Tetrahedron Vol. 38, No. 7, pp. 871 to 909, 1982
Printed in Great Britain.

0040-4020/82/070871-39$19.50/0
Pergamon Press Ltd.

TETRAHEDRON REPORT NUMBER 124

SUICIDE SUBSTRATES: MECHANISM-BASED ENZYME INACTIVATORS

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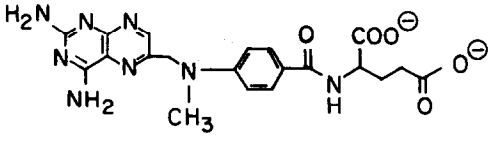
(*Received in the U.S.A. 15 May* 1981)

Abstract—This review article defines suicide substrates as a class of irreversible inactivators of specific target enzymes where the target enzyme participates in its own destruction by catalytic unmasking of a latent functional group at some stage in the catalytic cycle of the enzyme. Criteria for evaluation of suicide substrates are presented. Then, examples of the various types of latent functional groups and their likely enzymic routes of activation are presented for both natural and synthetic compounds. These examples include gabaculine, secobarbital, allopurinol, clavulanate and penicillin sulfones, tranylcypramine, rhizobitoxin, fluoroalanine, 5-fluorodeoxyuridylate and mitomycin.

1. INTRODUCTION

A large variety of molecules, both naturally occurring as well as synthetic, have been shown, over the past decade, to cause specific inactivation of some target enzyme. The outcome of this chemical intervention in the biological system can be beneficial, as in blockage of microbial penicillinases, or it can often produce toxic consequences. An understanding of the molecular bases of these phenomena in chemical terms can aid not only in the delineation of how chemical transformations are effected by nature's macromolecular protein catalysts, the enzymes, but also in the rational design of drugs with maximal *in vivo* specificity.

At the outset one can divide chemical agents that inhibit some specific enzyme into reversible and irreversible (inactivating) inhibitors. Reversible inhibitors can be quiet useful but, in general, require no chemical transformation by the target system to have their effect. For this reason we will not focus on them in this review. Rather, we will analyze the chemistry of irreversible inactivation of target enzymes which result from covalent derivatization of the enzyme protein (or of a tightly bound cofactor) by some reactive inhibitor species. However, one must note that at the limit where reversible inhibitors bind extremely tightly to an enzyme, they are functionally irreversible because their rates of dissociation are so slow. Thus, for example, the anticancer drug methotrexate **1** binds to dihydrofolate reductase with a $K_D = 10^{-11} M$[1] and dissociation of the drug enzyme complex is very slow, with *in vivo* half-times of hours, long enough to deprive the target tumor cells of required DNA biosynthetic intermediates for a period to induce cell death. We will elaborate on other "pseudo-irreversible" cases at the end of this review.



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In the broad category of irreversible inhibitors (inactivators) of target enzymes one can delineate two generations of molecules, the first represented by so-called affinity labels or active site-directed reagents.[2-4] These are structural analogs of normal substrates for the target enzyme but have built-in to them some reactive group such as a haloketone (tosyl phenyl chloromethyl ketone **3**),[2] an epoxide (phosphonomycin **4**),[5] or such groups as a sulfonyl halide (phenylmethanesulfonyl fluoride **5**) or phosphoryl halide (diisopropylfluorophosphate, **6**). These molecules take advantage of binding specificity and the consequent propinquity of some nucleophilic amino-acid side chain in the active site of a target enzyme to set up a displacement reaction for covalent modification and inactivation of the biocatalyst molecule. Although useful for active site structural mapping of purified enzymes, such compounds,

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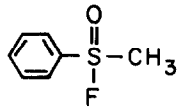

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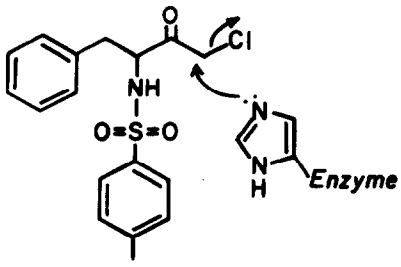

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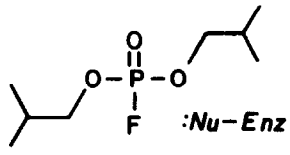

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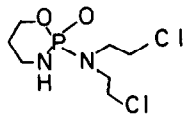
because the reactive functional groups are already present when the molecules are in cellular solutions before reaching the target protein, are in general too deleteriously and indiscriminately reactive to be of wide use except in extreme situations. Thus, some anticancer drugs that are uncovered alkylating



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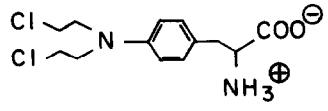

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agents,[6] such as cyclophosphamide **7**, or phenylalanine mustard **8**, and organophosphorus insecticides, targeted against acetylcholinesterases, as we shall note later in this article, fall into this group.

The second generation of enzyme-specific inactivation reagents have been in use for about the past decade and differ from the first in that the reactive functional group is *latent* in the molecules in solution. Only after binding to the target enzyme and after the enzyme begins catalysis is the reactive chemical grouping uncovered. The particular chemical reaction sequence of the given enzyme is required to unravel the inactivator. This activation occurs in a precise microenvironment only, the active site of the target enzyme. If covalent capture is efficient, then only the conscripted enzyme molecule is modified and its catalytic activity destroyed. This class of inactivation has received several terminologies, including mechanism-based inactivation, k_{cat} inhibitors, and "suicide substrates".[7-13] Although the term suicidal inactivation conveys the role of the enzyme molecule in catalyzing its own destruction, the reasonable objection has been raised that the enzyme is not doing this deed willingly but rather by mistake.[14] The suicide substrate is really a Trojan horse reagent.

Nomenclature debate notwithstanding, the purpose of this review is to focus on these mechanism-based enzyme inactivators and examine, in both natural products and synthetic substrate analogs, what kinds of precursor or latent functional groups have been used. We shall inquire what are the chemical mechanisms of latent group unraveling in the active site of a given enzyme and what kind of alkylating or acylating species is generated and, where known, what covalent modified structure results. In general, it will be an electrophilic species uncovered and, therefore, a nucleophilic side chain of an amino-acid residue that "bites".[10,11] Occasionally, though, the polarity is reversed, the inactivator uncovered is nucleophilic. Since there are almost no amino acid side chains that are electrophilic (except the guanidinium group of arginine), covalent derivatization will then occur in general with those enzymes that have tightly bound organic or inorganic low molecular weight cofactors with electrophilic sites.

1.1 *Criteria for mechanism-based inactivation*

Before delving into the functional group activations summarized in Table 1, we will address briefly the questions of some simple criteria to determine that irreversible loss of catalytic activity on exposure of enzyme to some compound is specific, mechanism-based, and so chemically interpretable in that framework.[9,11,15] These are straightforward kinetic and chemical criteria. As with any substrate where

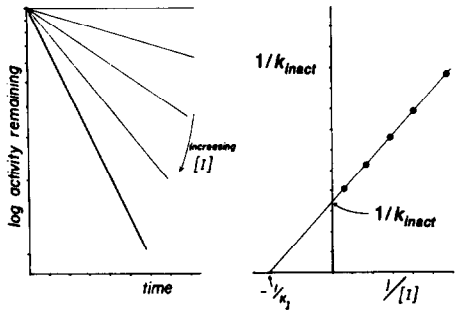
Table 1. Types of enzymically activatable functional groups

Acetylenes
Olefins
Halogenated Substrates
Cyclopropanes
Quinones
Carbonium Ion Precursors
Penicillin Analogs
Thionosulfur Compounds

physical binding to the enzyme active site precedes catalysis, the inactivator should follow the simple minimal scheme, where there is a binding equilibrium followed by first-order (the kinetics can deviate from first order, depending on particular rate constants in complex cases[16]) chemical process leading to inactivation. One can readily extract these two macroscopic kinetic parameters, a K_D and a k_{inact}, by conducting inactivations at one fixed enzyme concentration and various fixed inactivator concentrations $I_1 - I_n$. As inhibitor concentration is increased from one incubation to the next, greater mass action pressure accrues on the pre-equilibrium and a greater fraction of total enzyme is present as $E \cdot I$. Since $k_{obs} = [E \cdot I]k_{inact}$, the family of first-order rate constants seen in Fig. 1 are obtained. An extrapolation to infinite concentrations of [I] and thereby the limiting k_{inact} is obtained in a double reciprocal plot of $1/k_{obs}$ vs 1/I shown in idealized form also in Fig. 1. The existence of a finite vertical intercept is characteristic for saturation of enzyme with ligand and validates the kinetic specificity of a binding step prior to inactivation chemistry. The values of k_{inact} reveals the speed of the killing step. In alkylation of an active site histidine residue of the intestinal protease chymotrypsin by haloketone, **3**, k_{inact} is 0.2 min^{-1}, some 10^6-fold accelerated over a corresponding non-enzymic model displacement reaction.[17]

$$E + I \rightleftarrows E \cdot I \longrightarrow E - X$$

If the covalent modification that leads to enzyme activity destruction is a mechanism-based process that derivatizes a key amino acid residue of the enzyme, the chemical stoichiometry of modification should be the minimal 1:1 adduct/enzyme ratio. (In some cases of multimeric enzymes which show



negative cooperativity, sub-stoichiometric labeling can cause complete activity loss. Thus, when the bacterial enzyme involved in methionine degradiation, methionine-γ-lyase, is inactivated by propargylglycine, **9**, 2 labels/tetramer block all activity. Presumably, the two modified subunits show altered conformations that are transmitted by intersubunit contacts to the unmodified subunits.[18]) Higher stoichiometry values probably reflect non-specific alkylation processes[19] and will obscure any simple

mechanistic interpretation of the correspondence of inactivation to the active site chemistry presumed to be rerouted from normal catalysis to mechanistically related inactivation.

A final criterion which is both useful mechanistically in identifying the nature of the inactivating species at the active site of the enzyme and also in evaluation of any *in vivo* therapeutic potential is the *partition ratio*,[15,20] the number of times a suicide substrate is processed to released product without harm to an enzyme molecule per inactivation event. In the simplified scheme shown, this is the ratio k_3/k_4. Since the mechanism-based class of inactivators requires the enzyme to go at least partway through its normal chemical catalytic cycle before the latent functional group is uncovered ($E \cdot I \rightarrow E \cdot X$), the question devolves to how often the normal catalytic cycle is completed compared to how often it is rerouted into covalent derivatization of the enzyme. Maximal specificity will be represented by complete routing of flux via k_4. It may be problematic when flux goes via k_3 since molecules of P′ that are released are, unlike the latent groups in the substrate, fully uncovered and reactive. These reactive product molecules can diffuse to other cellular components and alkylate or acylate them. This is, on the microscopic scale, a loss of specificity and, on the macroscopic scale, would be a source of side effects or toxicity. We shall enumerate these partition ratios for several cases below.

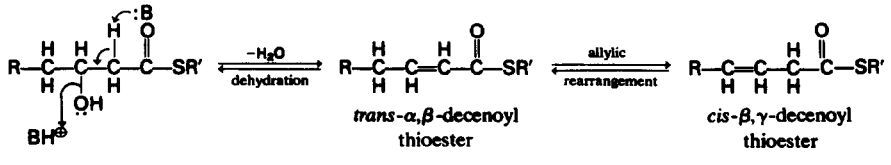
$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdots I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E \cdots X \begin{matrix} \overset{k_3}{\rightarrow} E + P & \text{covalent;} \\ \underset{k_4}{\rightarrow} E - X & \text{inactive} \end{matrix}$$

2. CLASSES OF MECHANISM-BASED INACTIVATORS

2.1 *Acetylenic functional groups*

There are at least three ways by which the essentially inert acetylenic group can be suicidally activated at an enzyme active site: (1) by carbanion formation at an adjacent carbon and propargylic rearrangement to an allene, (2) by oxidation of an adjacent functional group to a ketone equivalent, or (3) by monooxygenation to an oxacyclopropene equivalent. Each case will be exemplified. A fourth category may represent enzymic uncovering of an allenic anion equivalent as nucleophilic rather than electrophilic killing species and will also be considered.

2.1.1 *Carbanion formation and propargylic rearrangement.* The now classical example of this subset and one which spurred the development of the whole class of mechanism-based inactivators over the past decade was the study by Bloch *et al.*[21,22] on an enzyme in bacterial fatty acid biosynthesis responsible for dehydration of β-hydroxyacyl thiolesters to first the α,β-enolyl acyl-S-CoA, then isomerization out of conjugation to the β,γ-enoyl-CoA. The responsible enzyme, β-hydroxydecanoyl thiolester dehydrase, is thus a dehydrase and a 1,3-allylic isomerization catalyst. When presented with the product β,γ-olefinic thiolester, the enzyme will catalyze reversible isomerization back into conjugation. But exposure to the corresponding β,γ-acetylene, the 3-decynoyl thiolester **10** leads to



first-order inactivation kinetics due to covalent modification of an active site histidine residue. Use of 2-$^2H_2$-**10** shows a kinetic isotope effect (k_H/k_{2H}) of 2 on k_{inact}, suggesting the enzyme misrecognizes **10** as substrate for allylic isomerization, yielding conjugated allene **11** by net propargylic rearrangement at the active site. Model studies by Bloch's group[23] demonstrated a high degree of electrophilicity at the central carbon of 2,3-butadienyl esters to Michael-type additions by such nucleophiles as imidazole derivatives. Suicidal blockage of this enzyme *in vivo* leads to bacterial mutants that can no longer manufacture unsaturated fatty acyl chains in memranes.[22] It was recognized that two allene isomers were possible, but the stereospecificity (or unlikely lack thereof) of the enzymic propargylic reearrangement for this enzyme is yet undetermined.

The observation that enzyme-mediated carbanion formation at a site adjacent to an acetylene linkage can be followed by propargylic rearrangement to set up an electrophilic, alkylating conjugated allene has been generalized to several other enzymes which carry out specific isomerizations by proton shifts. As

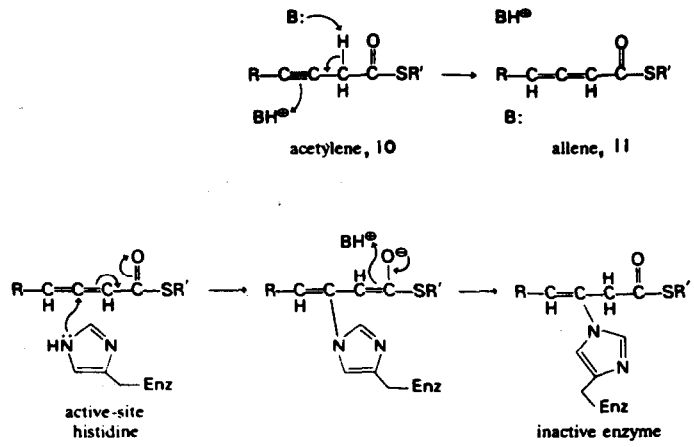
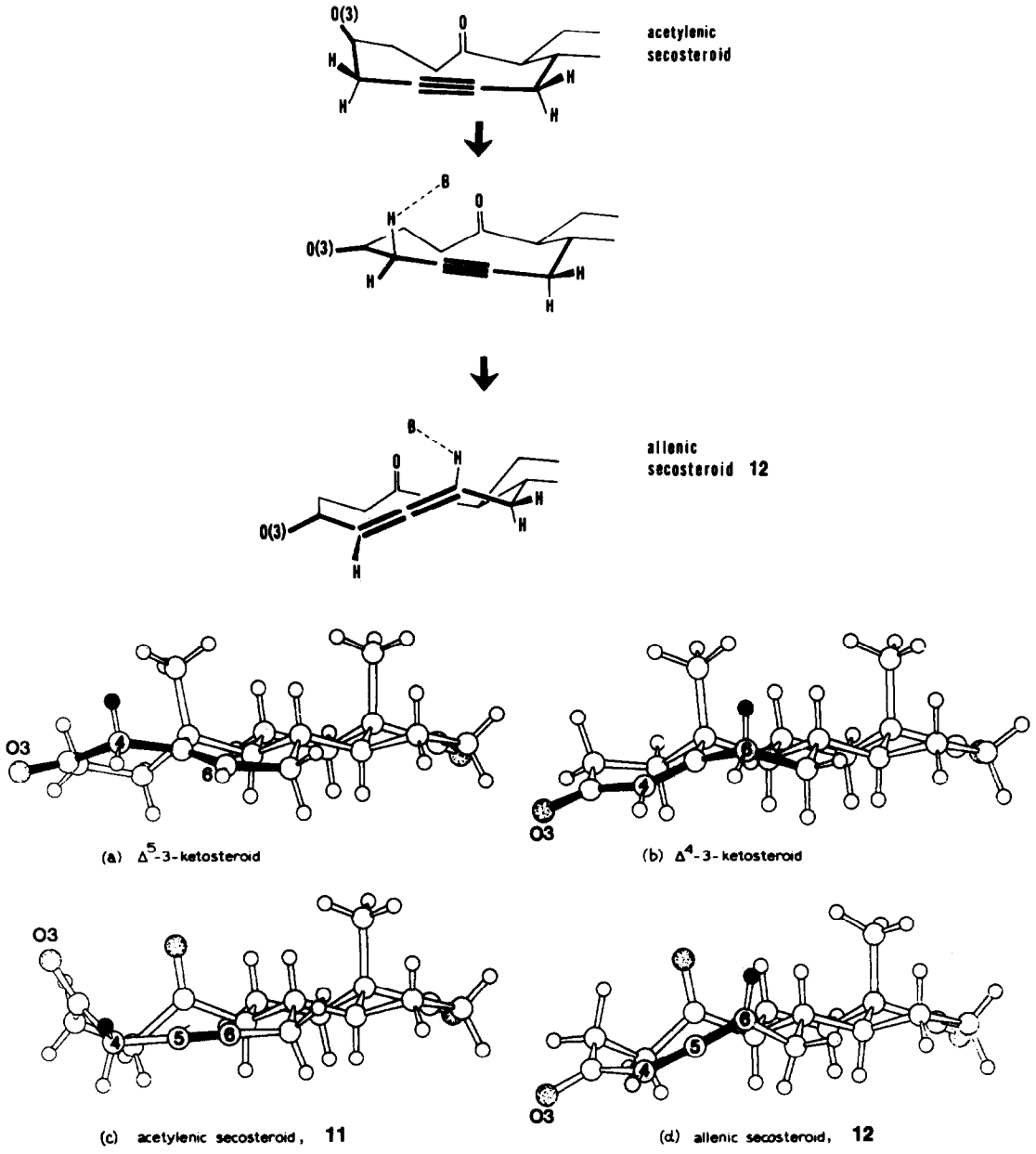


Table 2. Suicide substrates with acetylenic functional groups

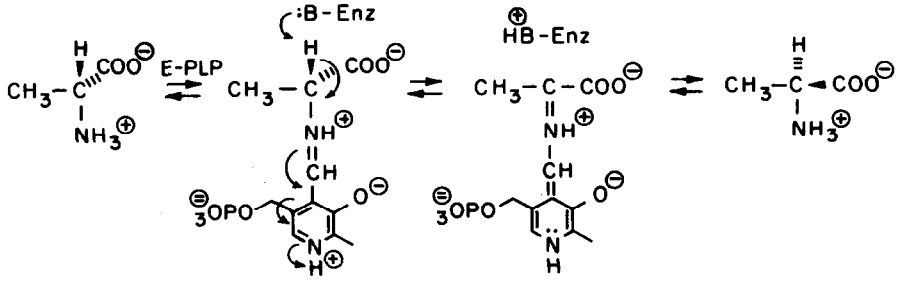
Entry	Compound	Target Enzyme	Partition Ratio	Comment	Reference
1	3-decynoyl-CoA, 10	β-hydroxydecanoyl thiolester dehydrase	n.d.	active site histidine residue	21
2	acetylenic seco-steroids, 11	Δ^5-3-ketosteroid isomerase	n.d.	via free allenic ketone products which rebind to active site	24-26
3	ethynylglycine, 13	alanine racemase	n.d.		29
4	propargylglycine, 9	γ-cystathionase	n.d.	rat liver	30-35
		cystathionine-γ-synthetase	4-8	plants and bacteria	
		methionine-γ-lyase	4-8	bacteria	
		L-aspartate transaminase	n.d.	rat liver enzyme in presence of 3M $HCOO^-$	
		L-alanine transaminase	2	rat liver enzyme	
		D-amino acid oxidase	several thousand	histidine residue predominantly	
5	γ-acetylenic-GABA, 19	GABA transaminase	n.d.		39,40
		glutamate decarboxylase	n.d.	different isomers for bacterial vs. mammalian enzymes	
		ornithine-δ-transaminase	n.d.		
6	4,5-lysyne	lysine-ε-transaminase	40	Achromobacter liquidum	179
		ornithine-δ-transaminase	n.d.	rat liver	
7	1,4-diaminobutyne	ornithine decarboxylase	n.d.		119
8	α-ethynylornithine	ornithine decarboxylase			180
9	α-ethynyl-DOPA	DOPA decarboxylase			181,182
10	3-butynyl CoA	β-ketoacyl thiolase			183
		glutaryl CoA dehydrogenase			184
		butyryl CoA dehydrogenase			
11	N-propynylglycine	sarcosine oxidase			185
12	butyne-1-ol	alcohol dehydrogenase			40
	butyne-1,4-diol	alcohol oxidase			41
13	ethylene	liver P-450 monooxygenase		phenobarbital-induced isozyme	44-48
14	danazol and other 17-ethynyl steroids	liver P-450 monooxygenase			
15	placidyl	liver P-450 monooxygenase			
16	19-propynyl and ethynyl-estrogen precursors, 22,22a	aromatase		placental enzyme	50-52
17	hydroxybutynoate	several flavin-linked α-hydroxy acid oxidizing enzymes	see Table 3		53-58
18	pargyline, 28	monoamine oxidase		mitochondrial flavoprotein	61-65
	clorgyline, 29				
	deprenyl, 30				
	dimethylpropargylamine, 31				

noted in Table 2, acetylenic secosteroids **11a–c**,[23,24] are isomerized to a 4:1 mixture of conjugated allenic ketones by a Δ^5-3-ketosteroid isomerase from *Pseudomonas testosteroni* and these come back from solution, rebind to the active site, and undergo conjugate addition from a residue of the enzyme. The X-ray structure[26] of the secosteroid **11** reveals remarkable homology to the normal steroid substrate, following the conformational change shown below.[25] The allenic secosteroid, generated by the enzyme's action on the acetylenic compound, also shows close conformational similarity to the normal Δ^4-3-ketosteroid product of the enzymatic reaction.[25]

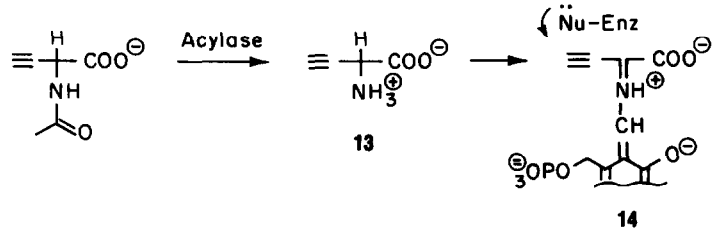


A related strategy for enzymic generation of these secosteroid allenic ketones has been used by enzymic dehydrogenation of the precursor allenic alcohols. The 3-β-alcohols are suicide substrates for bovine adrenal and human placental Δ^5-3-β-hydroxysteroid dehydrogenases, the alcohol → ketone oxidation step generating electrophilic product in the active site.[26a]

A notable target enzyme for antibacterial agents is the bacterial enzyme alanine racemase, involved in provision of D-alanine as the first step in bacterial cell wall mucopeptide synthesis.[27] This enzyme uses vitamin B_6, pyridoxal-P, as tightly bound cofactor to stabilize a substrate-carbanion equivalent. The planar p-quinoid contributor to the carbanion structure, if achirally reprotonated, yields the racemic

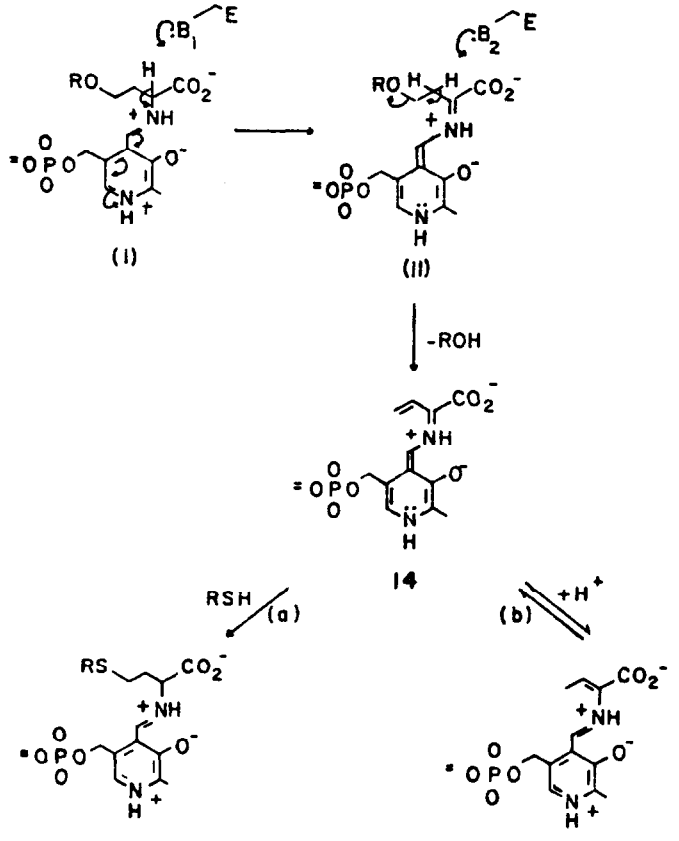


alanine mixture back from either starting enantiomer.[28] Recently, L-ethynylglycine, **13**, has been isolated as its N-acetyl derivative from a fermentation broth as an antimicrobial agent.[29] Although rather unstable as the free amino acid, **13** was reported to inactivate alanine racemase. It could do so *via* Michael addition to the carbanion equivalent **14**, but other possibilities exist as well.

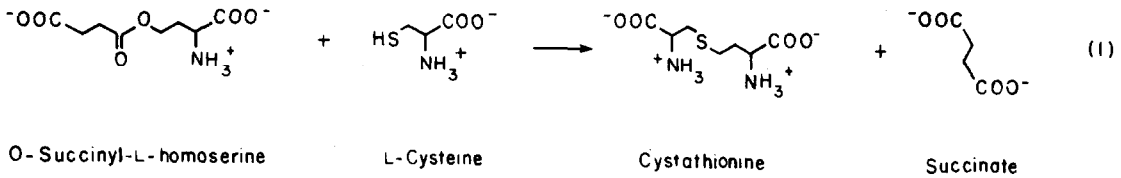


13 **14**

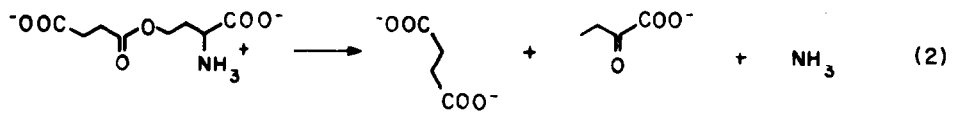
A propynylogous extension of carbanion reactivity can also be exploited in inactivation of two classes of enzymes by a natural product of mushrooms, the γ, δ-acetylenic propargylglycine **9**.[30] The γ, δ-acetylene is insulated from the α-carbon by a β-methylene group and so may resist activation by α-carbanion equivalent but not by β-carbanion equivalents. We validated this expectation with three pyridoxal-P-dependent enzymes that carry out replacement or elimination reactions from γ-substituted amino acids by sequential α-, then β-substrate-PLP stabilized anion equivalents.[18,31] The γ, δ-olefinic



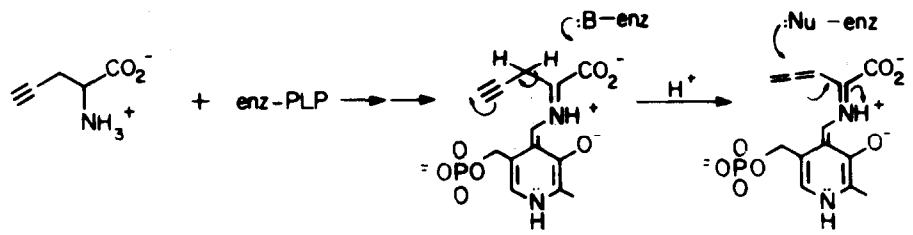
paraquinoid **14** is the key partitioning intermediate in bacterial methionine biosynthesis from O-succinyl homoserine, in methionine breakdown to 2-ketobutyrate and methane thiol, and in mammalian degradation of cystathionine.[18,23,33] As noted in Table 2, L-propargylglycine inactivates all three target enzymes presumably by a two-step catalytic unraveling via the same sequential α,β-carbanion process followed by propargylic protonation at C_5 to give the β, γ, δ-allenic paraquinoid analog of normal olefinic intermediate **15**. This species is again apparently much more electrophilic than the conjugated olefin and inactivates these enzymes with very low partition ratios, as few as 4–8 product molecules generated per inactivation event. The molecular basis of this heightened reactivity has not yet been thoroughly explored or (probably) exploited. The killing site of propargylglycine in its (weak) action as an antibiotic is likely the methionine biosynthetic enzyme, cystathionine-γ-synthase.[18]



O-Succinyl-L-homoserine L-Cysteine Cystathionine Succinate (1)

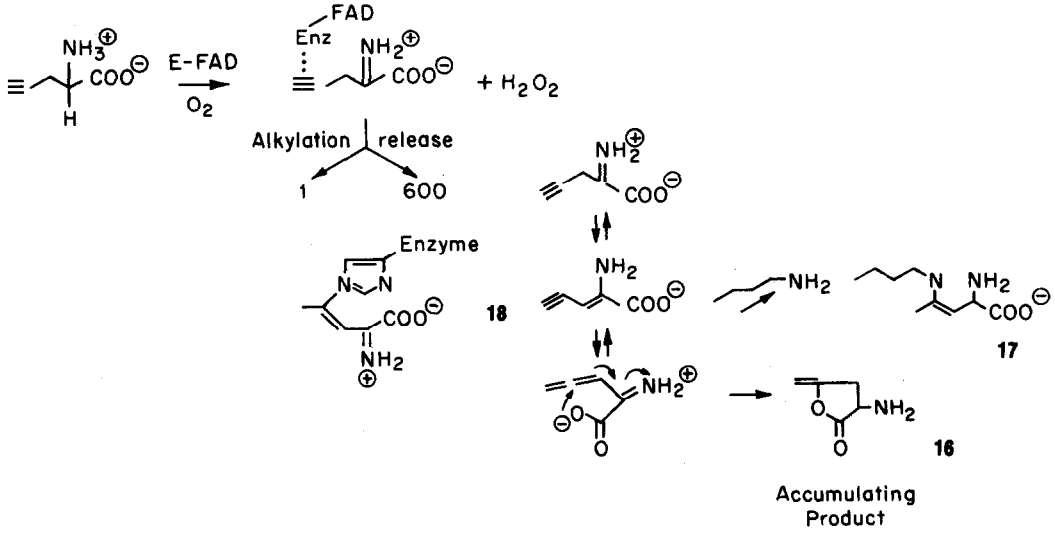


O-Succinyl-L-homoserine Succinate α-Ketobutyrate (2)

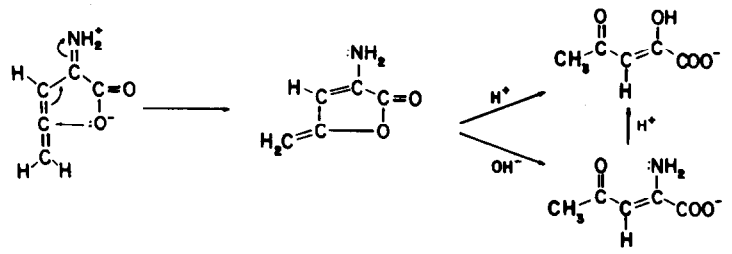


9 **15**

2.1.2 *Acetylene activation: oxidation at an adjacent locus.* Propargylglycine also alkylatively modifies a different type of enzyme during catalytic processing; this is the broad-specificity D-amino-acid oxidase, an FAD-enzyme, of kidney cells.[34-36] D-Propargylglycine is an effective substrate, passing two electrons first to enzyme-bound flavin, and then to O_2 in a second half-reaction. On average, after some 600 turnovers, a histidine residue captures a product molecule alkylatively, leading to an enzyme

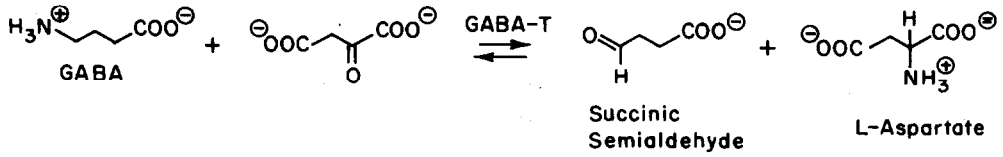


that is "wounded" but not "dead."[36] D-Alanine oxidation is decreased by a factor of 10 in maximal rate and its affinity is lessened as the modified catalyst limps along. This alkylation appears to hit a non-essential histidine residue stoichiometrically and, in fact, the modification appears to occur on rebinding of a rearranged allenic product molecule to the active site. Examination of accumulating product moelcules in solution reveals cyclic eneamino lactone **16** as the predominant species.[37] In the presence of a trapping nucleophile butylamine, a species that is most likely the conjugated eneamino-acid **17** builds up. Both results strongly suggest the initial 2-imino-4-pentynoate product rapidly loses a now acidic C_3-H to yield the yneamino acid. Protonation at C_5 completes propargylic rearrangement, and the 2-imino-3, 4-allene **18**, is captured intramolecularly with high efficiency by the adjacent $-COO^-$ unless a

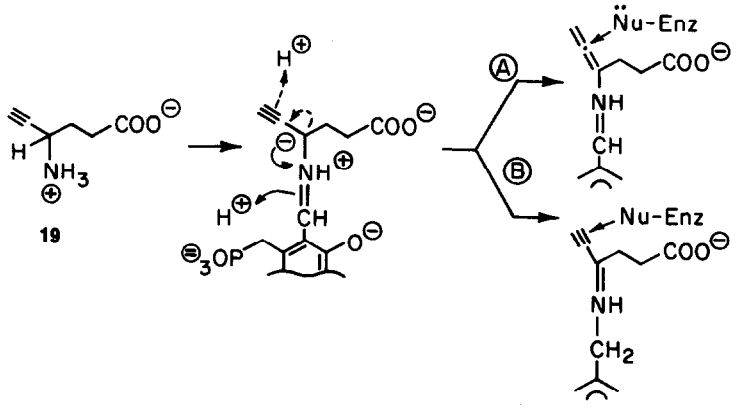


18 **16**

completing strong nucleophile is present in high concentration. In several other cases, acetylenic substrate analogs have been fashioned to inactivate target enzymes. When transaminases such as γ-aminobutyrate transaminase (GABA-T) a brain enzyme target for antiepileptic drugs, is inactivated by γ-acetylene GABA **19** it is unclear yet if rearrangement of a carbanion to a conjugated allene or



oxidation to the conjugated acetylenic imino acid-PLP complex is the step leading to the alkylating species.[38,39] This mechanistic uncertainty can be resolved by structural determination of the adducted



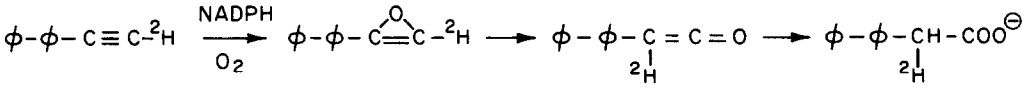
enzyme since an allene would be attacked at C_5 but a bound acetylenic product would be attacked at C_6 before its release. In the case of butyne-1-ol or 1, 4-diol processing and inactivation by nicotinamide coenzyme-dependent[40] or by flavoenzyme oxidases,[41] the capture of acetylenic aldehyde products, in kinetic competition with release, is the likely killing process.

2.1.3 *Acetylene activation by monooxygenation.* A third route of acetylene activation appears to occur during monooxygenative processing of substrates by hemeprotein monooxygenases which shows the following general stoichiometry. There are tissue specific differences, with liver hemeprotein cytochrome P_{450} monooxygenases engaged in detoxification roles in processing of xenobiotic compounds

$$R\text{-}X + NADPH + O_2 \longrightarrow R\text{-}X\text{-}OH + NADP + H_2O$$

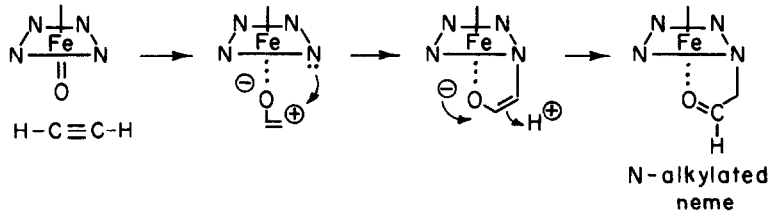
while in adrenal and reproductive tissues, the P_{450} enzymes carry out a series of regio- and stereospecific oxygenations in steroid hormone biosynthesis.

In liver some acetylenes are apparently harmless substrates, such as ethynyl biphenyl **20**, which yields biphenylacetic acid[42] one carboxylate oxygen deriving from O_2.[43] Equally intriguing, 1-[^{2}H]-ethynyl biphenyl experiences quantitative migration of ^{2}H to C_2 of product, consistent with the vinyloxiran as initial oxygenation product, followed by allowed 1,2-rearrangement to the ketene.[44] Hydration would

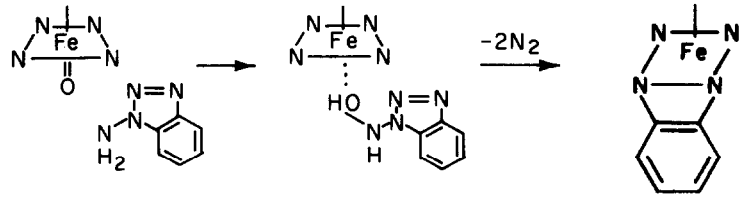


20

then produce the observed acid. Other alkynes, even as simple as acetylene,[45-47] can be problematic though, inducing suicidal inactivation of the P_{450} enzyme forms that work on them. The nature of the inactivating reagent is unclear, although the ketene could be one possibility. Some elegant studies of Ortiz de Montellano and colleagues point to alkylation of one of the heme cofactor's tetrapyrrole nitrogens as the inactivation event.[48] They point out that a vinyloxiran has substantial carbonium ion character and could be readily intercepted by a nucleophile in proximity. It is likely that this type of

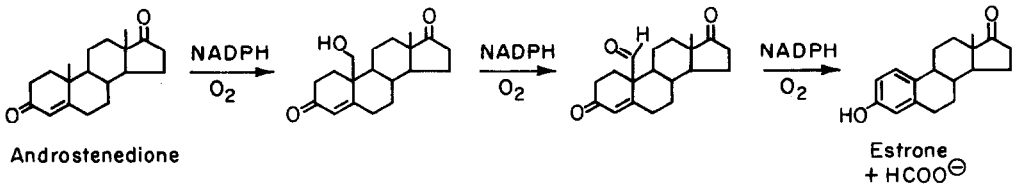


enzyme chemistry in insect P_{450} enzymes accounts for the synergistic effects of various acetylenes as insecticides. Ortiz de Montellano[49] has also found that 1-amino benzotriazole **21** can be N-hydroxylated by the hemeprotein monooxygenase and then unravel to benzyne which appears to add across two pyrrole nitrogens of the heme group of the enzyme.



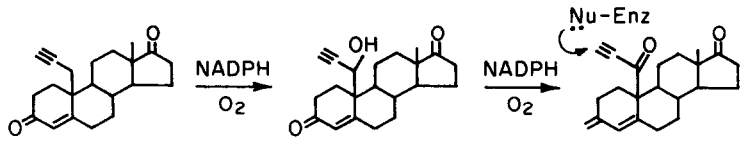
21

Two notable recent entries for the enzyme aromatase complete our discussion of this subset of alkynes.[50,51] Aromatase is a poorly characterized (i.e. not yet extensively purified) hemeprotein monooxygenase responsible for aromatizing the A ring of steroids in estrogen biosynthesis in reproduc-



tive tissue. The chemistry is of interest, since three molecules of O_2 and NADPH are consumed as the angular methyl at C_{19} is oxidatively carved out of the skeleton by a series of hydroxylations first to

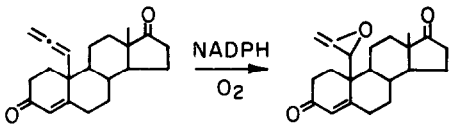
alcohol then aldehyde. The third O_2-consuming step could be a biological Baeyer–Villiger sequence *via* a formate ester or by a ring hydroxylation at C_2.[51a] Recent work[51b] suggests that the first two hydroxylations occur at one site and that the third step occurs at a different site on the enzyme.



22 **22b** **22c**

Covey *et al.*[50] and then Metcalfe and colleagues[51] recently reported synthesis of the ethynyl analog **22**, and both tight affinity for and time-dependent irreversible inactiviation of placental aromatase. The possibility of estrogen biosynthesis blockage via oxygenation of **22** to a reactive oxacyclo propene by this hemeprotein enzyme looks quite interesting. The analogous **22a** has also been reported by them as an aromatase inactivator, possibly via oxygenation to the indicated methylene oxiran. Related acetylenic steroids have also been prepared recently by two other troups.[50,52]

The Covey group[50] also prepared the epimeric alcohols of **22b** and found one epimer to be a competitive inhibitor, the other to be a suicide substrate. Previous stereochemical studies of Arigoni, Akhtar *et al.*[52a] established that in the normal alcoholic substrate the pro R hydrogen is removed. This stereochemistry is consistent with subsequent oxygenation of the active isomer of **22b** to the presumed proximal electrophile **22c**.



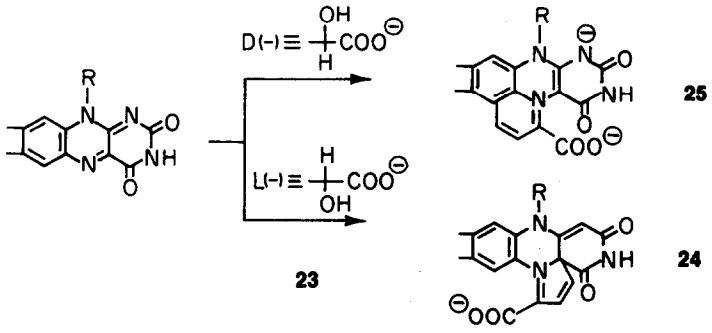
22a

2.1.4 *Acetylene activation to nucleophilic anion equivalents.* The hydroxy acid, **23**, 2-hydroxy-3-butynoate (ethynyl lactate) is a synthetic analog of natural product ethynylglycine **13**. This hydroxy acid (and 3-alkynyl homologs) has been observed to inactivate flavoproptein α-hydroxy acid oxidizing enzymes from bacteria, yeast, plants and animals.[19,53–58] In several cases examined, partition ratios for oxidative turnover to 2-keto-3-butynoate per inactivation event range from 5–150/1 as collected in Table 3. Thus, conjugated electrophilic product molecules are generated, but these may not be the inactivating species. In two examples of L-hydroxy acid oxidases examined, one from *Mycobacterium phlei*[56] and one from rat liver,[19] the covalent modification in inactive enzyme molecules is not to an active site residue but to the flavin coenzyme. Structural analysis in the bacterial case indicated a cyclic 4, 5-adduct **24** of flavin and substrate **23**. While this adduct could arise from attack of dihydroflavin eneamine C_{4a} on

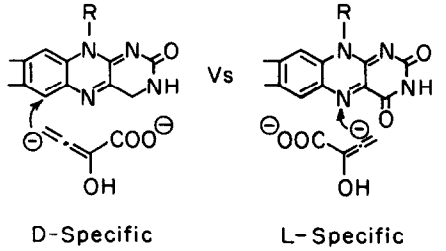
Table 3. Partition ratio for flavoenzyme hydroxy acid oxidases by 2-hydroxy-3-butynoate

Enzyme	Substrate Enantiomer	Turnovers/ Inactivation
1. M. Smegmatis Lactate Oxidase	L-	110
2. Rat Kidney L-Hydroxy Acid Oxidase	L-	25
3. E. coli D-Lactate Dehydrogenase	D-	15-30
4. M. elsdenii D-Lactate Dehydrogenase	D-	5

the acetylenic terminus of bound-2-keto-3-butynoate, it could also arise from addition of the allenic anion contributor of a C_2 anion onto C_{4a} of oxidized flavin coenzyme, the only electrophilic species around. Nucleophilic amino-acid side chains would be incompetent in such a capture.

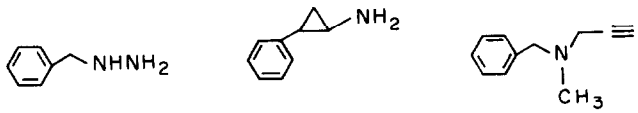


Perhaps most consistent with an inactivating substrate anion equivalent are data with a bacterial D-α-hydroxy acid dehydrogenase from *Megasphera elsdenii*.[58] This flavoprotein processes the D-enantiomer of 2-hydroxy-3-butynoate for turnover and suicidal inactivation also exclusively via bound flavin coenzyme modification. Now, though, the adduct is a N_5, C_6 cyclic structure **25**, which most logically arises from attack of a C_4 substrate allenic anion equivalent at C_6 of the dimethylbenzene ring of flavin followed by ring closure by now basic N_5 of the flavin. Precedent for anionic attack on C_6 derives from isolation of 6-cysteinyl flavin as a cofactor in the bacterial enzyme trimethylamine dehydrognase.[59] The district regiospecificity of cyclization can be accommodated in the D- and L-specific enzymes by orientation of the ethynyl group of bound substrate in opposite orientations at the respective active sites.[60] The allenic anion interpretation is consistent with normal catalytic oxidation of substrate alcoholic groups by carbanionic mechanisms as well as explaining why only the flavin coenzyme, and not any amino-acid

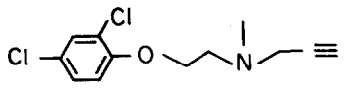


residues is modified. In an allenic anion mechanism the oxidized flavin cofactor is the most readily available electrophile at the active site.

The enzyme monoamine oxidase (MAO) is widely distributed through animal organs involved in oxidative deamination and thereby deactivation, of various exogenous (foodstuffs) and endogenous neuroactive amines such as phenylethylamine, noradrenalin, dopamine, tryptamine, histamine. Since the detection in 1952 that iproniazid is an MAO inhibitor, hundreds of irreversible inhibitors have been developed. Three major types of functional groups have evolved, hydrazines, cyclopropylamines, and propargylamines, typified by phenelzine, **26**, tranylcypramine **27**, and pargyline **28**.[61] Two highly potent



acetylenic amine inactivators are clorgyline, **29**, and (–)-deprenyl **30**.[62] Clorgyline shows selectivity in destruction of MAO activity involved in 5-hydroxytryptamine deamination (MAO-A), while deprenyl is selective at inactivation of MAO-activity responsible for benzylamine deamination (MAO-B).[61,65] The molecular distinctions between MAO-A and B are as yet unknown. All the acetylenic amines inactivate



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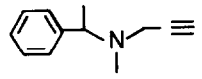

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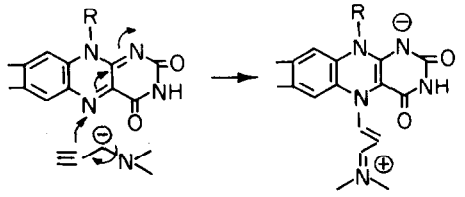
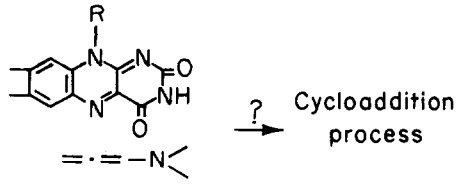
via covalent derivatization at N_5 of the flavin.[63,64] For example, dimethylpropargylamine **31** yields the indicated flavocyanine adduct and thus is likely a representative case.[63,65] Again there is the unresolved



31

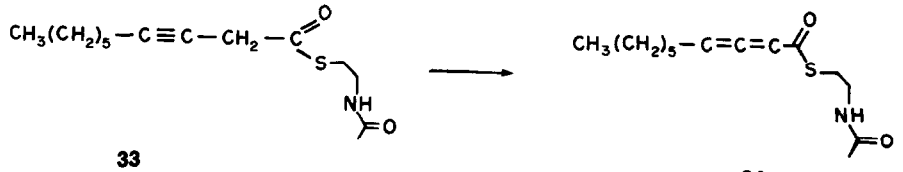
dichotomy in these amine oxidases whether inactivation occurs before redox catalysis between substrate allenic anion equivalent attacking electrophilic N_5 of oxidized flavin or after redox catalysis between conjugated acetylenic amine and now nucleophilic N_5 of dihydroflavin. Whether selective acetylenic MAO inactivators have clinical potential is still unclear due to past difficulties with people on diets with high intake of pressor amines who suffered hypertensive crises because tyramine in the foods could no longer be oxidatively removed due to inactivation of intestinal MAO by the specific inhibitors.[68] A final comment on amine inactivation is that while allenic amine **32**, causes irreversible and stoichiometric inactivation[69] by adduct formation with the bound flavin coenzyme, the flavin adduct is distinct from (but as yet unidentified) the N^5-flavocyanine formed from N,N-dimethyl propynylamine **31**. A speculation of



32

a cycloaddition mechanism in the allene case is intriguing and could suggest a reaction pathway for allene groups isolated from other activating functionality.

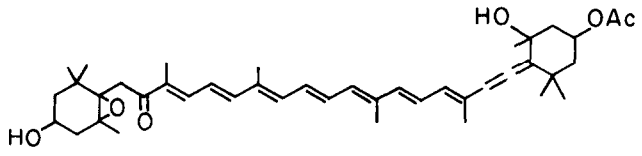
One should note that not all enzymes processing conjugated allenes inevitably commit suicide during turnover. Thus, a hog liver olefinic thiolester isomerase isomerizes 3-decynoyl-N-acetyl cysteamine **33** to



33 34

the (+)-isomer of 2, 3-decadienyl-N-acetylcysteamine **34** without incident.[14] Also the hydration enzyme crotonase from beef liver will hydrate the same decadienyl thiolester to the enol product without inactivation. There may be no nucleophilic enzyme groups appropriately placed for covalent addition reactions in these cases.

Several allenic natural products[70] are known including the major carotenoid fucoxanthrin **35**, and the frog arrow poison component isodihydrohistrionicotoxin **36**, but no specific biological consequence is yet



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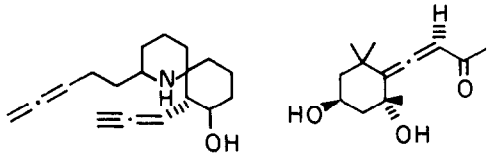
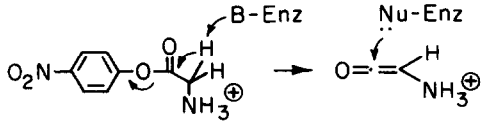


36 37

attributed to the allene. In the case of the defensive secretion **37**, of the wingless grasshopper *Romalea microptera* though, the allenic ketone moiety may be an alkylating irritant.

2.2 *Enzymic activation of nitrile groups: ketene imine equivalents*

There exists in animals an amine oxidase circulating in plasma that is distinct from the intracellular flavoprotein amine oxidase noted above. The plasma enzyme requires Cu(II) and an as yet unidentified prosthetic group that may function as electron sink. In addition to being inactivated by the simple alkynylamine, propargylamine, the plasma enzyme is inactivated by p-nitrophenylglycine **38**, or by aminoacetonitrile **39**. Abeles *et al.*[71] suggest the ester may decompose, after enzymic α-H abstraction to



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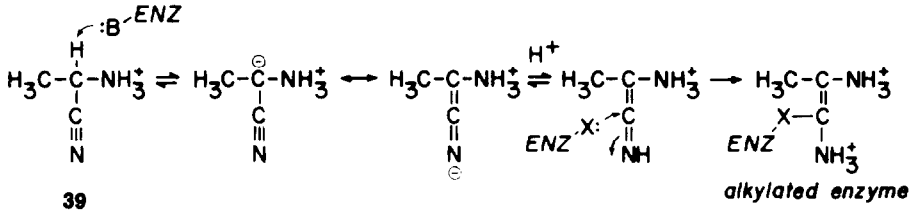
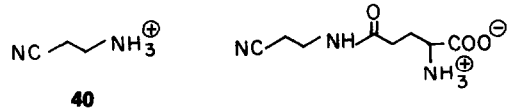
a ketene equivalent which is the killing agent. In analogy, enzymic α-H abstraction from amino-acetonitrile as substrate analog may yield the analogous ketene imine equivalent at the enzyme active site.



39 alkylated enzyme

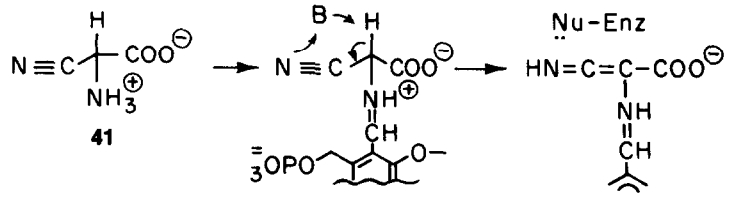
The homologue, β-aminopropionitrile **40**, is a natural toxin produced by plants as the γ-glutamyl amide **40a**.[55] Ingestion of the toxin or β-aminopropionitrile directly yields an osteolathyrism manifested as a



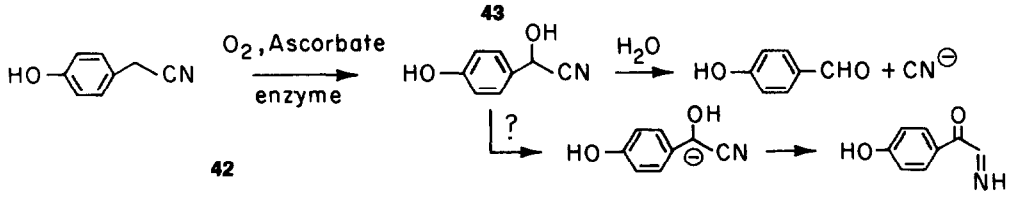
40 40a

connective tissue weakness, resulting from molecular blockage of cross-linking of collagen fibers, an enzymic process required for normal tensile strength in connective tissue. The responsible cross-linking enzyme is an oxidase working on the ϵ-NH_2 groups of lysine residues in collagen. This is a Cu-pyridoxal-linked protein.[72,73] Studies with ^{14}C-BAPN show irreversible inactivation and stoichiometric labeling consistent with a ketene imine inactivation route. An alternative enzyme-mediated α,β-loss of HCN to yield a substrate-pyridoxal eneamine seems ruled out by the labeling result.[74]

Incorporation of a cyano group into an α-amino-acid skeleton yields cyanoglycine **41** as simplest representative, and it is an inactivator of a pyridoxal-P dependent enzyme, tryptophan synthetase, which can abstract the α-H of the substrate in PLP aldimine adduct.[75]



A final recent example is the inactivation[76] of the copper-dependent enzyme, dopamine-β-hydroxylase, involved in adrenalin biosynthesis, by p-hydroxybenzylcyanide **42**. In fact, this substrate is converted



to product p-hydroxymandelonitrile **43** on average, 800 times per inactivation event. Indeed, addition of that product to isolated enzyme leads to inactivation; control studies with para-hydroxybenzaldehyde ruled out its participation as inactivator, nor is free cyanide responsible. Whether the cyanohydrin can be enzymically converted to a ketenimine equivalent, then the glyoxamine and lead to arginine residue guanidinium moiety modification is yet to be probed. The possibility of a quinone species as killer has been ruled out since benzylcyanide is also oxygenated to an inactivating product.[76]

2.3 *Enzymic activation of olefinic groups*

There are hundreds of natural products and dozens of primary metabolites with olefinic functionality and even several cases where α,β-unsaturated conjugated systems are used without incident in enzymic catalyses. Enzymes which process such substrates and intermediates have probably evolved to keep active site nucleophiles away from the electrophilic intermediates. However, when one unleashes a conjugated olefin in an active site microenvironment which does not deal with them routinely, mechanism-based alkylative inactivations can occur.

2.3.1 ***Oxidative activation at an adjacent site.*** A clear-cut set is provided by the natural products vinylglycine **44**, and the coressponding vinylether derivatives such as rhizobitoxin **45**,[77] and the methoxy **46**[78] and ethanolamine **47**[79] derivatives noted in Table 4. When L- or D-vinylglycine is transaminated by a PLP-dependent transaminase of requisite chirality, the bound product can either be released or captured adventitiously in an inactivating Michael attack as shown.[80,81] Partition ratios for several important

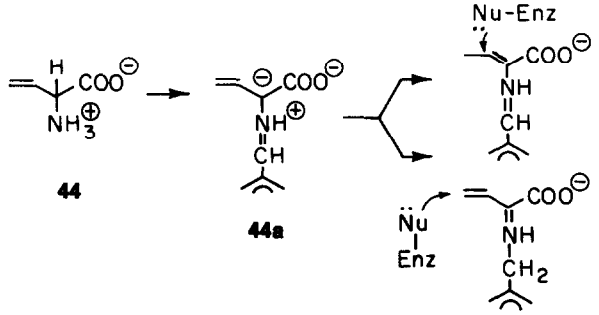
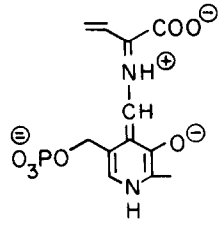


Table 4. Suicide substrates with olefinic functional groups

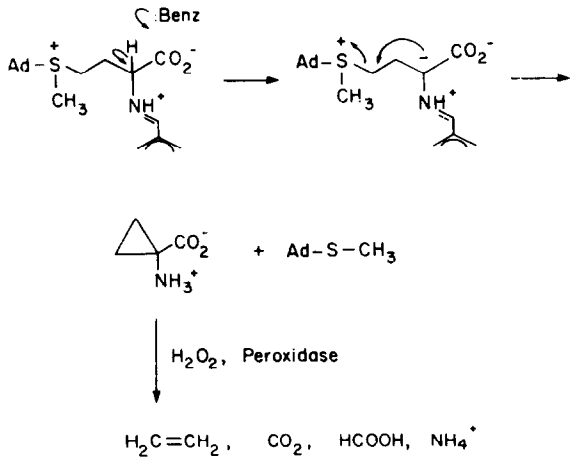
Entry	Compound	Target Enzyme	Partition Ratio	Comment	Reference
1	N-allylglycine	sarcosine oxidase	n.d.		185
2	allyl alcohol	alcohol dehydrogenase	n.d.		186
3	vinylglycolate	L-hydroxy acid oxidase	>1000	multiple labeling of rat liver enz, E.coli membranes	19,187
		enzyme I of hexose phosphotransferase system			
4	vinylglycine, 44	L-aspartate transaminase	1		80
		D-amino acid transaminase	400-800		81
		L-amino acid oxidase	2000		
5	4-trans-methoxy vinylglycine, 46	L-aspartate transaminase	n.d.		79
6	4-trans-aminoethyloxy-vinyglycine, 47	PLP-enzyme in ethylene biosynthesis	n.d.		78
7	rhizobitoxin, 45	β-cystathionase	n.d.		77
8	3-chloroallylamine	plasma amine oxidase	n.d.		9
9	acrolein	5-deoxyribose phosphate aldolase	n.d.		7
10	gabaculine	GABA transaminase	1		86-89
11	γ-vinylGABA	GABA transaminase	n.d.		90,91
12	cis-4,5-lysene	lysine-ε-transaminase	1700		179
	trans-4,5-lysene	lysine-ε-transaminase	160		
13	3,4-dehydro-L-glutamate	glutamate decarboxylase	n.d.		188
14	allylglycine	glutamate decarboxylase	n.d.		93
15	2-amino-cis-3-pentenoate	methionine-γ-lyase	n.d.		84
16	ethylene	liver P-450 monooxygenase	n.d.		44-48
17	allylisopropylacetamide	liver P-450 monooxygenase	220		96
18	secobarbital and many other terminal olefins	liver P-450 monooxygenase	100		189
19	α-vinylDOPA	DOPA decarboxylase	n.d.		181,182
20	α-vinyl ornithine				180

animal and bacterial transaminases vary greatly, reflecting the distinct kinetic fates in the particular enzymic microenvironments.[81] In the L-aspartate transaminase case the ϵ-NH_2 of lysine 258 has added apparently to C_3, not C_4, of vinylglycine suggesting the killing species is not transaminated, bound product but rather forms from allylic-1,3-isomerization of the initial substrate-PLP α-anion and attack on the resultant aminocrotonate-PLP complex.[82] To iterate that one enzyme's poison may be another enzyme's substrate, recall that in γ-replacements and γ-eliminations on γ-substituted α-amino-acids, the vinylglycine-PLP-α-anion **44a** is the normal key intermediate in catalysis, highlighting the possibility for

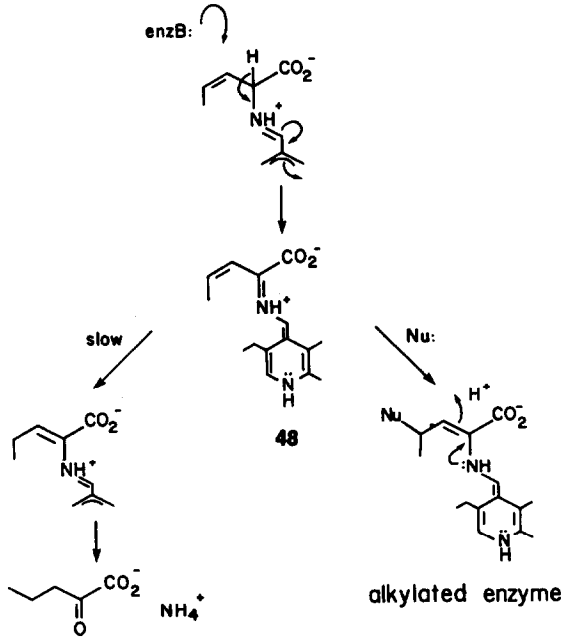


44a

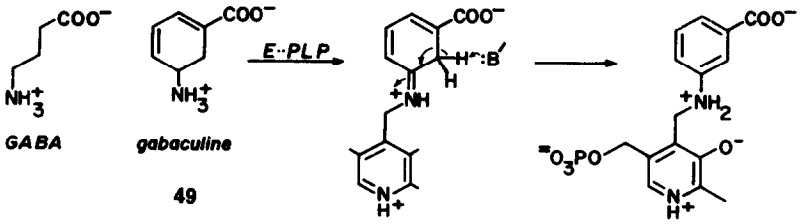
target enzyme specificity.[28] Rhizobitoxin blocks a PLP-linked enzyme in methionine biosynthesis[77] while the other vinylethers **46** and **47** block production of the fruit-ripening hormone ethylene,[78,79] possibly by inactivation of the enzyme converting s-adenosylamethionine to the ethylene precursor, 1-amino-1-carboxycyclopropane.[83] How frequently the allylic carbanion rearrangement process uncovers an olefin-based inactivator, analogous to the acetylenic propargylic rearrangements noted above, compared



to α-carbon oxidation to the conjugated olefin as reactive electrophile remains to be determined. Table 4 lists a series of other, synthetic olefinic amino-acids and their specific pyridoxal-P-dependent enzyme targets. Of some interest is the dichotomy between *cis*- and *trans*-2-amino-3-pentenoates with bacterial methionine-γ-lyase.[84] The *trans* isomer shows a 20-fold higher V_{max} as substrate than the *cis*-isomer for 2-ketobutyrate production, consistent with distinct rate-determining steps. Indeed the enzymic solution in the presence of *cis*-isomer turns from yellow to red in steady state as the fully conjugated intermediate **48** accumulates; for the *trans*-isomer some other step is slow and no red intermediate builds up. With the increased lifetime of the electrophilic **48** in the *cis*-case, irreversible inactivation specifically ensues suggesting that given enough kinetic opportunity, the enzyme cannot resist the forbidden Michael attack.



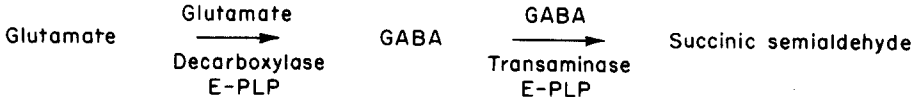
An unusual unsaturated amino acid worth note is the dihydroaromatic gabaculine **49** a dihydroanthranilate first isolated from a mold[85] and shown recently to have antiepileptic properties by virtue of brain GABA-transaminase inactivation.[86–89] A rigid cyclic analog of GABA, the cyclohexadienoid is enzymically transaminated to the product exocyclic imine. Here anticipated normal hydrolysis of the imine linkage does not compete kinetically with facile aromatization to yield a meta-anthranilate in *non-hydrolyzable* secondary amine-linkage to the PLP cofactor. Since PLP does not dissociate readily from the apoprotein, the enzyme molecules are dead, again by virtue of structural modification of a tightly,



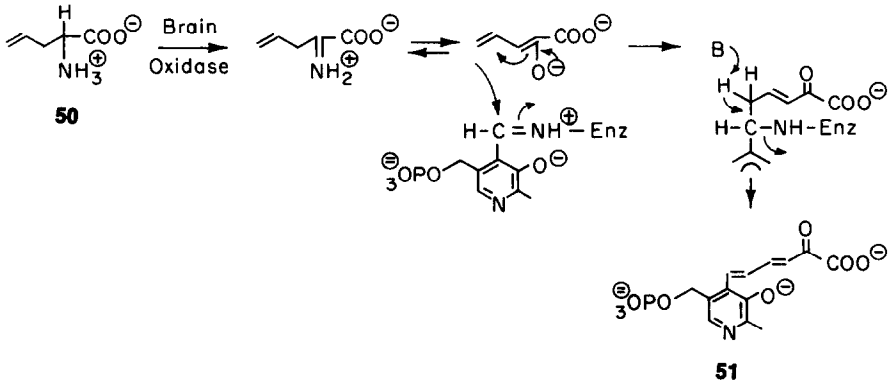
49

but not covalently bound cofactor at the active site rather than by derivatization of an apoprotein residue. With the pseudomonad transaminase gabaculine is a completely efficient suicide substrate.[89] No product molecules get away, all flux is to inactivation, every gabaculine molecule titrates out an enzyme molecule, a useful partition ratio for *in vivo* specificity. Gabaculine double bond isomers have now been synthesized[39] and, not unexpectedly, they also are suicide substrates for GABA transaminase, suggesting aromatization need not be regiospecifically (or even enzymically) effected.

While olefinic inactivators of GABA-transaminase, the enzyme responsible for oxidative removal of this inhibitory neurotransmitter in brain, are antiepileptic,[90,91] inhibitors of the PLP-enzyme producing GABA in the brain, glutamate decarboxylase, are neuroconvulsive. Thus, the natural allylglycine, **50**, on entry into brain tissue is first oxidized by brain amino acid oxidases to amino acid and then to 2-keto-4-pentenoate.[92] This product is neuroconvulsant by virtue of glutamate decarboxylase blockage.



To elucidate the mechanism of such inhibition, we used readily accessible *E. coli* glutamate decarboxylase to analyze irreversible inactivation by solutions of 2-keto-4-pentenoate.[93] The obvious Michael addition candidate, 2-keto-3-pentenoate, by prototropic shifts, was kinetically incompetent to account for observed inactivation rates. Instead, the active site reagent appears to be the dienolate, a nucleophilic not an electrophilic equivalent. A likely active site electrophile in high local concentration cannot be an amino acid side chain, other than an arginine guanidinium group, but rather the aldehyde carbonyl of the vitamin B_6 cofactor. Indeed, labeling studies show only the bound coenzyme, not the apoprotein, is

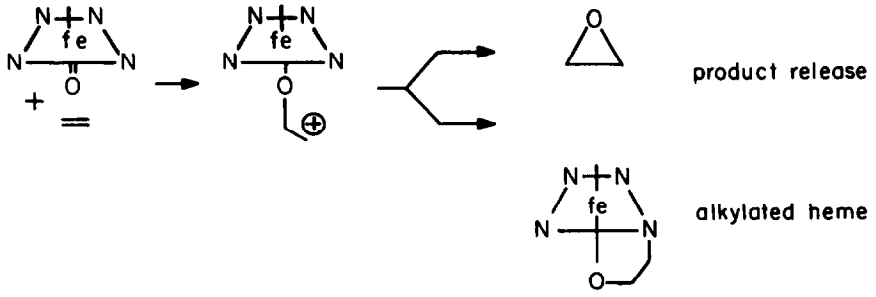


50 51

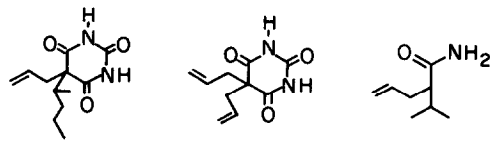
derivatized[93] to what is probably conjugated species **51**, $\lambda_{max} = 430$ nm. The allyl glycine sequence represents a polarity reversal from the gabaculine inactivation case but both lead to exclusive derivatization of bound B_6 coenzyme at the glutamate decarboxylase or the GABA transaminase active site, respectively.

2.3.2. *Activation during epoxidation.* A distinct type of enzyme-mediated autoinactivation during olefin processing operates again with a subset of the liver hemeprotein cytochrome P_{450} monooxygenases. Even the most elementary ethylene is a killer molecule during oxygenation.[47] The inactivation pattern, a most interesting one, has been itself unraveled elegantly by Ortiz de Montellano *et al.*, who showed that only the heme prosthetic group is blocked, at one of the tetrapyrrole nitrogens.[48] Combined mass spectroscopic and high field NMR data suggest the same basic adduct structure as seen with acetylene (*vide supra*) which may arise from the carbonium ion which forms on initial oxygen transfer to olefin. The carbonium ion could be quenched internally to produce epoxide product or be intercepted by nearby

pyrrole nitrogen to yield the tell-tale green heme derivative. Studies exposing the heme enzyme to epoxide products from olefinic substrates reveal they are not the alkylating agents themselves.[94] In the

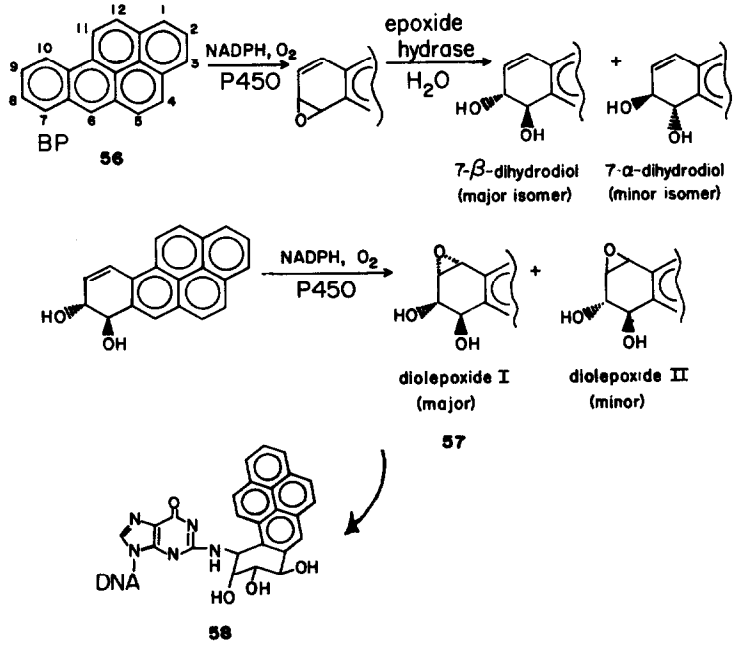


case of the much-studied model sedative allylisopropylacetamide **53**, the partition ratio of epoxidative turnover/inactivation is about 200/1.[95,96] Barbiturates such as secobarbital **54** and aprobarbital **55** also



54 **55** **53**

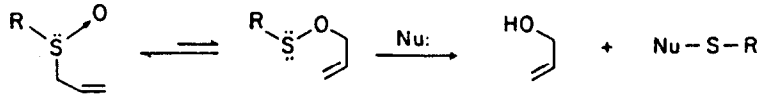
yield heme group derivatization in these P_{450} enzymes.[97] Another major class of P_{450} isozymes in liver epoxidizes aromatic double bonds to yield arene oxide products. These enzymes do not process simple olefins and are unsusceptible to this brand of autoinactivation. On the other hand, the product arene oxides thus turned out, although not specifically killing the generating enzyme, can wreak molecular havoc in the cell.[98] Thus, benzo[a]pyrene **56** is epoxidized regio- and stereospecifically at the 7, 8-locus, but is then enzymically opened hydrolytically (epoxide hydrolase) and harmlessly to the trans 7,8-dihydrodiol. Then a second round of epoxidation at the highly *reactive* 9,10-double bond can produce, as metabolic grandchild of the initial polycyclic **56**, the diol epoxide I, **57**, thought currently to be the proximal carcinogenic metabolite by virtue of DNA alkylation in liver and lung cells.[98] Several adducts are formed, the major one being the indicated guanine adduct **58**. This is a case where an enzymic detoxification strategy (to remove benzpyrene) leads instead to a toxification outcome.



56 **57** **58**

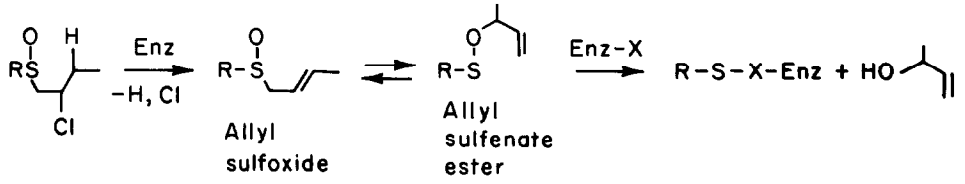
2.3.3 *Inactivation by 2,3-sigmatropic rearrangement of allylsulfoxides.* A novel route to convert an unreactive grouping to an uncovered electrophilic functionality with an olefinic component at the active site of a target enzyme has been to conscript the 2,3-sigmatropic rearrangement of allylsulfoxides to allylsulfenate esters.[99] After rearrangement, the sulfur is readily susceptible to nucleophilic attack. In fact, both S and O capture are known in organic chemistry during sulfenate ester trapping,[90] but protein

PREDICTED THIOPHILIC INTERCEPTION

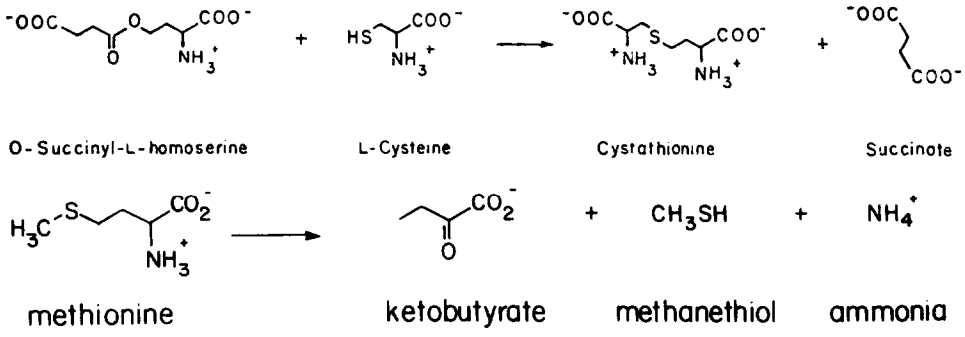


nucleophiles are likely to attack sulfur with thiolation of the enzyme and release of the allylic alcohol fragment.

The one case so far reported[99] involves two pyridoxal-P-enzymes involved in bacterial methionine metabolism, catalyzing elimination of a γ-substituent either in methionine biosynthesis (cystathionine-γ-synthetase) or degradation of the γ-sulfur amino-acid via the key common β, γ-quinonoidal-PLP adduct

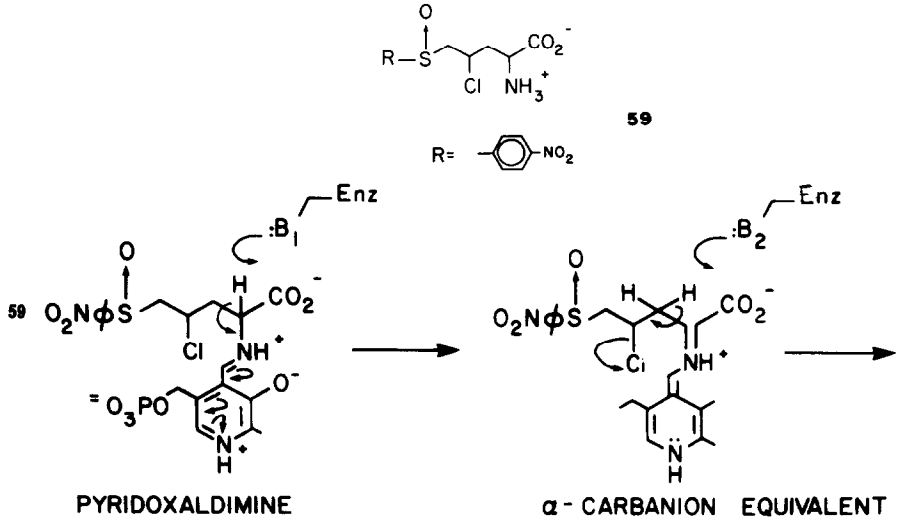


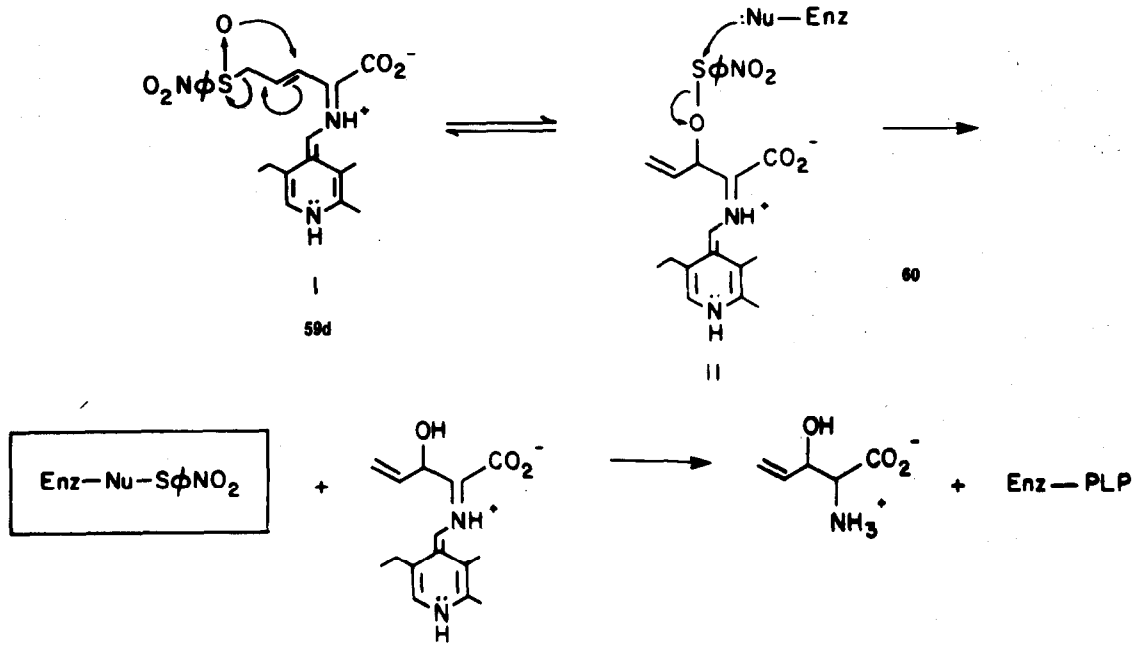
44a which arises by sequential α- and β-carbnion formation, then γ-elimination from the initial



substrate-coenzyme adduct. Synthesis of the γ-chloro-δ-S-paranitrophenyl-2-aminovalerate **59** produced a suicide substrate for both these enzymes by mistaken processing of **59** for α-then β-carbanion formation, followed by γ-elimination to set up the β, γ-double bond in a position allylic to the aryl

PROPOSED INACTIVATION MECH



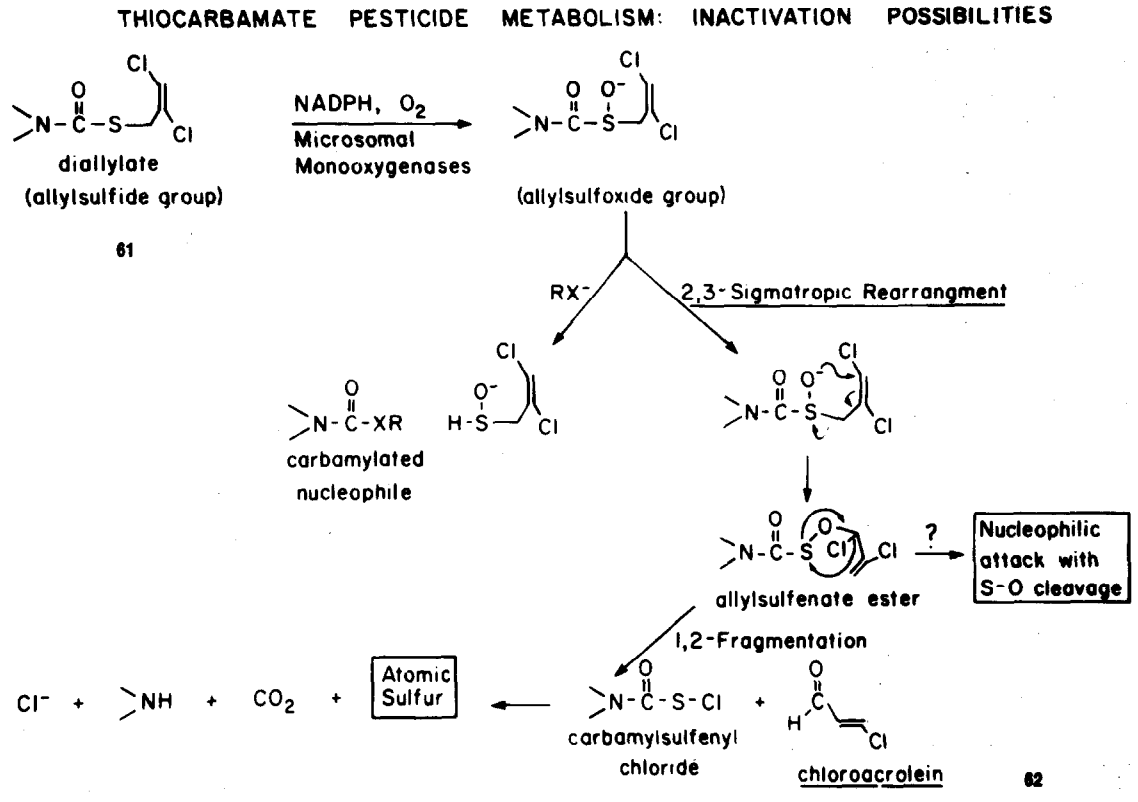


I
59d

II
60

sulfoxide, so uncovering the major partner in the 2, 3-sigmatropic rearrangement equilibrium. Enzymic capture of the minor allylsulfenate ester component **60** of the equilibrium led to an inactive enz-cys-S-S-NO_2 mixed disulfide, and ultimately 2-keto-3-pentenoate after unraveling of the allylic alcohol-PLP adduct fragment.

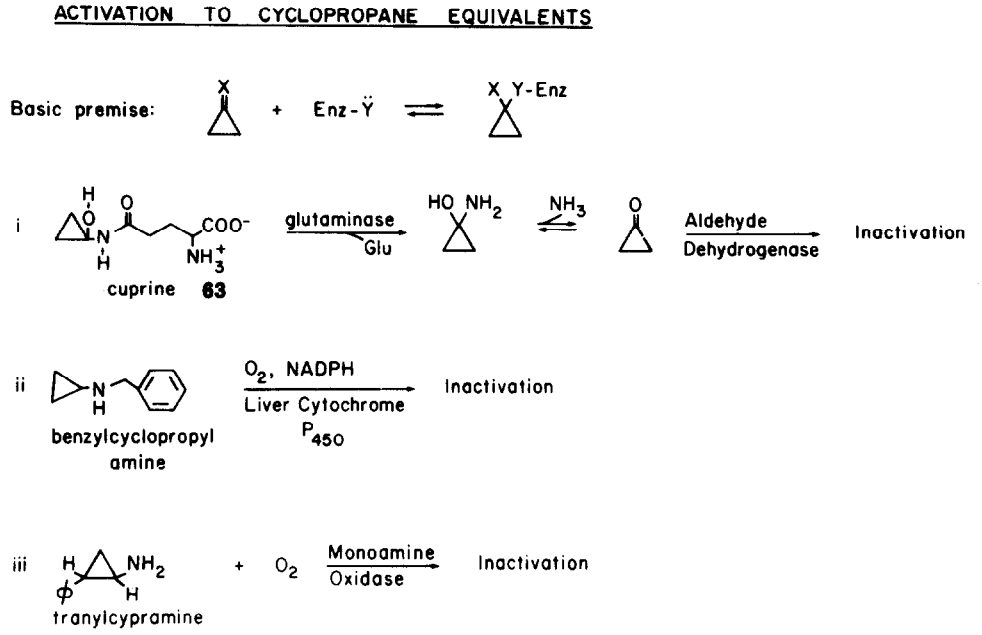
This example used a saturated sulfoxide precursor with an enzyme that carries out HX-elimination to set up the allylic group during the catalytic cycle. An alternate strategy would be enzymic oxygenation of an allylsulfide, and this may be the case in liver P_{450} oxygenative processing of the thiocarbamate herbicides of the diallylate **61** type as noted below.[100] These herbicides are mutagenic in the Ames test, and chloroacrolein **62** may be the culprit.



2.4 *Cyclopropanoid suicide substrates*

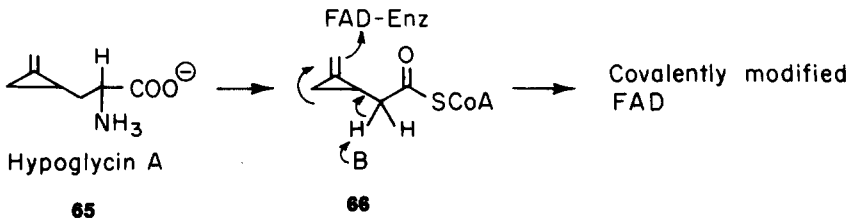
There exist both naturally occurring and synthetic cyclopropanoid compounds that are irreversible inhibitors of particular target enzymes. Three of the four well-documented examples to date probably involve enzymic processing of a precursor molecule to a cyclopropanone equivalent,[101-104] and inactivation may derive from the favorable equilibrium position of the carbonyl tetrahedral addition product relative to the cyclopropanone itself. If the nucleophilic group is a part of the enzyme covalent derivatization has been achieved.

Thus, the toxic mushroom constituent cuprine **63** undergoes hydrolysis of the amide bond probably by a glutaminase enzyme, and the hemiaminal rapidly equilibrates to free cyclopropanone which binds to the active site of aldehyde dehydrogenase, possibly as hemithioacetal with an active site cysteinyl SH group.[93] An especially tight complex is formed with the normal redox cofactor NAD^+ to yield inactive aldehyde dehydrogenase molecules.[105]

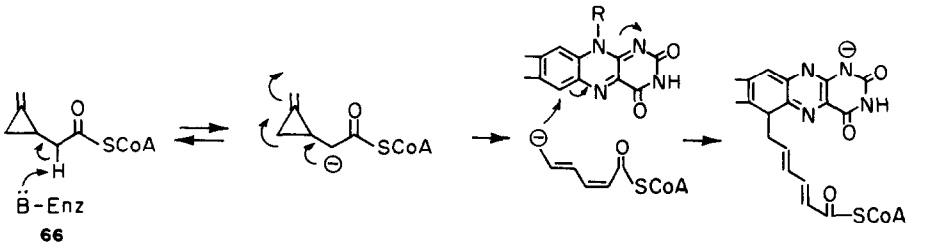


Some classical antidepressant inhibitors of monoamine oxidase are cyclopropylamines, among them tranylcypramine.[102] These primary and secondary cyclopropylamines are apparently oxidized to the cyclopropylimine equivalents by the amine oxidase. It may be that these imines capture an active site sulfhydryl group to form a stable, inactive hemithioaminal tetrahedral adduct with the enzyme[102] although it has been argued that a labile adduct to N^5 of the flavin coenzyme may be involved.[103] In either case, the structural evidence for the enzyme-adduct is yet lacking and in particular the instability of phenylcyclopropanones raise the possibility of Favorski-type ring-opening routes. Similarly, the benzylcyclopropylamine inhibitor of a drug-inducible cytochrome monooxygenase P_{450} isozyme in mammalian liver probably oxygenates the cyclopropyl substrate to a cyclopropanone equivalent.[104] This may offer a general strategy for redox enzyme inactivation.

The last example deals with a metabolite of hypoglycin A **65**, itself a toxic metabolite of the Jamaica ackee fruit.[106] The methylene cyclopropane amino acid **65** is converted by an oxidative decarboxylation route eventually to the acyl CoA thiolester and this species blocks isovaleryl CoA dehydrogenase reversibly.

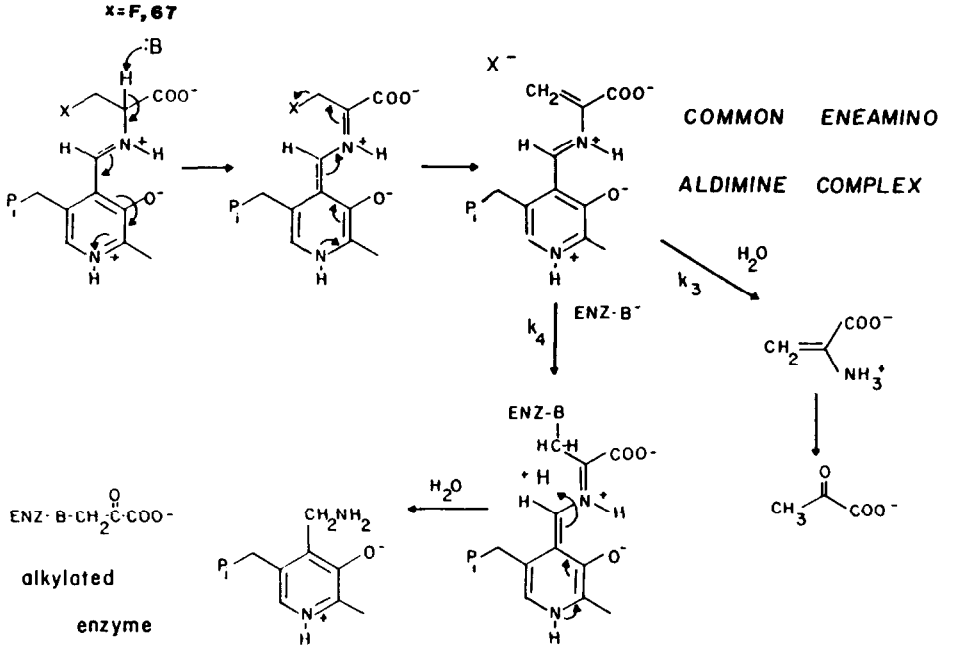


Additionally, Ghisla[60] has recently shown the acyl CoA **66** is a suicide substrate for the flavoenzyme desaturase, butyryl CoA dehydrogenase (butyryl-SR→crotonyl-SR), apparently by covalent derivatization of the FAD cofactor, possibly by reaction at C_6 of the benzene ring. This is reminiscent of hydroxybutynoate-flavin adducts discussed above and could suggest an anionic opening of an initial enzyme-generated C_2-anion (the normal intermediate in desaturation) to produce a conjugated anion as nucleophilic attacking reagent on C_6 of FAD. Whether enzymes generally can open methylene cyclopropanes by adjacent carbanion formation remains to be seen.



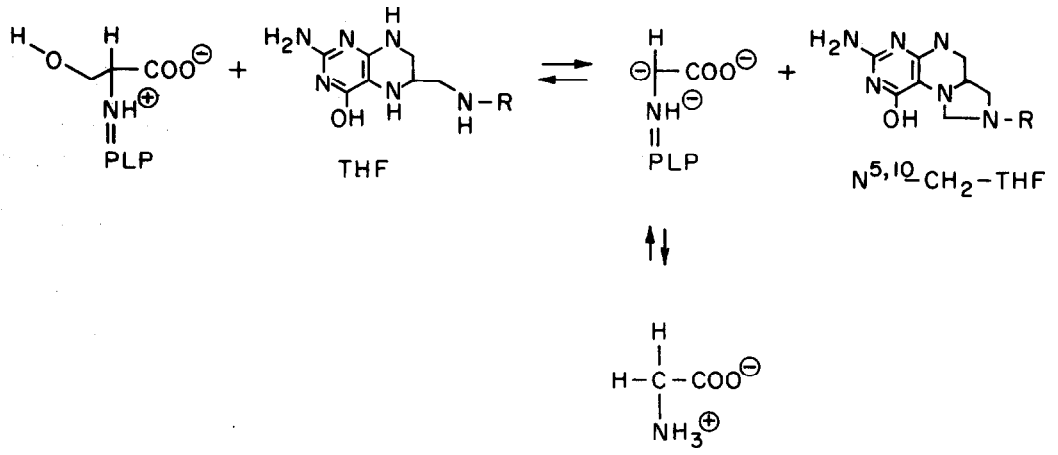
2.5 *Halogens and other leaving groups adjacent to carbanion sites*

A large number of compounds are now known that function as suicide substrates by enzyme-mediated elimination of a halide or other good leaving group following carbanion formation at an adjacent locus to yield a conjugated olefinic system set up for Michael addition.[7-13] Almost all the target enzymes for this class are pyridoxal-P-linked enzymes since they specialize in generating α-carbanion equivalents during catalysis. Thus, β-halo-α-amino-acids, since PLP-enzymes work on amino-acid substrates, are the suicide substrates. The paradigmatic β-fluoroalanine **67** illustrates the strategy in inactivation of bacterial alanine racemase and explains its molecular action as an experimental antibiotic.[107,108] The *E. coli* enzyme processes either isomer of β-fluoroalanine with a partition ratio of



about 800 eliminative turnovers to pyruvate for every Michael attack and alkylative inactivation.[109] Bacterial asparate α-decarboxylase, generating β-alanine for coenzyme A biosynthesis, inactivates itself with the D-isomer only of β-fluoroalanine[110] as does mammalian serine transhydroxymethylase.[111] This

enzyme normally interconverts L-serine and tetrahydrofolate (THF) by aldol cleavage on the β-hydroxy-α-amino-acid, with glycine and the biological formaldehyde equivalent, $N^{5,10}$–CH_2–THF. Although this process does not involve a α-carbanion equivalent in the cleavage direction, the reaction in



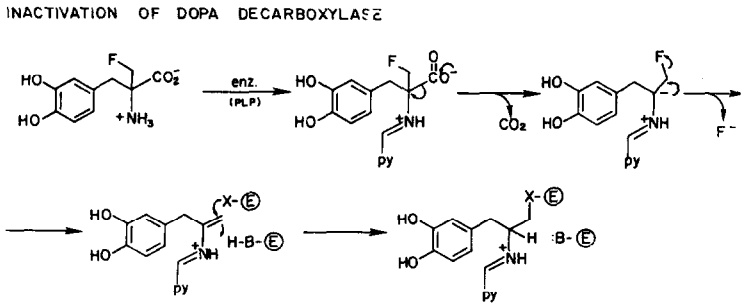
the back direction requires a stabilized glycine-PLP-α-anion as the carbon nucleophile in C_β–C_α bond construction in product L-serine. The α-H of glycine sterospecifically removed by the enzyme in fact corresponds to the α-H of a D-amino acid not an L-amino acid. Thus, we predicted catalytic HF-elimination from D-fluoroalanine and indeed have observed a 60/1 partition ratio for pyruvate molecules formed per enzyme molecule inactivated by enzymic cysteinyl attack on the aminoacrylyl-PLP complex. The normal cofactor tetrahydrofolate accelerates both turnover and inactivation a dramatic 660-fold by selectively accelerating the C_α–H cleavage step on bound D-fluoroalanine. Table 5 collects some representative β-substituted amino-acids, target PLP-enzymes, and partition ratios where known. The polyhaloalanines also have been examined: β, β-difluoroalanine yields an unstable adduct

Table 5. α- or β-Substituted amino acids as suicide substrates

Compound	Enzyme	Reference
1. β-fluoroalanine, D- or L-	alanine racemase serine transhydroxymethylase	109,113 110
2. β,β-difluoroalanine, D- or L-	alanine racemase	112
3. β,β,β-trifluoroalanine, D,L-	several PLP-enzymes	121
4. 5-fluoroGABA	GABA transaminase	174
5. α-fluoromethylDOPA	DOPA decarboxylase	116,113
6. α,α-difluoromethylDOPA	DOPA decarboxylase	115
7. α-fluoromethyl histidine	histidine decarboxylase	113,117
8. α-fluoromethyl ornithine	ornithine decarboxylase	113
9. α,α-difluoromethyl ornithine	ornithine decarboxylase	120
10. α-fluoromethyl glutamate	glutamate decarboxylase	118

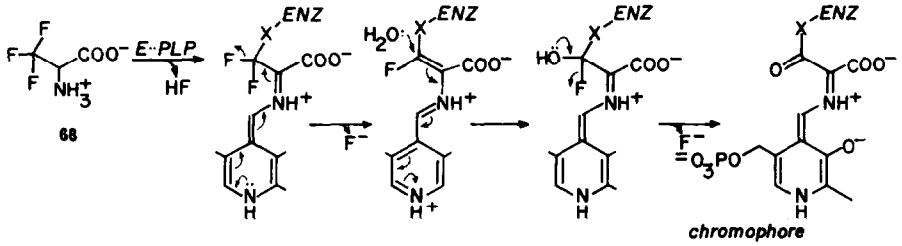
with *E. coli* alanine recemase, while β, β, β-trifluoroalanine has a very low partition ratio but is not an effective antibiotic, probably because the CF_3 group lowers the amino group pKa from a normal 9.8 down to 5.8, such that trifluoroalanine is not zwitterionic at physiological pH and may not be transported into the cell.[112]

Since amino-acid decarboxylases are often involved in the biogenesis of pharmacologically active amines, e.g. DOPA, histamine, tryamine, selective inhibitors have been sought and α-fluoromethyl amino acids are suicide substrates by virtue of enzymic decarboxylation, halide elimination, and Michael addition.[113–117] Glutamate decarboxylase is similarly inactivated by α-fluoromethyl glutamate,[118] The α,



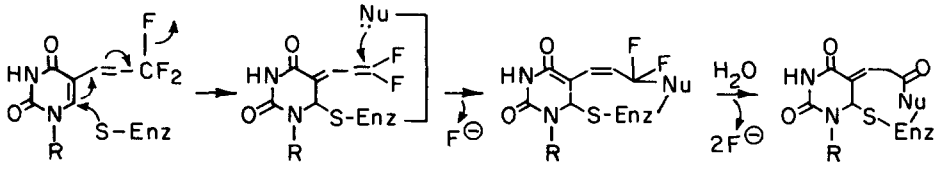
α-difluoromethylornithine appears particularly effective for inactivation of ornithine decarboxylase,[119] a key enzyme is polyamine biosynthesis, and its curative effects in trypanosomal and other parasitic infections have recently been reported.[120]

With the polyhalo derivatives, it is likely that all fluorine substituents can be consecutively eliminated as shown recently by Silverman and Abeles for β, β, β-trifluoroalanine **68** with the γ-elimination enzyme cystathionase.[121] The covalent inactive enzyme derivative is at the acyl oxidation state at the β-carbon, found as an amide derivative to an active site amino group, with a possible mechanistic course as shown.



68

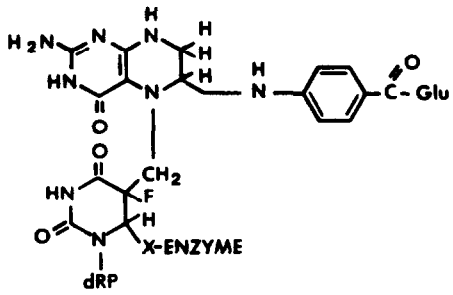
The enzyme thymidylate synthetase, responsible for provision of one of the four buildings blocks in DNA, is a target for cancer chemotherapy. The classical suicide substrate 5′-fluoro-2′-dUMP **69**[122,123] is used clinically for certain epithelial cell cancers. We shall note its action below but point out here that the 5-vinyl trifluoromethyl 2′-dUMP **70**[124] also inactivates, with eventual loss of all three fluorines and conversion of the CF_3 group to a C–X-enzyme linkage in analogy to the trifluoroalanine route noted above.



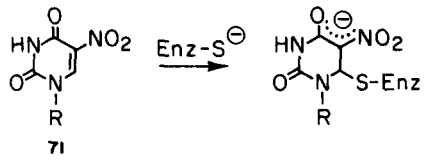
70

Thymidylate synthetase enables carbon-carbon bond formation by addition of an enzyme cysteinyl SH across the 5, 6-double bond of the 2′-dUMP substrate to yield a C_5-enolate equivalent that attacks the CH_2 group of 5, 10-methylene tetrahydrofolate. This ternary complex of substrate, pyrimidine, folate then unravels in (a) an apparent 1, 3-hydride-shift (suprafacial) and (b) expulsion of the initially added enz-SH to produce 5-CH_3-2′-dUMP (thymidylate).[125,126]

For the vinyl-CF_3 inactivator **70** the key species is the 5-enolate equivalent which can undergo F^- loss to yield the electrophilic difluoro olefin for covalent capture.



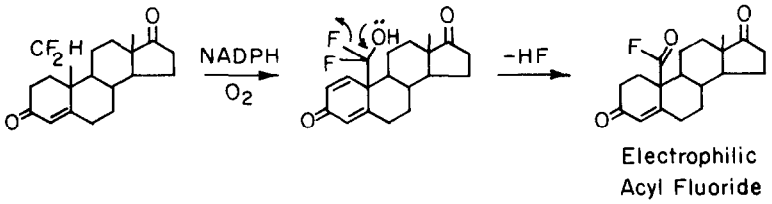
For 5-F-dUMP itself, the ternary covalent complex of enz-6-thio-5-F-dihydro-5-CH_2-tetrahydrofolate accumulates with an *in vivo* half-life for decomposition of ~14 hr, long enough to curtail DNA synthesis effectively in the affected cells. 5-Nitro-dUMP **71** sets up an even more stable enolate and here



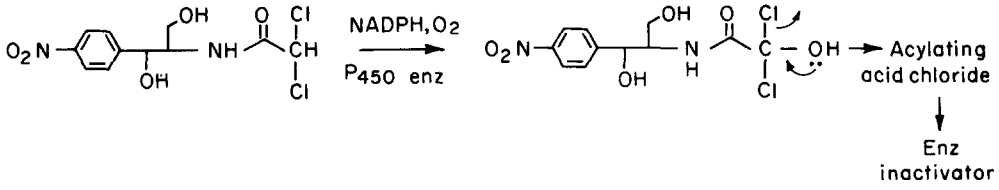
71

the binary enz-pyrimidine adduct accumulates.[127, 127a]

Another example of a fluorinated suicide substrate in this section is the 19-difluoromethyl testosterone derivative which inactivates the hemeprotein oxygenase aromatase noted earlier in this review. A likely sequence is hydroxylation at the difluoromethyl group, decomposition of the fluorohydrin to an acyl fluoride and subsequent acylation of the aromatase.[52]

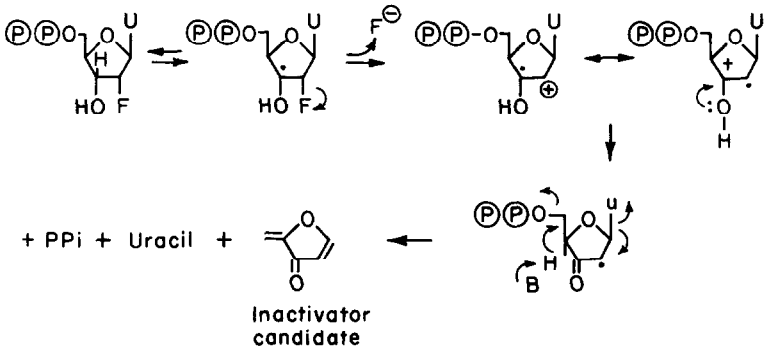


An analogous autoinactivation of a cytochrome P_{450} hemeprotein monooxygenase occurs in liver by the antibiotic chloramphenicol. The dichloroacetylamide portion is hydroxylated enzymically at the indicated dichloromethyl group to yield the tetrahedral adduct precursor to the chloramphenicol oxalyl chloride which is the proximal acylating and inactivating reagent.[147, 152] About 50% of the radioactivity from [^{14}C]-chloramphenicol becomes attached to the ϵ-NH_2 of a lysine residue of the apoprotein. No other derivatives are yet identified, but there is no evidence for heme group modification.[152a] The partition ratio is 5 turnovers per inactivation.



The last case of a fluorinated substrate analog to be discussed involves the rerouting of flux in ribonucleotide reductase catalysis from turnover to inactivation. The *E. coli* reductase is an iron-enzyme converting ribonucleoside diphosphates to the 2'-deoxy series, the direct precursors of the 2'-deoxytriphosphates used in DNA biosynthesis. It has been observed that the 2'-chloro or 2'-fluoro analogs of

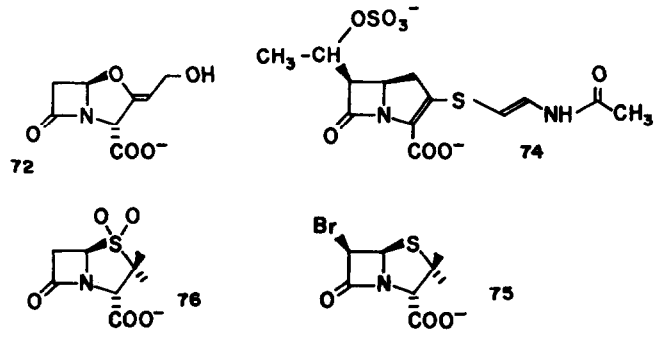
UDP in fact cause time-dependent inactivation of this key enzyme[128] and the mechanistic pathway has been carefully analyzed by Stubbe and coworkers who find F^-, inorganic pyrophosphate, and uracil as product fragments while the ribose moiety apparently unravels to an inactivating agent. The following mechanism involving a radical cation cleavage of the C_2'-F linkage and subsequent elimination from the 2'-cation-3'-keto species is hypothesized to produce an alkylating ribose fragment as the cause of enzyme inactivation.[129–131]



2.6 *Mechanism-based inactivators of β-lactamases*

The enzyme β-lactamase is generally coded for by DNA on independently replicating plasmids which inhabit resistant strains of bacteria. Penicillins and cephalosporins are rendered ineffective as antibiotics by enzymic hydrolysis of the β-lactam amide groups. The promiscuous spread of β-lactamase-elaborating plasmids among bacterial populations rapidly limits utility of a given β-lactam. Thus, specific inactivators of β-lactamases could be very important, but until recently, no effective compounds had been found. Now, though, there are both naturally occurring and synthetic suicide substrates which proceed partway through the normal hydrolytic catalytic cycle, to the acyl-enzyme intermediate, and then partition between deacylation (normal hydrolysis) and various intramolecular fragmentation or elimination routes to uncover electrophilic species which inactivate the enzyme.[132]

In this suicide substrate group are the natural oxapenam clavulanic acid **72**, the natural carbapenems thienamycin **73** and olivanic acid **74**, and also synthetic 6-β-bromopenicillanic acid **75**, and various sulfone derivatives, the first example being Pfizer's 899, **76**. Mechanistic investigators by the groups of Knowles,[132–135] Pratt,[136–138] Waley,[139,140] Coulson,[141] and the Beecham[142,143] and Pfizer groups[144] have



been very fruitful in deciphering the chemistry of enzyme alkylation. A generalized scheme for both clavulanate and penicilline sulfones, involving fragmentation at the acyl enzyme intermediate stage is shown below. In the case of various penicillin sulfones, there is a good correlation between k_{cat} for hydrolytic turnover and the number of turnovers which occur on average before inactivation (Table 6), consistent with argument of Knowles *et al.*, that the longer the lifetime of the acyl-enzyme intermediate, the greater the probability it will fragment and then alkylatively inactivate before it has a chance to hydrolyze in a second-order reaction.[145]

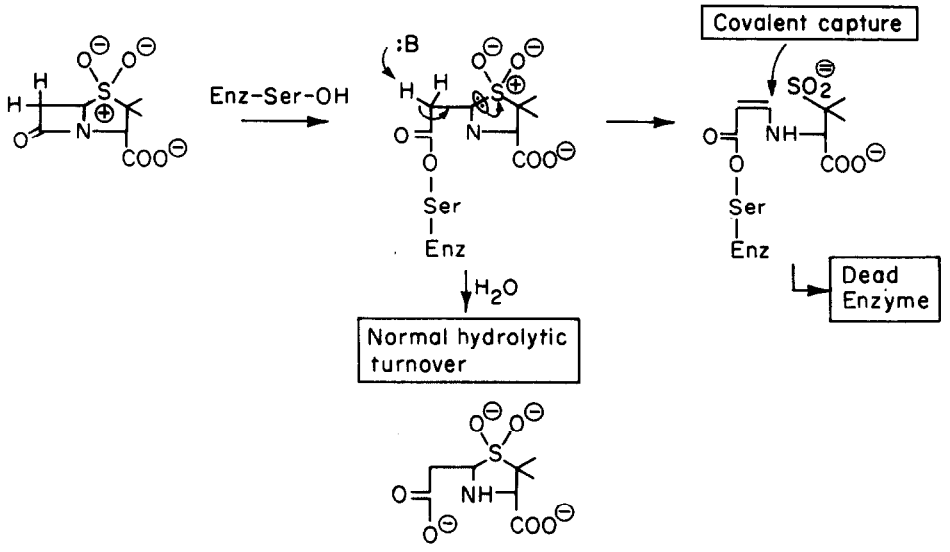
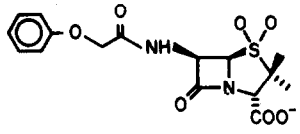
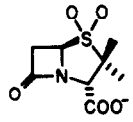
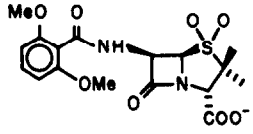
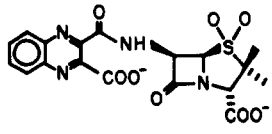
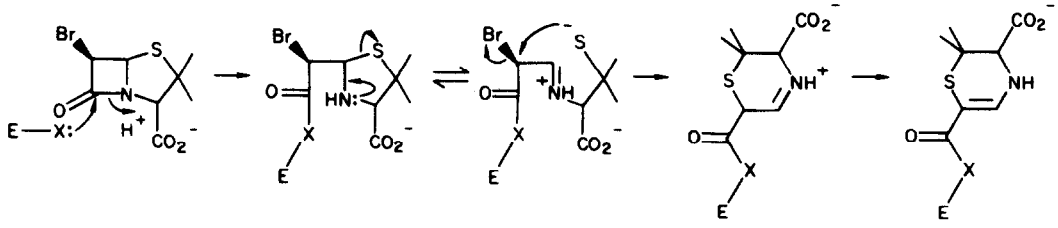


Table 6. Kinetic characteristics of the interaction of various penam derivatives, with the β-lactamase from *E. coli* RTEM

	k_{cat} of parent penam (s^{-1})	$t_{1/2}$ for inactivation (min)	Turnovers before inactivation
	1000	–	> 100 000
	40	44	4500
	10	~ 1	22 500
	7	~ 1	400

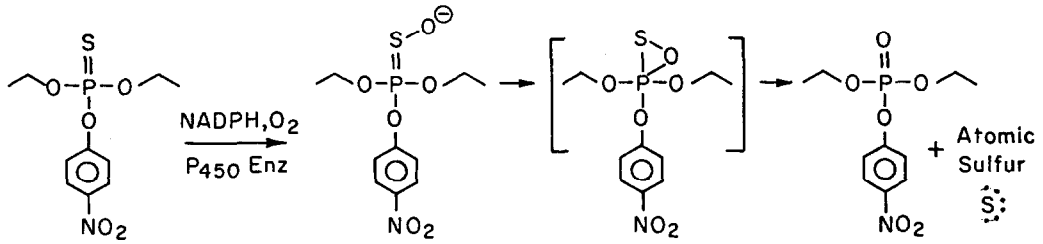
The fate of 6-β-bromopenicillin has been analyzed by Pratt, Cohen, and Loosemore[136–138] and by Waley[139,140] and the following scheme suggested to account for observed thiazolidinone-enzyme adduct formation.



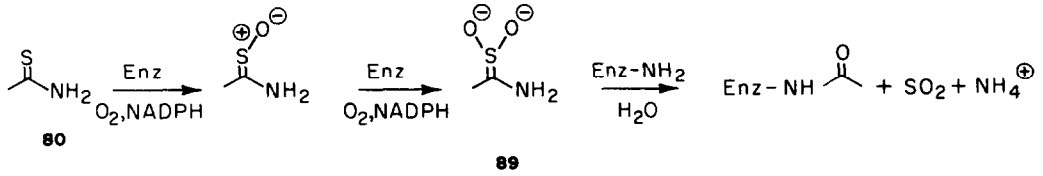
2.7 *Thionosulfur compounds as inactivators of hemeprotein monooxygenases*

Compounds with thionosulfur groups (P = S or C = S) are common in pesticides[146] on the one hand and serve as several industrial chemicals[147] on the other. These functionalities can engender both acute and chronic hepatotoxicity on enzymic metabolism during oxygenative desulfurization by liver cytochrome P_{450} hemeprotein monooxygenases. Due largely to the work of Neal *et al.*[147–151] it is held likely that electrophilic atomic sulfur generated as a product at the active site may be the deleterious species for formation of hydridodisulfides of cysteinyl groups.

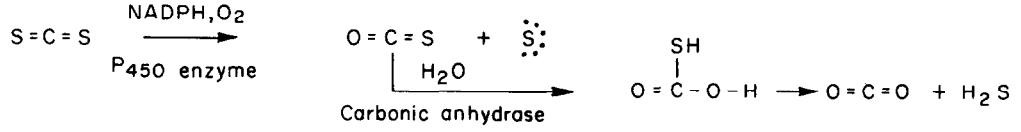
The insecticide parathion **77** is, in fact, a proinsecticide and must first be converted oxygenatively to paraoxon **78** in the liver before paraoxon can inactivate its target acetycholinesterase at neuromuscular junctions (with *some* selectivity for insects over man). Initial monooxygenation at sulfur is thought to be



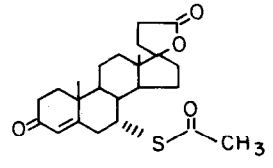
followed by cyclization to the phosphooxathiran **79**, and then collapse of the 3-membered ring with extrusion of electrophilic sulfur.[147] The atomic sulfur can bind covalently to the P_{450} enzyme, predominantly as hydridosulfide linkages with cysteinyl groups, and leading to enzyme inactivation.[151] Similar sequences are evidenced with the industrial carcinogen thioacetamide **80**, where the S-oxide is relatively stable and its processed iteratively to the S-dioxide **89** which then decomposes to SO_2 and an



acetylating agent. Carbon disulfide is analogously oxygenated first to carbon suboxide and atomic sulfur, then COS is hydrated enzymically by carbonic anhydrase, and so the second sulfur ends up at the H_2S



oxidation state.[147] The diuretic spironolactone **90** also inactivates heme-protein oxygenases in adrenal and reproductive tissues possibly by such a electrophilic sulfur deposition mechanism.[152]



Spironolactone

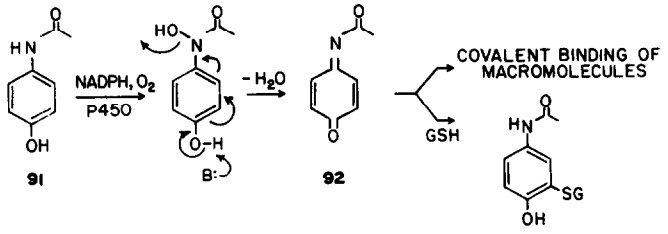
90

2.8 *Alkylation from quinones or quinones methide equivalents*

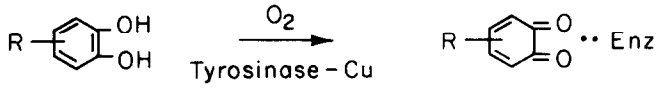
Two types of enzymic activation mechanisms (oxidative and reductive) have been proposed for generation of quinone methides as alkylating agents, albeit these electrophiles then appear to behave as nonspecific alkylating agents, modifying species other than just the generating enzyme.

The oxidative activation sequences are delineated by the processing of acetaminophen **91**, the

analgesic ingredient in Tylenol, again by the liver cytochrome P_{450} monooxygenases to the N-hydroxy product.[154] This alone is not toxifying, but subsequent conjugation of the new N-hydroxy group by sulfuryl or acetyl transfer produces the N-acetoxy or N-sulfates, respectively. These derivatizations deleteriously facilitate low-energy N–OR cleavage to yield the imino quinone **92** as proximal alkylating electrophile. As long as liver glutathione levels are high, the tripeptide–SH group is an efficient

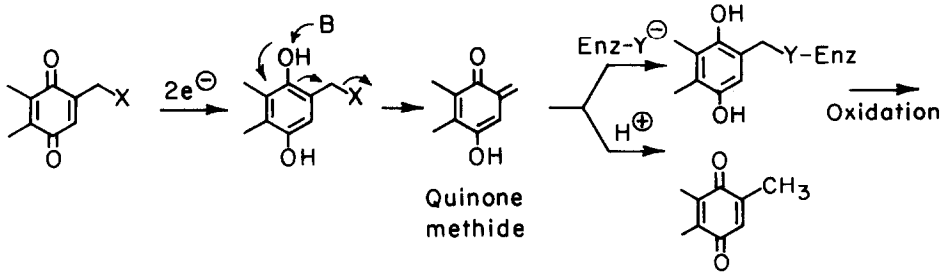


scavenger. But at overdoses ($\geq$ 10 g/day) of acetaminophen the gluathione levels are depleted, protein alkylation then ensues and severe hepatic necroses and liver failure can ensue. A second, related example is the occasional suicidal processing of catechols to orthoquinones by the cuproenzyme tyrosinase,[155] a key

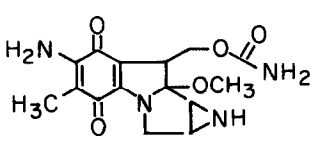


enzyme in biosynthesis of skin pigment melanin in vertebrates, sclerotization of insect cuticles, and in biosynthesis of polyphenolic compounds in plants and microbes. Inactivation probably arises by adventitious nucleophilic attack on the product 0–quinone prior to its release from the enzyme active site.

A quite distinct mode of catalytic unmasking of an electrophilic quinone species has been postulated recently *via* a bioreductive activation [156] of quinones with leaving groups at the α-carbons. Reduction to the hydroquinone level makes available electron density to facilitate α-elimination to the quinone methide which can then alkylate.



Reoxidation would then trap the newly added nucleophile in this net elimination/addition sequence. Such a sequence has been postulated to account for the alkylating activity of mitomycin C **93**, which occurs only after bioreduction[157] and for substituted naphthoquinones **94**, which are particularly effective



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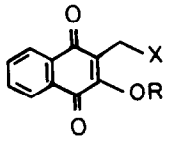
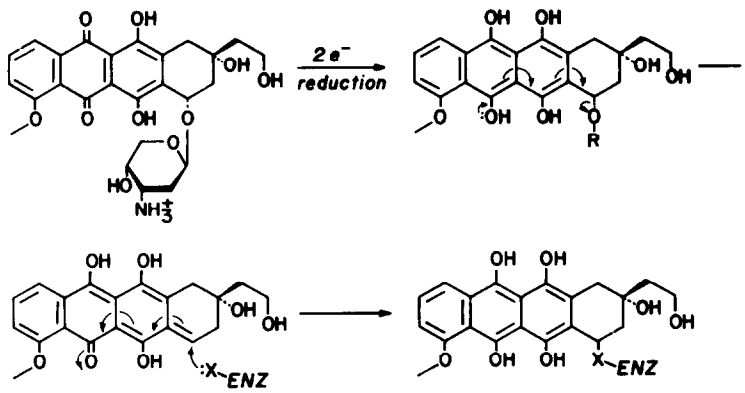
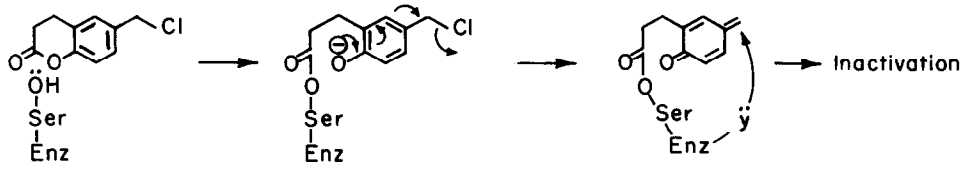


94

as antitumor agents in hypoxic cells where the hydroquinone lifetime may be long enough for elimination/addition to compete with reoxidation of the hydroquinone species.[158] Also there has been speculation that such a net two-electron reduction occurs with antitumor anthracyclines to yield quinone methide equivalents as the source of species incorporated covalently into liver cell protein.[156]



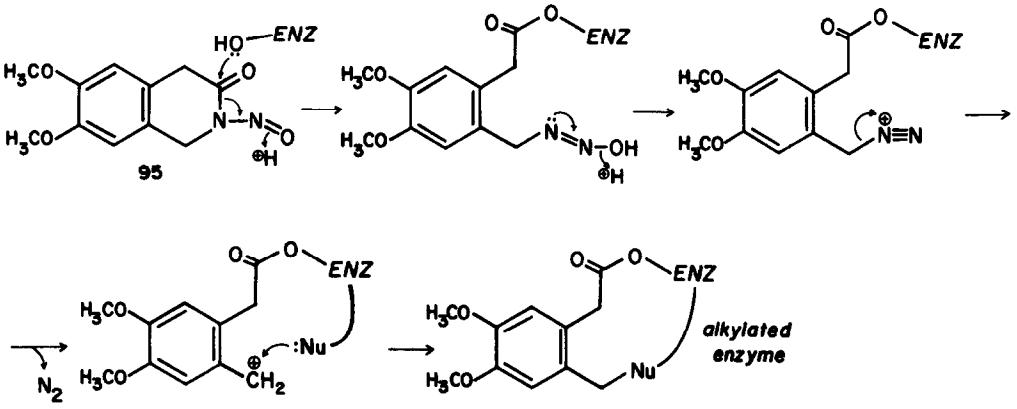
A last example of controlled generation of a quinone methide equivalent is drawn from the work of Vilkas *et al.* who used p-chloromethylphenol ester as a suicide substrate which could be enzymically activated by hydrolysis of the ester linkage at the active site of a susceptible peptidase. Incorporation of this group into a cyclic structure ensured tethering of the precursor group at the active site in the acyl enzyme intermediate stage.[159]



2.9 *Carbonium ion precursors*

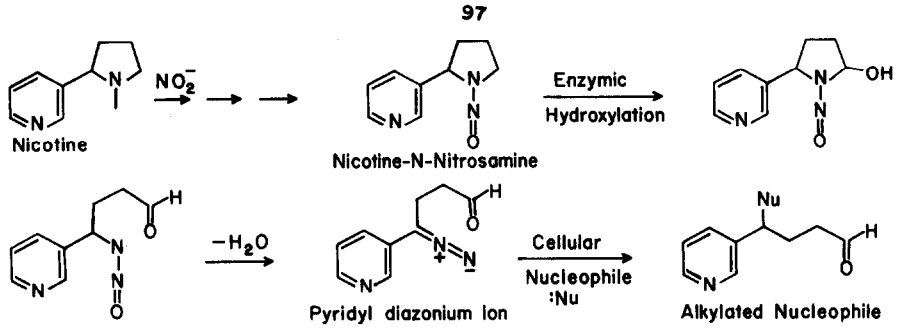
There are several examples of structurally unrelated functional groups in suicide substrates which apparently inactive target enzymes by unraveling to electrophilic carbonium ion intermediates during catalytic processing. Among them are the olefinic substrates such as allylisopropylacetamide **53** which inactive certain liver cytochrome P_{450} monooxygenases by heme group alkylation on nitrogen noted-above.

White *et al.*[160] have used a nitrolactam **95** as a suicide substrate for the protein hydrolytic enzyme chymotrypsin. This enzyme normally hydrolyzes amide bonds in substrates via an intermediate acyl enzyme. With the cyclic nitrolactam attact by the enzymic active serine residue's β-hydroxyl group produces a ring-opening diazohydroxide which can decompose facilely to the diazonium ion and then to N_2 and the benzylic carbonium ion which alkylates the enzyme active site. Because of the cyclic nature of the initial substrate, the carbonium ion fragment is still covalently tethered to the acyl-enzyme intermediate and is so maintained at the active site until capture by some enzymic residue. This is the same tethering strategy used by Vilkas in the quinone methide case above.[159]

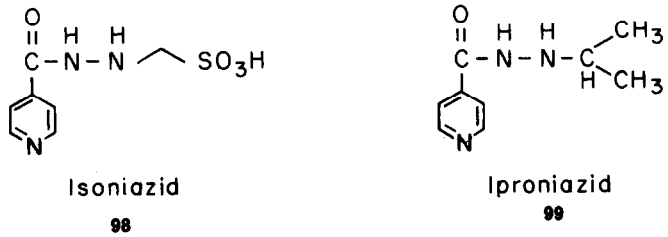


A similar diazonium ion type of sequence underlines the conversion of such nitrosamines as dimethylnitrosamine (DMN) **96** and nicotine-N-oxide (NNN) **97** into carcinogenic carbonium ion

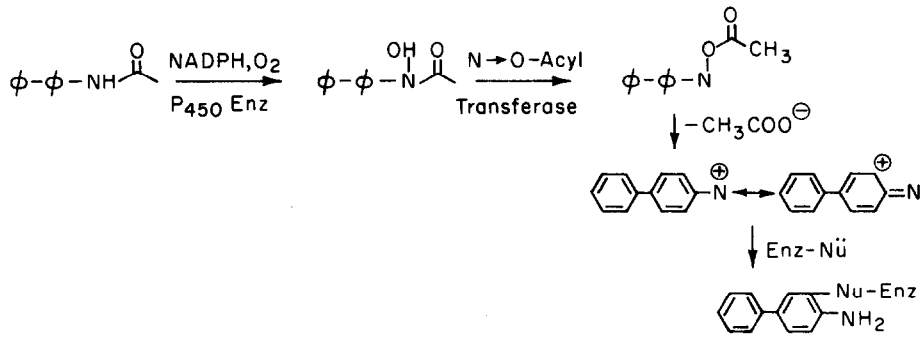
equivalents in mammalian cells.[161,162] In these sequences hemeprotein P_{450} monooxygenation at the α-carbon is the fateful enzymic step since this tetrahedral product decomposes, in the case of DMN, to formaldehyde and methyl diazohydroxide, and, hence, a methyl carbonium ion equivalent leading to methylation of DNA and proteins. The proposed α-hydroxy nicotine-N-oxide unraveling is shown below.



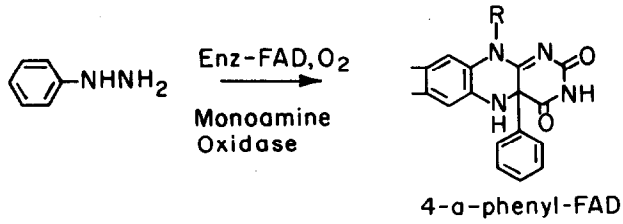
It appears that the antitubercular agents isoniazid **98**, and iproniazid **99**, also yield



carbonium ion equivalents after oxygenative processing, this time by cytochrome P_{450}-mediated N-hydroxylation sequences. A subsequent enzyme in the liver xenobiotic detoxification pathway is an acetyl transferase which catalyzes an N→O acetyl shift on the N-hydroxy-N-acetyl aromatic substrates. This action converts the N-hydroxy group to an N-acetoxyl amine with a new labile N–O bond (analogous to the acetaminophen metabolic route outlined earlier). S_N1–N–O cleavage before product dissociation yields a stabilized aryl nitrenium ion which then derivatizes the acetyl transferase and inactivates it.[163]

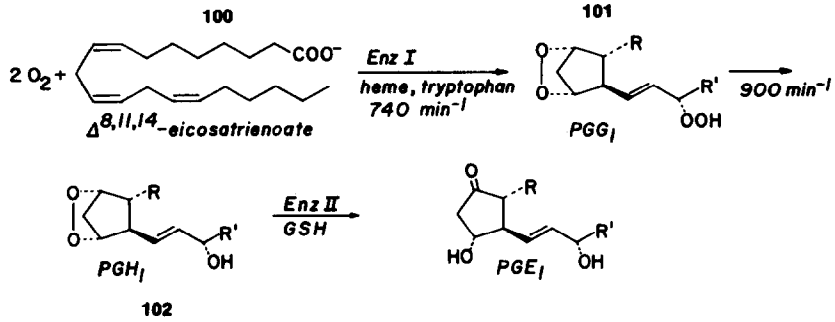


As a last example in this mechanistic set, phenelzine **28**, and congeneric hydrazines, is a potent inhibitor of monoamine oxidase. Oxidative processing of the model compound phenylhydrazine by purified monoamine oxidase leads to stoichiometric modification, not of an amino acid residue on the apoprotein, but of the flavin cofactor at the C_{4a} bridgehead carbon.[164] The process may involve a phenyldiazonium ion as attacking electrophile.



2.10 *Enzyme inactivation by autoxidation sequences from O_2*

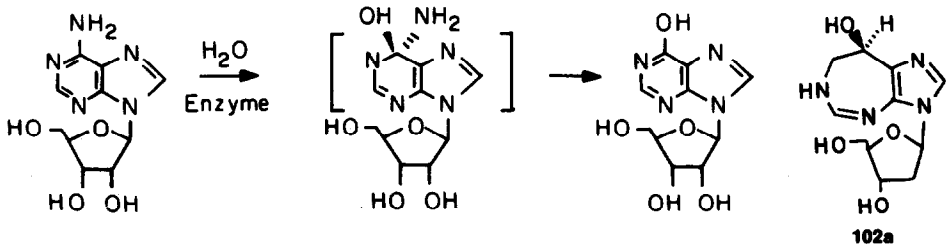
Many of the enzyme-specific inactivations denoted in the preceding sections result from oxygenative sequences producing activated organic intermediates. An additional example of obvious physiological relevance occurs during catalytic turnover of prostaglandin cyclooxygenase on its *normal* substrate, arachidonic acid or the related trienoate **100**. This enzyme, the first in the common biosynthetic path to prostaglandins, prostacyclins, and thromboxanes, is a hemeprotein dioxygenase[165] which carries out a double dioxygenation sequence, explicable as successive hydroperoxylations (with the first cyclizing to the 5,8-endoperoxide) to yield prostaglandin G_1 **101** as enzyme-bound intermediate. This endoperoxy-15-α-hydroperoxide is then cleaved reductively to the 15-α-alcohol, PGH_1 **102**. About once in every 5000



turnovers the cyclooxygenase self-destructs by undergoing an as yet uncharacterized oxidation, probably by way of a peroxy radical species.[166] This autoinactivation sequence turns off any purified enzyme molecule after 1–2 minutes; whether this represents a physiologically significant regulatory process or represents a catalytic short-circuiting by a species too hot to handle safely in the enzymic microenvironment is unclear. It also appears that the arachidonate-5-lipoxygenase, introducing a hydroperoxy group at C_5 in biosynthesis of slow reacting substances (2RS) also self-destructs during turnover, probably by a similar scheme.

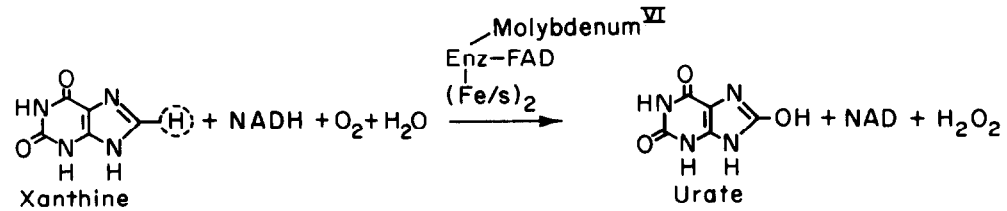
2.11 *Irreversible enzyme inactivation can occur by tight but noncovalent binding of substrate, transition state, or product analogs*

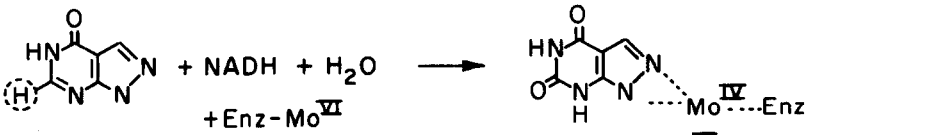
To this point we have focused on catalytic autoinactivation of a target enzyme by covalent modification of an active site residue or a tightly associated cofactor from an uncovered reactive species in the active site. In principle, though, actual covalent bond formation need not occur for observable time-dependent inactivation of an enzyme population if dissociation of the associated inhibitory small molecule is extremely slow. Slow dissociations are, of course, a consequence of very tight binding constants, a reflection of high affinity (large favorable energy of interaction with the enzyme). The point is easily made for the general case where one allows the on rate for combination of enzyme and inhibitor to be at the upper collisional limit, that is, a biomolecular rate constant of *ca.* $10^9 M^{-1}\,sec^{-1}$ for a diffusion-controlled encounter. Now if the observed $K_D = 10^{-12}M$, then the unimolecular rate constant



k_{off} is uniquely set at $k_{off} = 10^{-3}$ sec, a half-time for dissociation = 693 secs. In fact, k_{on} values are often as low as $10^6\ M^{-1}\ sec^{-1}$. A case in point is the natural product pentostatin (2'-deoxycoformycin) **102a** which inhibits adenosine deaminase apparently as transition state analog to the tetrahedral species in substrate hydrolysis. In this case for the erythrocyte adenosine deaminase interaction with pentostatin, $k_{on} = 6 \times 10^6 M^{-1} sec^{-1}$, $K_D \approx 6 \times 10^{-12} M$, so $k_{off} \approx 10^{-6}\ sec^{-1}$. That is, the half-time for a dissociation event is 25–30 hr, an interval so long that on the biological time scale, this is an irreversible inhibition.[167-170]

In this light, one classifies allopurinol **103** the agent of choice in the treatment of many forms of gout, as a suicide substrate for its target enzyme xanthine oxidase. Indeed, allopurinol is oxidized at C_2 to the alloxanthine product **104**, reducing the enzyme's active site molybdenum atom from Mo(VI) to Mo(IV). It is to this specific Mo(IV) oxidation state that the pyrazolopyrimidine product binds in tenacious but noncovalent fashion.[171] The half-time for product dissociation is ≈300 min, long enough to provide the

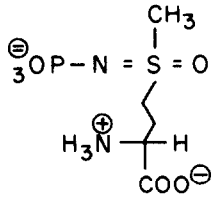




103 104

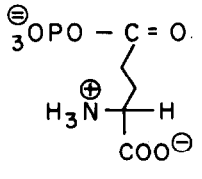
molecular basis for therapeutic action of allopurinol.

A third noncovalent example involves tight binding of 2S, 5S-methionine sulfoximine-phosphate to brain glutamine synthase.[172] The methionine sulfoximine **105** generated from bleaching of flour by nitrosylchloride, is a neuroconvulsant by virtue of being mistaken for a substrate by the enzyme. Phosphoryl transfer from ATP to the sulfoximine nitrogen yields the enzyme-sulfoximine-P-ADP complex which does not noticeably dissociate. This constitutes a stoichiometric titration of active enzyme to inactive product complex. The tight binding of sulfoximine-P is explained by structural similarity to the proposed normal reaction intermediate, γ-glutamyl-P **106**, on the way from glutamate to normal product glutamine.



2S,5S-Methionine Sulfoximine-P

105



2S-γ-glutamyl-P

106

3. CONCLUSIONS

This review has grouped together a large number of molecules of disparate structures, both natural products and synthetic compounds, which have the common property of irreversible inactivation of some enzyme of physiological or pharmacological significance. The great majority of the cases qualify clearly as suicide substrates where a rerouting of the target enzyme's normal catalytic flux leads to activation of a latent functional group in the active site microenvironment and subsequent covalent modification and inactivation of the biological catalyst prior to physical release of the activated species.

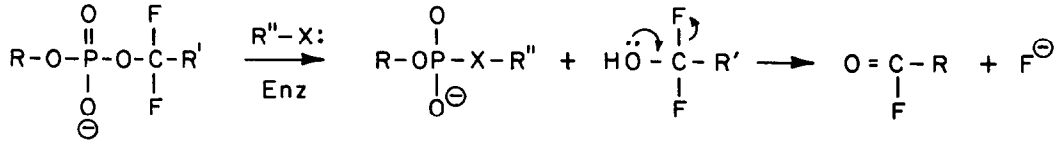
This rubric explains the toxic properties of some natural products including rhizobitoxin, allylglycine, and chloramphenicol and also the potential therapeutic effects of propargylglycine, gabaculine, and clavulanate to name a few examples.

The organizational framework chosen has been to emphasize the small number of latent functional groups yet detected and the comnonality of the routes for enzymic unraveling. Most inactivators are processed to uncovered electrophiles which capture enzymic or coenzymic oxygen, nitrogen, or sulfur atoms. By contrast, enzymes have few electrophilic groups in the twenty amino acid units that are the building blocks of proteins to react with nucleophilic suicide substrates. In this reverse polarity mode only enzymes with coenzymes that contain electrophilic sites such as pyridoxal phosphate (aldehyde carbon) or flavin cofactors (N_5 and C_{4a} of oxidized flavins) are chemically competent to become derivatized. This may explain the reaction of bound flavins with hydroxybutynoate, acetylenic amines and phenylhydrazines, and of PLP with allylglycine in the glutamate decarboxylase active site.

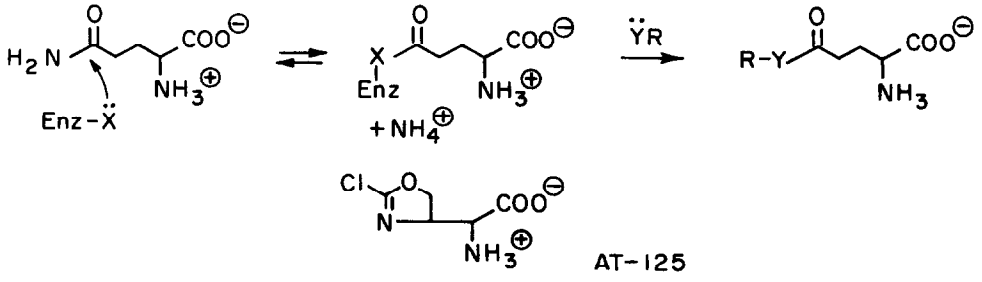
In the enzymic generation of electrophilic intermediates or bound products, there is a kinetic balance between covalent capture and physical release steps. Enzymes which generate conjugated olefinic species in normal turnover have probably evolved active sites with no nucleophilic groups close enough to engage in Michael capture and inactivation requires a much "hotter" electrophile, e.g. vinylglycine is a substrate, propargylglycine, *via* the allene, an inactivator of several PLP-enzymes. For maximal *in vivo* specificity, this partition ratio between release of uncovered product molecules and inactivation (turnovers/inactivation event) should be as low as possible. Thus, gabaculine[85] and 5-fluoroGABA[174] appear to be completely efficient at inactivation of GABA transaminase and L-vinylglycine nearly so for L-aspartate transaminase. With *E. coli* alanine recemase, systematic progression from mono- to trifluoroalanine lowers k_3/k_4 from 800/1 to $< 10/1$.

When one considers the processing of such molecules as the analgesic acetaminophen or the precarcinogen benzo[*a*]pyrene, the functional group-activating P_{450} monooxygenases are themselves not inactivated. All flux is by k_3, to release activated product molecules which ultimately wreak molecular havoc on other cellular targets, a clear case where enzymic generation of molecules too hot to handle produces molecular toxicology.

At this point in the development and analysis of suicide substrates a number of types of enzymes are susceptible based on the kinds of chemistry they employ in catalysis, e.g. generation of carbanionic intermediates, oxidative transformations, oxygen transfer processes. Enzymes catalyzing group transfer reactions such as acyl, glycosyl, and phosphoryl transfers to water or some other nucleophilic acceptor are extremely important in biosynthesis and degradation process and still require attention. The recent discovery of natural and synthetic lactam suicide substrates for penicillinases is a special case of induced abnormal fragmentation in a normal acyl-enzyme covalent intermediate where the longer the life of the intermediate, the greater the chance for inactivation to compete with hydrolytic flux. The nitrolactam and quinone methide studies on chymotrypsin acyl enzymes are not yet specific enough for *in vivo* utility but suggests ways of generalizing the strategy. In this regard, Ortiz de Montellano *et al.*[175] have recently synthesized phosphate esters of difluoro alcohols with the premise that enzymic phosphoryl transfer to some cosubstrate nucleophile would liberate the difluoroalcohol moiety that is, in aqueous environment, a rapidly decomposed precursor of an acylfluoride.



Also the antitumor chlorisoxaxole amino-acid AT-125[176–178] may be a prototype for inactivators of enzymes engaged in transfer of γ-glutamyl groups to cosubstrates.



One can anticipate that careful attention of mechanistic and synthetic chemists to the synthesis of and mechanism for uncovering latent functionalities coupled to a detailed knowledge of the specificity and mechanistic pathways of key target enzymes will lead to fruitful new advances. Already the suicide substrate concept has proved a powerful new approach for the rational design of drugs in the hands of medicinal chemists such as the Merck and Merrell groups. Further, the mode of action of many natural products may be collectable under this rubric. Thus, the design of a suicide substrate for almost any category of enzyme may soon be feasible, allowing one to go into the *in vivo* phase where the vagaries of pharmacokinetics and pharmacodynamics, as always, condition delivery and availability of compounds to the target tissue and enzyme.

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