Competitive inhibition of the inverting β -xylosidase of *Bacillus pumilus* 12 by monosaccharide derivatives of different structural and conformational types

A possible natural substrate

Philip J. MARSHALL and Michael L. SINNOTT

Department of Organic Chemistry, University of Bristol, Cantock's Close, Bristol BS8 1TS, U.K.

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1. The title enzyme is competitively inhibited by compounds, for example α -D-xylopyranosylpyridinium salts and 1,6-anhydro-D-glucopyranose, for which the normal 4C_1 conformation of the xylopyranose ring is precluded. 2. It is competitively inhibited by compounds, for example β -D-xylopyranosylpyridinium salts and 1,6-anhydro-L-idopyranose, for which the 1C_4 conformation is precluded, and which have no accessible conformations in common with the first set of inhibitors. 3. It is also competitively inhibited by α -L-arabinofuranosides. 4. Inhibition by 1,6-anhydro-glucopyranose, 1,6-anhydro-L-idopyranose and L-arabinono- γ -lactone is competitive with respect to each other. α -D-Xylopyranosyl fluoride is not a detectable substrate, by itself or in the presence of a representative of any of the three types of inhibitor. 5. On the basis of these and literature data, it is proposed that the natural substrate is a hemicellulose fragment containing the D-Xylp β (1 $\rightarrow$ 4)-[L-Araf α (1 $\rightarrow$ 3)]D-Xylp structure. Tentative inferences about the catalytic mechanism can also be drawn.

The production of an extracellular pentosanase by *Bacillus pumilus* 12 was discovered by Simpson (1954). An apparently intracellular β -xylosidase was later isolated by De Bruyne and co-workers (Kerstens-Hilderson *et al.*, 1969; Claeysens *et al.*, 1970) and subjected to intensive chemical investigations (Saman *et al.*, 1976; Kersters-Hilderson *et al.*, 1977; Claeysens & De Bruyne, 1978; Kersters-Hilderson *et al.*, 1980; Van Doorslaer *et al.*, 1980).

The enzyme is a dimer of 60 kDa subunits, although at high enzyme concentration there is some evidence for the presence of tetramers; there is one active site per subunit (Claeysens *et al.*, 1975). Titration with 5,5'-dithiobis-(2-nitrobenzoic acid) indicated that, of the six cysteine residues per monomer, two form a non-reducible intramolecular disulphide bond (Saman *et al.*, 1978).

The enzyme is of mechanistic interest since it gives α -D-xylopyranose as first product (Kerstens-Hilderson *et al.*, 1976), i.e. the anomeric configuration is inverted, whereas most glycosidases work with retention. Other mechanistically significant observations are of α -deuterium kinetic isotope effects on $V_{\max}$ of about 1.05 (Van

Doorslaer *et al.*, 1979) and of a modest increase in $V_{\max}$ for aryl β -D-xylopyranosides caused by electron-withdrawing groups in the aglycone (K_m showing no systematic variation) (Kerstens-Hilderson *et al.*, 1978).

The present work has its origins in our unexpected observation that α -D-xylopyranosylpyridinium salts, in which the pyranose ring is constrained to the normally disfavoured 1C_4 conformation by the reverse anomeric effect and the bulk of the pyridinium ring (Marshall, 1982), are nonetheless competitive inhibitors of the enzyme. More extended studies now reported here provide clues as to the natural substrate and catalytic mechanism of the enzyme.

Materials and methods

Ligands

α - and β -D-Xylopyranosylpyridinium bromides are described elsewhere (Marshall, 1982). α -D-Xylopyranosyl fluoride, m.p. 117–120°C, was made by the method of Micheel (1957), who gives m.p. 105°C (Found: C, 39.75%; H, 6.25%; $C_5H_9FO_4$ requires C, 39.45%; H, 5.95%; 1.0 equiv. of F^- was

liberated on hydrolysis). 1,6-Anhydro-D-glucopyranose, m.p. 180°C after phase-change 120°C [lit. (Nikitin *et al.*, 1968) m.p. 179°C], was made by the method of Montgomery *et al.* (1943). 1,6-Anhydro-D-galactopyranose (Case *et al.*, 1973), cyclopent-1-ene-3,5/4-triol and 1,2-anhydro-cyclopentane-1,2,3,5/4-pentol (Sinnott & Widdows, 1981) and 4-nitrophenyl and 4-phenylphenyl α -L-arabinofuranosides (Widdows, 1979) are described elsewhere. 1,6-Anhydro-L-idopyranose, m.p. 123.5–125.5°C [lit. (Sorkin & Reichstein, 1945) m.p. 126–127°C], was made by Zemplén deacetylation of the tri-*O*-acetyl derivative, a gift from Dr. N. Baggett, University of Birmingham, Birmingham, U.K. 1,6-Anhydro-D-allopyranose was a gift from Dr. L. C. N. Tucker, University of Dundee, Dundee, Scotland, U.K. 2-Nitrophenyl β -D-xylopyranoside, m.p. 160.5–163°C, was made by the method of Looftiens & De Bruyne (1964), who give m.p. 159–161°C.

Enzyme

Bacillus pumilus 12, a gift from Professor C. K. De Bruyne, Rijksuniversiteit te Gent, Gent, Belgium, was cultured as described by Kersters-Hilderson *et al.* (1969). β -Xylosidase was isolated by the modification of their procedure described by Marshall *et al.* (1981). Enzyme activity chromatographed on Sephadex G-150 as a single symmetrical peak in the presence of dithiothreitol, but chromatography in its absence, followed by subsequent dithiothreitol activation, gave several peaks. Densitometer traces of polyacrylamide-slab-gel electrophoretograms, made in the presence of sodium dodecyl sulphate, indicated that 70% of the Coomassie Blue-stainable material was accounted for by a single band of molecular mass approx. 60kDa. Enzyme was stored at 4°C as a slurry in aqueous (NH₄)₂SO₄, and was activated as required by dialysis (overnight at room temperature or at 4°C for several days) against 100 vol. of 10 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA and 1 mM-dithiothreitol. The specific activity attained with the oldest sample, activated after 4 years' storage, was 2 units/mg, assayed under conditions described by Claeysens *et al.* (1970), who give 6.2 units/mg as the specific activity of the freshly isolated enzyme. One unit of activity is defined as liberating 1 μ mol of *p*-nitrophenol/min from its β -D-xylopyranoside under the conditions described.

Kinetic measurements

All kinetic data pertain to 10 mM-sodium phosphate buffer, containing 1 mM-EDTA, final pH 7.2, at 25°C. Inhibition of the hydrolysis of 2-nitrophenyl β -D-xylopyranoside was measured by monitoring absorbance changes at 400 nm in a Unicam SP.1800 spectrophotometer, fitted with a thermo-

statically controlled cell-block, through which water, maintained at 25.0 $\pm$ 0.1°C by a Tecam TV-14 Tempunit, was circulated. Cells of 1 cm pathlength and 1 ml capacity were used, and reactions were initiated by the addition of activated enzyme solution (approx. 10 μ l) from a microlitre syringe.

Where possible, K_i measurements were estimated from 30 measurements of initial rate [six inhibitor concentrations (including zero) each at five substrate concentrations]. The initial-rate data were fitted iteratively to, first, the expression for mixed inhibition:

$$V = V_{\max} \cdot [S] / \{ [S] (1 + [I]/K_{i(u)}) + K_m (1 + [I]/K_{i(c)}) \}$$

and then, if $K_{i(u)} \gg K_{i(c)}$, to the expression for pure competitive inhibition:

$$v = V_{\max} \cdot [S] / \{ [S] + K_m (1 + [I]/K_i) \}$$

by a program for a Hewlett–Packard 9821A calculator written by Dr. P. J. England, Department of Biochemistry, University of Bristol, Bristol, U.K. K_i values calculated in this way are reported with standard errors. However, with some difficultly-won ligands this extensive treatment was not possible, and K_i values had to be estimated from two Lineweaver–Burk plots, with and without a fixed concentration ($\approx K_i$) of inhibitor.

Initial rates of hydrolysis of 2-nitrophenyl β -D-xylopyranoside in the presence of α -D-xylopyranosyl-3-carboxaminopyridinium bromide were estimated by constructing a perpendicular to the beginning of the chart-recorder trace by using a mirror.

1,6-Anhydro-D-glucopyranose was tested as a substrate polarimetrically with a Perkin–Elmer 241 MC spectropolarimeter at the Na D line. α -D-Xylopyranosyl fluoride was tested as a substrate in a jacketted vessel thermostatically controlled by a Tecam Tempunit, by monitoring F[−] electrochemically, with a Philips fluoride electrode, a double-junction calomel reference electrode (KNO₃ in the outer bridge) and a Philips PW9414 pH/ion-meter. β -D-Xylopyranosyl-3-carboxamidopyridinium bromide was tested as a substrate in a Unicam SP.8-200 u.v.–visible kinetic system, with repeat scanning.

Results and discussion

Table 1 gives K_i values for all the inhibitors tested. At first sight the structural types are so diverse that a trivial cause, such as the ability to bind pyridinium salts or anything with three hydroxy groups, must be suspected. However, neither *N*-methylpyridinium iodide ($K_i > 80$ mM) nor glycerol ($K_i > 100$ mM) is a detectable inhibitor; neither is xylitol (Kersters-Hilderson *et al.*, 1969). Inhibition must therefore be due to a specific property of the various glycones (or

Table 1. *Competitive inhibition of β -xylosidase of Bacillus pumilus at 25°C and pH 7.2*
For details see the text.

	K_i (mM)
¹ C ₄ -type inhibitors	
α -D-Xylopyranosyl-3-bromopyridinium bromide	1.38 ± 0.08
α -D-Xylopyranosyl-4-bromoisoquinolinium bromide	0.24 ± 0.01
α -D-Xylopyranosyl-3-carboxamidopyridinium bromide	$0.92 \pm 0.04^*$
	$2.73 \pm 0.25^\dagger$
α -D-Xylopyranosylpyridinium bromide	6.16 ± 0.25
α -D-Xylopyranosylisoquinolinium bromide	2.66 ± 0.14
α -D-Xylopyranosyl-4-methylpyridinium bromide	8.20 ± 0.38
1,6-Anhydro-D-glucopyranose	0.551 ± 0.023
1,6-Anhydro-D-allopyranose	2.9
1,6-Anhydro-D-galactopyranose	>80
1,2-Anhydrocyclopentane-1,2,3,5/4-pentol	1.80 ± 0.22
⁴ C ₁ -type inhibitors	
β -D-Xylopyranosyl-4-bromoisoquinolinium bromide	0.36
β -D-Xylopyranosylpyridinium bromide	6.76 ± 0.71
β -D-Xylopyranosylisoquinolinium bromide	1.23 ± 0.08
1,6-Anhydro-L-idopyranose	15
α -D-Xylopyranosyl fluoride	$14^\ddagger$
L-Araf-type inhibitors	
4-Phenylphenyl α -L-arabinofuranoside	0.029 ± 0.002
4-Nitrophenyl α -L-arabinofuranoside	0.19
L-Arabinono- γ -lactone	0.63 ± 0.02
	$9.1 \pm 1.4^\S$
Cyclopent-1-ene-3,5/4-triol	4.7
Substrate	
2-Nitrophenyl β -D-xylopyranoside	0.80 (K_m value)
* Calculated on steady-state rates [K_i (slow)].	
† Calculated on initial rates [K_i (fast)].	
‡ Weak uncompetitive component of the inhibition ($K_{i(u)} \approx 10 K_{i(c)}$) detected.	
§ Uncompetitive inhibition constant.	

glycone-like structures). These fall into three general classes, whose conformational characteristics are illustrated in Fig. 1.

¹C₄-type inhibitors

A representative set of Lineweaver–Burk plots for an α -D-xylopyranosylpyridinium salt is illustrated in Fig. 2. In all the cases tested the inhibition was strictly competitive, although the inhibition by α -D-xylopyranosyl-3-carboxamidopyridinium ion exhibited hysteresis. When enzyme was added to a mixture of inhibitor and substrate, a slow deceleration to a steady-state rate was observed; when enzyme, preincubated with inhibitor (10 mM) for 20 min, was diluted into substrate, an acceleration to a steady-state rate was observed. Phenomenologically, the behaviour is exactly the same as that described by Legler *et al.* (1979) for the interaction of β -glucosidase of *Aspergillus wentii* with D-glucal, the approach to the steady states being in both cases exponential. The behaviour can be described by the kinetic scheme:

k_{off} is given by the rate constant for approach to the steady state when enzyme, preincubated with inhibitor, is diluted into substrate ($7.9 \times 10^{-3} \text{ s}^{-1}$). The K_i value calculated from steady-state rates [K_i (slow)] is given by

$$K_i(\text{slow}) = K_i(\text{fast}) \cdot \left(1 + \frac{k_{\text{on}}}{k_{\text{off}}}\right)$$

By using K_i (fast) estimated from initial rates, k_{on} is estimated at $1.5 \times 10^{-2} \text{ s}^{-1}$. [The rate constant for approach to the steady state in the presence of both substrate and inhibitor depends in a complex way on the concentrations of both species (Legler *et al.*, 1979), and the precision of the data did not warrant detailed analysis of this dependence.]

No other xylopyranosyl salt or neutral inhibitor exhibited this behaviour, which in all probability arises from the slow reversible nucleophilic addition of some group on the enzyme to the pyridinium ring. Such nucleophilic additions are particularly facile when the ring is substituted with conjugatively electron-withdrawing groups such as $-\text{CONH}_2$.

The K_i values for the four α -D-xylopyranosylpyridinium salts derived from monocyclic pyridines

¹C₄-type inhibitors

α -D-Xylopyranosylpyridinium ion in the preferred ¹C₄ conformation. A range of the pseudo-rotational itinerary of the twist-boat conformation [approximately ^{2,5}B through ¹S₃, the conformation adopted by the α -D-glucopyranosylpyridinium ions (Marshall, 1982), to B_{2,3}] is also available.

1,6-Anhydro-D-aldohexopyranoses (top 1,6-anhydro-D-glucopyranose, with numbering system of the ring) are rigidly confined to the ¹C₄ and B_{3,0} conformations, as is 1,2-anhydrocyclopentane-1,2,3,5/4-pentol, regarded as a pyranose derivative.

⁴C₁-type inhibitors

β -D-Xylopyranosylpyridinium ion. A portion of the pseudo-rotational itinerary of the twist-boat form (approximately ^{2,5}B through ³S to B_{2,3}) is also accessible if hydroxy groups are placed pseudo-axial.

1,6-Anhydro-L-idopyranose, confined to the ⁴C₁ and ^{3,0}B conformations.

Fig. 2. Inhibition by α -D-xylopyranosyl-4-methylpyridinium bromide of the β -xylosidase-catalysed hydrolysis of 2-nitrophenyl β -D-xylopyranoside

The concentrations of α -D-xylopyranosyl-4-methylpyridinium bromide used were: ●, 25 mM; ▲, 10 mM; ■, 5 mM; ○, 2.5 mM; △, 1.0 mM; □, 0 mM. Reaction mixtures were incubated at pH 7.2 at 25°C as indicated in the text.

increase regularly with the pK_a of the pyridine, with a β value of 0.23 ($r=0.98$) [we take K_i (fast), governing the first, and putatively non-covalent, complex, in the case of the 3-carboxamidopyridinium salt]. This betokens some electrostatic or charge-transfer interaction between the pyridinium ring and the protein.

L-Araf-type inhibitors

Fig. 1. Structures of inhibitors
Conformational nomenclature: *Pure Appl. Chem.* **53**, 1902–1905 (1981).

4C_1 -type inhibitors

The 4C_1 conformation is the preferred conformation of β -D-xylopyranosides because the three hydroxy groups are equatorial. In the case of β -D-xylopyranosylpyridinium salts this preference is reinforced by the bulk of the pyridinium ring and the reverse anomeric effect, and in the case of α -D-xylopyranosyl fluoride by the anomeric effect of fluorine, which is powerful enough to make tri-*O*-acetyl- β -D-xylopyranosyl fluoride adopt the 1C_4 conformation with all substituents axial (Paulsen *et al.*, 1979). 1,6-Anhydro-L-idopyranose cannot adopt conformations common to 1,6-anhydro-D-glucopyranose without the rupture of covalent bonds. The above inhibitors are nonetheless all bound to the enzyme.

Binding subsites for 4C_1 -type inhibitors and for 1C_4 -type inhibitors, and catalysis

Xylobiose [4-*O*-(β -D-xylopyranosyl)-D-xylose] is about as good a substrate as is 2-nitrophenyl β -D-xylopyranoside (Kerstens-Hilderson *et al.*, 1977), yet the enzyme does not hydrolyse alkyl xylosides, despite the rough similarity of leaving-group pK_a (Kerstens-Hilderson *et al.*, 1980). Clearly some specific interaction with the reducing saccharide residue is involved. [Extensive studies of the binding of alkyl xylosides led to the conclusion that there was a specific aglycone-binding site (Van Doorslaer *et al.*, 1980).] That this specific interaction is expressed largely at the transition state for glycoside cleavage is shown by the only modest difference between K_i for xylose (26 mM; Kerstens-Hilderson *et al.*, 1969) and K_m for xylobiose (4.6 mM; Kerstens-Hilderson *et al.*, 1977) (cf. Jencks, 1975). The hypothesis that these specific interactions are with the reducing xylopyranose residue of xylobiose bound in the normally disfavoured 1C_4 conformation rationalizes the following facts.

(i) 1,6-Anhydro-D-glucopyranose, rigidly precluded from adopting the 4C_1 conformation, is not a substrate, despite being a homoeomorph of a β -xylopyranoside and possessing a formal fissile bond between C-1 and O-6.

(ii) β -D-Xylopyranosyl-3-carboxamidopyridinium ion, anchored in the 4C_1 conformation, is a substrate, if a very poor one. Solutions of it at <1 mM in neat activated enzyme (approx. 1 mg/ml) are approx. 30% hydrolysed overnight, but stable in buffer.

(iii) Whereas epimerization at C-3 of 1,6-anhydro-D-glucopyranose (to give 1,6-anhydro-D-allopyranose) has only a modest effect on inhibitory power (Table 1), epimerization at C-4 (to give 1,6-anhydro-D-galactopyranose) completely destroys it. On our hypothesis epimerization at C-4 is likely to introduce steric clashes with catalytic groups, which epimerization at C-3 is not.

(iv) 1,2-Anhydrocyclopentane-1,2,3,5/4-pentol, a 1C_4 -type inhibitor, shows no sign whatever of inactivating the enzyme, despite similar structures with six-membered carbocyclic rings, when bound in the active sites of glycosidases, alkylating catalytic groups (Legler, 1973; Braun *et al.*, 1977; Legler & Herrchen, 1981).

(v) D-Ribopyranosides are particularly powerful competitive inhibitors (K_i for 4-methylumbelliferyl β -D-ribopyranoside is 0.6 μ M) (Claeysens & De Bruyne, 1978), but they are not hydrolysed. This enhanced binding is not caused by a specific interaction in the 1C_4 subsite, since a corresponding structural change applied to 1,6-anhydro-D-glucopyranose (to give 1,6-anhydro-D-allopyranose) results in a slight decrease in binding (Table 1). Neither is it due to a lower free-energy difference between the 1C_4 conformation and the preferred 4C_1 conformation, arising from the axial OH at C-3 in this conformation, since D-lyxose, with an axial OH at C-2, is not especially tightly bound (Kerstens-Hilderson *et al.*, 1969).

Inverting glycosidases, insofar as their catalytic mechanism has been addressed, are considered to work by a one-step nucleophilic displacement by a water molecule, the departure of the aglycone OR being assisted by an acidic group AH, and the water being made more nucleophilic by partial deprotonation by a base B (Thoma & Koshland, 1960; Thoma, 1968). The nucleophilic water molecule displacing the aglycone of a β -D-xylopyranoside in the 4C_1 conformation is in approximately the same position as the axial OH of a β -ribopyranoside in the 4C_1 conformation (Fig. 3). The similarity is increased if the chair is distorted towards co-planarity of C-2, C-1, O-5 and C-5, as is required for efficient conjugation of oxygen lone pairs of electrons with the reaction centre. This distortion would also decrease steric hindrance to S_N2 displacement of an equatorial leaving group, which is virtually prohibitive in perfect chair conformations (Fry *et al.*, 1970).

β -D-Ribopyranosides are thus probably transition-state analogues (Wolfenden, 1972).

L-Ara-f-type inhibitors

The binding of two xylose units with the pyranose ring containing the reaction centre in the 4C_1 conformation, and the leaving group in the 1C_4 conformation, has no chemical rationale if the function of the enzyme is merely to hydrolyse xylose oligomers. It is therefore useful to enquire further as to the probable biological function of this enzyme.

It is intracellular: this is confirmed by its functioning only in the thiol reduced form. A long-chain xylose polymer is therefore unlikely to be the natural substrate, since these are largely of plant origin and exogenous macromolecules are unlikely

Fig. 3. Possible reason for the tight binding of D-ribofuranosides

Illustrating the similar positions of a 3-OH of a ribopyranoside in the 4C_1 conformation and the attacking water molecule in the putative enzymic transition state for β -xyloside hydrolysis.

to be taken up into the bacterial cell. Circumstances must arise in which the enzyme is important to primary metabolism, since it can be produced in fairly large quantities (Kerstens-Hilderson *et al.*, 1969).

Bacillus pumilus is a soil bacterium (Buchanan *et al.*, 1966; Goodfellow *et al.*, 1968). The major xylose derivatives in soil are xylans from decaying plant material (see, e.g., Cheshire, 1979). Simple xylans [linear $\beta(1\rightarrow4)$ -linked polymers] are rare, glycosylation at O-3 being common (Whistler & Richards, 1970; Wilkie, 1979). These hemicellulose xylans are degraded to small fragments by extracellular xylanases of soil fungi (Skujins, 1975). A common residue at O-3 of the $\beta(1\rightarrow4)$ -linked xylan backbone is the α -L-arabinofuranose residue (Whistler & Richards, 1970), although diglycosylation at O-2 and O-3 is also observed (Wilkie, 1979). The breakdown of hemicelluloses by fungal xylanases to arabinofuranosylated xylose oligomers has been observed many times under controlled conditions (Bishop, 1956; Aspinall *et al.*, 1960; Goldschmid & Perlin, 1963; Takenishi & Tsujisaka, 1973; Frederick *et al.*, 1981); particularly relevant is the identification of xylotriose and xylotetraose α -L-arabinofuranosylated on position 3 of the second xylose residue from the non-reducing end (Shambe, 1983). This train of reasoning leads to the speculative proposal that α -L-arabinofuranosylated xylose oligomers are the natural substrates of the enzyme. Substantiation of the proposal comes from the observation that the α -L-arabinofuranosides are good competitive inhibitors of the enzyme, but are not hydrolysed (Table 1). [Inhibition by L-arabin-

Table 2. Hydrolysis of 0.80 mM-2-nitrophenyl β -D-xylopyranoside by *Bacillus pumilus* β -xylosidase inhibited by mixtures of two inhibitors

For details see the text.

1,6-Anhydro-L-iodopyranose (77.0 mM)	1,6-Anhydro-D-glucopyranose (2.76 mM)	L-Arabinono- γ -lactone (2.89 mM)	Relative rate
+	—	—	1.24
—	+	—	0.98
—	—	+	0.90
+	+	—	0.64
+	—	+	0.60
—	+	+	0.54

ono- γ -lactone is not strictly competitive, there being apparently a small uncompetitive component (Table 1). However, in the light of the lability of aldono-lactones, and the possible presence of quantities of the δ -lactone, it is most unlikely that this has mechanistic significance.]

The di-(or possibly tri-)glycosylation pattern of a xylopyranose ring of an arabinoxylan fragment is related to the binding of 1C_4 xylopyranose rings by the enzyme in a non-catalytic way. Examination of space-filling CPK molecular models reveals that there is severe steric crowding in 4-O-(β -D-xylopyranosyl)-3-O-(α -L-arabinofuranosyl)-D-xylopyranose when the diglycosylated xylopyranose ring is in the 4C_1 conformation, but that this strain is relieved when this ring adopts the 1C_4 conformation. There is an experimental precedent for glycosylation (on O-2) altering the preferred conformation of a pentopyranose ring from 4C_1 to 1C_4 (Ishii *et al.*, 1981). Finally, even in β -D-xylopyranose itself, the free-energy difference between 4C_1 and 1C_4 conformations is only 10.0 kJ/mol (2.4 kcal/mol) [as estimated from Angyal's (1969) instability factors].

The hypothesis that the natural substrate of the enzyme is a xylan fragment (or fragments) containing α -L-arabinofuranosyl branches thus provides a rational explanation for what the enzyme is doing in the bacterium in the first place, and why it binds 1C_4 and 4C_1 xylopyranose, and L-Araf-type inhibitors. Its confirmation must wait upon the major task of synthesizing these oligosaccharide fragments.

Mutually exclusive binding of the different types of inhibitor

1C_4 -type, 4C_1 -type and L-Araf-type inhibitors (with the exception of α -L-xylopyranosyl fluoride and L-arabinono- γ -lactone) are rigorously competitive with respect to 2-nitrophenyl β -D-xylopyranoside substrate. Table 2 displays the results of experiments to test whether they are also competitive with respect to each other, i.e. whether EI_1I_2 complexes

can be formed. The simultaneous binding of two inhibitors is treated by Segel (1975), the rate law being:

$$v = \frac{v_{\max} [S]}{[S] + K_m \left(1 + \frac{[I_1]}{K_{I1}} + \frac{[I_2]}{K_{I2}} + \frac{[I_1][I_2]}{\alpha K_{I1}K_{I2}} \right)}$$

where $\alpha = 0$ if no EI_1I_2 complexes are formed and $\alpha = 1$ if the two inhibitors bind completely independently. It is clear from the data in Tables 1 and 2 that $\alpha = 0$ for all three pairs of inhibitors and that no EI_1I_2 complexes are formed. The finding that different types of inhibitor are mutually competitive with respect to each other means that the diversity of inhibitor type is not trivially due to the presence of several isoenzymes, or to two dissimilar enzyme subunits with different characteristics. This is in any event unlikely in the light of the constant value of K_m for 2-nitrophenyl β -D-xylopyranoside obtained from the full, 30-initial-rate-measurement, treatment of inhibition by different types of compound (0.80 ± 0.06 mM from seven α -D-xylopyranosyl pyridinium salts; 0.77 mM from two β -D-xylopyranosyl pyridinium salts; 0.81 ± 0.03 mM from 1,6-anhydro-D-glucopyranose; 0.80 ± 0.04 mM from L-arabinono- γ -lactone; 0.75 ± 0.05 mM from 4-phenylphenyl α -L-arabinofuranoside).

Even though the derivatives chosen for the mutual competition experiment had the minimal steric demands possible in the aglycone region, the possibility of clashes not present in the putative natural substrate (e.g. between the anomeric hydrogen of 1,6-anhydro-L-idopyranose and the 4-OH of 1,6-anhydro-D-glucopyranose) still remains. We therefore made use of the discovery (Hehre *et al.*, 1979, 1982; Kitahata *et al.*, 1981) that 'inverting' glycosidases hydrolyse glycosyl fluorides of the 'wrong' anomeric configuration via formation of a ternary glycosyl fluoride-enzyme-acceptor complex. The hydrolysis is (very plausibly) considered to involve a tautomer of the usual catalytically competent form of the enzyme (cf. Fig. 3): first a single nucleophilic displacement of F^- , electrophilically assisted by $-BH^+$, by the OH of a saccharide acceptor, made more nucleophilic by partial proton removal by A^- , gives a new saccharide of the 'right' anomeric configuration. This is then hydrolysed, via assistance from AH and B, in accordance with the normal catalytic mechanism of the enzyme.

However, with a concentration of enzyme sufficient to transform 4 mM 2-nitrophenyl β -D-xylopyranoside at a rate of $0.3 \mu M \cdot s^{-1}$, there was no detectable enzyme-catalysed F^- liberation from 5 mM α -D-xylopyranosyl fluoride (spontaneous hydrolysis rate approx. $5 \times 10^{-6} s^{-1}$), by itself or in the presence of 0.5 mM 1,6-anhydro-D-glucopyranose, 0.6 mM L-arabinono- γ -lactone, 1.4 mM α -D-xylo-

pyranosyl-3-bromopyridinium bromide or 0.4 mM β -D-xylopyranosyl-4-bromoisoquinolinium bromide. The required ternary complex is not being formed.

We are thus presented with the paradox that monosaccharides held in mutually exclusive conformations are nonetheless bound mutually exclusively. Although separate allosteric sites cannot be ruled out, they would be most unusual on a simple hydrolase. An attractive proposition is that the enzyme is so constructed that, once one saccharide residue is bound, the intrinsic binding energy of the others is expressed only at the transition state (cf. Jencks, 1975): ground-state binding of two or more independent saccharide residues is therefore not possible.

There is some evidence for this in the data for xylobiose discussed above. Indeed, it is possible to calculate an approximate K_1 for xylobiose, on the assumption that the binding of the two saccharide residues is additive, and the additional binding energy is not used for lowering the free-energy barrier to reaction. The free energy of binding of a molecule AB (ΔG_{AB}^θ) can be split into contributions from the binding of the A portion (ΔG_A^θ), contributions from the binding of the B portion (ΔG_B^θ), and a contribution ΔG^s , which represents a change in the probability of binding that results from connection of A and B (Jencks, 1981):

$$\Delta G_{AB}^\theta = \Delta G_A^\theta + \Delta G_B^\theta - \Delta G^s$$

For xylobiose, ΔG_A^θ is the free energy of binding of a 4C_1 xylopyranose residue, which the data in Table 1 and the adoption of a standard state of 1 M give as about -9.6 kJ/mol (-2.3 kcal/mol). ΔG_B^θ estimated as -7.1 kJ/mol (-1.7 kcal/mol) is the free energy of binding of a 1C_4 xylopyranose residue, less the energy required to place a 4C_1 xylopyranose residue in the 1C_4 conformation [10.0 kJ/mol (2.4 kcal/mol), as calculated from instability factors (Angyal, 1969)]. Precedented values of ΔG^s correspond to a favouring of the binding of connected molecules by approx. 10^5 M [$\Delta G^s = 25$ – 29 kJ/mol (6 – 7 kcal/mol)]. Were this factor to operate in the case of xylobiose, it would be an inhibitor of K_1 40 nM rather than a substrate of K_m 4.6 mM.

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References

- Angyal, S. J. (1969) *Angew. Chem. Int. Ed. Engl.* **8**, 157–166
- Aspinall, G. O., Cairncross, I. M., Sturgeon, R. J. & Wilkie, K. C. B. (1960) *J. Chem. Soc.* 3881–3885

- Bishop, C. T. (1956) *J. Am. Chem. Soc.* **78**, 2840–2841
- Braun, H., Legler, G., Deshusses, J. & Semenza, G. (1977) *Biochim. Biophys. Acta* **483**, 135–140
- Buchanan, R. E., Holt, J. G. & Lessel, E. F. (1966) *Index Bergeyana*, p. 241, E. and S. Livingstone, Edinburgh and London
- Case, G. S., Sinnott, M. L. & Tenu, J.-P. (1973) *Biochem. J.* **133**, 99–104
- Cheshire, M. V. (1979) *Nature and Origin of Carbohydrates in Soils*, pp. 1–22, Academic Press, London and New York
- Claeyssens, M. & De Bruyne, C. K. (1978) *Biochim. Biophys. Acta* **533**, 98–104
- Claeyssens, M., Kersters-Hilderson, H., Van Wauwe, J.-P. & De Bruyne, C. K. (1970) *FEBS Lett.* **11**, 336–338
- Claeyssens, M., Saman, E., Kersters-Hilderson, H. & De Bruyne, C. K. (1975) *Biochim. Biophys. Acta* **405**, 475–481
- Frederick, M. M., Frederick, J. R., Fratzke, A. R. & Reilly, P. J. (1981) *Carbohydr. Res.* **97**, 87–103
- Fry, J. L., Harris, J. M., Bingham, R. C. & Schleyer, P. von R. (1970) *J. Am. Chem. Soc.* **92**, 3540–3542
- Goldschmid, H. R. & Perlin, A. S. (1963) *Can. J. Chem.* **41**, 2272–2277
- Goodfellow, M., Hill, I. R. & Gray, T. R. G. (1968) in *The Ecology of Soil Bacteria* (Gray, T. R. G. & Parkinson, D., eds.), pp. 500–515, Liverpool University Press, Liverpool
- Hehre, E. J., Brewer, C. F. & Genghof, D. S. (1979) *J. Biol. Chem.* **254**, 5942–5950
- Hehre, E. J., Sawai, T., Brewer, C. F., Nakano, M. & Kanda, T. (1982) *Biochemistry* **21**, 3090–3097
- Ishii, H., Kitagawa, I., Matsushija, K., Shirakawa, K., Tori, K., Tozyo, T., Yoshikawa, M. & Yoshimura, Y. (1981) *Tetrahedron Lett.* **22**, 1529–1532
- Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol.* **43**, 219–410
- Jencks, W. P. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4046–4050
- Kersters-Hilderson, H., Loontjens, F. G., Claeyssens, M. & De Bruyne, C. K. (1969) *Eur. J. Biochem.* **7**, 434–441
- Kersters-Hilderson, H., Claeyssens, M., Van Doorslaer, E. & De Bruyne, C. K. (1976) *Carbohydr. Res.* **47**, 269–273
- Kersters-Hilderson, H., Van Doorslaer, E., De Bruyne, C. K. & Yamanaka, K. (1977) *Anal. Biochem.* **80**, 41–50
- Kersters-Hilderson, H., Van Doorslaer, E. & De Bruyne, C. K. (1978) *Carbohydr. Res.* **65**, 219–227
- Kersters-Hilderson, H., Van Doorslaer, E. & De Bruyne, C. K. (1980) *Carbohydr. Res.* **78**, 163–170
- Kitahata, S., Brewer, C. F., Genghof, D. S., Sawai, T. & Hehre, E. J. (1981) *J. Biol. Chem.* **256**, 6017–6026
- Legler, G. (1973) *Mol. Cell. Biochem.* **2**, 31–38
- Legler, G. & Herrchen, M. (1981) *FEBS Lett.* **135**, 139–144
- Legler, G., Roeser, K. R. & Illig, H. K. (1979) *Eur. J. Biochem.* **101**, 85–92
- Loontjens, F. G. & De Bruyne, C. K. (1964) *Naturwissenschaften* **51**, 359
- Marshall, P. J. (1982) Ph.D. Thesis, University of Bristol
- Marshall, P. J., Sinnott, M. L., Smith, P. J. & Widdows, D. (1981) *J. Chem. Soc. Perkin Trans. 1* 366–376
- Micheel, F. (1957) *Chem. Ber.* **90**, 1612–1616
- Montgomery, E. M., Richtmyer, N. K. & Hudson, C. S. (1943) *J. Am. Chem. Soc.* **65**, 3–7
- Nikitin, V. N., Levdiuk, I. Yu. & Ivanov, M. A. (1968) *J. Struct. Chem. (Engl. Transl.)* **9**, 901–906
- Paulsen, H., Luger, P. & Heiker, F. R. (1979) in *The Anomeric Effect: Origin and Consequences* (Szarek, W. A. & Horton, D., eds.), p. 63, American Chemical Society, Washington
- Saman, E., Claeyssens, M., Kersters-Hilderson, H. & De Bruyne, C. (1976) *FEBS Lett.* **63**, 211–214
- Saman, E., Claeyssens, M. & De Bruyne, C. K. (1978) *Eur. J. Biochem.* **85**, 301–307
- Segel, I. H. (1975) *Enzyme Kinetics*, pp. 474–487, John Wiley and Sons, New York
- Shambe, T. (1983) *Carbohydr. Res.* **113**, 125–131
- Simpson, F. J. (1954) *Can. J. Microbiol.* **1**, 131–139
- Sinnott, M. L. & Widdows, D. (1981) *J. Chem. Soc. Perkin Trans. 1* 401–402
- Skujins, J. (1975) *CRC Crit. Rev. Microbiol.* **4**, 383–421
- Sorkin, E. & Reichstein, T. (1945) *Helv. Chim. Acta* **28**, 1–17
- Takenishi, S. & Tsujisaka, Y. (1973) *Agric. Biol. Chem.* **37**, 1385–1391
- Thoma, J. A. (1968) *J. Theor. Biol.* **19**, 297–310
- Thoma, J. A. & Koshland, D. E. (1960) *J. Mol. Biol.* **2**, 169–170
- Van Doorslaer, E., Kersters-Hilderson, H. & De Bruyne, C. K. (1979) *Arch. Int. Physiol. Biochim.* **87**, 853–854
- Van Doorslaer, E., Kersters-Hilderson, H. & De Bruyne, C. K. (1980) *Carbohydr. Res.* **78**, 317–326
- Whistler, R. L. & Richards, E. L. (1970) in *The Carbohydrates* (Pigman, W. W. & Horton, D., eds.), 2nd edn., vol. 2B, pp. 452–458, Academic Press, New York and London
- Widdows, D. (1979) Ph.D. Thesis, University of Bristol
- Wilkie, K. C. B. (1979) *Adv. Carbohydr. Chem. Biochem.* **36**, 215–264
- Wolfenden, R. (1972) *Acc. Chem. Res.* **5**, 10–18