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Configurationally selective transition state analogue inhibitors of glycosidases. A study with nojiritetrazoles, a new class of glycosidase inhibitors

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ABSTRACT

"Mannonojiritetrazole" (**7**), a novel mannosidase inhibitor, has been synthesized in six steps from 2,3,4,6-tetra-*O*-benzyl-D-mannose oxime. The structure of **7** has been established by X-ray analysis. The solid state conformation of **7** is $^{6}H_{7}$ ($=^{4}H_{3}$, numbering based on carbohydrate nomenclature), and the conformation in CD_3OD is close to S_7 (sofa; $= S_3$, numbering based upon carbohydrate nomenclature), while the conformation of the previously synthesized analogue with the *gluco* configuration (**6**) is $^{6}H_{7}$, both in the solid state and in solution in D_2O or CD_3OD. Both **6** and **7** have been tested as inhibitors of each of a series of five α- and β-glucosidases and -mannosidases as well as of a β-galactosidase, and inhibition constants have been determined. A good correlation ($\rho = 0.9$) was found between log K_i for each inhibitor–enzyme pair and log (V_m / K_m) for the corresponding substrate–enzyme pair, thereby providing the first such proof for any glycosidase inhibitor being a transition state analogue. This clearly demonstrates a case where true transition state analogue inhibitors of glycosidases are configurationally selective.

INTRODUCTION

Glycosidase inhibitors are tools for studying the mechanism of action of glycosidases and are of therapeutic and biotechnological relevance[1]. The known, tight-binding, competitive inhibitors are characterized by one or several of the following features: a positive charge, a trigonal anomeric center, a half-chair-like conformation, and the proper configuration, factors that have long been proposed as potentially important features of transition state analogue inhibitors, as reviewed recently[2]. However, since some inhibitors bind well to several different glycosidases[2–5], and since several glycosidases are inhibited by a range of configura-

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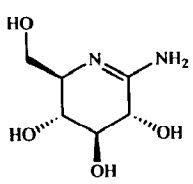
tional or constitutional isomers of a given type of inhibitor[6,7], there is some question about the factors which are primarily responsible for strong inhibition on the one hand, and for a lowered degree of inhibitor selectivity, on the other hand. It also raises the question of the extent to which glycosidase inhibitors, particularly the unselective ones, are transition state analogues. Wong et al.[8,9] concluded, from a correlation between calculated dissociation energy and the K_i values of a range of glucosidase inhibitors, irrespective of their transition state character, that charge and half-chair conformation both contribute to strong inhibition, and that the electrostatic interaction is the most important single factor. They also concluded[6,8,9], largely on the basis of inhibition studies with a series of pyrrolidine and piperidine inhibitors, that the relative configuration was important for basic inhibitors adopting a chair conformation, but of much less importance for those in a half chair conformation. Ganem et al.[3] reported that the amidine **1** inhibits sweet almond β-glucosidase, jack-bean α-mannosidase, and bovine liver β-galactosidase, each with a K_i value of $\sim 10^{-5}$ M. They termed the amidine a "broad spectrum inhibitor", and categorised it as a transition state analogue. The amidrazone **2** (ref 4) and the lactam oxime **3** (ref 4) were similarly categorised, implying that transition state analogues are, at least in some cases, nonselective inhibitors. However, if such inhibitors are indeed transition state analogues, their inhibitory efficiencies must parallel the corresponding substrate specificities[10]. Given the relatively high substrate specificities of many glycosidases, the broad spectrum inhibition proposed would seem unlikely for transition state analogues. Ganem et al.[4] also compared the inhibition of β-glucosidase by **1**, **2**, and **3**, and concluded that a flattened anomeric conformation is more important for transition state binding by the enzyme than achieving a full fledged charge of the cation. This conclusion is based to a large extent on the presumed half-chair conformation of the lactam oxime, as implied by the endocyclic double bond, the neutral character of the lactam oxime **3**, and a comparison of its inhibition with that of the lactone oxime **4** (ref 11), which is conformationally relatively labile[12]. All X-ray analyses, however, as available from the Cambridge Data File, show that lactam oximes possess the structure of a true hydroximolactam, such as **5**, and not of a hydroxylaminoimine, such as **3**. This has also been demonstrated for amide oximes in solution[13]. The pK_a value of the lactam oxime[4] (5.6) is in the same range as that of nojirimycin[14] (5.3) and that of pyridine[15] (5.1–5.4). We consider these aspects to be in keeping with the conclusion that the amidine **1**, amidrazone **2**, and lactam oxime **3** act as basic inhibitors, and that the stronger inhibition (as compared to the lactone oxime **4**) of sweet almond β-glucosidase by **3** is caused by its basic character. This again raises the question, not only of whether the lowered discrimination of these inhibitors is mostly due to their basic character, as the conformation of **3** cannot be unequivocally deduced from its constitution, but also whether the more or less strongly basic gluconolactam-derived inhibitors are transition state analogues. In other words, the character of the basic gluconolactam derivatives as transition state analogues cannot be considered as established.



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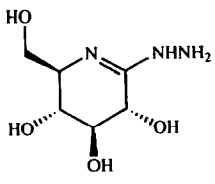

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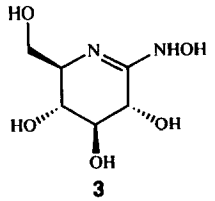

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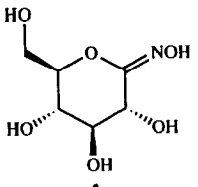

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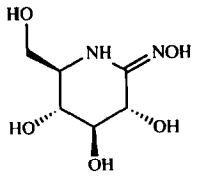

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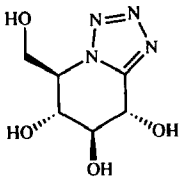

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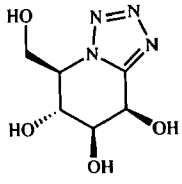

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It is important, therefore, to assess the inhibitory potential of neutral compounds which unambiguously possess a half-chair or closely related conformation, to determine the extent to which the inhibition depends upon their configuration, and to examine their character as transition state analogues. For this purpose, the fused D-*gluco* and D-*manno* tetrahydropyrido[1,2-*d*]tetrazoles **6** (ref 16) and **7** are promising candidates since several previous studies have indicated that interactions of the enzyme with the substrate at the 2-position are the most critical for selective transition state stabilisation[17–20]. A modification of such interactions should therefore provide the greatest kinetic and thermodynamic consequences. The former compound, termed "nojiritetrazole" has already been shown to possess a half-chair conformation and to inhibit sweet almond β-glucosidase to a moderate extent ($IC_{50} = 8 \cdot 10^{-5}$ M at pH 4.5 (ref 16); $K_i = 1.5 \cdot 10^{-4}$ M at pH 6.8; cf. experimental).

We now describe the synthesis of the analogous "mannonojiritetrazole" **7**, the determination of its structure, and the inhibition by **6** and **7** of the β-glucosidase from *Agrobacter*, a β-mannosidase (snail), an α-glucosidase (yeast), two α-mannosidases (jack bean, almonds), and a β-galactosidase (bovine liver). In addition, Michaelis–Menten parameters for the hydrolysis of *p*-nitrophenyl α- and β-D-glucopyranosides (PNPGlc) and *p*-nitrophenyl α- and β-D-mannopyranosides (PNP-

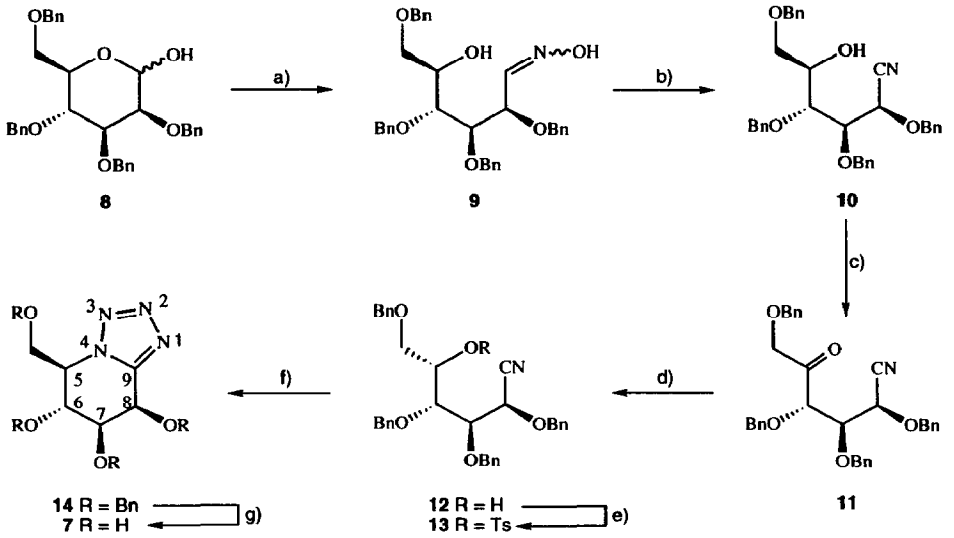
Man) by each enzyme are provided along with a correlation between these rates and the inhibition constants.

RESULTS AND DISCUSSION

"Mannonojiritetrazole" **7** was prepared in 6 steps (cf. Scheme 1) from the (*E*/*Z*)-oximes **9** (ref 21), obtained almost quantitatively from **8** (ref 22), following the route for the synthesis of "nojiritetrazole" **6** (ref 16). The oximes **9** were dehydrated with CBr_4–PPh_3 to yield 68% of the mannononitrile **10** (ref 23). Oxidation of **10** with pyridinium chlorochromate (PCC) gave ketone **11** (85%).

The structure of **10** was supported by the mass spectrum, showing $[M + NH_4]^+$ and $[M + H]^+$ at *m*/*z* 555 and 538. In the NMR spectra, the resonance of the CN group was observed at 117.75 ppm and the signal of a CD_3OD-exchangeable H at 2.43 ppm Table I indicates an OH group. The IR spectrum of **11** shows a new CO band at 1730 cm^{-1}, and ^{13}C NMR signals at 117.14 and 207.26 ppm indicating the presence of the CN and the CO group, respectively.

Reduction of **11** with $NaBH_4$–$CeCl_3 \cdot 6H_2O$ yielded mostly the L-gulononitrile **12** and some **10** (85%, 93 : 7). The L-gulononitrile **12** was tosylated to yield 87% of **13**. The signal of H-5 in the ^1H NMR spectrum of **13** Table I is now shifted downfield by 0.73 ppm. The ^{13}C NMR spectrum shows the presence of the CN group (s at 117.17 ppm). The *manno*-tetrazole **14** was obtained in 85% yield by



Scheme 1. (a) According to the procedure of Aebischer et al.[21], (b) CBr_4, PPh_3, MeCN, room temperature, 30 min, 68%; (c) 3A molecular sieves, PCC, CH_2Cl_2, room temperature, 2 h, 85%; (d) $CeCl_3 \cdot 6H_2O$, $NaBH_4$, MeOH, −60 to −40°C, 70 min, 79%; (e) TsCl, pyridine, 40–50°C, 22 h, 87%; (f) NaN_3, Me_2SO, 110–120°C, 4 h, 85%; (g) H_2, Pd–C, MeOH, AcOH, 98%.

TABLE 1

Selected ^{1}H NMR spectroscopic data of **10**, **12** and **13** (300 MHz, $CDCl_3$)

Compound	Chemical shift (ppm)							Coupling constants (Hz)						
	H-2	H-3	H-4	H-5	H-6	H'-6	OH	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6'}$	$J_{6,6'}$	$J_{5,OH}$
10	4.44	4.15	3.70	3.96	3.59	3.64	2.43 [a]	7.5	2.7	8.3	4.7	3.4	9.7	6.6
12	4.56	3.95	3.68	4.04	3.38	3.44	2.40 [a]	4.5	5.0	3.0	5.8	6.2	9.5	6.3
13 [b]	4.31	3.86	3.91	4.77	3.35	3.64		6.5	3.7	6.5	4.8	3.1	11.5	

[a] Exchanged after addition of CD_3OD. [b] Assignments were confirmed by homonuclear irradiation.

treating the L-*gulo* tosylate **13** with NaN_3 in Me_2SO at 110–125°C. This result compares favourably with the analogous reaction of the L-*ido* tosylate, which produced the *gluco*-tetrazole in only 70% yield, in addition to 10% of a 2,5-anhydro-D-gluclononitrile derivative[16]. The structure of **14** is confirmed by elemental analysis and the mass spectrum where $[M + H]^+$ is found at m/z 563. The resonance of the CN group of **13** is replaced by a singlet at 151.24 ppm.

Compounds **8–14** were obtained as oils, thus, the intermediates **9–13** were not purified when **14** was prepared on a larger scale, starting from 160 g of crude **8**. Except for the oxidation, which was carried out with Me_2SO–$(COCl)_2$ (instead of PCC), conditions similar to those described above were used; the quenching and workup of the mixtures were occasionally modified (cf. experimental). MPLC purification after 6 steps afforded **14** in an overall yield of 9%.

Hydrogenolytic debenzylation of **14** yielded 98% of the desired mannonojiritetrazole **7**. Its structure was established by X-ray analysis (cf. Fig. 1 *). The torsion angle C-5–N-1–C-1–C-2 is −1.4°, i.e., C-5, N-1, C-1, and C-2 are coplanar. The torsion angles N-1–C-1–C-2–C-3 and C-4–C-5–N-1–C-1 are 22.0 and 11.1°, re-

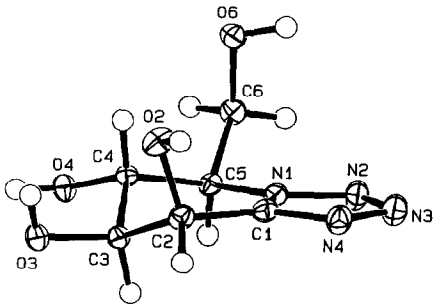


Fig. 1. ORTEP representation of compound **7**.

* The numbering of the atoms in Figs. 1 and 2 is different from the systematic numbering (cf. Scheme 1) used to discuss the NMR spectra. Atomic coordinates, bond lengths, and bond angles have been deposited with the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK.

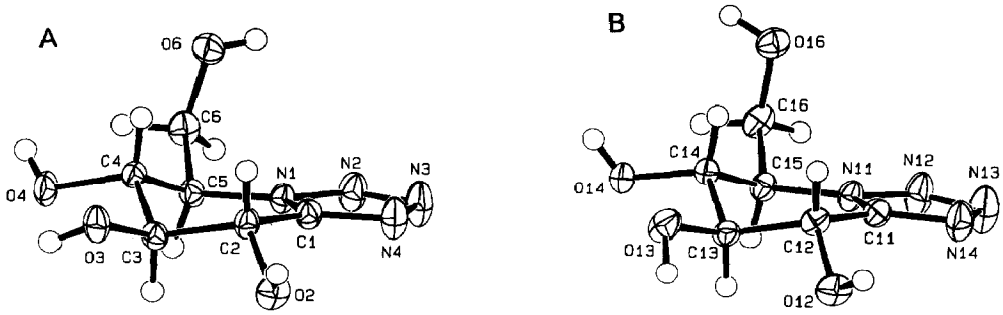
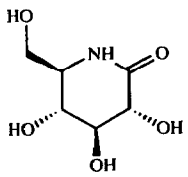


Fig. 2. ORTEP representation of the two crystallographically independent molecules **A** and **B** of compound **6**.

spectively, indicating a $^4H_3$ solid state conformation. The values of the coupling constants ($J_{7,8}$ 3.9, $J_{6,7}$ 7.4, and $J_{5,6}$ 4.8 Hz) for a soln of **7** in CD_3OD (strong overlap of the signals in D_2O) are consistent with a S_7 (sofa) conformation[24].



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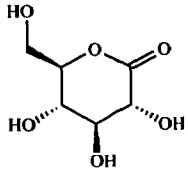

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In the solid state, nojiritetrazole **6** (ref 16) (cf. Fig. 2 *), as well as nojirilactam **15** (ref 25) adopt a half-chair conformation, while D-glucono-1,5-lactone **16** (ref 26) exists in a distorted half-chair conformation (cf. Table II). A coplanar arrangement of C-2, C-1, N or N-1, respectively, and C-5 is found for **15** and **6**; in **16**, C-5 is not

TABLE II

Selected structural data for compounds **6**, **7**, **15** and **16**

	6A	**6B**	**7**	**15**	**16** [a]
	Torsion angle (°)				
C-5–X [b]/C-1–C-2	−4.5°	−3.4°	−1.4°	−4.8°	−15.2°
C-1–C-2–C-3–C-4	−44.5	−47.6	−52.2	−50.0	−46.9
C-1–X–C-5–C-4	21.8	19.2	11.1	16.2	28.0
X–C-1–C-2–C-3	15.2	16.9	22.0	21.7	24.8
C-3–C-4–C-5–X	−49.6	−48.2	−42.2	−43.8	−51.5
	Bond length (Å)				
C-1–X	1.333(3)	1.335(3)	1.341(2)	1.326(8)	1.324
X–C-5	1.477(3)	1.475(3)	1.483(2)	1.463(8)	1.468
C-1–N-4	1.311(3)	1.314(3)	1.322(2)		
C-1–O-1				1.231(7)	1.208

[a] Torsion angles have been calculated from the coordinates of Hackert et al.[26]. [b] X corresponds to the hetero atom of the 6 membered ring, N or O respectively.

in the same plane as the oxycarbonyl group. In solution (^{1}H NMR) **15** (ref 25, $CDCl_3$!), **6** (ref 16, CD_3OD, D_2O, cf. experimental), and **16** (ref 11, D_2O) appear to adopt a similar conformation to that in the solid state.

The ("endocyclic") C-1–N-1 bond of the tetrazoles **6** and **7** (cf. Figs. 2 and 1, respectively) is of approximately the same length as the ("exocyclic") C-1–N-4 bond and significantly shorter than the N-1/C-5 bond, indicating that C-1–N-1 and C-1–N-4 possess about the same (aromatic) double bond character. In **15** and **16** the carbonyl CO bond is much shorter than the endocyclic CO or CN bond, respectively, as expected for these systems with a partially localized (exocyclic) double bond. The C-1–N-1 bond in **6** and the corresponding bonds in **15** and **16** possess about the same length.

Michaelis–Menten parameters were determined for each of the glucosidases and mannosidases with both PNPGlc and PNPMan of the correct anomeric configuration. In one case only (jack bean α-mannosidase with PNPGlc), the K_m value was too high for a reliable determination of the parameters, and in that case the V_m/K_m value was determined from the slope of a plot of the rate against substrate concentration. In addition to the above, the bovine liver β-galactosidase was studied with the two substrates PNPGal and PNPGlc and results for all these studies are presented in Table III.

Inhibition constants for the two tetrazole inhibitors **6** and **7** were measured with each of the enzymes studied, and these are also presented in Table III. In addition, a K_i value of 200 μM was determined for the inhibition of the Glu358Asp mutant of *Agrobacter* β-glucosidase by nojiritetrazole **6**. Full K_i determinations were carried out as described in the experimental section for each of the enzymes with the "correct" inhibitor, and competitive inhibition kinetics were observed in all cases but one, this being the inhibition of yeast α-glucosidase with nojiritetrazole **6**. In that case, the inhibition pattern observed was neither competitive nor noncompetitive. Full K_i determinations were not carried out for the "wrong" inhibitors of each enzyme since the limited quantities of material precluded such a study, given the high K_i values anticipated. Rather, an approximate K_i value was determined by measurement of rates at a single substrate concentration (around K_m) in the presence of different inhibitor concentrations, as described in the Experimental. Previous experience has shown that K_i values determined in this manner fall within 50% of values determined from full K_i measurements.

In order to ensure that the weaker substrate activity observed was indeed a true activity of the enzyme, and not due to a contaminant, the K_i value for each inhibitor–enzyme combination was, wherever possible, determined against each substrate using the Dixon plot method. In each case, the K_i values measured against both substrates were identical.

Each of the glucosidases and mannosidases investigated was found to be capable of hydrolysing both glucosides and mannosides, though there was clearly a much greater activity (as quantified through V_m/K_m values) with the "correct"

TABLE III

Comparison of glycosidase activities with regard to substrate vs inhibitor

Enzyme	Substrate–inhibitor	V_m [a] (μmol·min^{-1}·mg^{-1})	K_m [a] (mM)	V_m/K_m (L·min^{-1}·mg^{-1})	K_i [a] (mM)
α-Mannosidase (jack bean)	α-PNPMan	16.9 (0.3)	1.69 (0.07)	10	
	α-PNPGlc [b]	N/A	N/A	0.079	
	6 [c]				8.5
	7				0.18 (0.01)
α-Mannosidase (almonds)	α-PNPMan	23 (1)	3.69 (0.49)	6.2	
	α-PNPGlc	13(2)	184(34)	0.07	
	6 [c]				13.8
	7				0.70 (0.08)
β-Mannosidase (snail)	β-PNPMan	40.3 (0.4)	0.49 (0.04)	82	
	β-PNPGlc	0.11 (0.01)	1.2 (0.1)	0.09	
	6 [c]				47
	7				0.16 (0.01)
α-Glucosidase (yeast)	α-PNPMan	6.4 (0.3)·10^{-4}	0.08 (0.02)	0.008	
	α-PNPGlc	184 (2)	0.18 (0.01)	1020	
	6 [d]				1.3–5.6
	7 [c]				100
β-Glucosidase (*Agrobacter*)	β-PNPMan	0.158 (0.003)	0.028 (0.001)	5.6	
	β-PNPGlc	197 (2)	0.071 (0.002)	2770	
	6				0.0014 (0.0001)
	7 [c]				6
β-Galactosidase (bovine liver)	β-PNPGal	0.28 (0.01)	0.24 (0.02)	1.17	
	β-PNPGlc	0.15 (0.01)	0.063 (0.07)	2.38	
	6 [e]				0.0015
	7 [c]				14

[a] Value in parentheses is the standard error. [b] V_m/K_M Estimated from the slope of Lineweaver–Burk plot. [c] K_i Reported is approximate. [d] Inhibition behavior observed is neither strictly competitive nor noncompetitive. [e] K_i Reported is approximate and was measured in duplicate using first β-PNPGal as substrate and then β-PNPGlc as substrate.

substrate. In correspondence with this, all enzymes were found to be inhibited by both the nojiritetrazole **6** and the mannonojiritetrazole **7**. Again, the "correct" tetrazole inhibitor was found to be considerably more effective than the "incorrect" one. This correspondence is best represented in the form of a linear free energy relationship relating the activation free energy for turnover of each substrate (from V_m/K_m) with the free energy of binding of the inhibitor (from K_i) as shown in Fig. 3. A good correlation is observed, with a slope of 1.1 and a correlation coefficient of $\rho = 0.9$. Such plots have been used previously[27,28] to correlate the binding behaviour of modified transition state analogue inhibitors with the catalytic activity found for the correspondingly modified substrates. Very similar levels of correlation were found in those cases to that seen here, and this, coupled with the lack of any significant correlation of log V_m/K_m with substrate K_m value, has been used

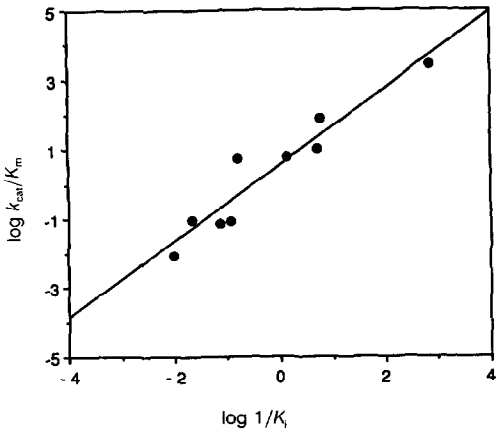

Fig. 3. Correlation of the activation free energy for turnover of each substrate (log V_m/K_m) with the free energy of binding of the tetrazole inhibitor (log $1/K_i$).

as proof of the transition state analogue nature of the inhibitor type. The basis for this conclusion, as described in detail elsewhere[28,29], is the fact that enzymes function by selectively binding to, and therefore stabilising the transition states for the reactions which they catalyse. The extent by which they stabilise the transition state over the ground state substrate structure is determined by the extent by which they catalyse the reaction faster than the nonenzymic process, according to the following relationship:

$$K_{TS} = (k_{noncat}/k_{cat}) \cdot K_D$$

where K_{TS} is the "dissociation constant" for the enzyme–substrate complex at the transition state, K_D is the substrate dissociation constant, and k_{noncat} and k_{cat} are the rate constants for the uncatalysed and the enzyme-catalysed reactions, respectively. This correlation of rate enhancement with transition state binding should therefore translate into a correlation with the enhanced binding of transition state analogue inhibitors, such that a perfect transition state analogue inhibitor should bind to the enzyme with a K_i value lower than the substrate or product K_D by a factor equal to the ratio of the catalysed and uncatalysed rates. Since it is clearly impossible to produce perfect mimics of the transition state, such enhancements are not found. However, very large binding enhancements are frequently observed. Indeed, in this work, nojiritetrazole **6** binds to *Agrobacterium* β-glucosidase some 4000 times more tightly than does the reaction product D-glucose[30]. An important corollary is that changes in the substrate V_m/K_m value occasioned by changes in the substrate structure should be reflected in equivalent changes in the binding of the correspondingly modified transition state analogue inhibitor. Thus, a plot of log V_m/K_m versus log $(1/K_i)$ should be a straight line plot with a good correlation

coefficient. However, there should be no equivalently strong correlation of inhibitor K_i value with substrate K_m value.

The plot in Fig. 3 therefore provides direct evidence that the tetrazoles can be truly classified as transition stage analogue inhibitors. Indeed, this is the first time that such a correlation has been provided for any glycosidase inhibitor. Furthermore, the plot of log K_m versus log K_i (not shown) was found to be a scatter plot with a correlation coefficient $\rho = 0.2$, thereby confirming the absence of a significant correlation with ground state structure. Further evidence that these are transition state analogous comes from the K_i value determined for a mutant of *Agrobacter* β-glucosidase in which a residue which is believed to play a major role in transition state binding (i.e., glutamic acid 358, the catalytic nucleophile), but a minimal role in ground state binding, has been mutated to an aspartic acid. Such a mutation results in a decrease in V_m/K_m of 10000-fold for PNPGlc, corresponding to a 5 kcal mol^{-1} change in activation free energy[31]. However, the binding of substrates and ground state analogue inhibitors is only weakened 5-fold[31] (1 kcal mol^{-1}) while the binding of nojiritetrazole **6** is weakened some 200-fold (3.2 kcal mol^{-1}), consistent with the notion that these tetrazoles are indeed transition state mimics, if yet imperfect. The fact that the full difference in binding free energy is not seen is quite consistent with the fact that this compound binds only 10^3–10^4-fold more tightly than D-glucose, rather than the approximately 10^8-fold expected for a perfect mimic. This approach of use of mutants with impaired transition state binding was recently also applied to a protease[32].

This finding therefore disproves the general validity of the notion forwarded recently[3,4,8] that the inhibitor configuration is unimportant for the binding of transition state analogue inhibitors to glycosidases. One of the major pieces of evidence on which this proposal of broad spectrum inhibition was based, was the observation that the D-*gluco*-amidine **1** inhibits the bovine liver β-galactosidase very effectively. However, as seen in Table III, this enzyme is in fact an even better β-glucosidase than a β-galactosidase, thus it is not surprising that the D-*gluco*-amidine **1** bound well. Indeed the nojiritetrazole **6** also bound extremely well to this enzyme, and mannonojiritetrazole **7** did not, as expected.

These results therefore suggest that transition state analogue inhibitors for glycosidases should possess a conformation close to that of a flattened chair with an sp^2 hybridised anomeric centre and the correct configuration at C-2, -3, -4, and -5. Formal charge on the molecule is clearly not important since most of the best inhibitors of this type are neutral compounds. However, a significant dipole at the anomeric centre resulting in partial charge development at C-1 and/or at O-5 (or its equivalent), may well be important, since resonance forms can be drawn for all these neutral inhibitors in keeping with a partial charge separation. This conclusion is also consistent with the fact that secondary deuterium kinetic isotope effect studies have shown that the transition states of glycosidases are generally quite pre-associative in nature and do not involve much charge development at the "anomeric" carbon[33,34].

EXPERIMENTAL

General methods.—Solvents were distilled before use. Normal workup implies distribution of the crude product between the indicated organic solvent and H_2O, drying of the organic layer ($MgSO_4$), filtration, and evaporation of the filtrate. TLC used Merck Silica Gel 60F-254 plates; detection by heating with 5% vanillin in concd H_2SO_4 or with mostain[35] [400 mL of 10% H_2SO_4 soln, 20 g of $(NH_4)_6Mo_7O_{24} \cdot 6H_2O$, 0.4 g of $Ce(SO_4)_2$]. Flash chromatography (FC) used Silica Gel (Merck 60, 0.04–0.063 mm). Filtration through silica gel implies use of a sinter funnel charged with Silica Gel (Merck 60, 0.04–0.063 mm) and the indicated solvent. Mp's are uncorrected. ^1H and ^{13}C NMR (spectra were recorded at 300 and 50 MHz, respectively.

(5R,6R,7S,8S)-5,6,7,8-Tetrahydro-5-(hydroxymethyl)pyrido[1,2-d]tetrazole-6,7,8-triol (**6**) *(ref 16).*—Additional data: ^1H NMR (D_2O): δ 3.87 (dd, 1 H, $J_{7,8}$ 8.9, $J_{6,7}$ 10.0 Hz, H-7), 4.05 (dd, 1 H, $J_{5,6}$ 9.3, $J_{6,7}$ 9.9 Hz, H-6), 4.13 (dd, 1 H, $J_{5,CH2\text{-}C5}$ 2.3, J_{gem} 12.8 Hz, CH_2-C5), 4.37 (dtd, 1 H, $J_{5,8}$ 1.0, $J_{5,CH2\text{-}C5} = J_{5,CH2\text{-}C5}$ 2.3, $J_{5,6}$ 9.2 Hz, H-5), 4.48 (dd, 1 H, $J_{5,CH2\text{-}C5}$ 2.5, J_{gem} 12.8 Hz, CH_2-C5), 4.86 (dd, 1 H, $J_{5,8}$ 1.0, $J_{7,8}$ 8.9 Hz, H-8).

(5R,6R,7S,8R)-5,6,7,8-Tetrahydro-5-(hydroxymethyl)pyrido[1,2-d]tetrazole-6,7,8-triol (**7**).—A solution of **14** (11.6 g, 20.6 mmol) in MeOH (400 mL) and AcOH (17 mL) was hydrogenated at 5 bar at room temperature in the presence of 10% Pd–C (2.0 g). After 21 h, more 10% Pd–C (2.0 g) was added. Since the reaction was not complete after 70 h, Pd–C was replaced by fresh catalyst (10% Pd/C, 6.5 g). Hydrogenation at 5 bar was continued for ca. 40 h (after 22 h 11.4 g of 10% Pd–C was added). The mixture was filtered through celite. The residue was washed with MeOH and the filtrate and washings were concentrated. Crystallisation of the residue (4.25 g) from MeOH afforded **7** (0.59 g, 14%). FC (17:3 EtOAc–MeOH) of the mother liquor yielded **7** (3.5 g, 84%) as a colorless powder; R_f 0.22 (17:3 EtOAc–MeOH); $[\alpha]_D^{25}$ −64.0° (*c* 0.995, MeOH); mp 159–161°C; ν_{max}: 3400, 3250 (OH), 3000, 2960 (CH), 1120, 1070 cm^{-1} (CO); ^1H NMR (CD_3OD): δ 3.95 (dd, 1 H, $J_{7,8}$ 3.9, $J_{6,7}$ 7.4 Hz, H-7), 4.02 (m, 1 H), 4.20–4.30 (m, 2 H), 4.37 (dd, 1 H, $J_{5,6}$ 4.8, $J_{6,7}$ 7.4 Hz, H-6), 5.03 (d, 1 H, $J_{7,8}$ 3.9 Hz, H-8); irrad. at 3.95: changed at 4.37 and at 5.03; ^{13}C NMR (D_2O): δ 59.29 (t), 61.33 (d), 64.11 (d), 65.72 (d), 70.83 (d), 154.51 (s); CIMS (NH_3): 220 (37, $[M + NH_4]^+$), 204 (7), 203 (100, $[M + H]^+$). Anal. Calcd for $C_6H_{10}N_4O_4$: C, 35.65; H, 4.99; N, 27.71. Found: C, 35.81, H, 4.86, N, 27.92.

2,3,4,6-Tetra-O-benzyl-D-mannononitrile (**10**).—Crude **9** (ref 21) (2.03 g, 3.65 mmol) was converted into **10** by the procedure used for the synthesis of 2,3,4,6-tetra-*O*-benzyl-D-gluconononitrile[16], with CBr_4 (2.96 g, 8.9 mmol), PPh_3 (2.34 g, 8.9 mmol), MeCN (54 mL), and MeOH (40 mL). FC (3:1 hexane –EtOAc) of the crude afforded **10** (1.33 g, 68%); R_f 0.15 (8:2 hexane–EtOAc); $[\alpha]_D^{25}$ −31.1° (*c* 0.954, $CHCl_3$); ν_{max}: 3490 (OH), 3060, 3030, 2910, 2870 (CH), 1095, 1070 cm^{-1} (CO); ^1H NMR data: see Table I; ^{13}C NMR ($CDCl_3$): δ 68.56 (d), 69.29 (d) 70.52

(t), 72.10 (t), 73.23 (t), 73.79 (t), 75.24 (t), 77.43 (d), 78.62 (d), 117.75 (s), 127.48-128.71 (d, Ar), 135.46 (s), 137.17 (s), 137.46 (s), 137.53 (s); CIMS (NH_3): m/z 556 (35), 555 (91, $[M + NH_4]^+$), 539 (36), 538 (100, $[M + H]^+$), 430 (47), 108 (22). Anal. Calcd for $C_{34}H_{35}NO_5$: C, 75.95; H, 6.56; N, 2.61. Found: C, 75.81; H, 6.37; N, 2.45.

*2,3,4,6-Tetra-O-benzyl-*D-lyxo-*5-hexulononitrile* (**11**).—3A Molecular sieves (Union carbide, powder, 995 mg) were dried under vacuum in the reaction vessel at 120-140°C for 18 h. At room temperature, PCC (1.19 g, 5.5 mmol), dry CH_2Cl_2 (15 mL), and a solution of **10** (985 mg, 1.83 mmol) in CH_2Cl_2 (25 mL) were added. Stirring was continued for 120 min. After addition of PCC (0.2 g, 0.92 mmol), the mixture was filtered through silica gel and the residue was thoroughly washed with EtOAc. The combined filtrate and washings were concentrated. FC (8:2 hexane–EtOAc) gave **11** (835 mg, 85%); R_f 0.19 (8:2 hexane–EtOAc); $[\alpha]_D^{25}$ −68.1° (*c* 0.878, $CHCl_3$); ν_{max}: 3120, 3090, 2920 (CH), 1730 (CO, ketone), 1095 cm^{-1} (CO); ^1H NMR ($CDCl_3$): δ 4.13 (d, 1 H, $J_{6,6'}$ 18.5 Hz, H-6), 4.20 (d, 1 H, $J_{3,4}$ 3.0 Hz H-4), 4.22 (d, 1 H, $J_{6,6'}$ 18.5 Hz, H'-6), 4.23 (dd, 1 H, $J_{3,4}$ 3.0, $J_{2,3}$ 8.0 Hz, H-3), 4.28 (d, 1 H, *J* 11.5 Hz, PhCH_2), 4.35 (d, 1 H, *J* 11.1 Hz, PhCH_2), 4.38 (d, 1 H, *J* 11.7 Hz, PhCH_2), 4.42 (d, 1 H, $J_{2,3}$ 7.9 Hz, H-2), 4.45 (d, 1 H, *J* 11.4 Hz, PhCH_2), 4.46 (d, 1 H, *J* 11.9 Hz, PhCH_2), 4.48 (d, 1 H, *J* 10.4 Hz, PhCH_2), 4.79 (d, 1 H, *J* 11.1 Hz, PhCH_2), 4.85 (d, 1 H, *J* 10.5 Hz, PhCH_2), 7.11–7.35 (m, 20 H, Ar H); ^{13}C NMR ($CDCl_3$): δ 67.47 (d), 72.33 (t), 73.15 (t), 74.16 (t), 74.36 (t), 75.45 (t), 79.16 (d), 82.14 (d), 117.14 (s), 127.75–128.85 (d, Ar), 135.10 (s), 136.17 (s), 136.26 (s) 136.89 (s), 207.26 (s); CIMS (NH_3): m/z 554 (28), 553 (100, $[M + NH_4]^+$), 536 (14, $[M + H]^+$). Anal. Calcd for $C_{34}H_{33}NO_5$: C, 76.22; H, 6.21; N, 2.61. Found: C, 76.33; H, 6.46; N, 2.71.

*2,3,4,6-Tetra-O-benzyl-*L-*gulononitrile* (**12**).—A solution of **11** (130 mg, 0.24 mmol) in MeOH (5 mL) was treated with $CeCl_3 \cdot 6H_2O$ (86 mg, 0.243 mmol) and $NaBH_4$ (28 mg, 0.74 mmol), as reported for the synthesis of 2,3,4,6-tetra-*O*-benzyl-L-idononitrile[16]; the reaction time was 70 min. FC (7:3 hexane–AcOEt) yielded **10** (8 mg, 6%) and **12** (103 mg, 79%); R_f 0.21 (7:3 hexane–EtOAc); $[\alpha]_D^{25}$ −38.5° (*c* 1.435, $CHCl_3$); ν_{max}: 3530 (OH), 3060, 3030, 3000, 2930, 2870 (CH), 1090 cm^{-1} (CO). ^1H NMR: see Table I; ^{13}C NMR ($CDCl_3$): δ 68.91 (d), 69.69 (d), 70.82 (t), 72.19 (t), 73.08 (t), 74.06 (t), 74.30 (t), 77.07 (d), 78.66 (d), 116.95 (s), 127.65–128.71 (d, Ar), 135.67 (s), 137.17 (s), 137.44 (s), 137.69 (s); CIMS (NH_3): m/z 556 (31), 555 (84, $[M + NH_4]^+$), 539 (37), 538 (100, $[M + H]^+$), 430 (36), 108 (28), 91 (22). Anal. Calcd for $C_{34}H_{35}NO_5$: C, 75.95; H, 6.56; N, 2.61. Found: C, 76.15; H, 6.64; N, 2.78.

*2,3,4,6-Tetra-O-benzyl-5-*O*-(*p*-tolylsulfonyl)-*L-*gulononitrile* (**13**).—A mixture of TsCl (330 mg, 1.73 mmol) and **12** (93 mg, 0.173 mmol) in pyridine (5 mL) was kept at room temperature for 22 h, as described for 2,3,4,6-tetra-*O*-benzyl-5-*O*-(*p*-tolylsulfonyl)-L-idononitrile[16]. FC (8:2 hexane–EtOAc) of the crude product afforded **13** (104 mg, 87%) and **12** (5 mg, ca. 5%, containing a trace of an unknown compound); R_f 0.26 (8:2 hexane–EtOAc); $[\alpha]_D^{25}$ −38.4° (*c* 0.839, $CHCl_3$); ν_{max}:

3060, 3020, 2925, 2870 (CH), 1365, 1175 (SO), 1095 (CO), 915 cm^{-1}; ^1H NMR: see Table I; ^{13}C NMR ($CDCl_3$): δ 21.51 (q), 67.99 (t), 68.81 (d), 72.18 (t), 73.03 (t), 74.47 (t), 74.54 (t), 76.36 (d), 77.62 (d), 80.55 (d), 117.17 (s), 127.65–129.53 (d, Ar), 133.43 (s), 135.45 (s), 136.87 (s), 137.27 (s), 137.30 (s), 144.66 (s); CIMS (NH_3): *m/z* 709 (4, $[M + NH_4]^+$), 448 (14), 447 (49), 281 (17), 280 (100), 108 (10). Anal. Calcd for $C_{41}H_{41}NO_7S$: C, 71.18; H, 5.97; N, 2.02; S, 4.63. Found: C, 71.13; H, 5.80; N, 1.99; S, 4.89.

(5R,6R,7S,8R)-6,7,8-Tris(benzyloxy)-5-[(benzyloxy)methyl]-5,6,7,8-tetrahydropyrido[1,2-d]tetrazole (**14**).—(*a*) A solution of **13** (98 mg, 0.142 mmol) and NaN_3 (125 mg, 1.92 mmol) in dry Me_2SO (1.5 mL) was stirred for 4 h at 110–120°C, diluted with H_2O, and worked up as usual (EtOAc, H_2O). FC (3:1 hexane–EtOAc) afforded **14** (68 mg, 85%); R_f 0.09 (8:2 hexane–EtOAc); $[\alpha]_D^{25}$ −57.0° (*c* 1.165, $CHCl_3$); ν_{max}: 3060, 3030, 3000, 2920, 2870 (CH), 1100 cm^{-1} (CO); ^1H NMR ($CDCl_3$): δ 3.93 (dd, 1 H, $J_{7,8}$ 3.5, $J_{6,7}$ 8.0 Hz, H-7), 3.98 (dd, 1 H, $J_{5,CH2\text{-}C5}$ 3.4, $J_{gem.}$ 10.0 Hz, CH_2-C5), 4.15 (dd, 1 H, $J_{5,CH2\text{-}C5}$ 5.5, J_{gem} 9.9 Hz, CH_2-C5), 4.41 (d, 1 H, *J* 11.9 Hz, PhCH_2), 4.46 (d, 1 H, *J* 12.2 Hz, PhCH_2), 4.50 (dt, 1 H, $J_{5,CH2\text{-}C5}$ 3.5, $J_{5,CH2\text{-}C5} = J_{5,6} = 5.6$ Hz, H-5), 4.57 (d, 1 H, *J* 12.0 Hz, PhCH_2), 4.58 (d, 1 H, *J* 11.3 Hz, PhCH_2), 4.64 (d, 1 H, *J* 11.7 Hz, PhCH_2), 4.64 (dd, 1 H, $J_{5,6}$ 5.5, $J_{6,7}$ 7.9 Hz, H-6), 4.74 (d, 1 H, *J* 12.1 Hz, PhCH_2), 4.81 (d, 1 H, *J* 11.3 Hz, PhCH_2), 4.98 (d, 1 H, $J_{7,8}$ 3.4 Hz, H-8), 4.99 (d, 1 H, *J* 12.1 Hz, PhCH_2), 7.17–7.48 (m, 20 H, Ar H); irrad. at 4.50: changed at 3.98, and at 4.15, and at 4.64.; irrad. at 3.93: changed at 4.64, and at 4.98; ^{13}C NMR ($CDCl_3$): δ 60.61 (d), 66.12 (d), 67.26 (t), 71.94 (t), 72.44 (d), 72.72 (t), 73.16 (t), 74.06 (t), 77.49 (d), 127.51–128.52 (d, Ar), 136.86 (s), 137.16 (s, double intensity), 137.28 (s), 151.24 (s); CIMS (NH_3): *m/z* 564 (38), 563 (100, $[M + H]^+$). Anal. Calcd for $C_{34}H_{34}N_4O_4$: C, 72.58; H, 6.09; N, 9.96. Found: C, 72.53, H, 6.05, N, 9.82.

(*b*) At 45°C, $NH_2OH \cdot HCl$ (104.8 g, 1.5 mol) was added to a stirred solution of Na (17.8 g, 0.77 mol) in aq 96% EtOH (3 L). Stirring was continued for 15 min, followed by the addition of a solution of crude **8** [160.4 g, content 82%, determined by FC (3:1 hexane–EtOAc) of 240 mg of crude **8** which gave 197 mg of pure **8**; 0.24 mol] in 96% EtOH (0.5 L). The mixture was stirred for 6 h at 60°C and filtered. The residue was washed with EtOAc, and the filtrate and washings were concentrated. Normal workup (EtOAc, H_2O) afforded crude **9** (161.8 g).

A solution of CBr_4 (236.6 g, 0.71 mol) in dry MeCN (0.4 L) was added dropwise at a rate such that the temperature did not exceed 50°C (over 30 min) to a solution of crude **9** (161.0 g) and PPh_3 (149.2 g, 0.57 mol) in MeCN (1.5 L). Stirring was continued for 90 min, then a solution of PPh_3 (37.4 g, 0.14 mol) in MeCN (0.5 L) and MeOH (1.0 L) were added. After 30 min, evaporation of the solution and filtration of the the residue (337 g) through silica gel (1:1 hexane–EtOAc) gave crude **10** (134.7 g).

A solution of dry Me_2SO (97.7 g, 1.25 mol) in dry CH_2Cl_2 (0.28 L) was added dropwise over 50 min to a cooled (−65°C) solution of oxalyl chloride (72.6 g, 0.57 mol) in CH_2Cl_2 (0.85 L). After 15 min, a solution of crude **10** (134.7 g) in CH_2Cl_2

(0.65 L) was added over 45 min at −65°C. The mixture was stirred for 10 min, allowed to warm to −40°C (over 20 min), and cooled to -65°C within 10 min. At this temperature, Et_3N (158.1 g, 1.56 mol) was added dropwise over 20 min. The mixture was stirred for 10 min at −65°C and warmed over 2 h to 25°C. Normal workup (CH_2Cl_2, H_2O, satd aq NaCl) and filtration of the resulting brown oil (189.6 g) through silica gel (1:1 hexane–EtOAc) gave crude **11** (103.8 g).

A solution of crude **11** (103.0 g) in MeOH (3.0 L) was treated with $CeCl_3 \cdot 6H_2O$ (68.4 g, 0.19 mol) and cooled to −65°C. $NaBH_4$ (22.0 g, 0.58 mol) was added in portions over 20 min, so that the temperature did not exceed −60°C. The mixture was maintained at −65 to −60°C for 105 min, allowed to warm to −40°C over 30 min, and treated with phosphate buffer (0.6 L; to a solution of 100 g of Na_2HPO_4 in 1 L of H_2O, was added H_3PO_4 until pH 6 was attained). The mixture was concentrated to ~1 L and worked up as usual (EtOAc, phosphate buffer, H_2O) to give crude **12** (99.1 g).

A solution of crude **12** (99.0 g) and TsCl (351.0 g, 1.8 mol) in dry pyridine (1.4 L) was stirred for 15 h at 50°C, and concentrated to a volume of ~0.4 L, which was cooled in an ice bath, treated with satd aq $NaHCO_3$ (2 L), and stirred over 30 min. Normal workup ($CHCl_3$, satd aq $NaHCO_3$, H_2O) afforded a residue which was co-evaporated with toluene. A solution of the residue in $CHCl_3$ was filtered through Celite, concentrated and filtered through silica gel (3:1 hexane–EtOAc) to give crude **13** (67.9 g).

A solution of crude **13** (67.0 g) and NaN_3 (63.0 g, 0.97 mol) in dry Me_2SO (0.94 L) was stirred for 3 h at 120°C, diluted with H_2O and worked up as usual (EtOAc, H_2O). The residue was filtered through silica gel (3:1 hexane–EtOAc) to give crude **14** (59.8 g) as a brown oil. MPLC (silica gel Merck 60, 0.015–0.04 mm, 2 kg; 3:1 hexane–EtOAc, flow 50 mL/min) afforded **14** (12.6 g, ca. 9% based on **8**).

Enzymology.—Agrobacter β-glucosidase was purified as described previously[34] and the Glu358Asp mutant was generated and purified according to Withers et al.[31]. All other enzymes were obtained from Sigma Chemical Company and used without further purification. Buffer chemicals and substrates were obtained from Sigma Chemical Company or BDH.

Enzyme assays at neutral pH were performed by continuous monitoring of the release of nitrophenolate anion by UV/Vis spectroscopy through measurements at 400 nm. Those at low pH were performed as stopped assays by incubation of the enzyme–substrate mixture (0.1 mL) for a 5 min period (less than 10% substrate depletion), then stopping the reaction by addition of 200 mM sodium borate buffer, pH 9.8 (0.8 mL) and measuring the absorbance at 400 nm. Buffer systems and reaction temperatures employed were as follows. *Agrobacter* β-glucosidase, 50 mM sodium phosphate, 0.1% BSA, pH 7.0, 37°C; yeast α-glucosidase (Type III), 50 mM sodium phosphate, pH 6.8, 37°C; jack bean α-mannosidase, 45 mM sodium citrate, 0.1 mM Zn^{2+}, pH 4.5, 25°C; almond α-mannosidase, 45 mM sodium citrate, pH 4.5, 25°C; snail β-mannosidase, 45 mM sodium citrate, pH 4.0, 25°C;

bovine liver β-galactosidase, 50 mM sodium phosphate, 0.1% BSA, 1 mM $MgCl_2$, pH 7.0, 37°C, emulsin β-glucosidase, 80 mM potassium phosphate, pH 6.8, 37°C.

Estimates of K_i values were obtained by measuring rates in a series of cells at a fixed substrate concentration (approximately equal to the K_m value) in the presence of a range of inhibitor concentrations (6–10 concentrations) which encompassed the K_i value ultimately determined. The observed rates were plotted in the form of a Dixon plot ($1/v_{obs}$ vs [I]) and the K_i value determined from the intercept of this line with the horizontal line drawn through $1/V_{max}$. Full K_i determinations were performed by measurement of rates at a series of substrate concentrations (typically 7 concentrations) which bracket the K_m value in the presence of a range of inhibitor concentrations (typically 5 concentrations) which bracket the K_i value ultimately determined. Data were analysed by nonlinear regression using the programme GraFit (Leatherbarrow, 1990).

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