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A Mechanism-Based Inactivator Of *E. coli* β-Hydroxydecanoyl Thiolester Dehydrase Designed To Crosslink Active Site Amino Acids

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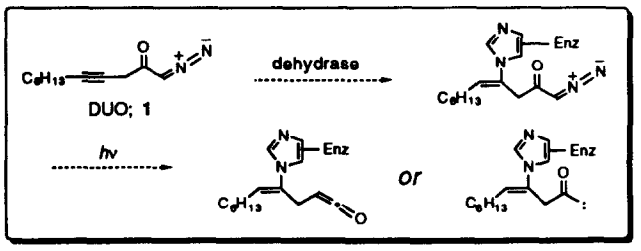
Abstract: 1-Diazo-4-undecyn-2-one, a potential active site crosslinking agent, is shown to be a mechanism-based inactivator of *E. coli* β-hydroxydecanoyl thiolester dehydrase.

β-Hydroxydecanoyl thiolester dehydrase, the pivotal enzyme in the biosynthesis of unsaturated fatty acids in *Escherichia coli*, equilibrates thiolesters of (*R*)-3-hydroxydecanoic acid, *E*-2-decenoic acid, and Z-3-decenoic acid.[1,2] Subsequent elongation of Z-3-decenoyl-ACP (i.e., the acyl carrier protein thiolester) leads to the normal complement of monounsaturated fatty acids. During the 1960s, it was discovered that dehydrase is inactivated by the substrate analog 3-decynoyl-NAC (i.e., the *N*-acetylcysteamine thiolester).[3,4] This compound undergoes a dehydrase-catalyzed, rate-limiting propargylic rearrangement to the allenic thiolester, 2,3-decadienoyl-NAC,[5] which irreversibly alkylates histidine-70.[6,7] This is now recognized as the seminal example of mechanism-based enzyme inactivation.[8]

Although stereochemical studies have suggested that His-70 is the only active site acid/base that mediates substrate protonation/deprotonation at C-2, C-3, and C-4,[9,10] the weak acidity of dehydrase's substrates coupled with the moderate basicity of histidine suggests that substrate deprotonation may be facilitated via protonation of, or hydrogen bonding to, the substrate carbonyl oxygen. The present study has been initiated in the hope of identifying an active site amino acid that might promote substrate deprotonation by serving as an electrophilic catalyst.[11]

As a β,γ-acetylenic carbonyl compound (and therefore an analog of 3-decynoyl-NAC), 1-diazo-4-undecyn-2-one (DUO) was expected to alkylate His-70 of dehydrase. In addition, through photoirradiation DUO's α-diazoketone moiety could be converted into an α-ketocarbene or a ketene, either of which might react with a second active site functional group, leading to protein crosslinking.



DUO, which is readily prepared[12] from 3-decynoic acid,[13] irreversibly inactivates dehydrase. Inactivation is a saturable phenomenon and obeys pseudo-first order kinetics (Figure 1). Additionally, the fact that substrate affords protection indicates that inactivation is an active site-directed process.

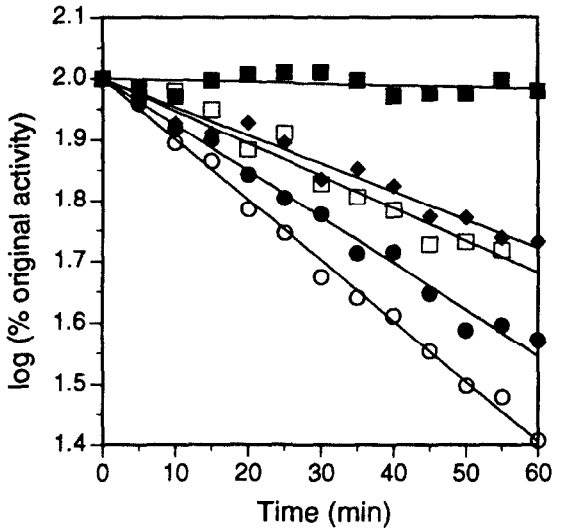
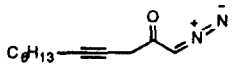
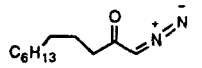
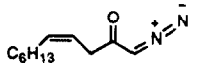
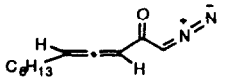
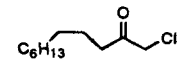
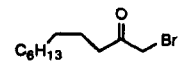
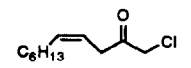


Figure 1. Time course for the inactivation of dehydrase by DUO. Duplicate samples of dehydrase (0.18 nmol protein) were incubated with DUO at the concentrations shown. Aliquots were removed at regular intervals and assayed for isomerase activity.[15]

In order to determine the stoichiometry of the inactivation process, dehydrase was incubated with [2-^{14}C]DUO (prepared from 3-[1-^{14}C]decynoic acid[14]). Following removal of excess inactivator either by gel filtration or extended dialysis, 1.0 equivalent of DUO was found to have been incorporated per equivalent of dehydrase subunits (dehydrase is a homodimeric protein[6]). All of the radioactivity remained with the protein following denaturation of the inactivated enzyme in 8 M urea. This confirms the covalent nature of the enzyme-inactivator adduct.

While the foregoing results are consistent with the original hypothesis (that DUO would initially alkylate His-70), they are also consistent with a second hypothesis: that alkylation of the enzyme might involve initial protonation at C-1 of DUO, giving a diazonium ion, which could alkylate an enzyme nucleophile via displacement of N_2. This is the mechanism by which various cysteine-dependent hydrolases are inactivated by α-diazoketone substrate analogs.[16-18] In an effort to discriminate between these mechanisms, compounds **2-8** were prepared and evaluated as inactivators of dehydrase. If the mechanism by which DUO inactivates dehydrase were to proceed via the diazonium ion, then all of these compounds might be expected to serve as inactivators.

Table I. Evaluation of Dehydrase Inactivators

Compound	Compound Number	Percent Enzyme Activity Remaining
	**1**	0
	**2**	97
	**3**	100
	**4**[b]	0
	**5**	99
	**6**	89
	**7**	100
	**8**	0

[a] Following 5 h incubations with dehydrase
[b] A mixture of 40% 4 and 60% DUO (see text)

Dehydrase was incubated separately, in the dark with compounds **2-8**, under conditions known to result in complete inactivation by DUO. An aliquot of each incubation mixture was then assayed for isomerase activity. As seen in Table I, only DUO, allenic diazoketone **4**, and acetylenic chloromethyl ketone **8** inactivate dehydrase. It should be noted that although the sample of **4** contained 60% DUO, the kinetic competence of **4** itself was demonstrated by a comparison of the pseudobimolecular rate constants, k_{inact}/K_I[19] for DUO (2057 $M^{-1}min^{-1}$) and allene **4** (10223 $M^{-1}min^{-1}$). These combined data strongly suggest that DUO does not inactivate dehydrase by simple alkylation; rather, the mechanism appears to parallel that by which 3-decynoyl-NAC inactivates dehydrase (see above).

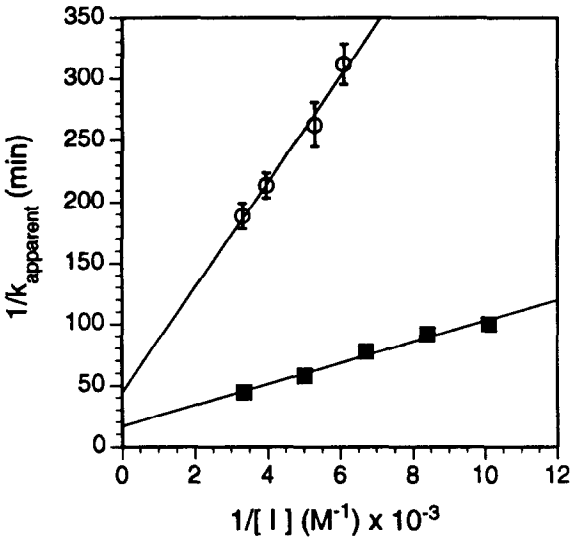


Figure 2. Kitz and Wilson plot, illustrating inactivation of dehydrase by DUO (■) and [3,3-$^2H_2$]DUO (○). Inactivators were preincubated (0-60 min for DUO; 0-240 min for [3,3-$^2H_2$]DUO) with dehydrase (0.18 nmol protein; 0.36 nmol active sites) in buffer (500 μL; 10 mM KPO_4, pH 7.0) at 8 °C.

As a further test of the foregoing hypothesis, the rates of inactivation of dehydrase by DUO and [3,3-$^2H_2$]DUO[20] were compared (Figure 2). By using the methods of Cleland,[21] values of K_I = 637 ± 126 μM and 797 ± 225 μM, and k_{inact} = 0.0707 ± 0.0106 min^{-1} and 0.0137 ± 0.00302 min^{-1} were determined for DUO and [3,3-$^2H_2$]DUO, respectively. (The k_{inact} value for [3,3-$^2H_2$]DUO is corrected[22] for ca. 10% residual protium.) This reveals a primary kinetic deuterium isotope effect of 5.2, which is consistent with the suggestion that allenic diazoketone **4** is an intermediate in the inactivation of dehydrase by DUO.

A full evaluation of the photochemistry of the DUO-dehydrase adduct will be presented in due course.

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