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Hydrolytic activity of α-galactosidases against deoxy derivatives of *p*-nitrophenyl α-D-galactopyranoside

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Abstract

The four possible monodeoxy derivatives of *p*-nitrophenyl (PNP) α-D-galactopyranoside were synthesized, and hydrolytic activities of the α-galactosidase of green coffee bean, *Mortierella vinacea* and *Aspergillus niger* against them were elucidated. The 2- and 6-deoxy substrates were hydrolyzed by the enzymes from green coffee bean and *M. vinacea*, while they scarcely acted on the 3- and 4-deoxy compounds. On the other hand, *A. niger* α-galactosidase hydrolyzed only the 2-deoxy compound in these deoxy substrates, and the activity was very high. These results indicate that the presence of two hydroxyl groups (OH-3 and -4) is essential for the compounds to act as substrates for the enzymes of green coffee bean and *M. vinacea*, while the three hydroxyl groups (OH-3, -4, and -6) are necessary for the activity of the *A. niger* enzyme. The kinetic parameters (K_m and V_{max}) of the enzymes for the hydrolysis of PNP α-D-galactopyranoside and its deoxy derivatives were obtained from kinetic studies.

Keywords: α-Galactosidase; Substrate specificity; Deoxy galactoside; Synthesis

1. Introduction

Generally, different types of *exo*-glycosidases are distinguished on the basis of specificity for the glycon structure of their substrates, and their substrate specificities are expressed in their relative activities against substrates having various aglycons. Indications exist that the specificity of glycosidases for the glycon moiety of the substrate is not absolute. Several studies reported that weak hydrolytic reactions with deoxy analogs of the appropriate glycon were catalyzed by several D-glucosidases [1–7] and by two of the three α-galactosidases used in the present study [8,9]. In addition, we have reported [10] that jack bean and almond α-mannosidase both hydrolyzed *p*-nitrophenyl (PNP) α-D-rhamnopyranoside at a rate approaching that for PNP α-D-mannopyranoside.

The aim of the present study was to gain further knowledge about the glycon specificity of α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22). α-Galactosidases catalyze the hydrolytic cleavage of terminal α-D-galactopyranosyl residues from oligosaccharides; preparations from some sources also release D-galactose from glycopeptides and glycolipids. Many α-galactosidases have been purified from various sources [11], and the amino acid sequences of the enzymes from *Mortierella vinacea* [12], *Saccharomyces carlsbergensis* [13], and *Streptococcus mutans* [14] have been determined. Complementary and genomic DNAs encoding α-galactosidases

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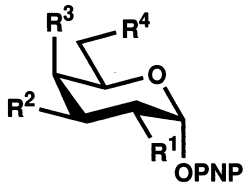


1: $R^1=R^2=R^3=R^4=OH$
7: $R^1=H$, $R^2=R^3=R^4=OH$
16: $R^1=R^3=R^4=OH$, $R^2=H$
23: $R^1=R^2=R^4=OH$, $R^3=H$
27: $R^1=R^2=R^3=OH$, $R^4=H$
PNP = *p*-nitrophenyl

Fig. 1. PNP α-D-galactopyranoside and its deoxy derivatives.

were cloned from coffee bean [15], guar [16], human [17], yeast [13], *Esherichia coli* [18], *Aspergillus niger* [19], *S. mutans* [14], and *M. vinacea* [20]. Finally, the aglycon specificities of many α-galactosidases have been investigated, but the glycon specificities have not been systematically checked.

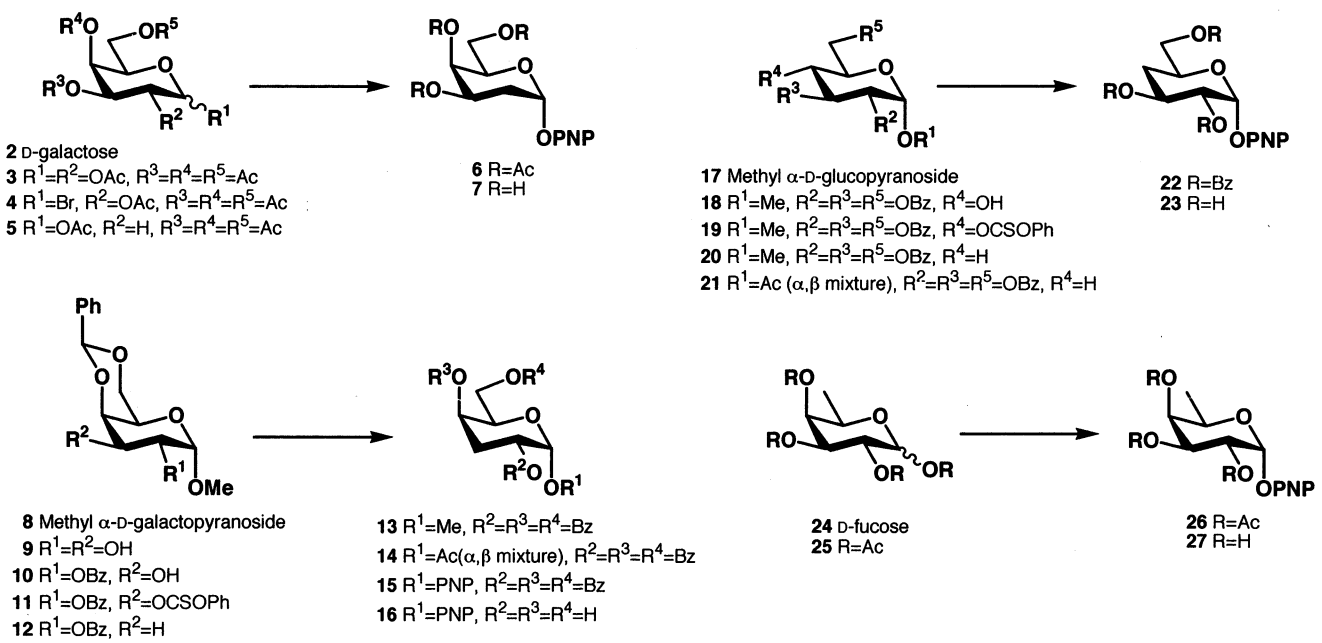
Here, we report the synthesis of monodeoxy derivatives of PNP α-D-galactopyranoside (**1**) (Fig. 1) and the hydrolytic activities of α-galactosidases from green coffee bean, *M. vinacea*, and *A. niger* against them, and we discuss differences in the substrate specificities of these enzymes.

2. Results and discussion

Synthesis of deoxy substrates.—The structures of PNP α-D-galactopyranoside (**1**) and of the newly synthesized deoxy derivatives (**7**, **16**, **23**, and **27**, Scheme 1) used in the present study are compared in Fig. 1. Each derivative, namely PNP 2-deoxy-α-D-*lyxo*-hexopyranoside (**7**), PNP 3-deoxy-α-D-*xylo*-hexopyranoside (**16**), PNP 4-deoxy-α-D-*xylo*-hexopyranoside (**23**), and PNP α-D-fucopyranoside (**27**), was obtained in crystalline form. The vicinal diaxial proton coupling constants ($J_{2,3}$) observed in the ^{1}H NMR spectra of **1**, **7**, **16**, **23**, and **27** are 10.5, 12.0, 13.0, 10.4, and 10.4 Hz, respectively. These values indicated that these deoxygenated glycosides retained the $^4C_1$ chair conformation in D_2O. In addition, low-energy conformations of **1**, **7**, **16**, **23**, and **27** were calculated[1]. These computed data supported the results of ^{1}H NMR (data not shown).

Substrate specificity of α-galactosidase.—We investigated the specific activities of α-galactosidases from green coffee beans and from the molds *M. vinacea* and *A. niger* on the hydrolysis of **1** and the foregoing deoxy derivatives. Since different reaction conditions

[1] MM2 force field by CAChe (Sony Tektronix).



Scheme 1. Synthesis of 2-, 3-, 4-, and 6-deoxy derivatives of PNP α-D-galactopyranoside.

were used for each enzyme, the findings allow only a rough comparison to be made between the activities of one enzyme relative to those of the other α-galactosidases. Of the four deoxy derivatives of **1**, the *A. niger* α-galactosidase hydrolyzed only the 2-deoxy 'galactopyranoside' (**7**); its activity with **7** appeared to be substantially higher than against **1**. On the other hand, the green coffee bean and *M. vinacea* enzymes showed activity not only for the 2-deoxy compound **7** (substantially lower than for **1** in each case), but also some still lower activity for the 6-deoxy derivative PNP α-D-fucopyranoside (**27**). The failure of all three enzymes to hydrolyze detectably the 3- and 4-deoxy derivatives suggests that the C-3 and C-4 hydroxyl groups of a galactopyranoside may be important for substrate recognition by the α-galactosidases.

To examine further the several apparently large differences between the activities against 2- and 6-deoxy substrates shown by green coffee bean and *M. vinacea* α-galactosidases versus the *A. niger* enzyme, kinetic studies of the hydrolysis of **1**, **7**, and **27** were carried out (Table 1). From reaction velocities measured at different substrate concentrations, a Lineweaver–Burk plot of $1/v$ versus $1/S$ was used to derive K_m and V_{max} for each enzyme–substrate combination. Reaction conditions (pH and/or incubation temperature) were different for the three enzymes. Values of K_m for the hydrolysis of **1**, **7**, and **27** by the green coffee bean enzyme were 0.85, 27.4, and 38.5, and those with the *M. vinacea* enzyme were 0.96, 10.2, and 27.0, respectively. The K_m values of the *A. niger* enzyme for the hydrolysis of **1** and **7** were 0.54 and 2.63. The data presented indicate that the increase of K_m for **7** relative to **1** is 4.8-fold with the *A. niger*, 10.6-fold with *M. vinacea*, and 32-fold with the green coffee bean α-galactosidases. The closeness of the first two values emphasizes the need to provide standard errors of the parameters in Table 1 and indicate that the data were collected only from early-stage reactions. These results show that the affinity for the deoxy 'galactopyranoside' differentiates the green coffee bean and *M. vinacea* α-galactosidases from the *A. niger* enzyme. The high K_m values of the green coffee bean and *M. vinacea* enzymes against 2- and 6-deoxy galactopyranosides are probably due to the loss of a hydrogen bond, which affects formation of the enzyme–substrate complex. However, we think that the foregoing inference is not applicable to the *A. niger* enzyme because of the slight increase of the K_m value against **7**. This fact indicates that the C-2 hydroxyl group of **1** is not concerned with the hydrogen-bonding interaction between the substrate and the *A. niger* enzyme. We think that the high activity of the *A. niger* enzyme against **7** is due to the inductive effect of OH-2, which depresses the formation of the carbonium–oxonium ion. These results indicate that the three α-galactosidases are separable into two classes based on the hydrolysis of the 2- and 6-deoxy substrates.

Amino acid sequences of α-galactosidases from green coffee bean [15], *M. vinacea* [20], and *A. niger* [19] have been reported. The sequence homologies of the α-galactosidase from *A. niger* with the enzymes from green coffee bean and *M. vinacea* are 35 and 32%, respectively, and the sequence homology between the green coffee bean and *M. vinacea* enzymes is high (43%), suggesting a close relationship. Various types of glycosyl hydrolases have been classified into families on the basis of amino acid sequence similarities [21–23]. α-Galactosidases of *A. niger* and green coffee bean were classified into the same family (**27**) by this classification system. A different view of the relationship among α-galactosidases appears when specificity for the entire substrate is used as a basis of comparison. Suzuki et al. [8] noted that, unlike the coffee bean and *A. niger* enzymes, the *M. vinacea* enzyme does not liberate D-galactose from legume galactomannan [8]. Classification of α-galactosidases has been carried out by Kaneko et al. [24]. They classified microbial α-galactosidases into two groups based on the substrate specificity against galacto-*manno*-oligosaccharides.

Canellakis et al. reported that mammalian α-glucosidase hydrolyzes PNP 2-deoxy-α-D-'glucopyranoside' [25], and Nakano et al. reported that *A. niger* α-glucosidase has 2-deoxy-D-'glucose' condensation activity [26]. Purified α-glucosidases from various sources, including the crystalline enzyme from *A. niger*,

Table 1
Kinetic study on the hydrolysis of PNP α-D-galactopyranoside (**1**), and 2- and 6-deoxy derivatives of **1** by α-galactosidases [a]

Substrate	Green coffee been			*M. vinacea*			*A. niger*		
	K_m (mM)	V_{max} (μmol/min/mg protein)	V_{max}/K_m	K_m (mM)	V_{max} (μmol/min/mg protein)	V_{max}/K_m	K_m	V_{max} (μmol/min/mg protein)	V_{max}/K_m
PNP α-D-galactopyranoside (**1**)	0.85 (0.0025)	11.7 (0.537)	13.8	0.96 (0.0566)	47.1 (1.77)	49.1	0.54 (0.0148)	19.3 (0.148)	35.7
PNP 2-deoxy-α-D-*lyxo*-hexopyranoside (**7**)	27.4 (0.672)	16.9 (0.459)	0.62	10.2 (0.881)	95.2 (2.26)	9.3	2.63 (0.212)	175.0 (1.51)	66.5
PNP α-D-fucopyranoside (**27**)	38.5 (0.460)	4.8 (0.141)	0.12	27.0 (0.891)	111.1 (2.56)	4.1			

[a] Standard errors given in parentheses.

have been found to catalyze the hydration of D-glucal and, thus, do not require an OH group at C-2 [27]. This is true of the α-galactosidase from green coffee beans, which has been found to hydrate D-galactal [9]. Our data are consistent with these various observations and suggest that α-galactosidases in general will show 2-deoxy-D-'galactopyranoside' hydrolyzing ability. Recently, Notenboom et al. reported that the C-2 hydroxyl group of the substrate acts to stabilize the transition state in the action of β-glucosidases [28], but the above data with α-galactosidase and α-glucosidase suggest that the glycon C-2 hydroxyl group of the substrate is not important with the α-glycosidase.

Further studies using various substituted substrates, including deoxy glycosides, should be done to elucidate the substrate specificities of various *exo*-α-glycosidases.

3. Experimental

Materials and methods.—New compounds were characterized by elemental analysis and ^{1}H NMR spectra. Melting points were determined with a Yamato model MP-21 capillary apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer 141 polarimeter at 20 °C. ^{1}H NMR spectra were recorded with a Varian VXR-400 spectrometer. Chemical shifts are expressed in ppm downfield shift from Me_4Si. Mass spectra were obtained with a Jeol JMX SX-102A instrument under positive FAB conditions. Column chromatography was performed on Silica Gel 60 (230–400 mesh, E. Merck). The progress of all reactions was monitored by thin-layer chromatography (TLC) on Silica Gel 60 F_{254} (0.25 mm, E. Merck).

To prepare each deoxy derivative of PNP α-D-galactopyranoside (**1**), the following methods were used.

Method A. To a solution of the sugar derivative (55 mmol) in pyridine (150 mL) at room temperature (rt) was added Ac_2O (50 mL). The mixture was stirred overnight and poured into water (200 mL). The product was extracted with EtOAc (3 × 150 mL), washed with water, 1 M HCl, satd $NaHCO_3$, and

brine, and then dried over Na_2SO_4. Removal of the solvent afforded the corresponding acetate.

Method B. 40:40:1 AcOH–Ac_2O–H_2SO_4 (16.2 mL) was added to a stirred solution of the sugar derivative (2.5 mmol). The mixture was stirred overnight and then poured into water (200 mL). The product was extracted with EtOAc (200 mL). The organic layer was washed with water (3×200 mL), satd $NaHCO_3$, and brine, and then dried over Na_2SO_4. Removal of the solvent afforded the corresponding acetate.

Method C [29]. To a melt of the acetate (1 equiv) and *p*-nitrophenol (4 equiv) was added 0.33 g anhyd $ZnCl_2$ (1 equiv) dissolved in 1 mL of 19:1 AcOH–Ac_2O. The flask was kept in an oil bath at 125 °C for 5 min and evacuated with a water pump. After dissolving the resulting brown syrup in 300 mL of EtOAc, the $ZnCl_2$ and *p*-nitrophenol were removed by successive washing with satd $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated.

Method D. 5:1:1 MeOH–Et_3N–water (10 mL) was added to a stirred solution of the sugar derivative (16 mmol). After the mixture had been stirred for 3 h at rt, the solvent was evaporated.

Method E. Sodium methoxide (28%) in MeOH (1.0 mL) was added to a stirred solution of the sugar derivative (10 mmol) in dry MeOH (50 mL). After the mixture had been stirred for 5 h at rt, Amberlite IR-120 (H^+ form) was added to neutrality.

*p-Nitrophenyl 2-deoxy-3,4,6-tri-*O*-acetyl-α-*D*-xylo-hexopyranoside* (**6**).—Compound **3** was prepared from D-galactose (10.21 g, 55.1 mmol) according to Method A. To a solution of pentaacetate **3** in 200 mL CH_2Cl_2 was added 25% HBr–AcOH (67 mL). The mixture was stirred for 4 h and poured into water (250 mL). The organic layer was washed with water (2×250 mL), satd $NaHCO_3$, and brine, and then concentrated after drying over Na_2SO_4. The product was purified by column chromatography on silica gel (2:1 hexane–EtOAc) to give **4** (19.9 g, 87.1%). A solution of compound **4** (21.6 g, 55.2 mmol) in benzene (100 mL) was added dropwise over 14 h to a stirred solution of refluxing benzene (50 mL) and Bu_3SnH (19 mL, 66.3 mmol) [30]. After the solution had been refluxed for 4.5 h, the solvent was evaporated. The residue was dissolved in 1:9 MeCN–Et_2O (200 mL) and KF (5.0 g) was added. The mixture was filtered and the solvent was evaporated. The product was purified by column chromatography on silica gel (1:1 hexane–EtOAc) to afford 13.3 g (72.7%) of **5**. Boron trifluoride ethyl ether complex (0.61 mL, 4.82 mmol) was added to a stirred solution of **5** (0.53 g, 1.61 mmol) followed by *p*-nitrophenol (0.45 g, 3.21 mmol) in dry toluene (50 mL) under N_2, and the mixture was stirred for 1.5 h at rt. The mixture was washed with satd $NaHCO_3$ and brine, and then concentrated after drying over Na_2SO_4. After evaporation of the solvent, the product was purified by column chromatography on silica gel (3:2 hexane–EtOAc) to afford 0.20 g (29.8%) of **6**, which was recrystallized from hot EtOH; mp 140.0–141.0 °C; $[\alpha]_D$ +187° (*c* 0.98, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δ 1.93, 2.04, 2.17 (3 s, 9 H, $3 \times OCOCH_3$), 2.16 (ddd, 1 H, $J_{1,2eq}$ 1.2, $J_{2ax,2eq}$ 12.8, $J_{2eq,3}$ 5.2 Hz, H-2eq), 2.32 (ddd, 1 H, $J_{1,2ax}$ 3.14, $J_{2ax,3}$ 12.8 Hz, H-2ax), 4.07 (m, 1 H, H-6a), 4.08 (m, 1 H, H-6b), 4.18 (m, 1 H, H-5), 5.41 (dd, 1 H, $J_{3,4}$ 3.0, $J_{4,5}$ 2.4 Hz, H-4), 5.48 (ddd, 1 H, H-3), 5.85 (d, 1 H, H-1), 7.17 (d, 2 H, *J* 9.2 Hz, aromatic H), 8.22 (d, 2 H, aromatic H); MS: 412 [MH^+].

*p-Nitrophenyl 2-deoxy-α-*D*-xylo-hexopyranoside* (**7**).—Compound **7** was prepared from **6** (64.4 mg, 0.16 mmol) according to Method C, and recrystallized from EtOAc–hexane (40.6 mg, 90.9%): mp 146.5–148.0 °C; $[\alpha]_D$ +227° (*c* 0.50, MeOH); ^1H NMR (400 MHz, D_2O): δ 2.09 (ddd, 1 H, $J_{1,2eq} = J_{2eq,3}$ 3.6, $J_{2ax,2eq}$ 12.6 Hz, H-2eq), 2.16 (ddd, 1 H, $J_{1,2ax}$ 4.8, $J_{2ax,3}$ 12.0 Hz, H-2ax), 3.66 (dd, 1 H, $J_{5,6a}$ 4.4, $J_{6a,6b}$ 12.1 Hz, H-6a), 3.72 (dd, 1 H, $J_{5,6b}$ 7.8 Hz, H-6b), 3.90–3.94 (m, 2 H, H-4 and H-5), 4.32 (ddd, 1 H, $J_{3,4}$ 6.0 Hz, H-3), 7.28 (d, 2 H, *J* 9.2 Hz, aromatic H), 8.26 (d, 2 H, aromatic H); MS: 286 [MH^+]: Anal. Calcd for $C_{12}H_{15}O_7$: C, 50.53; H, 5.30; N, 4.91. Found: C, 50.33; H, 5.25; N, 4.90.

*Methyl 4,6-*O*-benzylidene-α-*D*-galactopyranoside* (**9**).—Compound **8** (25 g, 117.8 mmol) was dissolved in a mixture of formic acid (125 mL) and benzaldehyde (125 mL). The mixture was stirred for 20 min at rt and poured into

800 mL of 3.12 M K_2CO_3 solution and petroleum ether (1 L). The crystalline product in the solution was recrystallized from CH_2Cl_2–hexane to afford 22.4 g (67.3%) of **9**: mp 171.0–171.5 °C; $[\alpha]_D$ +141° (*c* 0.59, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.46 (s, 3 H, –OCH_3), 3.71 (ddd, 1 H, $J_{4,5} = J_{5,6b}$ 1.4, $J_{5,6a}$ 2.0 Hz, H-5), 3.90 (dd, 1 H, $J_{2,3}$ 9.9, $J_{3,4}$ 3.7 Hz, H-3), 3.93 (dd, 1 H, $J_{1,2}$ 3.0 Hz, H-2), 4.09 (dd, 1 H, $J_{6a,6b}$ 12.7 Hz, H-6a), 4.28 (dd, 1 H, H-4), 4.30 (dd, 1 H, H-6b), 4.94 (d, 1 H, H-1), 5.56 (s, 1 H, PhCH–), 7.36–7.51 (m, 5 H, –Ph); MS: 283 [MH^+].

Methyl 2-O-*benzoyl-4,6*-O-*benzylidene*-α-D-*galactopyranoside* (**10**).—Aqueous NaOH (40%, 10 mL) and Bu_4NCl (0.28 g, 0.78 mmol) were added to a solution of **9** (4.40 g, 15.59 mmol) in CH_2Cl_2 (60 mL) at rt, followed by a dropwise addition of BzCl (1.9 mL, 16.37 mmol). The mixture was stirred for 20 min at rt and poured into CH_2Cl_2 (300 mL) and water (200 mL), the organic layer was washed with satd $NaHCO_3$ and brine, and then concentrated after drying over Na_2SO_4. The product was purified by column chromatography on silica gel (3:2 toluene–Et_2O) to afford **10** (3.03 g, 39.6%): mp 192–193 °C, lit. 202–204 °C [26], lit. 206–207 °C [16]; $[\alpha]_D$ +146° (*c* 1.1, $CHCl_3$), lit. +146° [26], lit. +153° [16]; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.44 (s, 3 H, OCH_3), 3.81 (d, 1 H, H-5), 4.13 (dd, 1 H, $J_{5,6a}$ 1.8, $J_{6a,6b}$ 12.6 Hz, H-6a), 4.29 (dd, 1 H, $J_{2,3}$ 10.0, $J_{3,4}$ 3.6 Hz, H-3), 4.34 (dd, 1 H, $J_{5,6b}$ 1.4 Hz, H-6b), 4.36 (dd, 1 H, $J_{4,5}$ 1.2 Hz, H-4), 5.12 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 5.38 (d, 1 H, H-2), 5.61 (s, 1 H, PhCH–), 7.39–8.11 (m, 10 H, aromatic H); MS: 387 [MH^+].

Methyl 2-O-*benzoyl-3-deoxy-4,6*-O-*benzylidene*-α-D-xylo-*hexopyranoside* (**12**).—To a solution of **10** (1.27 g, 3.30 mmol) in CH_2Cl_2 (25 mL) was added phenylchlorothionoformate [31] (0.7 mL, 4.94 mmol) at rt, followed by dropwise addition of pyridine (2 mL). The mixture was stirred for 19 h and poured into CH_2Cl_2 (200 mL) and 1 M HCl (100 mL), washed with satd $NaHCO_3$ and brine, and then concentrated after drying over Na_2SO_4. The product was purified by column chromatography on silica gel (2:1 hexane–EtOAc). The unstable thiocarbonyl derivative **11** was immediately dissolved in toluene (100 mL), and then Bu_3SnH (1.5 mL, 5.58 mmol) was added to the solution. The mixture was refluxed for 15 h and the solvent was evaporated. The product was dissolved in MeCN (80 mL) and washed with hexane (2 × 40 mL) to remove the remaining Bu_3SnH. After evaporation of the solvent, the product was purified by column chromatography on silica gel (2:1 hexane–EtOAc) to afford **12** (1.18 g, 96.4%): mp 115.5–116.0 °C; $[\alpha]_D$ +91° (*c* 0.61, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.25 (ddd, 1 H, $J_{2,3eq}$ 5.0, $J_{3ax,3eq}$ 13.2, $J_{3ax,4}$ 3.0 Hz, H-3eq), 2.36 (ddd, 1 H, $J_{2,3ax}$ 12.6, $J_{3ax,4}$ 3.4 Hz, H-3ax), 3.47 (s, 3 H, OCH_3), 3.72 (m, 1 H, H-5), 4.12 (dd, 1 H, $J_{5,6a}$ 1.8, $J_{6a,6b}$ 12.6 Hz, H-6a), 4.24 (m, 1 H, H-4), 4.29 (dd, 1 H, $J_{5,6b}$ 1.2 Hz, H-6b), 5.13 (d, 1 H, $J_{1,2}$ 3.2 Hz, H-1), 5.47 (ddd, 1 H, H-2), 5.57 (s, 1 H, PhCH–), 7.35–8.07 (m, 10 H, aromatic H); MS: 371 [MH^+].

Methyl 2,4,6-tri-O-*benzoyl-3-deoxy*-α-D-xylo-*hexopyranoside* (**13**).—Compound **12** (1.45 g, 3.92 mmol) was dissolved in a mixture of MeOH (60 mL) and 1 M HCl (12 mL). The mixture was stirred for 3.5 h at 50 °C, and then satd $NaHCO_3$ was added to neutrality. The solution was evaporated and the product was extracted with EtOAc from the residue. After evaporation of the solvent, pyridine (60 mL) was added, followed by a dropwise addition of BzCl (2.1 mL, 17.5 mmol). The mixture was stirred overnight and concentrated, and the solution was washed with 1 M HCl, satd $NaHCO_3$, and brine, and then concentrated after drying over Na_2SO_4. The product was purified by column chromatography on silica gel (3:1 hexane–EtOAc) to afford **13** (1.57 g, 81.7%), which was recrystallized from hot EtOH: $[\alpha]_D$ +69° (*c* 1.3, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.38 (ddd, 1 H, $J_{2,3eq}$ 4.6, $J_{3ax,3eq}$ 13.6, $J_{3ax,4}$ 3.4 Hz, H-3eq), 2.47 (ddd, 1 H, $J_{2,3ax}$ 13.0, $J_{3ax,4}$ 3.0 Hz, H-3ax), 3.49 (s, 3 H, OCH_3), 4.42 (ddd, 1 H, $J_{4,5}$ 1.4, $J_{5,6a}$ 5.0, $J_{5,6b}$ 10.0 Hz, H-5), 4.44 (dd, 1 H, $J_{6a,6b}$ 8.4 Hz, H-6a), 4.56 (dd, 1 H, H-6b), 5.17 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 5.43 (ddd, 1 H, H-2), 5.57 (ddd, 1 H, H-4), 7.40–8.14 (m, 15 H, aromatic H); MS: 419 [MH^+].

p-Nitrophenyl 2,4,6-tri-O-*benzoyl-3-deoxy*-α-D-xylo-*hexopyranoside* (**15**).—Compound **15** was prepared from **13** (1.26 g, 2.57 mmol)

according to Methods B and C. The product was purified by column chromatography on silica gel (4:1 hexane–EtOAc) to afford **15** (0.95 g, (74.6%), which was recrystallized from EtOH: mp 133.5–134.0 °C; $[\alpha]_D$ +76° (*c* 0.60, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.56 (ddd, 1 H, $J_{2,3eq} = J_{3eq,4}$ 4.0, $J_{3ax,3eq}$ 13.8 Hz, H-3eq), 2.66 (ddd, 1 H, $J_{2,3ax}$ 12.4, $J_{3ax,4}$ 3.0 Hz, H-3ax), 4.41–4.53 (m, 3 H, H-5, H-6a, and H-6b), 5.60 (ddd, 1 H, $J_{1,2}$ 3.6 Hz, H-2), 5.62 (ddd, 1 H, $J_{4,5}$ 5.2 Hz, H-4), 6.06 (d, 1 H, H-1), 7.19–8.15 (m, 19 H, aromatic H); MS: 598 [MH^+].

*p-Nitrophenyl 3-deoxy-α-*D-xylo-*hexopyranoside* (**16**).—Compound **16** was prepared from **15** (0.67 g, 1.13 mmol) according to Method E. The product was purified by column chromatography on silica gel (8:1 CH_2Cl_2–MeOH) to afford **16** (0.22 g, 67.4%), which was recrystallized from hot EtOH: mp 162.0–163.5 °C, lit. 163.5–164.5 °C [27]; $[\alpha]_D$ +221° (*c* 0.56, MeOH), lit. +219° [27]; ^{1}H NMR (400 MHz, D_2O): δ 2.13 (ddd, 1 H, $J_{2,3eq} = J_{3eq,4}$ 4.4, $J_{3ax,3eq}$ 13.6 Hz, H-3eq), 2.23 (ddd, 1 H, $J_{2,3ax}$ 13.0, J_{3ax},4 3.0 Hz, H-3ax), 3.65–3.67 (m, 2 H, H-6a and H-6b), 3.89 (ddd, 1 H, $J_{4,5}$ 1.1, $J_{5,6a}$ 5.5, $J_{5,6b}$ 6.6 Hz, H-5), 4.13 (m, 1 H, H-4), 4.28 (ddd, 1 H, $J_{1,2}$ 3.6 Hz, H-2), 5.79 (d, 1 H, H-1), 7.33 (d, 2 H, *J* 9.2 Hz, aromatic H), 8.28 (d, 2 H, aromatic H); MS: 286 [MH^+]: Anal. Calcd for $C_{12}H_{15}O_7$: C, 50.53; H, 5.30; N, 4.91. Found: C, 50.37; H, 5.35; N, 4.91.

*Methyl 2,3,6-tri-*O*-benzoyl-α-*D*-glucopyranoside* (**18**).—Compound **17** (7.65 g, 39.4 mmol) and bistributyltin oxide [$(Bu_3Sn)_2O$, 25 mL, 59.1 mmol] were dissolved in toluene (750 mL). After refluxing the mixture overnight, the solvent was cooled to rt, and BzCl (20.6 mL, 177.4 mmol) was added. The mixture was stirred for 4 days at rt and the solvent was evaporated. The residue was dissolved in MeCN (400 mL) and washed with hexane (2×200 mL) to remove the remaining $(Bu_3Sn)_2O$. After evaporation of the solvent, the product was purified by column chromatography on silica gel (3:1 hexane–EtOAc) to afford **18** (10.9 g, 54.5%): $[\alpha]_D$ +140° (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.46 (s, 3 H, $-OCH_3$), 3.89 (m, 1 H, H-4), 4.12 (ddd, 1 H, $J_{5,6a}$ 2.2, $J_{5,6b}$ 4.6 Hz, H-5), 4.64 (dd, 1 H, $J_{6a,6b}$ 12.2 Hz, H-6a), 4.79 (dd, 1 H, H-6b), 5.15 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 5.27 (dd, 1 H, $J_{2,3}$ 10.2 Hz, H-2), 5.80 (dd, 1 H, $J_{3,4}$ 10.2 Hz, H-3), 7.35–8.11 (m, 15 H, aromatic H); MS: 507 [MH^+].

*Methyl 2,3,6-tri-*O*-benzoyl-4-deoxy-α-*D*-xylo-hexopyranoside* (**20**).—To a solution of **18** (10.9 g, 21.5 mmol) in CH_2Cl_2 (300 mL) was added 4-dimethylaminopyridine (7.89 g, 64.5 mmol) at rt, followed by dropwise addition of phenylchlorothionoformate [31] (3.6 mL, 25.8 mmol). The mixture was stirred for 4 days and the solvent was evaporated. The product was purified by column chromatography on silica gel (3:2 hexane–EtOAc). The unstable thiocarbonyl derivative **19** (11.1 g, 17.2 mmol) was immediately dissolved in toluene (700 mL), and then Bu_3SnH (5.8 mL, 20.6 mmol) was added to the solution. After refluxing the mixture overnight, the solvent was evaporated and the product was purified by column chromatography on silica gel (3:1 hexane–EtOAc) to afford **20** (7.95 g, 94.3%): mp 115.5–117.0 °C; $[\alpha]_D$ +132° (*c* 1.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.90 (ddd, 1 H, $J_{3,4ax} = J_{4ax,5} = J_{4ax,4eq}$ 11.9 Hz, H-4ax), 2.48 (ddd, 1 H, $J_{3,4eq}$ 5.4, $J_{4eq,5}$ 2.2 Hz, H-4eq), 3.46 (s, 3 H, $-OCH_3$), 4.36–4.50 (m, 3 H, H-5, H-6a and H-6b), 5.18 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 5.32 (dd, 1 H, $J_{2,3}$ 10.0 Hz, H-2), 5.79 (ddd, 1 H, H-3), 7.36–8.10 (m, 15 H, aromatic H); MS: 419 [MH^+].

*p-Nitrophenyl 2,3,6-tri-*O*-benzoyl-4-deoxy-α-*D*-xylo-hexopyranoside* (**22**).—Compound **21** was prepared from **20** (7.95 g, 16.2 mmol) according to Method B. The residue was purified by column chromatography on silica gel (4:1 hexane–EtOAc) to afford **21** (6.96 g, 82.8%). Compound **22** was prepared from **21** (1.44 g, 2.78 mmol) according to Method C. The product was purified by column chromatography on silica gel (4:1 hexane–EtOAc) to afford **22** (1.17 g, 70.4%), which was recrystallized from hot EtOH: mp 173.0–176.0 °C; $[\alpha]_D$ +148° (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.99 (ddd, 1 H, $J_{3,4ax} = J_{4ax,5} = J_{4ax,4eq}$ 12.0 Hz, H-4ax), 2.55 (ddd, 1 H, $J_{3,4eq}$ 5.0, $J_{4eq,5}$ 5.2 Hz, H-4eq), 4.36–4.47 (m, 3 H, H-5, H-6a and H-6b), 5.50 (dd, 1 H, $J_{1,2}$ 3.6, $J_{2,3}$ 10.4 Hz, H-2), 5.99 (ddd, 1 H, H-3), 6.06 (d, 1 H, H-1), 7.39–8.08 (m, 15 H, aromatic H); MS: 598 [MH^+].

p-Nitrophenyl 4-deoxy-α-D-xylo-hexopyranoside (**23**).—Compound **23** was prepared from **22** (0.30 g, 0.51 mmol) according to Method E. The product was purified by column chromatography on silica gel (7:1 CH_2Cl_2–MeOH) to afford **23** (0.13 g, 87.9%), which was recrystallized from EtOH: mp 152.5–155.0 °C; $[\alpha]_D$ + 243° (*c* 0.51, MeOH); ^{1}H NMR (400 MHz, D_2O): δ 1.58 (ddd, 1 H, $J_{3,4ax}$ 14.1, $J_{4ax,4eq} = J_{4ax,5}$ 12.1 Hz, H-4ax), 2.05 (m, 1 H, H-4eq), 3.52–3.64 (m, 2 H, H-6a and H-6b), 3.71 (dd, 1 H, $J_{1,2}$ 3.4, $J_{2,3}$ 10.4 Hz, H-2), 3.99 (m, 1 H, H-5), 4.21 (ddd, 1 H, $J_{3,4eq}$ 5.4 Hz, H-3), 5.86 (d, 1 H, H-1), 7.27–8.29 (m, 4 H, aromatic H); MS: 286 [MH^+]; Anal. Calcd for $C_{12}H_{15}O_7$: C, 50.53; H, 5.30; N, 4.91. Found: C, 50.81; H, 5.43; N, 4.75.

p-Nitrophenyl 2,3,4-tri-O-acetyl-α-D-fucopyranoside (**26**).—Compound **25** was prepared from **24** (1.01 g, 5.91 mmol) according to Method A. The product was purified by column chromatography on silica gel (2:1 hexane–EtOAc) to afford **25** (1.95 g, 99.3%). Compound **26** was prepared from **25** (4.55 g, 13.7 mmol) according to Method C. The product was purified by column chromatography on silica gel (3:1 hexane–EtOAc) to afford **26** (1.20 g, 21.3%), which was recrystallized from EtOH: mp 171.5–172.5 °C; $[\alpha]_D$ + 225° (*c* 1.0, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.13 (d, 3 H, $J_{5,6}$ 6.8 Hz, H-6), 2.04, 2.07, 2.21 (3 s, 9 H, 3 × $OCOCH_3$), 4.19 (dq, 1 H, $J_{4,5}$ 1.0 Hz, H-5), 5.31 (dd, 1 H, $J_{2,3}$ 11.2, $J_{3,4}$ 3.6 Hz, H-3), 5.37 (dd, 1 H, H-4), 5.57 (dd, 1 H, $J_{1,2}$ 3.5 Hz, H-2), 5.86 (dd, 1 H, H-1), 7.15–8.23 (m, 4 H, aromatic H); MS: 412 [MH^+].

p-Nitrophenyl α-D-fucopyranoside (**27**).—Compound **27** was prepared from **26** (1.00 g, 2.44 mmol) according to Method D. The product was recrystallized from hexane–EtOAc to afford **27** (0.624 g, 92.3%): mp 194.5–195.5 °C; $[\alpha]_D$ + 249° (*c* 1.0, MeOH); ^{1}H NMR (400 MHz, D_2O): δ 1.16 (d, 3 H, $J_{5,6}$ 6.8 Hz, H-6), 4.19 (dd, 1 H, $J_{3,4}$ 3.2 Hz, H-4), 4.01 (dd, 1 H, $J_{1,2}$ 3.8, $J_{2,3}$ 10.4 Hz, H-2), 4.11–4.17 (m, 2 H, H-3 and H-5), 5.82 (d, 1 H, H-1), 7.27–8.29 (m, 4 H, aromatic H); MS: 286 [MH^+]; Anal. Calcd for $C_{12}H_{15}O_7$: C, 50.53; H, 5.30; N, 4.91. Found: C, 50.36; H, 5.27; N, 4.85.

Assay of enzyme hydrolytic activity.—Pure-grade α-galactosidases from *M. vinacea* (Seikagaku Ind.), *A. niger* (Sigma Chemical Co.), and green coffee bean (Böehringer Mannheim Co.) were used in this study. PNP α-D-galactopyranoside was purchased from Tokyo Kasei Ind., and recrystallized from EtOH.

Hydrolytic activity of α-galactosidase was assayed by measuring the release of *p*-nitrophenol from each glycosidic derivative after incubation at the following conditions: *M. vinacea* in 50 mM sodium phosphate buffer (pH 5.9) at 40 °C, *A. niger* in 50 mM sodium acetate buffer (pH 4.0) at 25 °C, green coffee bean in 50 mM sodium phosphate buffer (pH 7.2) at 25 °C. The enzyme reaction was stopped by the addition of 0.3 M Na_2CO_3, and the *p*-nitrophenol released by the reaction was measured spectrophotometically at 405 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol/min at each assay condition. Kinetic studies on the hydrolysis of PNP α-D-galactopyranoside and its deoxy derivatives by the α-galactosidases were performed at concentrations between 0.8 and 2.4 mM, and values of K_m and V_{max} were calculated from reciprocal plots of the reaction curves.

Protein in the enzyme solution was determined by the method of Lowry using bovine serum albumin as a standard.

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