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Thiosugars II [1,2]. A novel approach to thiodisaccharides The synthesis of 3-deoxy-4-thiocellobiose from levoglucosenone

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Abstract

An expeditious methodology for the synthesis of β-(1 → 4)-3-deoxythiodisaccharides (3-deoxythiocellobiose) has been developed. The methodology is based on the stereoselective Michael addition of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucose to levoglucosenone, followed by stereoselective reduction at C-2 with L-Selectride® and DIBAH, followed by acetolysis to the target thiodisaccharide derivative. © 1997 Elsevier Science Ltd.

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1. Introduction

Thiosugars have attracted extremely wide attention as convenient probes for enzyme-inhibition studies [1–4]. As part of our continued interest in thiosugars [1] as enzyme inhibitors [2–4] and components of sugar antibiotics, we turned our attention to a new method of synthesis of α- and β-(1 → 4)-linked thiodisaccharides containing biologically important sugar moieties such as D-galactose, D-glucose, D-mannose, and L-fucose. Sulfur, relatively less electronegative than oxygen, also has a lower affinity for protons and less readily forms the conjugate acid intermediate involved in glucoside hydrolysis. This explains why 1-thioglycosides are competitive inhibitors of glycosidases, as well as specific targets for enzyme-inhibition studies.

Moreover, these disaccharides, where sulfur bridges replace glycosidic linkages, serve as excellent models for these targeted studies since they are conformationally similar to their natural counterparts. However, evidence has been provided recently against the generality of the conformational similarity between O- and S-glycosides [5] and O- and C-glyco-

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sides [6] in particular. Thiodisaccharides containing sulfur in the glycosidic linkage have been synthesized previously by a variety of methods [7–11], including S_N2-type reactions involving the action of a thiolate anion and a glycosyl halide [8], the displacement of a leaving group by a 1-thioglycopyranose [9], and, more recently, by the condensation of benzylated 1,6-anhydro-glucopyranose with a suitably protected 4-thioglucopyranoside to give a predominantly α-linked disaccharide [10]. These conventional methods are generally multi-step, low-overall-yield approaches. In our approach reported here, thiodisaccharides are produced stereoselectively in a one-step reaction from levoglucosenone.

Our recent results [1] on the Michael addition of sugar thiols to levoglucosenone (**2**) show highly stereoselective addition reactions due to its rigid bicyclic framework and steric shielding of the upper face of the pyranose ring by the 1,6-anhydro ring (Fig. 1).

Because this particular stereoselectivity is highly predictable, it was also observed in the Michael addition of organometallics [12] and other carbon nucleophiles [13,14] to levoglucosenone (**2**). The Michael addition of thiols to levoglucosenone has also been reported [15,16]. The sterically hindered 1,6-anhydro bridge in levoglucosenone effectively prevents the formation of the 4-equatorial (e) product. Only the 4-axial (a) product was consistently obtained as the single addition product.

As previously mentioned, (1 → 4)-thiodisaccharides are recognized as good glycohydrolase inhibitors. In an attempt to elucidate extremely important mechanistic aspects of retaining glycoside hydrolases by (1 → 4)-thiodisaccharides, we tested the inhibitory activity of the new thiocellobiose derivative **3** against β-glucosidase from sweet almond β-glucosidase. The synthesis of analogous thiocellobiose [17] and thiomaltose [18] derivatives has been reported, and 4-methylumbelliferyl 4-thiocellobioside was found to inhibit cellobiohydrolase I.

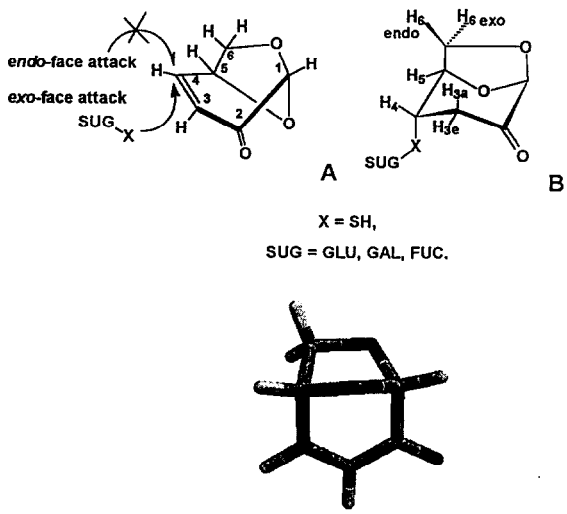


Fig. 1. The shielding effect of the 1,6-anhydro ring of levoglucosenone and stereoselective conjugate addition of thiols by *exo*-face attack.

2. Results and discussion

The important considerations presented here prompted us to expand our earlier [2] exploration of the synthetic utility of levoglucosenone (**2**) by selectively introducing a sulfur bridge, which will connect two sugar rings at C-(1 → 4) by Michael addition of 1-thiosugars. Indeed, the proton–proton coupling in the ^{1}H NMR spectrum of the conjugate addition product confirmed the D-*erythro* stereochemistry with a coupling constant of $J_{3e,5}$ 1.5 Hz. This coupling indicates that the substituent at C-4 is axial with a quasi-equatorial relationship between H-3 and H-5, as seen in compound **3**. Additionally, lack of coupling between H-4 and H-5, and coupling constants of adduct **3**, $J_{3a,4}$ 7.95 and $J_{3e,4}$ 3.18 Hz, indicate the axial disposition of the new substituent at C-4. The ^{1}H NMR spectrum of the adduct did not show signals corresponding to a potential D-*threo* isomer, indicating that the stereochemistry of the addition of thiol to enone **1** is completely governed by the steric bulk of the 1,6-anhydro bridge. The ^{13}C NMR spectrum showed no alkene signals, and the C-3 signal appeared upfield at 38.4 ppm.

Ketone **3** offers potential in the synthesis of precursors of certain amino sugars as reported by us earlier [1] through conventional oximation and highly stereoselective reduction of the acetamido function. Accessible in a single-step process from levoglucosenone and formed in a completely stereospecific manner, **3** is potentially useful for syntheses of amino sugars having three chiral centers in the D-*ribo* configuration.

The reduction of the C-2 keto function of ketone **3** with L-Selectride®, followed by conventional acetylation, proceeded stereoselectively with the formation of the D-*ribo* isomer **4a** in 91% yield. Only a trace amount of D-*arabino* isomer **4b** was detected by ^{1}H NMR spectroscopy. Unlike levoglucosenone (**2**), where the ketone reduction was predictably con-

trolled by the 1,6-anhydro bridge, the analogous reduction in **3** was expected to be governed by the relative steric contribution of the axial substituent at C-2, as well as by the 1,6-anhydro bridge. This is in agreement with an earlier observation [19] of high stereoselectivity and is yet another example of the preferential attack of the reducing agent from the top face on this bicyclic molecule (Scheme 1).

Moreover, the ^{1}H and ^{13}C NMR spectra of **4a** firmly support their assignments as the D-*ribo* configuration. Particularly, the coupling constants between the equatorially disposed H-2 and the axially disposed H-3 ($J_{2,3}$ 4.7 Hz) are of great diagnostic value. On the other hand, the more polar equatorial alcohol **4b** with the β-D-*arabino* configuration (isolated in only ~ 3% yield) displayed a coupling of 10.4 Hz, as anticipated for the diaxial disposition of H-2 and H-3.

In contrast, reduction of ketone **3** with DIBAH proceeded with reverse stereoselectivity [20] to form **4b** as the major epimer in 61% yield, and only a trace amount of D-*ribo* epimer **4a** was detected. The exclusive formation of alcohol **4b** in the DIBAH reduction suggests a different favored path of this reduction from the bottom face as depicted in Fig. 2.

The cleavage of the 1,6-anhydro ring in **4a** was examined under various reaction conditions. Treatment of compound **4a** with dilute 2% sulfuric acid in refluxing methanol resulted in slow degradation and recovery of most of the starting material. In contrast, acetolysis using trifluoroacetic acid and acetic anhydride afforded an anomeric mixture of heptaacetate **5**

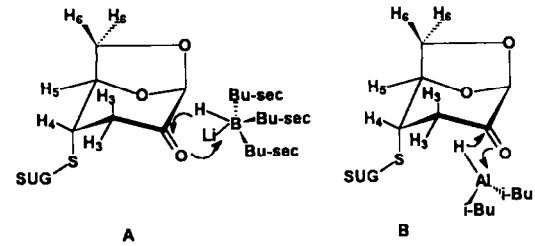
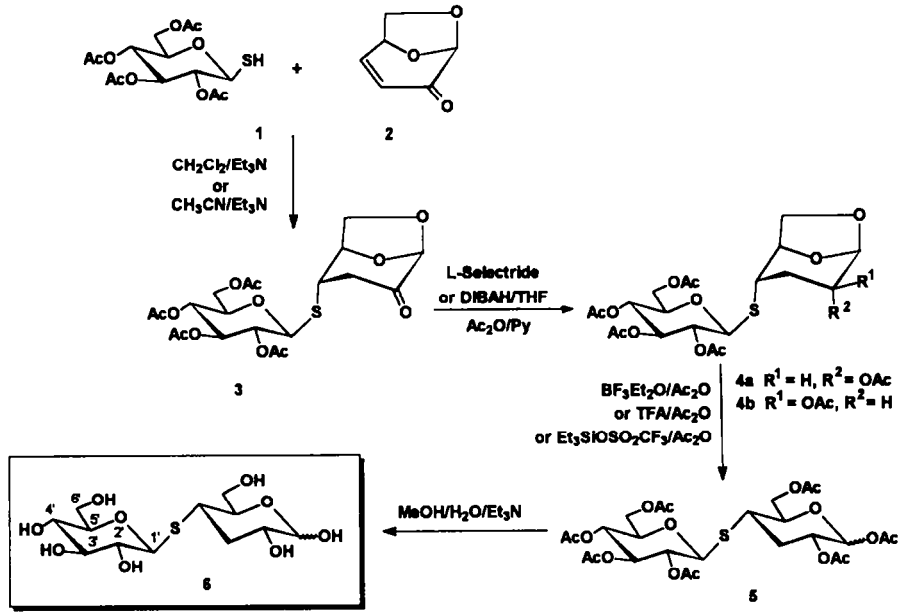


Fig. 2. Possible steric interaction in the reduction of ketone **3** with L-Selectride (A) and DIBAH (B).

in good yield (62%). Acetolysis, under similar conditions, such as acetic anhydride and a catalytic amount of borontrifluoride etherate ($BF_3 \cdot Et_2O$) also gave a good yield (58%). However, chromatographic purification of the crude material was required.

The method of choice was acetolysis using acetic anhydride solution and a catalytic amount of trifluoromethanesulfonate, performed according to the convenient methodology of Fraser-Reid and co-workers [21]. This resulted in the formation of an anomeric mixture of heptaacetate **5** (α:β 1:5) in 91% yield. The presence of the deoxy group at C-3 clearly indicates the strong influence of the of 1,6-anhydro ring on the cleavage, and the reaction requires a prolonged reaction time (up to 12 h) as compared with the literature data reported [21]. Separation of the anomers proved impossible because of their almost identical R_f values. However, the ^{1}H and ^{13}C NMR spectra and mass spectral data for the mixture firmly established their identity.



Scheme 1.

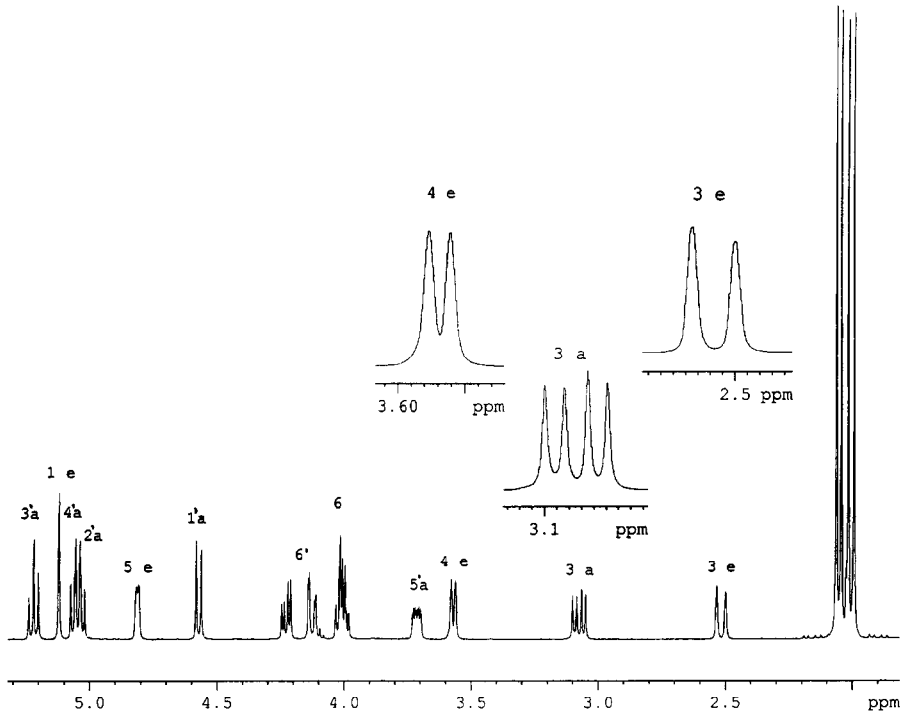


Fig. 3. 500-MHz ^{1}H NMR spectrum of compound **3** in $CDCl_3$.

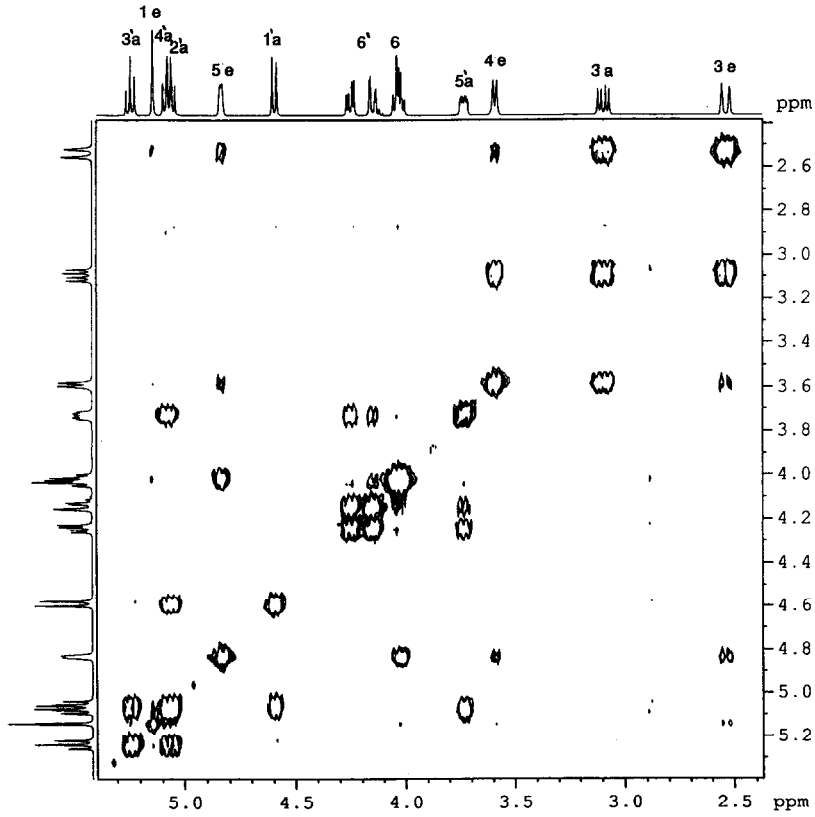


Fig. 4. ^{1}H–^{1}H COSY spectrum of **3**. The respective cross-sections for the individual sugars and the assigned signal are indicated.

Table 1
^{1}H NMR chemical shifts (δ in ppm) and coupling constants (J in Hz) for compounds **3–6** [a]

Compound	H-1′ $J_{1,2}$	H-2′ $J_{2,3}$	H-3′ $J_{2,3'}$	H-4′ $J_{3,4}$	H-5′ $J_{5,6'}$	H-6′ $J_{5,6''}$	H-6 $J_{6,6}$	H-1 $J_{1,2}$	H-2 $J_{2,3}$	H-3a $J_{3a,4}$	H-3e $J_{3a,3e}$	H-4 $J_{3e,4}$	H-5′ $J_{5,6ex}$	H-6endo $J_{5,6endo}$	H-6exo $J_{6ex,6en}$	-COCH$_3$
3	4.58d	5.17dd	5.22t	5.06d	4.82d	4.24dd	4.46dd	5.28s	–	3.04dd	2.52d	3.56d	4.86d	3.96dd	4.02dd	2.02s, 2.04s
	7.4	9.6	10.5	9.5	5.2	1.1	8.4			7.9	16.6	3.1	4.8	1.2	7.5	2.06s, 2.09s
4a	4.66d	5.33dd	5.2t	5.04d	4.86d	4.26dd	4.40dd	5.36s	3.76d	3.06dd	2.54d	3.52d	4.82dd	3.99dd	4.04dd	2.02–2.1,
	7.8	9.8	10.2	9.3	5.0	–	8.6		4.7	7.2	16.4	4.4	4.6	1.1	7.5	5s, 5x-OAc
4b	4.60d	5.25dd	5.24t	5.03d	4.84d	4.28dd	4.38dd	5.39s	4.08d	3.09dd	2.56d	3.58d	4.88dd	3.94dd	4.02dd	2.02–2.1
	8.2	9.6	9.8	9.6	5.2	–	8.4		10.4	7.3	16.8	4.9	5.0	1.2	7.0	5s, 5x-OAc
5	4.56d	5.19dd	5.23t	5.04d	4.82d	4.26dd	4.42dd	5.3d	3.66d	3.12dd	2.60d	3.56d	4.90dd	3.93dd	4.04dd	2.02–2.1
	8.8	9.6	9.9	9.5	5.0	–	8.6	7.2	5.0	7.6	16.6	8.0	4.9	1.1	8.2	6s, 6x-OAc
6 [b]	4.62d	5.21dd	5.26t	5.08d	4.84d	4.28dd	4.46dd	5.3d	3.58d	3.19dd	2.62d	3.59d	4.84dd	3.96dd	4.06dd	–
	8.6	9.8	10.0	9.7	5.2	–	8.6	7.5	5.2	7.4	16.4	8.1	4.8	1.2	8.4	

[a] Determined at 500 MHz in $CDCl_3$ with Me_4Si as internal reference.
[b] Determined at 500 MHz in D_2O with TMSPA-Na as internal reference.

Final deprotection of heptaacetate **5** was performed with an aqueous methanolic solution of triethylamine (4:1:5 MeOH–Et_3N–H_2O) at room temperature for 6 h, which resulted in the formation 3-deoxythiocellobiose **6** in 89% yield.

This new methodology should prove extremely useful for synthesizing a wider variety of other 3-deoxythiodisaccharides with various linkages, while using levoglucosenone as a convenient synthon to control the reaction's stereoselectivity. The readily procured representative **3** of β-(1 → 4)-linked 3-deoxythiodisaccharides is a versatile precursor to the other class of functionalized thiosugars and may be employed in a variety of transformations to amino-, thio-, and branch-chain sugars by functionalization of the remaining functional group. This procedure fully documents the promising, synthetic utility of levoglucosenone for preparing these compounds. Its use as a convenient synthon to various functionalized C–S- and cyclic oligosaccharides is currently under intense investigation in our laboratory.

NMR results.—A fully expanded scale of a one-dimensional NMR spectrum is shown in Fig. 3. The spectral assignments of 3-deoxythiocellobiose derivative **3** were made on the basis of the coupling constants and connectivities in the ^1H–^1H COSY spectrum (Fig. 4).

The axial connection of glucose moiety in 3-deoxythiocellobiose derivative **3** was confirmed on the basis of the measured coupling constants. The measured coupling constants of the $^3J_{4e,3a}$ and $^3J_{4e,3e}$ are 7.95 and 3.18 Hz, respectively, and are consistent with the values reported in the literature [18] (Tables 1 and 2).

Moreover, in a ^{13}C NMR spectrum of compound **3**, it is extremely important to notice the downfield signal of C-3, which occurs at about 38.4 ppm.

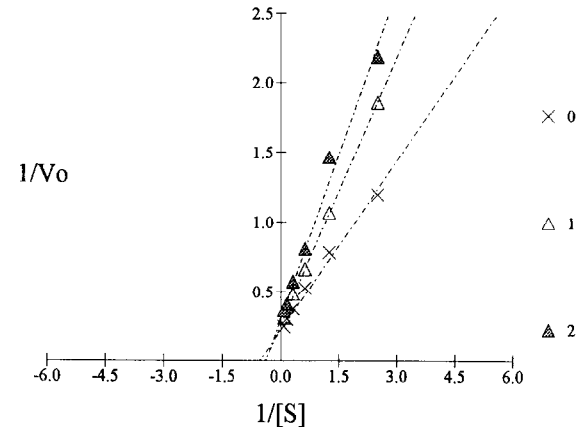


Fig. 5. Lineweaver–Burk plot for the inhibition of sweet almond β-glucosidase by compound **3** at concentration 0, 1, and 2 mM of the inhibitor.

Enzymatic study.—By analogy with alkyl and aryl 1-thioglycosides, which usually have been found to behave as competitive inhibitors of the corresponding glycosidases, interglycosidically S-linked thiodisaccharides were expected to behave similarly.

In the test determining the inhibitory activity of compound **3** against sweet almond β-glucosidase, *p*-nitrophenyl β-D-glucoside was used as a substrate. Based on a Lineweaver–Burk plot (Fig. 5), compound **3** proved to be a mixed type of inhibitor with its inhibitory activity defined as $K_i = 6.06$ mM with $K_m = 4.29$ mM. These results clearly warrant further study of the next generation of (1 → 4)-thiodisaccharide derivatives, specifically modified and functionalized at the C-2 or C-6 positions.

3. Experimental

General methods.—Unless otherwise noted, starting materials were obtained from commercial suppli-

Table 2
^{13}C Chemical shift data for compounds **3–6** [a]

Compound	C-1	C-2	C-3	C-4	C-5	C-6	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′	-OCO	-Ac
3	85.6	79.7	77.1	71.9	81.1	62.1	102.6	207.1	38.4	70.6	76.2	65.7	4 × 171.1	17.6, 17.5, 17.5, 17.4
4a	84.8	78.7	77.0	70.6	81.0	63.1	101.6	65.2	39.5	70.2	76.9	63.9	5 × 171.0	17.6, 17.6, 17.5, 17.4, 17.4
4b	84.1	78.6	76.8	69.3	80.4	63.3	101.8	73.8	39.1	70.1	76.8	63.2	5 × 178.8	17.8, 17.8, 17.6, 17.5, 17.4
5	85.8	75.7	73.1	68.9	71.0	63.1	101.9	65.7	35.7	69.9	75.0	61.8	7 × 176.0	7 × 17.6
6 [b]	84.5	75.4	75.6	68.4	77.7	63.8	102.1	65.0	35.1	69.8	78.4	62.0		

[a] Determined at 125 MHz in $CDCl_3$ with Me_4Si as the internal reference.
[b] Determined at 125 MHz in D_2O with TMSPA-Na as the internal reference.

ers and used without purification. All melting points were uncorrected and were measured in open capillary tubes. Optical rotations were determined on a Jasco Model DIP polarimeter in $CHCl_3$ solns. Mass spectra were obtained either in EI mode at 70 eV or using CI (NH_3). Thin-layer chromatography (TLC) was performed on precoated Silica Gel $60F_{254}$ plates from E. Merck that were developed by spraying with 10% ethanolic H_2SO_4 with subsequent heating.

Column chromatography was performed on Silica Gel 60 (70–230 mesh, E. Merck No. 7734). The starting 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose was prepared according to the procedure of Horton [22]. It is also commercially available from Aldrich (10,447-7). Levoglucosenone was prepared according to the reported procedure [23].

^1H NMR spectra.—NMR samples were prepared in $CDCl_3$ (99.8 atom-% D), filtered, freeze–thawed, and sealed in a 5-mm NMR tube. Tetramethylsilane (TMS) was used as an internal chemical shift reference. High-resolution 1D and 2D NMR spectra were obtained on a Bruker DMX 500 spectrometer. 1D ^1H 500-MHz NMR spectra were recorded using acquisition parameters: 90° pulse width, 6.2 μs; spectral width, 3255.2 Hz; data size, 16K; recycling delay, 15 s; number of transients, 32; temperature, 298 K. 2D ^1H–^1H chemical shift correlation spectra with double quantum filter (DQF–COSY) (14) were obtained at 500 MHz with the acquisition parameters: 90° pulse width, 6.2 μs; spectral width, 3255.2 Hz; recycling delay, 2 s. The data were 256w in the F1 dimension and 2k in the F2 dimension and were zero filled in F1 prior to 2D Fourier transformation to yield a 2k × 2k data matrix. The spectra were processed using a shift sine-bell window function in both dimensions.

*1,6-Anhydro-3-deoxy-4-*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-*D*-glycero-hexopyranos-2-ulose* (**3**). —A soln of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-glucopyranose [22] (**1**) (364 mg, 1.00 mmol) in acetonitrile (5 mL) was added dropwise to a soln of levoglucosenone [13,15,23] (**2**) (126 mg, 1.00 mmol) in acetonitrile (10 mL). The reaction mixture was stirred at room temperature for 24 h. After evaporation of the solvent, the syrupy residue was crystallized from ether–hexane to give **3** (436 mg, 89%): mp 157–158.5 °C; $[\alpha]_D^{30}$ −124.21° (*c* 0.84, $CHCl_3$); R_f 0.59 (1:4 hexane–EtOAc); MS $(M)^+$ *m/z*: Calcd for $C_{20}H_{26}O_{12}S$: 490.47. Found: 490.11. The ^1H NMR and ^{13}C NMR spectra of **3** are listed in Tables 1 and 2, respectively.

*2-O-Acetyl-1,6-anhydro-3-deoxy-4-*S*-(2,3,4,6-tetra-O-acetyl-4-thio-β-D-glucopyranosyl)-β-D-ribo-hexopyranose* (**4a**) *and 2-O-acetyl-1,6-anhydro-3-deoxy-4-*S*-(2,3,4,6-tetra-O-acetyl-4-thio-β-D-glucopyranosyl)-β-D-*arabino*-hexopyranose* (**4b**)

A. Reduction with L*-Selectride®.* To a cooled and stirred soln of **3** (210 mg, 0.428 mmol) in THF, L-Selectride® (1 M in THF, 1.0 mL) was added at −78 °C under an Ar atmosphere. The reaction mixture was stirred for 3 h, then pyridine (4 mL) and Ac_2O (5 mL) were added and stirred at room temperature overnight. The reaction mixture was poured into ice–water and extracted with EtOAc. The extract was washed and dried. Removal of the solvent in vacuo after coevaporation with 1:1 toluene–ethyl alcohol (5 × 30 mL) afforded an oily residue that was subjected to column chromatography on silica gel. The fraction that eluted with 20:80 EtOAc–hexane (v/v) gave syrupy **4a** (190 mg, 91%); $[\alpha]^{30}$ −34.2° (*c* 0.84, $CHCl_3$): R_f 0.42 (1:4 hexane–EtOAc); and **4b** (6 mg, 3%): $[\alpha]_D^{30}$ −12.1° (*c* 0.84, $CHCl_3$); R_f 0.29 (1:4 hexane–EtOAc); MS $(M)^+$ *m/z*: Calcd for $C_{22}H_{30}O_{13}S$: 534.53. Found: 534.14. The ^1H NMR and ^{13}C NMR spectra of **4a** and **4b** are listed in Tables 1 and 2, respectively.

B. Reduction with diisobutylaluminum hydride in THF. To a cooled and stirred soln of **3** (210 mg, 0.428 mmol) in THF, a 1.5 M soln of DIBAH in toluene was added slowly at −78 °C under an Ar atmosphere. The reaction mixture was stirred at −78 °C for 20 h. After the soln was warmed to room temperature and stirred for 1.5 h, water (1.2 mL) was carefully added. The reaction mixture was stirred for 3 h, then pyridine (4 mL) and Ac_2O (5 mL) were added and stirred at room temperature overnight, and the mixture worked up as described in the previous experiment to afford, after chromatography, alcohol **4b** as a colorless syrup (127 mg, 61%) along with a trace amount of **4a**.

*3-Deoxy-4-*S*-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-1,2,6-tri-O-acetyl-4-thio-α,β-D-glucopyranose* (**5**).—(a) Thiodisaccharide **4a** (0.2 g, 0.375 mmol) was dissolved in Ac_2O (8.5 mL) and CF_3COOH (6.2 mL), and the mixture was stirred at room temperature for 10 h. After the solvent was evaporated, the resulting brown syrup was chromatographed (2:1 hexane–Et_2OAc) to afford an anomeric mixture of heptaacetate **5** as colorless syrup (α:β 1:5), (160 mg, 62%).

(b) Thiodisaccharide **4a** (0.4 g, 0.75 mmol) was dissolved in Ac_2O (8.5 mL). Boron trifluoride etherate ($BF_3 \cdot Et_2O$, 0.1 mL) was added, and the mixture was stirred at room temperature for 10 h. After the solvent was evaporated, the resulting brown syrup

was chromatographed (2:1 hexane–Et_2OAc) to afford an anomeric mixture of heptaacetate **5** as a colorless syrup (α:β 1:5), (0.30 g, 58%).

(c) To a cooled soln (0 °C) of **4a** (0.5 g, 0.93 mmol) in Ac_2O (10 mL) stirred under argon, two drops (3 μL) of trimethylsilyl trifluoromethanesulfonate were added. TLC 1:1 EtOAc–hexane indicated the completion of the reaction in 12 h. A soln of satd $NaHCO_3$ was added, the mixture was stirred for 30 min, and the aq mixture was extracted three times (3 × 20 mL) with EtOAc. The combined extracts were washed with satd $NaHCO_3$ (20 mL) and brine (20 mL) and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yielded an inseparable anomeric mixture (α:β 1:6) of **5** (0.648 g, 91%) as a colorless syrup: $[\alpha]_D^{30}$ −12.4° (*c* 0.82, $CHCl_3$); R_f 0.42, and R_f 0.41 (1:4 hexane–EtOAc); MS $(M)^+$ *m/z*: Calcd for $C_{26}H_{36}O_{16}S$: 636.62. Found: 636.17. The ^1H NMR and ^{13}C NMR spectra of **5** (mainly the β anomer) are listed in Tables 1 and 2, respectively.

4-S-(β-D-Glucopyranosyl)-4-thio-D-glucopyranose (3-deoxycellobiose, **6**).—Thiodisaccharide **5** (0.250 mg, 0.15 mmol) was dissolved 4:1:5 MeOH–Et_3N–H_2O (15 mL) and stirred at room temperature. TLC indicated the completion of the reaction after 6 h. Evaporation of the solvent produced an inseparable anomeric mixture (α:β 1:6) of **6** (119 mg, 89%) as a colorless syrup $[\alpha]_D^{30}$ +12.26° → +36.2° (*c* 0.82, H_2O); MS $(M)^+$ *m/z*: Calcd for $C_{12}H_{22}O_9S$: 342.36. Found: 342.09. The ^1H NMR and ^{13}C NMR spectra of **6** are listed in Tables 1 and 2, respectively.

Enzymatic assay.—The inhibitory activity of compound **3** toward the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside by sweet almond β-glucosidase [24] (EC 3.2.1.21) was determined under the following conditions [25] in a typical assay: Final vol, 0.1 mL; used 0.01–0.03 U/mL of the enzyme (1 U = 1 enzyme unit hydrolyzes 1 μmole of glycoside per min from *p*-nitrophenyl β-D-glucopyranoside) and 5 mM aq soln of the appropriate *p*-nitrophenyl glycoside buffered to the optimum pH. The reaction was terminated by adding 0.25 mL of a 0.2 M aq soln of sodium borate (pH 9.8). The concns of the released *p*-nitrophenolate (initial velocities with less than 10% substrate consumed) were measured by visible absorption spectroscopy at 400 nm (with the reference as the same soln without enzyme). Under standard conditions, an optical density at 400 nm was reached after 20 min of glycosidase catalyzed hydrolysis at 37 °C. Inhibition evaluations used concns of 1.0 and 2.0 mM of the reference inhibitor and the potential inhibitors. The K_m and K_i values and inhibition type were calculated and determined by the computer software program, "Enzyme Kinetics" (Window Chem Software–HaloSoft®) and are presented as a Lineweaver–Burk plot (Fig. 5).

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