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Note

1-*O*-Acetyl-β-D-galactopyranose: a novel substrate for the transglycosylation reaction catalyzed by the β-galactosidase from *Penicillium* sp.

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Abstract

1-*O*-Acetyl-β-D-galactopyranose (AcGal), a new substrate for β-galactosidase, was synthesized in a stereoselective manner by the trichloroacetimidate procedure. Kinetic parameters (K_M and k_{cat}) for the hydrolysis of 1-*O*-acetyl-β-D-galactopyranose catalyzed by the β-D-galactosidase from *Penicillium* sp. were compared with similar characteristics for a number of natural and synthetic substrates. The value for k_{cat} in the hydrolysis of AcGal was three orders of magnitude greater than for other known substrates. The β-galactosidase hydrolyzes AcGal with retention of anomeric configuration. The transglycosylation activity of the β-D-galactosidase in the reaction of AcGal and methyl β-D-galactopyranoside (**1**) as substrates was investigated by ^{1}H NMR spectroscopy and HPLC techniques. The transglycosylation product using AcGal as a substrate was β-D-galactopyranosyl-(1→6)-1-*O*-acetyl-β-D-galactopyranose (with a yield of ~70%). In the case of **1** as a substrate, the main transglycosylation product was methyl β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside. Methyl β-D-galactopyranosyl-(1→3)-β-D-galactopyranoside was found to be minor product in the latter reaction.

Keywords: β-D-Galactosidase; *Penicillium* sp.; 1-*O*-Acetyl-β-D-galactopyranose; Transglycosylation; Disaccharides

1. Introduction

In recent years, enzymatic syntheses using glycosidases that possess transglycosylation activity are becoming more common.[1,2] This approach has the advantage of regioselective and stereoselective reactions without the protection and deprotection processes required with synthetic chemical methods.[3,4] Enzymatic synthesis has been utilized to obtain neoglycoproteins and neoglycoconjugates for use in medicine and other fields.[5,6] Enzymatic transglycosylation, the reverse of hydrolysis effected by hydrolases, has been more widely used than glycosyltransferase reactions since glycosidases are frequently used in the preparation of a variety of transglycosylation products.[7,8]

β-D-Galactosidases from various sources have been successfully applied in the enzymatic syntheses via transglycosylation of biologically active galactooligosaccharides. The β-D-galactosidase from *Bacillus circulans* was used in the synthesis of *N*-acetyllactosamine [β-D-Gal-(1→4)-D-GlcNAc], the main component of oligosaccharides from human milk.[9] Transglycosylation activity of the β-D-galactosidases from several sources was also used in the synthesis of β-D-Gal-(1→3)-D-GlcNAc,[10,11] the key oligosaccharide of sialyl Lewis a (sLe^a), which has been reported to be the ligand involved in the metastasis of cancer cells.[12] Also of note, β-D-Gal-(1→3)-D-GlcNAc is a component of the mucin-type glycoprotein side chains.[13] Finally, the β-D-galactosidase from *E. coli* was employed in the enzymatic synthesis of some di-β-D-galactosyl glycopeptides.[14]

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The choice of donor in the transglycosylation reactions is important, as it affects the yield of the product. Therefore, the search for new donors, as well as new glycosidases having high transglycosylation activity, is essential for increasing the yield and improving the stereospecificity of the reaction.[15,16]

In this work, we describe the synthesis of 1-*O*-acetyl-β-D-galactopyranose (AcGal), a novel substrate for β-D-galactosidases, and the transglycosylation reactions of this substrate. To evaluate the suitability of this compound for enzymatic syntheses, we used the β-D-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) having transglycosylation activity, isolated from filamentous fungi *Penicillium* sp. and for which preliminary investigation by X-ray crystallography has been reported.[17]

2. Results and discussion

Synthesis of 1-O-*acetyl-β*-D-*galactopyranose*.—To obtain a 1-*O*-acylated sugar, two distinct problems must be solved. First, a protective group removable under neutral conditions (such as Bn, chloroacetyl) is needed because of the hydrolytic liability of an anomeric acyl group. Second, if a specific anomer is required, the acyl group should be introduced stereoselectively or, at least, the two anomers should be readily separable.

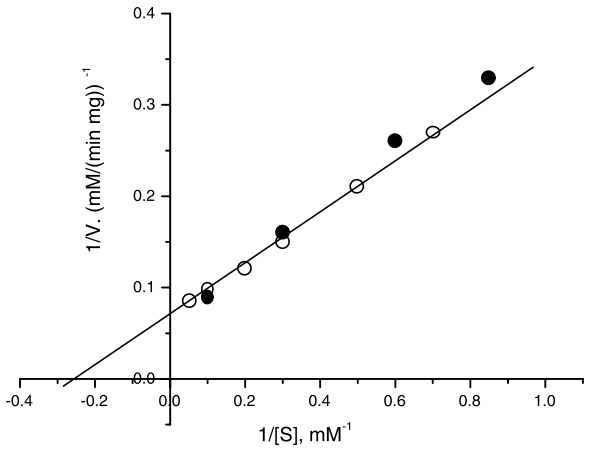

Fig. 1. Analysis of the hydrolysis of AcGal as a function of its concentration by the Lineweaver–Burk method: ○, data obtained by the HPLC method; ●, NMR data.

Table 1
Kinetic parameters of reactions catalyzed by β-galactosidase from *Penicllium* sp.

Substrate	K_m (mM)	k_{cat} (mmol min^{-1} mg^{-1})
p-Nitrophenyl β-D-galactopyranoside	1.0	0.125
Methyl β-D-galactopyranoside (**1**)	5.9	0.00067
1-*O*-Acetyl-β-D-galactopyranose (AcGal)	3.9	14
Lactose	11.1	0.04

Per(chloroacetylated) glucopyranosyl bromide reacts with silver carboxylates in CH_2Cl_2 to give 1-*O*-acyl derivatives in good yield and with excellent β-selectivity, although the yields at the deprotection step are moderate.[18] Most of the known 1-*O*-acyl derivatives of glucose were obtained via acylation of 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose, usually with poor stereoselectivity. The reaction of ethyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-galactopyranoside with *N*-iodosuccinimide and AcOH in CH_2Cl_2 was reported to give the 1-acetate in excellent yield as a 1:1 mixture of anomers that was not separated.[19]

The reaction of *O*-glycosyl trichloroacetimidates with carboxylic acids in a non-polar solvent affords 1-*O*-acyl sugars in high yield and complete inversion of configuration.[20,21] Since both anomers of 2,3,4,6-tetra-*O*-benzyl-D-galactopyranosyl 2,2,2-trichloroacetimidate are readily available,[22,23] we chose to apply the trichloroacetimidate method for synthesis of 1-*O*-acetyl-β-D-galactopyranose. Action of AcOH on 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl 2,2,2-trichloroacetimidate[22] in CH_2Cl_2 afforded the 1-*O*-acetylated product in 76% yield following column chromatography. To our surprise it contained significant amounts of the α anomer (10:1 β/α by NMR), non-detectable by TLC because of small differences in R_f, irrespective of the eluent used. We were able to isolate the desired β anomer by crystallization, which lowered the yield to 51%. Hydrogenolytic debenzylation gave crystalline 1-*O*-acetyl-β-D-galactopyranose in quantitative yield.

Analysis of the enzymatic hydrolysis of 1-O-*acetyl-β*-D-*galactopyranose*.—The β-galactosidase from *Penicillium* sp. catalyzes the hydrolysis of 1-*O*-acetyl-β-D-galactopyranose. D-Galactose and acetic acid were found by direct ^{1}H NMR examination to be the products of the hydrolysis (not accounting for the transglycosylation process). As shown in Fig. 1, the dependence of the initial hydrolytic rate on the substrate concentration over the range from 10 to 1 mM obeys Michaelis–Menten kinetics and can be expressed by Lineweaver–Burk presentation of the data. The kinetic parameters K_m and k_{cat} (Table 1) were evaluated by direct analysis of the reaction course in ^{1}H NMR experiments and by HPLC analysis of liberated D-galactose, as described in the Section 3. Independent experiments showed that spontaneous breakage of the *O*-acetyl linkage in AcGal did not take place under the conditions of enzymatic hydrolysis. AcGal was found to be a competitive inhibitor in the two-substrate process of hydrolysis of *p*-nitrophenyl β-D-galactopyranoside (**2**), with the inhi-

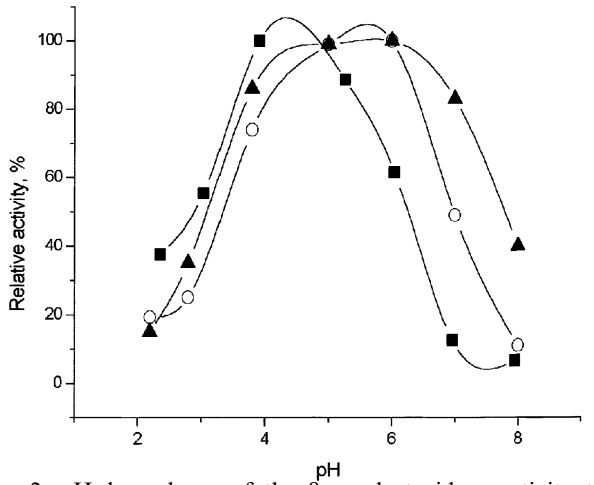

Fig. 2. pH-dependence of the β-D-galactosidase activity toward AcGal (■), **1** (○), **2** (▲).

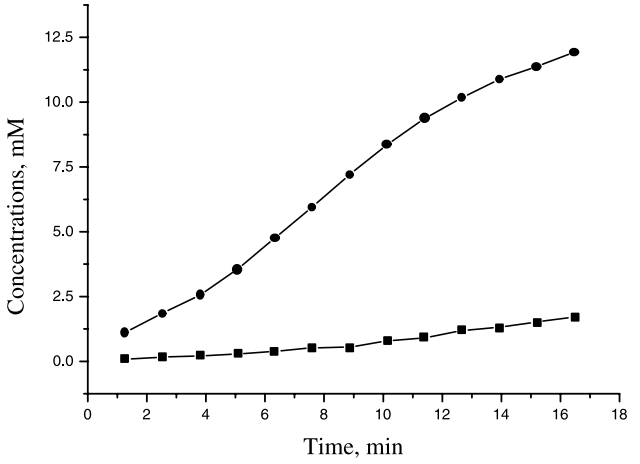

Fig. 3. Time course of D-galactose α (■) and β anomer (●) formation during the hydrolysis of AcGal.

bition constant (4.1 mM) close to the K_m value for the hydrolysis of AcGal (Table 1). Consequently, we can conclude that AcGal binds to the normal catalytic center β-D-galactosidase. As shown in Fig. 2, the pH optimum for the hydrolysis of AcGal coincides with that of the well-known substrates, **1** and **2**.

Stereochemical course of action.—Application of ^{1}H NMR provides a direct approach for determining the stereochemical course of hydrolysis catalyzed by glycanases.[24] Chemical shifts and coupling constants of the anomeric protons in α- and β-galactosides and in the product hemiacetals are distinct and readily observed. When sufficient quantities of the β-galactosidase was used to complete the hydrolysis of AcGal within 15 min, the initially formed β anomer could be detected before mutarotation had occurred to any significant extent. The time-course of the appearance of the α and β anomers of D-galactose resulting from hydrolysis of AcGal with the β-galactosidase from *Penicillium* is presented in Fig. 3. From these data, it is apparent that the β anomer of D-galactose is formed at the initial stage of the hydrolysis. At later stages, the α anomer appears as a result of mutarotation. Mutarotation was considered to be complete when the anomeric ratio ($\approx 30\%$ α, $\approx 70\%$β) was established (~ 20 h).[25] These data unequivocally demonstrate that hydrolysis catalyzed by the enzyme proceeds with the retention of the anomeric configuration.

Analysis of steady-state kinetics of the hydrolytic reaction catalyzed by the β-D-galactosidase.—Kinetics of the β-D-galactosidase hydrolysis of **1**, lactose, and **2** obeys Lineweaver–Burk equations in the concentration range from 20 to 5 mM for lactose, from 20 to 2 mM for **1**, and from 2 to 0.2 mM for **2** (Table 1). As may be seen from the data, the k_{cat} value in the hydrolysis of AcGal is 350 times higher than the corresponding value for lactose and 17,500 times higher than for **1**. It is about 100 times higher than the maximal velocity for the 'fastest' β-galacosidase substrate, **2**. At the same time, values for K_M in the hydrolysis of AcGal and galactooligosaccharides are quite close.

Glycosidases operate by a double-displacement mechanism in which a glycosyl–enzyme intermediate is formed first during the glycosylation step, followed by hydrolysis (deglycosylation step) in a general acid–base-catalyzed process through oxocarbenium ion-like transition states.[26] As just shown, the *Penicillium* β-D-galactosidase acts via a double-displacement mechanism.[27] A minimal kinetic scheme describing the two-step process of the hydrolysis (not taking into account transglycosylation reactions) can be written:

$$\begin{array}{ccccccc} & & & & P_1 & & \\ & & \underline{k_1} & & \uparrow & & \underline{k_5} \\ E+S & \longleftrightarrow & ES & \longrightarrow & ES' & \longrightarrow & E+P_2, \\ & & \underline{k_{-1}} & & \underline{k_2} & & \end{array}$$

where E is the enzyme; S is the substrate; ES is the enzyme–substrate complex (Michaelis complex); ES′ is the intermediate galactosyl–enzyme complex; P_1 is the reaction product formed from the aglycon portion of the substrate; P_2 is D-galactose; k_1, k_{-1}, k_2, and k_5 are the rate constants of the corresponding stages ($k_5 = k'_5 \cdot [H_2O]$). The solution to this scheme under the conditions of steady-state ES and ES′ complexes can be readily determined from the Michaelis–Menten equation:

$$v = \frac{d[P_1]}{dt} = \frac{d[P_2]}{dt} = \frac{k_{cat}[E_0][S]}{K_m + [S]} \quad (1)$$

where v is the initial reaction rate:

$$k_{cat} = \frac{k_2 k_5}{k_2 + k_5} \quad (2)$$

On the assumption of $k_2 \ll k_5$, the value for $k_{cat} = k_2$ for 'fast' substrates. This particular scheme was successfully

applied to describe the kinetic properties of an α-D-galactosidase from *Phanerochaete chrysosporium*,[28] *Bacillus* (1→3)-(1→4)-β-glucanase,[29] and the α-galactosidase from *Trichoderma reesei*.[30] It was established that there are two types of substrates for α-D-galactosidase: 'fast' (*p*-nitrophenyl α-D-galactopyranoside) and 'slow' (melibiose, raffinose, etc.).[30] Differentiation into fast and slow substrates was based on the dissociation constants for the enzyme–substrate complex at the stage of releasing the reaction product, which is the second stage of the two-step hydrolysis process.

Here, AcGal might be considered as a 'fast' substrate along with **2**, in contrast to 'slow' lactose and **1**. The stage of deglycosylation of the ES′ complex is the same for both 'slow' and 'fast' substrates, and only the difference in k_2 values, the formation-rate constant for the ES′ complex, affects the k_{cat} values for different substrates. Thus, in terms of the kinetic scheme presented, our data indicate that acetic acid is the best leaving group when compared with MeOH and *p*-nitrophenol. Using Eq. (4) from Section 3, we found that the k_H^+ value for acid hydrolysis of **1** is about 35 times lower than for AcGal (1.76 and 0.05 M^{-1} min^{-1}, for AcGal and **1**, respectively). Due to the acid–base nature of the β-galactosidase catalysis in this case, one possible explanation for the relatively high k_2 value for AcGal is the low stability of the substrate under acid conditions.[31]

Transglycosylating activity of the β-D-galactosidase with AcGal and **1** *as substrates.*—Transglycosylating activity in the reaction of substrate transglycosylation was investigated with AcGal as a substrate. Kinetics of formation of the transglycosylation product measured by ^{1}H NMR at different concentrations of AcGal is shown in Fig. 4. The time-course of the hydrolysis of AcGal at a concentration of 60 mM assayed by the same method is presented in Fig. 5. Based on these data, we used Eq. (3)[32] to estimate the autocondensation reaction yield.

$$\text{Transfer yield} = \frac{\text{Transfer product}}{\text{Transfer product} + \text{D-galactose}} \quad (3)$$

As seen from Fig. 6, the yield of transglycosylation product in the condensation reaction with AcGal is ~70% during the first 10 min of reaction. The corresponding value for the same concentration of **1**, also obtained using the ^{1}H NMR technique, was roughly 45%. This would suggest that AcGal may be a better donor for enzymatic synthesis that involves a transglycosylation reaction. It should be noted that the yield of 70% is rather high in comparison with exo-glycosidases from other sources exhibiting transglycosylating activity. The yield in the substrate transglycosylation reactions catalyzed by α-galactosidases from thermophilic sources was 20%;[33] this value for *E. coli* β-D-galactosidase was 20–30%,[34] and for α-galactosidase from

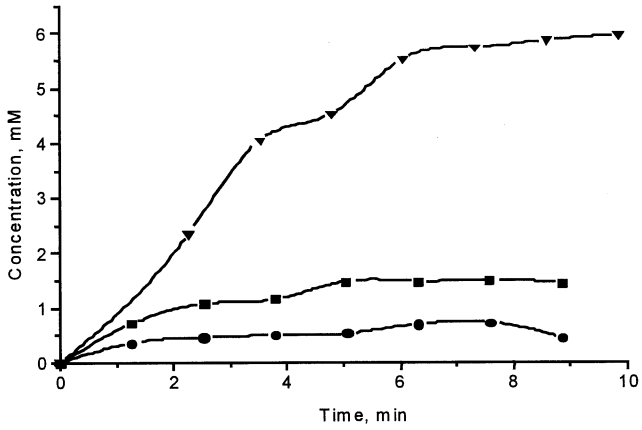

Fig. 4. Formation of transglycosylation products in the hydrolysis of AcGal at 6.9 (●), 13.8 (■), and 27.5 (▲) mM concentration of the substrate as a function of time.

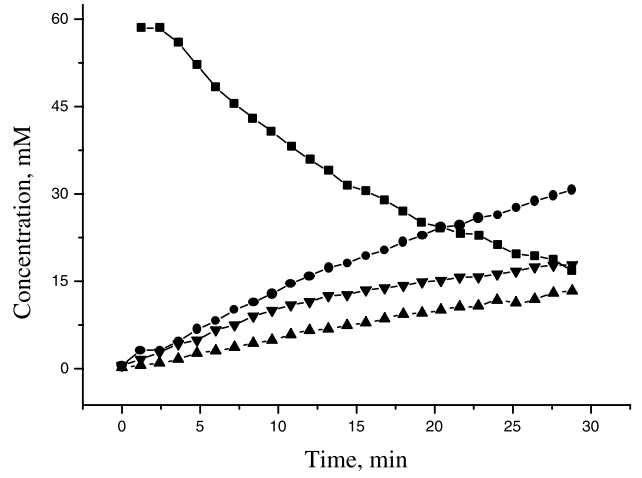

Fig. 5. Time-dependence of AcGal hydrolysis (AcGal concentration of 60 mM): ■, AcGal; ●, acetic acid; ▼, transglycosylation product; ▲, D-galactose.

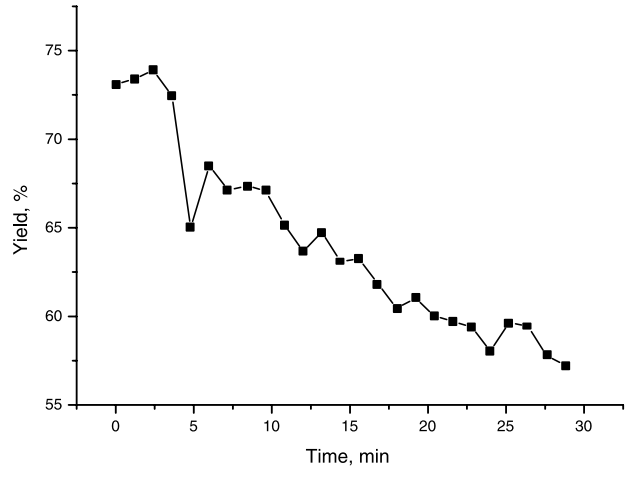

Fig. 6. Time-dependence of transglycosylation product yield in the hydrolysis of AcGal at 60 mM concentration.

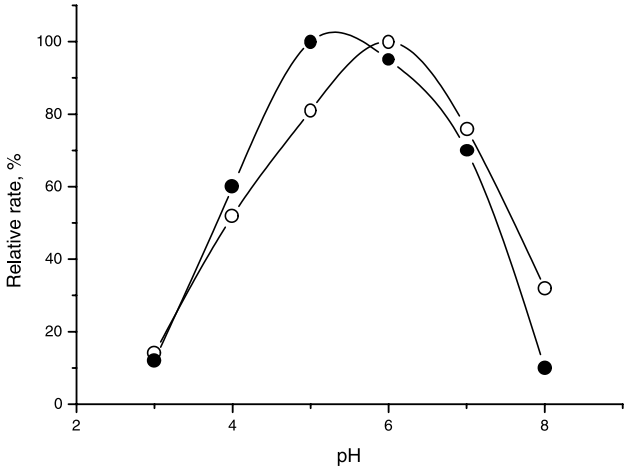

Fig. 7. pH-dependence of formation of transglycosylation products in the condensation reaction with AcGal (○) and **1** (●).

Bifidobactericum adolescentic — 15%.[35] As may be seen from the kinetics of product formation for the transglycosylation process with **1**, the rate of product formation is about three orders of magnitude lower than for AcGal. Rates of formation of transglycosylation products in the condensation reactions with AcGal and **1** as a function of pH were determined by HPLC. Fig. 7 shows that the pH-optima for both substrates are rather close.

Structures of transglycosylation products were determined after their HPLC purification from the reaction mixture. Based on the data of ^{1}H and ^{13}C NMR analysis, melting point, and optical rotation,[34] the major and minor products of transglycosylation reactions with **1** were identified as methyl β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside and methyl β-D-galactopyranosyl-(1→3)-β-D-galactopyranoside, respectively. They were obtained in 36% (major) and 9% (minor) yields, respectively. These data coincide with those for methyl β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside as synthesized by transglycosylation with β-D-galactosidase from *E. coli*.[34] The product of transglycosylation reactions with AcGal was β-D-galactopyranosyl-(1→6)-1-*O*-acetyl-β-D-galactopyranose. Minor products of the substrate transglycosylation were in trace amount. Acid hydrolysis of the main product gave β-D-galactopyranosyl-(1→6)-D-galactopyranose which was identified by TLC, HPLC, and ^{13}C NMR.[36] The β(1→6) configuration of the galactosyl bonds has been reported for only a few β-D-galactosidases.[36] The products of enzymatic syntheses with other β-D-galactosidases were β(1→3)- and β(1→4)-galactooligosaccharides.[9–11,34]

Further investigations will be focused on AcGal as a potentially effective donor in the substrate transglycosylation reactions for production of different biologically active galactooligosaccharides.

3. Experimental

Chemicals.—Methyl β-D-galactopyranoside, *p*-nitrophenyl β-D-galactopyranoside, lactose, β-D-galactopyranosyl-(1→6)-D-galactopyranose, salts, and bovine serum albumin (BSA) were purchased from Sigma, St. Louis, MO, reagents used for synthesis of AcGal and solvents were from Fluka and E. Merck (Germany).

General methods.—Protein concentrations were measured following the Lowry procedure with BSA as the standard.[37] Optical rotations were determined with a Jasco DIP-360 polarimeter. All ^{1}H and ^{13}C NMR spectra were recorded with an AMX-500 Bruker spectrometer. Prior to NMR analysis, the β-galactosidase, buffer materials, and substrates were freeze-dried twice from D_2O. The ^{1}H NMR measurements were made with MeCN as the internal standard, δ 2.1 ppm. To measure ^{13}C chemical shifts, the methyl acetone resonance at δ 30.2 ppm was used as an internal standard. Oligosaccharides, products of enzymatic hydrolysis, and products of transglycosylation were analyzed qualitatively by thin-layer chromatography on Kieselgel 60 plates (E. Merck) with a mobile phase of 2:2:1 ethanol–butan-1-ol–water. Plates were developed at room temperature, air dried, and sprayed with 5% H_2SO_4 in 2-propanol, followed by incubation at 120 °C for 8 min.

Enzyme and enzymatic assays.—β-D-Galactosidase from *Penicillium* sp. was purified according to the procedure reported in Ref. 17. Purified enzyme contained no more than 0.05% of admixtured exoglucosidase activity, measured with corresponding α/β-PNP glycosides.[38] One unit of β-galactosidase activity towards **2** was defined as the amount of the enzyme required to hydrolyze 1 μmol of the substrate per 1 min at 37 °C in 30 mM sodium acetate buffer, pH 5.0. The activity of β-galactosidase in the hydrolysis of **1** was determined by measuring the concentration of the reducing sugar during the reaction by Somogyi–Nelson's visualization method[39] and, in the case of the hydrolysis of lactose, by the glucose oxidase method.[40] Determination of the β-D-galactosidase activity in the hydrolysis of AcGal was carried out after the reaction has been terminated by freeze-drying the sample. Then the mixture was analyzed by HPLC on a Lichrosorb-NH_2 column (Pharmacia) with refractometric detection, using isocratic elution with 4:1 MeCN–water; a quantitative interpretation was accomplished by integrating the peaks for D-galactose and AcGal.

The effect of pH on the β-D-galactosidase activity was measured at 37 °C over the pH range 3–9 in 100 mM sodium citrate buffer. The values for K_M and k_{cat} were determined based on the initial rates of the hydrolysis of **1**, **2**, AcGal, and lactose. The hydrolysis was carried out in 30 mM NaOAc buffer, pH 5.0, at 37 °C and concentrations of **1** from 20 to 2 mM; **2** ranging from 2 to 0.2 mM; AcGal from 10 to 1 mM, and

lactose from 20 to 5 mM. Data were analyzed by the Lineweaver–Burk method. The inhibition constants were measured by the Dixon method, varying the concentration of the substrate in the reaction mixture.[41] As an alternative, the kinetics of AcGal hydrolysis were studied by ^{1}H NMR techniques as described next.

Acid hydrolysis of substrates.—Acid hydrolysis of **1** and AcGal was carried out at 80 °C in HCl in the concentration range of 16.7–83.3 mM for AcGal and 100–800 mM for **1**. The mixtures contained 13.5 mM of **1** or 2.5 mM of AcGal, along with the appropriate amount of HCl and were incubated for 16 or 4 min, respectively. Subsequently, the reaction samples were lyophilized and analyzed on a Lichrosorb-NH_2 column with the refractometric detection already described. Values for k_H^+ were calculated based on Eq. (4):

$$k_H^+ = [\ln([S_0]/[S])]/[[HCl]\tau] \quad (4)$$

where k_H^+ is an acid hydrolysis constant; [S] is a substrate concentration; [S_0] is the initial substrate concentration; [HCl] is the concentration of HCl, M; τ is the reaction time (min). Hydrolysis of β-D-galactopyranosyl-(1→6)-1-*O*-acetyl-β-D-galactopyranose was carried out in 20 mM HCl for 12 h at 60 °C.

Transglycosylating activity of the β-D-galactosidase.—Experiments on the transglycosylating activity of β-D-galactosidase were carried out in 30 mM NaOAc buffer, pH 5.0, or in 20 mM sodium phosphate, pH 6.0 at 37 °C. The reaction was terminated by freezing and lyophilization. TLC was used for qualitative analysis of transglycosylation products. A quantitative analysis of the transglycosylation products in the case of the hydrolysis of **1** and AcGal was carried out by HPLC chromatography on a Lichrosorb-NH_2 column or by ^{1}H NMR as described later. The effect of pH on the β-D-galactosidase transglycosylation activity was measured at 37 °C over the pH range 3–9 in 100 mM sodium citrate buffer.

Methyl β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside and methyl β-D-galactopyranosyl-(1→3)-β-D-galactopyranoside.—To **1** (50 mg) in 30 mM sodium phosphate buffer (pH 6.0, 0.4 mL), 27 units of the β-D-galactosidase were added to initiate the reaction, which was conducted at 37 °C for 90 min. Following termination of the reaction by freezing and lyophilization, the transglycosylation products were isolated by gel-permeation chromatography using a P2 column (5 × 100 cm) in water followed by HPLC purification on a Lichrosorb-NH_2 column.

Methyl β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside.—Mp 210–212 °C, $[\alpha]_D^{21}$ −9.5° (*c* 0.2, water); ^{13}C NMR (D_2O): δ 104.33 (C-1), 103.81 (C-1′), 75.67 (C-5), 74.32 (C-5′), 73.22 (C-3), 73.15 (C-3′), 71.26 (C-2), 71.15 (C-2′), 69.48 (C-6′), 69.21 (C-4′), 69.14 (C-4), 61.50 (C-6), 57.85 (O-Me); ^{1}H NMR (D_2O): δ 4.33 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1), 4.21 (d, 1 H, $J_{1,2}$ 7.71 Hz, H-1′), 3.93 (m, 1 H, H-5′), 3.84 (dd, 1 H, $J_{3,4}$ 3.4, $J_{4,5}$ 0.6 Hz, H-4), 3.78 (m, 3 H, CH_2, H-4′), 3.66 (dd, 1 H, $J_{5,6}$ 7.9, $J_{6,6}$ 11.6 Hz, H-6′), 3.63 (dd, 1 H, $J_{5,6}$ 4.3 Hz, H-6′), 3.57 (ddd, 1 H, H-5), 3.52 (dd, 2 H, $J_{2,3}$ 9.9 Hz, H-3, H-3′), 3.45 (s, 3 H, O-Me), 3.40 (dd, 1 H, H-2′), 3.38 (dd, 1 H, H-2).

Methyl β-D-galactopyranosyl-(1→3)-β-D-galactopyranoside.—Mp 201–202 °C, $[\alpha]_D$ +24.5° (*c* 1, water); ^{13}C NMR (D_2O): δ 104.32 (C-1), 103.43 (C-1′), 82.41 (C-3′), 75.03 (C-5), 74.74 (C-5′), 72.50 (C-3), 71.02 (C-2), 69.81 (C-2′), 68.55 (C-4), 68.43 (C-4′), 60.92 (C-6, C-6′), 57.09 (O-Me); ^{1}H NMR (D_2O): δ 4.47 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 4.24 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1′), 4.07 (dd, 1 H, $J_{4,3}$ 3.4, $J_{4,5}$ 1.1 Hz, H-4′), 3.79 (dd, 1 H, $J_{3,4}$ 3.3, $J_{4,5}$ 1.0 Hz, H-4), 3.68 (dd, 1 H, $J_{2,3}$ 9.8 Hz, H-3′), 3.67 (dd, 1 H, $J_{5,6}$ 7.9, $J_{6,6}$ 11.7 Hz, H-6a), 3.64 (67 (dd, 1 H, $J_{5,6}$ 7.7, $J_{6,6}$ 11.7 Hz, H-6b), 3.62 (dd, 1 H, $J_{5,6}$ 4.4, $J_{6,6}$ 10.3 Hz, H-6′), 3.617 (dd, 1 H, $J_{5,6}$ 4.0, $J_{6,6}$ 10.3 Hz, H-6′), 3.59 (ddd, 1 H, H-5′), 3.56 (ddd, 1 H, H-5), 3.54 (dd, 1 H, H-2′), 3.53 (dd, 1 H, $J_{2,3}$ 9.9 Hz, H-3), 3.48 (dd, 1 H, $J_{2,3}$ 9.9 Hz, H-2), 3.45 (s, 3 H, O-Me).

β-D-Galactopyranosyl-(1→6)-1-O-acetyl-β-D-galactopyranose.—This product was obtained in the same manner.

β-D-Galactopyranosyl-(1→6)-1-O-acetyl-β-D-galactopyranose.—Mp 190–205 °C, $[\alpha]_D^{21}$ −4.3° (*c* 0.2, water); ^{13}C NMR (D_2O): δ 104.52 (C-1), 95.81 (C-1′), 76.64 (C-5′), 76.24 (C-5), 74.07 (C-3), 73.66 (C-3′), 72.19 (C-4′), 70.90 (C-4), 70.22 (C-6′), 70.06 (C-2′), 69.90 (C-2), 62.46 (C-6), 21.87 (CH_3-Ac); ^{1}H NMR (D_2O): δ 5.53 (d, 1 H, $J_{1,2}$ 7.9 Hz, H-1′), 4.42 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1).

^{1}H NMR measurements.—To determine AcOH, AcGal, and transglycosylation product concentrations, signals having chemical shifts at δ 1.99, 5.50, and 4.43 ppm, respectively, were used. The initial hydrolysis rate of AcGal was measured in 30 mM NaOAc buffer made up in D_2O, pD 5.0, 37 °C. Concentrations of AcGal were in a range between 10 and 1 mM. The direct NMR analysis of transglycosylation activities toward AcGal and **1** was made in 30 mM sodium phosphate buffer prepared in D_2O, pD 6.0, 37 °C. Data were acquired using 64 scans per series, including the first four dummy scans; total acquisition time was 1 min. Stereospecificity of the AcGal hydrolysis with β-D-galactosidase was studied as follows: the reaction was carried out at 37 °C in 50 mM sodium phosphate buffer in D_2O, pD 6.0. The concentration of D-galactose was determined from the sum of the anomeric proton signals at δ 5.26 and 4.57 ppm (α and β anomer, respectively). The reaction was monitored by collecting ^{1}H NMR spectra once per min after the enzyme addition. Anomeric proton signals (α and β) were registered during the course of the reaction.

1-O-*Acetyl*-*2,3,4,6*-*tetra*-O-*benzyl*-*β*-D-*galactopyranose*.—Glacial AcOH (0.25 mL, 4.37 mmol) was added during 3 min to a stirred solution of 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl 2,2,2-trichloroacetimid-ate[23] (944 mg, 1.38 mmol) in anhyd CH_2Cl_2 (10 mL) under argon at −20 °C. The reaction mixture was allowed to warm to rt (25 °C) and was kept at rt for 2 h to complete the reaction (TLC). The resulting solution was diluted with $CHCl_3$ and washed with satd aq $NaHCO_3$. The organic layer was dried by passing through a pad of Na_2SO_4–Celite and concentrated at reduced pressure. Column chromatography (0→10% EtOAc in PhH) afforded 610 mg (76%) of a syrup containing the title compound, along with its α anomer (10:1 β/α by NMR). Crystallization from Et_2O–hexanes yielded 410 mg (51%) of pure title compound: mp 102–103 °C; $[\alpha]_D^{28}$ +3.8° (*c* 2.0, $CHCl_3$); R_f 0.38, 9:1 (v/v) PhH–EtOAc; ^{1}H NMR ($CDCl_3$): δ 7.45–7.25 (m, 20 H, 4 × C_6H_5), 5.60 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.96, 4.86, 4.76, 4.74, 4.65, 4.46, 4.41 (8 H, 8 × CH_2-Bn), 4.00 (dd, 1 H, $J_{4,5}$ 1.2 Hz, H-4), 3.98 (dd, 1 H, $J_{2,3}$ 9.7 Hz, H-2), 3.72 (m, 1 H, $J_{5,6a}$ 7.6, $J_{5,6b}$ 5.5 Hz, H-5), 3.64 (dd, 1 H, $J_{6a,6b}$ 8.9 Hz, H-6a), 3.63 (dd, 1 H, $J_{3,4}$ 2.7 Hz, H-3), 3.59 (dd, 1 H, H-6b), 2.04 (s, 3 H, CH_3-Ac). ^{13}C NMR ($CDCl_3$): δ 94.31 (C-1), 82.42 (C-3), 78.19 (C-2), 74.09 (C-4), 73.13 (C-5), 67.96 (C-6), 21.00 (CH_3-Ac), 75.30, 74.70, 73.51, 72.89 (4 × CH_2-Bn). Anal. Calcd for $C_{36}H_{38}O_7$: C, 74.21; H, 6.57. Found: C, 73.96; H, 6.65.

1-O-*Acetyl*-*β*-D-*galactopyranose*.—A suspension of 1-*O*-acetyl-2,3,4,6-tetra-O-benzyl-β-D-galactopyranose (360 mg, 0.618 mmol) in 95% EtOH (10mL) was hydrogenated under atmospheric pressure at 36 °C over 10% Pd–C (125 mg) for 12 h. The catalyst was removed by centrifugation and washed with 95% EtOH. The combined supernatants were concentrated under reduced pressure and the residue was crystallized from MeOH–EtOAc to give the title compound (136 mg, quant.): mp 186–191 °C (with partial decomposition), $[\alpha]_D^{30}$ +15.6 (*c* 1.5, MeOH), R_f 0.38, 4:1 (v/v) EtOAc–MeOH; ^{1}H NMR (D_2O): δ 5.53 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.01 (dd, 1 H, $J_{4,5}$ 1.0 Hz, H-4), 3.86 (m, 1 H, $J_{5,6a}$ 6.0, $J_{5,6b}$ 6.1 Hz, H-5), 3.79 (m, 2 H, H-6a,b), 3.76 (dd, 1 H, $J_{3,4}$ 3.0 Hz, H-3) 3.75 (dd, 1 H, $J_{2,3}$ 9.9 Hz, H-2), 2.23 (s, 3 H, CH_3-Ac). ^{13}C NMR (D_2O): δ 95.96 (C-1), 77.56 (C-5), 73.83 (C-2), 70.97 (C-3), 69.81 (C-4), 62.20 (C-6), 21.88 (CH3-Ac), 174.24 (CO-Ac). Anal. Calcd for $C_8H_{14}O_7$: C, 43.24; H, 6.35. Found: C, 49.92; H, 6.49.

For enzymatic assay, an additional step of compound purification was performed on a Lichrosorb-NH_2 column.

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References

1. Wong, C.-H.; Whitesides, G. M.; Baldwin, J. E.; Magnus, P. D. *Enzymes in Synthetic Organic Chemistry*; Tetrahedron Organic Chemistry Series: Oxford, 1994; pp. 252–311 ch. 5.
2. Toone, E. J.; Simon, E. S.; Bednarski, M. D.; Whitesiedes, G. M. *Tetrahedron* **1989**, *45*, 5365–5422.
3. Stowell, C. P.; Lee, Y. C. *Adv. Carbohydr. Chem. Biochem*. **1980**, *37*, 225–281.
4. Thiem, J. *FEMS Microbiol. Rev*. **1995**, *16*, 193–211.
5. Nilsson, K. G. I.; Bednarski, M. D.; Simon, E. S. *Enzymes in Carbohydrate Synthesis*; ACS Symposium Series: Washington, DC, 1991; pp. 51–62 ch. 4.
6. Guo, Z.; Wang, P. G. *Appl. Biochem. Biotechnol*. **1997**, *68*, 1–20.
7. Ichikawa, Y.; Look, G. C.; Wong, C.-H. *Anal. Biochem*. **1992**, *202*, 215–238.
8. Wymer, N.; Toone, E. J. *Curr. Opin. Chem. Biol*. **2000**, *4*, 110–119.
9. Vetere, A.; Paoletti, S. *Biochem. Biophys. Res. Commun*. **1996**, *219*, 6–13.
10. Fujimoto, H.; Miyasato, M.; Ito, Y.; Sasaki, T.; Ajisaka, K. *Glycoconjugate J*. **1998**, *15*, 155–160.
11. Ogawa, T.; Kitajima, T.; Nukada, T. *Carbohydr. Res*. **1983**, *123*, 8–11.
12. Hedbys, L.; Johansson, E.; Mosbach, K.; Larsson, P. O.; Gunnarsson, A.; Svensson, S.; Lonn, H. *Glycoconjugate J*. **1989**, *6*, 161–168.
13. Cantacuzene, D.; Attal, S.; Bay, S. *Biomed. Biochim. Acta* **1991**, *50*, 231–236.
14. Bay, S.; Namane, A.; Cantacuzene, D. *Carbohydr. Res*. **1993**, *248*, 317–325.
15. Johnson, K. F. *Glycoconjugate J*. **1999**, *16*, 141–146.
16. Palcic, M. M. *Curr. Opin. Biotechnol*. **1999**, *10*, 616–624.
17. Neustroev, K. N.; de Sousa, E. A.; Golubev, A. M.; Brandão Neto, J. R.; Eneyskaya, E. V.; Kulminskaya, A. A.; Polikarpov, I. *Acta Crystallogr., Sect. D* **2000**, *56*, 1508–1509.
18. Ziegler, T. *Liebigs Ann. Chem*. **1990**, 1125–1131.
19. Veeneman, G. H.; van Leeuwen, S. H.; van Boom, J. H. *Tetrahedron Lett*. **1990**, *31*, 1331–1334.
20. Schmidt, R. R. In *Carbohydrates–Synthetic Methods and Application in Medicinal Chemistry*; Hasegawa, A.; Ogura, H.; Suami, T., Eds.; Kodanasha Scientific: Tokyo, 1992; pp. 66–69.
21. Wang, R.; Steensma, D. H.; Takaoka, Y.; Yun, J. W.; Kajimoto, T.; Wong, C.-H. *Bioorg. Med. Chem*. **1997**, *5*, 661–672.
22. Schmidt, R. R.; Michel, J.; Roos, M. *Liebigs Ann. Chem*. **1984**, 1343–1357.
23. Wegmann, B.; Schmidt, R. R. *J. Carbohydr. Chem*. **1987**, *6*, 357–360.
24. Williams, S. J.; Withers, S. G. *Carbohydr. Res*. **2000**, *327*, 27–46.
25. Izumi, K. *Agric. Biol. Chem*. **1971**, *35*, 1816–1818.
26. Sinnott, M. L. *Chem. Rev*. **1990**, *90*, 1171–1202.
27. Gebler, J.; Gilkes, N. R.; Claeyssens, M.; Wilson, D. B.; Beguin, P.; Wakarchuk, W. W.; Kilburn, D. G.; Miller, R. C., Jr.; Warren, R. A. J.; Withers, S. G. *J. Biol. Chem*. **1992**, *267*, 12559–12561.

28. Brumer, H. III; Sims, P. F. G.; Sinnott, M. L. *Biochem. J.* **1999**, *339*, 43–53.
29. Abel, M.; Planas, A.; Christensen, U. *Biochem. J.* **2001**, *357*, 195–202.
30. Savel'ev, A. N.; Ibatullin, F. M.; Eneyskaya, E. V.; Kachurin, A. M.; Neutroev, K. N. *Carbohydr. Res.* **1996**, *296*, 261–273.
31. Konstantinidis, A.; Sinnott, L. *Biochem. J.* **1991**, *279*, 587–593.
32. Fujimoto, H.; Isomura, M.; Ajisaka, K. *Biosci. Biotech. Biochem.* **1997**, *61*, 164–165.
33. Spangenberg, P.; Andre, C.; Dion, M.; Rabiller, C.; Mattes, R. *Carbohydr. Res.* **2000**, *329*, 65–73.
34. Nilsson, K. G. I. *Carbohydr. Res.* **1987**, *167*, 95–103.
35. Van Laere, K. M. J.; Hartemink, R.; Beldman, G.; Pitson, S.; Dijkema, C.; Schols, H. A.; Voragen, A. G. *Appl. Microbiol. Biotechnol.* **1999**, *52*, 681–688.
36. Bhattacharjee, A. K.; Glaudemans, C. P. *Carbohydr. Res.* **1978**, *67*, 536–540.
37. Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
38. Bahl, O. P.; Agrawal, K. M. *J. Biol. Chem.* **1969**, *244*, 2970–2978.
39. Somogyi, M. *J. Biol. Chem.* **1952**, *195*, 19–23.
40. Lloyd, J. B.; Whelan, W. J. *Anal. Biochem.* **1969**, *30*, 467–470.
41. Dixon, M. *Biochem. J.* **1953**, *55*, 170–171.