 (1H, d, J=3.9 Hz), 5.28 (1H, d, J=4.2 Hz), and other signals as given in Table III. UV λ_{max}^{MeOH} nm (log ε): 228 (3.90), 268 (4.29), 483 (3.66).

Phenolindo-3′,5′-dibromophenyl-β-D-glucopyranoside (**2e**): Yield, 433 mg (41.7%). Melting point, elemental analysis: as given in Table II. IR (KBr): 1644, 1622, 1582, 1536, 1448, 1388, 1314, 1242, 1072, 870 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.03—3.84 (m), 4.11 (1H, br dd, J=5.5, 5.5 Hz), 4.79 (1H, br d, J=2.2 Hz), 4.99 (1H, br d, J=1.0 Hz), 5.25 (1H, br d, J=2.7 Hz), and other signals as given in Table III. UV λ_{max}^{MeOH} nm (log ε): 208 (4.55), 263 (4.37), 440 (3.57).

Preparation of 15 Deacetylation of **9a** (2.00 g, 4.56 mmol) was carried out as described in the general procedure for preparation of **2** (deacetylation of **5**).

4-Aminophenyl-β-D-glucopyranoside (**15**): Yield, 1.14 g (92.3%). mp 153.5—155.0 °C. *Anal.* Calcd for $C_{12}H_{17}NO_6$: C, 53.13; H, 6.32; N, 5.16. Found: C, 53.10; H, 6.35; N, 5.12. IR (KBr): 3510, 3490, 3410, 1516, 1226, 1082, 1060, 1044, 990, 820 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.30—3.78 (m), 4.44 (1H, dd, J=5.9, 5.3 Hz), 4.57 (1H, d, J=7.1 Hz), 4.85 (1H, d, J=4.6 Hz), 4.89 (1H, d, J=3.7 Hz), 5.10 (1H, d, J=4.2 Hz), 6.49 (2H, d, J=8.8 Hz), 6.77 (2H, d, J=8.8 Hz). UV λ_{max}^{MeOH} nm (log ε): 204 (3.91), 233 (3.96), 294 (3.24).

General Procedure for Preparation of Phenolindophenyl-β-D-glucopyranosides (2) (Condensation with Quinones) Compound **15** (1.22 g, 4.50 mmol) was added to a stirred solution of quinones (**10** and **11**) (45.0 mmol), TFA (1.2 ml) and molecular sieves (10.0 g) in 1,4-dioxane (50 ml), and the mixture was kept at room temperature for 1—10 h until TLC (CH_2Cl_2–MeOH, 1:1, v/v) indicated that the reaction was complete. Then the dark-colored mixture was filtered through a glass filter without neutralization. Insoluble material was thoroughly washed with AcOEt. The filtrate and washings were evaporated under reduced pressure at 40 °C. The syrupy residue was chromatographed on silica gel with CH_2Cl_2–MeOH (4:1, v/v) to give **2**. Recrystallization from MeOH afforded analytically pure **2**.

2a: Yield, 1.09 g (67.1%).

2d: Yield, 399 mg (22.8%).

Evaluation Procedure for 2 as β-Glucosidase Substrates A solution of **2** (2.0 ml) in H_2O was mixed with 30 mM phosphate buffer (pH 6.8, 0.5 ml) and a solution of β-glucosidase (0.06 U/ml, 0.5 ml) and the mixture was incubated at 37 °C for 1 min, 2.5 min, 4 min, and 5.5 min. At the designated time 1.2 ml of 350 mM Na_2CO_3 solution was added and the optical density at 620 nm against H_2O was measured immediately. For the blank, H_2O was added instead of the indicated volume of β-glucosidase solution. The K_m values and maximum velocities (V_{max}) of hydrolysis of **2** by β-glucosidase were obtained from Lineweaver–Burk plots.

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