Evaluation of Isofagomine and Its Derivatives As Potent Glycosidase Inhibitors†

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ABSTRACT: A pseudo-aza-monosaccharide and several pseudo-aza-disaccharide compounds were constructed based on replacement of the anomeric carbon with a nitrogen and the ring oxygen with a carbon. The inhibition constants of these compounds toward five different glycosidases, α-glucosidase, β-glucosidase, isomaltase, α-mannosidase, and glucoamylase, were obtained. Isofagomine, the pseudo-aza-monosaccharide, shows a broad spectrum of strong inhibition against glycosidases. It is the most potent inhibitor of β-glucosidase from sweet almonds reported to date and also a strong inhibitor of glucoamylase, isomaltase, and α-glucosidase. Isofagomine inhibits β-glucosidase, glucoamylase, and isomaltase more strongly than 1-deoxynojirimycin where the ring oxygen has been replaced with a nitrogen. The α-1,6-linked pseudo-disaccharide showed very strong inhibition toward glucoamylase, being nearly as potent an inhibitor as acarbose. Pseudo-disaccharides in which the anomeric nitrogen was methylated to favor formation of either the α or β substrate linkage generally had weakened inhibition for the glycosidases studied most likely due to steric interference with the various active sites. These results indicate that the presence of a basic group at the anomeric center is important for carbohydrase inhibition. The presence of a charged carboxylate group near the anomeric carbon which interacts with the basic nitrogen is suggested for these enzymes, particularly for β-glucosidase. The presence of a second α-linked glucosyl residue is also critical for strong inhibition of glucoamylase.

Glycosidases are crucial in many biological processes, including breakdown of edible carbohydrates (Marshal et al., 1974), eukaryotic glycoprotein processing (Kornfeld & Kornfeld, 1985), and polysaccharide and glycoconjugate anabolism and catabolism (Kobata, 1979). Glycosidases are also involved in a variety of metabolic disorders and other diseases such as diabetes (Lin et al., 1987), viral attachment (Sharon, 1986), bacterial infection (Paulson, 1985), and cancer formation (Dennis et al., 1987). Because of their importance, considerable effort has been expended to control glycosidase activity and to study the mechanisms involved (Sinnot 1990; Legler, 1990).

Some detail into the catalytic mechanism of glycoside hydrolysis has been obtained from studies of various three-dimensional glycosidase structures complexed with substrate analogs and also from kinetic studies of site-directed mutations of these enzymes [for reviews see Sinnot (1990), and Svensson and Søgaard (1993)]. The glycosidic bond is cleaved via a nucleophilic substitution at the saturated carbon of the anomeric center, resulting in either retention or inversion of the anomeric configuration. Glycosidases are thus classified as either retaining or inverting enzymes. The retaining enzymes are thought to react using a double-displacement mechanism, where a nucleophile of a side chain carboxylate group attacks from the opposite side of the pyranose ring to the leaving group, producing a covalent glycosyl ester intermediate. The transition state most likely has an oxocarbonium ion-like character which is electrostatically stabilized by an enzyme carboxylate. Inverting glycosidases are considered to utilize a single displacement mechanism, where protonation of the glycosidic oxygen by a general acid is followed by nucleophilic attack of the anomeric carbon by a water molecule which can be promoted by a carboxylate base.

Glycosidase inhibitors can be important tools for studying glycosidase mechanisms and are also prospective therapeutic agents (Winchester & Fleet, 1992). Glycosidase inhibitors such as acarbose and glucose analogs with the ring oxygen replaced with nitrogen among others have been studied for possible use to control diabetes mellitus (Truscheit et al., 1981; Robinson et al., 1991). Substantial effort has been directed toward the development of glycosidase inhibitors. Many naturally occurring azasugars in which the ring oxygen is replaced by nitrogen have been found to be potent glycosidase inhibitors and with only a few exceptions inhibit several hundred to more than 10 000-fold better than their oxygen analogs (Legler, 1990). Despite the many studies on glycosidases and their inhibitors, there is some doubt as to the correct electronic and steric configuration of the transition state structure. A trigonal anomeric center, positive charge, half chair-like conformation, and proper hydroxyl group configuration are all proposed to be important characteristics of a good inhibitor. Kinetic studies on the secondary isotope effects showed that the transition state of

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glycosidases may involve partial charge development on the anomeric carbon (Kempton & Withers, 1992).

Some uncertainty as to the relative importance of conformational and electrostatic characteristics of transition-state analogs complicates the criteria for evaluating transition-state analogs. The stereochemical configuration of the C-2, C-3, and 3-OH groups were shown to be important factors for glycosidase inhibition by glucose derivatives (Dale et al., 1985). Removal of 2-OH slightly weakened binding, whereas removal of the 1-OH group enhanced binding. Replacement of the ring oxygen with a nitrogen largely enhanced binding when comparing 1-deoxynojirimycin to 1-deoxyglucose. Two strong inhibitors of α- and *β*-glucosidases, 1-deoxynojirimycin and D-glucono-1,5-lactam, were concluded not to be transition state analogs, however, because the pH dependence of their affinities for the glucosidases did not resemble the dependence of the k_{cat}/K_M values obtained for substrate hydrolysis.

Assuming that the primary factors that influence inhibitor binding include positive charge, half-chair conformation, and the 2-OH and 6-OH groups, the following correlation for inhibitor binding energy based on analyses of inhibition kinetics was proposed to predict inhibition constants for new inhibitors: $\Delta G = \Delta G(\text{conformation}) + \Delta G(2\text{-OH}_{eq}) + \Delta G(6\text{-OH}_{eq}) + \Delta G(\text{charge})$ (Kajimoto et al., 1991; Liu et al., 1991). A good glycosidase inhibitor was concluded to require a half-chair conformation with positive charge and a ring heteroatom or a chair-form conformation with a positive charge and proper topographic orientation of hydroxyl groups.

Kinetic measurements of substrate analogs indicated that the 4-OH and 6-OH groups play dominant roles in glucose oxidase, glucose dehydrogenase, and glucoamylase activity, with the 3-OH playing a significant but lesser role (Sierks et al., 1992). These three groups, particularly the 4-OH and 6-OH, should be crucial for strong inhibitor binding. The 2-OH group did not have a significant role in activity.

Studies using a valioamine or valienamine group in which the ring oxygen has been replaced with a $=CH_2-$ or $=CH-$ group indicate that these compounds are not as good inhibitors of α- and *β*-glucosidase activity as glucosylamines or 1-deoxynojirimycin (Legler, 1990). However, longer pseudo-di- and tetrasaccharide inhibitors such as acarbose inhibit α-amylases, glucoamylase, and isomaltase very well due to interactions with the additional sugar groups (Truscheit et al., 1981). These results indicate that the half-chair conformation, which is not approximated well by the cyclohexane or cyclohexene group, is essential for good inhibition of glucosidase activity (Legler, 1990).

Inhibition studies using amidine, amidrazone and amidoxime, derivatives of glucose and mannose showed that the two amidoxime derivatives of glucose and mannose with flattened chair conformation were glycosidase transition-state analogs based on similar pH dependencies of the inhibition constants and substrate activity (Tong et al., 1990; Ganem & Papandreou, 1991; Papandreou et al., 1993). Strong inhibition by noncharged analogs further suggested that a flattened anomeric conformation is more important than a positive charge.

Assuming that the binding behavior of transition-state analogs to the enzyme should parallel that of substrates, two nojiritetrazoles were concluded to be transition state inhibitors of glycosidases (Ermert et al., 1993). On the basis of the linear correlation of free energy of activation for turnover of each substrate [log (V_m/K_M)] with the free energy of binding of the inhibitor [log$(1/K_i)$], the transition state inhibitors of glycosidases were concluded to be configurationally selective. A flattened anomeric region and partial charge development around the anomeric carbon were also shown to be important for strong binding.

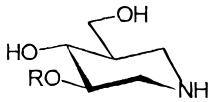

FIGURE 1: Isofagomine: **1**, R = H; **1a**, R = Bn.

A compound that is able to develop a positive charge or partial positive charge at the anomeric position of a flattened ring conformation may therefore be a good transition-state mimic. A sugar analog (isofagomine) and its derivatives, in which the anomeric carbon is replaced by a nitrogen, the ring oxygen is replaced by carbon and the 2-OH group is removed, were therefore designed and synthesized (Jespersen et al., 1994a,b). The inhibition kinetics of isofagomine and several disaccharide derivatives of this parent pseudomonosaccharide toward various glycosidases are presented here. These studies provide new information for the design of potent, novel glycosidase inhibitors.

MATERIAL AND METHODS

Analog Synthesis. Isofagomine (**1**, Figure 1) and 3-*O*-benzylisofagomine (**1a**) were prepared as previously described (Jespersen et al., 1994a). Alcohol **2** was prepared from D-glucose by four known steps (Figure 2) (Liptak et al., 1975). Swern oxidation of **2** to the aldehyde followed by Wittig reaction with $CH_2{=}PPh_3$ gave alkene **3** in 62% yield. Hydroboration with borane dimethylsulfide and oxidation with hydrogen peroxide converted **3** to the alcohol **4** in 91% yield. Swern oxidation of **4** gave the heptose aldehyde **5** in 91% yield. One to one reductive coupling of **5** with 3-*O*-benzylisofagomine (**1a**) using hydrogen and palladium on carbon catalyst gave the tertiary amine **6a** in 76% yield. Somewhat surprisingly no debenzylation occurred under these conditions. Debenzylation of **6a** could be effected, however, with H_2 and Pd/C when hydrochloric acid was added. The unprotected amine **6** was obtained in 82% yield.

Reaction of **6** with 35% hydrogen peroxide resulted in formation of a single *N*-oxide **7** having the oxygen in the axial position. The configuration at the nitrogen atom was determined from the ^{1}H chemical shift data of **7** by comparison with the ^{1}H chemical shift data of the hydrochloride of **6** (supporting information, Table 1). The axial protons H-2a′ and H-6a′ were downfield shifted 0.25−0.35 ppm in **7** compared to **6**, HCl, while the equatorial protons H-2e′ and H-6e′ were shifted 0.15−0.2 ppm upfield. This was consistent with chemical shift changes observed when *N*-methyl-1-deoxynojirimycin (Glaser & Perlin, 1988) was converted to the corresponding axial *N*-oxide (Kajimoto et al., 1991). In that case the neighboring axial H-1 was shifted downfield 0.16 ppm while the equatorial H-1 was shifted upfield 0.1 ppm (Glaser & Perlin, 1988; Kajimoto et al., 1991). Further evidence for the axial position of oxygen in **7** came from the downfield shift of H-3′ and H-5′ compared to **6**, HCl (supporting information, Table 1). It is known

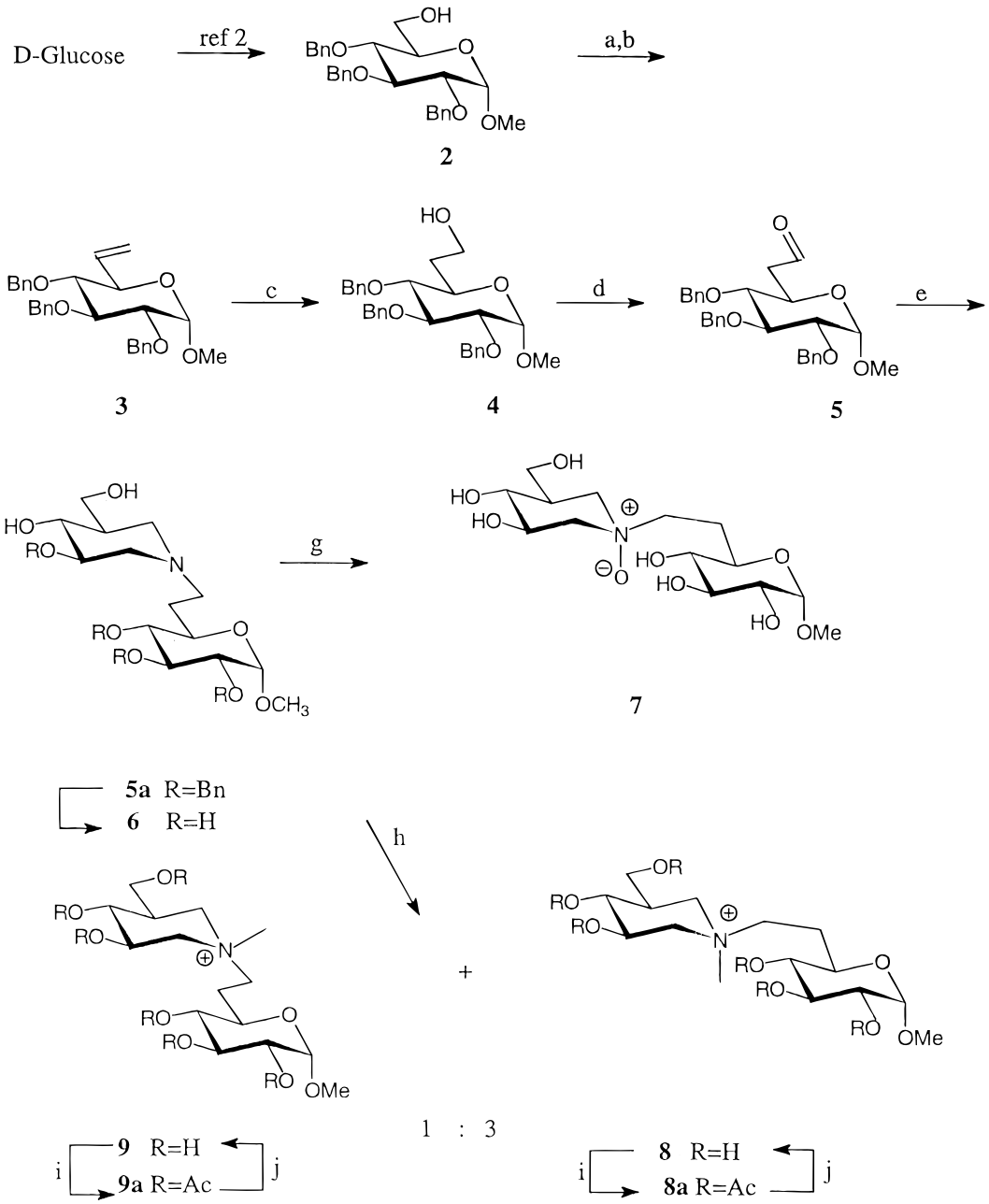

FIGURE 2: a, ($COCl_2$), Et_3N, DMSO; b, $Ph_3P{=}CH_2$, 62% (2 steps); c, 9-BBN, NaOH, H_2O_2, 91%; d, ($COCl_2$), Et_3N, DMSO, 93%; e, H_2, Pd/C, **1a**, 76%; f, H_2, Pd/C, HCl, 94%; g, H_2O_2, 68%; h, MeI, 97%; i, Ac_2O, C_6H_5N, 59%; j, NaOMe, 100%.

that introduction of an axial electronegative substituent causes a significant 1,3-diaxial downfield shifting of protons (Jackman & Sternhell, 1969; Lemieux & Stevens, 1966).

Methylation of **6** using methyl iodide in methanol resulted in formation of the two stereoisomeric quaternary ammonium iodides **8** and **9** in a 3:1 ratio and 97% yield. After conversion to the hexaacetates **8a** and **9a** with acetic anhydride and pyridine, the two isomers could be separated by chromatography. Deacetylation of the compounds with NaOMe/MeOH gave **8** and **9** in 46% and 6% yield from **6**, respectively. The configuration at nitrogen of **8** and **9**, were determined based on the ^{13}C chemical shift of the *N*-methyl group (supporting information, Table 3) and NOE measurements on the acetate **8a**. The ^{13}C chemical shifts of the *N*-methyl groups in **8** and **8a** were 46.5 and 47.3, ppm respectively, while the corresponding shifts in **9** and **9a** were 54.3 and 54.1 ppm, respectively. These data show that the *N*-methyl group of the major isomer **8** (and **8a**) is far more shielded than the minor isomer **9** (and **9a**). This shielding could only be explained as a result of more sterical crowding around the N-methyl group in **8** than in **9** and therefore that the *N*-methyl group in **8** was axial. This was confirmed by NOE measurements of **8a** that showed strong NOE between the *N*-methyl group and both H-3′ and H-5′.

^{13}C-NMR and ^{1}H-NMR spectra were recorded on Bruker instruments AC 200, AC 250 and AM 500 (supporting information, Tables 1−3). D_2O was used as solvent with DHO (^{1}H NMR: 4.7 ppm) and acetone (^{1}H-NMR, 2.05 ppm; ^{13}C-NMR, 29.8 ppm) as reference. With $CHCl_3$ as solvent TMS and $CHCl_3$ (^{13}C-NMR, 76.93 ppm) were used as references. Mass spectra were obtained on a VG TRIO-2 instrument. Optical rotations were measured on a Perkin Elmer 141 polarimeter. Concentrations were performed on a rotary evaporator at a temperature below 40 °C. Dry tetrahydrofurane and diethyl ether were prepared by distillation from sodium and benzophenone.

*Methyl 2,3,4-Tri-O-benzyl-6,7-dideoxy-α-D-glucohept-6-enopyranoside (**3**).* To a solution of oxalyl chloride (283 μL, 3.25 mM) in dichloromethane (4 mL) at −65 °C was added a solution of DMSO (533 μL, 7.52 mM) in dichloromethane (2 mL). After this solution had been stirred for 5 min at −65 °C, the alcohol **2** (1.0 g, 2.16 mM) in dichloromethane (2 mL) was added over 5 min. The mixture was then stirred for 5 min and then heated to −50 °C. Triethylamine (1.95 mL, 14 mM) was added, and the reaction was allowed to reach at room temperature over 1/2 h. Dichloromethane was added and the organic phase was washed with water (2×), NaCl solution (saturated), dried, and concentrated. Evaporation 3 times with toluene left the crude aldehyde, which was used directly as described below. A mixture of $MeP^+Ph_3Br^-$ (1.2 g, 3.36 mM) in THF (10 mL) was cooled to −40 °C, and butyl lithium (1.6 M, 2.45 mL, 3.52 mM) was slowly injected to give a dark orange solution. After this solution had stirred at −40 °C for 1 h the aldehyde (2.16 mM) dissolved in THF (5 mL) was slowly added, and the mixture was stirred for 30 min. It was then heated to 0 °C and stirred overnight. Aqueous NH_4Cl and ether were added, and the organic phase was washed with water (2×), NaCl solution, dried, and concentrated to a residue that was purified by flash chromatography to give colorless crystals of **3** (620 mg, 62%).

*Methyl 2,3,4-Tri-O-benzyl-6-deoxy-α-D-glucoheptopyranoside (**4**).* In a 100 mL flask the alkene **3** (2.17 g, 4.72 mM) was added to 0.5 M 9-BBN in THF (48 mL, 23.6 mM). The mixture was stirred for 2.5 h at 20 °C, after which time TLC showed complete conversion of starting material. The solution was cooled to 0 °C, and an NaOH solution (3 M, 8.7 mL, 26 mM) was added followed by 30% H_2O_2 (8.7 M, 9.5 mL, 94.4 mM). The mixture was stirred for 3 h at room temperature. NH_4Cl solution (saturated) and CH_2Cl_2 were added, and the organic layer was washed with NaCl solution (saturated), dried (Na_2SO_4), and concentrated to a residue. Flash chromatography in EtOAc−heptane 1:2 gave **4** as a colorless syrup (2.04 g, 91%).

*Methyl 2,3,4-tri-O-benzyl-6-deoxy-α-D-glucoheptodialdo-1,5-pyranoside (**5**).* A solution of oxalyl chloride (110 μL, 1.3 mM) in dry dichloromethane (1.55 mL) was stirred under nitrogen atmosphere and cooled to −78 °C. Dimethyl sulfoxide (0.178 mL, 2.5 mM) in dichloromethane (0.67 mL) was added dropwise over 10 min. After stirring for 5 min, methyl 2,3,4-tri-*O*-benzyl-6-deoxy-α-D-glucoheptopyranoside (**4**, 0.40 g, 0.8 mM) dissolved in dichloromethane (0.67 mL) was added dropwise over 5 min. After stirring for another 15 min at −78 °C, triethylamine (0.88 mL, 0.8 mM) was added. The reaction mixture was warmed to room temperature (30 min). Dichloromethane (1 mL) and water (4 mL) were added. The organic layer was extracted with water (4 mL) and brine (4 mL), dried ($MgSO_4$), filtered, and concentrated. The residue was co-concentrated with toluene (2 × 3 mL) to give a colorless syrup in 93% (0.37 g) yield $[\alpha]_D^{20}$: +18.07° (*c* 1.4, $CHCl_3$).

*Methyl 2,3,4-tri-O-benzyl-6,7-dideoxy-7-[(3R,4R,5R)-3-benzyloxy-4-hydroxy-5-hydroxymethylpiperidinyl]-α-D-glucoheptopyranoside, (**6a**).* To a solution of methyl 2,3,4-tri-*O*-benzyl-6-deoxy-α-D-gluco-heptodialdo-1,5-pyranoside (**5**, 961 g, 2.0 mM) in ethanol (30 mL) was added (3*R*,4*R*,5*R*)-3-benzyloxy-4-hydroxy-5-hydroxymethylpiperidine (0.475 g, 2.0 mM) dissolved in ethanol (30 mL) 5% Pd/C (300 mg). The mixture was hydrogenated at 300 psi and 20 °C for 66 h. The reaction mixture was filtered and concentrated. The remnants were flash chromatographed using ethyl acetate and ethyl acetate/methanol 10:1 as eluent to give the product in 76% yield (1.06 g). $[\alpha]_D^{20}$: −2.9° (*c* 0.95, $CHCl_3$). MS (CI, NH_3): *m*/*z* 298 ($M + H^+$).

*Methyl 6,7-Dideoxy-7-[(3R,4R,5R)-3,4-dihydroxy-5-hydroxymethylpiperidinyl]-α-D-glucoheptopyranoside (**6**).* To a solution of methyl 2,3,4-tri-O-benzyl-6,7-dideoxy-7-[(3*R*,4*R*,5*R*)-3-benzyloxy-4-hydroxy-5-hydroxymethylpiperidinyl]-α-D-glucoheptopyranoside (0.350 g, 0.58 mM) in ethanol (20 mL) was added 0.5 M HCl (1.8 mL) and 5% Pd/C (250 mg). The mixture was hydrogenated at 1 atm and 20 °C for 22 h. The reaction mixture was filtered and concentrated to give the hydrochloride of **6** as a colorless syrup in 94% (203 mg) yield. $[\alpha]_D^{20}$: +36.71° (*c* 0.85, MeOH).

Methyl 6,7-dideoxy-7-[3*R*,4*R*,5*R*)-3,4-dihydroxy-5-hydroxymethylpiperidinyl]-α-D-glucoheptopyranoside hydrochloride (**6**, 220 mg, 0.6 mM) was dissolved in water (5 mL). Amberlite IR 67, OH^- (8 mL), was added. After being stirred for 75 min, the ion exchange resin was filtered off and rinsed with water. Concentration gave the product as colorless syrup in 87% yield (173 mg, 82% from **6a**). $[\alpha]_D^{20}$: +77.1° (*c* 0.35, H_2O).

*Methyl 6,7-Dideoxy-7-[(1S,3R,4R,5R)-3,4-dihydroxy-5-hydroxymethylpiperidinyl]-α-D-glucoheptopyranoside N-oxide (**7**).* Methyl-6,7-dideoxy-7- [(3R, 4R, 5R)-3,4-dihydroxy-5-hydroxymethyl-piperidinyl (23 mg, 0.07 mM) was dissolved in water (0.6 mL), and 35% hydrogen peroxide (30 μL) was added. After 18 h the reaction mixture was concentrated to give the *N*-oxide as one isomer as a white foam (27 mg, 100%). $[\alpha]_D^{20}$: +74.3° (*c* 0.6, H_2O).

*Methyl 2,3,4-Tri-O-benzyl-6,7-dideoxy-7-[(1R,3R,4R,5R)-N-methyl-3-benzyloxy-4-hydroxy-5-hydroxymethylpiperidinium]-α-D-glucoheptopyranoside (**8**) and Methyl 2,3,4-Tri-O-benzyl-6,7-dideoxy-7-[(1S,3R,4R,5R)-N-methyl-3-benzyloxy-4-hydroxy-5-hydroxymethylpiperidinium]-α-D-glucoheptopyranoside (**9**).* To a solution of methyl 6,7-dideoxy-7-[(3R,4R,5R)-3,4-dihydroxy-5-hydroxymethylpiperidinyl]-α-D-glucoheptopyranoside (**6**, 64 mg, 0.19 mM) in methanol (0.6 mL) was added methyl iodide (0.16 mL) at 0 °C. The reaction mixture was stirred in darkness at 20 °C for 19 h. Evaporation gave a mixture of two isomers as a white foam in 97% yield (88 mg). This mixture (55 mg) was dissolved in acetic acid anhydride (1 mL) and pyridine (0.9 mL) and stirred at 20 °C for 18 h. The solvents were removed by evaporation followed by co-concentration with toluene (4 × 1 mL) to give a syrup.

The isomers were separated by chromatography using ethyl acetate/methanol 9:1 as eluent to give **8a** [40 mg, $[\alpha]_D^{20}$: +64.4° (*c* 1.75; $CHCl_3$)) and **9a** [7 mg, $[\alpha]_D^{20}$: +19.4° (*c* 0.38, $CHCl_3$)] and some mixture of **8a** and **9a** (3 mg). Total yield: 59% (50 mg). **8a** was first collected followed by **9a**.

Compound **8a** (40 mg, 0.05 mM) was dissolved in methanol (1.5 mL), and 0.17 M sodium methoxide (40 μL) was added. The solvent was removed by evaporation after stirring for 18 h to give a clear syrup of **8** (26 mg, ∼100%). $[\alpha]_D^{20}$: +67.3° (*c* 1.25; MeOH).

9a (6 mg) was deacetylated as described above for **8a** to yield 3 mg of **9** (ca. 73%, 6% from **6**). $[\alpha]_D^{20}$: +26.5° (*c* 0.25; MeOH). ^{13}C-NMR spectra of compounds **5a**, **6**, **6**−**HCl**, **7**, **8**, **8a**, **9**, and **9a** are included (supporting information, Table 3).

Enzyme Assays. α-Glucosidase (yeast), β-glucosidase (sweet almonds), isomaltase (yeast), and α-mannosidase (jack bean) were purchased from Sigma Chemical Co. Glucoamylase from *Aspergillus awamori* as expressed in *Saccharomyces cerevisiae* was obtained as described (Sierks et al., 1989). 1-Deoxynojimycin, 1-deoxymannojirimycin, *p*-nitrophenyl α-D-glucopyranoside, *p*-nitrophenyl β-D-glucopyranoside, and other chemicals were also purchased from Sigma. Acarbose was a gift from Dr. Alexander Scriabine, Miles Inc., Westhaven, CT.

p-Nitrophenyl α-D-glucopyranoside was used as substrate for α-glucosidase and isomaltase assays, *p*-nitrophenyl β-D-glucopyranoside was used as substrate for β-glucosidase assays, *p*-nitrophenyl α-D-mannopyranoside was used as substrate for α-mannosidase assays, and maltose was used as substrate for glucoamylase assays. α-glucosidase, β-Glucosidase (Halvorson, 1966), and isomaltase (Gorman & Halvorson, 1966) assays were performed in 50 mM phosphate buffer, pH 6.8 at 37 °C. A set of 1450 μL substrate solutions was prewarmed in reaction chambers for 10 min, and the reaction was initiated by adding 50 μL of enzyme stock solution to the prewarmed substrate solutions. The release of product, *p*-nitrophenol, was monitored continuously at 400 nm using a Shimadzu UV2101-PC instrument.

The α-mannosidase assay (Li, 1968) was performed in 50 mM citrate buffer, pH 4.5 at 25 °C. 1900 μL of substrate solutions was prewarmed at 25 °C for 10 min, and the reaction was initiated by adding 100 μL of enzyme stock solution to the prewarmed solutions. Samples were taken every 2 min and mixed with an equal volume of 0.2 M borate buffer, pH 9.8, to stop the reaction. The release of *p*-nitrophenol was followed at OD 405 nm using a BioTek microtiter plate reader (EL340).

Glucoamylase assays were performed in 50mM acetate buffer, pH 4.5 at 50 °C as described (Sierks et al., 1989). 1900 μL of substrate solutions was prewarmed at 50 °C for 10 min, and the reaction was initiated by adding 100 μL of enzyme stock solution to the prewarmed solutions. Samples were taken every 2 min and mixed with 2.5 M Tris-HCl buffer, pH 7.0, at a ratio of 3:2 to stop the reaction. The release of glucose was determined by using a glucose assay kit based on glucose oxidase activity (Sigma) using a BioTek microtiter plate reader (EL340) at 450 nm.

Inhibition studies were performed by adding the various inhibitors to a final concentration between 10^{-3} and 10^{-8} M to the respective buffer solutions along with substrate. The solutions were prewarmed before adding enzyme to initiate the reaction. Enzyme concentrations were 0.19 μg of α-glucosidase/mL, 2.98 μg of β-glucosidase/mL, 19.05 μg of isomaltase/mL, 0.95 μg of α-mannosidase/mL, and 4.5 μg of glucoamylase/mL.

Kinetic Analyses. Initial reaction rates were calculated from the slope of the first order plot of product concentration vs reaction time. K_M and v_m values were calculated by nonlinear regression of reaction rate vs substrate concentration using GraFit (Erithacus Software Ltd.). Inhibition constants, K_i, were determined by plotting apparent K_M values vs inhibitor concentrations using GraFit. K_M values were determined using six substrate concentrations between $^1/_6$ and 6 times K_M, and K_i values from three experiments using inhibitor concentrations between $^1/_2$ and $^3/_2$ times K_i.

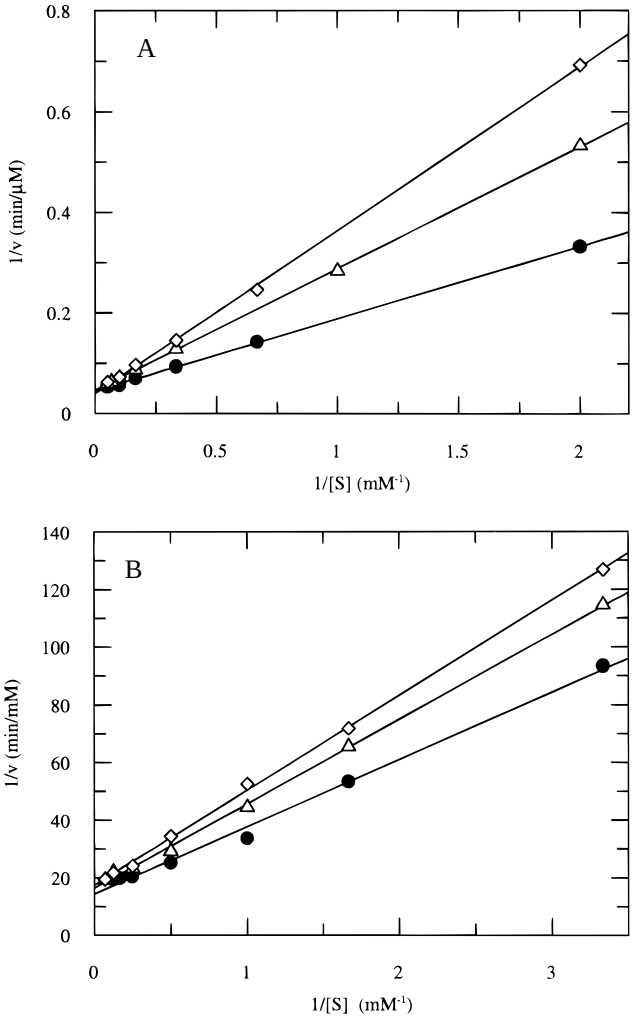

FIGURE 3: (A) Plot of $1/v$ vs 1/[S] for β-glucosidase activity at 37 °C, pH 6.7, with isofagomine (**1**) concentrations of 0 μM (●), 0.075 μM (△), and 0.1 μM (◇). (B) Plot of $1/v$ vs 1/S for glucoamylase activity at 50 °C, pH 4.5, with inhibitor (**6**) concentrations of 0 μM (●), 0.032 μM (△), and 0.054 μM (◇).

RESULTS AND DISCUSSIONS

Inhibition by Isofagomine. Isofagomine (**1**), the monosaccharide analog of glucose in which the anomeric carbon is replaced by a nitrogen and the ring oxygen replaced by a carbon, showed competitive inhibition against all five glycosidases tested. Typical plots of $1/v$ vs $1/S$ for β-glucosidase with varying isofagomine (**1**) and **6** concentrations showing competitive inhibition are shown in Figure 3. Isofagomine displays a broad spectrum of inhibition with K_i values ranging from 10^{-4} to 10^{-7} M for the five glycosidases studied (Table 1). Isofagomine inhibits β-glucosidase particularly strongly and is the most potent inhibitor of β-glucosidase from almonds known to date. Compared with the known strong glycosidase inhibitor, 1-deoxynojirimycin, isofagomine displayed more potent inhibition for three out of the five glycosidases tested. Both isofagomine and 1-deoxynojirimycin have chair-like conformations and a positive charge around the anomeric trigonal region, but they differ in the position of the replaced nitrogen and the removal of the 2-OH group in isofagomine. Since the lack of the 2-OH group increases substrate activity toward glucoamylase (Sierks et al., 1992) and slightly reduces the inhibition toward β-glucosidase (Dale et al., 1985), the increased binding of

Table 1: Inhibition Constants (K_i, M) Obtained at 45 °C

analogs	α-glucosidase	β-glucosidase	isomaltase	glucoamylase	α-mannosidase
acarbose	$(8.1 \pm 0.3) \times 10^{-5}$	$(>10^{-3})$	$(>10^{-3})$	$(2.4 \pm 0.2) \times 10^{-8}$	$(7.6 \pm 0.8) \times 10^{-3}$
1-deoxynojirimycin	$(2.5 \pm 0.4) \times 10^{-5}$	$(4.7 \pm 0.7) \times 10^{-5}$	$(1.1 \pm 0.3) \times 10^{-5}$	$(9.8 \pm 0.3) \times 10^{-6}$	$(2.7 \pm 0.1) \times 10^{-4}$
1-deoxymannojirimycin	$(>10^{-3})$	$(3.0 \pm 0.9) \times 10^{-4}$	$(4.9 \pm 0.7) \times 10^{-4}$	$(6.6 \pm 0.3) \times 10^{-5}$	$(2.8 \pm 0.2) \times 10^{-4}$
isofagomine, **1**	$(8.6 \pm 2.1) \times 10^{-5}$	$(1.1 \pm 0.2) \times 10^{-7}$	$(7.2 \pm 0.2) \times 10^{-6}$	$(3.7 \pm 0.9) \times 10^{-6}$	$(7.7 \pm 0.7) \times 10^{-4}$
analog **6**	$(5.9 \pm 0.2) \times 10^{-5}$	$(2.3 \pm 0.4) \times 10^{-6}$	$(1.0 \pm 0.1) \times 10^{-4}$	$(6.3 \pm 0.3) \times 10^{-8}$	$(1.2 \pm 0.1) \times 10^{-3}$
analog **7**	$(7.0 \pm 0.8) \times 10^{-5}$	$(3.8 \pm 0.2) \times 10^{-7}$	$(1.9 \pm 0.2) \times 10^{-5}$	$(2.4 \pm 0.4) \times 10^{-7}$	$(>10^{-3})$
analog **8**	$(2.8 \pm 0.6) \times 10^{-4}$	$(1.5 \pm 0.1) \times 10^{-4}$	$(>10^{-3})$	$(9.4 \pm 1.0) \times 10^{-5}$	$(>10^{-3})$
analog **9**	$(>10^{-3})$	$(5.1 \pm 0.3) \times 10^{-4}$	$(1.9 \pm 0.2) \times 10^{-4}$	$(1.6 \pm 0.2) \times 10^{-4}$	$(4.0 \pm 0.5) \times 10^{-4}$

isofagomine compared to 1-deoxynojirimycin can be attributed to the position of the replaced nitrogen and not removal of the 2-OH. Isomaltase and glucoamylase are also strongly inhibited by isofagomine indicating that this compound may resemble a transition state structure common to both α- and β-linkages. The 800-fold stronger inhibition of isofagomine for β-glucosidase compared to α-glucosidase indicates a much more favorable interaction with the β-glucosidase active site. Of the five enzymes studied, β-glucosidase is the only enzyme which displays more than an order of magnitude difference in inhibition between isofagomine and 1-deoxynojirimycin. The NH group in either position should specifically interact with a negatively charged carboxylate group in the active site of the various glycosidases. In β-glucosidase this carboxylate group is apparently situated more closely to the anomeric position, accounting for the increased inhibition by isofagomine. Since isofagomine and 1-deoxynojirimycin inhibit retaining (β-glucosidase, α-glucosidase, α-mannosidase, isomaltase) and inverting enzymes (glucoamylase) equally well, the charged carboxylate group can be located on either side of the anomeric group and still interact with the NH group.

A method to calculate K_i values for glucosidase inhibitors has been proposed (Liu et al., 1991). Using this equation to predict the inhibition constants of isofagomine for α- and β-glucosidase, a value of approximately 1 mM is obtained which is nearly 10^4 times higher than the experimental value. This result suggests that this equation does not account for the charge location resulting from sugar analogs with the anomeric carbon replaced by a nitrogen.

Inhibition Studies Using Disaccharide Derivatives of Isofagomine. The disaccharide derivative **6** of isofagomine was designed as a transition-state analog for hydrolysis of α-1,6-linkages. Analog **6** competitively inhibited all five glycosidases with inhibition constants ranging from 10^{-4} to 10^{-8} M. Even though this pseudo-disaccharide was designed to inhibit α-1,6 activity, inhibition toward isomaltase actually decreased compared to inhibition by the pseudo-monosaccharide, isofagomine. The aglycon group may favor an equatorial position for steric reasons and therefore not actually reflect the true structure of an α-1,6-linkage. Analog **6** was also a less potent inhibitor for β-glucosidase as was expected since the aglycon group attached to the anomeric nitrogen in the α-conformation may sterically interfere with binding at the active site. In contrast to the effect on β-glucosidase and isomaltase, analog **6** was a much stronger inhibitor toward glucoamylase than its precursor **1**. Glucoamylase, which hydrolyzes both α-1,4- and α-1,6-linkages, was inhibited 50-fold more strongly by the disaccharide. Analog **6** inhibits almost as well as acarbose, a known potent inhibitor of glucoamylase. Acarbose has been used for affinity purification and to study the catalytic mechanism of glucoamylase and related amylases. The greatly improved affinity of the disaccharide structure to glucoamylase is most likely due to interactions at an additional glucose binding site in the enzyme active site. The second sugar residue should bind at subsite 2 of glucoamylase which has a very high affinity, around 20 kJ/mol at 45 °C (Hiromi et al. 1983; Sierks et al., 1989). The additional binding at this subsite may account for the increased inhibition by the disaccharide. It is rather surprising that analog **6** has such potent inhibition toward β-glucosidase, a retaining enzyme, and glucoamylase, an inverting enzyme.

The *N*-oxide derivative **7** of analog **6**, with a zwitterionic type charge at the anomeric position, is also a potent inhibitor for glycosidases. Analog **7** is a more potent inhibitor than analog **6** for isomaltase, suggesting that either the addition of oxygen perturbs the steric pattern around the linkage to mimic an α-1,6-linkage better or a better electrostatic interaction with isomaltase is achieved. Analog **7** is also a 6-fold more potent inhibitor for β-glucosidase than analog **6** but has similar inhibition constants as **6** for α-glucosidase and α-mannosidase. The zwitterionic feature of the oxidized ring nitrogen may have a stronger electrostatic interaction with the negatively charged residues on the active site of β-glucosidase and isomaltase or may also have interactions with a positively charged group near the anomeric site.

Analog **7** is a less potent inhibitor for glucoamylase than analog **6**, but stronger than **1**. Compared to the pseudo-monosaccharide, the disaccharide structures bind more favorably to glucoamylase as expected with an extended active site. A specific interaction with the glucoamylase active site was noticed with two tight binding inhibitors, acarbose and analog **7**. With both of these inhibitors, k_{cat} increases with increasing inhibitor concentration. The 10%−20% increase in activity can be attributed to multiple binding in the glucoamylase active site. Glucoamylase hydrolyzes the glucosidic linkage between subsites 1 and 2, so a productively bound disaccharide substrate will fill these two subsites. A disaccharide inhibitor can also bind in subsites 1 and 2, inhibiting activity, or it can bind in the more distant subsites while substrate is bound in subsites 1 and 2. Glucoamylase displays an increased k_{cat} for longer substrates, and a different catalytic mechanism seems to control hydrolysis of short and long substrates (Sierks & Svensson, 1996). An increase in k_{cat} at high maltose concentrations was also noticed with glucoamylase (Swanson et al., 1977). Occupation of some of the distant subsites confers a conformational change (Svensson & Sierks, 1992) which apparently alters the glucoamylase catalytic mechanism to increase k_{cat}. The increase in k_{cat} with increasing concentration of acarbose or analog **7** is therefore likely due to inhibitor binding at a distant subsite which does not block the active site but does alter the catalytic mechanism from that normally

observed with maltose to that seen for longer substrates.

Analogs **8** and **9** are two isomers of methylated derivatives of analog **6**, with a methyl group attached to fix the compound in either the α- or β-position relative to the anomeric nitrogen. The α-methylation in analog **8** should favor binding to β-glycosidases more than α-glycosidases, while the β-methylation in analog **9** should favor binding to α-glycosidases more than for β-glycosidases. This trend is not consistently seen; in fact, methylation of the ring nitrogen actually reduces inhibition for four of the five glycosidases studied, α-mannosidase being the exception. The substantially reduced inhibition of **8** and **9** compared to **6** suggests that the added methyl group may sterically interfere with binding to the active sites or may restrict the flexible access of these compounds to the active sites. This steric interference is apparently more important than the structural conformation since the β-methylated form does not always inhibit the α-glycosidases better than the α-methylated form and vice versa.

Mechanistic Implications. The strong broad inhibition displayed by the pseudo-mono- and disaccharide derivatives studied here provide some insight into the various enzymatic mechanisms studied. Previous studies on inhibition of β-glucosidase suggest that a half-chair structure is critical for a strong inhibitor rather than generation of a positive charge (Ganem & Papandreou, 1991; Papandreou et al., 1993). Since isofagomine, where the nitrogen replaces the anomeric carbon, inhibits β-glucosidase 400-fold more strongly than compounds where the nitrogen replaces the ring oxygen, generation of a positive charge is important for strong inhibition of β-glucosidase. Isofagomine, the pseudo-disaccharide **6** and the *N*-oxide form **7** all show very potent inhibition of β-glucosidase. The active site of β-glucosidase contains two carboxylic acid groups positioned close to the anomeric center of the substrate. The carboxylic group situated on the β-face should be in the acid form and can protonate the nitrogen of isofagomine or the related compounds. Either the carboxylate on the α-face of the substrate or the generated carboxylate on the β-face can then interact electrostatically with the protonated nitrogen. If this charge is removed from the anomeric center, either to the ring oxygen or bound to the anomeric carbon, the electrostatic interaction is not realized (Ganem & Papandreou, 1991). A possible explanation for the *N*-oxide form of the disaccharide (**7**) binding almost 10-fold more strongly than the simple pseudo-disaccharide **6** is that the β-face acid group hydrogen bonds to the negatively charged oxygen and the very positive nitrogen then interacts electrostatically with the α-face carboxylate.

While compounds **1**, **6**, and **7** show strong inhibition toward α-glucosidase, the inhibition constants are 25- to nearly 800-fold lower than the corresponding constants toward β-glucosidase. The inhibition constants for all three of these compounds are similar, and are also similar to the values obtained with the known inhibitors 1-deoxynojirimycin and acarbose. Since both compounds **6** and **7** bind equally well, formation of the zwitterion does not effect interactions with the active site nor does the location of the nitrogen at the anomeric carbon or the ring oxygen. These results suggest that the carboxylate groups in the active site of α-glucosidase are not strongly influenced by the location of the positive charge on the substrate as is seen with β-glucosidase.

Isomaltase should have an active site similar to α-glucosidase, and similar inhibition profiles were obtained with both enzymes. Since **6** was designed to inhibit isomaltase activity, it is surprising that this compound binds less strongly than the parent compound, isofagomine or 1-deoxynojirimycin. As mentioned earlier, this may be due to steric interference of the aglycon group. The similar inhibition constants obtained with isomaltase suggest that its active site carboxylate groups also are not strongly influenced by the location of the positive charge. The half-chair conformation is apparently more critical for strong inhibition of isomaltase and α-glucosidase than electrostatic interactions.

Glucoamylase has an extended active site where interactions with distant subsites seem to contribute to strong binding (Svensson & Sierks, 1992). It is somewhat surprising that the pseudo-disaccharide **6** inhibits nearly as well as acarbose. This effect is diminished somewhat since acarbose actually increases enzyme activity as noted earlier which results in a calculated inhibition constant that is weaker than the actual value. The strong inhibition of **6** relative to other pseudo-disaccharides (Truscheit et al., 1981) suggests a specific interaction with the glucoamylase active site. The zwitterion interferes with these specific electrostatic interactions. Since glucoamylase is strongly inhibited by both 1-deoxynojirimycin and isofagomine, the location of the positive charge on the substrate during hydrolysis seems to have only a slight preference for the anomeric center over the ring oxygen. Presence of more than one glucosyl ring in the inhibitor is important for strong glucoamylase inhibition as noticed earlier (Svensson & Sierks, 1992).

Finally, α-mannosidase does not show particularly strong inhibition by any of the inhibitors studied. The half-chair conformation appears to be important, but location of the positive charge does not substantially affect inhibition nor surprisingly does the position of the 2-OH group. Further insight into the structure of α-mannosidase is required to facilitate design of potent inhibitors.

Implications for Inhibitor Design. Compounds with a nitrogen replacing the ring oxygen show strong inhibition toward glycosidase activity due to interactions between an enzyme carboxylate group and the protonated inhibitor (Legler, 1990) and structural complementarity resulting from the trigonal structure formed by the nitrogen (Papandreou et al., 1993). In this study, the carboxylate group is shown to form equally strong or, particularly in the cases of β-glucosidase and glucoamylase, stronger interactions with compounds where the basic nitrogen is located at the anomeric carbon position. An effective and novel approach to the design and synthesis of these azasugars and derivatives is demonstrated.

The positive charge which results from the nitrogen at the anomeric position likely accounts for much of the enhancement of inhibition for glycosidases. Methylation of the anomeric nitrogen of the pseudo-disaccharide to favor either the α- or β-linkage reduces the inhibition for glycosidases, either because the methyl group sterically obstructs access of inhibitors to the active site of the glycosidase or the needed flexibility of the substrate to fit into the various active sites has been restricted. The strong inhibition of isofagomine against β-glucosidase activity suggests an interaction with a carboxylate group which is located closer to the anomeric center than the ring oxygen. The strong inhibiton of **6** toward

glucoamylase activity is due to an interaction with a carboxylate group near the anomeric carbon and an additional strong interaction with more distant subsites. These inhibition studies provide useful information for design of new potent inhibitors for glycosidases. This information can be used to study the catalytic mechanisms of glycosidases or to generate novel enzymes for carbohydrate processing, such as catalytic antibodies. A catalytic antibody with glycosidase like activity has recently been isolated using an antigen consisting of a cyclohexane with an attached quaternary ammonium ion at the exo-oxygen (Suga et al., 1994). The resulting antibody did not show evidence of acid hydrolysis apparently because the charged ammonium ion is not positioned correctly for catalysis. A nitrogen in the anomeric position such as isofagomine may have a better orientation to induce a catalytic acid group. These inhibitors may also have practical applications in controlling various glycosidic processes such as in diabetes treatment or modification of glycosylation patterns.

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SUPPORTING INFORMATION AVAILABLE

Tables containing ^{1}H and ^{13}C NMR data for the compounds **5**, **6**, **6HCl**, **7**, **8**, **8a**, **9** and **9a** are available (3 pages). Ordering information is given on any current masthead page.

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