Inhibition of N-Acetylglucosaminyltransfer Enzymes: Chemical–Enzymatic Synthesis of New Five-Membered Acetamido Azasugars

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Two new acetamido azasugars have been synthesized and tested as inhibitors of β-*N*-acetylglucosaminase. Ozonolysis of enantiomerically pure *N*-(4-phenyl-2-azido-3-butenyl)acetamide, derived from cinnamic aldehyde, followed by lipase-catalyzed resolution of the amine intermediate **5**, gave 2-azido-3-acetamidopropanal which was then condensed with dihydroxyacetone phosphate by using FDP-aldolase. The condensed product was dephosphorylated and hydrogenated to afford the five-membered acetamido azasugar analogous to *N*-acetylglucosamine. Compounds **1** and **2** prepared in this manner were new competitive inhibitors of a β-*N*-acetylglucosaminidase with K_i values of 1.9 and 3.6 μM, respectively, and could be useful for the synthesis of *N*-acetylglucosaminyltransferase inhibitors.

Introduction

Seven *N*-acetylglucosaminyltransferases (GnTase) are involved in the synthesis of oligosaccharides of complex type, N-linked glycoproteins.[1] Among these transferases, GnTase V is the most interesting from a view point of inhibition, because the increase in the activity of this enzyme is associated with the metastatic potential of tumor cells.[2,3] Some trisaccharide acceptor analogs in which the nucleophilic hydroxyl group of the acceptor was masked or removed[4,5] have been prepared as inhibitors (K_i = 60–70 μM) of GnTase V. These types of acceptor-like inhibitors are, however, often nonselective and not very potent. To improve the selectivity and inhibition potency, *bisubstrate analogue inhibitors* which contain both the donor and the acceptor molecules involved in the enzymatic glycosylation have been developed, as illustrated in the inhibition of an α-1,3-fucosyltransferase (K_i = 2.3–16 μM).[6] The transition-state nature of the glycosyl donor, however, was not properly considered in the inhibitor design. As part of our interest in the development of inhibitors of glycosyltransferases, we have designed an inhibitor (Figure 1) which contains a five-membered acetamido azasugar considered to be the transition-state mimic of the GlcNAc moiety in the GnTase reaction. Like glycosidase reactions,[7a] glycosyltransferase reactions are believed to proceed through a transition state with positively-charged, half-chair conformation characteristics.[7b] A malonyl group is also incorporated into the inhibitor to mimic the pyrophosphate moiety.

A number of azasugars which mimic the transition-state nature of glycosyltransfer reactions have been reported to be potent inhibitors of glycosidases.[7] Azasugars are also inhibitors of glycosyltransferases,[7b] especially in the presence of nucleoside phosphates.[7g] The reason we chose the five-membered acetamido azasugars **1** and **2** is based on the observation by us[7d] and others[7c] that 5-membered azasugars are generally as good as or better than the corresponding 6-membered azasugars as inhibitors of glycosidases. Presumably the envelope shape and positive charge of protonated 5-membered aza sugars resemble the flattened chair shape of glycosyl cations.

To test the merit of our inhibitor design, we report here the synthesis of compounds **1** and **2** and the evaluation of the acetamido azasugars as inhibitors of β-*N*-acetylglucosaminases from two different sources.

Results and Discussion

Synthesis of 1 and 2. We have previously reported a facile enzymatic method to prepare the azasugar analog of *N*-acetylglucosamine, 1,2-dideoxy-2-acetamidonojirimycin.[7f] Condensation of (*S*)-3-azido-2-acetamidopropanal with dihydroxyacetone phosphate (DHAP) in the presence of FDP-aldolase, followed by cleavage of the phosphate ester of the aldol product with acid phosphatase and subsequent hydrogenation of the intermediate azasugar, 1,2-dideoxy-2-acetamidonojirimycin was obtained. This procedure also appears to be suitable for the synthesis of **1** and **2** (Scheme I) with the appropriate choice of chiral acetamidoaldehydes (**3**, **4**) as acceptors in the enzymatic aldol reactions.[8] The racemic amine **5**, the precusor of both **3** and **4**, was prepared using the classical method shown in Scheme II. Mesylation of azido alcohol

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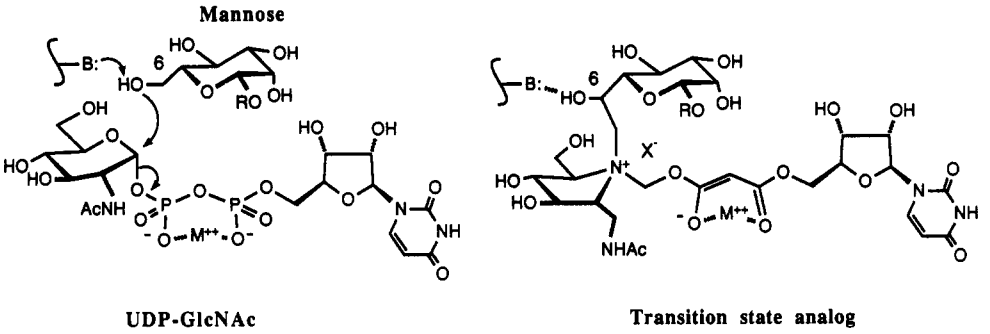
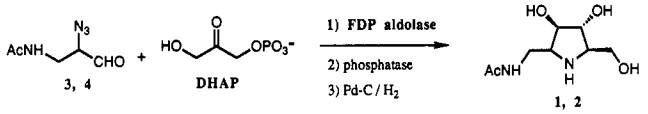
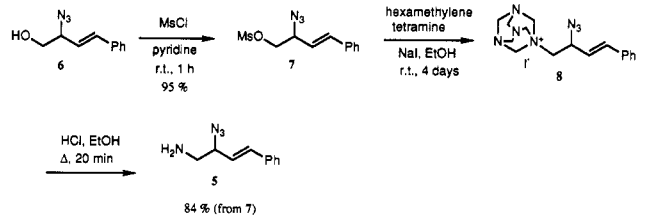


Figure 1. *N*-Acetylglucosaminyltransferase V inhibitor.

Scheme I. Strategy for the Synthesis of Compounds 1 and 2

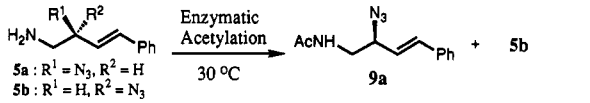


Scheme II. Synthesis of Compound 5



6[8f] afforded compound **7**, which was treated with hexamethylenetetramine to yield the ammonium salt **8**. Compound **8** was converted to primary amine **5** by hydrolysis with concd hydrochloric acid. Enzymatic resolution of amine **5** was then attempted; however, neither resolution of the acetamide of **5** with amidases nor the previously reported enantioselective enzymatic acetylation was successful.[9-13] However, the combination of using ethyl acetate as the acylating reagent and using *Pseudomononas* lipase (Amano PS) as catalyst efficiently resolved **5**. The chemical yield of acetamide **9a** from a mixture of **5a** and **5b** was 34% with 80% ee (Table I). The enantiomeric excess was determined by chiral HPLC,[14] and the configuration of **9a** was determined using Kakisawa's modification of Moscher's method.[15] Namely, acetamide **9a** was hydrogenated to afford **10a** (contaminated with **10b**) which was converted to both its (*R*)- and

Table I. Enzymatic Acetylation of Compound 5



enzyme[a]	acetyl donor	time	yield of **9a** (%)	ee (%)
PLE	EtOAc	1d	<5	0
PPL	## Ib ##	10 min	94	<5[b]
PPL	AcOH	3d	0	0
PPL	EtOAc	12 h	5	0
CCL	EtOAc	1 d	8	0
WGL	EtOAc	5 d	<5	3
Amano PS	EtOAc	17 h	34	80

[a] PLE:pig liver esterase. PPL:pancreatic lipase. CCL:*candida cylindracea* lipase. WGL:wheat germ lipase. Amano PS:*pseudomonas* lipase. [b] The reaction proceeded with or without the enzyme.

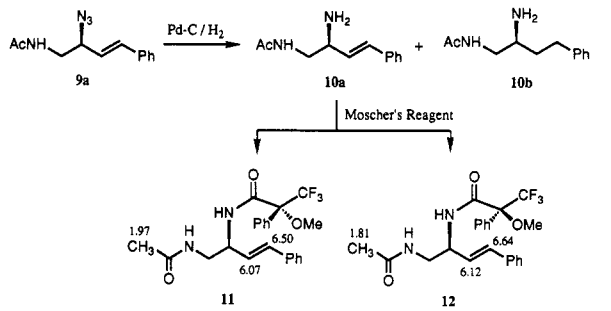


Figure 2. MTPA amide of compound **10a** and the values of chemical shift in their ^{1}H-NMR.

(*S*)-MTPA [2-methoxy-2-phenyl-2-(trifluoromethyl)acetyl]-amides **11** and **12**. The $\Delta\delta$ value ($\delta S - \delta R$) for the acetyl group is +0.16, while those for the olefinic protons are −0.14 and −0.05. According to the model, the acetyl group is oriented on the right side of MTPA plane and the olefin on the left side. Therefore, compound **9a** has the *S*-configuration and **5b** has the *R*-configuration (Figure 2). To prepare amide **9b**, compound **5b** was treated with isopropenyl acetate. Ozonolysis of compounds **9a** and **9b** in methanol followed by workup with dimethyl sulfide produced the respective aldehydes (**3**, **4**). Each aldehyde was condensed with dihydroxyacetone phosphate (DHAP), catalyzed by FDP-aldolase. The phosphate groups of each aldol product were removed by acid phosphatase (sweet potato) to yield **13** and **14**. Subsequent hydrogenation of the product over palladium provided the five-membered azasugars corresponding to *N*-acetylglucosamine (**1**, **2**) (Scheme III). The relative stereochemistry at C-5 of compound **1** was determined to be *trans* to H-2 based on the ^{1}H–^{1}H COSY spectrum and NOE effects between H-2 and H-4, and between H-3 and H-5. An assignment of the signals attributed to the two methylene groups (H-1, H-6)

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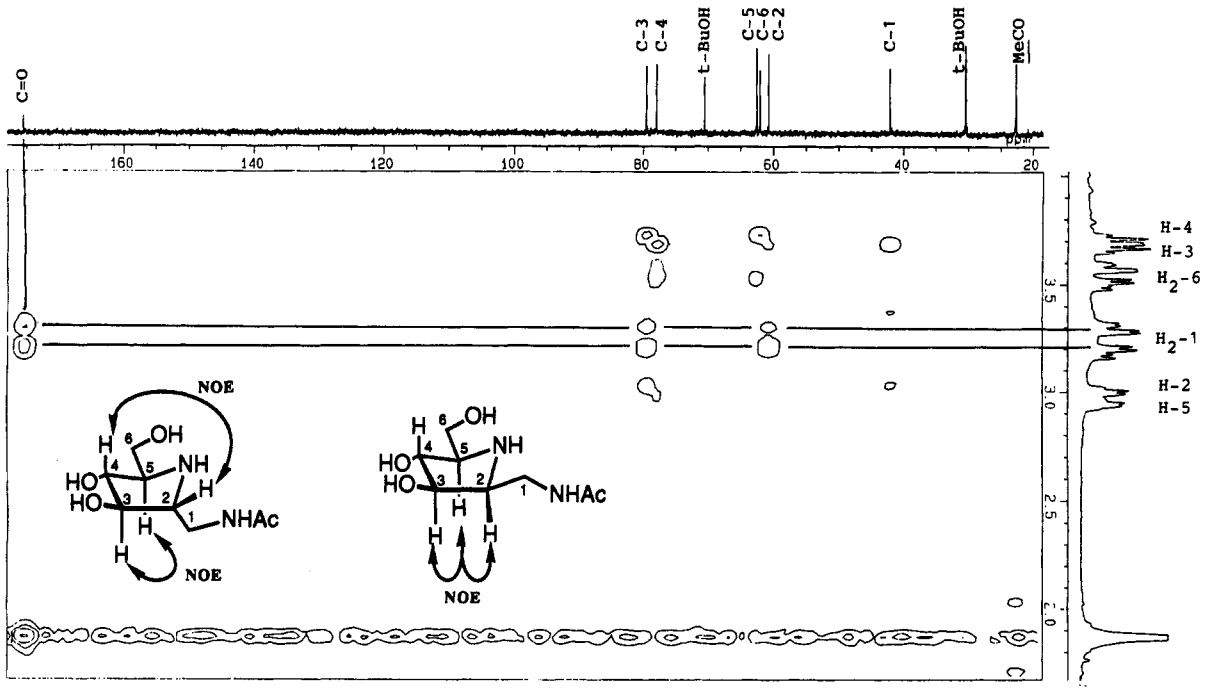
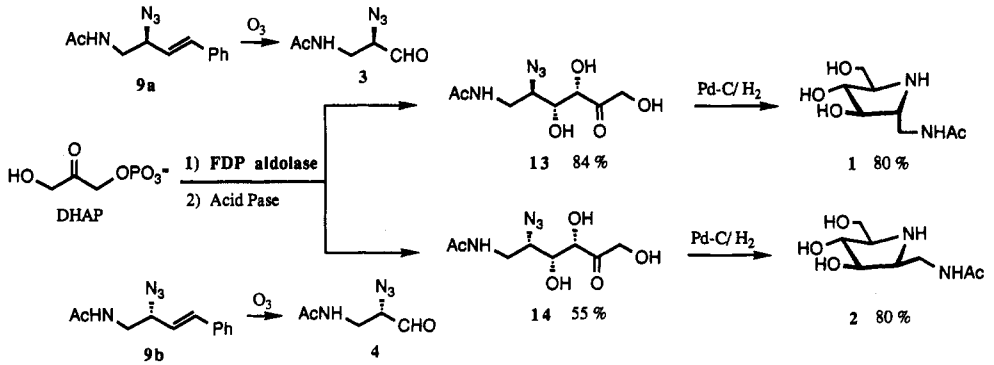


Figure 3. HMBC spectrum (in D_2O) of compound 1.

Scheme III. Synthesis of Five-Membered Aza-*N*-acetylglucosamine



was clearly established by the HMBC spectra (Figure 3). Similarly, the side chain at C-5 of compound **2** was determined to be *cis* relative to H-2. The hydrogenation is stereoselective and follows the same pattern as observed previously, i.e. it favors the products with the *trans* relationship between C-4 and C-5.

Inhibition Analysis. As expected, compound **1** was found to be a potent competitive inhibitor of β-*N*-acetylglucosaminidase from both bovine kidney (K_i = 9.8 μM) and from Jack beans (K_i = 1.9 μM). Compound **2** was also an inhibitor of the enzyme from bovine kidney (K_i = 68.6 μM) and from Jack beans (K_i = 3.6 μM).

In summary, this study demonstrates a facile synthetic method for the preparation of five-membered azasugars of *N*-acetylglucosamines. Both of the enantiomeric aldehydes, the acceptor substrates of the aldol condensation, were easily prepared from cinnamic aldehyde with an excellent yield. The resolution of the intermediate amine (**5a**, **5b**) was also accomplished using an enzymatic resolution with Amano PS. In only three more steps in aqueous medium; namely, the aldol condensation of these chiral aldehydes and DHAP catalyzed by FDP-aldolase, dephosphorylation by acid phosphatase, and catalytic hydrogenation on palladium, the acetamido azasugars were obtained. We believe that the method developed in this study is another practical route to pyrrolidines. In addition, that these five-membered azasugars of *N*-acetylglucosamine, especially compound **1**, are potent inhibitors of *N*-acetylglucosaminidase further supports the hypothesis of our inhibitor design. Work is in progress to prepare the designed GnTase V inhibitor.

Experimental Section

Mesylation of Compound 6. To a solution of **6**[8f] (10.0 g, 52.8 mmol) in pyridine (50 mL) was added mesyl chloride (4.9 mL, 63.4 mmol), and the mixture was stirred at room temperature for 1 h. The reaction mixture was extracted with dichloromethane, and the organic layer was treated as usual. The crude product was purified by silica gel chromatography (hexane/EtOAc = 4:1) to yield **7** (15.0 g, 95%): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 3.09 (3H, s), 4.20 (1H, dd, J = 7.6, 10.2 Hz), 4.32 (1H, dd, J = 3.9, 10.2), 4.44 (1H, dt, J = 3.9, 7.6 Hz), 6.09 (1H, dd, J = 7.6, 15.8 Hz), 6.78 (1H, d, J = 15.8 Hz), 7.31–7.43 (5H, m); ^{13}C-NMR ($CDCl_3$) 37.8 (q), 62.5 (t), 69.9 (d), 120.9 (d), 126.8 (d), 128.7 (d), 135.1 (s), 136.3 (d), HR MS 267.0651 (M^+ + H) [calcd 267.0676].

Preparation of Compound 5. An ethanol solution (200 mL) of **7** (16.5 g, 61.7 mmol) was slowly added to a suspension of hexamethylenetetraamine (9.52 g, 67.9 mmol) and sodium iodide (10.2 g, 61.7 mmol), and the reaction mixture was stirred for 4 days. The precipitates (27.0 g, compound **8**) that appeared were collected by filtration under a reduced pressure and carried on to the next reaction.

To an ethanol (270 mL) suspension of compound **8** (27.0 g, 62.0 mmol) was added 12 N hydrochloric acid (27 mL) and the mixture was heated at 65 °C for 20 min. After the reaction was complete,

the white precipitate was removed by filtration. The filtrate was adjusted to pH 8.0 with 10% NaOH and extracted with AcOEt. The organic layer was treated as usual, and the residue was purified by flash silica gel chromatography ($CHCl_3$/MeOH = 20:1) to yield **5** (9.6 g, 84%).

^{1}H-NMR ($CDCl_3$) δ 2.83, 2.87 (each 1H, dd, AB, J = 6.0, 12.5 Hz, CH_2NH_2), 4.06 (1H, q, J = 6.0 Hz, CHN_3), 6.14 (1H, dd, J = 8.0, 15.8 Hz), 6.69 (1H, d, J = 15.8 Hz), 7.25–7.43 (5H, m, C_6H_5); ^{13}C-NMR ($CDCl_3$) δ 46.2, 67.6, 124.7, 126.7, 128.3, 128.7, 134.6, 135.8.

Enzymatic Resolution of Amine 5. To a solution of compound **5** (530 mg, 2.81 mmol) in ethyl acetate (10 mL) was added the lipase Amano PS (200 mg) and the mixture was gently stirred for 17 h at 30 °C. After the reaction was complete, the enzyme was removed by filtration and the ethyl acetate was evaporated under reduced pressure. The residue was purified by silica gel chromatography ($CHCl_3$/MeOH = 20:1) to yield **9a** (221 mg, 34%, 80% ee) and **5b** (215 mg, 41%). **9a**: $[\alpha]^{23}_D$ = +91.3° (*c* 1.18, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 2.01 (3H, s, MeCO), 3.22 (1H, ddd, J = 5.0, 8.3, 13.9 Hz, C*H*$_2$NHAc), 3.58 (1H, ddd, J = 5.0, 6.6, 13.9 Hz, CH_2NHAc), 4.28 (1H, dt, J = 5.0, 7.9 Hz, C*H*N_3), 6.07 (1H, dd, J = 7.9, 15.8 Hz), 6.27 (1H, br s, NH), 6.69 (1H, d, J = 15.8 Hz), 7.23–7.42 (5H, m); ^{13}C-NMR ($CDCl_3$) δ 23.3 (q), 43.0 (t), 64.0 (t), 123.7 (d), 126.8 (d), 128.5 (d), 128.7 (d), 135.1 (d), 135.5 (s), 170.3 (s). HR MS 231.1238 (M^+ + H) [calcd 231.1245].

Stereochemistry of 9a. A solution of compound **9a** (44.0 mg) in ethanol (10 mL) was hydrogenated (4 kg/cm^2) on 10% Pd–C (10 mg). After 1 day, the catalyst was removed by filtration and the filtrate was concentrated. The residue was purified by silica gel chromatography ($CHCl_3$/MeOH/H_2O = 8:2:0.07) to yield amines **10a** (13.7 mg, 35%) and **10b** (12.1 mg, 31%).

10a: $[\alpha]^{23}_D$ = –8.02° (75% ee, *c* 1.72, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) 2.00 (3H, s), 2.40–2.60 (2H, m), 3.27 (1H, dt, J = 13.5, 7.0 Hz), 3.49 (1H, dt, J = 13.6, 5.6 Hz), 3.73 (1H, br q, J = 6.9 H), 6.16 (1H, dd, J = 16.2, 6.9 Hz), 6.22 (1H, br s, NHAc), 6.59 (1H, d, J = 16.2 Hz), 7.24–7.38 (5H, m); ^{13}C-NMR ($CDCl_3$) 23.3 (q), 44.7 (t), 53.4 (d), 126.4 (d), 127.9 (d), 128.6 (d), 129.5 (d), 131.7 (d), 136.2 (s), 170.9 (s); HR MS 205.1283 (M^+ + H) [calcd 205.1339].

10b: $[\alpha]^{23}_D$ = –6.40° (75% ee, *c* 1.21, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) 1.64 (1H, A part of AB type, m), 1.80 (B part of AB type, m), 1.99 (3H, s), 2.67 (1H, A part of AB type, ddd, J = 7.0, 9.8, 14.0 Hz), 2.75 (1H, B part of AB type, ddd, J = 5.9, 9.8, 14.0 Hz), 2.89 (1H, m), 3.05 (1H, ddd, J = 5.3, 8.2, 13.6 Hz), 3.44 (1H, ddd, 3.9, 5.9, 13.6 Hz), 6.20 (1H, br s, NH), 7.17–7.30 (5H, m); ^{13}C-NMR ($CDCl_3$) 23.3, 32.2, 37.4, 45.0, 50.6, 126.0, 128.3, 128.5, 141.4, 170.5; HR MS 207.1445 (M^+ + H) [calcd 207.1496].

Acetylation of 5b. Isopropenyl acetate (3 mL) was added to a solution of **5b** (215 mg, 1.14 mmol) in EtOAc, and the mixture was stirred for 30 min at room temperature. After the reaction was complete, the organic solvent was removed under vacuum, and the residue was purified by silica gel chromatography (hexane/AcOEt = 1:1) to yield **9b** (261 mg, 99%). The NMR data were the same as **9a**.

Compounds 13 and 14. Ozone gas was injected into a methanol solution of **9a** or **9b** (60 mL, 1.7 mmol) until the color of the reaction solution became pale blue. After bubbling nitrogen to remove excess amounts of ozone, the ozonide was treated with dimethyl sulfide (9 mL) for 12 h at room temperature. Methanol was evaporated and the residue was purified by MCI gel chromatography (H_2O) to yield **3** or **4** respectively (1.3 g), which was used directly in the next reaction.

A buffer solution (Tris, 200 mM, pH 9.0, 100 mL) of DHAP (60 mL, 1.7 mmol) was added to an aqueous solution of **3** or **4** (1.3 g, 8.3 mmol), and the pH was adjusted to 6.5 with 4 N HCl. Rabbit muscle FDP-aldolase (440 units) was added to this solution, and the mixture was stirred slowly for 1 h at 37 °C. After DHAP was consumed completely based on enzymatic assay,[16] the mixture was passed through Dowex 1 (HCO_2^-) (3.5 × 20 cm) and eluted with water (1 L), 0.2 M NaCl (1 L), and 0.4 M NaCl (1 L) solution, successively. The pH value of the fraction eluted with 0.2 M NaCl solution was adjusted to 4.7 with 1 N HCl, acid phosphatase (from sweet potato, type X, 530 units) was added, and the mixture was stirred slowly at 37 °C for 1 day. The pH was adjusted to 7.0 and water was removed by lyophilization. The residue was treated with methanol, and the soluble portion was collected and purified by silica gel chromatography ($CHCL_3$/MeOH/H_2O = 8:2:0.1) to yield **13** (351 mg, 84%) or **14** (230 mg, 55%), respectively.

13: $[\alpha]^{23}_D$ = –27.6° (*c* 1.70, MeOH); ^{1}H-NMR (D_2O) 2.00 (3H, s), 3.39 (1H, dd, J = 14.6, 7.4 Hz), 3.64 (1H, dd, J = 14.6, 2.9 Hz), 3.79 (1H, ddd, J = 9.6, 7.4, 2.9 Hz), 3.83 (1H, d, J = 9.6 Hz), 4.55 (1H, s), 4.48, 4.59 (each 1H, 19.8 Hz); ^{13}C-NMR (D_2O) 22.3, 40.7, 61.8, 66.5, 71.6, 75.8, 175.3, 213.5; HR MS 247.1036 (M^+ + H) [calcd 247.1043].

14: $[\alpha]^{23}_D$ = –35.0° (*c* 1.80, MeOH); ^{1}H-NMR (D_2O) 2.91 (3H, s) 3.35 (1H, A part of AB type, dd, J = 14.1, 7.8 Hz), 3.41 (1H, J = 14.1, 4.4 Hz), 3.78 (1H, dt, J = 7.8, 4.4 Hz), 3.97 (1H, dd, J = 7.8, 3.1 Hz), 4.47 (1H, d, J = 3.1 Hz), 4.48, 4.55 (each 1H, d of AB type, J = 19.4 Hz); ^{13}C-NMR (D_2O) 22.3, 40.3, 63.3, 66.6, 72.5, 75.9, 175.0, 212.7; HR MS 247.1040 (M^+ + H) [calcd 247.1043].

Reductive Amination of 13 and 14. A solution of compound **13** (120 mg, 0.49 mmol) or **14** (53.8 mg, 0.22 mmol) in ethanol (10 mL) was hydrogenated with 10% Pd/C (15 mg) under 4 kg/cm^2 of hydrogen for 24 h. The catalyst was removed by filtration and the solvent was evaporated under vacuum. The residue was purified by silica gel chromatography ($CHCl_3$/MeOH/H_2O = 6:4:1) to yield **1** (79 mg, 80%) or **2** (36 mg, 80%).

1: $[\alpha]^{23}_D$ = +32.5° (*c* 0.40, MeOH); ^{1}H-NMR (D_2O) 1.95 (3H, s), 3.02 (1H, m), 3.07 (1H, m), 3.25 (1H, dd of AB type, J = 14.0, 7.1 Hz), 3.38 (1H, dd of AB type, J = 14.0, 5.1 Hz), 3.57 (1H, dd of AB type, J = 11.9, 6.3 Hz), 3.66 (1H, dd of AB type, J = 11.9, 4.3 Hz), 3.72 (1H, t, J = 6.4 Hz), 3.79 (1H, t, J = 6.4 Hz); ^{13}C-NMR (D_2O) 23.4 (q), 43.0 (t), 61.3 (d), 63.0 (d), 63.4 (t), 78.9 (d), 80.5 (d), 176.2 (s); HR MS 205.1187 (M^+ + H) [calcd 205.1188].

2: $[\alpha]^{23}_D$ = +12.8° (*c* 0.43, MeOH); ^{1}H-NMR (D_2O) 1.95 (3H, s), 2.99 (1H, br q, J = 4.9 Hz), 3.31 (1H, m), 3.25 (1H, dd of AB type, J = 12.2, 5.9 Hz), 3.40 (1H, dd of AB type, J = 12.2, 5.4 Hz), 3.61 (1H, dd of AB type, J = 11.3, 5.9 Hz), 3.67 (1H, dd of AB type, J = 11.3, 4.9 Hz), 3.83 (1H, dd, J = 4.1, 2.4 Hz), 3.79 (1H, dd, J = 3.8, 2.4 Hz); ^{13}C-NMR (D_2O) 22.3 (q), 38.4 (t), 60.4 (d), 61.8 (t), 66.3 (d), 77.1 (d), 78.7 (d), 175.1 (s); HR MS 205.1187 (M^+ + H) [calcd 205.1188].

General Procedure for Enzyme Assay. For each inhibitor, five inhibitor concentrations, ranging from 0 to 10 times K_i, were used to determine the K_i value. At each inhibitor concentration, four substrate concentrations, spanning from 0.168 K_m to 0.325 K_m, were used to obtain a single Lineweaver–Burk plot. The amount of enzyme added in each assay was adjusted so that less than 10% of the substrate would be consumed within 15 s. Since *p*-nitrophenyl *N*-acetylglucosaminide was used as the substrate, the assays were monitored at 400 nm, where the molecular extinction coefficient ϵ was calibrated to be 3204.5 M^{-1} cm^{-1} at pH 6.5.

To a 1-mL disposable cuvette was added 950 μL of 0.1 M NaOAc–PIPES buffer solution, 20 μL of the inhibitor solution, and 20 μL of *p*-nitrophenyl β-D-*N*-acetylglucosaminide solution (100 mM in PIPES–NaOAc buffer, pH 6.5). The solution was well mixed, and 20 μL of the enzyme solution was injected into the cuvette to initiate the reaction. The reaction was monitored at 400 nm on a Beckmann DU-70 spectrophotometer for 15 s, and the initial rate of hydrolysis was calculated. The same procedure was repeated with three other substrate concentrations. After the initial rates were accumulated, the corresponding Lineweaver–Burk plot at that inhibitor concentration was constructed.

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Supplementary Material Available: Inhibition analysis and copies of ^{1}H and/or ^{13}C NMR spectra of **1**, **2**, **5**, **7**, **9a**, **10a**, 11–14 (24 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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