Product Stereochemistry and Some Inhibitors of the α-Arabinofuranosidases of *Monilinia fructigena*

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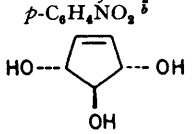
That the initial product of the title enzymes is α-L-arabinofuranose is shown by the detection of methyl α-L-arabinofuranoside when the hydrolysis of *p*-nitrophenyl α-L-arabinofuranoside is carried out at a concentration of 0.5—1.5M in methanol. A study of competitive inhibitors indicates that L-arabinono-1,4-lactone is unusually tightly bound.

ALTHOUGH the catalytic mechanisms of *O*-glycopyranosidases (such as lysozyme, β-glucosidase, and β-galactosidase) and *N*-glycofuranosidases (such as NAD-glycohydrolases) have received detailed attention,[1] little is known about *O*-glycofuranosidases; indeed the only enzyme in this class for which even the initial product is known is invertase.[2] Invertase however is mechanistically intractable because of very high substrate specificity [3] and synthetic difficulties in introducing isotopes. The fungus *Monilinia fructigena*, responsible for a brown rot of apples, produces three α-L-arabinofuranosidases, and the two extracellular enzymes, AF I and AF III,[4] seem suitable vehicles for an investigation of catalysis by furanosidases. We now report some basic mechanistic information on these enzymes.

K_i Values for various ligands binding to α-L-arabinofuranosidases from *Monilinia fructigena*

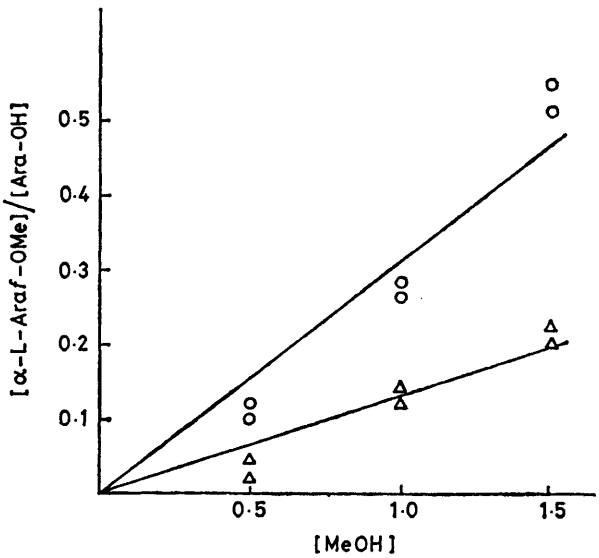
Ligand	K_i/mM AF I	AF III
α-L-Ara*f*-O-*p*-$C_6H_4NO_2$	0.09 ± 0.01 [a]	0.82 ± 0.01 mM [a]
Products of decomposition of α-L-Ara*f*$CH_2N{=}N{-}H$ [c] *p*-$C_6H_4NO_2$ [b]	0.016	>4
	*ca.* 10^2	*ca.* 10^2—10^3
α-L-Ara*f*$CH_2NH_3^+$ [b]	0.88 ± 0.08	5.8 ± 0.5
L-Arabinono-1,4-lactone	0.015 ± 0.01	0.12 ± 0.01

All data pertain to 0.1M-sodium acetate–acetic acid buffer, pH 5.0 at 30 °C.

[a] K_m values. [b] P. J. Marshall, M. L. Sinnott, P. J. Smith, and D. Widdows, *J. Chem. Soc., Perkin Trans 1*, 1981, 366. [c] M. L. Sinnott and D. Widdows, *J. Chem. Soc., Perkin Trans. 1*, 1981, 401.

RESULTS AND DISCUSSION

When *p*-nitrophenyl α-L-arabinofuranoside is hydrolysed by AF I and AF III in the presence of methanol, small quantities of methyl α-L-arabinofuranoside are produced (Figure). There are two reasons for confidence that this compound represents a kinetic rather than a thermodynamic product (*i.e.* it is not being synthesised from arabinose and methanol by the enzyme working backwards). First, liberation of *p*-nitrophenol was monitored and the reaction was stopped when this process was complete (*ca.* 2 h), and secondly incubation of a 1.5M solution of arabinose in methanol in the presence of enzyme for this time produced <0.5% methyl arabinoside. No *p*-nitrophenol was produced in the absence of enzyme. Therefore the enzyme is transferring the α-L-arabinofuranosyl residue from the *p*-nitrophenyl glycoside to methanol as well as to water; it gives products of retained anomeric configuration.



Methyl α-L-arabinofuranoside in products from the hydrolysis of *p*-nitrophenyl α-L-arabinofuranoside by (○) AF I and (△) AF III. Duplicate analyses are shown

The catalytic mechanism of this enzyme then, in all probability, involves a discrete covalent intermediate, although in view of the opening of the furanose ring in some pathways for the acid-catalysed hydrolysis of furanosides,[5,6] it may well not be a β-L-arabinofuranosyl enzyme.

The partitioning of the arabinosyl–enzyme intermediate from AF I between water and methanol favours methanol by a factor of 17 on a molar basis and that from AF III by a factor of 7. This is comparable with the discrimination exhibited by other glycosyl–enzyme intermediates (preferences for methanol of 110 being exhibited by the *lacZ* β-galactosidase of *E. coli*,[7] of 20 by the *ebg*° isoenzyme,[8] and of 7 by the α-mannosidase of *Medico sativa* [9]).

Recently, studies of the inhibition of the α-L-arabinofuranosidase of *Aspergillus niger* by L-arabinino-1,4-lactone were reported.[10] Certain glycopyranosidases are powerfully inhibited by the corresponding aldonolactones and Leaback [11] pointed out that a probable cause was the half-chair conformation constrained upon the pyranose ring by the lactone function; this was the same half-chair as would be adopted by an aldopyranosyl cation, and therefore the lactones were ' transition state analogues ' [12] for those enzymes whose catalytic pathway involved a glycopyranosyl cation-like transition state. This is not the whole story, however, since D-glucopiperidinolactam, isoelectronic and isosteric with the corresponding lactone, is nonetheless bound to sweet almond β-glucosidase B some 10^3 times less tightly.[13] In this case, the proposal that enhanced binding of aldonolactones arises from reversible addition of a nucleophile on the enzyme to form a hemiorthoester seems more plausible.[14]

The reversible formation of a covalent link is the only mechanism whereby enhanced binding of aldofuranolactones to furanosidases could arise, since ordinary envelope conformations of the furanose ring have four of the five atoms coplanar, and no significant additional advantage is to be gained by the lactone function further constraining the furanose ring to this coplanarity. The data in the Table indicate that L-arabinono-1,4-lactone is not an unusually good competitive inhibitor, K_i values being comparable to those for arabinofuranosylmethylamine or K_m values for *p*-nitrophenyl α-L-arabinofuranoside substrate. An addition mechanism is clearly not operating in this case.

EXPERIMENTAL

Analysis of Reaction Products.—Enzyme [4] was added to a solution (5 ml) of *p*-nitrophenyl α-L-arabinofuranoside [4] (10 mmol l^{-1}) in 0.1M-sodium acetate–acetic acid buffer (pH 4.0 for AF I and pH 5.5 for AF III) also containing methanol, and when the theoretical quantity of *p*-nitrophenol had been liberated, the enzyme was denatured by heating on a steam-bath. Nitrophenol was extracted with ether, and the aqueous layer was evaporated in a rotary evaporator, and then left for 18 h at 15 mmHg over P_2O_5. A mixture of pyridine, trimethylchlorosilane, and hexamethyl disilazane (10 : 4 : 2) was added, the solution was left for 1 h at 22 °C, and then analysed by g.l.c. on a Perkin-Elmer F-11 instrument (2 m column of 25% Embaphase on Diatoport, 170 °C, nitrogen elution). Retention times of the four arabinose peaks, relative to methyl arabinoside were 1.23, 1.39, 1.55, and 1.77, and the column had *ca.* 2 000 theoretical plates. Molar response factors, for arabinose and methyl arabinoside, were assumed to be identical, and the identity of the methyl arabinoside peak in the enzyme reaction-products was confirmed by co-injection of a genuine sample. The ability of this analysis system to distinguish between methyl α- and β-L-arabinofuranosides was confirmed as follows. A Fischer synthesis of methyl arabinosides [15] was stopped as soon as the reaction mixture no longer reduced Fehling's solution. After work-up [15] and derivatisation, g.l.c. showed two peaks only, relative retention times 1 : 1.20 and relative areas 3 : 1, the major peak being that of methyl α-L-arabinofuranoside. Under these conditions it is known [5,15] that furanosides greatly predominate in the reaction mixture. Therefore the minor, slower-moving peak is methyl β-L-arabinofuranoside, cleanly separated from its α-anomer and in the quantity expected.[5] Analysis of the reaction mixture from the Fischer synthesis after a 50-fold longer reaction time revealed that methyl β-L-arabinofuranoside had largely disappeared and had been replaced by a compound appearing as a shoulder on the methyl α-L-arabinofuranoside peak.

K_i Determinations.—Rates of production of *p*-nitrophenol from *p*-nitrophenyl α-L-arabinofuranoside were monitored continuously at 400 nm in the thermostatted cell-compartment of a Unicam SP 1800 spectrophotometer. At least 30 measurements at each of six substrate and five inhibitor concentrations were made for the lactone and α-L-arabinofuranosylmethylamine and the inhibition was shown to be cleanly competitive. K_i Values for other ligands were measured from plots of V_0/V against various inhibitor concentrations at one substrate concentration.

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