Latent Inhibitors. Part 3.[1] The Inhibition of Lactate Dehydrogenase and Alcohol Dehydrogenase by Cyclopropane-containing Compounds

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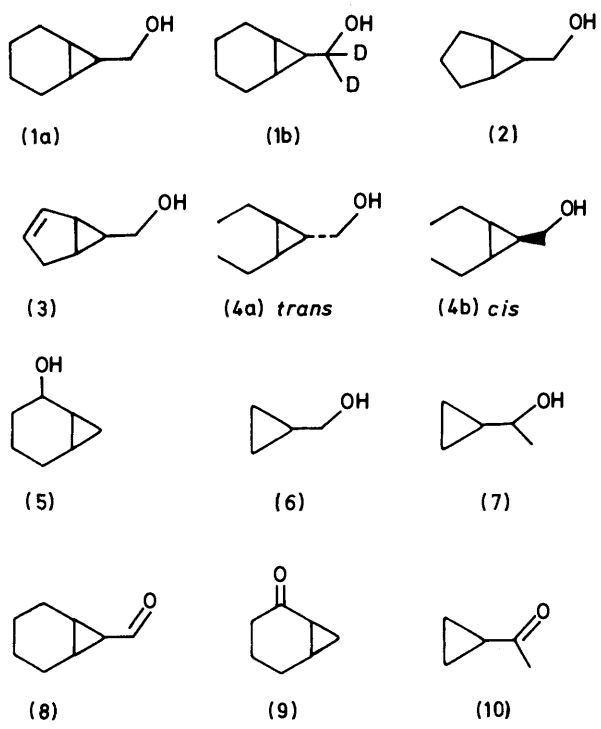
A series of monocyclic and bicyclic cyclopropylmethanols typified by bicyclo[4.1.0]heptan-7-ylmethanol is shown to comprise latent irreversible inhibitors of horse liver alcohol dehydrogenase (E.C.1.1.1.1) with inhibitory properties related to the ability of the inhibitor to bind to the enzyme. The time course of inhibition is biphasic, a property shared by a number of unsaturated aldehydes, ketones, and alcohols. Kinetic studies also suggest that inhibition occurs most effectively during the removal of hydride from the inhibitor as oxidation takes place. Analogous properties were found for the inhibition of lactate dehydrogenase (E.C.1.1.1.27) by cyclopropylglycolic acid.

The twin targets of high selectivity and prolonged duration of action of enzyme inhibitors as drugs have been most vigorously approached recently by means of latent or suicide inhibitors.[1,2] A general design strategy to obtain such inhibitors is to modify the substrate to include a small, readily activated group that will be converted into a reactive functionality by the enzyme's catalytic cycle. Thus alkenes, alkynes, and certain alkyl halides have been found to be effective in many cases.[1,2] The cyclopropane ring has some properties akin to alkenes and its small size suggests that it can be incorporated into normal enzyme substrates without seriously impairing binding to the enzyme. These two properties make it an attractive focus for the design of latent inhibitors. The susceptibility of cyclopropanones and their imine derivatives to nucleophilic addition has been exploited in the inhibition of alcohol dehydrogenase [3] and cytochromes P450.[4,5] A few years ago, we reported that a cyclopropylmethanol, *exo*-bicyclo[4.1.0]heptan-7-ylmethanol irreversibly inhibits horse liver alcohol dehydrogenase (HLADH, E.C.1.1.1.1) in a time-dependent manner consistent with covalent bond formation between inhibitor and enzyme.[6] In this paper we show that a similar reaction occurs with several cyclopropylmethanols and that the related enzyme, lactate dehydrogenase (LDH, E.C.1.1.1.27), is inhibited by substrate analogues containing a cyclopropane ring. For both enzymes, inhibition appeared to be most effective during hydride transfer to NAD^+ from the inhibitor.



Results and Discussion

Alcohol Dehydrogenase.—The series of compounds tested is listed in Table 1. Synthesis followed standard routes typically *via* cyclopropanation of the corresponding alkene. Separation of isomeric mixtures of intermediates of products was usually accomplished by preparative scale g.l.c. (see Experimental section). It was found that ^{13}C n.m.r. spectroscopy greatly assisted characterisation of stereoisomers (Table 2); the resonance of the *endo* (*cis*) carbon bearing the alcohol always appeared 8—9 p.p.m. to lower field than the analogous resonance of the *exo* (*trans*) isomer.

The kinetic properties of the compounds tested (shown in Table 1) were determined by normal procedures using the absorption of NADH at 340 nm to measure enzyme activity (see Experimental section). In no case was a highly inhibited enzyme sample re-activated in gel filtration (Sephadex G10). A typical time course for inhibition by a potent inhibitor is shown in Figure 1; a notable feature of this first-order plot is that the reaction is biphasic. This property will be discussed further below. Many of the binding and inhibitory properties of the compounds tested can be understood with regard to the known binding preferences of HLADH.[7] Thus the secondary alcohols (5) and (7) bound less than ten-times as well as the primary alcohols; strongest binding was found with the bicyclic compounds (1) and (2) which presumably interact favourably with the hydrophobic binding site of the enzyme. The closely related monocyclic compounds (4a) and (4b) have greater flexibility than (1) and hindrance to binding by the mobile ethyl groups becomes detectable, especially in the *cis*-isomer (4b) which binds five times more weakly than either (1) or (2). The maximum rate constants for oxidation of the compounds varied over a much narrower range than the Michaelis constants. The rate of oxidation of the secondary alcohols were close to those of the primary alcohols. A clear summary of the effects of structure upon binding and oxidation comes from the ratio $k_{ox.}/K_m$, the compounds lacking hydrophobic character, (6) and (7), having the lowest values.

The above variations are not large and the major effect of

Table 1. Inhibitory properties of cyclopropane-containing inhibitors of HLADH

Compound	$K_m \times 10^5$/ mol l^{-1}	$k_{ox.}/s^{-1}$	$k_{inhib.} \times 10^3/s^{-1}$	$\frac{k_{ox.}}{k_{inhib.}}$	$\frac{k_{ox.}}{K_m \times 10^3}$	$\frac{k_{inhib.}}{K_m}$
Ethanol	26.9	3.33				
(1a)	1.92	1.70	13.5	126	88.5	703.1
(1b) [a]						
(2)	1.95	1.75	10.6	165	89.7	543.6
(3)	4.95	2.25	5.10	442	45.5	103.0
(4a)	6.38	4.72	5.13	920	74.0	80.4
(4b)	10.7	4.72	2.79	1 700	44.1	26.1
(5)	18.6	2.42	2.27	1 070	61.5	62.8
Cyclohexanol	34.6	5.25				
(6)	6.89	4.24	4.33	980	13.0	12.2
(7)	70.3	7.75	2.19	3 540	11.0	3.11
	$K_m \times 10^2$	$k_{red.}$	$\frac{k_{red.}}{K_m}$			
(8) [b]						
(9)	3.11	1.88	60.5			
Cyclohexanone	1.86	67.5	3 630			
(10)	13.4	0.625	4.66			

K_m = Michaelis constant, $k_{ox.}$ = maximum initial rate constant for oxidation, $k_{inhib.}$ = rate constant for first phase of inhibition, $k_{red.}$ = initial rate constant for reduction.

[a] See Discussion section. [b] Inhibits too rapidly.

Table 2. ^{13}C N.m.r. resonances of CH_2OH carbons

Compound	δ/p.p.m. *exo,trans*	δ/p.p.m. *endo,cis*
(1a)	67.71	
(2)	65.83	
(3)	65.22	56.73
(4a)	67.34	
(4b)		59.59
(6)	67.28	

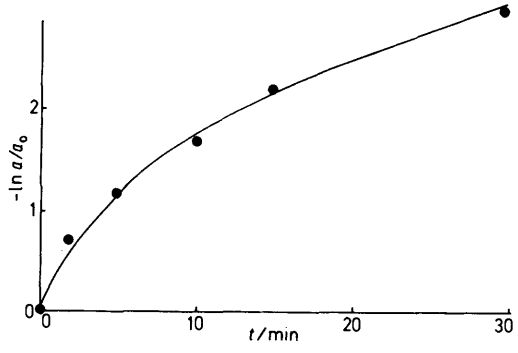


Figure 1. Inhibition of HLADH (7.5×10^{-5}M) by (1) (1.62×10^{-3}M) and NAD^+ (1.5×10^{-3}M) in 0.1M pH 9 phosphate buffer at 30 °C

structural changes was revealed when the efficiency of the compounds as inhibitors was assessed. A convenient measure of the inhibitory potency of a latent inhibitor is the average number of normal reactions, in this case oxidations, that take place for every inhibition; the ratio of the rate constants $k_{ox.}/k_{inhib.}$ gives the appropriate figure (Table 1). It has been reported that the rates of solvolysis of the 3,5-dinitrobenzoates of several of the alcohols tested or close relatives are very sensitive to structure. Thus the derivative of *exo*-bicyclo-[4.1.0]heptan-7-ylmethanol solvolysed 220 times faster than the parent compound, cyclopropylmethanol, and this rate difference was attributed to the greater strain present in the three-membered ring of the bicyclic compound.[8,9] We found that the bicyclic compounds (1) and (2) were better inhibitors than cyclopropylmethanol (6) but only by a factor of 3—7. The potency of a compound as an inhibitor therefore is not a simple function of the strain in the small ring in these compounds. The related secondary alcohols (5) and (7) clarify the patterns of reactivity. These compounds have similar inherent strain to the primary analogues but both are poorer inhibitors, despite their relative ease of oxidation. It can be concluded from these results that the relative position of the enzyme nucleophile with respect to the electrophilic carbon atom of the cyclopropane ring plays a major part in determining the efficiency of a compound as an inhibitor. An important feature of the results for the general application of cyclopropanes in enzyme inhibition is that an unfused cyclopropane ring has sufficient reactivity to be an active inhibitor. Both of these conclusions are strengthened by the results obtained for LDH described below.

Biphasic behaviour. Usually, latent irreversible inhibitors show good first-order kinetic behaviour over the time required for *ca.* 90% inhibition. The marked deviations from this behaviour for (1) merited further investigation. There has been discussion about whether HLADH, which is a dimeric enzyme, exhibits ' half of the sites ' reactivity; [10] if the enzyme can indeed behave in this way, then inhibition of one active site could lower the activity of the second leading to two phases of first-order kinetic behaviour as shown in Figure 1. In our previous work, we found that inhibition by an α,β-unsaturated aldehyde that could liberate a persistent competitive inhibitor, ethanethiol, showed good first-order kinetic behaviour. It was therefore of interest to examine the time course for inhibition of HLADH by more typical α,β-unsaturated carbonyl compounds for comparison with our earlier studies [1] and with this work. We found that crotonaldehyde and (−)-carvone both behaved in the same way as (1); the logarithmic inhibition plot for (−)-carvone is shown in Figure 2. Similarly, compounds that can give rise to α,β-unsaturated ketones or aldehydes at the enzyme's active site showed biphasic inhibition properties. 2-Vinylcyclohexanol and cyclohexenyl-2-acetaldehyde were examples tested; the activity of the former compound accounts for inhibition of HLADH by the methanol (1) in the presence of NADH noted

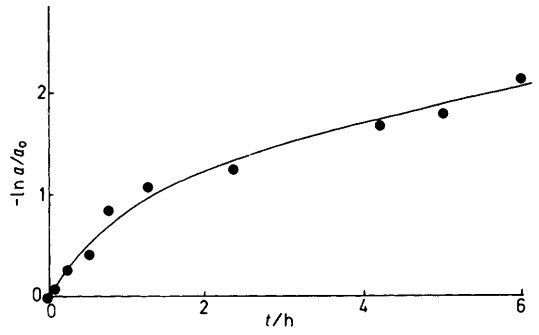


Figure 2. Inhibition of HLADH (7.5×10^{-5}M) by (−)-carvone (1.15×10^{-3}M) in 0.1M pH 9 phosphate buffer at 30 °C

in our preliminary report [6] (see also below). Finally *exo*-bicyclo[4.1.0]heptane-7-carbaldehyde (8) also showed biphasic inhibition, although in this case the first phase proceeded within 5 s to *ca.* 80% inhibition. It therefore appears that biphasic behaviour is normal for HLADH in these nucleophilic inhibition reactions.

The site of attack by the enzyme on the inhibitor. There are two possible sites for attack by the enzyme on each inhibitor, namely the oxygen-bearing carbon atom and the vinylogous or homovinylogous carbon atom. Our previous results with thioalkyl allylic alcohols [1] cannot easily be explained by attack at the oxygen-bearing atom and it is likely that the same situation obtains for the compounds described in this paper. We are carrying out experiments with isotopically labelled inhibitors to investigate this matter. Inhibition of 2-vinylcyclohexanol is likely to conform to this pattern of behaviour because, after oxidation to the ketone, enzyme-catalysed tautomerism again leads to an α,β-unsaturated ketone.

The production of 2-vinylcyclohexanol. Early in the work on the alcohol (1), we found that 2-vinylcyclohexanol was a by-product of the oxidation–inhibition reaction and was produced in 5—10% yield when the alcohol, HLADH, and the reducing coenzyme, NADH, were allowed to react.[6] This reaction corresponds to enzyme-catalysed solvolytic cleavage of the three-membered ring. Similar reactions have been observed with cyclopropyl alcohols in ether in the presence of Lewis acids,[11] but the enzyme-catalysed analogue that we observed was unusual in that it involved cleavage of an alkyl–oxygen bond. Two major mechanisms appeared possible for this reaction. Firstly, nucleophilic attack by the enzyme could lead to a covalently modified enzyme which, on hydrolysis by water at the active site, would lead to *cis*-2-vinylcyclohexanol (Figure 3a). Secondly, the enzyme could act as a general base catalyst (Figure 3b) and cause hydrolysis of the methanol (1) in one step; this would necessarily form *trans*-2-vinylcyclohexanol. An authentic sample of the *trans*-isomer was obtained from opening of cyclohexene epoxide by acetylide followed by reduction of the alkene, and a sample was compared with the product isolated from a large scale enzymic reaction. The two samples were found to be identical in all respects and there was no trace of an isomeric product in the enzyme-generated material. It is therefore probable that HLADH catalyses this hydrolysis reaction as a general base using the charge relay triad present at the active site.[10] The only other compound of those tested to generate by-products was the unsaturated bicyclic alcohol (3). Many products as yet uncharacterised were present in preparative scale incubations of this compound with HLADH. No doubt the wide range of reaction paths offered by the presence of the double bond accounts for this behaviour. The hydrolytic ability of

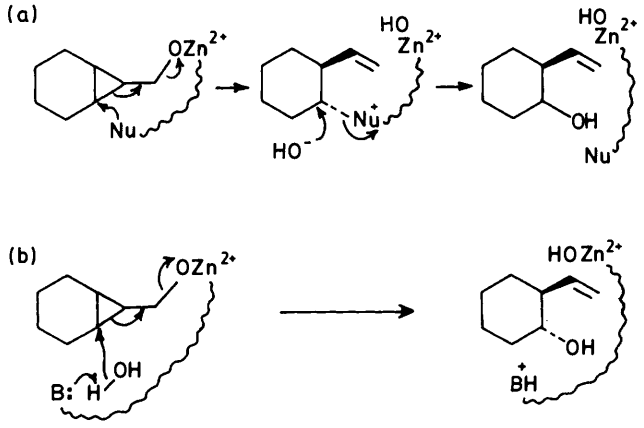


Figure 3. Mechanisms of solvolytic ring cleavage of the methanol (1) by HLADH: (a) nucleophilic cleavage followed by hydrolysis; (b) general base-catalysed hydrolysis

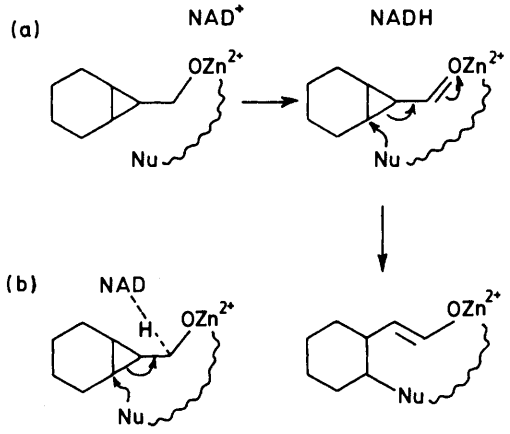


Figure 4. Design strategy. for cyclopropyl inhibitors: (a) planned, (b) observed

the enzyme has been noted in our earlier work [1] and by others.[12,13]

The timing of inhibition and the mechanism of oxidation. Our original concept in designing cyclopropyl inhibitors of HLADH was that, after oxidation of the alcohol, the corresponding carbonyl group would be sufficiently polarised by the Lewis acid zinc ion at the active site to encourage nucleophilic attack by the enzyme upon the inhibitor in a homo-Michael addition (Figure 4a). The potency of the aldehyde (8) as an inhibitor tended to support this concept. However, it turns out that this aldehyde is the only carbonyl compound that we have tested which is an effective inhibitor. The ketones (9) and (10) in particular scarcely caused inhibition at all even though the corresponding alcohols are oxidised at reasonable rates and are typical inhibitors. This contrasting behaviour is illustrated in Figure 5. It is notable that the ketones bind very poorly to the enzyme (Table 1) and this result together with their poor inhibitory potency suggested that inhibition occurs before the ketone is formed. The possibility then came to mind that inhibition was taking place as hydride was being removed from the inhibitor by the coenzyme. If full formation of the carbonyl double bond lagged behind this event, then the maximum generation of positive charge at the oxygen-bearing carbon would occur during oxidation; at this point on the reaction co-ordinate, the activation of the cyclopropane ring would also be maximal. Evidence consistent with this course of

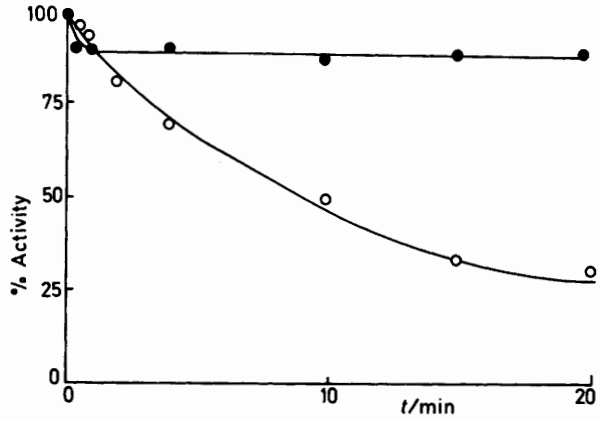
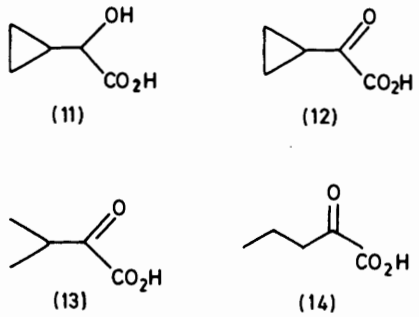


Figure 5. Inhibition of HLADH by cyclopropyl methyl ketone (3.57×10^{-3}M) ● and 1-cyclopropylethanol (2.49×10^{-3}M) ○ in 0.1M pH 9 phosphate buffer at 30 °C



events came from a study of the kinetics of inhibition of the protio alcohol (1a), described above, and the 8-dideuteriated analogue (1b). We found that the deuteriated compound (1b) inhibited the enzyme measurably more effectively than its protio analogue. There was a small isotope effect for oxidation, $k_{ox.H}/k_{ox.D} = 1.08$, but a surprisingly large inverse isotope effect for inhibition, $k_{Inhib.H}/k_{Inhib.D} = 0.75$. The latter result is contrary to what would be expected if the chief mechanism of inhibition was through the aldehyde (8); in that case the two isotope effects would be expected to be equal. The inverse deuterium isotope effect of 0.75 is larger than would be expected for a steric secondary isotope effect. There is in any case doubt as to whether the enzyme's active site is sufficiently rigid for such an effect to be detectable [14] on the binding of a substrate and we found that the K_m values of (1a) and (1b) were the same within experimental error. If bonding of the enzyme to the inhibitor occurs prior to full formation of the carbonyl group, then the slower oxidation of the deuterio alcohol (1b) might permit a higher rate of inhibition with that compound. Such an event would be facilitated by hydride (deuteride) transfer. The unusual isotope effects and the behaviour of the ketones therefore lend support to the hydridic nature of oxidation by NAD^+ and HLADH, as we had deduced from product studies with these cyclopropyl compounds.[15] As will now be described, results in complete agreement with this conclusion have been obtained with LDH.

Lactate Dehydrogenase.—Since LDH has more stringent substrate requirements than HLADH, we thought it worthwhile to assess the effect of the cyclopropane ring in the potential inhibitor, cyclopropylglycolic acid (11), on binding to the enzyme. Our data were principally assembled from keto acids (12)—(14) (Table 3) in comparison with the data of

Table 3. Kinetic properties of substrates and inhibitors of LDH. For reaction conditions, see Experimental section

Compound	$K_m \times 10^3$M	$k_{ox.}, k_{red.}/s^{-1}$	$k_{Inhib.}/s^{-1}$
(11)	0.72	3.83	0.014
(12)	3.73	61.7	
(13)	3.29	2.70	
(14)	1.56	69.3	
$CH_3CO{\cdot}CO_2H$	0.05 [15]		

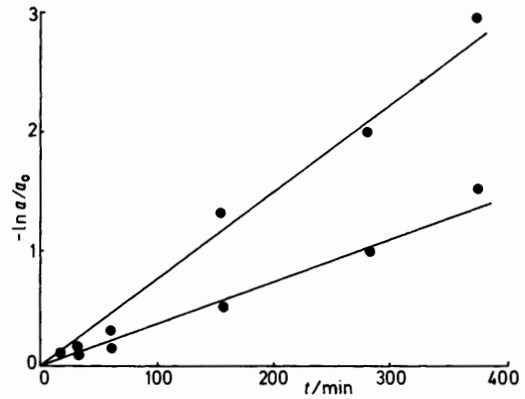


Figure 6. Inhibition of LDH (1.33×10^{-9}M) by potassium cyclopropylglyoxylate (1.04, 2.08×10^{-3}M) in 0.07M pH 7.4 phosphate buffer at 30 °C

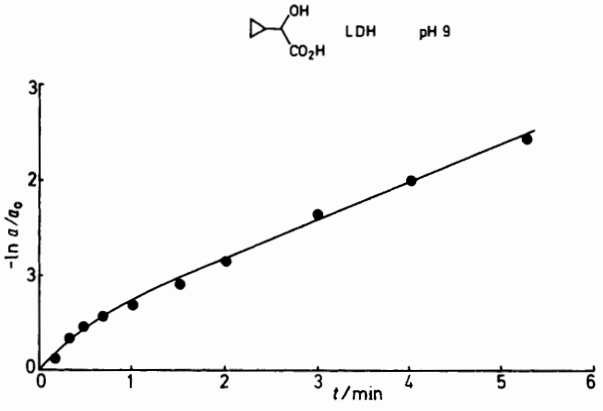


Figure 7. Inhibition of LDH (5.95×10^{-7}M) by cyclopropylglycolic acid (4.16×10^{-3}M) in 0.1M phosphate buffer, pH 9 at 30 °C

Meister for an extensive series of straight chain α-keto acids.[16] From the results, it was clear that the branched alkyl and cycloalkyl substituents interfere significantly with binding to LDH, although they are still satisfactory substrates. The first experiments that we carried out on the inhibition properties of the cyclopropyl compounds (11) and (12) used the keto acid (12). In the presence of NADH, this compound was found to be a slow inhibitor of LDH, the half life of the enzyme being several hours (Figure 6). However the corresponding hydroxy acid (11) proved to be an extremely potent inhibitor with 50% inhibition occurring in 1 min under standard conditions (see Experimental section). The inhibition was time-dependent, again with some biphasic character (Figure 7), irreversible, and could be prevented by the presence of sodium pyruvate. The ratio of oxidative reactions to inhibition reactions was 273 : 1, a number comparable with the most potent HLADH

inhibitors. Since LDH is stereoselective with respect to the L-isomer of lactic acid, it is possible that inhibition could be effected by the D-isomer of cyclopropylglycolic acid present in our racemic sample. However such inhibition would show no time dependence. The product cyclopropylglyoxylate was shown above to be a poor inhibitor, a major factor in its inactivity presumably being the relative weakness with which it binds to the enzyme compared with the corresponding alcohol. It therefore seems most probable that inhibition occurs during hydride abstraction from the hydroxy acid (11) in an analogous manner to inhibition of HLADH and in agreement with our product studies and model experiments.[17]

General Conclusions.—Our experience with HLADH and LDH leads to several significant conclusions. Firstly, the mechanism of hydrogen transfer by both these enzymes during oxidation is confirmed to be hydride-like. Secondly, a cyclopropane ring without additional strain is reactive enough to generate an effective latent irreversible enzyme inhibitor when suitably oriented at the active site. Thirdly, an enzyme with a fairly specific substrate requirement (LDH) is efficiently inhibited by a cyclopropyl analogue of its substrate. It can therefore be asserted that the cyclopropyl group meets the requirements for a basis for the design of the latent inhibitors outlined above. We are actively investigating the application of this strategy to several other enzymes.

Experimental

^{1}H N.m.r. spectra (δ scale) were recorded at 90 MHz on a Perkin-Elmer R 32 spectrometer, except where otherwise stated. ^{13}C N.m.r. spectra were recorded on a Jeol PS 100 n.m.r. spectrometer operating at 23.4 MHz. G.l.c. chromatography was carried out on a Perkin-Elmer F33 or Sigma 3B instrument using: A, 5% FFAP on Chromosorb G; and B, 5% Apiezon on Chromosorb G. Ether refers to diethyl ether.

Synthesis.—exo-*Bicyclo*[4.1.0]*heptan-7-ylmethanol* [18] (1a) was prepared from the corresponding carboxylic acid [19] by reduction with lithium aluminium hydride in ether as described below. The product obtained after work-up and distillation (b.p. 50 °C at 2 Torr) contained an impurity (*ca.* 5%) which was removed by preparative g.l.c. (20 ft; 25% Apiezon on Chromosorb G at 160 °C) to give an overall yield of 40% of the required alcohol (Found C, 77.0; H, 10.9; $C_{18}H_{14}O$ requires C, 76.2; H, 11.1%) (Found: M^+ 126.1058. $C_8H_{14}O$ requires M 126.1045). G.l.c.: A at 160 °C, single peak, 3.7 min; B at 120 °C, single peak, 7 min. δ ($CDCl_3$) 0.7—1.0 (3 H, m), 1.1—1.5 and 1.6—2.1, (8 H, m), 3.4 (2 H, d), and 3.0 (1 H, s). The dideuterio analogue (1b) was prepared in an analogous manner to the above compound with lithium aluminium deuteride, in 40% yield. δ ($CDCl_3$) 0.7—1.0 (3 H, m), 1.1—1.4 and 1.5—1.9 (8 H, m), and 2.1 (1 H, s). The resonance at δ 3.4 was absent.

exo-*Bicyclo*[3.1.0]*hexan-6-ylmethanol* (2) was prepared similarly in 56% yield from the corresponding pure *exo*-acid.[20] Preparative g.l.c. purification was unnecessary as the compound (b.p. 46—48 °C at 0.5 Torr) was pure by g.l.c. (A at 120 °C, 18.2 min; B at 120 °C, 9.0 min) (Found: M^+ 128.0892. $C_7H_{12}O$ requires M 128.0888), δ ($CDCl_3$) 0.7—1.0 (1 H, m), 1.0—1.2 (2 H, m), 1.3—1.9 (6 H, m), 2.2 (1 H, s), and 3.4 (2 H, d).

endo- *and* exo-*Bicyclo*[3.1.0]*hex-2-en-6-ylmethanol* (3) was prepared in the same way from the corresponding carboxylic acid [21] as a *ca.* 1 : 4 *endo* : *exo* mixture, b.p. 50 °C at 0.1 Torr (Found: C, 76.3; H, 9.7. $C_7H_{10}O$ requires C, 76.3; H, 9.2%) (Found: M^+ 110.0728. $C_7H_{10}O$ requires M 110.0732). δ ($CDCl_3$) 0.4—0.7 (1 H, m), 1.4—1.7, 1.7—1.85 (2 H, m), 2.0—2.15 (1 H, s), 2.35—2.6 (2 H, m), 3.3—3.6 (2 H, dd), 5.3—5.5 (1 H, m), and 5.8—6.0 (1 H, m). G.l.c.: A at 120 °C, single peak 10.8 min; B at 100 °C, peak 5.8 min with shoulder (*ca.* 20% area, *endo*-isomer), 6.4 min.

trans,trans- (4a) *and* cis,cis-2,3-*Diethylcyclopropylmethanol* (4b).—To *cis*-hex-3-ene [21] (21 g, 0.25 mol) in hexane (100 ml) was added dry copper (5 g, freshly precipitated from aqueous copper sulphate with zinc dust) and anhydrous copper sulphate (15 g, freshly dried). The mixture was heated to boiling and ethyl diazoacetate (30 ml, 0.25 mol) added. After the addition was complete, the mixture was heated under reflux for 1 h, filtered, and the hexane distilled off. The residue was distilled under reduced pressure to give a mixture of the isomeric esters (33%), b.p. 100—108 °C at 20 Torr.

To a stirred suspension of lithium aluminium hydride (3 g, 0.081 mol) in dry ether (100 ml) was added the mixture of the above esters (13.8 g, 0.081 mol) in dry ether (50 ml). After the addition was complete, the reaction mixture was stirred for 1 h. Sodium hydroxide (3 g as a 15% w/v aqueous solution) was added dropwise followed by water (12 ml). The precipitate was filtered off, the ether solution dried (Na_2SO_4), and the ether evaporated under reduced pressure. The residue was distilled (b.p. 70—80 °C at 10 Torr). The mixture of isomers was separated by preparative g.l.c. (20 ft, 25% Apiezon on Chromosorb G at 120 °C) affording a 50% yield of trans,trans-2,3-*diethylcyclopropylmethanol* (4a) and cis,cis-2,3-*diethylcyclopropylmethanol* (4b) in a 2 : 1 ratio. The isomers were identified by ^{13}C n.m.r. (Table 2). *trans,trans*-Isomer (4a) (Found: C, 74.9; H, 12.8. $C_8H_{16}O$ requires C, 74.9; H, 12.6%) (Found: M^+ 128.1201. $C_8H_{16}O$ requires M 128.1201). δ ($CDCl_3$) 0.4—0.7 (3 H, m), 0.9—1.1 (6 H, m), 1.2—1.5 (4 H, m), 2.1 (1 H, s), and 3.45 (2 H, d). G.l.c.: A at 100 °C, single peak 20.8 min; B at 100 °C, single peak, 12.0 min. *cis,cis*-Isomer (4b) (Found: M^+ 128.1208. $C_8H_{16}O$ requires M 128.1201). δ ($CDCl_3$) 0.8—1.1 (9 H, m), 1.2—1.5 (4 H, m), 1.5 (1 H, s), and 3.65 (2 H, d).

Bicyclo[4.1.0]heptan-2-ol [23] (5) was prepared by Miss L. Brown from cyclohexen-2-ol. The product had b.p. 57—60 °C at 10 Torr.

Cyclopropylmethanol (6) was prepared by reduction of cyclopropanecarboxylic acid (Aldrich) with lithium aluminium hydride.

1-*Cyclopropylethanol* (7). This was prepared by reduction of the corresponding ketone (Aldrich) with lithium aluminium hydride. Evaporation of the ether solvent during work-up was carried out with a Vigreux column. The *product* had b.p. 120—122 °C (atmospheric pressure) (Found: C, 70.1; H, 11.9. $C_5H_{10}O$ requires C, 69.8; H, 11.6%). δ 0.1—1.1 (5 H, m), 1.3 (3 H, d), and 2.95—3.25 (1 H, dq). G.l.c. on A at 100 °C, single peak in 4.9 min.

exo-*Bicyclo*[4.1.0]*heptane-7-carbaldehyde* (8).[24] To a mixture of freshly distilled dichloromethane (150 ml; from P_4O_{10}) and freshly distilled pyridine (26 ml; from BaO) at 0 °C was added chromium trioxide (16.2 g) with continuous stirring, the temperature being kept below 5 °C. After the addition was complete, the solution was stirred for 1 h and a solution of *exo*-bicyclo[4.1.0]heptan-7-ylmethanol (1) (2.5 g) in dry dichloromethane (12 ml) added dropwise, again keeping the temperature below 5 °C. Reaction was allowed to continue after the addition for 1½ h and the mixture was then poured into ether (200 ml). The organic solution was washed with aqueous sodium hydroxide (4 × 100 ml; 5% w/v), aqueous sodium hydrogen carbonate (100 ml, 5% w/v), and water (2 × 100 ml), dried ($Na_2S_2O_4$) and concentrated under reduced pressure. The residue was distilled to give the required *aldehyde*, b.p. 28 °C at 1 Torr (60%) (Found: M^+ 124.0909. $C_8H_{12}O$ requires M 124.0888). δ ($CDCl_3$) 1.1—1.5 and 1.6—

2.1 (11 H, m), and 9.05 (1 H, d). G.l.c. on A at 160 °C, single peak, 2.6 min; on B at 120 °C, single peak, 7.7 min.

Bicyclo[4.1.0]heptan-2-one (9) was prepared by Miss Linda Brown by oxidation of the corresponding alcohol with chromium trioxide in pyridine. The product had b.p. 56—58 °C at 10 Torr.

Cyclopropyl methyl ketone (10) was purchased from Aldrich and was redistilled before use.

2-Ethynylcyclohexanol was prepared by the method of Inhoffen.[25]

trans-2-Vinylcyclohexanol was prepared by hydrogenation of the preceding compound.[26] δ (250 MHz; Bruker WH 250; $CDCl_3$) 1.17—1.38 (4 H, m), 1.65—1.98 (4 H, m), 2.06 (1 H, m), 3.28 (1 H, m), 5.10—5.22 (2 H), and 5.62—5.78 (1 H, AB_2 pattern). G.l.c. on B at 120 °C single peak, 4.5 min.

Cyclopropylglycolic acid (11) was a gift from Burroughs Wellcome, Research Triangle Park, North Carolina, U.S.A.

Potassium cyclopropylglyoxylate (12) was prepared by oxidation of cyclopropyl methyl ketone with potassium permanganate.[27]

Kinetics.—Individual data points in kinetic experiments were determined at least in triplicate and the precision of the results was typically ±3%. Michaelis constants (K_m) were determined from initial rate measurements at a fixed enzyme concentration and at a minimum of five substrate concentrations. K_m was evaluated using the method of Cornish-Bowden [28] or using a numerical solution calculated by computer to an equation kindly supplied by Dr. Nimmo, Department of Biochemistry, University of Edinburgh.

The rate constants for oxidation $k_{ox.}$ and reduction $k_{red.}$ were derived from the steady state equation:

$$v_0 = \frac{k[E_0][S_0]}{K_m + [S_0]}$$

where v_0, $[E_0]$, and $[S_0]$ are the initial velocities, enzyme and substrate concentrations, respectively determined under conditions where the enzyme is saturated with substrate. A plot of v_0 against $[E_0][S_0]/K_m + [S_0]$ was a straight line of gradient $k_{ox.}$ or $k_{red.}$. Reactions in the oxidative direction were carried out at 30 °C in 0.1M-phosphate buffer, pH 9, and in the reductive direction at 30 °C in 0.1M-phosphate buffer, pH 7, following the reaction by the absorption of NADH at 340 nm. Rates of inhibition were determined from larger scale reactions, samples of which were removed at intervals, and the residual enzyme activity determined by saturating with a non-inhibiting substrate (ethanol or sodium lactate). Conditions were such that enzyme was saturated with inhibitor. A plot of $-\ln a_t/a_0$, where a_t is the enzyme activity remaining at time t and a_0 is the initial enzyme activity against time, afforded $k_{inhib.}$ from the gradient.

For HLADH. Typical conditions for K_m, $k_{ox.}$: enzyme 3.75×10^{-7}M in sample u.v. cell, NAD^+ 1.5×10^{-3}M in sample and reference cells. Substrate 2×10^{-3}—5×10^{-5}M in both cells. All the constants reported were with the same batch of enzyme (Sigma); different batches can give kinetic constants that differ by as much as 20%. For $k_{inhib.}$, concentrations in the u.v. cell were in the same range as above and the measurement was initiated by the addition of NAD^+ solution (0.1 ml; 4.5×10^{-2}M) followed by ethanol (0.05 ml) to the sample under test (2.85 ml).

For LDH. Typical conditions used 6×10^{-7}M enzyme (pig heart isozyme, Boehringer, Mannheim), but were otherwise analogous to those for HLADH.

Preparative scale enzymic reactions. These were carried out following Jones [29] using 1 mmol of substrate. In the oxidising direction, sodium riboflavin-5′-phosphate was used to recycle NAD^+. Products were isolated by continuous extraction with ethyl acetate from reaction solutions saturated with sodium sulphate. Control experiments omitting substrate were carried out to identify potential spurious by-products. In the reductive direction, sodium dithionite was used to recycle NADH.

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