Inhibition of the α -L-arabinofuranosidase III of *Monilinia fructigena* by 1,4-dideoxy-1,4-imino-L-threitol and 1,4-dideoxy-1,4-imino-L-arabinitol

Mohammed T. H. AXAMAWATY,* George W. J. FLEET,† Kevin A. HANNAH,* Sung K. NAMGOONG† and Michael L. SINNOTT*‡

*Department of Organic Chemistry, University of Bristol, Cantocks Close, Bristol BS8 1TS, U.K., and

†Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, U.K.

1. 1,4-Dideoxy-1,4-imino-L-threitol was synthesized and the synthesis of 1,4-dideoxy-1,4-imino-L-arabinitol was improved. 2. Both compounds are competitive inhibitors of *Monilinia fructigena* α -L-arabinofuranosidase III, the additional hydroxymethyl group in the arabinitol contributing about 17.8 kJ/mol (4.25 kcal/mol) to the Gibbs free energy of binding. 3. The affinities ($1/K_i$) of both compounds vary with pH in a classical bell-shaped way, the pK_a value being that of the acid-catalytic group on the enzyme [5.9; Selwood & Sinnott (1988) *Biochem. J.* **254**, 899–901] and the pK_b values being those of the free inhibitors, 7.6 and 7.8 respectively. 4. On the basis of these and literature data we suggest that efficient inhibition of a glycosidase at its pH optimum by an appropriate iminoalditol will be found when the pK_a of the iminoalditol is below that of the acid-catalytic group of the target enzyme.

INTRODUCTION

Although the powerful inhibition of glucosidases by the antibiotic derivative 1-deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol) has been known for over two decades (Inouye *et al.*, 1968), most of the reports of the synthesis and evaluation of glycosidase inhibitors of this type have appeared in the last 7 years, probably as a consequence of the potential of inhibitors of glycoprotein processing as anti-viral agents [for reviews see Legler (1987) and Fellows (1987)].

The physical basis for the powerful inhibition of glycosidases by iminoalditol derivatives was at first thought to be their resemblance in charge (though not in precise geometry) to the corresponding glycosyl cations (Lalegerie *et al.*, 1982). Since the balance of evidence is that glycosidases act through transition states in which the sugar moiety resembles a glycosyl cation (see, e.g., Sinnott, 1987), the protonated form of the iminoalditol was considered to be a transition-state analogue (Fig. 1).

This interpretation by itself failed to rationalize two patterns of behaviour exhibited by iminoalditol inhibitors. The first was that there seemed to be no straightforward relationship between the molecular structure of an iminoalditol inhibitor and the affinity for the target enzyme, when the affinity was measured at the optimum pH for catalysis (Tulsiani *et al.*, 1982; Saul *et al.*, 1983; Bischoff & Kornfeld, 1984; Cenci di Bello *et al.*, 1984; Legler & Jülich, 1984; Card & Hitz, 1985; Eis *et al.*, 1985; Evans *et al.*, 1985; Fleet *et al.*, 1985a,b; Iwama *et al.*, 1985; Rule *et al.*, 1985; Tulsiani *et al.*, 1985; Bischoff *et al.*, 1986; Kuszmann & Kiss, 1986; Legler & Pohl, 1986; Molyneux *et al.*, 1986; Elbein *et al.*, 1987; Noshimura *et al.*, 1988; Tadano *et al.*, 1988; Liotta *et al.*, 1989). The second was that the inhibitory potency of these inhibitors increased with increasing pH (Hanozet

Fig. 1. Analogy between (a) protonated deoxynojirimycin and (b) the D-glucopyranosyl cation

et al., 1981; Saul *et al.*, 1984), indicating that the species inhibiting the catalytically active form of the enzyme was the neutral form rather than the protonated form of the inhibitor. An incisive study was performed by Dale *et al.* (1985) of the inhibition of almond β -glucosidase by 1-deoxynojirimycin, in which it was shown that inhibitory potency was at a maximum at pH 6.8, the pK_a of both enzyme and inhibitor.

A more detailed consideration of the likely transition states for glycosidase catalysis suggests a reason for this behaviour. Proton donation to the leaving group is commonly invoked as a factor in glycosidase catalysis, and such proton donation will involve a catalytic group in the enzyme active site that will be partly deprotonated at the transition state (see, e.g., Sinnott, 1987; Kirby, 1987). Therefore, if protonated iminoalditol inhibitors do indeed resemble the glycosyl moieties at enzymic transition states, since at these transition states the catalytic group will be partly deprotonated, the transition-state analogues will bind most tightly to deprotonated enzyme. The effect will be reinforced by simple electrostatics: binding of a positively charged inhibitor is enhanced if the active site decreases in charge by 1 unit. These ideas are illustrated in Fig. 2 with respect to an α -L-arabinofuranosidase.

‡ To whom correspondence should be sent, at present address: Department of Chemistry (M/C 111), University of Illinois at Chicago, P.O. Box 4348, Chicago, IL 60680, U.S.A.

Fig. 2. (a) Probable transition-state structure for the first chemical step in the hydrolysis of an α -L-arabinofuranoside by the α -L-arabinofuranosidase III of *Monilinia fructigena* and (b) binding of protonated compound (II) in the manner of a transition-state analogue

In (a) the glycone-aglycone bond is largely broken and the acid-catalytic proton largely transferred (Kelly *et al.*, 1987).

If the complex of protonated inhibitor and deprotonated enzyme predominates, then the affinity of the inhibitor for the enzyme will show a bell-shaped dependence upon pH. If K_I and K_E are the pK_a values of inhibitor and enzyme respectively, then eqn. (1) should hold:

$$1/K_I = A/(1 + K_I/[H^+] + [H^+]/K_E) \quad (1)$$

If we set $B = A \times K_E/K_I$, then the dependence is described by eqn. (2):

$$1/K_I = B/(1 + K_E/[H^+] + [H^+]/K_I) \quad (2)$$

which could equally well be interpreted as binding of deprotonated inhibitor to protonated enzyme. Maximal inhibition is observed between the two pK_a values. This type of pH-dependence, if general, would explain why the pattern of inhibitory potencies at optimum pH observed with a series of compounds and a series of enzymes has a large random component, because of the effect of change of enzyme and of change of inhibitor structure on the crucial pK_a values.

We now therefore report a study of pH-dependence of the binding of iminoalditol inhibitors to a glycosidase in which the pK_a values of the acid-catalytic group of the enzyme and of the inhibitor can be assigned. The enzyme

Fig. 3. Formulae of 1,4-dideoxy-1,4-imino-L-threitol (I) and 1,4-dideoxy-1,4-imino-L-arabinitol (II)

is the α -L-arabinofuranosidase III of *Monilinia fructigena*, and the inhibitors the pyrrolidines (I) and (II) (Fig. 3). The enzyme works with retention of the anomeric configuration (Fielding *et al.*, 1981), probably via arabinofuranosyl cation-like transition states leading to and from an arabinofuranosyl-enzyme intermediate (Kelly *et al.*, 1987). In the first chemical transition state proton donation to the leaving group is far advanced, the proton probably being provided by a group on the enzyme with a pK_a of 5.9 (Selwood & Sinnott, 1988). The enzyme of course is fully active only below this pH, whereas ample precedent suggests that the pK_a values of inhibitors (I) and (II) should be around 8. In this system, therefore, maximal inhibition should be observed well above the pH optimum of the enzyme.

METHODS AND MATERIALS

Enzyme and substrates

M. fructigena α -L-arabinofuranosidase (Kelly *et al.*, 1987) and 4-nitrophenyl α -L-arabinofuranoside and 4-methylumbelliferyl α -L-arabinofuranoside (Kelly *et al.*, 1988) have been described previously.

Synthesis of 1,4-dideoxy-1,4-imino-L-threitol (I)

This compound was synthesized by reaction of the anion of toluene-*p*-sulphonamide with 2,3-isopropylidene-1,4-di(toluene-*p*-sulphonyl)-L-threitol, followed by removal of the *N*-toluene-*p*-sulphonyl group with Na in liquid NH_3 and the isopropylidene group with aqueous acid.

A mixture of 2,3-isopropylidene-1,4-di(toluene-*p*-sulphonyl)-L-threitol (5.0 g; Rubin *et al.*, 1952), toluene-*p*-sulphonamide (1.8 g) and NaH (0.51 g) in dry dimethylformamide (50 ml) was heated at 100 °C for 3 h, and then poured on to ice (200 g). The pH was raised to 11 with NaOH, and the alkali-insoluble solid was filtered off, washed with water and recrystallized from ethanol to yield 1,4-dideoxy-1,4-imino-2,3-isopropylidene-*N*-(toluene-*p*-sulphonyl)-L-threitol (1.65 g, 51% yield), m.p. 132–133 °C, $[\alpha]_D^{20} + 76.7^\circ$ (*c* 2.5 in chloroform), 1H n.m.r. (60 MHz) δ 1.45 (p.p.m.) (2H]chloroform) (6H, s, isopropylidene-Me), 2.4 (3H, s, Ar-Me), 2.95–3.85 (6H,

m, ring C-H), 7.4 (4H, AB, Ar-H). (Found: C, 56.78; H, 6.37; N, 4.53. Calc. for $C_{14}H_{19}O_4S$: C, 56.55; H, 6.44; N, 4.71 %). 1,4-Dideoxy-1,4-imino-2,3-isopropylidene-*N*-toluene-*p*-sulphonyl-L-threitol (1.01 g) was dissolved in dry tetrahydrofuran (25 ml), and was added to a solution of Na (0.31 g) in liquid NH_3 (40 ml), at $-70^\circ C$. After 50 min the solution was allowed to warm up to room temperature, and aq. 20 % (v/v) tetrahydrofuran (25 ml) was added, followed by water (20 ml). The solution was extracted with dichloromethane, and the dichloromethane extract was dried (over $MgSO_4$) and evaporated to give 1,4-dideoxy-1,4-imino-2,3-isopropylidene-L-threitol (0.26 g, 55 % yield), m.p. 111–112 $^\circ C$, $[\alpha]_D^{20} + 21.3^\circ$ (*c* 2.5 in chloroform). This compound (0.17 g) was heated under reflux in aq. 50 % (v/v) acetone (10 ml) with Duolite C-225 cation-exchange resin in the H^+ form (0.52 g) for 24 h. The cation-exchanger was filtered off, and washed with distilled water (15 ml), then aq. 5 % (w/v) NH_3 (15 ml) and finally water (5 ml). The aq.- NH_3 washings were evaporated and the resultant yellow oil, which crystallized on standing at $4^\circ C$, was sublimed under reduced pressure to give 1,4-dideoxy-1,4-imino-L-threitol (0.6 g, 49 % yield), m.p. 96–97 $^\circ C$, $[\alpha]_D^{20} + 12.4^\circ$ (*c* 2.5 in methanol), ^{13}C n.m.r. δ 76.3 and 50.6 p.p.m. with respect to acetonitrile, 1H n.m.r. (60 MHz), δ (p.p.m.) ($[^2H_3]$ acetonitrile) 4.7 (2H, dd, $H2 + H3$) 2.4–3.0 (4H, m, analysed as ABX, $J_{1a,b}$ 12 Hz, $J_{1a,2}$ 5 Hz, $J_{1b,2}$ 3 Hz, $H1 + H4$). (Found: C, 46.37; H, 8.45; N, 12.58. Calc. for $C_4H_9NO_2$: C, 46.79; H, 8.79; N, 12.58 %).

Synthesis of 1,4-dideoxy-1,4-imino-L-arabinitol (II)

This compound has been reported previously (Fleet & Smith, 1986), but we now report an improved synthesis, involving nucleophilic displacement of both methanesulphonate groups of 2,3,5-tri-*O*-benzyl-1,4-di-*O*-methanesulphonyl-D-xylitol by a single molecule of benzylamine with inversion of the configuration of the alditol at C-4, followed by hydrogenolytic removal of the benzyl groups.

2,3,5-Tri-*O*-benzyl-1,4-di-*O*-methanesulphonyl-D-xylitol (500 mg, 0.87 mmol; Fleet & Smith, 1986) in benzylamine (3.0 ml) was warmed at $60^\circ C$ for 72 h. The benzylamine was removed *in vacuo* and the residue was dissolved in chloroform (10 ml). The chloroform solution was washed with water (10 ml) and dried (over $MgSO_4$), and the solvent was evaporated to give, after purification by flash chromatography on silica gel with diethyl ether/hexane (1:5, v/v) as eluent, *N*-benzyl-2,3,5-tri-*O*-benzyl-1,4-dideoxy-1,4-imino-L-arabinitol (307 mg, 72 % yield) as a colourless oil, 1H n.m.r. δ (p.p.m.) ($[^2H]$ chloroform) 7.37–7.24 (20H, m, Ar-H), 4.52 (4H, m, $2PhCH_2$), 4.43 (2H, ABq, $J_{AB} = 12.2$, $PhCH_2$), 4.15 (1H, 1H of AB, $J_{AB} = 13.3$ Hz, $PhCH_2$), 3.92 (2H, m), 3.62 (2H, m), 3.50 (1H, 1H of AB, $PhCH_2$), 3.05 (1H, d, $J_{1-1'} = 10.7$ Hz, $H1'$), 2.88 (1H, dd, $J_{4,5} = 10.6$ Hz, $J_{4,5'} = 5.5$ Hz, $H4$), 2.58 (1H, dd, $J_{1,2} = 5.1$ Hz, $H1$).

This tetrabenzyl compound (305 mg, 0.62 mmol) in acetic acid (5 ml) was stirred under an atmosphere of H_2 in the presence of palladium black (50 mg) at room temperature until all the benzyl groups had been removed (about 3 days). The catalyst was removed by filtration and the solvent was evaporated. The resulting acetate salt was neutralized with dilute aq. NaOH and then purified by adsorption on a column of Dowex 50 X8-100 ion-exchanger (50–100 mesh) in the H^+ form and elution

with aq. 0.5 M- NH_3 to give compound (II); the free base was dissolved in water (3 ml) and the solution was adjusted to pH 4 with dilute aq. HCl to afford, after freeze-drying, 1,4-dideoxy-1,4-imino-L-arabinitol hydrochloride (81 mg, 98 % yield), m.p. 112–113 $^\circ C$, $[\alpha]_D^{20} - 34.3^\circ$ (*c* 0.51 in water); Fleet & Smith (1986) give m.p. 109–110 $^\circ C$ (from aq. acetone), $[\alpha]_D^{20} - 34.6^\circ$ (*c* 0.37 in water). The 1H -n.m.r., ^{13}C -n.m.r. and mass spectra of the sample were superimposable on those of a sample prepared by the previously described route (Fleet & Smith, 1986).

Buffer solutions

Buffer solutions employed were 0.1 M-sodium acetate/acetic acid, pH 3.8–5.85, 0.1 M- NaH_2PO_4/Na_2HPO_4 , pH 6.35–7.75, and 0.0333 M- $Na_2H_2P_2O_7$, pH 8.05, all made up with analytical-grade reagents. pH values were measured on a Radiometer PHM 62 pH-meter calibrated against commercial standard buffer solutions. These standard solutions were later calibrated against freshly made up standards. pK_a values for the iminoalditol inhibitors were measured from the half-neutralization points of titration curves and refer to $30^\circ C$.

Determination of kinetic constants

Inhibition constants were obtained by measurements of initial rate at five different inhibitor and five different substrate concentrations. The resultant 25 initial rates were fitted to eqn. (3) by the program COMP (Cleland, 1979) translated into BASIC and run on a BBC Master microcomputer. Standard deviations on V_{max} and K_m were always less than 10 %, and on K_i less than 5 %, indicating competitive inhibition.

$$v = V_{max} [S] / \{ [S] + K_m (1 + [I] / K_i) \} \quad (3)$$

The substrate normally employed was *p*-nitrophenyl α -L-arabinofuranoside, but above pH 8 the increase in spontaneous hydrolysis rate and decrease in enzyme activity made it necessary to change to a more stable and more sensitive substrate, the fluorogenic 4-methylumbelliferyl α -L-arabinofuranoside. The hydrolysis of the nitrophenyl glycoside was monitored in a Phillips PU 8800 spectrometer, in 1 mm-pathlength cells, at 347.3 nm (up to pH 6.35) or 400 nm (from pH 6.85 to pH 7.75); the spectrometer was fitted with a thermostatically controlled cell-block. The hydrolysis of the 4-methylumbelliferyl glycoside was followed by fluorescent emission at 450 nm consequent upon excitation at 360 nm in a Perkin-Elmer 3000 spectrofluorimeter; in this instrument the cell itself had an integral water-jacket. K_m values for *p*-nitrophenyl α -L-arabinofuranoside obtained in the present study were within experimental error the same as those reported by Selwood & Sinnott (1988).

RESULTS AND DISCUSSION

Fig. 4 shows the pH variation of the pK_i of the tetrose inhibitor (I) and Fig. 5 that of the pentose inhibitor (II). The continuous lines are the best fit to eqn. (4), as analysed by the program BELL (Cleland, 1979) translated into BASIC and run on a BBC Master microcomputer. For inhibitor (I), BELL gives $A = 764 \pm 40 M^{-1}$, $pK_E = 5.70 \pm 0.03$ and $pK_i = 7.66 \pm 0.05$; for inhibitor (II) the analogous parameters are $A = (9.27 \pm 0.60) \times 10^5 M^{-1}$, $pK_E = 5.67 \pm 0.04$ and $pK_i = 7.56 \pm 0.06$. Quoted errors are the standard deviations derived from each set of data, and take no account of

Fig. 4. Dependence of the binding constant of 1,4-dideoxy-1,4-imino-L-threitol upon pH

The units of K_i are M.

Fig. 5. Dependence of the binding constant of 1,4-dideoxy-1,4-imino-L-arabinitol upon pH

The units of K_i are M.

systematic error in, for example, pH measurements between different sets of measurements. The pK_E values derived from the inhibition experiments can thus be taken as the same, within experimental error, as the pK_a value of 5.9 ± 0.1 measured for the ionization of the acid-catalytic group from measurements of catalytic activity as a function of pH (Selwood & Sinnott, 1988). Likewise,

the derived pK_i measurements are within experimental error the same as the measured pK_a values for inhibitor (I) (7.80 ± 0.05) and for inhibitor (II) (7.58 ± 0.03). The predictions of the model are thus fulfilled.

Inhibitor (II), possessing an extra hydroxymethyl group, binds some 1200 times more tightly to the enzyme than does inhibitor (I). This is a reasonably large factor [corresponding to 17.8 kJ/mol (4.25 kcal/mol) in binding energy], and may be a consequence of the status of these inhibitors as transition-state analogues. Some hexopyranosidases will also hydrolyse the pentopyranoside homeomorphs of their primary substrates, e.g. *lacZ* (Marshall *et al.*, 1977) and *ebg* (Burton & Sinnott, 1983) β -galactosidases of *Escherichia coli* hydrolyse α -L-arabinopyranosides, and the mammalian ' β -glucosylceramidase' hydrolyses β -galactopyranosides, β -xylopyranosides and α -L-arabinopyranosides (Kobayashi & Suzuki, 1981), as does almond γ -glucosidase (Dale *et al.*, 1986, and references cited therein). In these cases, however, removal of the hydroxymethyl group rarely increases K_m values by much more than an order of magnitude, although effects on k_{cat}/K_m are commonly much bigger. It could well be that, as with these enzymes, the intrinsic binding energy of the hydroxymethyl group of the α -L-arabinofuranoside substrate of the *M. fructigena* α -L-arabinofuranosidase could be manifested at the transition state rather than in the Michaelis complex (cf. Jencks, 1975). If inhibitors (I) and (II) are transition-state analogues then the effect of the extra hydroxymethyl group of inhibitor (II) should more closely parallel the effects on transition states than on Michaelis complexes, and a large difference would be expected.

The quantitative success of our model in the present system, and its compatibility with the pH-dependences of inhibition of other glycosidases by other iminoalditol inhibitors, where these are known, suggest that it may be general. It is probable, therefore, that iminoalditol inhibitors will inhibit glycosidases at their pH optimum significantly more powerfully than substrate analogues (such as thioglycosides or anhydroalditols) only if the pK of the inhibitor lies below that of the acid-catalytic group of the glycosidase in question.

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