Studies of enzyme inhibition by compounds containing small rings

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Our interest in the enzyme chemistry of compounds containing small rings developed from a speculative study of the inhibition of alcohol dehydrogenase by allylic alcohols (MacInnes et al., 1981) in which we found that 3-thioethyloprop-2-en-1-ol (I) was a potent inhibitor of this enzyme. Our original strategy was that oxidation of (I) would lead to a α,β-unsaturated aldehyde to which a nucleophile at the active site would add leading to the inhibited enzyme. However, we found that, although inhibition accompanied oxidation of (I), the enzyme recovered activity on standing or on gel filtration apparently through hydrolysis. It turned out that the persistence of inhibition was due to ethanethiol, a hydrolysis product, bound at the active site. We argued that, in view of the similarities between alkene and cyclopropane chemistry, oxidation of cyclopropylmethanols (e.g. II) to the aldehydes would activate the cyclopropane ring to nucleophilic attack and ring opening. The reverse of this reaction would be improbable and inhibition should be permanent. This strategy proved successful (MacInnes et al., 1983a) as will be discussed below.

There are three reaction pathways of substituted cyclopropanes that are believed to be involved in enzyme inhibitors: (1) addition of nucleophiles to cyclopropanes (Wiseman et al., 1980; Dowd et al., 1984), (2) ring cleavage of hetero analogues of cyclopropylalkyl radicals (Silverman et al., 1980; Hanzík & Tullman, 1982; Macdonald et al., 1982; Wiseman et al., 1982; Silverman, 1983; Silverman & Yamaski, 1984; Dijkstra et al., 1984; Parkes & Abeles, 1984; Sherry & Abeles, 1985; Silverman & Zieske, 1985), and (3) nucleophilic ring opening of cyclopropylalkyl cations and their equivalents (unpublished work). In addition, cyclopropanes are susceptible to addition by electrophylic or radical reagents, as will be seen later.

Dehydrogenases and peptidases

Horse liver alcohol dehydrogenase (HLADH) has been our prototype system upon which to test chemical ideas. With activated cyclopropanes as alkylating agents, we thought that a small ring might easily be incorporated into substrate analogues of a number of enzymes and that a general strategy for the inhibition of enzymes, including those of chemotherapeutic significance, could be established. HLADH was found to be susceptible to inhibition by a wide range of cyclopropylmethanols (e.g. II–VIII) (MacInnes et al., 1983a), the most potent inhibitors being those that bound best to the hydrophobic pocket adjacent to the enzyme's active site (Dutler & Branden, 1980).

Kinetic studies suggested that the inhibition event was closely connected to the oxidation of the alcohols; conceptually it was therefore attractive to suggest that alkylation of the enzyme took place when hydride was essentially transferred to NAD\(^+\) at which point the positive charge adjacent to the cyclopropane ring would be maximal and hence the activation of the ring greatest. Our recent extensions of the studies using di- and tetramethyl cyclopropylalkylmethanols (MacInnes et al., 1980; Hanzlik et al., 1985) indicate that inhibition occurs by nucleophilic attack at the cyclopropane ring since 2,2,3,3-tetramethylcyclopropane methanol (VIII) is a very poor inhibitor but a good substrate.

Further light on the mechanism of this reaction can be shed by computer graphics techniques. We have modelled the active site of HLADH ternary complexes based upon an X-ray study (Eklund et al., 1976). Manipulating the inhibitors at the active site by rotating around the Zn—O, O—C and C—C< bonds strongly suggested that the hydroxyl group of Ser-48 is particularly well placed for alkylation of the enzyme to occur in close association with oxidation (R. J. Breckenridge & C. J. Sucking, unpublished work). The indications from molecular graphics must now be substantiated by experiments with labelled inhibitors. Such studies are also relevant to the question of the mechanism of hydrogen transfer by HLADH (MacInnes et al., 1982, 1983b).

Having established that the basic chemistry led to sufficient reactivity at an enzyme's active site to cause inhibition, we began to investigate the generality of our observations. We showed that lactate dehydrogenase underwent inhibition by the lactate analogue (IX) in a manner closely paralleling the inhibition of HLADH, (MacInnes et al., 1983a). This enzyme is not a metalloenzyme but uses an imidazolium group to activate the carbonyl group towards reduction and to receive the alcohol proton. Our inhibition strategy was therefore extendible, at least within the dehydrogenases. Returning to metalloenzymes, we have recently been studying carboxypeptidase A as a prototype for the inhibition of chemotherapeutically significant peptidases. This enzyme contains zinc at the active site and we designed our first range of inhibitors with the idea that co-ordination of the carbonyl group to zinc in the dipeptide (X) would enhance the activation of the cyclopropane ring sufficiently for nucleophilic attack to occur at the active site (A. Bell, S. K. Ner, C. J. Sucking & R. Wrigglesworth, unpublished work). The dipeptide (X) is a time-dependent inhibitor with kinetic properties typical of suicide substrates; it is also irreversible. However, it is not very reactive and we are currently studying compounds that should be more effective.

Abbreviation used: HLADH, horse liver alcohol dehydrogenase.
Cytochromes P-450

The chemistry of arylcyclopropanes and cytochromes P-450 makes an interesting contrast. Following the joint enzyme's active site and further oxidation is encouraged. We rationalized the enzyme's behaviour after two radicals which could add to the cyclopropane ring.

Phenol (D. C. Nonhebel) with a reconstituted cytochrome P-450 preparation from rat or rabbit liver, we found that the major oxidation product was benzoic acid (Suckling et al., 1982, 1985). This surprising product was also obtained from model non-enzymic experiments together with 2-cyclopropylphenol (D. C. Nonhebel & C. J. Suckling, unpublished work). We rationalized the enzyme's behaviour after Sligar's suggestion of the intermediacy of an acyl peroxide (Sligar et al., 1980); thus homolysis of the peroxide led to two radicals which could add to the cyclopropane ring. In this way, a molecule of substrate becomes fixed to the enzyme's active site and further oxidation is encouraged.

Overall, three oxidative steps must occur in the conversion of cyclopropylbenzene to benzoic acid; the remaining two carbon atoms, according to our hypothesis, should be converted into acetaldehyde. To investigate this further, we oxidized 1,2-diphenylocyclopropane under the same conditions and found benzoic acid together with phenyl acetaldehyde as principal products. Phenyl acetaldehyde is clearly the analogue of acetaldehyde formed through the oxidation sequence postulated. Again, the non-enzymic products from diphenylcyclopropane were similar to those produced by cytochrome P-450, although in the former case, the major neutral product was acetophenone. To account for these results we have postulated a covalent intermediate, but we observed no time dependent inhibition with these substrates. Both arylcyclopropanes are genuine substrates as shown by their ability to inhibit ethoxycoumarin oxidation and bind quite tightly (K_i: XI, 0.057 mM; XII, 0.011 mM).

The contrast in behaviour of these substrates with that of the cyclopropylamines that inhibit this enzyme is intriguing. Guengerich & McDonald (1984) have suggested that the reaction path taken by cytochrome P-450 with a particular substrate depends upon the ease with which either electrons or hydrogen atoms can be abstracted from the substrate. With the amines, electron transfer is easy but the arylcyclopropanes possess neither high energy occupied orbitals such as non-bonded electrons nor weak C-H bonds. Thus addition to the weakest part of the molecule, the cyclopropane ring, is favoured.

We have also studied an important endogenous biosynthetic cytochrome P-450, namely cholesterol 7α-hydroxylase. An extensive series of 5,6-methanocholsterols (XIII–XV) with an oxidized 7-position was synthesized. (L. Brown, W. J. S. Lyall & C. J. Suckling, unpublished work). The cholesterol analogue (XIII) was a good competitive substrate with respect to cholesterol (K_i: 15 μM) and, by means of dilution and recrystallization to constant activity, we were able to show that the cyclopropylcholate is oxidized by the enzyme. The 7α product at least in part without cleavage of the cyclopropane ring. There were, however, other products, and experiments to identify them are currently in progress.

Two other compounds investigated during this study were the epoxide analogue (XVI), which is a non-competitive inhibitor; steroidal epoxides have been shown to have enzyme inhibitory properties (Bantia et al., 1985). The novel amide (XVII) which showed non-competitive kinetics with respect to cholesterol and potent inhibition of 7-α-hydroxylase (K_i: 3.6 μM) interestingly failed to inhibit acylcholate: acyltransferase (J. D. Houghton, S. E. Beddows & K. E. Suckling, unpublished work). In no case was time dependent inhibition of the hydroxylase observed.

Pyrimidine biosynthesis

A recent interest of ours has been in the enzymes of pyrimidine biosynthesis and we have discovered, somewhat by chance, some new inhibitors of dihydro-orotate dehydrogenase from Clostridium oroticum (Buntain et al., 1985). The inhibitors are hydantoins derived from phenylalanine (XVIII) and they function only when the enzyme acts in an oxidative sense. Several lines of evidence led to a hypothesis that oxidation of the exocyclic benzylic group leads to an α,β-unsaturated carbonyl system (XIX) which traps an enzymic nucleophile irreversibly. Curiously, both enantiomers of this hydantoin are inhibitors, a fact that we have rationalized with the aid of computer graphics (Buntain et al., 1985).

Cyclopropanes have also figured in this study. The barbituric acid derivative (XX) is an interesting molecule in that it is a substrate analogue of orotic acid and also related to the commonly used inducer for cytochromes P-450,phenobarbital. It also has several pathways available by which it can cause enzyme inhibition, both via radical ring opening, and nucleophilic addition to the (doubly) activated cyclopropane ring. Unfortunately, the barbiturate (XX) binds too weakly to the appropriate cytochrome P-450 to have any significant inhibitory effect but it does inhibit dihydro-orotate dehydrogenase in a time-dependent manner, irreversibly (I. Buntain, J. Courtney, C. J. Suckling & H. C. S. Wood unpublished work).
lished work). The reaction occurs without the addition of cofactor, in contrast to the hydantoins. Although we have no further evidence, this observation alone suggests that a nucleophilic mechanism may be involved in the inhibition of dihydro- orotate dehydrogenase.

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\begin{align*}
\text{Ph} & \quad \text{N} \quad \text{CO}_2\text{H} \\
\text{O} & \quad \text{Ph} \quad \text{N} \quad \text{CO}_2\text{H}
\end{align*}
\]

The interplay between mechanism and inhibition is one of the fascinations of this field for the organic chemist, each aspect in turn supporting the other. We have so far only found cyclopropane derivatives that inhibit enzymes apparently by nucleophilic mechanisms; such radical reactions as have come along have treated our cyclopropanes as substrates. Currently, we are devoting most attention to clarifying the details of alcohol dehydrogenase inhibition and to applying our knowledge to the design of inhibitors of chemotherapeutically significant enzymes.


A new perspective on pyridoxal chemistry and its relevance to enzyme inhibition

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Pyridoxal phosphate-dependent enzymes occur widely and are responsible for the synthesis, racemization, degradation and interconversion of α-amino acids in living systems. (Bloch, 1972; Davis & Metzler, 1972; Walsh, 1977). Transamination of the holoenzyme by an α-amino acid produces the corresponding imine of the α-amino acid (1). Concurrently, the aza-allylic bonds are activated to cleavage by the facility with which the protonated pyridyl ring of the pyridoxal moiety can delocalize a negative charge. The rich chemistry of the enzyme-substrate complex derives from this activation moderated by the steric environment of the enzyme.

Many workers have studied aspects of pyridoxal chemistry (Walsh, 1979; Vederas & Floss, 1980) and strong interest continues in both systems in vivo (Liu et al., 1984) and model systems (Weiner et al., 1985). The initial reactive intermediates (2) or (3) are generated by cleavage of either bond (a) or (b) in protonation of the pyridine nitrogen atom. Stereoelectronic control requires that the breaking bond [bond (a) or bond (b) in (1)] is aligned with the pyridyl azomethine π-system (Dunathan, 1966; Fischer & Abbot, 1979).

Abbreviation used: GABA, γ-aminobutyric acid.

There are a large number of naturally occurring toxins which function as suicide substrates for pyridoxal enzymes (Rando, 1975) of which cycloserine (4) (Rando & Strominger, 1966) and gabaculine (5) (Rando & Bangert, 1976, 1977; Rando, 1977) are probably the best known. The former appears to generate an active acetylating agent which blocks the pyridoxal enzyme,