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Synthesis of aryl 3-*O*-β-cellobiosyl-β-D-glucopyranosides for reactivity studies of 1,3-1,4-β-glucanases

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Abstract

A series of substituted aryl β-glycosides derived from 3-*O*-β-cellobiosyl-D-glucopyranose with different phenol-leaving group abilities as measured by the pK_a of the free phenol group upon enzymatic hydrolysis has been synthesised. Aryl β-glycosides with a pK_a of the free phenol leaving group > 5 were prepared by phase-transfer glycosidation of the per-*O*-acetylated α-glycosyl bromide with the corresponding phenol, whereas the 2,4-dinitrophenyl β-glycoside was obtained by condensation of 1-fluoro-2,4-dinitrobenzene with the partially acetylated trisaccharide followed by acid de-*O*-acetylation. The aryl β-glycosides have been used for reactivity studies of the wild-type *Bacillus licheniformis* 1,3-1,4-β-D-glucan 4-glucanohydrolase. The Hammett plot log k_{cat} versus pK_a is biphasic with an upward curvature at low pK_a values suggesting a change in transition-state structure depending on the aglycon.

Keywords: Aryl glycosides; 1,3-1,4-β-Glucanase; Enzyme kinetics; Hammett analysis

1. Introduction

Synthetic chromophoric oligosaccharides are of widespread use as substrates and ligands to study the kinetic properties of glycosidases. 4-Methylumbelliferyl and aryl glycosides have been used to analyse the substrate specificity of a number of glycosidases (see, for example, refs. [1–4]). However, only few studies on linear free energy relationships (LFER, namely Hammett and Brønsted analyses) have been performed, the reported examples being mainly on monosaccharidases and *exo*-glycanases (e.g. β-glucosidase from *Agrobacterium faecalis* [5], β-galactosidase from *Escherichia coli* [6], exo-β-1,4-glycanase from *Cellulomonas fimi* [7], sialidases from influenza A virus and *Salmonella typhimurium* [8]), and lysozyme [9,10] and xylanase from *Bacillus circulans* [11,12] as representatives of *endo*-glycanases. Glycosidases are grouped in two families according to their mechanism: retaining and inverting enzymes, acting by double or single displacement mechanisms, respectively. Although members of one family share a common reaction mechanism, the few reported examples show

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remarkable differences in the shape and ρ (or β) values of their Hammett plots, providing evidence of subtle variations in the fine structure of their catalytic machineries and reaction mechanisms. Detailed mechanistic investigations for *endo*-acting enzymes have proved to be extremely difficult due in part to the complexity of the substrates and therefore the enormous amount of synthetic chemistry required. A clear need exists for LFER studies with *endo*-glycosidases, both acting with retention and inversion of configuration at the anomeric center to better understand the fine details and differences in their catalytic mechanisms.

1,3-1,4-β-D-glucan 4-glucanohydrolase (EC 3.2.1.73, 1,3-1,4-β-glucanase) is a retaining *endo*-depolymerase that catalyzes the regio and stereospecific hydrolysis of β-glucans containing mixed β-(1→3) and β-(1→4) glycosidic linkages such as cereal β-glucans and lichenan [13–15]. The bacterial 1,3-1,4-β-glucanases have significant commercial interest, particularly in the brewing and animal feedstuff industries [16,17]. The trisaccharide 3-*O*-β-cellobiosyl-D-glucopyranose (**1**) and the tetrasaccharide 3-*O*-β-cellotriosyl-D-glucopyranose are the final hydrolysis products of barley β-glucan, thus defining the enzymatic specificity of cleavage on β-(1→4) glycosidic linkages in 3-*O*-substituted glucopyranose units [15].

Following our structure/function studies of bacterial 1,3-1,4-β-glucanases [18–23], here we report on the synthesis of a series of aryl glycosides of 3-*O*-β-cellobiosyl-D-glucopyranose as specific substrates for reactivity studies. Their kinetic parameters for the enzyme-catalyzed hydrolysis with 1,3-1,4-β-glucanase from *Bacillus licheniformis* are reported.

2. Results

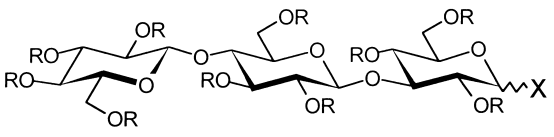
Synthesis.—The design of the target substrates was based on two criteria: (a) the glyconic portion should not be hydrolyzed by the enzyme, and should contain the minimum structural requirements for binding to the active site. Earlier kinetic work with low-molecular-weight β-glucan oligosaccharides having a 4-methylumbelliferyl aglycon [20,23] ($[G4]_nG3G\text{-}MU$[1], $n = 0$–3) showed that the trisaccharide derivative ($n = 1$) is a good substrate representing a good balance between synthetic complexity and reactivity; (b) the aglycons to be released as leaving groups in the enzymatic reactions are chosen by their phenol-leaving group ability as measured by the pK_a of the phenol (pK_a in parenthesis): phenol (10), 4-bromophenol (9.34), 3-nitrophenol (8.39), 4-nitrophenol (7.18), 3,5-dinitrophenol (6.69), 3,4-dinitrophenol (5.36), 2,3-dinitrophenol (4.96), and 2,4-dinitrophenol (3.96), which cover the pK_a range from 4 to 10 [5,6].

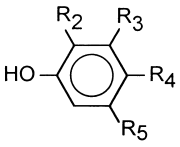
The oligosaccharide 3-*O*-β-cellobiosyl-D-glucopyranose (**1**) was obtained by enzymatic degradation of barley β-glucan (either as a purified polysaccharide or as a raw barley flour after treatment with ethanol) with recombinant 1,3-1,4-β-glucanase from *Bacillus licheniformis* [21]. After full acetylation of the reaction mixture with acetic anhydride–pyridine, chromatographic purification on silica gel afforded **2**.

The synthesis of aryl glycosides with a pK_a of the phenol-leaving group greater than 5 (**6a–g**) was based on a phase-transfer glycosidation according to the procedure reported by Dess et al. [24] for the preparation of aryl glycosides of monosaccharides, and previously used in our group for the synthesis of the trisaccharide G4G3G-MU [21]. In that case, the procedure improved substantially the condensation yield between the α-bromo derivative of the peracetylated trisaccharide **2** and 4-methylumbelliferone as compared to the classical procedure in a homogeneous system (aqueous NaOH–acetone mixture) [1,7]. As shown in Scheme 1, the α-glycosyl bromide **3**—prepared from the peracetate **2** by reaction with 33% hydrogen bromide in acetic acid—was treated with the phenols **4a–g** in a biphasic system chloroform-1.25 M aqueous NaOH in the presence of benzyltriethylammonium bromide as phase-transfer catalyst to afford **5a–g** in 25 to 65% yield (not optimized). Deacetylation of compounds **5a–f** with sodium methoxide in methanol yielded the aryl glycosides **6a–f**. Deacetylation of the base-sensitive 3,4- and 2,3-dinitrophenyl glycosides **5f,g** was performed with hydrogen chloride in anhydrous methanol [25]. The deprotected aryl glycosides **6a–g** were recrystalized from ethanol–water to give chromatographically homegeneous materials for enzymological studies (>99% purity by HPLC).

This strategy proved to be unsuccessful for the preparation of the 2,4-dinitrophenyl glycoside due to the lability of the glycosidic linkage with the

[1] MU: 4-methylumbelliferyl, G4G3G-X: aryl 3-*O*-β-cellobiosyl-D-glucopyranoside.





	R	X
1	H	(α/β)-OH
2	Ac	(α/β)-Ac
3	Ac	α-Br
5a	Ac	β-P
5b	Ac	β-4BrP
5c	Ac	β-3NP
5d	Ac	β-4NP
5e	Ac	β-3,5DNP
5f	Ac	β-3,4DNP
5g	Ac	β-2,3DNP
5h	Ac	β-2,4DNP
6a	H	β-P
6b	H	β-4BrP
6c	H	β-3NP
6d	H	β-4NP
6e	H	β-3,5DNP
6f	H	β-3,4DNP
6g	H	β-2,3DNP
6h	H	β-2,4DNP
6i	H	β-MU

	R_2	R_3	R_4	R_5	aglycon
4a	H	H	H	H	P
4b	H	H	Br	H	4BrP
4c	H	nitro	H	H	3NP
4d	H	H	nitro	H	4NP
4e	H	nitro	H	nitro	3,5DNP
4f	H	nitro	nitro	H	3,4DNP
4g	nitro	nitro	H	H	2,3DNP
4h	nitro	H	nitro	H	2,4DNP

Scheme 1.

aglycon (pK_a of the phenol 3.96) under the basic conditions used in the phase-transfer glycosidation step. Introduction of the 2,4-dinitrophenyl group at the anomeric center has been reported by reacting an acetylated glycosyl halide with 2,4-dinitrophenol in the presence of potassium carbonate [25]. A more efficient route, however, is based on the electrophilic aromatic substitution of 1-fluoro-2,4-dinitrobenzene (FDNB) by the free anomeric hydroxyl group of the saccharide in the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO) in *N,N*-dimethylformamide [7,26]. First, selective deacetylation of the anomeric hydroxyl group of the peracetylated trisaccharide **2** with hydrazine acetate led to **7** in 98% yield. Compound **7** was then reacted with 1-fluoro-2,4-dinitrobenzene in the presence of 1,4-diazabicyclo[2.2.2]octane and molecular sieves in anhydrous *N,N*-dimethylform-

Table 1
^{1}H NMR chemical shifts (δ), multiplicity, and coupling constants (Hz, in parenthesis) for compounds **6a–h** (D_2O, 300 MHz)

	6a	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**
H-2 phenyl	7.17 d (8.5)	7.07 d (8.0)	7.83 dd (3.0)	7.26 d (10)	8.34 d (1.8)	7.68 d (2.5)	—	—
H-6 phenyl	7.17 d (8.5)	7.07 d (8.0)	7.30 dd (8.0,3.0)	7.26 d (10)	8.34 d (1.8)	7.51 dd (9.0, 2.5)	7.86 dd (1.2, 8.5)	7.61 d (9.6)
H-3 phenyl	7.42 dd (8.0)	7.54 d (8.0)	—	8.27 d (10)	—	—	—	8.90 d (3.0)
H-5 phenyl	7.42 dd (8.0)	7.54 d (8.0)	7.47 t (8.0)	8.27 d (10)	—	8.21 d (9.0)	7.80 dd (8.5)	8.54 dd (9.3)
H-4 phenyl	7.15 t (8.5)	—	7.95 dd (8.0,3.0)	—	8.82 d (1.8)	—	8.06 dd (1.2, 8.5)	—
H-1	5.17 d (10)	5.13 d (7.5)	5.25 d (8.0)	5.31 d (7.5)	5.38 d (7.5)	5.34 d (8.0)	5.29 d (7.8)	5.44 d (8.0)
H-1′	4.84 d (8.0)	4.83 d (8.0)	4.83 d (8.0)	4.86 d (8.0)	4.85 d (7.8)	4.85 d (8.0)	4.84 d (8.0)	4.84 d (8.0)
H-1″	4.52 d (8.0)	4.52 d (8.0)	4.52 d (8.0)	4.52 d (8.0)	4.52 d (7.8)	4.52 d (8.0)	4.51 d (7.8)	4.52 d (8.0)
other H	4.03–3.30 m	4.03–3.30 m	4.04–3.30 m	4.04–3.30 m	4.060–3.30 m	4.06–3.30 m	4.03–3.30 m	4.05–3.30 m

Table 2
^{13}C NMR chemical shifts (δ) for compounds **6a–h** (D_2O, 75 MHz)

	6a	6b	6c	6d	6e	6f	6g	6h
C-1 phenyl	159.4	158.6	157.0	164.5	160.2	159.7	151.6	157.2
C-2 phenyl	119.5	117.9	110.8	119.4	120.6	112.2	135.2	141.8
C-3 phenyl	132.8	121.4	149.1	129.0	151.7	145.0	142.7	125.0
C-4 phenyl	126.3	135.4	118.1	145.5	116.1	136.7	122.4	144.4
C-5 phenyl	132.8	121.4	130.2	129.0	151.7	127.1	137.8	132.7
C-6 phenyl	119.5	117.9	123.8	119.4	120.6	120.4	126.7	120.6
C-1′,1″	105.5	105.4	105.4	105.4	105.5	104.0	105.4	105.4
C-1	102.8	102.9	101.9	102.1	102.9	101.9	103.7	102.9
C-3	86.8	86.7	86.7	86.5	86.4	86.7	86.1	86.3
C-4′	81.4	81.4	81.4	81.4	81.5	81.4	81.5	81.5
other C[a]	79–71	79–71	79–70	79–70	79–70	79–70	79–70	79-70
C-6,6′,6″	63.4, 63.3, 62.9	63.4, 63.3, 62.9	63.4, 63.3, 62.9	63.4, 63.3, 62.9	63.4, 63.3, 62.9	63.7, 63.3, 61.9	63.4, 63.3, 62.9	63.5, 63.2, 62.9

[a] C-2,2′,2″,3′,3″,4,4″,5,5′,5″.

Table 3
Kinetic parameters of aryl glycosides **6a–d,f,h,i** with *B. licheniformis* 1,3–1,4-β-glucanase at 55 °C

Compound aglycon		Concentration range (mM)	$\Delta\varepsilon$ (M^{-1}/cm) /λ (nm)	pK_a phenol	k_{cat} (s^{-1})	K_M (mM)	$k_{cat}/K_M \times 10^3$ (M^{-1}/s)
6a	P	0.5–12	920/277	9.99	3.0 ± 0.4	7.9 ± 0.6	0.38 ± 0.08
6b	4-BrP	1.3–10	683/288	9.34	3.8 ± 0.4	2.9 ± 0.5	1.31 ± 0.14
6c	3-NP	1.0–11	186/400	8.39	3.9 ± 0.1	6.5 ± 0.4	0.61 ± 0.06
6i	MU	0.4–15	5020/365	7.50	4.2 ± 0.3	3.3 ± 0.4	1.28 ± 0.27
6d	4-NP	1.2–10	6200/400	7.18	4.6 ± 0.5	2.6 ± 0.5	1.72 ± 0.52
6f	3,4-DNP	0.1–1.7	9496/425	5.36	39 ± 12	1.8 ± 0.7	21.4 ± 14.7
6h	2,4-DNP	0.02–1.0	6000/425	3.96	1880 ± 90	0.2 ± 0.03	(9 ± 2)×10^3

Conditions: citrate–phosphate buffer, pH 7.2, 0.1 mM $CaCl_2$, 55 °C.

Table 4
Kinetic parameters of aryl glycosides **6a,c–i** with *B. licheniformis* 1,3–1,4-β-glucanase at 30 °C

Compound aglycon		Concentration range (mM)	$\Delta\varepsilon$ (M^{-1}/cm) /λ (nm)	pK_a phenol	k_{cat} (s^{-1})	K_M (mM)	$k_{cat}/K_M \times 10^3$ (M^{-1}/s)
6a	P	0.7–20	1019/277	9.99	0.49 ± 0.02	7.6 ± 0.4	0.065 ± 0.006
6c	3-NP	0.3–6.5	221/400	8.39	0.85 ± 0.01	1.28 ± 0.04	0.67 ± 0.03
6i	MU	0.3–6.0	5136/365	7.50	0.75 ± 0.02	0.93 ± 0.04	0.80 ± 0.05
6d	4-NP	0.1–6.0	9867/402	7.18	0.81 ± 0.03	0.73 ± 0.05	1.1 ± 0.1
6e	3,5-DNP	0.1–4.1	1671/400	6.69	4.4 ± 0.2	0.46 ± 0.03	9.6 ± 0.9
6f	3,4-DNP	0.07–1.8	9616/400	5.36	14.2 ± 0.2	0.44 ± 0.023	2.0 ± 1.5
6g	2,3-DNP	0.1–4.0	2359/418	4.96	272 ± 6	1.28 ± 0.07	211 ± 15
6h	2,4-DNP	0.02–0.8	8954/400	3.96	590 ± 15	0.18 ± 0.02	3340 ± 300

Conditions: citrate–phosphate buffer, pH 7.2, 0.1 mM $CaCl_2$, 30 °C.

amide to give **5h** in 50% yield after chromatographic purification. Deprotection of **5h** was achieved with hydrogen chloride in anhydrous methanol [25] to afford the target compound **6h** in 67% yield. The β configuration of the glycosidic bond with the aryl aglycons of compounds **6a–h** was ascertained by ^1H NMR spectroscopy, which showed a characteristic doublet at δ 5.11–5.44 ppm with a coupling constant of 8–10 Hz assigned to the anomeric protons of the β-glycosides. Complete NMR data for **6a–h** are summarized in Tables 1 and 2.

Enzyme kinetics.—The aryl glycosides were used as substrates of *Bacillus licheniformis* 1,3–1,4-β-glucanase (recombinant wild-type enzyme expressed in *E. coli* [18]). Michaelis–Menten parameters for the hydrolysis of **6a–h**, as well as of the previously synthesized 4-methylumbelliferyl substrate **6i** [19], were determined by monitoring the release of the chromophoric aglycon by UV spectrophotometry

at the appropriate wavelength as described in the Experimental section. Kinetic results are presented in Table 3 (reactions at 55 °C) and Table 4 (reactions at 30 °C). As previously shown for the 4-methylumbelliferyl glycoside **6i**, substrate inhibition was observed at high substrate concentration. Since no transglycosidation was detected over the entire concentration range (as shown by TLC), the inhibition is likely due to binding of a second molecule of substrate to the binding site (composed of 6 or 7 subsites) [20,23]. Accordingly, data were fitted to an uncompetitive substrate inhibition model [eq (1)] where a second molecule of substrate binds with formation of unproductive ternary complexes ES_2.

$$v_o = \frac{k_{cat}[S][E_o]}{K_M + [S] + \frac{[S]^2}{K_I}} \quad (1)$$

All the aryl glycosides proved to be good substrates of *Bacillus licheniformis* 1,3-1,4-β-glucanase. They undergo a single glycosidic bond cleavage with release of the chromophoric aglycon and the trisaccharide **1** (TLC). Initially, the enzymatic hydrolyses of substrates **6a–d, 6f**, and **6h,i** were studied at 55 °C (Table 3), the temperature of maximum activity of the wild type enzyme with natural polymeric substrates [27]. The 2,4-dinitrophenyl glycoside **6f** is highly reactive; it has the highest k_{cat} and the lowest K_M values. However, the spontaneous hydrolysis (non-enzymatic) at the assay temperature (55 °C) is high. The enzyme concentration in the enzymatic assay had to be raised to obtain initial rates at least one order of magnitude faster than the spontaneous hydrolysis, thus necessitating great care in monitoring of initial reaction rates.

The steady-state kinetic parameters for the 1,3-1,4-β-glucanase-catalyzed hydrolysis of the aryl glycosides at 55 °C show no simple dependence on aglycon acidity. The Hammett plot (log k_{cat} versus pK_a) in Fig. 1(a) is biphasic with an upward curvature at low pK_a values. Only two substrates define the plot in the low pK_a region (**6f** and **6h**), so the slope defining the β value in the Hammett analysis can not be accurately determined.

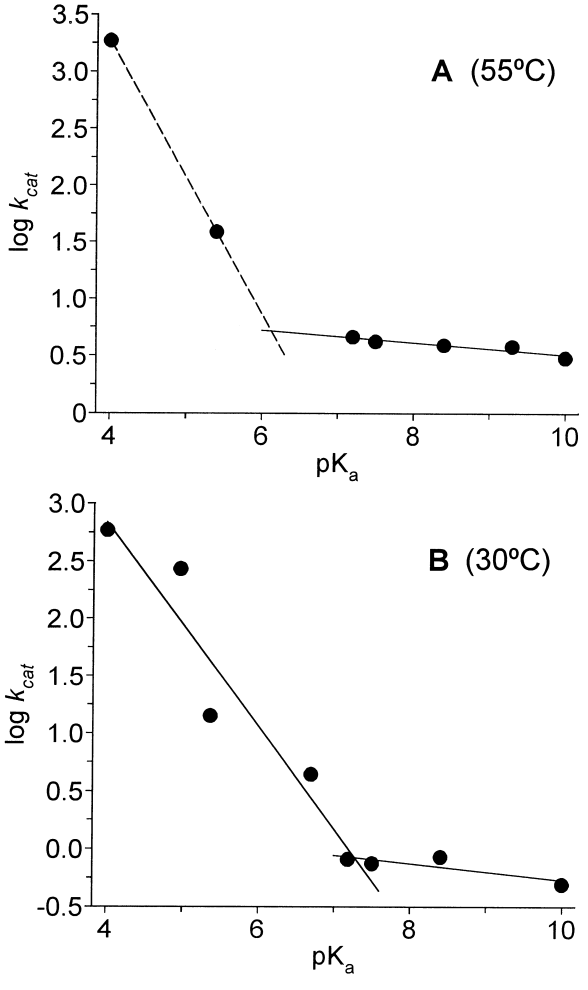


Fig. 1. Hammett relationship for the 1,3–1,4-β-glucanase-catalyzed hydrolysis of aryl glycosides **6a–h**. Data are presented in the form of a Brønsted plot (log k_{cat} versus pK_a of the aglycon phenol). Enzymatic reactions were carried out in citrate–phosphate buffer pH 7.2, 0.1 mM $CaCl_2$, (A) at 55 °C, and (B) at 30 °C.

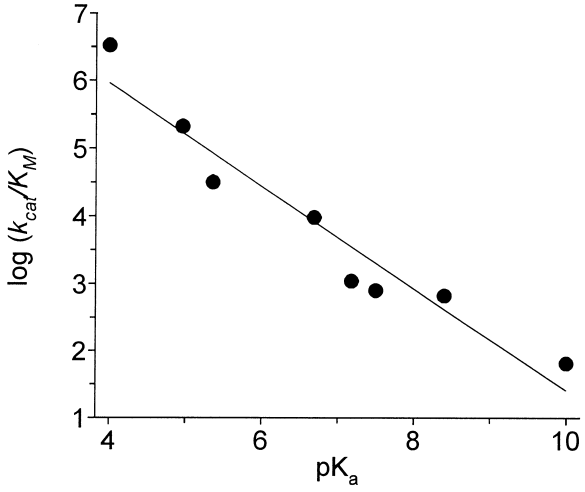
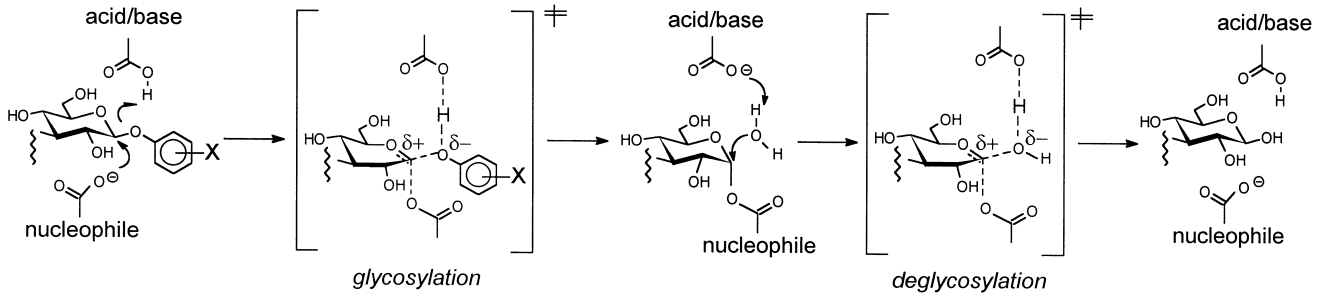


Fig. 2. Hammett plot log (k_{cat}/K_M) versus pK_a for the 1,3–1,4-β-glucanase-catalyzed hydrolysis of aryl glycosides **6a–h** at 30 °C.



Scheme 2.

To assess this behaviour and be able to evaluate the dependence of k_{cat} on aglycon acidity for aryl glycosides with good leaving groups, the kinetic parameters were determined at a lower temperature (30 °C) to reduce spontaneous hydrolysis of the reactive substrates, and two new aryl glycosides with a pK_a of the aglycon <7 (**6e** and **6g**) were included in the analysis. Kinetic data are summarized in Table 4, and the corresponding Hammett plot (log k_{cat} versus pK_a) is shown in Fig. 1(b). Again, the same biphasic plot is observed. Substrates with an aryl aglycon whose pK_a of the free phenol is higher than 7 show essentially no dependence of k_{cat} on aglycon acidity, whereas substrates with good leaving groups (pK_a of the free phenol <7) have increasing k_{cat} values as the pK_a decreases. Linear regression of the data log k_{cat} versus pK_a for the substrates **6d–h** gives a slope of -0.9. The Hammett plot for k_{cat}/K_M is also shown in Fig. 2. Although correlation is poor, data could be fitted to a single line with a slope of -0.8 approximately.

3. Discussion

Retaining glycosidases operate by a double-displacement mechanism [28–31] as shown in Scheme 2. In the first step (*glycosylation*) the amino acid residue acting as a general acid protonates the glycosidic oxygen with concomitant C–O cleavage of the scissile glycosidic bond to give a glycosyl-enzyme intermediate stabilized by the catalytic nucleophile. The second *deglycosylation* step involves the attack of a water molecule assisted by the conjugated base of the general acid to render the hydrolysis product with overall retention of configuration at the anomeric center. Both steps proceed through transition states with oxocarbenium ion character.

The electronic effect of substituents on rates (and equilibria) of organic reactions is formally expressed by the Hammett equation:

$$\log\ k = \text{constant} + \rho\cdot\sigma$$

where k is the rate constant, ρ is the reaction constant, and σ the substituent constant [32,33]. For the process being studied, enzymatic hydrolysis of a series of substituted aryl glycosides, the substituent constant is often expressed by the pK_a of the free phenol, and the Hammett equation is then written in the form of the Brønsted equation:

$$\log\ k = \text{constant} + \beta\cdot pK_a$$

where β is now the reaction constant reflecting the sensitivity of the reaction to the electronic effect of the substituents.

Previous studies with retaining glycosidases have shown that Hammett relationships for the enzyme-catalyzed hydrolyses of aryl glycosides of different leaving group ability fall into the following three categories:

(a) Linear Hammett relationship with negative β value for a wide range of leaving-group pK_a values (e.g. *Cellulomonas fimi* exo-β-1,4-glycanase [7], neuraminidase from influenza A virus [8], *Agrobacterium faecalis* β-glucosidase mutants [34] and *Bacillus circulans* xylanase [12]). The glycosylation step involving glycosidic bond cleavage is then the rate limiting step. A large negative β value reflects a large degree of negative charge accumulation on the phenolate oxygen. It indicates a late transition state with almost complete C–O bond breaking and relatively little proton donation. Conversely, a β value close to

zero may indicate an early transition state at the beginning of C–O bond breaking and significant proton donation.

(b) Biphasic Hammett relationship with a concave-downward shape (e.g. *Agrobacterium faecalis* β-glucosidase [5,35]) that reveals a change in the rate determining step. Less reactive substrates (high pK_a of the phenol) are leaving-group dependent with a negative β value, for which the glycosylation step is rate limiting as in case (a). Log k_{cat} (or log k_{cat}/K_M) levels off for substrates with good leaving groups, and no significant dependence of their reactivity on pK_a is observed. The glycosylation step is not rate determining any more but rather deglycosylation (that is aglycon-independent) becomes rate limiting for these more reactive substrates.

(c) Complex Hammett relationships where low correlations are obtained, the points being badly scattered (e.g. β-galactosidase from *E. coli* [6]). It indicates that electronic effects alone cannot provide an explanation for the variation of k_{cat} with aglycone structure, revealing a dependence on aglycone shape rather than acidity. Also a complex behaviour was obtained for lysozyme, which yielded a strong biphasic convex-upward shape where different mechanisms must obtain for electron-withdrawing and electron-donating substituents [9,10,36].

The observed Hammett relationship for 1,3-1,4-β-glucanase (Fig. 1) does not match any of the previous categories. Based on the reported examples summarized above, the mechanistic interpretation of this unusual behavior is obscure, but several working hypothesis can be proposed for further analysis:

1. The glycosylation step is rate determining for all the studied aryl glycosides; for deglycosylation to become rate limiting k_{cat} would level off for activated substrates. On the other hand, the plot $\log(k_{cat}/K_M)$ versus pK_a (Fig. 2) can be fitted approximately to a single line of slope -0.8, indicating that the first irreversible step, glycosylation, is rate-determining in k_{cat}/K_M. This slope is very similar to that obtained from the leaving group-dependent part of the log k_{cat} versus pK_a plot ($\beta = -0.9$), thus suggesting they monitor the same process.
2. If substrate binding to the active site is equivalent for all the aryl glycosides, e.g. binding constants within the same order of magnitude and the same binding mode in terms of E·S complex structure, the curved plot log k_{cat} versus pK_a then suggests a change in the transition state structure depending on the aglycone. For less reactive aryl glycosides (pK_a of the phenol leaving group > 7) β is approximately zero, consistent with an early transition state with little C–O bond breaking and little charge development. On the other hand, substrates with a pK_a of the phenol leaving group < 7 are aglycon-dependent and β becomes -0.9. It reflects a large degree of negative charge accumulation on the phenolate oxygen that corresponds to a late transition state with extensive C–O bond cleavage. In terms of proton donation to the leaving group, however, the curved plot may also reflect an increase in the degree of proton transfer as the leaving group becomes more basic, with the slope varying from about -0.9 with acidic leaving groups (2,4-dinitrophenyl to 3,5-dinitrophenyl, as would be expected for reaction with little proton donation) to zero or even slightly positive (as is seen with the specific acid-catalyzed hydrolysis of glycosides). Then the situation may be really complicated since both effects, extend of C–O bond cleavage and proton donation may overlap and vary themselves as leaving group acidity changes.
3. The premise of similar binding modes for all the aryl glycosides as stated in (ii) may not hold, rather the substrates may position the aryl aglycons in a different conformation into the active site cleft. Inspection of the structure of the aglycons reveals that those having a pK_a of the free phenol < 7 have a disubstituted phenyl ring (2,4-, 2,3-, 3,4-, and 3,5-dinitro substitution), whereas those having a $pK_a > 7$ are monosubstituted, except for the 4-methylumbelliferone which also has a disubstituted phenyl ring but as a part of a bicyclic system. It might be possible that steric constrains give rise to two different binding modes in subsite $+I$ for both groups of substrates, the aglycon being accommodated in different conformations, thus giving rise to two independent

structure–activity relationships. This is a highly speculative interpretation since no structural evidence on how the leaving group binds into subsite +I—either a glucose unit in the natural substrates or an aryl aglycon in these synthetic substrates—has been obtained yet. Work is in progress to solve the 3D-structure of a mutant enzyme–substrate complex by X-ray crystallography.

The Hammett relationship here obtained represents a novel mechanistic situation for retaining glycosidases. Whether these effects are a consequence of the *endo* action of the enzyme, as *endo*-glycosidases have been studied only sparingly, remains to be evaluated. Nevertheless other mechanistic probes (kinetic isotope effects, pre-steady state kinetics, etc.) should be used to unravel the fine details of the mechanistic pathways of *endo*-glycosidases.

4. Experimental

General.—NMR spectra were recorded on a Varian Gemini 300 spectrometer. ^{1}H chemical shifts (δ) were referenced to internal Me_4Si for solutions in $CDCl_3$, and to internal TSPNa for solutions in D_2O; ^{13}C chemical shifts were referenced to the solvent signal. ^{1}H and ^{13}C NMR data for compounds **6a–h** are listed in Tables 1 and 2. TLC was performed on precoated silica gel 60 F_{254} plates with detection by UV light or by charring with aq 50% H_2SO_4 and heating at 125 °C for 15 min. 1,3-1,4-β-glucanase was obtained from recombinant *Escherichia coli* HB101 harboring a *Bacillus licheniformis* β-glucanase expression plasmid as reported in refs. [18,27]. The protein was purified from the culture supernatant according to ref. [22]. Enzyme concentration was determined by UV spectrophotometry ($\varepsilon_{280} = 0.0145$ μg/mL/cm [37]). Absorbance measurements and kinetics were performed on a Varian Cary 4E spectrophotometer with a thermostated cell holder.

*(2,3,4,6-Tetra-*O*-acetyl-β-*D*-glucopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-*D*-glucopyranosyl)-(1→3)-1,2,4,6-tetra-*O*-acetyl-*D*-glucopyranose* (**2**).—This compound was obtained by enzymatic hydrolysis of barley β-glucan and further acetylation following a modification of the method described by Malet et al. [21]. A suspension of barley flour (100 g) and aq 80% EtOH (600 mL) was vigorously stirred under reflux for 12 h. The suspension was filtered, the solid washed with aq 80% EtOH and dried at room temperature. The solid thus obtained was suspended in H_2O (300 mL) and a fresh extracellular medium of a recombinant *E. coli* culture expressing *Bacillus licheniformis* 1,3-1,4-β-glucanase (100 mL) was added (final concentration of 0.3 U/mL). After incubation in an orbital shaker for 17 h at 55 °C, the supernatant was collected and concentrated up to 150 mL. Oligosaccharides were precipitated stepwise with aq 95% EtOH (2×150 mL), and acetone (450 mL). The solids were collected by centrifugation and dried. The final crude mixture (23 g) was dissolved in 1:1 Ac_2O–pyridine (350 mL) and stirred for 18 h at room temperature. After pouring the reaction mixture on ice-water (1.5 L), the yellow precipitate was collected by filtration, washed with H_2O, and purified by flash chromatography (1:2→1:1 EtOAc–$CHCl_3$), to yield **2** (6.78 g) and peracetylated 3-*O*-β-cellotriosyl-D-glucopyranose (1.24 g).

*General synthesis of aryl (2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-*D*-glucopyranosyl)-(1→3)-2,4,6-tri-*O*-acetyl-β-*D*-glucopyranosides* (**5a–g**).—The α-glycosyl bromide **3** was prepared as described in [21] by treatment of **2** with 4.1 M HBr in HOAc for 30 min at room temperature. After usual work-up, the product (98% yield) was used immediately in the next glycosidation steps without further purification. The purity of the crude product was controlled by TLC and ^{1}H NMR ($CDCl_3$; δ 6.5, d, 1 H, $J_{1,2}$ 4 Hz , H-1). A solution of **3** (9.86 g, 10.0 mmol) in CH_2Cl_2 (100 mL) was added to the corresponding phenol (20.0 mmol) and triethylbenzylammonium bromide (2.30 g, 8.46 mmol) in aq 5% NaOH (20 mL). The biphasic system was heated under reflux with vigorous stirring for 4 to 5 h. The mixture was diluted with H_2O (40 mL), and the aqueous layer extracted with CH_2Cl_2. The combined organic phases were washed with aq 5% NaOH, neutralized with diluted HCl, washed with H_2O, and dried ($MgSO_4$). After evaporation of the solvent, chromatographic separation gave **5a–g** in 25–65% yields.

*Phenyl (2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-*D*-glucopyranosyl)-(1→3)-2,4,6-tri-*O*-acetyl-β-*D*-glucopyranoside* (**5a**).—NMR: ^{1}H (300 MHz, $CDCl_3$), δ 1.98–2.14 (10 s, 30 H, 10 Ac), 3.62–4.42 and 4.82–5.15 (H-2,2′,2″,3′,3″, 4,4′,4″,5,5′,5″, 6a,6′a,6″a,6b,6′b,6″b), 4.47 (d, 1 H,

$J_{1,2}$ 8.0 Hz, H-1″), 4.59 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1′), 5.05 (d, 1 H, $J_{1,2}$ 10.0 Hz, H-1), 5.26 (dd, 1 H, $J_{2,3}=J_{3,4}=8.0$ Hz, H-3), 6.95 (d, 2 H, $J_{2,3}$ 8.5 Hz, phenyl H-2,6), 7.06 (t, 1 H, $J_{3,4}=J_{4,5}=7.5$ Hz, phenyl H-4), 7.29 (dd, 2 H, $J_{2,3}=J_{3,4}=8.0$ Hz, phenyl H-3,5); ^{13}C (75 MHz, $CDCl_3$), δ 20.4–20.9 ($COCH_3$), 61.5 and 62.2 (C-6,6′,6″), 67.7, 68.2, 71.1, 71.6, 72.0, 72.5, 72.6, 72.7, and 72.8 (C-2,2′,2″,3′,3″,4,4″,5,5′,5″), 76.2 and 78.8 (C-3,4′), 99.1 (C-1), 100.8 and 100.9 (C-1′,1″), 116.8 (phenyl C-2,6), 123.2 (phenyl C-4), 129.5 (phenyl C-3,5), 156.9 (phenyl C-1), 168.8–170.7 ($COCH_3$).

4-Bromophenyl (2,3,4,6-tetra-O-*acetyl-β*-D-*glucopyranosyl)-(1→4)-(2,3,6-tri*-O-*acetyl-β*-D-*glucopyranosyl)-(1→3)-2,4,6-tri*-O-*acetyl-β*-D-*glucopyranoside* (**5b**).—NMR: ^{1}H (300 MHz, $CDCl_3$), δ 1.96–2.19 (10 s, 30 H, 10 Ac), 3.61–4.42 and 4.82–5.16 (H-2,2′,2″,3′,3″,4,4′,4″,5,5′,5″,6a,6′a,6″a,6b,6′b,6″b), 4.47 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1″), 4.59 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1′), 5.07 (d, 1 H, $J_{1,2}$ 10.0 Hz, H-1), 5.24 (dd, 1 H, $J_{2,3}=J_{3,4}=8.0$ Hz, H-3), 6.84 (d, 1 H, $J_{2,3}$ 9.0 Hz, phenyl H-2), 7.39 (d, 2 H, $J_{2,3}$ 9.0 Hz, phenyl H-3); ^{13}C (75 MHz, $CDCl_3$), δ 20.5–20.9 ($COCH_3$), 61.5 and 62.1 (C-6,6′,6″), 67.7, 68.1, 71.1, 71.6, 72.0, 72.1, 72.4, 72.6, 72.7, and 72.8 (C-2,2′,2″,3′,3″,4,4″,5,5′,5″), 76.2 and 78.8 (C-3,4′), 99.1 (C-1), 100.8 and 100.9 (C-1′,1″), 115.8 (phenyl C-2,6), 118.7 (phenyl C-4), 132.4 (phenyl C-3,5), 155.9 (phenyl C-1), 168.8–170.6 ($COCH_3$).

3-Nitrophenyl (2,3,4,6-tetra-O-*acetyl-β*-D-*glucopyranosyl)-(1→4)-(2,3,6-tri*-O-*acetyl-β*-D-*glucopyranosyl)-(1→3)-2,4,6-tri*-O-*acetyl-β*-D-*glucopyranoside* (**5c**).—NMR: ^{1}H (300 MHz, $CDCl_3$), δ 1.99–2.17 (10 s, 30 H, 10 Ac), 3.62–4.43 and 4.63–5.16 (H-2,2′,2″,3′,3″,4,4′,4″,5,5′,5″,6a,6′a,6″a,6b,6′b,6″b), 4.48 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1″), 4.59 (d, 1 H, $J_{1,2}$ 8.0, H-1′), 5.03 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 5.30 (dd, 1 H, $J_{2,3}=J_{3,4}=8.5$ Hz, H-3), 7.30 (d, 1 H, $J_{5,6}$ 8.0 Hz, phenyl H-6), 7.47 (dd, 1 H, $J_{4,5}=J_{5,6}=8.0$ Hz, phenyl H-5), 7.83 (dd, 1 H, $J_{2,4}=J_{2,6}=3.0$ Hz, phenyl H-2), 7.95 (dd, 1 H, $J_{3,4}$ 8.0, $J_{2,4}$ 3.0 Hz, phenyl H-4); ^{13}C (75 MHz, $CDCl_3$), δ 20.3–20.9 ($COCH_3$), 67.7, 68.0, and 68.2 (C-6,6′,6″), 71.1, 71.4, 71.6, 72.0, 72.2, 72.4, 72.6, 72.7, 72.8, and 72.9 (C-2,2′,2″,3′,3″,4,4″,5,5′,5″), 76.2, and 78.6 (C-3,4′), 98.0 (C-1), 100.8 and 101.0 (C-1′,1″), 110.8 (phenyl C-2), 118.1 (phenyl C-4), 123.8 (phenyl C-6), 130.2 (phenyl C-5), 149.1 (phenyl C-3), 157.0 (phenyl C-1), 168.1–170.7 ($COCH_3$).

4-Nitrophenyl (2,3,4,6-tetra-O-*acetyl-β*-D-*glucopyranosyl)-(1→4)-(2,3,6-tri*-O-*acetyl-β*-D-*glucopyranosyl)-(1→3)-2,4,6-tri*-O-*acetyl-β*-D-*glucopyranoside* (**5d**).—NMR: ^{1}H (300 MHz, $CDCl_3$), δ 1.99–2.16 (10 s, 30 H, 10 Ac), 3.60–4.35 and 4.63–5.17 (H-2,2′,2″,3′,3″,4,4′,4″,5,5′,5″,6a,6′a,6″a,6b,6′b,6″b), 4.48 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1″), 4.60 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1′), 5.09 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 5.29 (dd, 1 H, $J_{2,3}=J_{3,4}=8.0$ Hz, H-3), 7.04 (d, 2 H, $J_{2,3}$ 9.6 Hz, phenyl H-2), 8.20 (d, 2 H, $J_{2,3}$ 9.6 Hz, phenyl H-3); ^{13}C (75 MHz, $CDCl_3$), δ 20.3–20.8 ($COCH_3$), 61.5, 62.0, and 62.1 (C-6,6′,6″), 67.7, 67.9, 71.1, 71.6, 72.0, 72.1, 72.4, 72.7, 72.8, and 72.9 (C-2,2′,2″,3′,3″,4,4″,5,5′,5″), 76.2 and 78.6 (C-3,4′), 98.0 (C-1), 100.8 and 100.9, (C-1′,1″), 116.5 (phenyl C-2,6), 125.7 (phenyl C-3,5), 143.2 (phenyl C-4), 161.2 (phenyl C-1), 168.1–170.4 ($COCH_3$).

3,5-Dinitrophenyl (2,3,4,6-tetra-O-*acetyl-β*-D-*glucopyranosyl)-(1→4)-(2,3,6-tri*-O-*acetyl-β*-D-*glucopyranosyl)-(1→3)-2,4,6-tri*-O-*acetyl-β*-D-*glucopyranoside* (**5e**).—NMR: ^{1}H (300 MHz, $CDCl_3$), δ 1.97–2.21 (10 s, 30 H, 10 Ac), 3.58–4.46 and 4.82–5.17 (H-2,2′,2″,3′,3″,4,4′,4″,5,5′,5″,6a,6′a,6″a,6b,6′b,6″b), 4.48 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1″), 4.60 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1′), 5.08 (d, 1 H, $J_{1,2}$ 9.3 Hz, H-1), 5.33 (dd, 1 H, $J_{2,3}=J_{3,4}=9.3$ Hz, H-3), 8.15 (d, 2 H, $J_{2,4}$ 2.1 Hz, phenyl H-2,6), 8.77 (dd, 1 H, $J_{2,4}=J_{4,6}=1.8$ Hz, phenyl H-4); ^{13}C (75 MHz, $CDCl_3$), δ 20.2–20.9 ($COCH_3$), 61.5, 62.1, and 62.2 (C-6,6′,6″), 67.7, 67.8, 71.0, 71.6, 71.9, 72.0, 72.6, 72.8, 72.8, and 73.0 (C-2,2′,2″,3′,3″,4,4″,5,5′,5″), 76.2, and 78.5 (C-3,4′), 98.4 (C-1), 100.8, and 101.0 (C-1′,1″), 113.0 (phenyl C-4), 117.2 (phenyl C-2,6), 149.1 (phenyl C-3,5), 157.3 (phenyl C-1), 168.5–170.7 ($COCH_3$).

3,4-Dinitrophenyl (2,3,4,6-tetra-O-*acetyl-β*-D-*glucopyranosyl)-(1→4)-(2,3,6-tri*-O-*acetyl-β*-D-*glucopyranosyl)-(1→3)-2,4,6-tri*-O-*acetyl-β*-D-*glucopyranoside* (**5f**).—NMR: ^{1}H (300 MHz, $CDCl_3$), δ 1.99–2.17 (10 s, 30 H, 10 Ac), 3.62–4.36 and 4.83–5.17 (H-2,2′,2″,3′,3″,4,4′,4″,5,5′,5″,6a,6′a,6″a,6b,6′b,6″b), 4.47 (d, 1 H, $J_{1,2}$ 9.0 Hz, H-1″), 4.59 (d, 1 H, $J_{1,2}$ 9.0 Hz, H-1′), 5.09 (d, 1 H, $J_{1,2}$ 9.0 Hz, H-1), 5.29 (dd, 1 H, $J_{2,3}=J_{3,4}=8.0$ Hz, H-3), 7.23 (dd, 1 H, $J_{2,6}$ 3.5, $J_{5,6}$ 9.0 Hz, phenyl H-6), 7.36 (d, 1 H, $J_{2,6}$ 3.5 Hz, phenyl H-2), 7.99 (d, 1 H, $J_{5,6}$ 10.0 Hz, phenyl H-5); ^{13}C (75 MHz, $CDCl_3$), δ 20.3–20.8 ($COCH_3$), 61.5 and 62.1 (C-6,6′,6″), 67.7, 67.8, 71.1, 71.9, 72.0, 72.6, 72.8, and 72.9 (C-2,2′,2″,3′,3″,4,4″,5,5′,5″), 76.2 and 78.3 (C-3,4′), 98.0 (C-1), 100.8 and 101.0 (C-1′,1″), 112.2 (phenyl C-2), 120.4 (phenyl C-6), 127.1 (phenyl C-5), 136.7 (phenyl C-4), 145.0 (phenyl C-3), 159.7 (phenyl C-1), 168.7–170.5 ($COCH_3$).

2,3-Dinitrophenyl (2,3,4,6-tetra-O-*acetyl-β*-D-*glucopyranosyl)-(1→4)-(2,3,6-tri*-O-*acetyl-β*-D-*glucopyr-*

anosyl)-(1→3)-2,4,6-tri-O-*acetyl-β*-D-*glucopyranoside* (**5g**).—NMR: ^{1}H (300 MHz, $CDCl_3$), δ 1.98–2.24 (10 s, 30 H, 10 Ac), 3.56–4.42 and 4.83–5.29 (H-2,2′,2″,3′,3″,4,4′,4″,5,5′,5″,6a,6′a,6″a,6b,6′b,6″b), 4.47 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1″), 4.58 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1′), 5.00 (d, 1 H, $J_{1,2}$ 9.3 Hz, H-1), 5.26 (dd, 1 H, $J_{2,3}=J_{3,4}=9.0$ Hz, H-3), 7.62 (dd, 1 H, $J_{4,5}=J_{5,6}=8.1$ Hz, phenyl H-5), 7.71 (d, 1 H, $J_{5,6}$ 7.5 Hz, phenyl H-6), 7.99 (d, 1 H, $J_{4,5}$ 8.1 Hz, phenyl H-4); ^{13}C (75 MHz, $CDCl_3$), δ 20.4–20.7 ($COCH_3$), 61.5, 61.7, and 62.1 (C-6,6′,6″), 67.7, 67.8, 71.0, 71.5, 71.6, 72.0, 72.7, 72.7, 72.7, and 72.9 (C-2,2′, 2″,3′,3″,4,4″,5,5′,5″), 76.1 and 78.2 (C-3,4′), 100.8 (C-1), 101.0 and 101.0 (C-1′,1″), 120.2 (phenyl C-4), 126.4 (phenyl C-6), 130.9 (phenyl C-2), 137.2 (phenyl C-5), 140.0 (phenyl C-3), 148.6 (phenyl C-1), 168.9–170.5 ($COCH_3$).

(2,3,4,6-Tetra-O-*acetyl-β*-D-*glucopyranosyl)-(1→4)-(2,3,6-tri*-O-*acetyl-β*-D-*glucopyranosyl)-(1→3)-2,4,6-tri*-O-*acetyl*-D-*glucopyranose* (**7**).—A solution of peracetylated trisaccharide **2** (6.00 g, 6.21 mmol) in DMF (25 mL) was treated with hydrazine acetate (720 mg, 7.82 mmol) at 50 °C with stirring during 8 min. The reaction mixture was diluted with EtOAc (200 mL), washed with aq satd NaCl and H_2O, and dried ($MgSO_4$). Evaporation of the solvent afforded **7** as a white solid (4.83 g, 97%). NMR: ^{1}H (300 MHz, $CDCl_3$), δ 1.99–2.14 (10 s, 30 H, 10 Ac), 3.62–5.26 (H-2,2′,2″,3′,3″,4,4′,4″,5,5′, 5″,6a,6′a,6″a,6b,6′b,6″b), 4.54 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1″), 4.63 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1′), 5.14 (d, 1/5 H, $J_{1,2}$ 9.3 Hz, H-1β), 5.37 (d, 4/5 H, $J_{1,2}$ 3.6 Hz, H-1α), 5.51 (dd, 1 H, $J_{2,3}=J_{3,4}=9.6$ Hz, H-3).

2,4-Dinitrophenyl (2,3,4,6-tetra-O-*acetyl-β*-D-*glucopyranosyl)-(1→4)-(2,3,6-tri*-O-*acetyl-β*-D-*glucopyranosyl)-(1→3)-2,4,6-tri*-O-*acetyl-β*-D-*glucopyranoside* (**5h**).—Compound **7** (5.18 g, 6.44 mmol) and diazabicyclo[2.2.2]octane (2.53 g, 22.43 mol) in anhydrous DMF (75 mL) were stirred for 3 h under Ar in the presence of 4 Å molecular sieves. 1-Fluoro-2,4-dinitrobenzene (1.59 g, 8.27 mmol) was added, and the mixture was stirred for 18 h. The mixture was filtered through Celite, $CHCl_3$ (200 mL) was added, and the liquid was distilled off. The resulting syrup was dissolved into $CHCl_3$, washed with aq $NaHCO_3$, dried ($MgSO_4$), and the solvent was evaporated. The solid was purified by column chromatography to yield **5h** (3.54 g, 50%). NMR: ^{1}H (300 MHz, $CDCl_3$), δ 1.98–2.22 (10 s, 30 H, 10 Ac), 3.59–4.43, and 4.83–5.16 (H-2,2′,2″, 3′,3″,4,4′,4″,5,5′,5″,6a,6′a,6″a,6b,6′b,6″b), 4.48 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1″), 4.60 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1′), 5.35 (dd, 1 H, $J_{2,3}=J_{3,4}=9.5$ Hz, H-3), 7.48 (d, 1 H, $J_{5,6}$ 9.6 Hz, phenyl H-6), 8.40 (dd, 1 H, $J_{3,5}$ 2.4, $J_{5,6}$ 9.6 Hz, phenyl H-5), 8.68 (d, 1 H, $J_{3,5}$ 2.4 Hz, phenyl H-3); ^{13}C (75 MHz, $CDCl_3$), δ 20.4–20.8 (CH_3), 61.5, 61.7, and 62.1 (C-6,6′,6″), 67.7, 67.8, 71.0, 71.3, 71.6, 72.0, 72.6, and 72.8 (C-2,2′,2″,3′,3″,4,4″,5,5′,5″), 76.2, and 78.2 (C-3,4′), 99.1 (C-1), 100.8, and 101.0 (C-1′,1″), 119.6 (phenyl C-6), 121.2 (phenyl C-3), 128.4 (phenyl C-5), 140.7 (phenyl C-2), 142.5 (phenyl C-4), 153.4 (phenyl C-1), 168.7–170.5 (CO).

General synthesis of aryl β-cellobiosyl-β-D-*glucopyranosides* (**6a–h**) *by deacetylation of* **5a–h**.—(a) NaOMe in MeOH deacetylation: To a stirred solution of the specific chromophoric oligosaccharide **5a–f** (3.37 mmol) in anhydrous MeOH (114 mL) was added 0.1 M NaOMe in MeOH (28 mL), and the mixture was stirred in the dark for 4.5–24 h at room temperature. The solid formed was filtered and washed with cold MeOH to yield **6a–f**. An additional fraction of **6a–f** was obtained from the mother liquors after neutralization with excess Amberlite IR 120 ($H^{\oplus}$) followed by chromatography, with an overall yield of 60–94%, except for **6f** (30% yield). Deacetylation of **5f** to afford **6f** was achieved in higher yield following method (b) (see below).

(b) HCl in MeOH deacetylation: A solution of compounds **5f–h** (3.20 mmol) in anhydrous MeOH (100 mL) was treated in the dark with 26% HCl in anhydrous MeOH (75 mL) under Ar at 4 °C. After 16 h, a new aliquot of HCl in MeOH (25 mL) was added, and the system was stirred for 2 h. The solvent was distilled off at low temperature (< 20 °C) and the resulting syrup was treated with Et_2O. The solid was washed twice with cold acetone and crystallized from MeOH-acetone to afford **6f–h** (50–70% yields).

^{1}H and ^{13}C NMR data for compounds **6a–h** are given in Tables 1 and 2, respectively.

Phenyl β-D-*glucopyranosyl-(1→4)-β*-D-*glucopyranosyl-(1→3)-β*-D-*glucopyranoside* (**6a**).—mp (MeOH) 235–250 °C (dec.); $[\alpha]_D^{20}$ −35.5 (*c* 0.0984, water). Anal. Calcd for $C_{24}H_{36}O_{16}\times 2H_2O$: C, 46.75; H, 6.54. Found: C, 46.29; H, 6.49.

4-Bromophenyl β-D-*glucopyranosyl-(1→4)-β*-D-*glucopyranosyl-(1→3)-β*-D-*glucopyranoside* (**6b**).—mp (MeOH) 215–220 °C; $[\alpha]_D^{20}$ n.d. Anal. Calcd for $C_{24}H_{35}O_{16}Br_1\times 4H_2O$: C, 39.52; H, 5.67. Found: C, 39.71; H, 5.32.

3-Nitrophenyl β-D-*glucopyranosyl-(1→4)-β*-D-*glucopyranosyl-(1→3)-β*-D-*glucopyranoside* (**6c**).—

mp (MeOH) 233–234 °C; $[\alpha]_D^{20}$ −43.4° (*c* 0.09675, water). Anal. Calcd for $C_{24}H_{35}O_{18}N_1 \times 4H_2O$: C, 41.34; H, 6.21; N, 2.01. Found: C, 41.34; H, 6.08; N, 1.90.

4-Nitrophenyl β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside (**6d**).—mp (EtOH–H_2O) 187–189 °C (dec.); $[\alpha]_D^{20}$ −35.54° (*c* 0.110, water). Anal. Calcd for $C_{24}H_{35}O_{18}N_1 \times 4H_2O$: C, 41.34; H, 6.21; N, 2.01. Found: C, 40.92; H, 5.98; N, 1.95.

3,5-Dinitrophenyl β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside (**6e**).—mp (MeOH) 163–165 °C (dec.); $[\alpha]_D^{20}$ −51.43° (*c* 0.101, water). Anal. Calcd for $C_{24}H_{34}O_{20}N_2 \times 3H_2O$: C, 39.78; H, 5.56; N, 3.87. Found: C, 39.34; H, 5.48; N, 3.63.

3,4-Dinitrophenyl β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside (**6f**).—mp (MeOH) 180–185 °C (dec.); $[\alpha]_D^{20}$ −36.8° (*c* 0.09785, water). Anal. Calcd for $C_{24}H_{34}O_{20}N_2 \times 3H_2O$: C, 39.78; H, 5.56; N, 3.87. Found: C, 39.58; H, 5.09; N, 3.48.

2,3-Dinitrophenyl β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside (**6g**).—mp (MeOH) 169–171 °C (dec.); $[\alpha]_D^{20}$ −50.48° (*c* 0.105, water). Anal. Calcd for $C_{24}H_{34}O_{20}N_2 \times H_2O$: C, 41.87; H, 5.27; N, 4.07. Found: C, 41.43; H, 5.02; N, 4.13.

2,4-Dinitrophenyl β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside (**6h**).—mp (MeOH–MeCOMe) 225–228 °C (dec.); $[\alpha]_D^{20}$ −65.4° (*c* 1.000, water). Anal. Calcd for $C_{24}H_{34}O_{20}N_2 \cdot 1/2\ H_2O$: C, 42.40; H, 5.19; N, 4.12. Found: C, 42.41; H, 5.30; N, 4.01.

Enzyme kinetics.—Absortivity coefficients of the chromophoric substrates and aglycons were determined at the appropriate wavelength under the same conditions (temperature, buffer, pH and ionic strength) used in the enzyme kinetic experiments [wavelength and $\Delta\varepsilon$ ($= \varepsilon_{phenol} - \varepsilon_{substrate}$) for **6a–i** are listed in Table 3 (at 55 °C) and Table 4 (at 30 °C)]. Kinetic constants for the enzyme-catalyzed hydrolysis of **6a–i** were determined by measuring the release of the aglycon [23]. All experiments were run at 55 or 30 °C in 6.5 mM citrate–87 mM phosphate buffer (pH 7.2) in the presence of 0.1 mM $CaCl_2$ at substrate concentrations ranging from $0.25 \times K_M$ to $8.5 \times K_M$. The reactions were started by addition of enzyme to a 5 min pre-incubated mixture of buffer, $CaCl_2$ and substrate in the case of **6a–f,i**, or by addition of substrate in the case of **6h**. Initial velocities were derived from the slope Abs versus time after correction for blank rates (non-enzymatic hydrolysis). Kinetic parameters were calculated by fitting the data to eq (1) by non-linear regression.

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