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Cyclic Guanidino-Sugars with Low pK_a as Transition-State Analog Inhibitors of Glycosidases: Neutral Instead of Charged Species Are the Active Forms

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Abstract: Cyclic guanidino-sugars with different pK_a values are designed and synthesized as transition-state analog inhibitors of galactosidases. Characterization of these structures (**7**, **10**, **12**) reveals that **7** and **10** are in a pH-dependent equilibrium between a furanose form and a mixture of neutral and protonated tetrahydropyrimidine forms. In contrast, the *O*-linked guanidino-sugar **12** exists as the tetrahydropyrimidine forms above pH 5. The furanose−tetrahydropyrimidine equilibrium can thus be modulated with the appropriate *N*-substituent which affects the guanidino-sugar pK_a value. Enzymatic inhibition by **7**, **10**, and **12** is also pH-dependent, indicating that the enzymes recognize the tetrahydropyrimidine form. Evidence is presented to support a dominant role for the uncharged form of the six-membered cyclic guanidino-sugar in the inhibition of galactosidases. Though the inhibition potency is moderate (K_i range 4−50 μM), the use of cyclic guanidino-sugars in the study provides new insights into the mechanism of inhibition of glycosidases.

Oligosaccharide biosynthesis and degradation pathways are crucial to such diverse processes as HIV-1 replication, inflammation, blood clotting, cell differentiation, tumor development, cell routing, diabetes, and other metabolic disorders. Mechanistic understanding[1] and inhibition[2−5] of the enzymes that direct these reactions (glycosidases, glycosyltransferases) are areas of considerable interest. Both classes of enzymes are thought to proceed through a flattened half-chair (or twist-boat) conforma-

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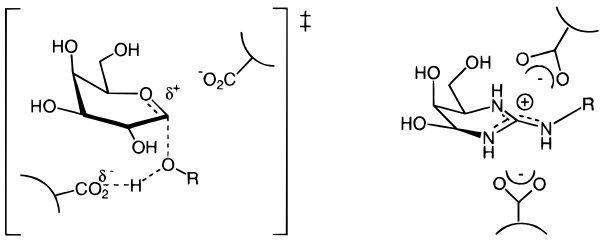


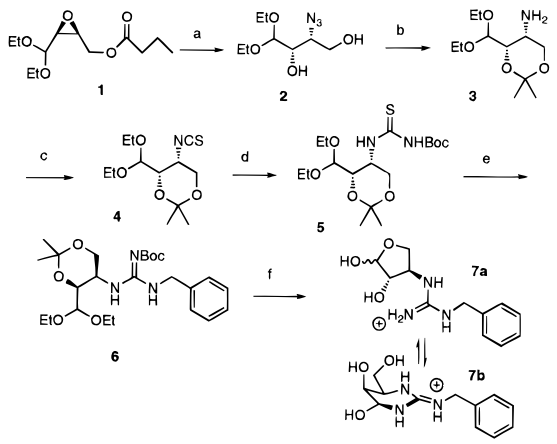
Figure 1. Expected transition state (left) and its mimic (right) in a α-galactosidase reaction. It is proposed that the neutral form of the cyclic guanidino-sugar is accepted by the enzyme followed by protonation from one of the two carboxyl groups to form a tight charge complex.

tion transition state with substantial sp^2 character at the anomeric position[1] (Figure 1).

In an effort to develop new transition-state analog inhibitors of glycosidases, we have reported a preliminary study of a cyclic guanidino-sugar as inhibitor of glycosidases.[5c] This type of compound possesses a number of attractive features. The cyclic guanidine moiety may mimic the electronic character and conformation of the transition state of both glycosidases and glycosyltransferases. Guanidino-sugars are stable over a wide pH range, and their pK_a values may be modulated with appropriate substituents. The cyclic guanidine moiety may interact favorably with the two putative carboxylic residues in the active site of glycosidases. For example, a potent inhibitor of influenza virus sialidase contains a guanidine which coordinates two active-site carboxylates ($K_i = 0.2$ nm).[6] Additionally, the hydroxyl topography may be designed into the cyclic guanidine molecule to mimic the specific topography of different types of sugars. 3-Deoxy-guanidino-sugars have been reported;[7] however, these compounds may have an insufficient hydroxyl topography for the targeting of specific glycosidases. A proper transition-state analog should mimic both the steric and electrostatic properties of the transition state. All cyclic guanidino-sugars known so far are very basic (pK_a > 10) and are completely charged under the assay condition. It is therefore not clear whether the charged or neutral species is active as the inhibitor. Here, we describe the synthesis of cyclic guanidino-sugars with different substituents equivalent to the leaving group and different pK_a values to investigate the nature of the transition state in glycosidase reactions.

The synthesis of a representative guanidino-sugar was carried out as shown in Scheme 1 from the mono-protected *cis*-2,3-epoxybutane-1,4-diol which is readily available as a racemic mixture[8] or as the optically pure form prepared *via* the Sharpless epoxidation[9] or *via* lipase reaction.[10] The dibutyrate of *cis*-2,3-epoxybutane-1,4-diol was a good substrate for pancreatic lipase-catalyzed enantioselective hydrolysis to give the mono-protected epoxybutanediol (ee > 90%). The Sharpless epoxidation required the TBDPS protecting group instead of butyrate (ee > 90%).[9] Epoxide **1** which was prepared from the monobutyrate was reacted with sodium azide regioselectively, and the butyrate was removed to give diol **2** in a 52% overall yield. Hydrogenation of the protected diol provided amine **3** in a good yield. Exposure of the ethyl acetate solution of **3** to thiocarbonyldiimidazole gave isothiocyanate **4** with an 87% isolated yield. Treatment of **4** with ammonium hydroxide followed by BOC protection yielded compound **5** exclusively. Reaction of thiourea **5** with benzylamine in the presence of mercuric chloride[11] led to the formation of guanidine **6** in a good yield. Removal of all of the protecting groups of **6** with trifluoroacetic acid resulted in the expected guanidinium trifluoroacetate in a good yield. The desired compound **7** was obtained after neutralization with Dowex-1 (OH^-). A similar synthetic route was carried out to prepare **10** and **12**, as shown in Scheme 2. Compound **12**, in which the guanidine moiety is linked to an oxygen group, was prepared *via* **5** using *O*-benzylhydroxylamine hydrochloride in the presence of mercuric chloride followed by deprotection with trifluoroacetic acid (79% overall yield).

Scheme 1[a]



[a] Conditions: (a) (i) NaN_3, NH_4Cl, EtOH, H_2O reflux, overnight 66%. (ii) K_2CO_3, MeOH, RT, 10 min, 80%. (b) (i) 2,2-Dimethoxypropane, PPTS, DMF, RT, 2 d, 97%. (ii) Pd/C, H_2 (1 atom), EtOH, 3 h, 82%. (c) Thiocarbonyldiimidazole, EtOAc, RT, 30 min, 87%. (d) (i) NH_4OH, RT, 1 h, quantitative. (ii) Boc_2O, Et_3N, DMAP, CH_2Cl_2, 0 °C to RT, 0.5 h, 72%. (e) Benzylamine, $HgCl_2$, Et_3N, DMF, 0 °C to RT, 4 h, 75%. (f) Trifluoroacetic acid, CH_2Cl_2, H_2O (10:10:1), RT, 6 h, Dowex-1 (OH^-), 95%.

Compound **12** exists as only one structure predominantly under several pD conditions as determined by ^1H and ^{13}C NMR spectra (see Figure 2 for ^{13}C). We assign the structure of compound **12** to the tetrahydropyrimidine form rather than the furanose form (see Table 1), because the peak of C4 at 59.7 ppm is a triplet in the coupled ^{13}C NMR spectra and shows a large deuterium-induced shift.[12]

Compound **7**, however, exists as a pH-dependent mixture of two isomers. The peak pattern of ^{13}C NMR spectra of compound **7** under the basic condition is similar to the peak pattern of compound **12**. We believe that the isomer under the basic condition corresponds to the tetrahydropyrimidine form. At lower pH, the other isomer detected by ^{13}C NMR is assigned

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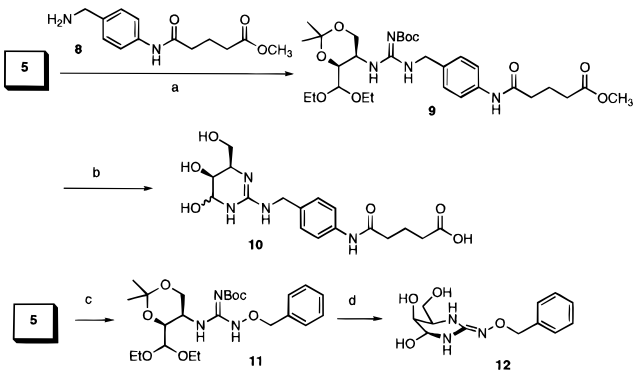
Scheme 2[a]



[a] Conditions: (a) Amine 8, $HgCl_2$, Et_3N, DMF, 0 °C to RT, 9 h, 65%. (b) (i) LiOH, MeOH, H_2O, RT, 2 h. (ii) Trifluoroacetic acid, CH_2Cl_2, H_2O (10:10:1), RT, overnight. (iii) Dowex-1 (OH^-), overall 75%. (c) *O*-Benzylhydroxylamine hydrochloride, $HgCl_2$, Et_3N, DMF, 0 °C to RT, 5 h, 83%. (d) (i) Trifluoroacetic acid, CH_2Cl_2, H_2O (10:10:1), RT, overnight. (ii) Dowex-1 (OH^-), overall 95%.

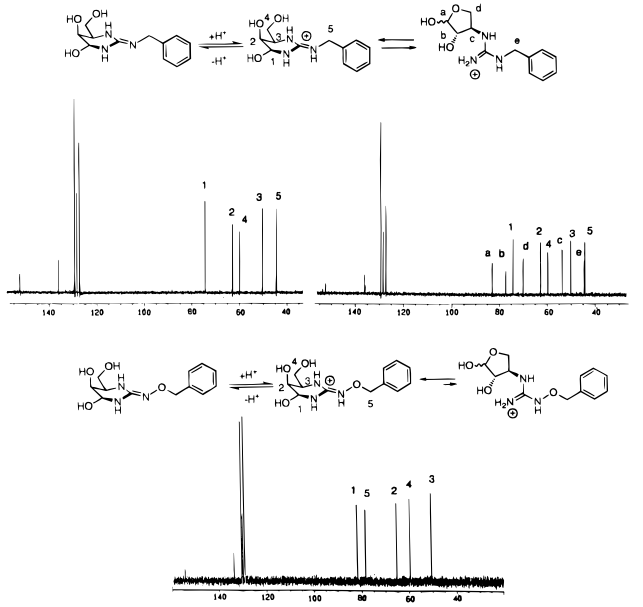


Figure 2. ^{13}C NMR (125 MHz) spectra of **7** and **12** (deuteriated phosphate buffer, internal standard: CH_3CN, 1.6 ppm): (top left) pD 10, (top right), pD 5.5, (bottom) pD 5.5.

to a furanose form. A 1:1 mixture of the furanose form and the tetrahydropyrimidine forms (charged and neutral species) at pD 5.5 and a 1:4 mixture at pD 7.0 were observed on the ^1H NMR spectra in deuteriated phosphate buffer. Only the tetrahydropyrimidine form is, however, observed at pH 10. A similar equilibrium mixture is observed for **10** at the same pH values. These results appear to indicate that protonation of the cyclic guanidine moiety destabilizes the ring system and results in the ring openings to form a mixture of charged six- and five-membered species due to the loss of the anomeric effect ($HO-CH_2-NH^+-$),[13] and the charged tetrahydropyrimidine form is slightly more stable than the charged furanose form probably due to the reverse anomeric effect.[13] Under the basic conditions, the noncharged tetrahydropyrimidine form is favored (Scheme 3), probably because the charged furanose form **7b** is more basic. We are investigating the stereochemistry of C1 (refer to Table 1), and at this point, we believe that the axial form may exist preferentially by 1D NMR spectra and minimized energy calculations.

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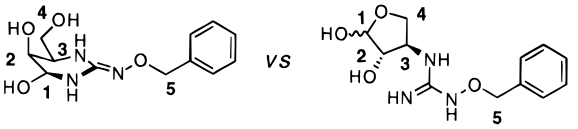
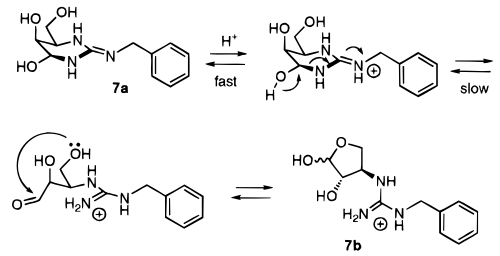
Table 1. The Characterization of **12** Was Established as the Tetrahydropyrimidine Form (left) on the Basis of the Observed Deuterium-Induced Chemical Shift at C4



assigned carbon atom	deuterium effect in the ^{13}C NMR spectra (ppm)	coupling pattern of the coupled ^{13}C NMR spectrum
C1	0.171	81.9 (doublet)
C2	0.170	65.4 (doublet)
C3	0.168	50.7 (doublet)
C4	0.207	59.7 (triplet)
C5	0.060	78.5 (triplet)
guanidine carbon	0.035	154.6 (singlet)
phenyl ring carbon	0.025	133.8
	0.028	130.5
	0.001	130.0
	0.020	129.2

Scheme 3



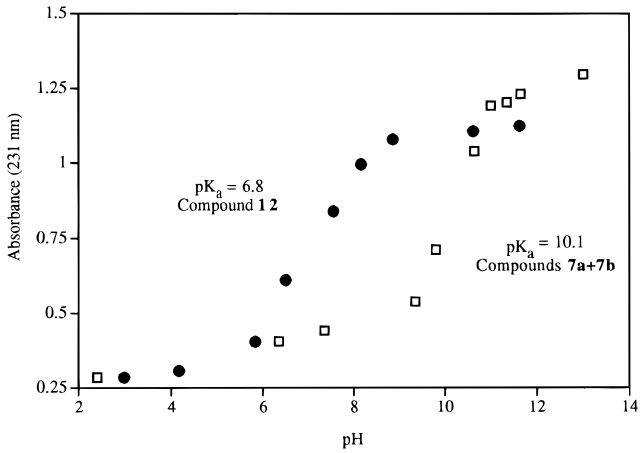


Figure 3. Spectrophotometric pH titrations of compounds **7** and **12**.

To better understand the pH-dependent behavior of **12** and **7**, the pK_a values of these guanidino-sugars were spectrophotometrically determined. The absorbance of the guanidino-sugars was measured at 231 nm as a function of pH. The absorbance of cyclic guanidine compounds such as 2-iminoimidazolidine has been determined (pH 12.0, $\lambda_{max} = 213$ nm, $\epsilon = 4250$).[14] At low pH, the spectrum exhibited a minimum at 231 nm. At high pH, the UV absorbance maximum of the guanidino-sugar shifted to a higher wavelength and the wavelength 231 nm was on the shoulder of the guanidino-sugar absorbance. Plotting the absorbance at 231 nm vs pH yielded a titration curve for a monoprotic acid (Figure 3). The titrations were initiated from both ends of the pH range, resulting in the same pK_a value for each compound. The pK_a values of **7** and

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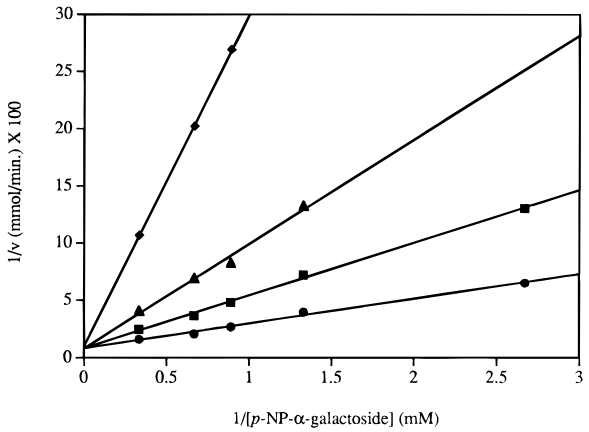

Figure 4. Inhibition of α-galactosidase with compound **12**. The K_i was determined to be 5.3 μM. The initial inhibitor pH was 10.7, and the assay pH was 7.5.

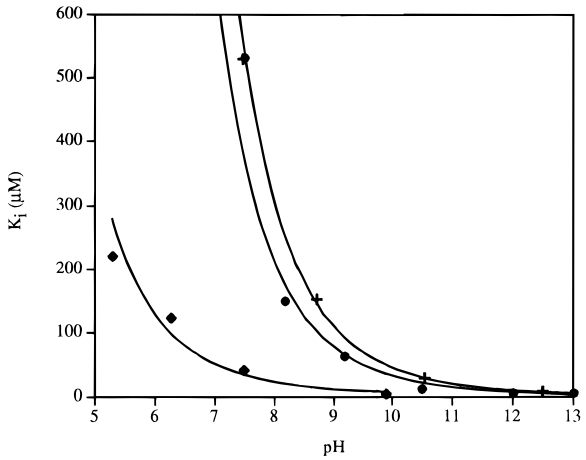

Figure 5. Inhibition of α-galactosidase with guanidino-sugars at different pH values: (+) compound **7**, (●) compound **10**, (◆) compound **12**. The inhibitor at a given pH value was added to the assay solution at pH 7.5 containing the enzyme. Addition of the substrate (*p*-nitrophenyl α-D-galactoside) initiated the assay reaction.

12 were determined to be 10.1 and 6.8, respectively. The change of the guanidine substituent from an *N*-benzyl to an *N*-benzoxy group resulted in the decrease of the pK_a value for the guanidine group from 10.1 to 6.8 (Figure 2). For comparison, the unsubstituted guanidino-sugar[5c] was found to have a pK_a = 9.7, and the cyclic guanidine compound 1,3,4,6,7,8-hexahydro-2*H*-pyrimido[1,2-*a*]pyrimidine, which is unable to undergo ring reorganization, was determined to have a pK_a = 11.6. The substituent effect on the pK_a values for substituted ammonia (ammonia, 9.2; benzylamine, 9.4; hydroxylamine, 6.0)[15] is also similar to the observed effect in the substituted guanidino-sugars. Therefore, the change in the guanidino-sugar *N*-substituent can be used to modulate the pK_a of the guanidino-sugars.

The nature of the guanidino-sugar chromophore was further probed by measuring the loss of the chromophore absorbance at 231 nm as a function of time in low pH buffer in the absence of enzyme. A concentrated solution of **12** (10 mM phosphate buffer, pH 11.0) was injected into a 100 mM phosphate buffer (pH 3.7). The absorbance at 231 nm decreased 1.2 absorbance units in less than 1 s. Since this observance was used for the spectrophotometric titration and protonation of a guanidine moiety should cause a red shift, it is reasonable to believe that this is a fast protonation step.

Inhibition analysis indicates that cyclic guanidino-sugars are competitive inhibitors of coffee bean α-galactosidase (Figure 4). Coffee bean α-galactosidase has been used extensively to test glycosidase inhibitors. In this and subsequent studies, an aliquot of the guanidino-sugar incubated at different pH values was taken and added to an assay solution containing the enzyme of interest and its substrate at a certain pH. Coffee bean α-galactosidase from *Coffea canephora* is a mixture of two enzymes. The two enzymes, α-galactosidase I and II, have molecular masses of 28 000 and 36 500 Da, respectively.[2g] The partially purified preparation purchased from Sigma Chemical Co. was analyzed by SDS−PAGE and found to contain only α-galactosidase II with no detectable amount of α-galactosidase I. All data concerning coffee bean α-galactosidase, therefore, refer to the type II form. Coffee bean α-galactosidase exhibits optimal activity at pH 6.1. Kinetic analysis revealed that there are two active site residues with pK_a values of 2.8−3.5 and 5.1−5.9. The latter group undergoes a pK_a shift to 6.6−6.8 upon substrate binding.[16] More recent studies have suggested that coffee bean α-galactosidase has only one protonating group in its active site.[17]

The inhibition of coffee bean α-galactosidase with **12** (initial pH 10.7, 10 mM phosphate buffer) was monitored at two different assay pH values. The guanidino-sugar was shown to be a competitive inhibitor at both assay pH values by Lineweaver−Burk analysis at fixed inhibitor concentrations. **12** inhibited coffee bean α-galactosidase better in the buffer at pH 7.5 (K_i = 6.4 ± 0.9 μM) compared to in an assay buffer at pH 6.0 (K_i = 15.2 ± 2.8 μM). Since the pK_a of **12** is 6.8, a shift in the pH from 6.0 to 7.5 changes the protonated fraction of **12** from 94% to 33%. Similar observations have been reported for the inhibition of rabbit small intestinal sucrase with nojirimycin and deoxynojirimycin. For example, nojirimycin (pK_a = 5.3) has a K_i = 0.33 ± 0.03 μM at pH 4.5 and a K_i = 0.127 ± 0.007 μM at pH 5.85.[18]

The initial studies demonstrate that guanidino-sugars significantly inhibit α-galactosidases. Assays of high buffer capacity were used to evaluate the effect of the addition of guanidino-sugar inhibitors at different pH values. This arrangement allowed for the variation of the initial pH of the guanidino-sugar inhibitor while maintaining constant assay pH. Three types of guanidino-sugar inhibitors were evaluated as inhibitors of coffee bean α-galactosidase II: **7**, **10**, and **12**. Compounds **7** and **10** exhibited a similar pH−K_i profile (Figure 5). Both guanidino-sugar compounds were potent inhibitors when they were taken at high pH values (pH 13) and added to the assay solution at pH 7.5 (K_i = 4.9 μM). When the inhibitors **7** and **10** were preincubated at pH 7.5, the inhibition of coffee bean α-galactosidase was poor; however, **12** had significant inhibition at pH 7.5 (K_i = 41 μM). The pH−K_i profile for **12** was shifted to lower pH values (Figure 5). When **12** was taken from a pH 9.9 solution and mixed with the assay solution at pH 7.5, the K_i was 5.3 μM. The inhibition is therefore pH-dependent and can be modulated through the selection of the appropriate guanidino-sugar substituent. A similar pH−K_i dependence was obtained for **12** with a bacterial α-galactosidase from *Aspergillus niger*.

The inhibition of coffee bean α-galactosidase by **7** was then evaluated at its IC_{50} concentration as a function of the

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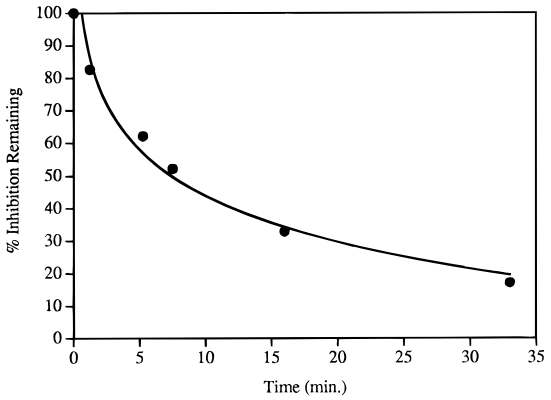

Figure 6. Inhibition of coffee bean α-galactosidase by compound **7**. Compound **7** (pH 10.7) was preincubated at pH 7.5 for various amounts of time prior to the addition of the galactosidase.

Table 2. Inhibition of Coffee Bean α-Galactosidases by **7** (pK_a = 10.1) Was Correlated with the Fraction of the Guanidino-Sugar That Was Initially Uncharged[a] (K_i in μM)

pH	K_i	% neutral	pH	K_i	% neutral
7.5	527	0.25	12	6.3	98.7
8.8	151	4.50	13	4.9	99.9
10.5	28.1	74.0			

[a] The inhibitor buffer had no contribution to the inhibition.

preincubation of **7** (pH 10.5, 10 mM phosphate buffer) in the assay buffer (100 mM HEPES, pH 7.5) prior to the addition of coffee bean α-galactosidase. Only the preincubation time was varied, and the percent inhibition was measured. The time to loss of half the initial inhibitory potency of **7** at 23.5 °C was 8.7 min with greater than 80% of the inhibitory potency lost in 40 min (Figure 6). **12** lost less than 20% of its inhibitory potency with a 30 min preincubation at pH 7.5 with no additional loss after 70 min. A solution of **7** at low pH regained its inhibitory properties upon a pH shift to a higher pH, suggesting that the loss of inhibition was not due to guanidino-sugar decomposition. The NMR experiments designed to investigate the ring reorganization equilibrium gave results comparable to those of the time-dependent inhibition experiments. The pD of compound **7** (pD 10, deuteriated phosphate buffer) was adjusted to pD 7.5 with the addition of a dilute deuterium chloride solution. It took approximately 1 h at room temperature to achieve the equilibrium between tetrahydropyrimidine and furanose forms.

Guandino-sugars are also potent inhibitors of β-galactosidases. Compound **12** (initial inhibitor pH 10) exhibited a K_i of 13.2 μM at pH 7.5 for *Aspergillus oryzae* β-galactosidase and a K_i of 24.7 μM for *A. niger* β-galactosidase. The K_i of **7** for *A. niger* β-galactosidase was 62 μM at pH 7.5 (initial inhibitor pH 10.5).

The pH dependence of the guanidino-sugars as determined by NMR analysis and the pH dependence on inhibition suggest that the neutral tetrahydropyrimidine form of the guanidino-sugars is the active species. Further analysis of the enzyme activities also correlated the neutral guanidino-sugar as the active inhibitory species (Table 2). Guanidino-sugars can be thought to have three species in equilibrium: neutral tetrahydropyrimidine, protonated tetrahydropyrimidine, and furanose forms. At a certain pH value, a ratio of tetrahydropyrimidine to furanose forms will be established. Thus, the initial pH of the guanidino-sugar dictates the percentage of guanidino-sugar molecules initially in the six-membered forms. Analogous to mutarotation, the protonation is a fast step and the ring reorganization is comparably slow. On the time scale employed in the K_i−pH studies, the ring reorganization should be minor. When shifted to a lower pH environment, the guanidino-sugar will have the ratio of neutral to charged tetrahydropyrimidine forms dictated by the new pH value but the concentration of the six-membered forms will be predetermined by the initial pH value. For example, the concentrations of neutral tetrahydropyrimidine in solutions of 50 and 300 μM compound **7** (pH 10.5) are calculated from the pK_a to be 37 and 222 μM. Upon a shift of **7** to pH 7.5, the fast protonation will produce concentrations of charged tetrahydropyrimidine of 36.9 and 221.4 μM. These conditions produced 15.8% and 73.0% inhibition of CB α-galactosidase even though the concentration of charged tetrahydropyrimidine was 8 and 45 times the lowest K_i measured for **7**. Alternatively, the uncharged tetrahydropyrimidine concentration at pH 7.5 would change from 0.09 to 0.55 μM, substantially less than the lowest determined K_i value. The accurate measurement of the CB α-galactosidase K_i for **12** (pK_a = 6.8) at an assay pH of 7.5 was significantly lower than when the assay pH was 6.0. Control experiments that monitored the enzyme activity with the addition of the inhibition buffer excluded the possibility that the inhibition was due to the variable pH of the inhibitor buffer. It is suggested that the neutral six-membered cyclic guanidino-sugar is first bound to the enzyme active site, followed by protonation from one of the two catalytic carboxylic groups to form the tight complex shown in Figure 1.

In conclusion, this study demonstrates the use of a new class of glycosidase inhibitors in which the inhibition is pH-dependent. The pH dependence was found to be a function of the pK_a of the guanidino-sugar. The results presented are consistent with the conclusion that the neutral, tetrahydropyrimidine form is the most potent form of the guanidino-sugars. The *O*-linked guanidino-sugar provides a new direction to the synthesis of sequence-specific inhibitors. The *N*-substituted groups of the guanidino-sugar compounds can be considered to be nonreactive analogs of the leaving group of enzymatic hydrolysis of *p*-nitrophenyl glycosides. The inhibition of **7**, **10**, and **12** at a pH high enough to shift essentially all of the guanidino-sugar to the tetrahydropyrimidine form is equal even though the *exo*cyclic *N*-substituent changes. The absence of an *N*-substituent effect on the inhibition in this study and leaving group effects in previous studies[5c] supports the proposal that the transition state of the glycosidic cleavage reaction is late.[19] Although the inhibition potency described in this study is moderate (K_i values are in the micromolar range for the *O*-linked cyclic guanidino-sugar at neutral pH), the use of cyclic guanidino-sugars with different pK_a provides new insights into the mechanism of inhibition of glycosidases.

Experimental Section

General Procedures. Reactions were generally carried out under a positive pressure of dry nitrogen. Anhydrous solvents and reagents were distilled before use. Reagents were typically purchased from Aldrich, Fisher, or Sigma. Dichloromethane and triethylamine were distilled from calcium hydride. *N*,*N*-Dimethylformamide was purchased from Aldrich as a dry reagent. Infrared spectra were recorded in a 0.1 mm path length sodium chloride cell on a Perkin-Elmer 1420. UV spectra were monitored on a Cary 3E UV−vis spectrophotometer. Inhibition studies were carried out on coffee bean (CB) α-galactosidase, *A. niger* α-galactosidase, *A. niger* β-galactosidase, and *A. oryzae* β-galactosidase (Sigma). Liberation of *p*-nitrophenolate was monitored at 400 nm on a Beckman DU-6 spectrophotometer. The ^{1}H NMR

(19) (a) Bennet, A. J.; Sinnott, M. L. *J. Am. Chem. Soc.* **1986**, *108*, 7287−7294. (b) Rosenberg, S.; Kirsch, J. F. *Biochemistry* **1981**, *20*, 3196. (c) Graze, G.-A.; Kirby, A. J. *J. Chem. Soc., Perkin Trans. 1* **1978**, *2*, 354. (c) Kempton, J. B.; Withers, S. G. *Biochemistry* **1992**, *31*, 9961−9969.

spectra were recorded on a Bruker AM-300 or a Bruker AMX-500 in the indicated solvent at the indicated field. Silica gel 60 (230−240 mesh) from Mallinckrodt was used in flash chromatography.

Preparation of *threo*-3-Azido-4-(butyryloxy)-1,1-diethoxy-2-hydroxybutane and *threo*-3-Azido-1,1-diethoxy-2,4-dihydroxybutane (2). To a solution of (2*S*,3*R*)-4-(butyryloxy)-1,1-diethoxy-2,3-oxiranylbutane (**1**) (12.8 g, 52 mmol, 1 equiv) in ethanol (250 mL) and water (25 mL) were added sodium azide (16.9 g, 260 mmol, 5 equiv) and ammonium chloride (13.9 g, 260 mmol, 5 equiv). This mixture was refluxed overnight. Next, the reaction mixture was cooled with an ice bath prior to the addition of 300 mL of a mixture of dichloromethane and water (1:1). The aqueous layer was extracted with dichloromethane (150 mL × 3). The combined organic layers were washed with water, dried with magnesium sulfate, concentrated *in vacuo*, and purified further by flash silica gel chromatography (hexane:ethyl acetate = 10:1) to afford 9.92 g of an oily product (*threo*-3-azido-4-(butyryloxy)-1,1-diethoxy-2-hydroxybutane) (yield 66%). R_f = 0.25 (hexane:ethyl acetate = 2:1). IR (neat): 3479, 2975, 2518, 2103, 1738, 1455, 1347, 1272, 1062 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.56 (d, J = 6.8 Hz, 1H), 4.32−4.40 (m, 2H), 3.75−3.84 (m, 3H), 3.57−3.64 (m, 3H), 2.35 (t, J = 7.4 Hz, 2H), 1.63−1.74 (m, 2H), 1.26 (*t*, J = 7.0 Hz, 3H), 1.23 (t, J = 7.0 Hz, 3H), 0.97 (t, J = 7.4 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.1, 102.5, 71.1, 64.3, 63.8, 63.7, 60.0, 35.8, 18.2, 15.3, 15.1, 13.5. MS (FAB$^+$): *m/e* (relative intensity) 312 (M + Na$^+$, 100), 244 (15), 228 (12), 201 (15), 198 (26) HRMS (FAB$^+$) for $C_{12}H_{23}N_3O_5$ + Na$^+$: calcd 312.1535, found 312.1530.

Potassium carbonate (23.8 g, 172 mmol) was added to a solution of *threo*-3-azido-4-(butyrylcarbethoxy)-1,1-diethoxy-2-hydroxybutane) (4.96 g, 17.2 mmol) in 60 mL of methanol. The reaction mixture was stirred for 10 min at room temperature. Next, ethyl acetate (100 mL) and water (100 mL) were added. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (100 mL × 3). The combined organic layers were dried with magnesium sulfate and concentrated *in vacuo* to afford a pale yellow oily product (3.0 g, yield 80%). R_f = 0.40 (hexane:ethyl acetate = 1:1). IR (neat): 3418, 2977, 2890, 2103, 1059 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.71 (d, J = 7.5 Hz, 1H), 3.92−3.96 (m, 1H), 3.78−3.86 (m, 2H), 3.69−3.72 (m, 1H), 3.59−3.67 (m, 4H), 1.26 (t, J = 7.0 Hz, 3H), 1.25 (t, J = 7.0 Hz, 3H). MS (FAB$^+$): *m/e* (relative intensity) 242 (M + Na$^+$, 100), 216 (10), 194 (10). HRMS (FAB$^+$) for $C_8H_{17}N_3O_4$ + Na$^+$: calcd 242.1117, found 242.1110.

Preparation of *threo*-5-Azido-4-(diethoxymethyl)-2,2-dimethyl-1,3-dioxacyclohexane and *threo*-5-Amino-4-(diethoxymethyl)-2,2-dimethyl-1,3-dioxacyclohexane (3). Compound **2** (6.0 g, 27.4 mmol) was dissolved in a mixture of DMF (120 mL) and acetone (40 mL). 2,2-Dimethoxypropane (14.4 mL, 117 mmol) and PPTS (477 mg, 1.8 mmol) were added to the reaction mixture at room temperature. The reaction mixture was stirred at room temperature for 1 d. Another portion of 2,2-dimethoxypropane (14.4 mL, 117 mmol) was added to the reaction mixture and stirred for another day. A sodium bicarbonate solution (10 mL), which was diluted by the same volume of water from saturated sodium bicarbonate solution, was added to the reaction mixture. The solvent was removed on the rotary evaporator which was connected with a vacuum pump. The residue was diluted with sodium bicarbonate solution (20 mL) and extracted with ethyl acetate (100 mL × 3). The combined organic layers were washed with a brine solution, dried with magnesium sulfate, and concentrated *in vacuo*. Silica gel column chromatography (hexane:ethyl acetate = 10:1) provided a clean, oily product (6.88 g, yield 97%). R_f = 0.50 (hexane:ethyl acetate = 2:1). IR (neat): 2977, 2931, 2885, 2107, 1456, 1383, 1284, 1238, 1202, 1124, 1071 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.60 (d, J = 7.2 Hz, 1H), 4.18 and 4.08 (ABq, d, J_{AB} = 13.0 and J = 2.1 Hz, 2H), 3.99 (d, d, J = 7.2, 2.0 Hz, 1H), 3.84−3.55 (m, 4H), 3.10 (d, J = 2.0 Hz, 1H), 1.48 (s, 3H), 1.45 (s, 3H), 1.24 (t, J = 6.4 Hz, 3H), 1.22 (t, J = 7.1 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 101.2, 99.2, 72.1, 63.9, 63.7, 62.4, 53.8, 28.4, 18.2, 15.3, 15.0. MS (FAB$^+$): *m/e* (relative intensity) 392 (M + Cs$^+$, 50), 381 (8), 260 (M + H$^+$, 5), 244 (8), 214 (16). HRMS (FAB$^+$) for $C_{11}H_{21}N_3O_4$ + Cs$^+$: calcd 392.0586, found 392.0600.

threo-5-Azido-4-(diethoxymethyl)-2,2-dimethyl-1,3-dioxacyclohexane (3.3 g, 12.9 mmol, 1 equiv) was dissolved in 150 mL of ethanol. Palladium on carbon (10%, 1.4 g, 1.3 mmol, 0.1 equiv) was carefully added to the reaction flask. A balloon filled with hydrogen was used to replace the septum of the reaction flask. The reaction was stirred at room temperature and monitored by TLC every 30 min. After 3 h, the reaction was complete. The reaction mixture was filtered through a 3 cm pad of Celite, and the filtrate was concentrated *in vacuo*. The crude oily product was further purified by silica gel column chromatography (hexane:ethyl acetate:triethylamine = 5:10:1) to give 2.5 g of oily product (yield = 82%). R_f = 0.10 (hexane:ethyl acetate = 1:1). IR (neat): 2976, 2943, 2885, 1380, 1201, 1064, 849 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.57 (d, J = 7.1 Hz, 1H), 4.09 (d, d, J = 12.0, 2.0 Hz, 1H), 3.89 (d, d, J = 6.8, 1.8 Hz, 1H), 3.78 (q, d, J = 9.2, 7.1 Hz, 1H), 3.69 (d, d, J = 12.0, 1.8 Hz, 1H), 3.63 (m, 2H), 3.58 (q, d, J = 9.6, 7.0 Hz, 1H), 2.80 (d,d, J = 3.8, 2.0 Hz, 1H), 1.45 (s, 3H), 1.43 (s, 3H), 1.24 (t, J = 7.0 Hz, 3H), 1.23 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 101.2, 98.7, 67.1, 62.7, 61.4, 45.4, 29.2, 18.2, 15.0, 14.9. HRMS (FAB$^+$) for $C_{11}H_{23}NO_4$ + Cs$^+$: calcd 366.0681, found 366.0680.

Preparation of *threo*-4-(Diethoxymethyl)-2,2-dimethyl-5-isothiocyano-1,3-dioxacyclohexane (4). Compound **3** (450 mg, 1.93 mmol) was slowly added to a solution of thiocarbonyldiimidazole (350 mg, 1.93 mmol) in ethyl acetate (15 mL) at 0 °C and the mixture allowed to warm to room temperature for 1 h. The precipitate from the reaction mixture, which was the imidazole byproduct, was filtered out. The filtrate was concentrated and purified by silica gel column chromatography (hexane:ethyl acetate = 2:1) to afford a yellow oil product (462 mg, yield 87%). R_f = 0.65 (hexane:ethyl acetate = 2:1). IR (neat): 2977, 2931, 2884, 2052 (br), 1455, 1382, 1201, 1103, 1061 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.50 (d, J = 7.0 Hz, 1H), 4.05 and 3.96 (ABq, d, J_{AB} = 12.5 Hz, J = 2.0 Hz, 2H), 3.90 (d, d, J = 7.0, 1.9 Hz, 1H), 3.83 (d, d, J = 9.5, 7.0 Hz, 1H), 3.71−3.52 (m, 5H), 1.49 (s, 3H), 1.45 (s, 3H), 1.23 (t, J = 7.0 Hz, 3H), 1.22 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 137.6, 101.5, 99.4, 72.0, 64.1, 63.9, 63.2, 52.7, 29.0, 18.4, 15.5, 15.1. MS (FAB$^+$): *m/e* (relative intensity) 298 (M + Na$^+$, 14), 281 (28), 276 (M + H$^+$, 27), 267 (35), 260 (20), 236 (100), 230 (85), 207 (31). HRMS (FAB$^+$) for $C_{12}H_{21}NO_4S$ + H$^+$: calcd 276.1270, found 276.1258.

Preparation of *threo*-4-(Diethoxymethyl)-2,2-dimethyl-5-(aminomethanthioamido)-1,3-dioxacyclohexane and *threo*-4-Diethoxymethyl-2,2-dimethyl-5-[[*N*-(*tert*-butoxycarbonyl)amino]methanthioamido]-1,3-dioxacyclohexane (5). Into a 10 mL two-necked flask, equipped with a reflux condenser, was placed 131 μL (32 mg, 1.88 mmol of ammonia) of concentrated ammonium hydroxide solution. A solution of compound **4** (334 mg, 1.22 mmol) in 3 mL of ethyl acetate was added over a period of 1 h. After the addition was completed, the reaction mixture was heated to 90 °C for 20 min to remove excess ammonia. The reaction mixture was diluted with 30 mL of ethyl acetate and washed sequentially with 1 M sodium bicarbonate solution and a brine solution. The organic layer was dried with magnesium sulfate and concentrated *in vacuo* to afford an oily product (354 mg, yield quantitative). R_f = 0.30 (hexane:ethyl acetate = 1:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.71 (6.86) (d, J = 9.1 Hz, 1H), 4.61 (d, J = 8.8 Hz, 1H), 4.44 (4.35) (d, J = 7.0 Hz, 1H), 4.09 (m, 3H), 3.94−3.43 (m, 4H), 1.57 (1.47) (s, 3H), 1.50 (1.44) (s, 3H), 1.20 (1.28) (t, J = 7 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 183.1, 100.7 (100), 99.9 (99.5), 70.9 (72.3), 64.7, 64.3 (63.8), 60.1 (62.0), 49.5 (48.9), 29.1 (29.2), 18.3 (18.2), 15.3 (15.0). MS (FAB$^+$): *m/e* (relative intensity) 293 (M + H$^+$, 41), 247 (100), 231 (8). HRMS (FAB$^+$) for $C_{12}H_{24}N_2O_4S$+H$^+$: calcd 293.1535, found 293.1540.

threo-4-(Diethoxymethyl)-2,2-dimethyl-5-(aminomethanthioamido)-1,3-dioxacyclohexane (766 mg, 2.6 mmol), which was obtained from the above procedure, was dissolved in 26 mL of dichloromethane. Continuously, triethylamine (747 μL, 3.9 mmol) and 4-(dimethylamino)-pyridine (220 mg, 1.3 mmol) were added to the solution. The reaction mixture was cooled to 0 °C, and BOC anhydride (464 μL, 2.6 mmol), in 5 mL of dichloromethane, was added over a period of 30 min. The reaction mixture was warmed to room temperature and diluted with 30 mL of dichloromethane. The diluted reaction mixture was then washed in the following sequence: pH 4 phosphate buffer solution, water, 1 M sodium bicarbonate solution, and brine solution. The organic layer was dried with magnesium sulfate, and concentrated *in vacuo* to afford an oily product (733 mg, yield 72%). ^{1}H NMR (500

MHz, $CDCl_3$): δ 10.41 (d, J = 8 Hz, 1H), 8.04 (s, 1H), 4.61 (d, J = 8.7 Hz, 1H), 4.39 (d, J = 7.3 Hz, 1H), 4.03 (m, 3H), 3.77−3.46 (m, 4H), 1.49 (s, 15H), 1.22 (t, J = 7 Hz, 3H), 1.20 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.9, 151.2, 100.2, 99.4, 83.4, 70.9, 64.0, 63.6, 60.6, 49.9, 29.4, 27.8, 18.3, 15.3, 14.9. MS (FAB^+): *m/e* (relative intensity) 393 ($M + H^+$, 31), 347 (32), 291 (100), 249 (14), 232 (17). HRMS (FAB^+) for $C_{17}H_{32}N_2O_6S + H^+$: calcd 393.2059, found 393.2063.

Preparation of *threo*-4-(Diethoxymethyl)-2,2-dimethyl-5-[3′-benzyl-2′-(*tert*-butoxycarbonyl)guanidino]-1,3-dioxacyclohexane (6). Mercuric chloride (76 mg, 0.28 mmol, 1.1 equiv) was added in one portion to a solution of thiourea **5** (98 mg, 0.25 mmol, 1 equiv), triethylamine (174 μL, 1.25 mmol, 5 equiv), and benzylamine hydrochloride (61 μL, 0.56 mmol, 2.2 equiv) in 1.5 mL of DMF at 0 °C. The color of the reaction mixture changed to yellow, and the reaction was then warmed to room temperature. After 24 h, a combination of a brine solution and dichloromethane was added to the reaction. The aqueous layer was extracted with dichloromethane (15 mL × 3). The combined organic layers were washed with a 1 M sodium bicarbonate solution, filtered through Celite, dried with magnesium sulfate, and concentrated *in vacuo* to afford a pale yellow oil product (87 mg, yield 75%). The product was further purified by silica gel column chromatography (hexane:ethyl acetate = 2:1). R_f = 0.55 (hexane:ethyl acetate = 1:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38−7.28 (s, 5H), 5.13 (m, 1H), 4.36 (m, 2H), 4.29 (m, 1H), 3.90−3.79 (m, 3H), 3.70−3.56 (m, 2H), 3.50−3.33 (m, 2H), 3.30−3.25 (m,1H), 1.47 (s, 9H), 1.38 (s, 6H), 1.15 (t, J = 7.0 Hz, 6H). HRMS (FAB^+) for $C_{24}H_{39}N_3O_6 + H^+$: calcd 466.2919, found 466.2923.

Preparation of 2-(Benzylamino)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-1,3-pyrimidine (7a,) and 2,3-Dihydroxy-4-(3′-benzylguanidino)tetrahydrofuran (7b,). Trifluoroacetic acid (1 mL) and water (100 μL) were added to a solution of compound **6** (102 mg, 0.22 mmol) in dichloromethane (1 mL). The reaction mixture was stirred at room temperature for 2 d, and the solvent was removed *in vacuo*. The residue was dissolved in water (10 mL), and a pH meter probe was placed in the solution. Purified resin Dowex-1 (OH^-) was added slowly to the stirring solution until the solution reached pH 7.0. The solvent was evaporated to give water-soluble solid (52.5 mg, yield 95%). ^{1}H NMR (500 MHz, D_2O) [two isomers (tetrahydropyrimidine form **7b** and furanose form **7a**)]: δ 7.40−7.24 (m, 5H), 4.98 (s, 1H, **a**), 4.86 (s, 1H, **b**), 4.51 (s, 1H, **a**), 4.41 (s, 2H, **b**), 4.33 (s, 2H, **a**), 4.30 and 4.18 (ABq, J_{AB} = 9.0 Hz, 2H, **a**), 3.97 (s, 1H, **b**), 3.86 (s, 1H, **a**), 3.78 (m, 1H, **b**), 3.68 (m, 2H, **b**). ^{13}C NMR (125 MHz, D_2O) [two isomers (tetrahydropyrimidine form **7b** and furanose form **7a**)]: δ 153.3 (**a**), 152.3 (**b**), 136.1 (**b**), 135.8 (**a**), 129.2, 128.3, 127.2 (**b**), 127.1 (**a**), 83.0 (**a**), 77.4 (**a**), 74.4 (**b**), 70.2 (**a**), 63.0 (**b**), 59.9 (**b**), 53.9 (**a**), 50.4 (**b**), 44.8 (**a**), 44.4 (**b**). MS (FAB^+): *m/e* (relative intensity) 252 ($M + H^+$, 100), 234 (5). HRMS for $C_{12}H_{17}N_3O_3 + H^+$: calcd 252.1348, found 252.1340.

Preparation of *threo*-4-(Diethoxymethyl)-2,2-dimethyl-5-[3′-[4″-[[4‴-(methoxycarbonyl)butyryl]amino]benzyl]-2′-(*tert*-butoxycarbonyl)guanidino]-1,3-dioxacyclohexane (9). Mercuric chloride (76 mg, 0.28 mmol, 1.1 equiv) was added in one portion to a solution of thiourea **5** (98 mg, 0.25 mmol, 1 equiv), triethylamine (174 μL, 1.25 mmol, 5 equiv), and amine **8** (140 mg, 0.56 mmol, 2.2 equiv) in 1.5 mL of DMF at 0 °C. The reaction mixture changed to yellow in color, and warmed to room temperature. After 24 h, the combination of a brine solution and dichloromethane was added to the reaction. The aqueous layer was extracted with dichloromethane (15 mL × 3). The combined organic layers were washed with 1 M sodium bicarbonate solution, filtered through Celite, dried with magnesium sulfate, and concentrated *in vacuo* to afford a pale yellow oil product (99 mg, yield 65%). The product was further purified by silica gel column chromatography (hexane:ethyl acetate = 1:1). R_f = 0.25 (hexane:ethyl acetate = 1:4). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.65 (s, 1H), 7.62 (s, 1H), 7.54 (m, 2H), 7.30 (m, 2H), 5.22 (s, 1H), 4.35 (br s, 2H), 3.95 (br s, 1H), 3.86 (s, 1H), 3.70 (s, 3H), 3.65 (m, 2H), 3.47 (br s, 2H), 3.33 (br s, 1H), 2.44 (q, J = 7.0 Hz, 4H), 2.05 (m, 4H), 1.70 (br s, 2H), 1.48 (s, 9H), 1.40 (s, 6H), 1.27 (br s, 2H), 1.14 (t, J = 7.0 Hz, 6H). MS (FAB^+): *m/e* (relative intensity) 609 ($M + H^+$, 100), 535 (10), 509 (20), 463 (8), 417 (8), 234 (21). HRMS (FAB^+) for $C_{30}H_{48}N_4O_9 + H^+$: calcd 609.3500, found 609.3471.

Preparation of *threo*-4-(Diethoxymethyl)-2,2-dimethyl-5-[3′-(benzyloxy)-2′-(*tert*-butoxycarbonyl)guanidino]-1,3-dioxacyclohexane (11). Mercuric chloride (72 mg, 0.27 mmol, 1.1 equiv) was added in one portion to a solution of thiourea **5** (95 mg, 0.24 mmol, 1 equiv), triethylamine (167 μL, 1.20 mmol, 5 equiv), and *O*-benzylhydroxylamine hydrochloride (86 mg, 0.54 mmol, 2.2 equiv) in 1.5 mL of DMF at 0 °C. When the color of the reaction mixture changed to yellow, the reaction was allowed to warm to room temperature. After 24 h, the combination of a brine solution and dichloromethane was added to the reaction and the aqueous layer was extracted with dichloromethane (15 mL × 3). The combined organic layers were washed with 1 M sodium bicarbonate solution, filtered through Celite, dried with magnesium sulfate, and concentrated *in vacuo* to afford a pale yellow oil product (96 mg, yield 83%). The product was further purified by silica gel column chromatography (hexane:ethyl acetate = 5:1). R_f = 0.75 (hexane:ethyl acetate = 2:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.62 (s, 1H), 7.37−7.30 (m, 5H), 4.84 and 4.80 (ABq, J_{AB} = 11.7 Hz, 2H), 4.55 (d, J = 7.0 Hz, 1H), 3.95 (d, d, J = 7.2, 1.8 Hz, 1H), 3.93 and 3.88 (ABq, d, J_{AB} = 11.7 Hz, J = 2.0 Hz, 2H), 3.74−3.48 (m, 5H), 1.47 (s, 15H), 1.21 (t, J = 7.0 Hz, 3H), 1.16 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.8, 148.2, 128.4, 128.2, 127.7, 109.0, 100.7, 99.0, 81.9, 75.5, 71.3, 64.0, 63.3, 60.9, 44.4, 29.5, 28.0, 19.4, 18.3, 15.4, 15.0. MS (FAB^+): *m/e* (relative intensity) 614 ($M + Cs^+$, 100), 482 ($M + H^+$, 38), 452 (8), 436 (12), 426 (14), 410(8), 292 (7), 266 (16), 221 (14). HRMS (FAB^+) for $C_{24}H_{39}N_3O_7 + H^+$: calcd 482.2866, found 482.2883.

Preparation of 2-[*N*-(Benzyloxy)amino]-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-1,3-pyrimidine (12). A similar procedure which was described in the preparation of compound **7** was repeated. ^{1}H NMR (500 MHz, D_2O): δ 7.48 (br s, 2H), 7.42 (br s, 3H), 5.05 and 4.98 (ABq, J_{AB} = 11.0 Hz, 2H), 5.03 (m, 1H), 4.86−4.77 (m, 2H), 4.00 (s, 1H), 3.76−3.70 (m, 1H), 3.64 (m, 2H). ^{13}C NMR (125 MHz, D_2O): δ 154.6, 133.8, 130.4, 130.0, 129.2, 81.9, 78.5, 65.4, 59.7, 52.3, 50.7. MS (ESI^+) for $C_{12}H_{17}N_3O_4$: *m/e* (relative intensity) 268 ($M + H^+$, 48), 177 (80), 118 (9), 104 (100).

pK_a Determination. The UV−vis spectra of the guanidino-tetrose compounds were monitored on a Cary 3E UV−vis spectrophotometer in 1 mL quartz cuvettes. Guanidino-sugars were dissolved in 10 mL of distilled water (0.299 mM), and the pH was adjusted with 0.1 M hydrochloric acid or 0.1 M sodium hydroxide. Titrations were made from low to high and from high to low pH. The pK_a value determined was invariant on the initial pH. The absorbance at 231 nm was recorded as a function of pH. The titration curves were fitted to the equation for a monoprotic acid. A control cyclic guanidine compound unable to undergo ring opening was evaluated: 1,3,4,6,7,8-hexahydro-2*H*-pyrimido[1,2-*a*]pyrimidine (Aldrich). The pK_a of this compound was evaluated at 205 nm and found to be 11.6.

Time-Dependent Chromophore Shift. An experiment was developed as an extension of the spectrophotometric pK_a determination. The rate of the shift of the absorbance to shorter wavelength values was measured as a function of time. A stock **12** solution was prepared by dissolving **12** in filtered, distilled, deionized water to a final concentration of 3.0 mM, and the pH was adjusted to 11.0. The assay was conducted in a 100 mM phosphate buffer at pH 7.0 that did not absorb light at a wavelength of 231 nm. A typical assay is as follows: 0.9 mL of phosphate buffer and 0.1 mL of stock **12** solution were added to a quartz cuvette, and then the cuvette was vigorously shaken. The absorbance at 231 nm was monitored as a function of time. The initial time point is defined as the first data point collected after the addition of the guanidino-sugar.

K_i vs pH Inhibition Studies. Inhibition studies were carried out on coffee bean α-galactosidase (Sigma Chemical Co., lot no. 19C03851). Coffee bean α-galactosidase as derived from *C. canephora* is a mixture of two enzymes. The two enzymes, α-galactosidase I and II, have molecular masses of 28 000 and 36 500 Da, respectively.[2g] The preparation purchased from Sigma Chemical Co. was analyzed by SDS−PAGE and found to contain only α-galactosidase II with no detectable amount of α-galactosidase I. Enzymatic assays were based on *p*-nitrophenyl α-D-galactoside (*p*-NP-α-gal) hydrolysis. The molar extinction coefficient of 4-nitrophenylate at the wavelength 400 nm and pH 6.8 was reported to be 7280 M^{-1} cm^{-1}.[19c] The mode of galactosidase inhibition was evaluated by double reciprocal analysis

of data derived from monitoring the enzyme velocity as a function of *p*-nitrophenyl α-D-galactoside concentration at fixed **12** concentrations (inhibitor was initially pH 10.7). The assay buffer was 100 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES; pK_a = 7.5), 37 °C. **12** was prepared at a concentration of 2 mM in a 10 mM phosphate buffer (pH 10.7). This analysis was done at two assay pH values: 6.00 and 7.50. An aliquot of a 1 mM **12** solution (5−80 μM) was added to the assay buffer containing the α-galactosidase (0.05 unit/mL). The assay was initiated with the addition of an aliquot of a 75 mM *p*-NP-α-galactose solution (0.375−3.0 mM) to a final volume of 1 mL. Liberation of *p*-nitrophenolate anion over 2 min time periods was monitored at a wavelength of 400 nm on a Beckman DU-6 spectrophotometer. Inhibition constants were derived by non-linear regression analysis with the FORTRAN Compo program for competitive inhibition.[20] A 10 mM phosphate (pH 11) buffer blank of a volume (0.2 mL) exceeding the maximal volume of inhibitor used had no effect on the activity of the α-galactosidase.

The pH dependence of the guanidino-sugar compounds was determined with CB-α-galactosidase II (0.011 units/mL) in 100 mM HEPES buffer at pH 7.5 (25 °C) with 1.5 mM *p*-nitrophenyl α-D-galactoside as the substrate. The pH of the guanidino-sugar inhibitor solutions prior to their addition to the enzymatic assay solution was varied. A 10 mM phosphate buffer (pK_1 = 2.15, pK_2 = 7.2, pK_3 = 12.33) was used to maintain the pH of the guanidino-tetrose solutions. A typical assay protocol would have 5 μL of enzyme solution added to the assay buffer in a 1.5 mL cuvette. The inhibitor (5−100 μL, at pH 5−13) was added to the enzyme solution. The assay was initiated with the addition of an aliquot of a 75 mM substrate solution (in 100 mM HEPES, pH 7.5). The final volume of the assay mixture was 1 mL. Enzyme kinetic parameters were calculated by nonlinear regression analysis with the FORTRAN Hypero program.[20] Inhibition constants were derived by the method of Dixon.[21] The pH value of the assay system was unchanged upon the addition of the basic inhibitor solution. The pH value of a 1 mL, pH 7.49 buffer solution of 100 mM HEPES (pK_a = 7.5) was unchanged upon the addition of 0.4 mL of 10 mM phosphate buffer (pH 11). A 10 mM phosphate (pH 11) buffer blank of a volume (0.2 mL) exceeding the maximal volume of inhibitor used had no effect on the activity of the α-galactosidase. The same protocol was applied to *A. niger* α-galactosidase and *A. oryzae* β-galactosidase.

Time-Dependent Inhibition of CB-α-Galactosidase. Compound **7**, initially at a pH value of 10.5 (10 mM phosphate buffer), was preincubated in the glycosidase assay buffer (100 mM HEPES, pH 7.5) for fixed amounts of time. Coffee bean α-galactosidase was then added, and the inhibition was subsequently measured. A typical assay would contain 955 μL of 100 mM HEPES buffer (pH 7.5), 5 μL of CB-α-galactosidase (0.05 unit), 20 μL of *p*-nitrophenyl α-galactoside (1.5 mM), and 20 μL of compound **7** (0.2 mM). The hydrolysis of *p*-nitrophenyl α-galactoside was measured at a wavelength of 400 nm as a function time. The preincubation period of the high-pH guanidinotetrose in the low-pH assay solution was varied from 1 to 70 min. The enzyme concentration, substrate concentration, inhibitor concentration, and assay pH value were all held constant.

Supporting Information Available: NMR spectra of **1**−**12** (15 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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