Tetrahedron Vol. 42, No. 20, pp. 5665 to 5677, 1986
Printed in Great Britain.

0040-4020/86 $5.00+.00
Pergamon Journals Ltd.

ENZYME INHIBITION BY ELECTROPHILIC CYCLOPROPANE DERIVATIVES

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Abstract: Electrophilic cyclopropane-containing compounds have been found in our previous studies to be latent, irreversible inhibitors of horse liver alcohol dehydrogenase, lactate dehydrogenase, and carboxypeptidase A. In addition, cyclopropyl methanols have provided information concerning the mechanism of hydrogen transfer by nicotinamide coenzymes and dehydrogenases. These results are evaluated with the aid of molecular graphics and frontier orbital considerations in the light of the established stereochemical courses of reactions involving cyclopropane ring opening. It is concluded that mechanisms for nucleophilic inhibition consistent with chemical theory and precedent are available at each enzyme's active site. Potential enzymic nucleophiles are identifed.

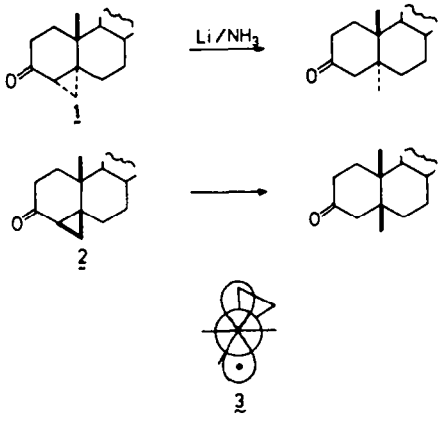
Our recent studies of the enzyme chemistry of cyclopropane containing compounds[1] have shown that they can act as inhibitors of alcohol and lactate dehydrogenases[2] and of carboxypeptidase A[3]. With alcohol dehydrogenase, it was also possible to use cyclopropyl methanols to probe the mechanism of hydrogen transfer between substrate and the coenzyme NAD(H).[4] The structure of each of these enzymes is known at atomic resolution thus providing the opportunity to investigate the likely interactions of the cyclopropane derivatives with the appropriate enzyme. There is also a considerable body of information concerning the stereochemical course of ring opening reactions of cyclopropanes[5] and such observations have been related to the expectations of theoretical arguments. It was therefore of interest to correlate these experimental and theoretical considerations with the environment and reactivity of the cyclopropane-containing compounds at the enzymes' active sites.

The molecular models for each enzyme were constructed using the molecular graphics package, INTERCHEM, developed at the University of Strathclyde by Bladon and Breckenridge,[6] starting from coordinates in the Brookhaven Protein Data Bank. The active site region was excised and the cyclopropane-containing compounds inserted into the active site by superimposition upon the substrate or inhibitor in the position bound in the crystal. The substrate or inhibitor was then deleted and the molecular array containing the cyclopropane derivative examined for unfavourable steric interactions by rotation about its available rotation axes and assessment of steric interactions using a Lennard Jones potential function. It was found that conformations were available in which the inhibitors can take up reasonable geometries for the expected reactions on the basis of precedent and theory without severe steric penalties. Three events were examined:
1. the availability of space and stereoelectronic requirements for the ring opening of a putative cyclopropylalkyl radical intermediate during hydrogen transfer by horse liver alcohol dehydrogenase and NAD(H), 2. the relationship between nucleophile and cyclopropane ring during inhibition, and 3. the relationship between the leaving group (H^-) in the

dehydrogenases reactions and the C-C bond of the cyclopropane ring that undergoes cleavage during inhibition. The relevant experimental and theoretical background will firstly be summarised.

Background and frontier orbital considerations

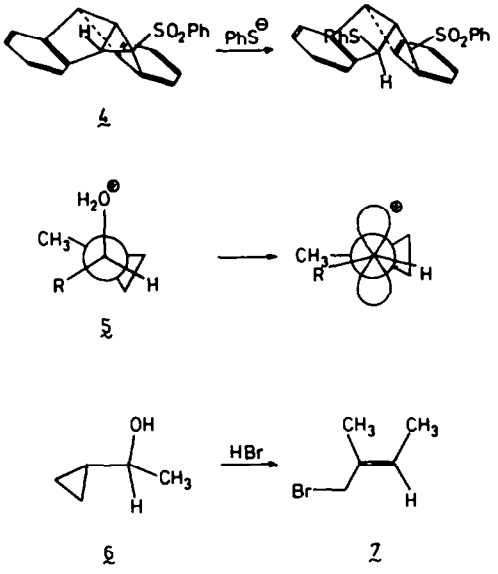
Ring opening of cyclopropylalkyl radicals The rapid ring opening of cyclopropylalkyl radicals to yield homoallyl radicals[7] is a reaction of mechanistic and synthetic interest and a stereoelectronic requirement for ring opening has been established from reactions occurring in fused ring systems. This is well illustrated by the contrasting courses of the dissolving metal reduction of the cyclopropyl steroids **1** and **2** studied by Dauben (Scheme 1).[8] Such reactions show that the bond that is broken is coplanar with the developing half-occupied p-orbital of the radical centre as shown in **3**.



1 2 3

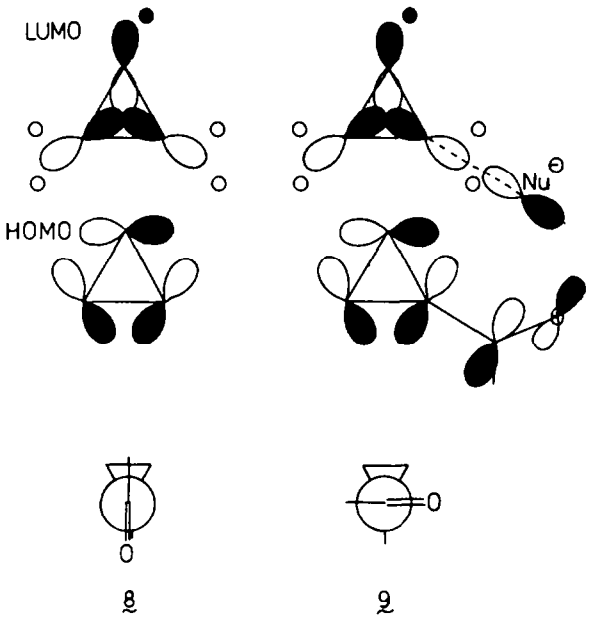
Scheme 1 Stereoselectivity of ring opening of cyclopropyl alkyl radicals

Nucleophilic ring opening of cyclopropanes The enzyme-catalysed transformation of presqualene into squalene is a classic example of the nucleophilic ring opening of a cyclopropane in biological chemistry[9] and the relevant nucleophile is hydride from NADPH. It has been demonstrated that the reaction takes place with inversion of configuration at the site of hydride attack and this result correlates well with the course of the ring opening of dibenzotricyclooctane derivatives such as **4** (Scheme 2).[10] Further, the cleaved cyclopropane bond has been shown to be the bond that better overlaps with the developing positive charge as the leaving group departs; thus the leaving group and the cleaved bond have been assigned the antiperiplanar conformation **5**.[11] The stereoselectivity of the reaction is expressed in the synthesis of E-substituted alkenes first introduced by Julia[12] and extended by Johnson[13] (e.g. **6** -> **7**), the less crowded of the two possible arrangements to the incipient cation being favoured. This stereochemical requirement is closely analogous to that for the ring opening of cyclopropylalkyl radicals as would be expected.



Scheme 2 Stereochemical course of nucleophilic ring opening of substituted cyclopropanes

Frontier orbital considerations In all of the inhibition events in the enzyme mediated processes to be discussed, the reaction can be considered as involving a cyclopropane conjugated to an electron withdrawing substituent such as a carbonyl group. So that all reaction types can be included with the same set of orbitals, the frontier orbitals of cyclopropane shown have been constructed from all the available s and p orbitals and are as shown in Scheme 3. These orbitals correlate well with those of the familiar Walsh model.[5] The effect of the acceptor group upon the cyclopropane can be associated with an interaction between the antisymmetric highest occupied bonding orbital of the cyclopropane and the antibonding orbital of the carbonyl group, which is also antisymmetric. Calculations on the related cyclopropylmethyl cation[14] indicate that the bisected conformation **8** is favoured over the orthogonal rotamer **9** by as much as 70 kJ mol^{-1}. Together, these arguments provide a firm guide for the geometry to be expected of an inhibitor in which the cyclopropane is activated by an electron withdrawing substituent.

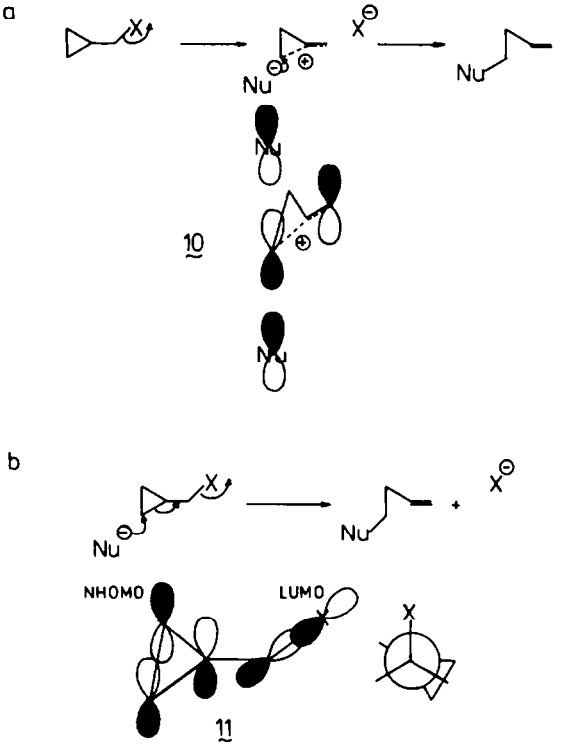


Scheme 3 Frontier molecular orbitals of cyclopropane and their interactions with σ-donors and π-acceptors.

Although this mixing of orbitals provides a useful indication of the relationship between electron acceptor or leaving group and the cyclopropane ring, it is silent concerning the direction of attack of the nucelophile. By analogy with the well-studied case of the S_N2 reaction,[15] a low lying unoccupied orbital of suitable symmetry to interact with a non-bonded electron pair such that occupation of the orbital leads to bond weakening is required. The LUMO of cyclopropane (Scheme 3) is of suitable symmetry. Maximum overlap of these two orbitals will occur when the nucleophile approaches the cyclopropane ring in the plane of the ring and making an angle of 150° with the target carbon atom and the substituted carbon atom of the cyclopropane ring. Although the nucleophile is not therefore expected to approach the target carbon atom from the same direction as an S_N2 reaction, such an attack would still lead to inversion of configuration, as has been observed.

Alternative mechanisms In addition to these factors applicable directly to cyclopropyl aldehydes and ketones, it is also necessary to consider the possible extremes of mechanism in an inhibition reaction involving ring opening of a cyclopropane with loss of a leaving group from the α-carbon (Scheme 4). If polarisation of the ring is so great at the active site that ring cleavage is essentially complete before nucleophilic attack occurs, then inhibition can take place by reaction of a homoallylic cation whose stereochemical

requirements are governed by a different set of orbitals (Scheme 4a). In such a case, the arguments above concerning the acceptor orbitals of the cyclopropane ring are irrelevant and one must examine the π-orbitals of a homoallyl cation. The LUMO of this system is the non-bonding orbital **10** and its symmetry leads to the conclusion that a nucleophile will attack the partially cleaved cyclopropane ring from above or below its plane. The obvious consequence of this possibility is that either inversion or retention of configuration could occur with ring opening and the reaction is analogous with an S_N1 substitution. There is, however, no experimental support for racemisation.

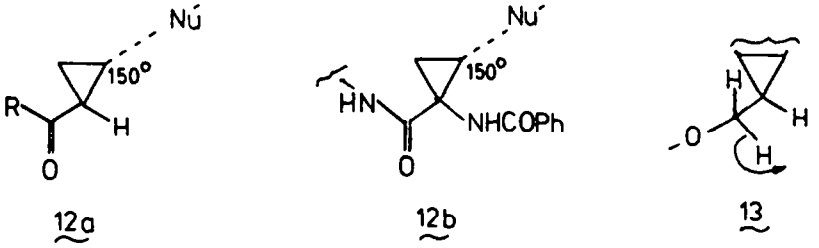


Scheme 4 Extreme mechanisms for nucleophilic ring opening of substituted cyclopropanes

At the other extreme, concerted loss of leaving group and attack of nucleophile could be envisaged and the geometrical requirements of such a reaction are closely defined by the cyclopropane ring's properties (Scheme 4b). Such a reaction has been shown to be favoured by a conformation in which the cleaved cyclopropane bond is antiperiplanar to the leaving group as discussed above, **5**. This conformation allows stabilisation of the developing

positive charge by the cyclopropane ring if the LUMO of the CH_2X fragment is mixed with a high energy (next in energy to HOMO, or NHOMO) π-bonding orbital of the cyclopropane as shown in **11**. It is notable that the relationshp of the C-X bond to the cyclopropane ring in this case differs from the bisected conformation preferred by the C=O group (**8**). Similar arguments may be advanced to rationalise the experimentally preferred conformation for ring opening of cyclopropylalkyl radicals discussed above. It is also apparent that the orbitals participating in ring opening (NHOMO) and nucleophilic attack (LUMO) in the model discussed above do not have compatible symmetries and it may therefore be argued that inhibition cannot be concerted with loss of the leaving group.

We can therefore define the expected conformations for inhibition as shown in Scheme 5. Conformation **12** is a sawhorse such as an aldehyde or ketone for the dehydrogenases (a) and a peptide for carboxypeptidase (b); the carbonyl group bisects the cyclopropane and the nucleophile makes an angle of 150° with the ring. Conformation **13** represents the arrangement for inhibition by an alcohol in which an extensively broken C-H bond provides the electron-accepting ability. The question now to be investigated is whether these conformations are available to the inhibitors at the enzymes' active site.



12a 12b 13

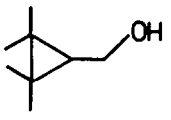
Scheme 5 Favourable conformations for ring opening reactions of cyclopropanes at the enzymes' active sites

Ring opening of cyclopropylalkyl radicals at the active site of horse liver alcohol dehydrogenase

We have found that cyclopropyl methanols are oxidised by horse liver alcohol dehydrogenase (HLADH) to the corresponding aldehydes without ring opening[4] suggesting that radical intermediates are not involved in the reaction. In order that such a conclusion can be drawn, there are several requirements that the probe molecule must satisfy. Firstly, the putative radical intermediate must not be electronically stabilised, stabilisation that can be great in the case of capto-dative substitution.[16] We have shown by extensive measurements of the rates of opening of substituted cyclopropylalkoxy radicals that a simple oxygen substituent does not stabilise the radical by more than an order of magnitude.[4,17] This is insufficient to invalidate the use of such mechanistic probes. For example, for the tetramethyl cyclopropylmethanol **14**, it was impossible to detect the unopened radical at temperatures as low as 90K implying that the rate of ring opening at room temperature must exceed $10^9 s^{-1}$ which compares with the rate of enzyme-catalysed hydrogen transfer of $10\text{-}100 s^{-1}$.[18] Secondly, the bond cleaved during ring opening must be coplanar with the singly occupied orbital of the radical as has been clearly shown in many studies mentioned.[8,19] Thirdly, the fragmented radical must be able to relax its conformation such that the product radical is not well placed to recombine and reclose the ring. In the case of the nortricyclyl radical,[20] the bicyclic structure prevents escape of the ring-opened radical and the equilibrium favours the ring-closed species. Clearly

nortricyclyl derivatives are not suitable mechanistic probes.[21] Having satisfied the electronic requirements experimentally,[17] conformational factors can be assessed with the aid of computer graphics.

Using the active site model of Branden's group,[22] we replaced the alcohol of the crystal with the probe molecule **14**. We found that it bound to the active site without introducing any severe steric interactions in the conformation for hydrogen transfer suggested by Branden. In particular, it was possible to obtain the stereoelectronically preferred conformation for ring opening with the cleaved bond coplanar with the p-orbital of a putative radical without apparent steric penalty thereby satisfying requirement two (Figure 1). To investigate the third requirement, the cyclopropane ring was cleaved in the model and rotations of the two nearly generated acyclic single bonds were examined for steric pressure for ring closure. No such pressure was found. The computer model therefore indicates that there is no impediment for a radical, if formed, to undergo ring opening at the active site of HLADH. This result reinforces our previous conclusion that the oxidation of alcohols and reduction of aldehydes and ketones by nicotinamide-dependent dehydrogenases occurs without the intermediacy of substrate radicals.[4]



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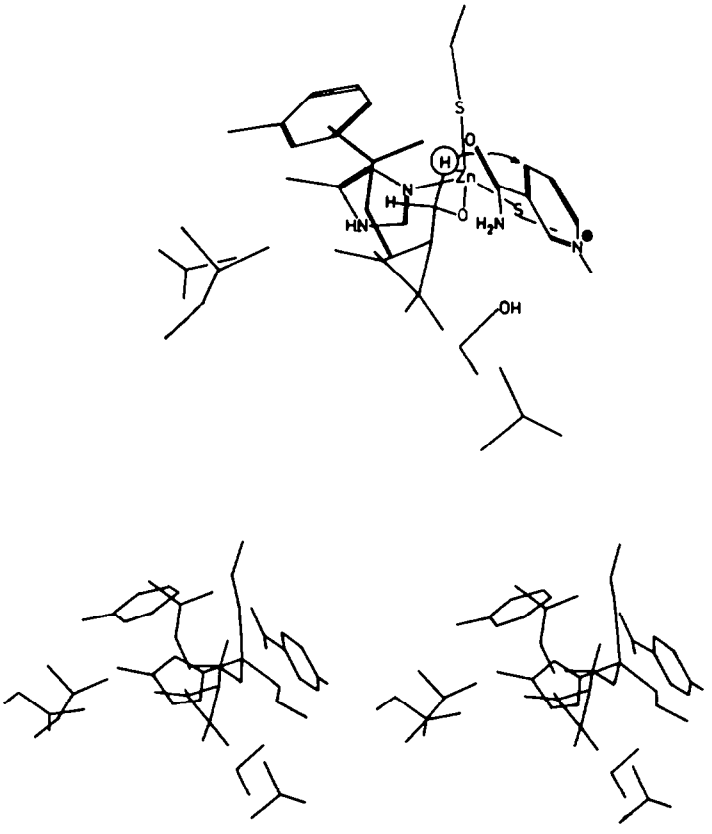
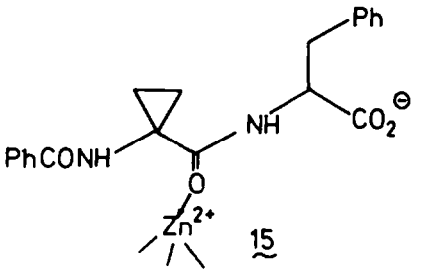


Figure 1 Mechanistic probe **14** at the active site of HLADH : diagram and stereopair

The inhibition of carboxypeptidase A

We have found that carboxypeptidase A (CPA) is inhibited by the peptide **15** in which the cyclopropane is activated to nucleophilic attack by the carbonyl group the polarisation of which is enhanced by coordination to zinc at the enzyme's active site (Scheme 6).[3] The crystal structure of CPA with a small peptide, glycyl-L-tryosine, bound to the active site has been determined[23] and this data formed the basis of our mode. Bearing in mind the predictions of theory and precedent, the peptide **15** was incorporated into the active site by superimposition of corresponding atoms upon the substrate and adjusting the conformation so that the carbonyl group bisected the cyclopropane ring (Figure 2). This conformation was found to be a satisfactory fit to the active site. It was immediately apparent that the carboxylate of Glu-270 lay ideally placed to act as a nucleophile and attack the cyclopropane ring leading to the formation of an ester at the active site. Indeed taking advantage of the flexibility of the last two C-C bonds of the glutamate, it was evident that the glutamate could attack the cyclopropane ring at the preferred angle of 150° and could approach within 2A of the ring at this angle. The computer model therefore strongly suggests that a chemically reasonable mechanism for inhibition by nucleophilic attack at a cyclopropane ring can take place at the active site of CPA.



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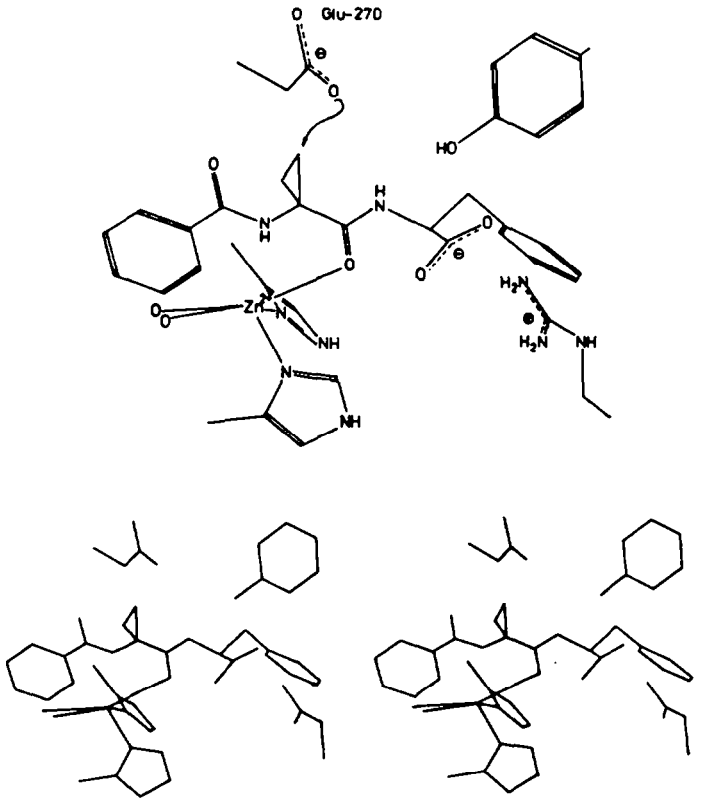
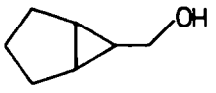


Figure 2 Inhibition of carboxypeptidase : diagrams and stereopair

Inhibition of HLADH

The computer model used to investigate the ability of cyclopropylalkyl radicals to undergo ring opening at the active site of HLADH is also appropriate for searching for reasonable mechanisms of inhibition. The theoretical arguments and precedent described above suggested that the leaving group, in this case hydride, should be placed in a conformation close to antiperiplanar to the cyclopropane bond that undergoes cleavage during inhibition. Such a conformation can be illustrated by **13** (Scheme 5) for an unsubstituted cyclopropyl methanol. This is the same conformation that leads to ring opening. However, for an aldehyde inhibitor, a slightly different conformation will be preferred, that in which the carbonyl group bisects the cyclopropane ring **12a**.

The first point of interest is the availability of nucleophiles at the active site of HLADH; the model reveals two cysteines (46 and 174) and a serine (48) as the most likely candidates. The mechanism of inhibition was studied with the bicylo[3,1,0]hextan--6-methanol inhibitor **16** which was one of the most reactive discovered.[2] Bearing in mind that oxidation of the inhibitor was kinetically associated with inhibition, the inhibitor was first inserted into the active site in a conformation appropriate for hydrogen transfer analogous to that used for the probe molecule **14** (Figure 3).



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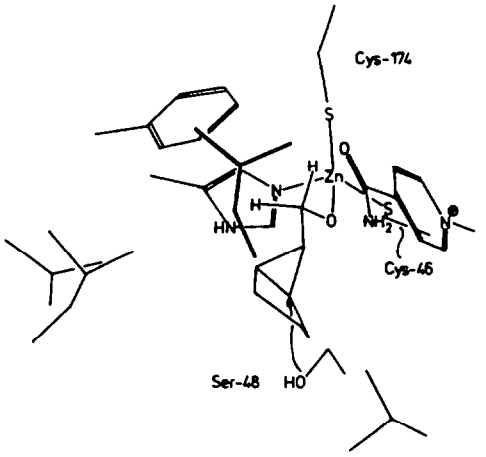


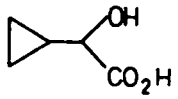
Figure 3 Inhibition of HLADH by cyclopropylmethanols : diagrams and stereopair

However, manipulation of the inhibitor by rotation around the Zn-O, O-C, and C-C< bonds showed that it was not possible for either of the two sulphur atoms to approach within a reasonable bonding distance of the electronically susceptible apices of the cyclopropane ring without entering an energetically unfavoured region. Indeed, with alcohols as substrates, the sulphur atoms could not act reasonably as nucleophiles unless the stereochemistry of hydrogen transfer by the enzyme during inhibition involved the pro-S hydrogen and not the pro-R as is normal. In contrast, the hydroxyl group of Ser-48 is well placed to attack the cyclopropane ring activated by extensive cleavage of the C-H bond. The fact that the tetramethyl probe molecule **14** was such a poor inhibitor but a good substrate[4b] argues strongly in favour of inhibition taking place by attack directly on the cyclopropane ring and not on the oxidised carbon atom. Further, rotation of the side chain of Ser-48 shows that the preferred angle of 150° for attack on the ring is readily accessible. Thus nucleophilic attack on the cyclopropane ring is clearly a competent mechanism for inhibition, although for the reasons stated earlier, oxidation and nucleophilic attack would not be expected to be concerted. This model suggests that the enzyme nucleophile attacks the cyclopropane ring when a high degree of positive charge has been induced on the α-carbon by substantial transfer of hydride to NAD^+. Our earlier kinetic results were consistent with this conclusion.[2] Inhibition thus probably follows hydride transfer and is largely complete before a carbonyl double bond is fully formed. However, we cannot ignore the possibility of alkylation of the enzyme by a ring-cleaved homo-allyl cation (Scheme 4a). The most significant experimental observation that argues against this possibility is the fact that the tetramethyl cyclopropane **14** was such a poor inhibitor.[4b] A homo-allyl cation would be favoured by ring opening of this compound and the planarity at the resulting terminal carbon atom would reduce steric hindrance. Both of these factors would be expected to enhance the inhibition reaction whereas the opposite was observed.

Aldehydes and ketones showed different, usually weaker, inhibition properties compared with alcohols[2] and the possibility of alternative mechanisms for inhibition merits consideration. Firstly, as mentioned earlier, an aldehyde would be expected to adopt a different conformation from the alcohol in which the carbonyl group bisects the cyclopropane ring **12b**. When this variation is introduced into the active site, inhibition by attack of the Ser-48 hydroxyl group is still available. Another significant point is that like the inhibitor of carboxypeptidase, an aldehyde or ketone needs only to be activated by coordination to zinc and accordingly, inhibition may occur at a different point on the reaction profile from that for alcohols. Alternative mechanisms are also available. Thus, both sulphur atoms of the cysteine ligands for the zinc ion come within range of nucleophilic addition to the carbonyl group with the inhibitor in the conformation illustrated in Figure 3. It is possible that these alternative pathways may account for the different inhibitory properties of the aldehydes and ketones.[2]

Inhibition of lactate dehydrogenase

The starting point for the construction of the model for inhibition of lactate dehydrogenase (LDH) was a crystal structure of the enzyme complexed to a covalent conjugate of substrate and cofactor.[24] To obtain the active site model, the conjugating bonds were removed and the liberated lactate substrate replaced by the inhibitor **17**. LDH is noteworthy in this study as it is the only one of the three enzymes that does not have a



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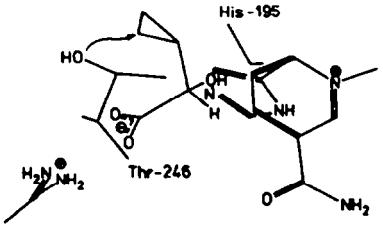
functional metal ion at the active site; a histidine (His-195) takes the place of zinc as an acid base catalyst. Inspection of the active site led to fewer obvious nucleophiles at the active site. The most prominent was the hydroxyl group of Thr-246. When the inhibitor was adjusted in the active site to the stereoelectronically required conformation for oxidation and ring opening (cf. 13) it was found that the hydroxyl group of Thr-246 could be adjusted to attack the cyclopropane ring at 150° from a distance of 2.8Å (Figure 4) in a manner similar to that discussed for alcohol dehydrogenase above. His-195 is also a potential nucleophile but it is unlikely to act as a such because it is required to accept a proton during oxidation. Unlike alkoxides, alcohols are known to be resistant to oxidation by nicotinamidium derivatives[25] and since inhibition is most effective during oxidation in a similar manner to HLADH[2] the presence of a base such as His-195 is essential.



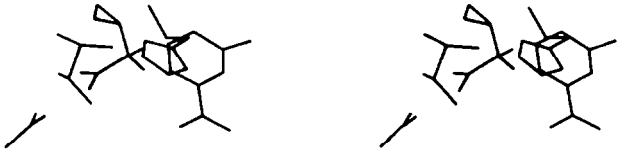


Figure 4 Inhibition of LDH : diagram and stereopair

Conclusions

The computer models are helpful to interpret the behaviour of substrates and inhibitors at enzyme active sites. However it is important to realise that their suggestions cannot be regarded as facts in themselves. In particular, the limitations of energy calculations and the exclusion of much of the protein molecule must be acknowledged. Such computer models are valid only as rationalisations of established experimental data or as generators of hypotheses to be tested, in this case, the identification of the nucleophiles involved in inhibition. Within these limitations, nevertheless, it can be asserted that the enzymes carboxypeptidase A, horse liver alcohol dehydrogenase, and lactate dehydrogenase can reasonably undergo inhibition by mechanisms consistent both with their initial design concept and with the expected chemistry of nucleophilic attack on cyclopropane ring. These results are also of significance in the design of further cyclopropane based inhibitors for other well-defined target enzymes.

Acknowledgement

We thank Dr. Peter Bladon for helpful discussions and for his contribution to the development of the INTERCHEM molecular modelling package.

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