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AFFINITY LABELLING OF *E. COLI* DEHYDROQUINASE

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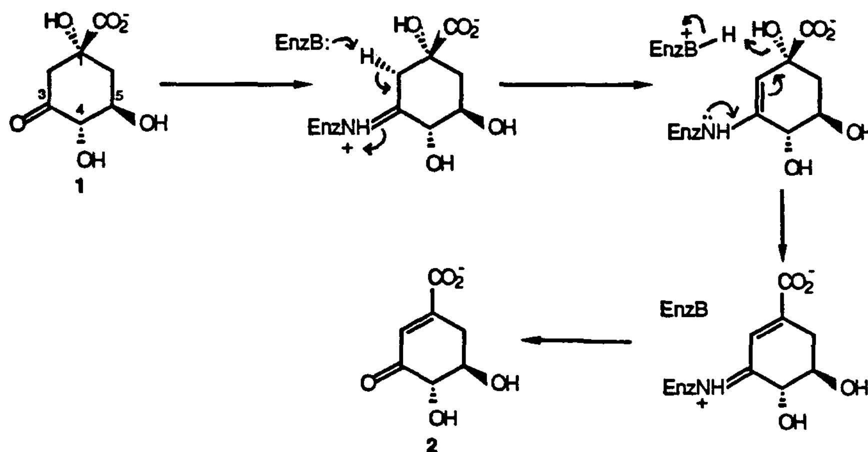
Summary: Three irreversible inhibitors of E. coli dehydroquinase have been synthesised; mechanisms for their mode of action are proposed.

The stereospecific *cis*-dehydration of 3-dehydroquinic acid (**1**) to 3-dehydroshikimic acid (**2**) catalysed by dehydroquinase (EC 4.2.1.10) is the third step of the shikimate pathway.[1] Since the shikimate pathway is found only in plants and micro-organisms, inhibitors of enzymes on the pathway are of considerable commercial interest as potential herbicides.[2] However, to date no substrate-based inhibitors of dehydroquinase have been reported. *E. coli* dehydroquinase has recently been purified to homogeneity, overexpressed and sequenced,[3] which has made possible active site studies. In this paper we describe the design and synthesis of three active site-directed irreversible inhibitors of *E. coli* dehydroquinase.

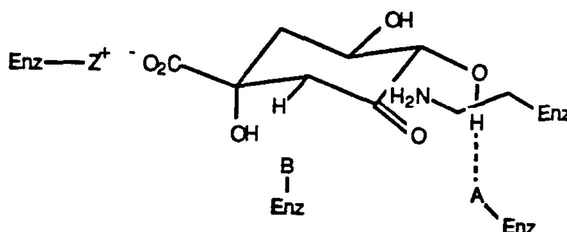
The active site of *E. coli* dehydroquinase is known to contain a lysine residue which forms a Schiff's base with the ketone at C-3 of the substrate.[4] Mechanisms have been proposed for the enzymic reaction involving a conformational change of the substrate, followed by abstraction of the C-2 proton by a general base (Scheme 1).[5] Evidence from the pH/rate profile and inactivation using diethyl pyrocarbonate has been used to infer that the general base may be a histidine residue.[6]

Scheme 1



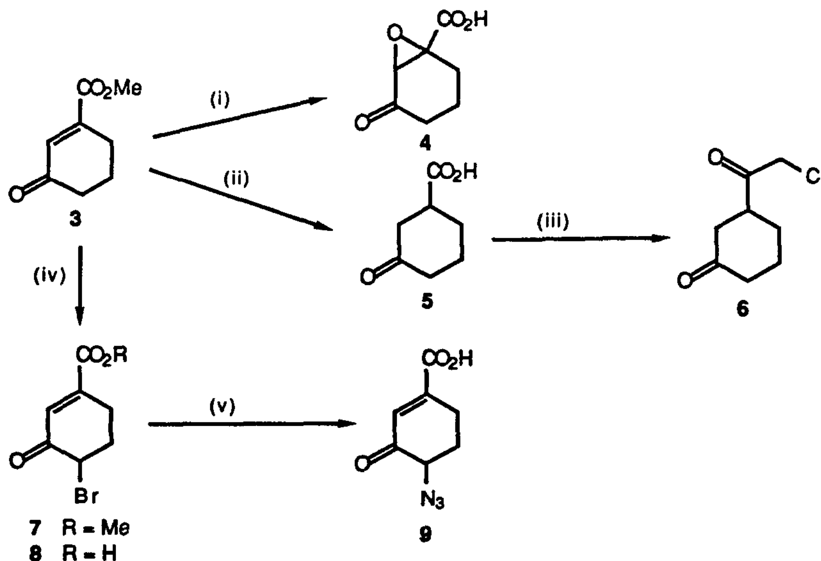
In addition to the general base and the active site lysine, a positively-charged active site residue to bind the carboxylate is implicated by the failure of dehydroquinase to bind the methyl ester of the natural substrate.[5] It is likely that there are also several residues involved in hydrogen bonding interactions with the C-1, C-4, and C-5 hydroxyl groups. Studies on *E. coli* shikimate dehydrogenase have already revealed hydrogen bonding interactions between the C-4 hydroxyl group and this enzyme.[7] A putative model for the dehydroquinase active site is shown in Figure 1.

Figure 1



In order to investigate this model of the active site, a series of affinity labels have been designed and synthesised (Scheme 2). The inhibitors incorporate reactive functional groups on a cyclohexane-carboxylate skeleton possessing a ketone at C-3. These were considered to be the minimal structural requirements necessary for binding at the active site.

Scheme 2



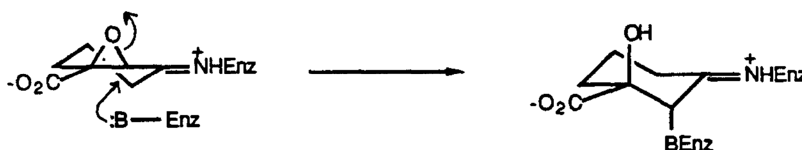
(i.) H_2O_2, Na_2CO_3, H_2O, 75%.
(ii.) H_2/Pd/C, MeOH, 60%; K_2CO_3, MeOH/H_2O, 60%.
(iii.) EtOCOCl, Et_3N, THF; CH_2N_2, Et_2O; 50% HCl/EtOH; 62%.
(iv.) LDA, TMSCl, THF, -78°C; NBS, CH_2Cl_2; 64%.
(v.) NaN_3, acetone, 60%; pig liver esterase, H_2O pH 7.0, 74%.

1) Labelling of the Active Site General Base.

The first compound to be synthesised was a racemic analogue (4) containing an epoxide between C-1 and C-2, which it was thought might be opened by nucleophilic attack of the active site general base. This compound was synthesised by treatment of methyl 3-oxo-cyclohex-1-enecarboxylate (3) with alkaline hydrogen peroxide (Scheme 2). When assayed against dehydroquinase, (4) showed rapid irreversible inhibition, which displayed saturation kinetics. Lineweaver/Burk plots[8] gave an inhibition constant K_i of 400 μM,[9] and a maximal rate of inactivation k_i of 2.5×10^{-3} s^{-1}. Inhibition was reduced by 90% on addition of 0.5 mM dehydroquinic acid, indicating that (4) is an active-site directed inhibitor.

A possible mechanism for inactivation of dehydroquinase by (4) involves nucleophilic attack on one enantiomer of the epoxide by the active site general base, which in the case of natural substrate would remove a C-2 proton from the lower face of the molecule (Scheme 3). Experiments to investigate this mechanism using the separate enantiomers of (4) are in progress. In particular we wish to establish if only one enantiomer leads to irreversible inhibition, as this mechanism implies.

Scheme 3



2) Labelling of the Carboxylate Binding Site.

There are indications in the literature that the carboxylate binding sites of other enzymes on the shikimate pathway contain a lysine residue.[10] As this may be a common feature of the pathway as a whole, it was envisaged that an analogue containing a reactive chloromethyl ketone functional group,[11] instead of the carboxylate group, might alkylate the putative lysine and so act as an irreversible inhibitor of dehydroquinase.

The Arndt-Eistert homologation procedure[12] was used to synthesise the chloromethyl ketone from the corresponding carboxylic acid. Using the dihydro-analogue (5) it was possible to synthesise racemic 3-(chloroacetyl)cyclohexanone (6), which was found to inhibit dehydroquinase irreversibly. Again saturation kinetics were observed, with an inhibition constant K_i of 0.68 mM,[9] and a maximal rate of inactivation k_i of 5.6×10^{-4} s^{-1}. Inhibition was completely prevented by the presence of 80 μM dehydroquinic acid.

3) Labelling of the C-4 Hydroxyl Binding Site.

Previous work on *E. coli* shikimate dehydrogenase has revealed the importance of the C-4 hydroxyl group for the specificity of this enzyme.[7] It was therefore considered of interest to study the C-4 hydroxyl binding site of *E. coli* dehydroquinase.

Failure of an analogue (8) containing a bromine atom at C-4 to inhibit the enzyme suggested perhaps a lack of nucleophilic residues at this site. Therefore, a racemic substrate analogue (9) containing an azide group at C-4 was synthesised, by displacement of the bromide with sodium azide. This azide was tested as a photoaffinity label. Under broad band illumination, the azide was found to inhibit dehydroquinase irreversibly, showing saturation kinetics, with a K_i of 1.1 mM,[9] and a k_i of 3.9×10^{-4} s^{-1}. Inhibition was reduced by 80% in the presence of 80 μM dehydroquinic acid. The mechanism of inactivation is thought to involve *in situ* generation of a nitrene diradical, followed by insertion into a nearby active site residue.[13]

In summary, three simple substrate analogues incorporating reactive functional groups have been shown to inhibit *E. coli* dehydroquinase irreversibly, and their proposed mode of action supports the current ideas concerning its mechanism and active site structure. Supporting biochemical experiments to identify the residues modified, and hence start to map the active site of *E. coli* dehydroquinase, are in progress.

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9. Values of K_i refer to the the concentration of racemic material required for half-maximal rate of inactivation, and as such are not corrected for enantiomeric specificity. K_m for (-)-3-dehydroquinic acid is 16 μM for comparison. The inhibition experiments were such that it was not feasible to isolate unreacted inhibitor in order to determine the chirality of the reactive enantiomer.
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