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Irreversible Inhibition of Type I Dehydroquinase by Substrates for Type II Dehydroquinase

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Abstract—Mechanistic differences between type I and type II dehydroquinases have been exploited in the design of type specific inhibitors. (2*R*)-2-Bromo-3-dehydroquinic acid (**3**), (2*R*)-2-fluoro-3-dehydroquinic acid (**5**) and 2-bromo-3-dehydroshikimic acid (**4**), all excellent substrates for type II dehydroquinase, are shown to be irreversible inhibitors of type I dehydroquinase.

Dehydroquinase (3-dehydroquinate dehydratase) catalyzes the dehydration of 3-dehydroquinic acid (**1**) to 3-dehydroshikimic acid (**2**) (Scheme 1). This transformation is the third reaction on the shikimic acid pathway,[1] and also a step on the quinic acid pathway.[2] There are two types of dehydroquinase, type I, found only on the shikimic acid pathway, and type II found on both pathways.[3]

The type I dehydroquinase (typified by the *Escherichia coli* enzyme)[4] and the type II dehydroquinase (e.g. from *Mycobacterium tuberculosis*)[5] use different catalytic mechanisms. The reaction catalyzed by the type I enzyme has been shown to proceed through an imine intermediate.[6] In contrast there is no evidence for a covalent intermediate in the type II reaction mechanism which appears to proceed by an E_1CB mechanism.[3,7] Furthermore, the type I catalyzed reaction involves loss of the *pro-R* hydrogen from C-2, corresponding to an overall *syn* elimination,[8] whereas the more acidic axial *pro-S* hydrogen is lost in both the non-enzyme catalyzed reaction,[9] and the type II dehydroquinase catalyzed reaction (Scheme 1).[10]

This mechanistic dichotomy has been used as the starting point for the design of type-specific mechanism-based inhibitors. Here we describe studies on (2*R*)-2-bromo-3-dehydroquinic acid (**3**), (2*R*)-2-fluoro-3-dehydroquinic acid (**5**) and 2-bromo-3-dehydroshikimic acid (**4**) which do show this differential reactivity with the type I and type II dehydroquinases. (2*R*)-2-Bromo-3-dehydroquinic acid (**3**) and (2*R*)-2-fluoro-3-dehydroquinic acid (**5**), were each synthesized in six steps from (−)-quinic acid.[11] 2-Bromo-3-dehydroshikimic acid (**4**) was also synthesized from (−)-quinic acid in nine steps.[12] We have previously reported that incubation with type II dehydroquinase from *M. tuberculosis* rapidly converts (2*R*)-2-bromo-3-dehydroquinic acid (**3**) into 2-bromo-3-dehydroshikimic acid (**4**) ($K_{eq} = 7.3$), and (2*R*)-2-fluoro-3-dehydroquinic acid (**5**) into 2-fluoro-3-dehydroshikimic acid (**6**) ($K_{eq} = 4.0$).[12] Somewhat surprisingly both **3** and **5** have similar specificity constants to 3-dehydroquinic acid (**1**) (k_{cat}/K_m (**3**) $= 3.67 \times 10^5$ M^{-1} s^{-1}, k_{cat}/K_m (**5**) $= 0.86 \times 10^5$ M^{-1} s^{-1}, k_{cat}/K_m (**1**) $= 2.50 \times 10^5$ M^{-1} s^{-1}). The presence of the equatorial halogen does not prevent binding to the enzyme, and the enhanced acidity of the axial C-2 hydrogen may actually facilitate the E_1CB mechanism.

In direct contrast, we now report that (2*R*)-2-bromo-3-dehydroquinic acid (**3**), (2*R*)-2-fluoro-3-dehydroquinic acid (**5**), and 2-bromo-3-dehydroshikimic acid (**4**) inhibit type I dehydroquinase.[13] (2*R*)-2-Bromo-3-dehydroquinic acid (**3**) was found to be a weak competitive inhibitor ($K_i = 3.7$ mM), and slowly inactivated the enzyme irreversibly (70% inactivation after 24 h at 10 mM). The

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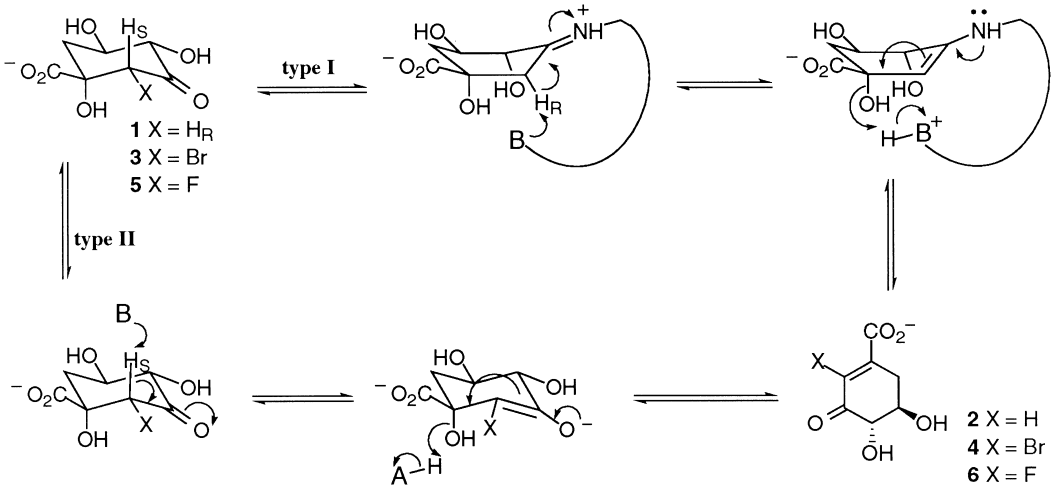


Scheme 1. The conversion of 3-dehydroquinic acid (**1**) to 3-dehydroshikimic acid (**2**) catalyzed by type I and type II dehydroquinases. The corresponding conversion of (2*R*)-2-bromo-3-dehydroquinic acid (**3**) to 2-bromo-3-dehydroshikimic acid (**4**) and (2*R*)-2-fluoro-3-dehydroquinic acid (**5**) to 2-fluoro-3-dehydroshikimic acid (**6**) is only catalyzed by type II dehydroquinase.

mass spectrum of a sample of partially inactivated enzyme consisted of a peak for unmodified protein (calculated mass 27,467) and a second peak for covalently modified protein, corresponding to an additional mass of 123 ± 5 and 126 ± 5 on separate samples. A representative mass spectrum is shown in Figure 1. Parallel studies were carried out using (2*R*)-2-fluoro-3-dehydroquinic acid **5**. It was also found to be a competitive inhibitor ($K_i = 80$ μM), and also slowly inactivated the enzyme irreversibly (45% inactivation after 22 h at 2.4 mM). The mass spectrum of the partially inactivated enzyme similarly had a new peak for modified protein corresponding to an additional mass of 126 ± 5.

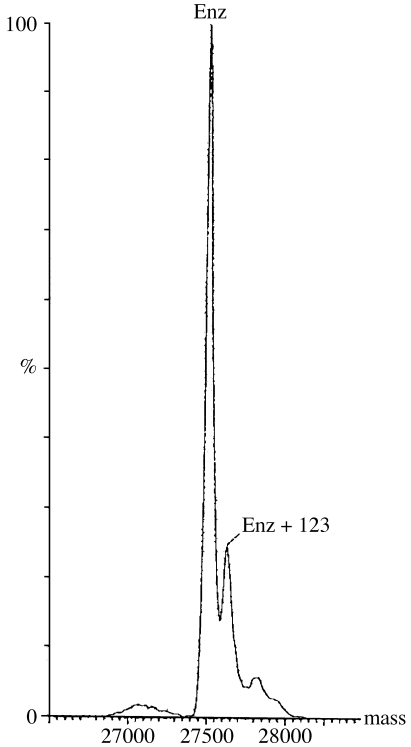
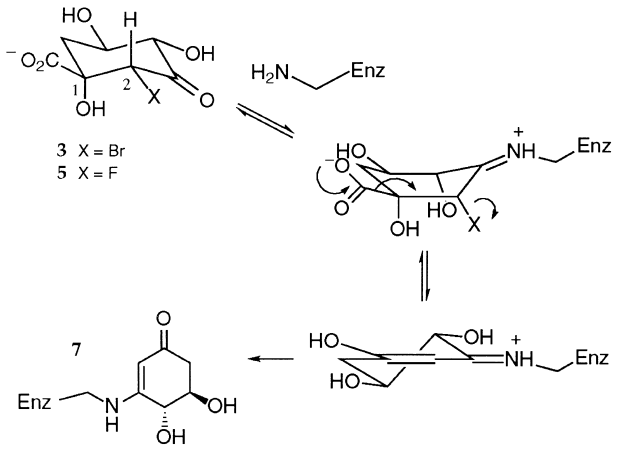


Figure 1. Mass spectrum inactivation of type I dehydroquinase by (2*R*)-2-bromo-3-dehydroquinic acid (**3**).

The modest competitive inhibition ($K_i = 3.7$ mM) observed with (2*R*)-2-bromo-3-dehydroquinic acid (**3**) may be due to the steric bulk of the large equatorial bromine blocking a catalytically sensitive volume adjacent to the position of the removed *pro-R* hydrogen of 3-dehydroquinic acid (**1**). This interaction was predicted to be much smaller for (2*R*)-2-fluoro-3-dehydroquinic acid (**5**), and a correspondingly much lower K_i of 80 μM was observed.

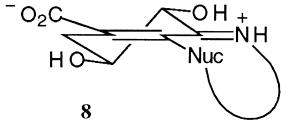
The formation of an enzyme–inhibitor adduct with an associated increase in mass of 126 ± 5 for both inhibitors, suggests the formation of a common adduct in which the halogen substituent has been lost from the inhibitor. The observed increase in molecular mass can be explained by the formation of the vinylogous amide **7** (additional mass 126) at the active site. A possible mechanistic rationale for the formation of this adduct is shown in Scheme 2. The loss of the less acidic *pro-R* hydrogen from 3-dehydroquinic acid (**1**) requires some distortion of **1** from its preferred chair conformation in the imine intermediate, to align the bond to the *pro-R* hydrogen at C-2 with the π-acceptor orbital of the imine and thereby increase its acidity. A corresponding distortion of the (2*R*)-2-bromo-3-dehydroquinic acid (**3**) or (2*R*)-2-fluoro-3-dehydroquinic acid (**5**) bound to type I dehydroquinase by imine formation at C-3 would move the C-2-halogen closer to an antiperiplanar orientation with respect to the C-1-carboxyl bond, and may trigger a decarboxylative elimination reaction. Moving the bond to the halogen into a more axial position also reduces any unfavorable dipole interaction between it and the polarized C=N of the iminium functionality. Finally tautomerization of the enol iminium adduct forms the vinylogous amide **7**, which is irrreversibly bound to the enzyme, and prevents further catalysis.[14]

Incubation of type I dehydroquinase with 2-bromo-3-dehydroshikimic acid (**4**) also resulted in slow irreversible inactivation (60% inactivation after 24 h at 10 mM). However the enzyme–inhibitor adduct observed by electrospray mass spectrometry now corresponded to



Scheme 2. Proposed mechanism for the irreversible inactivation of type I dehydroquinase by (2*R*)-2-bromo-3-dehydroquinic acid (**3**) and (2*R*)-2-fluoro-3-dehydroquinic acid (**5**).

an additional mass of 154 ± 5. The additional mass of 154 ± 5 for the protein modified by **4** may be due to formation of the adduct **8**, with the inhibitor bound via both an imine linkage and an active site nucleophile (additional mass 153). This could arise by imine formation followed by conjugate attack at C-2, and subsequent elimination of bromide. A decarboxylative elimination reaction similar to that proposed for **3** and **5** would not be expected with 2-bromo-3-dehydroshikimic acid (**4**) since *cis*-hydration of the imine intermediate on the enzyme would result in formation of imine-bound (2*S*)-2-bromo-3-dehydroquinic acid with the opposite stereochemistry at C-2.



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These experiments in which identical covalently-modified enzyme species are formed with substrate analogues **3** and **5**, and a different adduct is formed with the product analogue **4**, act as internal controls for each other. The proposed mechanism for inhibition of type I dehydroquinase by (2*R*)-2-bromo-dehydroquinic acid (**3**) and (2*R*)-2-fluoro-3-dehydroquinic acid (**5**) implies that these compounds are suicide inhibitors, where the activation is provided by a subtle conformational change in the inhibitor on forming the imine intermediate. This is in contrast to the finding that the same compound is an excellent substrate for type II dehydroquinase, and exemplifies the opportunities that are presented by having two enzymes that have evolved to catalyze the same reaction by entirely different mechanisms.

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13. Type I dehydroquinase from *E. coli* was purified as in Duncan, K.; Chaudhuri, S.; Campbell, M. S.; Coggins, J. R. *Biochem. J.* **1986**, *238*, 475. Incubations of type I dehydroquinase (170 μg from a stock protein concentration of 21.5 mg/ml stored in 50% glycerol) with (2*R*)-2-bromo-3-dehydroquinic acid (**3**) (10 mM), (2*R*)-2-fluoro-3-dehydroquinic acid (**5**) (2.4 mM) and 2-bromo-3-dehydroshikimic acid (**4**) (10 mM) were carried out in phosphate buffer (100 mM for **3** and **4** and 50 mM for **5**) at pH 7.0, 25 °C. Aliquots were removed and diluted into a standard assay mix containing 3-dehydroquinic acid (**1**) (500 μM for **3** and **4** and 560 μM for **5**) in phosphate buffer (100 mM for **3** and **4** and 50 mM for **5**) at pH 7.0, 25 °C. The formation of 3-dehydroshikimic acid (**2**) was monitored at 234 nm. Upon completion of the inhibition experiment, the protein samples were desalted with successive concentration and dilution using ammonium bicarbonate (4 mM) at 4 °C and in an Amicon Centricon-10 microconcentrator prior to loading on a electrospray mass spectrometer. Electrospray mass spectra were recorded on a VG BioQ quadrupole mass spectrometer.
14. Under the conditions of the enzyme incubation, but in the absence of enzyme, no decarboxylative loss of bromide or fluoride was observed from **3** or **5**, respectively.