Synthesis of Methyl 5′-Thio-α-isomaltoside via an Acyclic Monothioacetal and Its Behavior toward Glucoamylase

Hironobu Hashimoto,* Masashi Kawanishi, and Hideya Yuasa

Abstract: Methyl 5′-thio-α-isomaltoside (**1**), which contains the ring-sulfur analogue of the nonreducing glucoside of isomaltose, was synthesized from gentiobiose through a novel ring opening–recyclization approach. The nonreducing glucoside of per-*O*-benzylated phenyl 1-thio-β-gentiobioside underwent O-5′–C-1′ bond cleavage with dimethylboron bromide and thiolacetic acid to give the acyclic monothioacetal **4** with the 1-thioglucopyranoside at the reducing end intact. The HO-5′ group in **4** was inverted by a standard oxidation–reduction process with good efficiency. Recyclization under Mitsunobu condition allowed C-5′–S-1′ bond formation with inversion of configuration at C-5′, to give **1** after functional group interconversion. TLC analysis showed that **1**, unlike isomaltose, was not hydrolyzed by glucoamylase from *Rhizopus niveus*. A fluorometric assay confirmed that the dissociation constant (K_d) for **1** with the enzyme was 39 mM at 20 °C, which is comparable with that for isomaltose. A binding assay involving fluorescence titration of the enzyme–**1** complex with gluconolactone indicated that the disaccharide **1** was bound to the catalytic and noncatalytic subsites. Since isomaltose is known to bind only to the noncatalytic subsites, this result indicates a relatively high affinity of the 5-thioglucose moiety for the catalytic subsite.

Keywords
binding studies · glucoamylase · isomaltoses · oligosaccharides · thiosugars

Introduction

The ring oxygen of pyranose plays an essential role in biosyntheses and metabolisms of oligosaccharides by facilitating substitution reactions at the anomeric carbon. A number of studies were accordingly performed on the bioactivity of sugar analogues having a heteroatom in the ring. Among them, azasugars have been drawing much attention, since they are usually strong inhibitors of glycosidases.[1] On the other hand, biological properties of 5-thiosugars are not significantly different from those of the natural sugars.[2] This suggests that 5-thiosugars can be used as probes for studying the biological role of the ring oxygen of carbohydrates by comparing, for example, binding abilities to sugar-binding proteins. In particular, disaccharides that have a 5-thiosugar at the nonreducing end are of interest,[3] because the ring sulfur may perturb the *exo* anomeric conformation,[4] which has an O-5–C-1–O-1–C aglycon dihedral angle of 60°, thereby altering its affinity to the proteins.

In this report, we investigate the binding of methyl 5′-thio-α-D-isomaltoside (**1**) to glucoamylase from *Rhizopus niveus*. The enzyme has seven subsites (subsites 1–7) that recognize the heptasaccharide [Glcα(1 → 4)]$_7$ at the nonreducing end of amylose (Fig. 1); the catalytic group that hydrolyzes the nonreducing glucoside is located in subsite 1.[5] Amylose initially binds only to subsite 2 with the nonreducing glucoside (nonproductive binding), and then rearranges to the heptasaccharide binding mode (productive binding).[5a, b] The enzyme has such a high specificity that glucono-1,5-lactone and glucose exclusively bind to subsites 1 and 2, respectively.[5b, c] It is noteworthy that although isomaltose is hydrolyzed by the enzyme, it exclusively binds to the noncatalytic subsites (subsites 2 and 3).[5d] From the above examples, it seems that the grooves of subsites 1 and 2 are complementary to the transition and ground states of the substrate, respectively. In this respect, it is of interest to determine whether **1** acts as a substrate to the enzyme and, if so, at which subsites and to what degree **1** is bound to the enzyme. This study aims to provide information about the significance of the ring oxygen in the recognition of sugars by proteins.

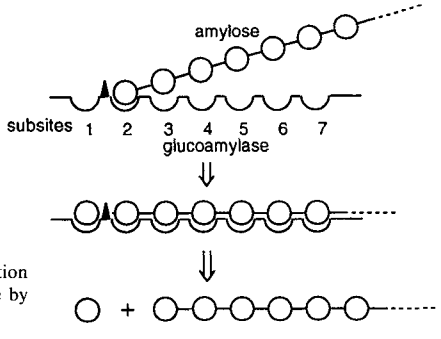

Fig. 1. Schematic representation of the hydrolysis of amylose by glucoamylase.

Results and Discussion

Synthesis of 5′-thioisomaltose: A 5′-thioisomaltose derivative was synthesized by Mehta et al. through 5-thioglucosylation of a glucose derivative by the trichloroimidate method.[3c] The

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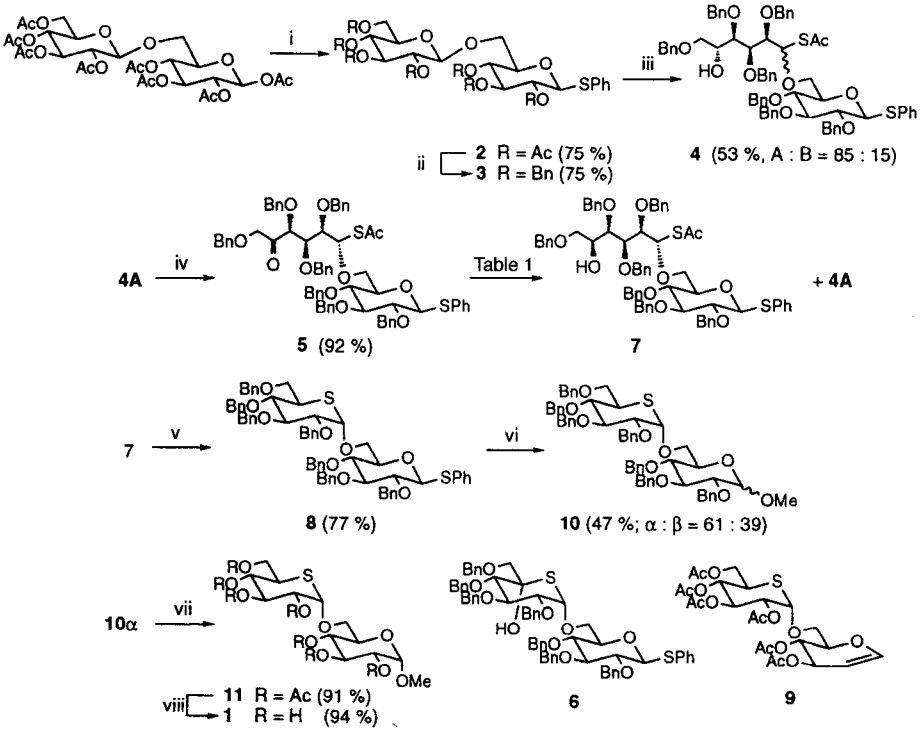
standard synthesis of 5-thioglucose is rather cumbersome. We therefore developed a new method[6] for synthesizing the disaccharide analogue. This method features the opening of the β-pyranoside ring with dimethylboron bromide and thiolacetic acid according to the method described by Guindon and Anderson,[7] yielding the 5-hydroxymonothioacetal derivative, and recyclization with formation of a C-5–S-1 bond.

Gentiobiose was chosen as the starting material. The aglycon moiety must remain intact during the ring opening of the pyranoside at the nonreducing glucoside. We therefore selected phenyl 1-thio-β-D-glucopyranoside as an aglycon moiety, since a preliminary examination indicated that this substrate is nearly inert under the ring-opening reaction conditions. Thus, per-*O*-benzylated phenyl 1-thiogentiobioside (**3**), which was obtained from per-*O*-acetylated gentiobiose[8] in two steps, was treated with dimethylboron bromide followed by thiolacetic acid to give the monothioacetal as a diastereomeric mixture (**4A** and **4B**; Scheme 1). Although we could not assign the configuration at C-1′ at this stage, the (*S*) configuration was deduced for **4A** at the end of the synthesis on recyclization.



Scheme 1. Synthesis of **1**: i) PhSH, BF_3OEt_2; ii) NaOMe; NaH; BnBr; iii) Me_2BBr, −78 °C; AcSH, iPr_2NEt; iv) $(COCl)_2$, DMSO, −78 °C; TEA; v) Ph_3P, DEAD; vi) MeOH, NBS; vii) Na, liq. NH_3, −78 °C; Ac_2O, pyridine; viii) NaOMe.

The first attempt at recyclization ended in failure: **4A** was oxidized by the Swern method to give the ulose **5** in good yield; the thioacetyl group was deprotected with 2-aminoethanethiol, and the resulting thiol cyclized spontaneously to give the hemithioacetal **6** in 92% yield. However, attempts to remove the 5′-OH of **6** by reduction with triethylsilane and trifluoroborane resulted in the formation of a multitude of products. A conventional oxidation–reduction method was therefore used to invert the configuration at C-5′ of **4A**. The results of the reduction of **5** with three types of reagent, namely, nonchelating $NaBH_4$, chelating $Zn(BH_4)_2$, and nonchelating bulky $Li(tBuO)_3AlH$, are listed in Table 1. Both of the nonchelating reagents provided the desired Cram product **7** in good yields and with high

Table 1. Results of the reduction of **5**.

Reagent [a]	*T* (°C)	Yield (%)	7:4A
$NaBH_4$	0	92	65:35
$NaBH_4$	−20	98	70:30
$Zn(BH_4)_2$	0	78	51:49
$Li(tBuO)_3AlH$	RT [b]	86	92:8
$Li(tBuO)_3AlH$	−10	91	96:4

[a] For details, see Experimental Procedure. [b] Room temperature.

diastereoselectivities; $Li(tBuO)_3AlH$ gave diastereoselectivities as high as 92%. $Zn(BH_4)_2$, which was expected to preferentially give the anti-Cram product **4A** as a result of chelation, was incapable of bringing about a significant stereoselectivity. Cyclization of **7** was successfully performed under Mitsunobu conditions with inversion of the configuration at C-5′ to afford the disaccharide **8**.[9]

Unfortunately, the Birch reduction of **8** with Na in liquid ammonia followed by acetylation exclusively gave the undesired isomaltal **9** in 57% yield. Since the glycal formation seemed to be due to the sulfur atom at C-1′, **8** was converted into methyl α-glycoside **10α** by means of standard procedures; some β-glycoside **10β** was also formed (α:β = 3:2). Deprotection of the benzyl groups of **10α** by the Birch reduction followed by the usual acetylation gave the peracetate **11**. The $^3J_{HH}$ values for the 5-thioglucoside moiety of **11** ($^3J_{1',2'} = 3.0$, $^3J_{2',3'} = 9.9$, $^3J_{3',4'} = {}^3J_{4',5'} = 10.1$ Hz) are indicative of a $^4C_1$ conformation with an α-anomeric configuration; this indicates that C-1′ in **4A** and C-5′ in **7** have the (*S*) configuration. The Zémplen deacetylation of **11** afforded 5′-thioisomaltose **1**.

Enzyme assays: The lability of 5′-thioisomaltose **1** toward the hydrolysis by glucoamylase from *Rhizopus niveus* was examined by TLC. Exposing **1** to the enzyme solution for as much as 600 min produced no hydrolyzed products, while isomaltose was completely hydrolyzed in 100 min under the same conditions. This indicates that 5-thiosugars are inferior hydrolase substrates, which is in agreement with the previous finding that 5′-thio-*N*-acetyllactosamine is hydrolyzed by a galactosidase at a retarded rate.[3a] These studies reveal a distinctive characteristic of the enzymes as catalysts, since the acid hydrolysis of methyl 5-thio-D-xylopyranoside proceeds faster than that of the corresponding ring oxygen analogue.[10]

The dissociation constant of **1** from the enzyme was evaluated by the change in fluorescence associated with enzyme–ligand binding.[5] The fluorescence isotherm obtained in a titration of **1** was applied to the Scatchard equation [Eq. (1)],[11] where

$$[\mathbf{1}]/\Delta F = [\mathbf{1}]/\Delta F^{S}_{max} + K_d/\Delta F^{S}_{max} \quad (1)$$

ΔF is the observed fluorescence change, K_d the dissociation constant, ΔF^{S}_{max} the fluorescence change at saturation, and [**1**] is the

concentration of **1**. From the regression analysis for the isotherm (Fig. 2), a K_d of 39 mM (at 20 °C) and a ΔF^{S}_{max} of 21 % were obtained. The affinity of **1** for the enzyme is comparable with that of isomaltose (K_d = 18 mM at 5 °C).[5d]

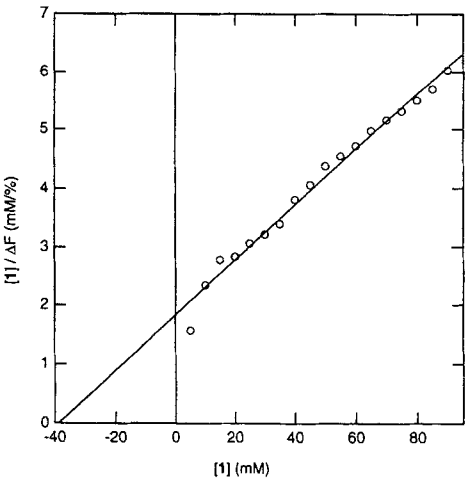
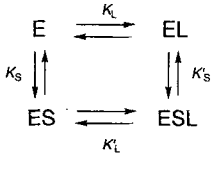


Fig. 2. Scatchard plot for the binding of **1** with the glucoamylase. $[E]_0$ = 3.3 μM; pH 4.5, 20 °C, λ_{ex} = 280 nm.

The subsite specificity of the enzyme for **1** was investigated with the method of Hiromi et al.[5a] This method is based on the analysis of a fluorescence change that occurs concomitantly with titration of the enzyme–**1** complex with glucono-1,5-lactone.[12] Since glucono-1,5-lactone (L) is known to bind exclusively to subsite 1, the dissociation constant (K'_S) for the binding of **1** (S) to the enzyme–lactone complex (EL) represents the affinity of **1** for the subsites other than subsite 1 (Scheme 2). If **1** has an affinity for subsite 1, K'_S should be larger than K_S (= K_d), which represents the sum of the affinities of **1** for each subsite.



Scheme 2. Three possible complexes for the mixture of glucoamylase (E), gluconolactone (L), and **1** (S).

ΔF values, obtained by titration of the mixture of **1** ($[S]_0$ = 39 mM) and the enzyme with the lactone, were analyzed through the fluorescence isotherm[12] with a curve-fitting program (Fig. 3).[13] Thus, a ΔF^{SL}_{max} of 51 %, a K'_S of 128 mM, and a K'_L of 5.2 mM were obtained. The results indicate that **1** binds to subsites 1 and 2 as well as to the other subsites, because K'_S is larger than K_S (39 mM). In addition, the affinity of **1** for the subsites other than subsite 1 is much lower than that of isomaltose (K'_S = 128 vs 18 mM); this in turn demonstrates the importance of the ring oxygen of isomaltose for binding to the noncatalytic subsites. On the other hand, the affinity of **1** for subsites 1 and 2 is much higher than that of isomaltose. This indicates that 5-thio-α-glucoside has a higher affinity for the catalytic subsite than does α-glucoside. It is noteworthy that this trend is similar to those found for 5-thiofucose[14] and 5-thioglucose[15] toward α-glycosidases.

Experimental Procedure

General: Melting points were measured with a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were determined with a JASCO DIP-4 polarimeter. Column chromatography was performed on Merck Kieselgel 60 (Art 7734) or Wako gel C-300 with the solvent systems specified. ^{1}H NMR spectra were recorded with a JEOL JNM-FX-90Q, JNM-PS-100, JNM-EX-270, or JNM-GX-500 spectrometer. ^{13}C NMR spectra were recorded with a JEOL JNM-FX-90Q or JNM-EX-270 spectrometer. Chemical shifts were recorded as δ values in parts per million (ppm) from tetramethylsilane as an internal standard in $CDCl_3$. In D_2O, acetone ($\delta(^1H)$ = 2.23 and $\delta(^{13}C)$ = 30.6) was used as an internal standard.

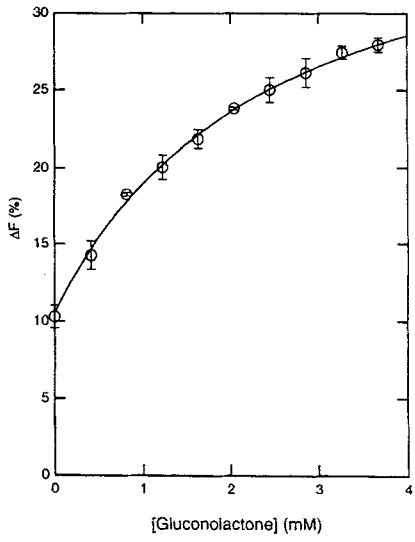


Fig. 3. Dependence the fluorescence intensity decrease (%, ΔF) at 340 nm on the initial concentration of gluconolactone in the presence of **1** (39 mM) and the enzyme. $[E]_0$ = 3.3 μM; pH 4.5, 20 °C, λ_{ex} = 280 nm. The curve was fitted with the isotherm described in ref [12].

Phenyl 2,3,4,2′,3′,4′,6′-Hepta-*O*-acetyl-1-thio-β-gentiobioside (2): To a stirred solution of 1,2,3,4,2′,3′,4′,6′-octa-*O*-acetyl-β-gentiobiose [8] (19.88 g, 29.3 mmol) in CH_2Cl_2 (200 mL) was added thiophenol (3.8 mL, 35.2 mmol) and BF_3·OEt_2 (18.4 mL, 147 mmol) at 0 °C. After 15 min, ice-water was added and the mixture was extracted with CH_2Cl_2. The organic layer was washed successively with 1 % NaOH and water, dried over $MgSO_4$, and evaporated to give crystals, which were recrystallized from EtOH to give **2** (16.0 g, 75%): M.p. 168–170 °C (from EtOH); $[\alpha]_D^{23}$ = −19.1 (*c* = 1.07 in $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ = 7.5–7.2 (m, 5H; aromatic protons), 5.3–4.7 (m, 6H; H-2, -3, -4, -2′,-3′, -4′), 4.67 (d, $^3J_{1,2}$ = 12.2 Hz, 1H; H-1), 4.53 (d, $^3J_{1',2'}$ = 7.7 Hz, 1H; H-1′), 4.25 (dd, $^3J_{5',6'a}$ = 4.5 Hz, $^3J_{6'a,6'b}$ = 12.6 Hz, 1H; H-6′a), 4.08 (dd, $^3J_{5',6'b}$ = 2.9 Hz, 1H; H-6′b), 3.9–3.4 (m, 4H; H-5, -6a, -6b, -5′), 2.1–1.9 (m, 21H; OAc); $C_{32}H_{40}O_{17}S$ (728.7): calcd C 52.74, H 5.53, S 4.40; found C 52.96, H 5.64, S 4.39.

Phenyl 2,3,4,2′,3′,4′,6′-Hepta-*O*-benzyl-1-thio-β-gentiobioside (3): NaOMe/MeOH (0.5 M, 4.4 mL, 2.2 mmol) was added dropwise to a stirred solution of **2** (16.02 g, 22.0 mmol) in methanol (200 mL). The solution was stirred for 3 h at room temperature. After neutralization with Dowex 50 (H^+), the resin was removed by filtration. The filtrate was evaporated and dried under high vacuum. The resultant amorphous solid was dissolved in DMF (500 mL). Benzyl bromide (36.6 mL, 308 mmol) and 55% NaH (3.4 g, 77.9 mmol) were added at 0 °C, and the reaction mixture was sonicated. After 20 min, a larger portion of 55% NaH (16.7 g, 384 mmol) was added. After a further 40 min, benzyl bromide (18.3 mL, 154 mmol) was added, and the mixture was stirred for 3 h. Methanol was carefully added to quench the reagents, and neutralization was carried out with acetic acid. The mixture was evaporated and chromatographed on a silica gel (*n*-hexane:ethyl acetate, 10:1–4:1) to give crystals, which were recrystallized from EtOH to give **3** (17.59 g, 75%): M.p. 120–122 °C (from EtOH); $[\alpha]_D^{23}$ = +10.5 (*c* = 1.8 in $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ = 7.6–7.1 (m, 40H; aromatic protons), 5.0–4.0 (m, 16H; H-1, -1′, CH_2Ph), 4.0–3.3 (m, 12H; H-2, -3, -4, -5, -6, -2′, -3′, -4′, -5′, -6′); $C_{67}H_{68}O_{10}S$ (1065.3): C 75.47, H 6.43, S 3.01; found C 75.90, H 6.49, S 3.44.

Phenyl 6-*O*-{(1*S*)- and (1*R*)-1-*C*-Acetylthio-2,3,4,6-tetra-*O*-benzyl-D-glucitol-1-yl}-2,3,4-tri-*O*-benzyl-1-thio-β-D-glucopyranoside (4A, 4B): Me_2BBr in CH_2Cl_2 (1.0 M, 19.5 mL, 19.5 mmol) was added dropwise to a stirred solution of **3** (5.18 g, 4.87 mmol) in CH_2Cl_2 (100 mL) under Ar at −78 °C. After 2 h, *i*Pr_2NEt (3.8 mL, 21.9 mmol) and AcSH (1.4 mL, 19.5 mmol) were added successively, and the mixture was further stirred for 2 h at −78 °C. Ice-water was added and the mixture extracted with CH_2Cl_2. The organic layer was successively washed with aq. $NaHCO_3$, 5% HCl, and water, dried over $MgSO_4$, and evaporated. The resultant syrup was chromatographed on a silica gel (*n*-hexane:ethyl acetate 10:1–5:1) to give **4A** (2.49 g, 45%) and **4B** (0.433 g, 7.8%) as a syrup, respectively.

4A: $[\alpha]_D^{21}$ = −7.9 (*c* = 3.2 in $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ = 7.6–7.1 (m, 40H; aromatic protons), 5.68 (d, $^3J_{1',2'}$ = 2.6 Hz, 1H; H-1′), 5.0–3.2 (m, 27H; H-1, -2, -3, -4, -5, -6, -2′, -3′, -4′, -5′, -6′), 2.96 (br s, 1H; OH-5′), 2.18 (s, 3H; SAc); ^{13}C NMR ($CDCl_3$): δ = 195.4 (SC=O), 138.6–127.4 (17 peaks; aromatic carbons), 87.8, 87.7,

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86.7, 82.2, 80.9, 80.0, 78.1, 77.9, 77.3, 75.3, 75.2, 75.1, 74.8, 73.4, 73.2, 71.4, 70.5 (C-1, -2, -3, -4, -5, -1′, -2′, -3′, -4′, -5′, -6′, CH_2Ph), 67.4 (C-6), 30.9 (SAc); $C_{69}H_{72}O_{11}S_2$ (1141.5): calcd C 72.61, H 6.36, S 5.62; found C 72.63, H 6.67, S 4.96.

4B: $[\alpha]_D^{21} = +3.3$ ($c = 4.9$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 7.6-7.1$ (m, 40H; aromatic protons), 5.75 (d, $^3J_{1',2'} = 3.3$ HZ, 1H; H-1′), 5.0–3.2 (m, 27H; H-1, -2, -3, -4, -5, -6, -2′, -3′, -4′, -5′, -6′, CH_2Ph), 2.66 (brs, 1H; OH-5′), 2.27 (s, 3H; SAc); ^{13}C NMR ($CDCl_3$): $\delta = 195.5$ (SC=O), 138.5–127.5 (17 peaks; aromatic carbons), 88.2, 87.5, 86.6, 82.2, 80.8, 78.2, 78.1, 75.7, 75.5, 75.1, 75.0, 74.9, 73.4, 71.8, 71.2, 70.5 (C-1, -2, -3, -4, -5, -1′, -2′, -3′, -4′, -5′, -6′, CH_2Ph), 67.3 (C-6), 31.1 (SAc); $C_{69}H_{72}O_{11}S_2$ (1141.5): calcd C 72.61, H 6.36, S 5.62; found C 72.75, H 6.42, S 6.08.

Phenyl 6-*O*-{(1*S*)-1-*C*-Acetylthio-2,3,4,6-tetra-*O*-benzyl-D-*xylo*-5-hexulose-1-yl}-2,3,4-tri-*O*-benzyl-1-thio-β-D-glucopyranoside (5): A solution of DMSO (3.9 mL, 55.1 mmol) in CH_2Cl_2 (40 mL) was slowly added to a stirred solution of oxalyl chloride (2.4 mL, 27.6 mmol) in CH_2Cl_2 (40 mL) under Ar at −78 °C, followed by a solution of **4A** (7.87 g, 6.89 mmol) in CH_2Cl_2 (40 mL). After 1 h, triethylamine (9.6 mL, 68.9 mmol) was slowly added, and the mixture allowed to reach room temperature over 1 h. Ice-water was added and the mixture extracted with CH_2Cl_2. The organic layer was washed successively with 5% HCl, sat. $NaHCO_3$, and water, dried over $MgSO_4$, and evaporated. The resultant syrup was chromatographed on silica gel (*n*-hexane:ethyl acetate 4:1) to give **5** (7.25 g, 92%) as a syrup: $[\alpha]_D^{21} = -17.9$ ($c = 2.8$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 7.6-7.1$ (m, 40H; aromatic protons), 5.53 (d, $^3J_{1',2'} = 3.3$ Hz, 1H; H-1′), 5.0–3.2 (m, 26H; H-1, -2, -3, -4, -5, -6, -2′, -3′, -4′, -6′, CH_2Ph), 2.19 (s, 3H; SAc); ^{13}C NMR ($CDCl_3$): $\delta = 206.8$ (C-5′), 195.5 (SC=O), 138.4–127.5 (14 peaks; aromatic carbons), 87.7, 86.9, 86.7, 82.5, 81.6, 80.8, 78.0, 77.2, 75.4, 75.3, 74.8, 74.0, 73.3 (C-1, -2, -3, -4, -5, -1′, -2′, -3′, -4′, -6′, CH_2Ph), 67.4 (C-6), 31.0 (SAc); $C_{69}H_{70}O_{11}S_2$ (1139.4): calcd C 72.73, H 6.19, S 5.63; found C 72.79, H 6.03, S 5.97.

Phenyl 6-*O*-{(1*S*)-1-*C*-Acetylthio-2,3,4,6-tetra-*O*-benzyl-L-iditol-1-yl}-2,3,4-tri-*O*-benzyl-1-thio-β-D-gluco pyranoside (7): **Method A**: To a stirred suspension of $NaBH_4$ (9 mg, 229 μmol) in isopropanol/CH_2Cl_2 (2 mL, 1/1 *v*/*v*) was added a solution of **5** (65 mg, 57 μmol) in CH_2Cl_2 (1 mL) at 0 °C. After 30 min, water and 5% HCl were successively added, and the mixture was extracted three times with CH_2Cl_2. The organic layer was dried over $MgSO_4$, evaporated, and chromatographed on silica gel (*n*-hexane:ethyl acetate 3:1) to give **7** and **4A** (60 mg, 92%) in a ratio of 65:35.

Method B: To a stirred solution of **5** (126 mg, 111 μmol) in Et_2O (2 mL) was added $Zn(BH_4)_2$/Et_2O (0.14 M, 4.0 mL, 560 μmol) under Ar at 0 °C. After 30 min, water and 30% AcOH were added, and the mixture was extracted twice with Et_2O. The organic layer was washed with sat. $NaHCO_3$, dried over $MgSO_4$, evaporated, and chromatographed on silica gel (*n*-hexane: ethyl acetate 3:1) to give **7** and **4A** (98 mg, 78%) as a mixture in the ratio of 49:51.

Method C: To a stirred solution of **5** (7.15 g, 6.28 mmol) in Et_2O (300 mL) was added $Li(tBuO)_3AlH$ (3.19 g, 12.6 mmol) under Ar at −10 °C. After 20 min, the excess reagent was decomposed with cold 1 M HCl, and the mixture extracted with Et_2O. The organic layer was washed with sat. NaCl, dried over $MgSO_4$, evaporated to give a 96:4 mixture of **7** and **4A**, which was chromatographed twice on silica gel (*n*-hexane: ethyl acetate 4:1) to give **7** (6.11 g, 85%) and a syrupy mixture of **7** and **4A** (0.428 g, 6%).

7: $[\alpha]_D^{20} = -0.9$ ($c = 4.1$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 7.6-7.2$ (m, 40H; aromatic protons), 4.94 (d, $^3J_{1',2'} = 3.3$ Hz, 1H; H-1′), 5.0–3.2 (m, 27H; H-1, -2, -3, -4, -5, -6, -2′, -3′, -4′, -5′, -6′, CH_2Ph), 2.66 (brs, 1H; OH-5′), 2.18 (s, 3H; SAc); ^{13}C NMR ($CDCl_3$): $\delta = 195.3$ (SC=O), 138.5–127.4 (19 peaks; aromatic carbons), 88.0, 87.4, 86.6, 81.7, 80.8, 79.3, 78.2, 77.6, 75.7, 75.4, 74.9, 74.6, 74.1, 73.2, 71.4, 69.5, 67.2 (C-1, -2, -3, -4, -5, -6, -1′, -2′, -3′, -4′, -5′, -6′, CH_2Ph), 30.9 (SAc); $C_{69}H_{72}O_{11}S_2$ (1141.5): calcd C 72.61, H 6.36, S 5.62; found C 72.42, H 6.14, S 6.16.

Phenyl 6-*O*-(2,3,4,6-Tetra-*O*-benzyl-5-thio-α-D-glucopyranosyl)-2,3,4-tri-*O*-benzyl-1-thio-β-D-glucopyranoside (8): To a stirred solution of **7** (5.90 g, 5.17 mmol) and Ph_3P (4.07 g, 15.5 mmol) in benzene (300 mL) was added diethyl azodicarboxylate (2.3 mL, 15.5 mmol) at 0 °C. The mixture was stirred for 20 h at room temperature, concentrated, and chromatographed on silica gel (*n*-hexane:ethyl acetate 9:1–5:1) to give **8** (4.30 g, 77%) as a syrup: $[\alpha]_D^{21} = +72.6$ ($c = 3.7$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 7.6-7.0$ (m, 40H; aromatic protons), 5.03–4.38 (m, 16H; H-1, -1′, CH_2Ph), 4.08–3.10 (m, 12H; H-2, -3, -4, -5, -6, -2′, -3′, -4′, -5′, -6′); ^{13}C NMR ($CDCl_3$): $\delta = 139.0-127.5$ (14 peaks; aromatic carbons), 87.7, 86.7, 84.8, 83.1, 82.1, 81.1, 80.6, 79.2, 77..8, 76.1, 74.9, 73.2, 72.4, 68.0, 66.8 (C-1, -2, -3, -4, -5, -6, -1′, -2′, -3′, -4′, -5′, -6′, CH_2Ph), 41.3 (C-5′); $C_{67}H_{68}O_9S_2$ (1081.4): calcd C 74.42, H 6.34, S 5.93; found C 74.64, H 6.41, S 5.94.

6-*O*-(2,3,4,6-Tetra-*O*-acetyl-5-thio-α-D-glucopyranosyl)-3,4-di-*O*-acetyl-1,5-anhydro-2-deoxy-D-arabino-hex-1-enitol (9): A flask containing a stirred solution of **8** (89 mg, 82 μmol) in THF (4 mL) was charged with liq. NH_3 under Ar at −78 °C. Sodium (100 mg, 4.3 mmol) was added in portions over 1 h. The mixture was stirred for a further 1 h, at which point the blue color did not disappear anymore. NH_4Cl (428 mg, 8 mmol) was carefully added, and the mixture was stirred for 30 min. After evaporation, the resultant syrup was acetylated in the usual manner with pyridine and acetic anhydride. The resultant compound was purified by column chromatography on silica gel (*n*-hexane:ethyl acetate 3:1–1:1) to give **9** (27 mg, 57%) as crystals: M.p. 195–196 °C (from EtOH); $[\alpha]_D^{21} = +176$ ($c = 0.89$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 6.46$ (dd, $^3J_{1,2} = 6.1$ Hz, $^3J_{1,3} = 1.2$ Hz, 1H; H-1), 5.49 (bt, $^3J_{2',3'} = 10.2$ Hz, $^3J_{3',4'} = 9.3$ Hz, 1H; H-3′), 5.34–5.19 (m, 3H; H-3, -4, -4′), 5.13 (dd, $^3J_{1',2'} = 3.0$ Hz, 1H; H-2′), 4.87–4.84 (m, 2H; H-2, -1′), 4.39 (dd, $^3J_{5',6'a} = 4.6$ Hz, $^3J_{6'a,6'b} = 12.2$ Hz, 1H; H-6′a), 4.29 (dt, $^3J_{4,5} = {}^3J_{5,6a} = 5.9$ Hz, $^3J_{5,6b} = 3.8$ Hz, 1H; H-5), 4.03 (dd, $^3J_{5',6'b} = 3.1$ Hz, 1H; H-6′b), 3.99 (dd, $^3J_{6a,6b} = 11.1$ Hz, 1H; H-6a), 3.64 (dd, 1H; H-6b), 3.50 (ddd, $^3J_{4',5'} = 10.9$ Hz, 1H; H-5′), 2.11–2.01 (m, 18H; OAc); ^{13}C NMR ($CDCl_3$): $\delta = 170.6$, 170.3, 169.6 (OC=O), 145.7 (C-1), 98.8 (C-2), 80.1 (C-1′), 74.8, 74.6, 72.3, 70.9, 67.7, 67.1, 66.5, 61.7 (C-3, -4, -5, -6, -2′, -4′, -6′), 38.6 (C-5′), 21.0, 20.9, 20.8, 20.6 (OAc); $C_{24}H_{32}O_{14}S$ (576.6): calcd C 50.00, H 5.59, S 5.56; found C 50.27, H 5.56, S 5.69.

Methyl 6-*O*-(2,3,4,6-Tetra-*O*-benzyl-5-thio-α-D-glucopyranosyl)-2,3,4-tri-*O*-benzyl-α- and β-D-glucopyranoside (10α and 10β): *N*-Bromosuccinimide in CH_2Cl_2 (50 mM, 3.2 mL, 0.175 mmol) was added dropwise to a stirred solution of **8** (76 mg, 70 μmol) and methanol (200 μL) in CH_2Cl_2 over 2 h under Ar at 0 °C. The reaction was quenched by adding 10% $NaHSO_3$ and the mixture extracted with CH_2Cl_2. The organic layer was washed with water, dried over $MgSO_4$, evaporated, and chromatographed on silica gel (*n*-hexane:ethyl acetate 8:1) to give **10α** (20 mg, 28%) and **10β** (13 mg, 18%) as crystals, respectively.

10α: M.p. 118–120 °C (from EtOH); $[\alpha]_D^{23} = +101.0$ ($c = 2.3$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 7.3-7.1$ (m, 35H; aromatic protons), 4.98–4.45 (m, 16H; H-1, -1′, CH_2Ph), 4.01–3.41 (m, 11H; H-2, -3, -4, -5, -6, -2′, -3′, -4′, -6′), 3.35 (S, 3H; OMe), 3.20 (m, 1H; H-5′); ^{13}C NMR ($CDCl_3$): $\delta = 138.9-127.4$ (16 peaks; aromatic carbons), 98.0 (C-1), 84.5, 83.0, 82.2, 81.9, 80.1, 77.9, 76.0, 75.7, 75.5, 75.0, 73.4, 73.2, 72.4, 70.5, 67.8, 66.9 (C-2, -3, -4, -5, -6, -1′, -2′, -3′, -4′, -6′, CH_2Ph), 55.2 (OMe), 41.2 (C-5′); $C_{62}H_{66}O_{10}S$ (1003.3): calcd C 74.23, H 6.63, S 3.20; found C 74.19, H 6.64, S 3.37.

10β: M.p. 110–111 °C (from EtOH); $[\alpha]_D^{20} = +97.9$ ($c = 1.8$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 7.4-7.1$ (m, 35H; aromatic protons), 4.95–4.48 (m, 15H; H-1′, CH_2Ph), 4.27 (d, $^3J_{1,2} = 7.9$ Hz, 1H; H-1), 3.93–3.27 (m, 12H; H-2, -3, -4, -5, -6, -2′, -3′, -4′ -5′, -6′), 3.53 (s, 3H; OMe); ^{13}C NMR ($CDCl_3$): $\delta = 138.9-127.4$ (21 peaks; aromatic carbons), 104.7 (C-1), 84.6, 83.1, 82.4, 82.0, 80.1, 78.0, 77.2, 76.1, 75.6, 75.5, 75.0, 74.9, 74.8, 73.1, 72.4, 67.9, 66.6 (C-2, -3, -4, -5, -6, -1′, -2′, -3′, -4′, -6′, CH_2Ph), 57.0 (OMe), 41.1 (C-5′); $C_{62}H_{66}O_{10}S$ (1003.3): calcd C 74.23, H 6.63, S 3.20; found C 73.85, H 6.62, S 3.17.

Methyl 6-*O*-(2,3,4,6-Tetra-*O*-acetyl-5-thio-α-D-glucopyranosyl)-2,3,4-tri-*O*-acetyl-α-D-glucopyranoside (11): The same method as that of the synthesis of **9** was employed for **11** (416 mg, 0.414 mmol) with **10α** as starting material. The product was purified by silica gel column chromatography (benzene:acetone 8:1) to give **11** (251 mg, 91%) as an amorphous solid: $[\alpha]_D^{21} = +206$ ($c = 1.1$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 5.52-5.45$ (m, 2H; H-3, -3′), 5.29 (t, $^3J_{3',4'} = {}^3J_{4',5'} = 10.1$ Hz, 1H; H-4′), 5.15 (dd, $^3J_{1',2'} = 3.0$, $^3J_{2',3'} = 9.9$ Hz, 1H; H-2′), 5.04 (t, $^3J_{3,4} = 9.9$, $^3J_{4,5} = 10.3$ Hz, 1H; H-4), 4.93 (d, $^3J_{1,2} = 3.6$ Hz, 1H; H-1), 4.86 (dd, $^3J_{2,3} = 10.2$ Hz, 1H; H-2), 4.85 (d, 1H; H-1′), 4.36 (dd, $^3J_{5',6'a} = 5.0$ Hz, $^3J_{6'a,6'b} = 12.2$ Hz, 1H; H-6′a), 4.05 (dd, $^3J_{5',6'b} = 3.0$ Hz, 1H; H-6′b), 3.99 (ddd, $^3J_{5,6a} = 5.6$ Hz, $^3J_{5,6b} = 2.4$ Hz, 1H; H-5), 3.89 (dd, $^3J_{6a,6b} = 10.9$ Hz, 1H; H-6a), 3.55–3.48 (m, 2H; H-5′, -6b), 3.44 (s, 3H; OMe), 2.1–2.0 (m, 21H; OAc); ^{13}C NMR ($CDCl_3$): $\delta = 170.6$, 170.3, 170.2, 170.1, 169.6 (OC=O), 96.5 (C-1), 79.9 (C-1′), 74.7, 72.1, 70.9, 70.8, 70.2, 69.3, 67.9, 67.0, 61.2 (C-2, -3, -4, -5, -6, -2′, -3′, -4′, -5′, -6′), 55.4 (OMe), 38.4 (C-5′), 20.8, 20.7, 20..6 (OAc) ; $C_{27}H_{38}O_{17}S$ (666.7): calcd C 48.65, H 5.75, S 4.81; found C 48.57, H 5.91, S 4.72.

Methyl 6-*O*-(5-Thio-α-D-glucopyranosyl)-α-D-glucopyranoside (1): To a stirred solution of **11** (242 mg, 363 μmol) in methanol (5 mL) was added NaOMe/MeOH (0.5 M, 36 μL, 18 μmol) at room temperature. The mixture was stirred for 3 h and neutralized with Dowex 50 (H^+). The resin was removed by filtration and the filtrate evaporated. The resultant syrup was purified by gel permeation column chromatography on Sephadex G-25 and lyophylized to give **1** (128 mg, 94%): $[\alpha]_D^{20} = +245$ ($c = 0.37$ in H_2O); ^{1}H NMR (D_2O): $\delta = 4.8$ (overlapped with HOD, 1H; H-1 or H-1′), 4.77 (d, $^3J_{1,2} = 5.9$ Hz, 1H; H-1 or H-1′), 4.16 (dd, $^3J = 4.5$, 11.1 Hz, 1H; H-6), 3.96–3.46 (m, 10H; H-2, -3, 4, -5, -6 , -2′, -3′, -4′, -6′), 3.43 (s, 3H; OMe), 3.10 (ddd, $^3J_{4',5'} = 10.0$ Hz, $^3J_{5',6'} = 5.4$, 3.5 Hz, 1H; H-5′); ^{13}C NMR (D_2O): $\delta = 99.7$ (C-1), 82.1 (C-1′), 75.6, 74.5, 73.81, 73.76, 71.5, 70.4, 69.8, 66.7, 60.4 (C-2, -3, -4, -5, -6, -2′, -3′, -4′, -6′), 55.5 (OMe), 43.4 (C-5′); $C_{13}H_{24}O_{10}S$ (372.4): calcd C 41.93, H 6.50, S 8.61; found C 41.48, H 6.81, S 8.93.

Enzyme Assay and Materials: Glucoamylase from *Rhizopus niveus* was purchased from Seikagaku Kogyo and used without further purification. The enzyme concentration was determined spectrophotometrically by using the absorption coefficient, E_{280}^1, of 16.3 cm^{-1} and a molecular weight of 58000 [5a]. Isomaltose (fine grade), maltose, and glucono-1,5-lactone were purchased from Seikagaku Kogyo, Kokusan Kagaku, and Nacalai Tesque, respectively. Gluconolactone was used within 20 min after its dissolution to minimize its hydrolysis into gluconic acid. Acetate buffer (20 mM, pH 4.5) was used for all cases. TLC analyses of the enzyme reaction were performed with an enzyme concentration of 41 μM and a substrate concentration of 12 μM at 20 °C. The reaction mixture was spotted with a capillary on Merck Kieselgel 60-F254, eluted with an *n*-butanol:ethanol:water (4:4:1) solvent system, and visualized by spraying aqueous $Ce(SO_4)_2$ (1%)/$(NH_4)_6Mo_7O_{24}$ (1.5%)/H_2SO_4 (10%) and heating. The enzyme fluorescence spectra and intensity at 340 nm were measured with a Hitachi 850 fluorescence spectrophotometer at an excitation wave-

length of 280 nm. To keep the enzyme concentration (3.3 μM) constant, the enzyme–ligand mixture (10–40 μL) was added with a micropipet to the enzyme solution (200 μL) in the quartz cell thermostated at 20 °C, and the fluorescence intensity was measured after each addition of the ligand solution.

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$$\Delta F = \Delta F^{S}_{max}[ES]/[E]_0 + \Delta F^{L}_{max}[EL]/[E]_0 + \Delta F^{SL}_{max}[ESL]/[E]_0 \quad (2)$$

fluorescence at saturation of S, L, and SL, respectively. Since $[E]_0 = [E] + [ES] + [EL] + [ESL]$, $K_L = [E][L]/[EL]$, $K_S = [E][S]/[ES]$, $K'_L = [ES][L]/[ESL]$, and $K'_S = [EL][S]/[ESL]$, the above equation is transformed into Equation (3), where K_S (39 mM), ΔF^{S}_{max} (21%) (vide supra), and K_L (1.6 mM), and

$$\Delta F = (\Delta F^{S}_{max}[S]_0/K_S + \Delta F^{L}_{max}[L]_0/K_L + \Delta F^{SL}_{max}[S]_0[L]_0/K_S K'_L)/(1 + [S]_0/K_S + [L]_0/K_L + [S]_0[L]_0/K_S K'_L) \quad (3)$$

ΔF^{L}_{max} (36%) are known values. Though a K_L of 1.08 was obtained at 10 °C [3a], we otherwise measured the value at 20 °C to avoid the formation of dew.

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