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Design and Mechanism of GABA Aminotransferase Inactivators. Treatments for Epilepsies and Addictions

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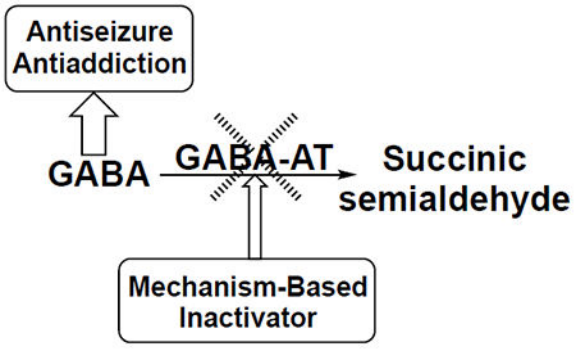
Abstract

When the brain concentration of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) diminishes below a threshold level, the excess neuronal excitation can lead to convulsions. This imbalance in neurotransmission can be corrected by inhibition of the enzyme γ-aminobutyric acid aminotransferase (GABA-AT), which catalyzes the conversion of GABA to the excitatory neurotransmitter L-glutamic acid. It also has been found that raising GABA levels can antagonize the rapid elevation and release of dopamine in the nucleus accumbens, which is responsible for the reward response in addiction. Therefore, the design of new inhibitors of GABA-AT, which increases brain GABA levels, is an important approach to new treatments for epilepsy and addiction. This review summarizes findings over the last 40 or so years of mechanism-based inactivators (unreactive compounds that require the target enzyme to catalyze their conversion to the inactivating species, which inactivate the enzyme prior to their release) of GABA-AT with emphasis on their catalytic mechanisms of inactivation, presented according to organic chemical mechanism, with minimal pharmacology, except where important for activity in epilepsy and addiction. Patents, abstracts, and conference proceedings are not covered in this review. The inactivation mechanisms described here can be applied to the inactivations of a wide variety of unrelated enzymes.

Graphical Abstract



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1. INTRODUCTION

1.1. γ-Aminobutyric Acid Aminotransferase (GABA-AT): Catalytic Mechanism

γ-Aminobutyric acid aminotransferase (GABA-AT) has been assigned Enzyme Commission Number EC 2.6.1.19, which indicates that the enzyme catalyzes the transfer (2) of a nitrogenous group (6), in a family of enzymes known as transaminases (also called aminotransferases) (1), specifically GABA aminotransferase (19). GABA-AT is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that catalyzes the degradation of the inhibitory neurotransmitter GABA to succinic semialdehyde (**5**, Scheme 1A). During this enzymatic process the Lys^{329}-bound PLP coenzyme (**1**) is converted to pyridoxamine 5'-phosphate (PMP, **6**) via intermediates **2-4**; consequently, when PLP is converted to PMP, the enzyme is not acting as a catalyst, since the enzyme (i.e., its coenzyme) has been modified. To attain catalyst status, a second catalytic step is required (Scheme 1B) in which the enzyme uses a second substrate, α-ketoglutarate (**7**), to return the PMP to PLP so that catalysis can resume. As a result of that process, the α-ketoglutarate is converted to the excitatory neurotransmitter *L*-glutamate (**8**). Therefore, one molecule of inhibitory neurotransmitter (GABA) is converted to one molecule of excitatory neurotransmitter (*L*-Glu) in the overall process. The mechanism in Scheme 1B to convert PMP back to PLP is the converse of the mechanism in Scheme 1A, which converts PLP to PMP. The PLP-dependent enzyme *L*-glutamic acid decarboxylase (GAD) catalyzes the conversion of *L*-glutamate to GABA; therefore, the two PLP-dependent enzymes, GABA-AT and GAD, are essential for the regulation of the brain levels of these two neurotransmitters.[1]

1.2. γ-Aminobutyric Acid Aminotransferase (GABA-AT): Function in the Nervous System

Normal functioning of the brain requires a finely balanced inhibitory/excitatory neuronal network. GABA is the principal inhibitory neurotransmitter in the central nervous system of mammals, and L-glutamate is the major excitatory neurotransmitter.[2] GABA is produced from L-glutamate in presynaptic (GABAergic) neurons (Figure 1); [3] depolarization of presynaptic neurons stimulates GABA release and transport into the synaptic cleft by several GABA transporters (GAT-1, GAT-2, GAT-3, and betaine-GABA transporter)[4] for neurotransmission. Once released into the synapse, GABA can bind to one of two principal GABA receptors ($GABA_A$ and $GABA_B$) on the postsynaptic neuron. Allosteric GABA binding to $GABA_A$ receptors causes opening of the receptor's central chloride ion channel, which establishes hyperpolarization of the neuronal membrane, resulting in reduced cell excitability and, therefore, neuronal inhibition. [5] $GABA_B$ receptors are metabotropic; activation by GABA results in a G-protein coupled receptor mediated opening of linked potassium channels, which results in hyperpolarization and neuronal inhibition similar to $GABA_A$ receptors.[6] After synaptic neurotransmission, the action of GABA is terminated either by reuptake into presynaptic neurons or into glial cells via GABA transporters. In glial cells GABA enters the GABA shunt (Scheme 2),[4] where it is catabolized by GABA-AT in two coupled half reactions, as noted above. In the first half reaction (Scheme 1A) GABA is catabolized to succinic semialdehyde (SSA) with concomitant conversion of the PLP coenzyme to PMP. The SSA is oxidized by succinic semialdehyde dehydrogenase (SSADH) to succinate, which enters the citric acid (Krebs) cycle for conversion to α-ketoglutarate.[7] The second half reaction (Scheme 1B) involves the regeneration of the PLP coenzyme

by GABA-AT catalyzed conversion of α-ketoglutarate to the excitatory neurotransmitter, L-glutamate. However, because there is no glutamic acid decarboxylase (GAD) in glial cells, the L-glutamate produced cannot be directly converted back to GABA. Therefore, it is converted by glutamine synthetase to glutamine, which is transported to presynaptic neurons, where it is converted back to L-glutamate by glutaminase. The GAD in the presynaptic neurons produces GABA by decarboxylation of L-glutamate as noted above. This convoluted process of conversion of the excitatory neurotransmitter, L-glutamate, to the inhibitory neurotransmitter, GABA, in presynaptic neurons and then conversion of GABA back to L-glutamate in glial cells allows the central nervous system to have more control over the levels of these important neurotransmitters.

1.3. γ-Aminobutyric Acid Aminotransferase: Properties

GABA-AT is found in many organs, including brain, liver, kidney, and pancreas; it is present at higher specific activity in glial cells and presynaptic neurons.[8] GABA-AT is a homodimer (vide infra) with each subunit containing an active site PLP, but the two binding sites are nonequivalent.[9] Following inactivation with one equivalent of an inactivator, it is possible to incorporate a second cofactor molecule and regain activity. This may explain why some inactivators become incorporated at the level of one equivalent per enzyme dimer and others incorporate two equivalents. GABA-AT undergoes a reversible unfolding process in urea at low pH, and enzyme activity is almost completely recovered upon dilution with buffers at neutral pH.[10] GABA-AT is one of a large family of PLP-dependent aminotransferases [11] that catalyzes similar mechanisms on different substrates, consisting of the two coupled half reactions described above for GABA-AT, in which the PLP is converted to PMP, and the PMP is converted back to PLP. Aminotransferase enzymes, in general, are classic examples of enzymes that proceed by a Bi-Bi Ping Pong mechanism. [12] Bi-Bi means that two substrates are involved (in the case of GABA-AT, GABA and α-ketoglutarate), which are converted to two products (succinic semialdehyde and L-glutamate, respectively), and Ping Pong refers to a mechanism in which the enzyme reacts with one substrate (GABA) to form one product (succinic semialdehyde), giving an intermediate modified enzyme (in this case, the PMP form), which then reacts with the second substrate (α-ketoglutarate) to form the second product (L-glutamate), thereby regenerating the original active enzyme (the PLP form).

1.4. γ-Aminobutyric Acid Aminotransferase: Structure

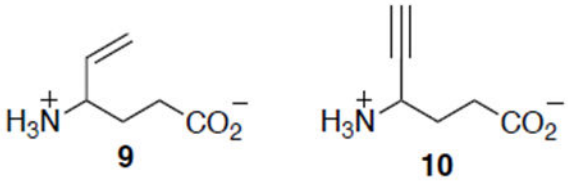
The primary structure of GABA-AT has been deduced from the cDNA of pig brain[13] and from peptide fragments of the pig liver enzyme;[14] the pig enzyme sequence has 96% *identity* (98% homology) with the human enzyme,[15] which validates studies with the pig enzyme as being highly relevant to humans.

GABA-AT is a homodimeric enzyme with each subunit containing 472 amino acid residues and having a molecular weight of 56 kDa per monomer. The two monomers are intertwined with the PLP cofactor deeply buried close to the subunit interface (Figure 2), which contains a [2Fe-2S] iron-sulfur cluster. The PLP cofactor of the pig brain enzyme is linked to Lys329 via its ε-amino group, thereby forming a Schiff base.[16] Upon treatment with $NaBH_4$ and proteolysis, a nonadecamer peptide containing reduced PLP becomes attached to Lys329

in pig brain.[17] The numerous interactions of the PLP with active site residues are shown in Figure 3. The phosphate group of the cofactor is held tightly by a network of hydrogen bonds to Gly136, Ser137 (both from one subunit), and Thr353 (from the other subunit), an active site region known as the "phosphate-binding cup".[18] Asp298 stabilizes the pyridinium nitrogen in a salt bridge, and the pyridinium ring is sandwiched between Val300 and Phe189 in edge-to-face π-π stacking. The external aldimine of PLP formed with GABA during substrate turnover is held in place with an electrostatic interaction between the GABA carboxyl group and Arg192. Lys329, the lysine residue that forms a Schiff base with PLP in native GABA-AT is the base that stereospecifically abstracts the *pro-S* proton from the gamma methylene of GABA (see Scheme 1A).

Molecular dynamics simulations of *apo*- and *holo*-GABA-AT were carried out to predict the ionization states and roles of the active site residues. [19] An important conclusion was that the pyridine ring of the PLP is protonated, resulting from the stability of deprotonated Asp298, which interacts with nearby His190. This protonation of PLP is responsible for the enzyme's ability to remove the γ-proton of GABA. The effects of numerous other residues, especially around the PLP in the active site, also were interrogated, and their functions were suggested.

In 1999 the X-ray crystal structure of native pig liver GABA-AT was reported to 3.0 Å resolution by the Schirmer group in Basel.[20] Five years later that group reported the crystal structure of the native pig liver enzyme to 2.3 Å resolution and the structures of this enzyme inactivated by the epilepsy drug, vigabatrin (**9**), and by γ-ethynylGABA (**10**) to 2.3 Å and 2.8 Å resolution, respectively.[21]



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1.5. γ-Aminobutyric Acid Aminotransferase: Relevance to Epilepsy

When the concentration of the inhibitory neurotransmitter GABA diminishes below a threshold level in the brain, convulsions result;[22] raising the brain GABA levels terminates the seizure.[23] Inhibiting the enzyme responsible for GABA catabolism (GABA-AT) has been shown to effectively dampen excessive neural activity without affecting basal neuronal firing. Most GABA agonists continuously interact with receptor or transporter proteins, leading to hyper GABAergic side effects, such as depression, somnolence, memory loss, difficulty concentrating, and ataxia. The incidence of seizure activity is widespread in the world. In fact, when epilepsy is defined broadly as any disease characterized by recurring seizures, then over one percent of the entire world population (including >3 million Americans) can be classified as having epilepsy. [24] Consequently, anticonvulsant agents have been sought for centuries. Not until diphenylhydantoin (Dilantin) was introduced onto the drug market 65 years ago was any particular anticonvulsant drug widely used.[25] However, this drug is not generally applicable. In fact, more than 30% of epileptic patients

worldwide do not respond to *any* marketed anticonvulsant drug.[26] Therefore, the need for new anticonvulsant drugs is great.

A reduction in the concentrations of GABA and of the enzyme GAD has been implicated not only in the symptoms associated with epilepsy[27,28] but also with several other neurological diseases such as Huntington's chorea,[29,30] Parkinson's disease,[31,32] Alzheimer's disease,[33] and tardive dyskinesia.[34] Administration of GABA peripherally is not effective because GABA, under normal conditions, does not cross the blood-brain barrier well[35] and readily effluxes from the brain, [36] presumably as a result of its polarity and hydrophilicity; [37] however, several other approaches have been taken to increase the brain concentrations of GABA. Some approaches include GABA receptor agonists, [38] glutamic acid decarboxylase activators, [39] GABA uptake inhibitors,[40] prodrugs,[41] GABA-AT inhibitors,[42] and mechanism-based GABA-AT inactivators.[43]

1.6. γ-Aminobutyric Acid Aminotransferase: Relevance to Addiction

Vigabatrin (**9**) has been found to possess another remarkable activity, namely, it prevents cocaine addiction in rats and baboons.[44] This activity also was established with other forms of addiction, including nicotine addiction[45] and methamphetamine, alcohol, and heroin addictions.[46] Self-administration of cocaine by rats decreased or was prevented by vigabatrin administration in a dose-dependent fashion, without affecting the craving for food.[47] It is well established that the neurochemical response to cocaine and other drugs of abuse is a sharp increase in dopamine levels in the nucleus accumbens (NAcc),[48] which activates the neurons responsible for pleasure and reward responses. The release of dopamine and effect on associated behaviors can be antagonized by an increase in the brain concentration of GABA. Therefore, the mechanism of vigabatrin that is responsible for the prevention of drug addiction is the same as that for epilepsy, namely, it inactivates GABA-AT, thereby increasing the GABA concentration in the brain (by blocking the degradation of brain GABA). Increased GABA levels produce the anticonvulsant effect directly. Positron emission tomography (PET) was used in primates to show that vigabatrin inhibits these cocaine-induced dopamine increases. [49] Vigabatrin treatment also is effective for stimulant addiction in humans, [50] including a randomized, double-blind, placebo-controlled trial of 103 subjects,[51] in which 28% of subjects treated with vigabatrin achieved abstinence compared to 7.5% of subjects treated with placebo.

However, because of the poor GABA-AT inhibition and ability to cross the blood-brain barrier, large doses (1-3 g/day) of vigabatrin are needed; as a result of these large doses over an extended period of time, retinal damage is observed in 25-40% of patients using vigabatrin.[52]

1.7. Mechanism-Based Inactivation

A mechanism-based inactivator[53] is an unreactive compound whose structure is related to that of the substrate (or product) of the target enzyme and initially acts as an alternate substrate. However, during the normal catalytic mechanism, the compound is converted into a molecule that causes irreversible inhibition of the target enzyme prior to it leaving the active site. Because the inactivator is activated by the catalytic mechanism of the target

enzyme, these compounds can be used as tools to reveal potential catalytic mechanisms of the target enzyme. These inactivators can be designed so they are activated by particular chemistry;[54] by identification of the structure of the inactivated enzyme [55] or determination of metabolites produced, [56] reasonable chemistry can be rationalized then validated with other structures[57] that can or cannot undergo that chemistry. Often the species that is formed produces a covalent bond to the enzyme, but this is not necessary; it may lead to tight-binding inhibition, to modification of the enzyme's cofactor, or any functionally irreversible inhibition mechanism. What is essential for this type of inactivation is that the compound is converted by the target enzyme's catalytic mechanism and the species causing inactivation does not escape the enzyme prior to it inactivating it. A simplified kinetic expression for this type of inactivator is shown in Scheme 3. If k_4 and the equilibrium set up by k_1 and k_{-1} are rapid, k_2 is the inactivation rate constant (k_{inact}), which determines the overall rate of inactivation. Most often, inactivation, as described by k_4, does not occur every time the inactivator (I) is converted to the inactivating species (E · I'); this species may escape the active site (k_3). When that occurs, it may release a reactive molecule that could cause damage elsewhere or undergo hydrolysis. This ratio of product release to inactivation (k_3/k_4) is called the partition ratio and is a measure of the efficiency of the inactivation process. In the ideal case, the partition ratio would be zero, but this is rare.[58]

Mechanism-based inactivators differ from reactive molecules that inactivate enzymes called affinity-labeling agents in that mechanism-based inactivators are unreactive and require the target enzyme to activate them by its normal catalytic mechanism. Because of their initial reactivity, affinity-labeling agents can react with other enzymes or biomolecules, leading to toxicity and side effects. If the mechanism-based inactivator is activated only by the target enzyme, and the partition ratio is zero, then a highly selective enzyme inactivator can be attained.[59]

2. GENERALIZATION OF GABA-AT INACTIVATOR MECHANISMS

As shown in Scheme 1A, Schiff base formation of substrates to the active-site PLP of GABA-AT lowers the pK_a of the gamma-proton for enzyme abstraction and formation of a resonance-stabilized carbanion. Because a mechanism-based inactivator initially acts as a substrate, inactivation mechanisms for GABA-AT generally proceed via initial deprotonation, followed by a variety of carbanion-initiated mechanisms. The mechanistic function of the PLP is to acidify the adjacent proton for removal, but it is the ensuing chemistry of the carbanion that determines the inactivation mechanism. Therefore, any enzymes that catalyze deprotonation of their substrate, such as many PLP-dependent enzymes but also other enzymes that do not contain PLP, or that catalyze decarboxylation, which also can lead to carbanion formation, should be capable of catalyzing, at least, some of the inactivation mechanisms discussed in this review. All that is needed is the appropriate structure, using one of the approaches described here, to design mechanism-based inactivators of a wide variety of enzymes.

3. DIVERSE GABA-AT INACTIVATOR MECHANISMS

3.1. Most Common Mechanisms for Mechanism-Based Inactivation of GABA-AT

Because mechanism-based inactivators act as substrates initially, deprotonation of the PLP-bound inactivator to give a resonance-stabilized carbanion (see Scheme 1A) is generally the first chemical step. From here, there are four common inactivation mechanisms[60]: 1) tautomerization followed by Michael (conjugate) addition; 2) tautomerization followed by enamine formation and enamine addition; 3) elimination followed by either Michael addition or enamine formation and then enamine addition; 4) tautomerization followed by aromatization. There are other minor mechanisms, which are discussed within the sections as unexpected or side mechanisms. More often than not, reasonable mechanisms are proposed without sufficient experimental evidence to support the mechanism; I will provide mechanistic information if available or propose alternative options. Each of these mechanisms is discussed below with relevant examples.

3.2. Principle of Microscopic Reversibility Applied to Mechanism-Based Inactivation

According to the Principle of Microscopic Reversibility, [61] for a reversible reaction, the mechanisms in the forward and reverse directions are the same (except for the direction). Because each step in the GABA-AT-catalyzed reaction is reversible, the Principle of Microscopic Reversibility can be applied to the design of mechanism-based inactivators. In this case, the cofactor of GABA-AT would be in the pyridoxamine 5'-phosphate (PMP) form, so the inactivator would need to contain a ketone or aldehyde carbonyl group to give the initial complex with PMP. Consider the reverse of the substrate reaction (Scheme 1A): aldehyde **5** (succinic semialdehyde) reacts with PMP to give the imine (**4**); deprotonation of the PMP followed by tautomerization, gives the initial GABA-PLP complex (**2**). Notice that intermediates **3** and **4** can be attained from either direction. That is the goal of this type of mechanism-based inactivation, namely, to reach an intermediate responsible for inactivation via an alternate pathway, using a compound related to the product structure rather than the substrate structure. Examples of this approach are described below as well.

3.3. Michael Addition Mechanisms

The most common inactivation mechanism is a Michael addition, exemplified by (*Z*)-4-amino-2-fluorobut-2-enoic acid (**11**, Scheme 4). [62] This compound acts mostly like an alternative substrate because GABA-AT turns it over about 750 times per inactivation event (i.e., the partition ratio is about 749) with concomitant loss of that many fluoride ions (measured with a fluoride ion specific electrode). With the use of [^{3}H]PLP-reconstituted GABA-AT, it was found that 708 ± 79 fluoride ions are released concomitant with 737 ± 15 transamination events per inactivation. Denaturation of inactivated enzyme does not contain any radioactive coenzyme; all is released as [^{3}H]PMP. From these experiments a Michael addition mechanism was proposed (Scheme 4). Schiff base formation between PLP and **11** gives **12**. Tautomerization to **13** followed by nucleophilic attack by an active site residue with elimination of fluoride ion would produce **14**; hydrolysis would give proposed inactivated enzyme **15** (with no experimental evidence).

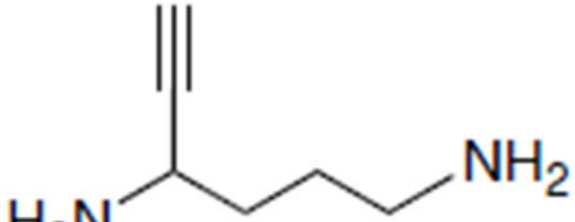
4-Aminohex-5-ynoic acid (**16**, Scheme 5), also referred to as γ-ethynylGABA or GEG for short, undergoes tautomerization, although mostly through the alkyne group, followed by Michael addition. Three different potential inactivation mechanisms were considered, from which eight different possible inactivated enzyme adducts could have emerged.[63] With the use of radioactively-labeled inactivator and PLP and a variety of chemical reactions involving [^{3}H]$NaBH_4$, acid, base, periodate, and spectroscopy, multiple pathways and metabolites were identified, all of which derived from Schiff base formation of **16** with PLP to give **17**. This intermediate partitions between pathways a and h; pathway a converts **17** by tautomerization through the alkyne to allene **18**, which partitions by pathways b, i, and j. Pathway b is a Michael addition with Lys329, leading to **19**, which tautomerizes to **20** (pathway c). Upon denaturation, **20** undergoes hydrolysis to **21** (pathway d), which tautomerizes via pathway e to **22**, which is hydrolyzed to covalent adduct **23**. Tautomerization of **21** via pathway f gives an unstable intermediate that hydrolyzes to metabolite **24**. Intermediate **20** also can undergo tautomerization via pathway g to **25**; hydrolysis of **25** to **26** followed by transimination releases metabolite **24**. Intermediate **17** also can tautomerize via pathway h to **27**; hydrolysis gives metabolite **28**. Pathway i from **18** gives allene **29**; hydrolysis produces metabolite **30**. Intermediate **18** also undergoes hydrolysis, via pathway j, to **31**; following enol tautomerization to **26**, transimination produces metabolite **24**.

The inactivation mechanism from **16** to **19** was the basis for the original design of **16**.[64] The X-ray crystal structure of GABA-AT inactivated by **16** confirmed the inactivation mechanism leading to **20** shown in Scheme 5.[65] On the basis of spectroscopic measurements during GABA-AT inactivation by **16**, intermediate structures were proposed, which demonstrated the flow of electrons into the PLP pyridine ring.[66] It is known that GABA-AT stereospecifically removes the *pro-S* gamma-proton of GABA; initially, it was found that only the (*S*)-isomer of **16** inactivated GABA-AT.[67] However, it was later found that both rat brain and bacterial GABA-AT are inactivated by (*S*)- and (*R*)-**16**, although inactivation requires about 20 times the concentration of (*R*)-**16** compared to (*S*)-**16**.[68] By incorporation of deuterium at the 4-position (gamma-position) of **16**, an isotope effect of 2.5 was observed on the apparent Michaelis constant (K_D/K_H), but there was no isotope effect on the inactivation rate constant, indicating that deprotonation occurs before inactivation, but proton removal is not fully rate determining; presumably the rate-determining step is enzyme attack of the incipient electrophile (**18→19**, Scheme 5). Also, the rate of incorporation of [^{14}C] into GABA-AT from [2,3-^{14}C]-**16** correlates with the rate of inactivation of the enzyme, and terminates with 0.9 equivalents of [^{14}C] per active site.[69]

In an attempt to mimic intermediate **25** (Scheme 5), which might go on to inactivate GABA-AT, the hydrolysis product (**24**) was synthesized and tested as an inactivator.[70] Although **24** did not inactivate GABA-AT, the corresponding aldehyde (**32**) did. The hypothetical inactivation mechanism proposed leads to **33** or **34** (Scheme 6), which mimic intermediates **25** and **20**, respectively, in Scheme 5.

When [2-^{3}H]-**16** was administered to mice, the amount of radioactivity bound to brain proteins was about the same as the amount of GABA-AT in mouse brain, suggesting the

selectivity of **16** for GABA-AT inactivation in vivo.[71] Peripheral administration of **16** to mice demonstrated that it caused an increase in brain GABA levels by almost 5-fold and protected the animals from seizures induced by five different methods, but not by two other methods, suggesting that increased brain GABA may not affect seizures in all animal models.[72] Unexpectedly, the administration of 5-hexyne-1,4-diamine (**35**) to rats led to inactivation of brain GABA-AT as a result of monoamine oxidase-catalyzed oxidation of the distal primary amino group of **35** to the corresponding aldehyde, which presumably was then oxidized to a carboxylic acid, producing **16** in vivo; **35** then could be considered a prodrug of **16**.[73] Administration of both (*R*)- and (*S*)-**35** inactivated GABA-AT in rat brain, although the (*S*)-isomer was more effective.[74] Compound **16** also inactivates ornithine aminotransferase and aspartate aminotransferase.[66]



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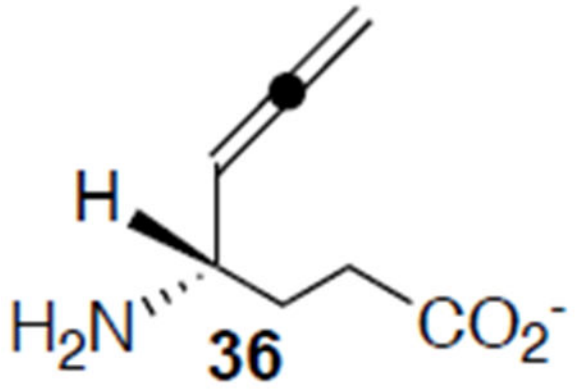
Related to **16** is the corresponding allene, 4-amino-5,6-heptadienoic acid (**36**), which also inactivates GABA-AT;[75] an inactivation mechanism has been proposed, resulting in **39** and **40** without any experimental support (Scheme 7).[76] The (*S*)-enantiomer of **36** is a potent inactivator of GABA-AT; the (*R*)-enantiomer does not inactivate, even at mM concentrations.[77] Oral administration of (*S*)-**36** to mice produced time- and concentration-dependent inactivation of GABA-AT, with an increase in brain GABA concentration; it also exhibited anticonvulsant activity in some mouse seizure models.[78]



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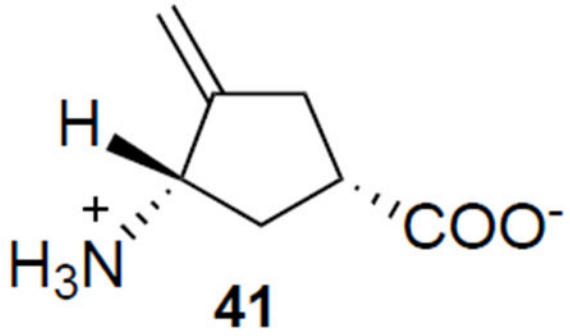
To improve on the potency of the only FDA-approved drug that inactivates GABA-AT, namely, 4-aminohex-5-enoic acid (generic name vigabatrin, trade name Sabril; see section 3.4), (1*S*,3*S*)-3-amino-4-methylenyl-1-cyclopentanoic acid (**41**) was designed as a Michael acceptor with the double bond facing Lys329 (based on a computer model using coordinates

from a crystal structure) so that following tautomerization, nucleophilic attack of **41** could occur readily. [79] However, the intermediate formed after tautomerization was not sufficiently reactive, so it only acted as a substrate. To make it more reactive after tautomerization, two fluorine atoms were added to the terminus of the alkene, giving (1*S*,3*S*)-3-amino-4-difluoromethylenyl-1-cyclopentanoic acid (**42**, Scheme 8), also known as **CPP-115**. This molecule was a potent irreversible inhibitor of GABA-AT. The mechanism proposed, initially without any experimental support, was Michael addition by Lys329 to **43**, leading to **44** (Scheme 8). Years later, the inactivation mechanism for **42** was elucidated, and it was found not to be a covalent Michael addition mechanism.[80] Time- and concentration-dependent inactivation occurs with a turnover number of 1.3 ± 0.3 per active site; however, upon dialysis, partial enzyme activity returns. As the concentration of **42** was increased, less enzyme activity returned upon dialysis; no enzyme activity returned when about 2000 equivalents of **42** was used. These results are consistent with at least two mechanisms, one that is reversible (major) and one irreversible. In the absence of α-ketoglutarate, so only one turnover was possible, 4.3 ± 0.5 fluoride ions were released per enzyme dimer, indicating that both fluoride ions are released in the first turnover. Inactivation of [^{3}H]PLP-reconstituted GABA-AT converted all of the coenzyme to PMP. Mass spectrometry identified three metabolites (**45-47**). With all three metabolites, the initial difluoromethylenyl group has been hydrolyzed to a carboxylic acid. To account for a hydrolysis reaction as the “reversible reaction” pathway in the mechanism, Scheme 8 was modified so that for most of the turnovers, an enzyme-catalyzed attack by water on reactive intermediate **43** occurred instead of Lys329 attack (Scheme 9). This is reasonable because GABA-AT catalyzes hydrolysis of the normal intermediate in its turnover of GABA (**4** in Scheme 1A). However, the X-ray crystal structure of GABA-AT inactivated by **42** followed by dialysis, so as to isolate the irreversibly inhibited enzyme, gave a surprising result (Figure 4A): **42** was not covalently bound to GABA-AT, but rather the bound species was hydrolysis product **45**, which was held tightly by two strong electrostatic interactions with the two carboxylates interacting with Arg192 and Arg445. In the native enzyme Arg445 interacts with Glu270, but when α-ketoglutarate enters the active site to convert PMP back to PLP, it requires both Arg192 and Arg445 (α-ketoglutarate has two carboxylates) to direct it for the reaction. Apparently, when **42** is used as the inactivator, during one in about 2000 turnovers, the enzyme responds as if **45** is the α-ketoglutarate-PMP complex and undergoes a conformational change, which locks it into the active site. This may be the first example of a mechanism-based inactivator that inactivates by enzyme-catalyzed conversion to a tight-binding inhibitor. On the basis of molecular dynamic simulations, the reason Lys329 does not attack **43** is because tautomerization, which puts a second sp^2 carbon into the ring, causes the difluoromethylenyl group to bend too far away from Lys329 to reach, so, apparently, water attack is the only option.



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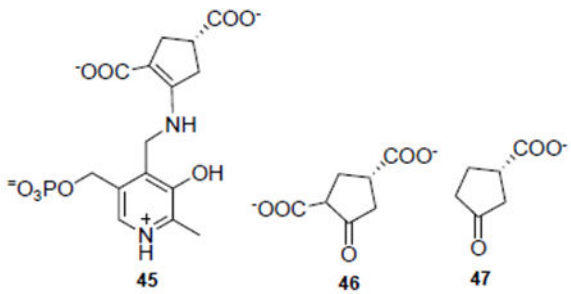
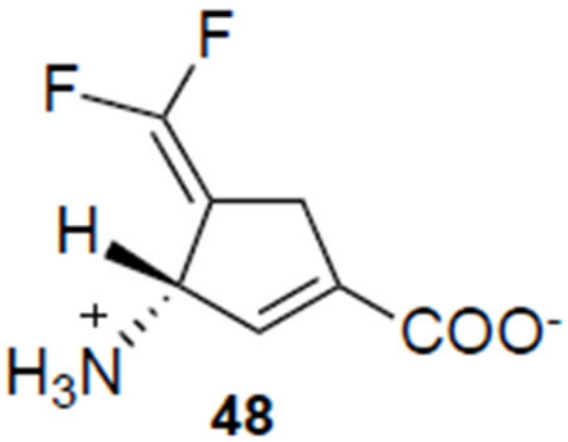


45 46 47

Molecular modeling and molecular dynamic simulations were carried out to determine what change to **42** could be made so that the difluoromethylenyl group did not bend away from Lys329, which might bring this electrophilic group closer to Lys329 for greater potency.[81] The results indicated that insertion of a double bon6d into **42** would flatten the molecule and bring the difluoromethylenyl group to within 4.6 Å of Lys329 after tautomerization (from 7.5 Å for the tautomer of **42**). Consequently, (*S*)-3-amino-4-(difluoromethylenyl)cyclopent-1-ene-1-carboxylic acid (**48**) was synthesized and was, in fact, shown to be 10 times more efficient as an inactivator of GABA-AT than **42**. Surprisingly, though, the X-ray crystal structure of **48**-inactivated GABA-AT (Figure 4B) was almost identical to that of **42**-inactivated GABA-AT (Figure 4A). In the case of **48**, however, there are eight possible tautomeric forms of the bound inactivator. Five of those tautomers can be disregarded because, as shown in Figure 4B, the carbon adjacent to the newly-formed carboxylate is nonplanar, and only three of the tautomers would accommodate that condition, but they cannot be distinguished from the electron density map of the crystal structure. Density functional theory and natural bond orbital analysis were carried out, and the most stable tautomer complex (if kinetics is ignored) is **49**.



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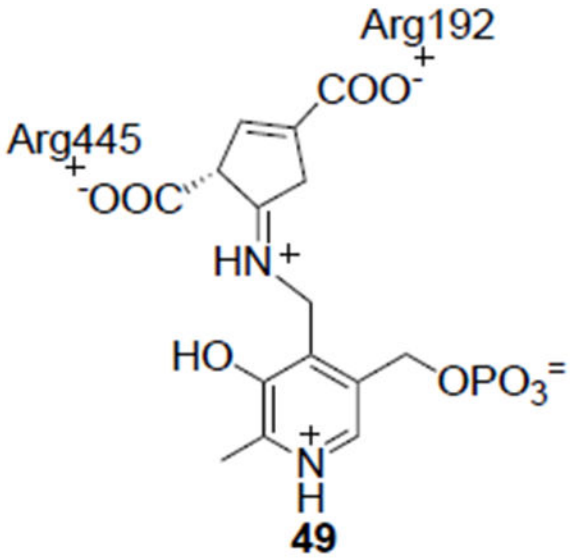


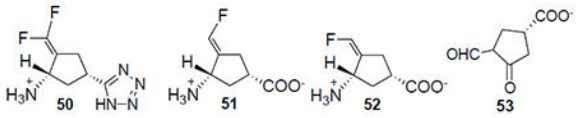
49

The question still remains as to why water, rather than Lys329, was the nucleophile. On the basis of many mechanistic studies of L-aspartate aminotransferase,[82] it appears that the base that deprotonates a water molecule for hydrolysis of the intermediate in the enzyme-catalyzed turnover is the lysine residue that holds the PLP in the enzyme (shown as Lys329 deprotonating water for attack of intermediate **4** in Scheme 1A). Therefore, if the difluoromethylenyl group of **48** is closer to Lys329, it also would be closer to the catalytic water molecule, which would allow hydrolysis to occur more easily. A relevant water molecule was demonstrated using classical molecular dynamic simulations; a comparison of the radial distribution function of water molecules around Lys329, Lys330, and Lys207 showed that only Lys329 has a close, well-ordered hydration sphere. This is consistent with favored water attack of intermediate **43** in Scheme 9.

Pharmacological studies with **42** indicated no GABAergic effects or off-target activities, and it demonstrated excellent anti-addictive properties.[83] In vivo microdialysis experiments in freely moving rats using microPET imaging techniques with **42** produced similar inhibition of cocaine-induced increases in extracellular dopamine and in synaptic dopamine in the

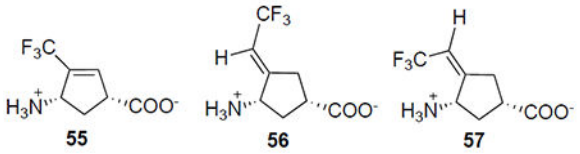
nucleus accumbens at 1/600th the dose of vigabatrin (**62**; see Section 3.4) (0.5 mg/kg of **42** vs. 300 mg/kg of **62**). Also, the expression of cocaine-induced conditioned place preference was blocked by **42** at a dose 1/300th that of **62**. Conditioned place preference is when an addicted animal that is no longer taking the addictive substance is returned to the place where it used to take the stimulus and becomes reminded of that substance; this results in the release of dopamine and the reward response. Compound **42** did not bind to any of the common mouse or human GABA transporters or receptors at 100 μM concentration; did not bind to any of 111 common biological targets in a Cerep panel (a company that carries out in vitro and in vivo assays) at 30 μM concentration; did not inhibit L-alanine aminotransferase or L-aspartate aminotransferase, even at 6 mM concentration; did not inhibit or induce the seven most common cytochrome P450 enzymes at 100 μM concentration (important to avoid drug-drug interactions); did not inhibit the hERG (human ether-a-go-go related gene) channel at 300 μM concentration (a potassium channel in the heart that can cause arrhythmias if blocked). In vivo it is rapidly orally absorbed and eliminated without metabolism. This drug also was much more potent than vigabatrin in the treatment of infantile spasms in rats[84] and humans.[85] Compound **48** was more potent than **42**; it suppressed the release of dopamine in the corpus striatum subsequent to either a cocaine or nicotine challenge in freely moving rats, even at a 0.01 mg/kg dose (>10 times the potency of **42**), which also attenuated increased metabolic demands (neuronal glucose metabolism) in the hippocampus, a brain region believed to encode conditioned place preference.[81] It also had favorable pharmacokinetic properties and low off-target effects.

The tetrazole bioisostere of **42** (compound **50**) was a time- and concentration-dependent inactivator of GABA-AT as well, but its potency was only 1/20th that of **42**; however, a calculation of its lipophilicity indicated that it was 69 times more lipophilic than **42** and 55 times more lipophilic than vigabatrin.86 The corresponding monofluorinated analogues, (*E*)-(**51**)- and (*Z*)-(1*S*,3*S*)-3-amino-4-fluoromethylenyl-1-cyclopentanoic acid (**52**) also were time-dependent inactivators of GABA-AT, comparable to vigabatrin; however, the dichloro analogue of **42** was only a weak reversible inhibitor.[87]



The mechanism of inactivation of GABA-AT by **51** and **52** (Scheme 10) was studied using the same experiments as described above for **42** (Scheme 9).[88] The metabolite detected (**53**) was one oxidation state lower than that detected from **42** (**46**, Scheme 9), namely, the corresponding aldehyde, because **51** and **52** are one oxidation state lower than **42**. Again, it was the X-ray crystal structure (Figure 5) that made the mechanistic hypothesis reasonable. The inactivated enzyme is a noncovalent complex with the hydrolysis product (**54**). This complex is not as stable as that produced from the difluoromethylenyl compound (**42**) because the aldehyde interaction with Arg445 is not as strong as the carboxylate interaction when **42** inactivates GABA-AT.

Once it was determined that **42** was a potent irreversible inhibitor, but before the inactivation mechanism was known, a series of other fluorinated compounds was designed that could potentially inactivate GABA-AT by a Michael addition mechanism.[89] Of the six compounds synthesized and assayed, three of them (**55-57**) were time- and concentration-dependent inactivators; the others were weak reversible inhibitors. Dialysis of inactivated enzyme led to partial return of activity, suggesting that these compounds also may involve irreversible and reversible pathways. Enzyme inactivation was concomitant with fluoride ion release. Compound **55**, but with a fluorine atom in place of the trifluoromethyl group, did not inactivate the enzyme; the compound with a pentafluoroethyl group in place of trifluoromethyl in **56** also did not inactivate the enzyme, but fluoride ions were released. Compound **56**, except with two trifluoromethyl groups on the alkene instead of one trifluoromethyl and one hydrogen atom, was a weak reversible inhibitor. A mechanism was hypothesized for inactivation of GABA-AT by **55** (Scheme 11). Following tautomerization, a nucleophile (X), which could be water, Lys329, or another active site residue, undergoes Michael addition, which could lead to fluoride ion elimination, giving an intermediate (**58**) whose structure is very similar to that of **43** in Schemes 8 and 9. This could undergo attack (Y) by either Lys329 (if X is not Lys329) to give the irreversibly inactivated enzyme (**59**) or by water to give **60**, which could hydrolyze further to **61** and PMP. Of course, now that the mechanism for **42** is known to inactivate GABA-AT exclusively by the hydrolysis pathway, **60** is more likely the inactivated enzyme complex.



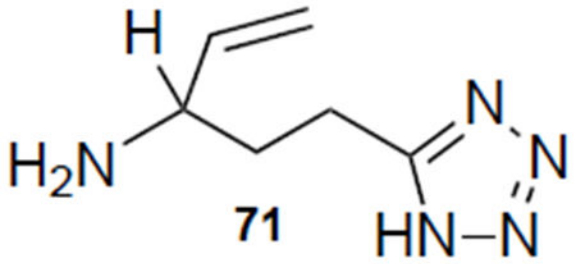
3.4. Michael Addition and Enamine Mechanisms via Tautomerization

The only GABA-AT inactivator that is FDA-approved for the pharmaceutical market is 4-aminohex-5-enoic acid (**62**; generic name vigabatrin, Trade name Sabril,[90] also referred to as γ vinylGABA or GVG for short),[91] which was designed at Merrell International in Strasbourg on the basis of the known inactivation of aspartate aminotransferase by the natural product α-vinylglycine.[92] Vigabatrin became the first rationally-designed mechanism-based inactivator to be approved for commercial use. It was shown to be an active-site directed inhibitor because GABA protected the enzyme from inactivation. A Michael addition mechanism was proposed without experimental evidence other than time-dependent irreversible inhibition and the demonstration that [4-^{2}H]-**62** displayed a primary isotope effect on inactivation (Scheme 12, pathway a). This mechanism was later found to be responsible for only about 70% of the inactivation of GABA-AT; the remaining 30% proceeded via an enamine intermediate (**67**), which led to inactivation (**68**) by a different mechanism (Scheme 12, pathway b).[93] The enamine mechanism was an inactivation mechanism originally proposed for the inactivation of glutamate decarboxylase [94] and aspartate aminotransferase [95] by serine-*O*-sulfate. The enamine inactivation mechanism for **62** with GABA-AT was elucidated by a number of experiments, e.g., inactivation of GABA-AT reconstituted with [^{3}H]PLP, followed by denaturation, produced 61% [^{3}H]PMP

(pathway a) and 39% of **69** (Scheme 13). Also, inactivation with [6-^{14}C]-**62**, followed by dialysis gave 1.02 equivalent of [^{14}C] bound to the enzyme, but after denaturation only 0.71 equivalent of [^{14}C] remained bound to the enzyme (pathway a). The structure of the released [^{14}C] compound was consistent with that of **69**. Structure **65** (Scheme 12) was confirmed by X-ray crystallography of **62**-inactivated GABA-AT (Figure 6).[65]

The authors of another mechanism study using [^{14}C]-**62** (although the location of the label was not revealed) came to an apparent incorrect conclusion regarding the inactivation mechanism.[96] One equivalent of radioactivity per monomer was identified upon inactivation; upon denaturation and tryptic digestion, only 60% of the radioactivity remained bound, but the lysine that binds PLP in place was identified as the site of its covalent attachment (now known to be Lys329). Although the PLP coenzyme was not radiolabeled, the conclusion based on denaturation and chromatography was that the remaining coenzyme was converted to PMP. Any enamine product would not have been detected by this experiment. An enamine mechanism was ruled out by these authors because no coenzyme was identified bound to the lysine (**68**). However, they only identified the product of 60% of the radioactivity; the other 40% (presumably the enamine product) could have resulted from the instability of **68**. These authors also favor a different Michael addition mechanism leading to **70** (Scheme 14) than that shown in Scheme 12 (pathway a), although they concede that the mechanism in Scheme 12 (pathway a) could also explain their data because the mass spectrum could not differentiate them. Now that the crystal structure of **62**-inactivated GABA-AT is known (Figure 6), it is clear that a mechanism like that in Scheme 12 (pathway a) is the correct one for the Michael addition pathway. Interestingly, although 4-aminohex-5-ynoic acid (**16**) also inactivates ornithine aminotransferase, **62** does not.[66,97]

A carboxylate bioisostere of vigabatrin, the corresponding tetrazole (**71**) had a K_I (5.6 mM) twice that of vigabatrin (2.6 mM) and an inactivation rate constant k_{inact} (0.73 min^{-1}) about a third that of vigabatrin (2.2 min^{-1}).[98] No mechanistic studies were carried out, except to show that the compound with the tetrazole NH methylated to NMe, thereby prohibiting anion formation, was not an inactivator of GABA-AT.



71

Density functional theory at the B3LYP/6-31+G(d,p) level of theory was used to differentiate the energies of the three inactivation mechanisms (Scheme 12, pathway a; Scheme 12, pathway b; and Scheme 14).[99] The conclusions, that the mechanism in Scheme 14 is least likely, the enamine mechanism (Scheme 12, pathway b) is next likely, and the Michael addition mechanism (Scheme 12, pathway a) is the most energetically favorable, are consistent with the experimental findings. Their calculations of the geometry of Michael

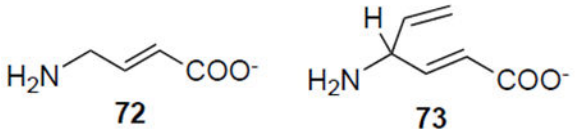
adduct **65** also agree well with the X-ray structure. Molecular dynamics simulations on the active site residues of **62**-inactivated GABA-AT show that interactions among the residues in *holo*-GABA-AT are conserved after inactivation, although the hydrogen-bond network is more stable as a result of the steric effect from **62** binding.[19]

Intraperitoneal administration of **62** to mice produces a dose- and time-dependent irreversible inhibition of GABA-AT and an increase in brain GABA levels; the half-life of GABA-AT was determined to be 3.4 days.[100] All of the activity is derived from the (*S*)-enantiomer;[101] also, only the (*S*)-isomer is actively transported into neurons, but not into astrocytes.[102] Treatment of mice with high doses of **62** led to time- and dose-dependent increases in brain GABA levels and an anticonvulsant effect against some, but not all, mouse epilepsy models.[103] Vigabatrin (**62**) has been used clinically since 1979; currently it is marketed as a monotherapy for pediatric patients one month to two years of age with infantile spasms (West's Syndrome) and as an adjunctive therapy for adults with refractory complex partial seizures in 65 countries, including the United States.[104] However, **62** was not approved by the FDA until 2009 because of a serious side effect with its clinical use, namely, visual field defects (VFDs) from retinal toxicity.[105] This occurs in 25-40% of patients following chronic administration of vigabatrin[106] because 1-3 g of this drug has to be taken daily to be effective. Shorter duration exposure in connection with studies of the treatment of stimulant addiction with vigabatrin (see below) does not show any occurrence of VFDs, which corroborates the prevailing belief that the development of VFDs results from prolonged exposure to large doses of vigabatrin. [107] The mechanism leading to the VFDs is not known. Although it has been hypothesized that GABA itself may be involved in vigabatrin-elicited retinal damage,[108] it has been demonstrated that in albino rats acute vigabatrin exposure damages the outer retina by a GABA-*independent* and vigabatrin-specific mechanism, resulting in sensitization of photoreceptors to light-induced damage.[109] In this case, it is probable that reactive oxygen species are involved, because they participate in light-mediated retinal toxicity.[110]

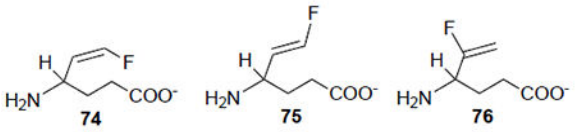
Vigabatrin (**62**) has another remarkable activity in that it prevents cocaine addiction in rats and baboons;[111] all of the activity arises from the (*S*)-enantiomer.[112] This activity also was demonstrated with other forms of addiction, including nicotine addiction,[113] methamphetamine, alcohol, and heroin addictions,[114] and inhalation addiction.[115] The effects of self-administration of cocaine by rats were decreased or blocked by vigabatrin administration dose-dependently, without an effect on the craving for food.[116] Current findings indicate that the resulting neurochemical response to cocaine and other abusive drugs is a significant increase in the levels of dopamine in the nucleus accumbens (NAc);[117] this activates the neurons producing pleasure and reward responses. The rise in dopamine and associated behaviors can be antagonized by an increase in the concentration of GABA. The mechanism of vigabatrin responsible for the prevention of drug addiction was determined to be the same as that for the prevention of epilepsy, namely, inactivation of GABA-AT, which increases the GABA levels in the brain and produces the anticonvulsant effect. It was demonstrated by positron emission tomography (PET) in primates that vigabatrin inhibits the rise in cocaine-induced dopamine levels, which blocks the reward response.[111] Vigabatrin treatment also is effective against drug addiction in humans;[118] a

randomized, double-blind, placebo-controlled trial of 103 subjects[119] revealed that 28% of subjects treated with vigabatrin achieved abstinence compared to only 7.5% treated with placebo.

Because (*E*)-4-aminocrotonic acid (**72**), the α,β-unsaturated analogue of GABA, was transaminated 1.8 times the rate of GABA,[120] (*E*)-4-amino-2,5-hexadienoic acid (**73**), the corresponding α,β-unsaturated analogue of vigabatrin, was synthesized. However, it inactivated GABA-AT at only one-sixth the rate of vigabatrin.[121] No mechanism was proposed, but it is likely similar to that of vigabatrin.

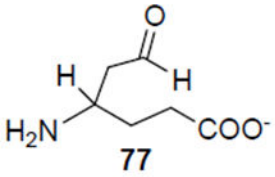


The addition of a single fluorine atom at any position of the vinyl group of vigabatrin[122] alters the mechanisms, resulting in multiple different mechanisms for each isomer. (*Z*)-(**74**) and (*E*)-4-amino-6-fluoro-5-hexenoic acid (**75**)[123] each inactivate GABA-AT by different mechanisms, which are also different from inactivation of GABA-AT by 4-amino-5-fluoro-5-hexenoic acid (**76**).[124] A variety of experiments were used to define the mechanisms of each of these isomers.



The kinetics of inactivation for **74** and **75** were about the same. Also, results of inactivation of GABA-AT reconstituted with [7-^{3}H]PLP were approximately the same: about two-thirds (63% for **74** and 66% for **75**) of the radioactivity remained covalently bound to the enzyme; what was released was established to be mostly PLP (31% for **74** and 29% for **75**) with a small amount (5% for **74** and 4% for **75**) of PMP. Furthermore, inactivation of GABA-AT with either [^{3}H]-**74** or [^{3}H]-**75** incorporated 1.0 equivalent of tritium into the enzyme. Urea denaturation released about a third of the tritium, 85% of which was identified as 4-amino-6-oxohexanoic acid (**77**), and the rest as 4-oxo-5-hexenoic acid (**78**) and the Michael adduct of **78** with 2-mercaptoethanol (a reductant that was added in solution). All appeared to be identical between the two isomers until it was shown that **74** inactivation produced two nonamine metabolites but **75** produced only one; the metabolite in common was the transamination product (or something derived from it), and the additional metabolite from **74** was **78**. In fact, both compounds undergo transamination, but **74** produces twice the amount of product as **75** (1.4 vs 0.7 equivalent). Also, **74** releases 1.4. F^- vs 0.9 F^- for **75**, which is not surprising as **74** gives two metabolites and **75** only one. After inactivation by both **74** and **75** an absorption peak in the UV-visible spectrum at 430 nm appears.
To rationalize these results, multiple mechanisms are necessary; the principal inactivation mechanisms for **74** and **75** appear to be different. Scheme 15 gives the major proposed inactivation mechanisms for **74**, which resemble the enamine inactivation mechanism seen

for vigabatrin (Scheme 12, pathway b), except leading to enamine **79**. However, unlike the enamine from vigabatrin (**67**, Scheme 12), **79** can readily undergo fluoride ion elimination to give iminium **80**, which can either hydrolyze to give metabolite **78** or suffer attack by an active site nucleophile (not Lys329 because it is bound to PLP), producing enamine **81**, which can undergo enamine addition to the coenzyme to give **82**. Hydrolysis of **82** would produce **83**, which could represent the 430 nm absorption. A minor pathway is represented by hydrolysis of enamine **81** to give **84**. Another significant mechanism for **74** (Scheme 16) is normal tautomerization to the reactive Michael acceptor (**85**), which undergoes nucleophilic attack by Lys329 to give **86**, which can either become protonated to **87** (pathway a) or eliminate F^- to **88** (pathway b), further tautomerization and hydrolysis of which would produce metabolite **77** and PLP (pathway c). Alternatively, **87** can eliminate F^- to give **89**, which can either tautomerize to **90** (pathway d) and hydrolyze to **77** and PLP (pathway e) or hydrolyze to **91** (pathway f), which would account for the small amount of PMP observed. PMP (and **91**) also could come from hydrolysis of **88** (pathway g).
The X group in Scheme 15 was identified as Cys294 (actually Cys293; there must have been a numbering error in the peptide map) by [2-^{3}H]-**74** inactivation of GABA-AT, tryptic digestion, HPLC, and mass spectrometry.[125]



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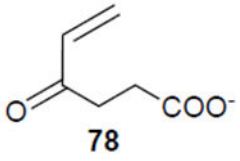

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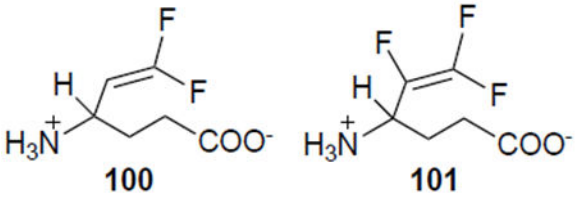
To account for the identification of only one metabolite produced from **75** (**77** not **78**), an S_N2 mechanism, leading to **82**, was invoked instead of elimination (Scheme 17 instead of Scheme 15; Scheme 16 can still occur for **75**). The difference in these two inactivator mechanisms could be rationalized by a difference in hydrogen bonding of the two isomers (Scheme 18). In addition to a difference in mechanisms between **74** and **75**, these both differ in inactivation mechanism from that of **76**.

Evidence from multiple experiments indicates that **76** inactivates GABA-AT by three different mechanisms, all of which are initiated by gamma-proton removal and elimination of F^- to **92** (Scheme 19).[124] By reconstitution of GABA-AT with [^{3}H]PLP it was found that after inactivation and denaturation, the coenzyme is released as PLP as well as a modified form in the ratio 7:3. Inactivation with [^{3}H-**76**] gave 0.7 equivalent bound; the other 0.3 equivalent is the released PLP, which also inactivates the enzyme. The first mechanism (Scheme 19) proceeds from **92** by Lys329 attack on the activated allene and tautomerization to **93**, which loses PLP to give **94**; hydrolysis of **94** gives **96** (detected as 0.5 equivalent starting from [^{3}H-**76**]) and inactivated enzyme (**95**). Another inactivation mechanism resembles that of **74** (Scheme 15) via **80**, which hydrolyzes to **78** (isolated in the amount of 1 equivalent) and **84**. The third inactivation mechanism is an enamine mechanism (Scheme 20). PLP is transferred to Lys329, resulting in allenamine **97**, which adds into the iminium bond of bound PLP to give **98**. Michael addition of water, followed by elimination of Lys329 produces **99**, which was isolated and identified by mass spectral analysis. With

the aid of a fluoride ion-selective electrode, 2.6 ± 0.1 equivalents of F^- were detected, consistent with loss of 1 F^- to give inactivation, 1 F^- to produce **78**, and 0.5 equivalent of F^- from formation of **96** (Scheme 19). Transamination also occurs 6.3 ± 0.6 times per inactivation event (determined by measuring the conversion of [5-^{14}C]-α-ketoglutarate to [5-^{14}C]-glutamate).

The corresponding 4-amino-6,6-difluoro-5-hexenoic acid (**100**) and 4-amino-5,6,6-trifluoro-5-hexenoic acid (**101**) also were synthesized; **100** has about one-tenth the potency of vigabatrin and **101** is the weakest of all of the fluorinated vigabatrin analogues, which was consistent with the in vivo results.[122] Compound **74** was the most potent in vitro and in vivo.

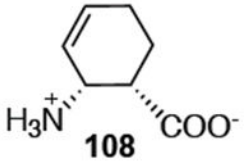


As discussed above (section 3.2), according to the Principle of Microscopic Reversibility, an inactivator that resembles the product in a reversible reaction might be used to reach a reactive intermediate that could be generated from a substrate-like inactivator in the forward direction. 4-Acryloylphenol (**102**), a ketophenol, produced time- and concentration-dependent inactivation of GABA-AT in the PMP form, protected by α-ketoglutarate, suggesting that it is active-site directed.[126] The phenolate group is an isostere of the carboxyethyl side chain of vigabatrin. No mechanism experiments were carried out, but a Michael addition mechanism was proposed; however, as this is a mimic of the keto form of vigabatrin, it could proceed by both a Michael addition and enamine mechanism (Scheme 21). Attack by Lys329 on **103** to give **104** is equivalent to attack on **64** in Scheme 12 (pathway a). The enamine mechanism would be equivalent to pathway b in Scheme 12, leading to **106**. Note that **102** reacts with the PMP form of GABA-AT to give **103**, but tautomerization produces **105**, which is equivalent to intermediate **66** in Scheme 12.

The same study as above was carried out by the same group with 3-chloro-1-(4-hydroxyphenyl)propan-1-one (**107**); following elimination of HCl, **103** is generated (Scheme 22), and the same mechanisms as in Scheme 21 could be drawn.[127]

On the basis of computer modeling, in conjunction with the crystal structure of GABA-AT,[20] it was determined that vigabatrin (**62**) bound to the active site lies with its vinyl group facing away from Lys329 (Figure 7A), the nucleophile in the Michael addition pathway (Scheme 12, pathway a); this conformation would only allow the enamine mechanism to proceed. However, rotation of the vinyl group bond would permit both Michael addition and enamine mechanisms (Figure 7B).[128] Therefore, it might be possible to prevent the Michael addition pathway without affecting the minor enamine pathway (Scheme 12, pathway b) just by restricting rotation of the vinyl group. To prevent the vinyl group from rotating toward Lys329, cyclic analogues **108** and **109** were synthesized. Both were

time-dependent inactivators of GABA-AT, but **108** still proceeded by both the Michael addition and enamine inactivation pathways. [129] Further molecular modeling of **108** revealed two low-energy conformations when bound to PLP, one (A) that can only proceed by the enamine mechanism because the double bond is faced away from Lys329, and one (B) that allows attack by Lys329 (Figure 8). Compound **109**, however, only inactivates GABA-AT by the minor enamine mechanism, because it cannot get into a conformation with the alkene facing Lys329.[128]



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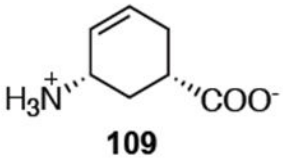
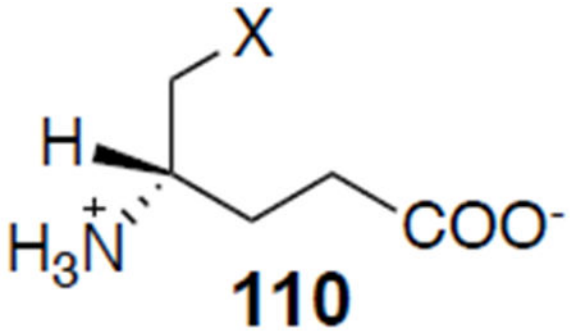


109

3.5. Michael Addition and Enamine Mechanisms via Elimination

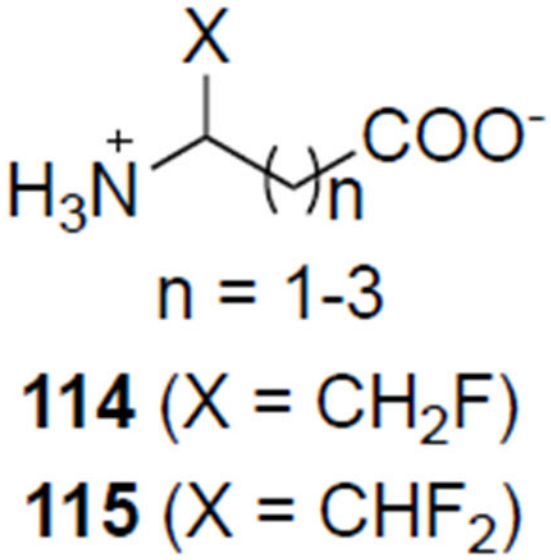
A series of (*S*)-5-substituted-4-aminopentanoic acid analogues (**110**) [130] was designed as inactivators of GABA-AT via a Michael addition mechanism (Scheme 23).[131] Elimination of HX from the Schiff base of **110** with PLP gave electrophile **111**, which could undergo attack by an active site nucleophile to give covalently bound **112**. No experiments were carried out to determine the actual mechanism, but, with the aid of [U-^{14}C]-4-amino-5-chloropentanoic acid (**110**, X = Cl), it was shown that 1.7 moles of inactivator becomes covalently attached per mole of enzyme dimer after inactivation, which suggests attachment was occurring at each active site of the homodimeric enzyme. Further studies were consistent with the mechanism in Scheme 23,[132] but did not rule out an enamine mechanism. Inactivation only occurred if the coenzyme was in the PLP form; (*S*)-[4-^{2}H]-**110** (X = Cl) exhibited a kinetic isotope effect on inactivation of 6.7, indicating that 4-proton removal was the rate-determining step for inactivation; a fluoride ion electrode was used with **110** (X = F) to demonstrate that 2.4 moles of F^- were released per mole of dimeric GABA-AT; inactivation by [U-^{14}C]-4-amino-5-chloropentanoic acid (**110**, X = Cl) resulted in 1.96 mole of inactivator per enzyme dimer; during inactivation there was a time-dependent increase in a 330 nm absorption and concomitant decrease in the 412 nm absorption, corresponding to the conversion of PLP to PMP. Intraperitoneal injection of **110** (X = F) in mice produced dose-dependent inactivation of brain GABA-AT concomitant with an increase in brain GABA levels. Four hours after a 100 mg/kg dose, brain GABA levels rose 16 fold with 80% loss of GABA-AT activity.[133]



110

The Michael addition inactivation mechanism was shown to be incorrect by inactivation of [4-^{3}H]PLP-reconstituted GABA-AT with **110** (X = F).[134] Denaturation released 6-[2-methyl-3-hydroxy-5-(phosphonoxymethyl)-4-pyridinyl]-4-oxo-5-hexenoic acid (Scheme 24, **113**), which is the expected product of the enamine mechanism depicted followed by denaturation. Inactivation of GABA-AT by **110** (X = F) was previously shown to proceed with a partition ratio (ratio of turnovers to give product per inactivation event) of zero.[58] It is very rare for a mechanism-based inactivator to have a partition ratio of zero, but it is especially unexpected in this case because inactivation requires the enamine generated from **110** (X = F) to be released and add to enzyme-bound PLP without escape from the active site!

ω-Monofluoromethyl- (**114**) and ω-difluoromethyl- (**115**) analogues of β-alanine, GABA, and 5-aminopentanoic acid, three known substrates of GABA-AT, were shown to be time-dependent inactivators; the corresponding ω-trifluoromethyl analogues were not inactivators.[135] No mechanistic studies were done; it was assumed that a Michael addition mechanism was operative. In vitro, the potency within a given fluorine substitution pattern increases with decreasing chain length. Also, the potency within a given chain length increases with decreasing number of fluorine atoms, except for the β-alanine series (n = 1), where mono- and difluoro analogues are equipotent. These results are consistent with the electron-withdrawing properties of fluorine, which, as the number of fluorine atoms increases, would affect Schiff base formation with PLP because of the lower basicity of the amino group, the increased acidity of the proton adjacent to the imine generated (the proton abstracted by the enzyme), and the decreased elimination of fluoride ion.[136] Intraperitoneal injection of 25 mg/kg difluoromethyl-β-alanine in mice reduced GABA-AT activity to almost zero within an hour, which remained at that level for about 48 hours, and increased brain GABA levels by 10 fold. This compound is about 100 times more potent than vigabatrin in vivo.

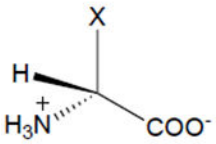


Further studies with **115** (n = 1) and the corresponding chlorofluoromethyl analogue demonstrated time- and concentration dependence to the inactivation.[137] Incorporation of a deuterium at the β-position of **115** (n = 1) produced a kinetic isotope effect on inactivation of 4.5, indicating that enzyme-catalyzed deprotonation is rate determining during inactivation. Only the (−)-isomer inactivates GABA-AT and at the same rate as double the concentration of the racemate. The stereochemistry of the (−)-isomer was not determined, but it presumably is the (*S*)-isomer, because that puts the proton that is abstracted in the correct position for deprotonation. The UV-visible spectrum shows a decrease in the 415 nm PLP peak and an increase in the 330 nm PMP peak, as was observed for **110** (X = F).[131] In vivo studies in mice with **115** (n = 1) and the corresponding deuterated analogue exhibited a deuterium isotope effect in vivo; a 2.5 mg/kg dose of the protio analogue produced a greater inhibition of GABA-AT and accumulation of brain GABA than a 10 mg/kg dose of the deuterio analogue. No definitive conclusion, however, could be made regarding the inactivation mechanism.

Mechanism studies on **114** (n = 1) were carried out, and, although an enamine mechanism was concluded, unlike **110** (X = F), which had a partition ratio of zero,[58] there were additional reactions that occurred with **114** (n = 1).[138] As in the case of **110** (X = F), [1,2-^{14}C]-**114** (n = 1) incorporated two equivalents of radioactivity per enzyme dimer with no transamination, as determined by the lack of conversion of [^{14}C]-α-ketoglutarate to [^{14}C]-glutamate during inactivation. All of the radioactivity that was covalently bound was released by base treatment, and two products were detected: the product of the reaction of PLP with acetoacetate (**116**) and the product of the reaction of PLP with acetone (**117**) (Scheme 25). Additionally, 1.70 equivalents of $^{14}CO_2$ were detected. The same two products (**116** and **117**) were obtained using [^{3}H]PLP-reconstituted GABA-AT with unlabeled **114** (n = 1). These results are consistent with the formation of the enamine inactivation pathway, giving **116** (after denaturation) and its decarboxylation product **117**. However, unlike **110** (X = F), 6.7 ± 0.7 F^- were released during inactivation, indicating more than inactivation

occurred. To account for the additional turnover, metabolites were investigated, and 5.5 equivalents of [^{14}C]-acetoacetic acid (**118**) was identified, which was concluded as being derived from release of the enamine in 5 out of 7 turnovers.

The corresponding (*R*)-α-amino acid derivatives (although the stereochemistry is the same as (*S*)-4-amino-5-halopentanoic acids, the nomenclature changes to (*R*) because the carboxylate group has higher (*R,S*) rule priority than a methylene group) (**119**, X = $CH_2OSO_3^{2-}$, $CH_2OPO_3^{2-}$, CH_2F, CH_2Cl, vinyl) also were shown to be time-dependent inactivators of GABA-AT; (*S*)-**119** (X = CH_2F) did not inactivate the enzyme.[139]



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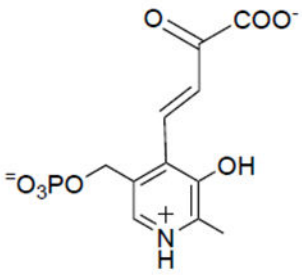

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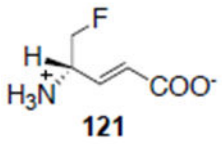
Mechanistic studies with serine *O*-sulfate (**119**, X = $CH_2OSO_3^{2-}$) were carried out to demonstrate that the mechanism is the same as that for 4-amino-5-fluoropentanoic acid (**110**, X = F), namely the enamine mechanism.[140] With the aid of [^{3}H]PLP, inactivation by **119** (X = $CH_2OSO_3^{2-}$) converted all of the radioactivity to 4-[2-methyl-3-hydroxy-5-(phosphonoxymethyl)-4-pyridinyl]-2-oxo-3-butenoic acid (**120**) by the same mechanism shown in Scheme 24.



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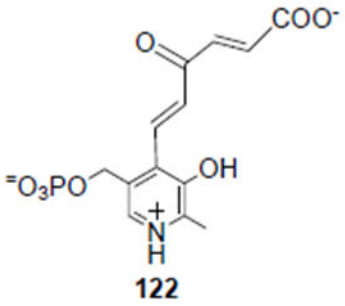
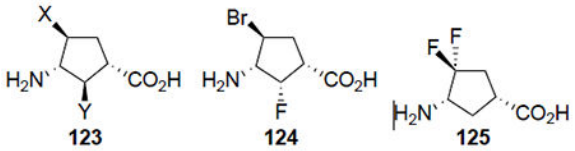


122

As noted above, because (*E*)-4-aminocrotonic acid (**72**), the α,β-unsaturated analogue of GABA, was transaminated 1.8 times the rate of GABA,[120] the corresponding unsaturated analogue of 4-amino-5-fluoropentanoic acid (**121**) was synthesized and was found to have a K_I 17 times lower than that of the saturated analogue **110** (X = F) with a comparable k_{inact}.[121] Another paper, published at about the same time, reported a difference in K_I of 52.5 times lower for **121** with a 5-fold decrease in k_{inact}, but this assay had to be run at much lower pH and temperature because the inactivation rate was so fast; this could account for the difference in kinetic results in the two papers.[141] It was found that inactivation released five fluoride ions, and titration of the enzyme using increasing equivalents of **121** confirmed that it takes five molecules of **121** to completely inactivate GABA-AT. Therefore,

the 5-fold decrease in rate compared with **110** (X = F) could occur because of the necessity of GABA-AT to turnover five times as many molecules per inactivation event. The partition ratio, therefore, is four, whereas that for **110** (X = F) is zero.[58] No transamination was observed using [^{14}C]-α-ketoglutarate in the assay, so there are presumably five turnovers with loss of HF, and four of the resulting intermediate molecules undergo hydrolysis and release for every one that releases the enamine. No attempt was made to detect the presumed hydrolysis product. In a follow-up paper, the mechanism of inactivation of GABA-AT by **121** was reported. [142] Inactivation of [^{3}H]PLP-reconstituted GABA-AT with **121** followed by release of the radioactivity with base gave a radioactive product with an HPLC retention time almost the same as that for **113** (Scheme 24) with no [^{3}H]PMP formed, suggesting, if that product really is **122**, that an enamine mechanism is the sole pathway.

Five-membered cyclic analogues of vigabatrin have been made as conformationally-rigid analogues, but none inactivated GABA-AT. [143] To determine if this would be a general phenomenon when converting acyclic inactivators to cyclic analogues, a series of cyclopentane analogues of mono- and di-halogenated 4-aminopentanoic acids (**123-125**) were made (to mimic compounds like **114** and **115**), and it was found that most of these compounds were good inactivators of GABA-AT (albeit not as potent as the open-chain molecules).[144] Compounds **123** (X = F or Br, Y = H; X = Br, Y = Br) and **124** were inactivators; **125** was not an inactivator nor did it undergo transamination, although it did very slowly release F^-. There was no difference in the k_{inact} for **123** (X = F or Br; Y = H), indicating that cleavage of the C-halogen bond is not the rate-determining step. Consistent with that conclusion was the observation that [^{2}H]-**123** (X = Y = Br) exhibits a kinetic isotope effect on inactivation of 3.3, indicating that C-H bond cleavage is, at least, partially rate-determining. Whereas open-chain analogue **110** (X = F) releases only one F^- per inactivation event, **123** (X = F, Y = H) releases 148 F^- per inactivation event, which may account for the 1/15 decrease in inactivation rate for the cyclic analogue. Interestingly, inactivation of GABA-AT with [5-^{3}H]-**123** (X = Y = Br) resulted in covalent attachment of 2 equivalents of tritium per dimeric enzyme, but after denaturation, there was still 1 equivalent of tritium associated with the protein. If this compound proceeded exclusively by the enamine mechanism, all of the radioactivity should have been released. This result suggests that more than one mechanism is occurring, possibly half by the enamine mechanism and half by the Michael addition mechanism (although neither was definitively established) as a result of conformational rigidity relative to the open-chain inactivator (**110** (X = F)). However, a crystal structure at 1.9 Å resolution of GABA-AT inactivated by **123** (X = F, Y = H) clearly showed the product of an enamine inactivation mechanism (Scheme 26 and Figure 9). [145] Either this crystal structure represents only the crystals that came from the enamine mechanism or the inactivation mechanism for **123** (X = F, Y = H) is only the enamine mechanism, and **123** (X = F, Y = Br) is both the enamine and Michael addition mechanisms.



Ethanolamine *O*-sulfate (**126**), a GABA mimic, was shown to be an active-site irreversible inactivator of GABA-AT in vitro and in vivo without inhibiting glutamate decarboxylase, alanine aminotransferase, or aspartate aminotransferase.[147] Administration to mice produced an anticonvulsant effect.[148] Mechanistic studies showed a spectral decrease in the 415 nm absorption signal and increase in the 330 nm absorption signal, indicative of a conversion of the PLP imine to a PMP imine.[149] [^{14}C]Ethanolamine *O*-sulfate is incorporated into GABA-AT to the extent of 1 mol/mol active site, but no radioactivity from ethanolamine [^{35}S]-*O*-sulfate is incorporated. Elimination of sulfate from **126** should give aminoethylene, which should decompose to ammonia and acetaldehyde; 11 equivalents of ammonia were detected, suggesting a partition ratio of 10. The acetaldehyde was detected using alcohol dehydrogenase, following the oxidation of NADH to NAD^+. The Michael addition mechanism in Scheme 27 to give **127** (pathway a) was suggested; however, this mechanism does not take into account the spectral result suggesting a conversion to PMP during inactivation. A Michael addition mechanism, which would give **128** (pathway b), could account for that; however, the results obtained do not exclude an enamine mechanism.

As mentioned above (Section 3.2), when an enzyme-catalyzed reaction is reversible, it is possible to invoke the Principle of Microscopic Reversibility and design an inactivator that is product-like. In the case of GABA-AT, complete in vivo inactivation has been difficult because as GABA-AT is inhibited and brain GABA levels rise, the equilibrium will favor the PMP form of the enzyme as a feedback inhibition mechanism. To attain complete inhibition, 5-fluorolevulinate (**129**) was designed to inactivate the PMP form of the enzyme.[150] Time- and concentration-dependent inactivation of GABA-AT occurred in vitro without inhibition of aspartate- or alanine aminotransferase at 10 mM; the presence of 2-mercaptoethanol to trap any electrophilic species released from the enzyme did not affect the rate of inactivation by **129**. However, there was a slow release of F^- in solution in the presence of 2-mercaptoethanol, so some of the inactivation may arise from S_N2 attack by an active site cysteine on **129**. The authors proposed a Michael addition mechanism, but after this work was published, the inactivation mechanism from **111** (Scheme 24) was shown to proceed via the enamine pathway (Scheme 28).[58] Administration of **129** to rodents resulted in a dose-dependent decrease in GABA-AT activity and increase in brain GABA.

(2*S*,4*S*)-4-Aminotetrahydrothiophene-2-carboxylic acid hydrochloride (**130**) was designed to inactivate GABA-AT by either an elimination-Michael addition mechanism (pathway a) or elimination-enamine mechanism (pathway b, Scheme 29) from **131**; it does something completely different.[151] The X-ray crystal structure of GABA-AT inactivated by (2*S*,4*S*)-**130** at 1.66 Å (Figure 10) revealed a nonplanar 5-membered ring covalently bound to the PLP; Lys329 was not modified, indicating that neither of the hypothesized inactivation pathways was occurring. Also, a heretofore unreported intermolecular nonbonded S·····O interaction between the sulfur atom of **130** and the carboxylate oxygen of Glu270 was

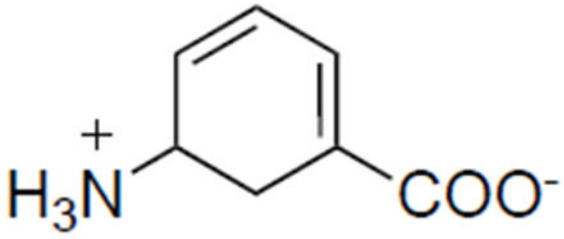
observed. This interaction is apparently important for binding because the corresponding cyclopentane analogue, (1*S*,3*R*)-3-aminocyclopentane-1-carboxylic acid, did not inactivate GABA-AT, but was a competitive reversible inhibitor. Denaturation caused hydrolysis of the active-site adduct, resulting in the formation of PMP and **133** (Scheme 30). A mechanism based on the crystal structure (Figure 10) involves tautomerization followed simply by deprotonation to **132**.

An even simpler mechanism could occur if tautomerization were not necessary, for example, with a ketone-containing molecule that forms a Schiff base with PMP. That would be another example of the Principle of Microscopic Reversibility,[61] as was hypothesized for the inactivation of GABA-AT by 3,5-dioxocyclohexanecarboxylic acid (**134**), leading to the ketone-stabilized enamine **135**; this would inactivate just by deprotonation (Scheme 31).

With the appropriate cyclic structure, a tautomerization-deprotonation mechanism can lead to the formation of an aromatic molecule; this is the genesis of the aromatization inactivation mechanism.

3.6. Aromatization Mechanisms

5-Amino-1,3-cyclohexadienylcarboxylic acid (known as gabaculine, **136**) is a natural product isolated from a *Streptomyces toyocaensis* subsp. 1039 culture; its structure was elucidated spectroscopically, the racemate was synthesized, and the racemate was shown to have half of the inhibitory activity toward GABA-AT as the natural product (stereochemistry not reported), but no mention was made if the inhibition was irreversible.[152] Time- and concentration-dependent inhibition was reported, although no mechanism was offered.[153] More definitive time- and concentration-dependent irreversible inhibition studies of GABA-AT from mouse brain were carried out, which demonstrated that **136** exhibited a pH-inactivation rate profile similar to the pH-rate profile of substrate; inactivation was protected by GABA and activated by α-ketoglutarate, thereby providing support for reaction of the PLP form of the enzyme.[154] Two mechanisms were proposed (Scheme 32); tautomerization of **136** leads to **137**, which can either undergo Michael addition to **138** (pathway a) or undergo deprotonation to aromatic adduct **139** (pathway b). The nonenzymatic reaction of PLP with **136** was shown to give **139**; with 4,5-dideuteriogabaculine there was a deuterium isotope effect of 4.26 on nonenzymatic product formation.[155]



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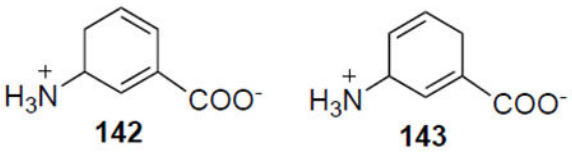
Studies to elucidate the inactivation mechanism for **136** were then carried out.[156] 4,5-Dideuteriogabaculine inactivated GABA-AT with a kinetic isotope effect of 2.27, indicating that deprotonation was partially rate determining enzymatically and less so than nonenzymatically. Time-dependent incorporation of radioactivity into GABA-AT from [2-^{3}H]gabaculine corresponded with the loss of enzyme activity; denaturation released the radioactivity, which was mixed with *m*-carboxyphenylpyridoxamine phosphate (**139**, Scheme 32) and chromatographed under several conditions, which showed that the radioactivity co-migrated with **139**. Dephosphorylation with alkaline phosphatase gave a radioactive product that co-migrated with *m*-carboxyphenylpyridoxamine. These results support the aromatization mechanism (Scheme 32, pathway b). Further confirmation of this mechanistic conclusion is as follows: (1) inactivation of [^{3}H]PLP-reconstituted GABA-AT with gabaculine and denaturation gave a product that co-migrated with **139** in two HPLC systems; (2) the product of inactivation had the same UV-visible spectrum as **139**; (3) electrospray ionization mass spectrometry gave the same fragmentation patterns as **139**; and (4) the NMR spectrum of the released inactivation product from gabaculine inactivation of GABA-AT was identical to that of **139**. [157] Further evidence against the Michael addition mechanism was that 1,2-dehydrogabaculine (**140**), which can be converted to a Michael acceptor (**141**) but cannot aromatize (Scheme 33), did not inactivate bacterial GABA-AT.[155] However, as we saw above, **109** (which is (*S,S*)-**140**, Section 3.4) is a mechanism-based inactivator of mammalian GABA-AT, which proceeds by the enamine mechanism exclusively.[128]

Gabaculine also inactivates ornithine aminotransferase,[97] alanine aminotransferase,[158,159] aspartate aminotransferase,[158,159] ω-amino acid aminotransferase, and D-amino acid aminotransferase but not alanine racemase and tryptophanase.[159] The enzymes that are inactivated by gabaculine can catalyze the exchange of the β-protons of their respective substrates, but the enzymes that are not inactivated by gabaculine cannot catalyze the exchange of the β-protons of their substrates. Therefore, it was proposed that deprotonation of the gabaculine intermediate (**137** in Scheme 32), leading to aromatization of gabaculine (**139** in Scheme 32), is enzyme catalyzed.[159] The promiscuity of gabaculine may account for its neurotoxicity.

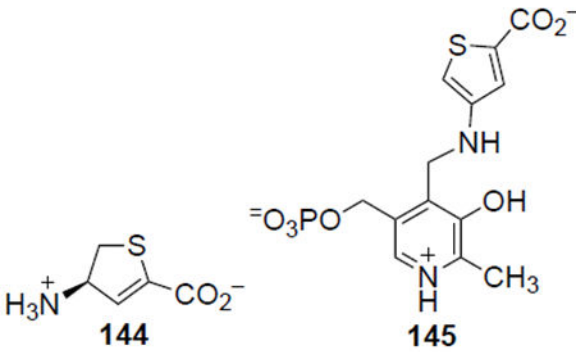
There are two other isomers of gabaculine, known as isogabaculines. 3-Amino-1,5-cyclohexadienylcarboxylic acid (**142**) was synthesized and shown also to be a time-dependent inactivator of GABA-AT in vitro; administration to mice led to inhibition of GABA-AT and an increase in brain GABA levels.[160] Although the affinity for GABA-AT is 10-20 times lower than that for gabaculine, the rate constant is much greater; therefore, the two isomers are almost equipotent. As expected, the nonenzymatic reaction of **142** and PLP gave **139**. The other gabaculine isomer, 4-amino-1,4-cyclohexadienylcarboxylic acid (**143**), was synthesized, but no biochemical studies were reported.[161]



Following inactivation of GABA-AT by gabaculine, the activity could be restored by treatment with PLP.[162] Furthermore, the two PLP binding sites in dimeric GABA-AT have different affinities for PLP (K_d 1 nM and 3 μM), as determined by formation of the dimeric enzyme with only one PLP. Inactivation of that form of the enzyme then allowed incorporation of a second PLP with total reactivation of the enzyme. Similar results were found using *m*-carboxyphenylpyridoxamine 5-phosphate (**139**, Scheme 32) as the active site titrant, except that the affinity for **139** was 10 times greater than that of PLP.[163] The binding of **139** to GABA-AT can be reversed by treatment with pyridoxine 5-phosphate oxidase, which converts **139** back to PLP.

Intraperitoneal [164] and intraventicular [165] administration of gabaculine (**136**) to mice produced a concomitant decrease in brain GABA-AT activity and increase in brain GABA. Although numerous side effects were apparent, chemical-induced, but not electroshock-induced, convulsions were prevented.[165] The in vivo effects of gabaculine (**136**) and isogabaculine (**142**) were investigated[166] and compared to those of 4-aminohex-5-ynoic acid (**16**) and 4-aminohex-5-enoic acid (**62**).[167] All four compounds inhibited GABA-AT in mouse brain and raised the GABA levels in a dose-dependent manner with potencies in the order **136~142>16>62**. Although all produced a variety of toxicities, they all protected mice against audiogenic seizures. In vivo edited ^{1}H NMR spectral studies, following an increase in the 4-methylene proton resonance of GABA, in rat brain demonstrated that treatment of rats with gabaculine produced an increase in GABA levels.[168]

Compounds that can be converted to heteroaromatic-PMP complexes also inactivate GABA-AT, but not all by an aromatization mechanism. 4-Amino-4,5-dihydrothiophene-2-carboxylic acid (**144**) was synthesized with the intent of producing a new mechanism-based inactivator utilizing the aromatization mechanism, but no enzyme studies were carried out by these authors.[169] (*S*)-**144** was later demonstrated to be a time- and concentration-dependent inactivator of GABA-AT that reacts with the active site PLP to give **145**, isolated by HPLC (>99% of radioactivity from [^{3}H]PLP-reconstituted GABA-AT was released) and characterized by UV-visible spectroscopy, electrospray ionization mass spectrometry, and tandem mass spectrometry using synthesized **145** as the standard.[170] These results support the aromatization inactivation mechanism in Scheme 32 (pathway b). The corresponding (*R*)-enantiomer does not inactivate the enzyme. Titration of the enzyme with increasing equivalents of (*S*)-**144** led to complete inactivation after only 1.7-1.8 equivalents were used. Using [^{14}C]-α-ketoglutarate, it was demonstrated that there were 0.6 ± 0.1 transamination events per inactivation event, consistent with a partition ratio of about 0.7.



L-Cycloserine (**146**, Seromycin™), a natural product isolated from Streptomyces, is used to treat tuberculosis and urinary tract infections.[171] It was reported to be an irreversible inhibitor of GABA-AT in vitro and to increase brain GABA concentration.[172] An inactivation mechanism (actually for D-cycloserine inactivating a variety of PLP-dependent enzymes) was proposed without any experimental support (Scheme 34).[173] Much later, the mechanism of inactivation of GABA-AT by L-cycloserine was established as an aromatization mechanism (Scheme 35).[174] L-[U-^{14}C]Cycloserine produced time- and concentration-dependent inactivation with incorporation of 1.1 equivalents of radioactivity bound covalently after rapid gel filtration. Dialysis or denaturation released all of the radioactivity from the enzyme. Inactivation of [^{3}H]PLP-reconstituted GABA-AT with **146** followed by dialysis or denaturation released all of the radioactivity, which comigrated by HPLC with the radioactive product from [U-^{14}C]-**146** and GABA-AT, indicating that the adduct contained both the inactivator and coenzyme. HPLC showed that the radioactive product was not the expected product of an enamine mechanism, but was very similar to the gabaculine-coenzyme adduct (**139**). Electrospray ionization tandem mass spectrometry was consistent with the aromatization mechanism product (**147**).

L-3-Chloroalanine hydroxamate (**148**) inactivates GABA-AT, but it was shown to be a prodrug of L-cycloserine (**146**) not a direct inactivator.[175] Inactivation of [^{3}H]PLP-reconstituted GABA-AT followed by denaturation did not produce PMP (the product of pathways a and b,c; Scheme 36) or the potential product of an enamine reaction (pathway b,d, **150**). Instead, **147** (Scheme 35), the aromatization product, was formed. Compound **148** was converted to L-cycloserine (**146**) in buffer (Scheme 37) with a small solvent deuterium isotope effect, indicating that deprotonation of the hydroxamate hydroxyl group in **148** is partially rate determining. This is consistent with the observation that the rate of inactivation of GABA-AT by **148** accelerates with time to a maximum rate that occurs if **148** is preincubated in buffer until it is all converted to L-cycloserine.

Given the aromatization mechanisms for gabaculine (**136**), 4-amino-4,5-dihydrothiophene-2-carboxylic acid (**144**), and L-cycloserine (**146**), it would seem redundant to investigate the mechanism of inactivation of GABA-AT by (*S*)-4-amino-4,5-dihydrofuran-2-carboxylic acid (**151**), which was synthesized and reported to be an inactivator of GABA-AT.[176] However, a detailed investigation into its inactivation mechanism revealed that this heterocycle does *not* proceed by an aromatization mechanism.[177] Inactivation occurs with a partition ratio of 3.7 as determined by titration of the enzyme with increasing concentrations

of **151** (4.7 equivalents required for complete inactivation). This is consistent with the number of transamination events per inactivation, namely, 3.2 ± 0.2). The partition ratio, however, changed when the pH was changed. Inactivation of [^{3}H]PLP-reconstituted GABA-AT followed by denaturation under different conditions only produced PMP, not a **151**-coenzyme adduct, such as the product of aromatization. Two alternative mechanisms were proposed, resulting in either metabolite **153** and PMP or covalent adduct **154** and PMP (Scheme 38). Presumably, because of the lower aromatic stabilization energy of furan (16 kcal/mol) relative to thiophene (29 kcal/mol), and benzene (36 kcal/mol),[178] deprotonation to give the aromatized adduct is not favored for **151**. A number of experiments were carried out in an attempt to differentiate pathways a and b in Scheme 38; for example, enzyme inactivated by pathway b should become reactivated by incubation with PLP, but that did not occur, although a control enzyme in the PMP form was reactivated. The conclusion was that pathway a is more likely than pathway b; a definitive answer might have been derived by inactivation with radiolabeled **151**, but that could not be synthesized. More recently, the X-ray crystal structure of *E. coli* L-aspartate aminotransferase inactivated by **151** was reported. The inactivated enzyme seems to be the product of intermediate **152**, either directly (pathway a) or via **153** (pathway b), giving a covalent complex with Lys329 (**155;** see Scheme 39 for a possible mechanism).[179] The same experiment with *E. coli* L-aspartate aminotransferase and 4-amino-4,5-dihydrothiophene-2-carboxylic acid (**144**) gave a mixture of the aromatization product (**145**) and the corresponding covalent complex with the active site lysine (**155** but thiophene instead of furan).[180] This, again, is consistent with the poorer aromatic stabilization energy of furan than thiophene.

The aromatization mechanisms to this point involved tautomerization and deprotonation to effect aromatization. Another approach is to design a molecule in which one of the double bonds is hydrohalogenated and can undergo elimination, the chemical equivalent of a double bond, such as the case with (±)-(1*S*,2*R*,5*S*)-5-amino-2-fluorocyclohex-3-enecarboxylic acid (**156**).[181] Inactivation of GABA-AT with **156** results in the release of one fluoride ion per active site and the formation of *N*-*m*-carboxyphenylpyridoxamine 5′-phosphate (**139**), the aromatization product of gabaculine (**136**, Scheme 32). A mechanism for inactivation is given in Scheme 40.

4. CONCLUSIONS

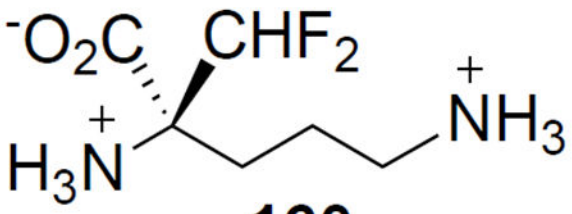
4.1. Mechanism-based Inactivation in Drug Discovery

The most common approach to drug discovery starts from a high-throughput screen to identify a hit, which is synthetically modified for enhanced binding and pharmacokinetic properties. In the mechanism-based inactivator approach, compound design stems from the catalytic mechanism of the target enzyme. With the application of physical organic chemical principles, new inactivators for a wide variety of enzymes can be designed, and standard methods for improving pharmacokinetic properties can be utilized once the inactivation chemistry is established. Mechanism-based inactivators also can be utilized to uncover new chemistry that enzymes are capable of catalyzing. The revealed new chemistry can then precipitate novel inactivator design ideas for innovative approaches to inactivate enzymes.

4.2. Selected Examples of How GABA-AT Inactivation Mechanisms Can Be Applied to Inactivation of Other Enzymes

The inactivation approaches discussed in this review can be incorporated into the design of a wide variety of molecules as mechanism-based inactivators of many other enzymes; a few examples are discussed here. The first mechanism-based inactivator for any enzyme, 3-decynoyl-*N*-acetylcysteamine (**157**), was reported in 1968,[182] although it was not known at the time that it was a mechanism-based inactivator. An inactivation mechanism for **157**, which inactivated β-hydroxydecanoyl thioester dehydrase, was reported two years later (Scheme 41). [183] α-Dideuterio-3-decynoyl-*N*-acetylcysteamine exhibited a kinetic isotope effect on inactivation of 2.6, whereas no isotope effect was observed with α-deuterio-2,3-decadienoyl-*N*-acetylcysteamine (**158**), although **158** also inactivated the enzyme. This suggested that β-hydroxydecanoyl thioester dehydrase catalyzed the isomerization of the acetylene of **157** to the allene in **158**, which underwent reaction with an active-site histidine residue (X = His in Scheme 41) to give covalent adduct **159**. Allene **158**, on the other hand, is not a mechanism-based inactivator, but, rather, is an affinity labeling agent because it does not require enzyme catalysis to activate it. The mechanism shown in Scheme 41 is equivalent to the mechanism for inactivation of GABA-AT by 4-aminohex-5-ynoic acid (**16**) in Scheme 5 (pathway b). The principal difference is that **157** already has an electron sink (the thioester carbonyl), whereas **16** needs to react first with PLP to make the electron sink (the PLP imine).

A carboxylate is chemically equivalent to a proton in the sense that decarboxylation leads to a carbanion as does deprotonation. Therefore, the approach of enzyme inactivation via a carbanion intermediate as it relates to GABA-AT can be incorporated into the design of α-amino acid inactivators as well. This is how α-difluoromethylornithine (**160**, eflornithine, DFMO) was designed to inactivate ornithine decarboxylase. The reaction catalyzed by the PLP-dependent enzyme ornithine decarboxylase is the conversion of ornithine (**161**) to putrescine (**163**, Scheme 42); putrescine is further converted to spermidine (**164**) and then to spermine (**165**), but ornithine decarboxylase is the rate-determining step in the polyamine biosynthetic pathway. These polyamines are regulators of cell growth, differentiation, and division and can enhance tumor cell and parasite growth; [184] Eflornithine is, in fact, a FDA-approved drug (Ornidyl) for the treatment of African sleeping sickness[185] and is in clinical trials for the treatment of relapsed or refractory neuroblastoma.[186] Eflornithine is a covalent inactivator of ornithine decarboxylase,[187] which appears to proceed by decarboxylation to **166**, elimination of both fluorine atoms, and attachment of either Lys 69 (the lysine originally bound to the PLP) or Cys360 or both (Scheme 43).[188] [1-^{14}C]-Eflornithine inactivated ornithine decarboxylase with concomitant release of $^{14}CO_2$; [5-^{14}C]-eflornithine led to inactivation with concomitant increase in radioactivity covalently bound to the enzyme.[189]



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The Principle of Microscopic Reversibility (Section 3.2) can be applied to the inactivation of ornithine decarboxylase because all steps are reversible except for the decarboxylation step. The last step in the ornithine decarboxylase mechanism is protonation of the stabilized carbanion of the putrescine-PLP complex (**162**, Scheme 42). On the basis of this principle, then, α-difluoromethylputrescine (**167**) also should inactivate ornithine decarboxylase, in which the first step (after Schiff base formation with the PLP) is deprotonation to give the same stabilized carbanionic intermediate (**166**, Scheme 44) that was obtained by decarboxylation of PLP-bound eflornithine (Scheme 43). In fact, this was shown to be the case.[190] These compounds are equivalent in inactivation mechanism to difluoromethylGABA (**115**), an inactivator of GABA-AT.

A final example of applications of a GABA-AT inactivator to other enzymes is the class of α-(1'-fluoro)vinyl amino acids;[191] depending on the amino acid scaffold used, these compounds could inactivate a variety of amino acid decarboxylases. The example reported was the inactivation of lysine decarboxylase, a PLP-dependent enzyme that catalyzes the decarboxylation of lysine to cadaverine (1,5-diaminopentane) by α-(1'-fluoro)vinyllysine (**168**). No mechanistic studies were carried out beyond demonstrating that **168** is an active-site irreversible inhibitor of lysine decarboxylase. Several mechanisms were proposed, including one similar to that for GABA-AT inactivator **76 (**Scheme 19); another mechanism proposed for **168** is shown in Scheme 45.

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Biography

Richard B. Silverman received his B.S. degree in chemistry from The Pennsylvania State University and his A.M. and Ph.D. degrees from Harvard University with David Dolphin in organic chemistry. Following postdoctoral studies with Robert Abeles in the Graduate Department of Biochemistry at Brandeis University, he started his academic career in the Department of Chemistry at Northwestern University in 1976, where he is currently the Patrick G. Ryan/Aon Professor in the Departments of Chemistry and Molecular Biosciences. He is the inventor of Lyrica™, marketed by Pfizer for fibromyalgia, neuropathic pains, epilepsy, and anxiety. A GABA-AT inactivator from the Silverman group has completed a Phase I clinical trial, and three other compounds are in advanced preclinical development. An author of >360 publications and 91 patents, he is a Fellow of the American Academy

of Arts and Sciences, the National Academy of Inventors, the American Chemical Society, the Royal Society of Chemistry, the American Association for the Advancement of Science, and the American Institute of Chemists. He has been recognized with the Creative Invention Award (ACS), iCON Innovator Award (iBIO), Medicinal Chemistry Prize (Israel Chemical Society), Centenary Prize (RSC), Bristol-Myers Squibb-Edward Smissman Award (ACS), Sato Memorial International Award (Pharmaceutical Society of Japan), E.B. Hershberg Award (ACS), Perkin Medal (Society of Chemical Industry), Arthur C. Cope Senior Scholar Award (ACS), and was inducted into the Medicinal Chemistry Hall of Fame (ACS).

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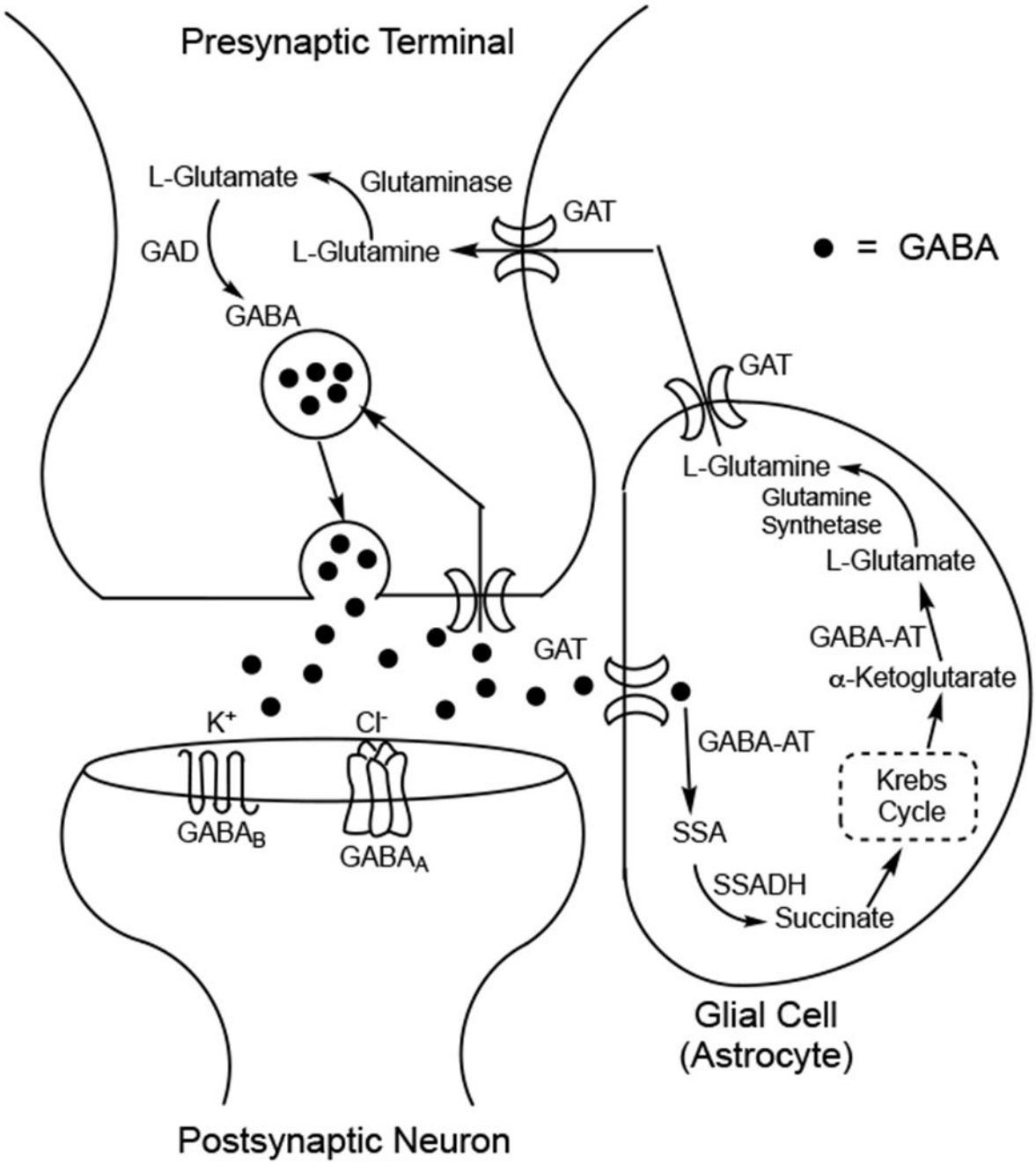


Figure 1.

GABAergic Synapse. Catabolism of GABA. GAD is glutamic acid decarboxylase; GAT is a GABA transporter; GABA-AT is GABA aminotransferase; SSADH is succinic semialdehyde dehydrogenase

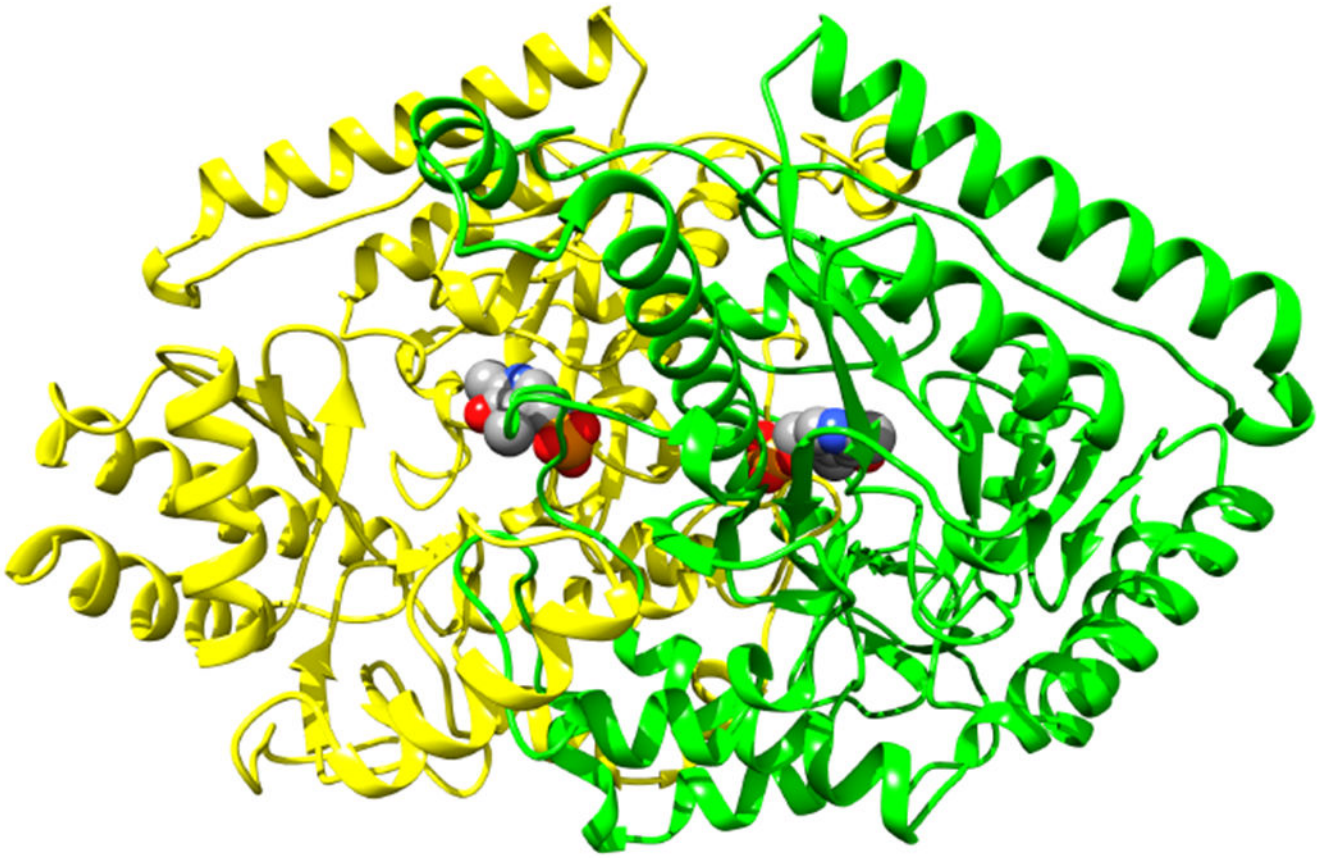


Figure 2.

Crystal structure of dimeric GABA-AT from pig liver (PDB code: 1OHV). The PLP coenzymes from each subunit are shown in gold (carbon), blue (nitrogen), red (oxygen), and orange (phosphorus). Figure was constructed from the PDB code with permission from Reference 22. Copyright 2004 The American Society of Biochemistry and Molecular Biology, Inc.

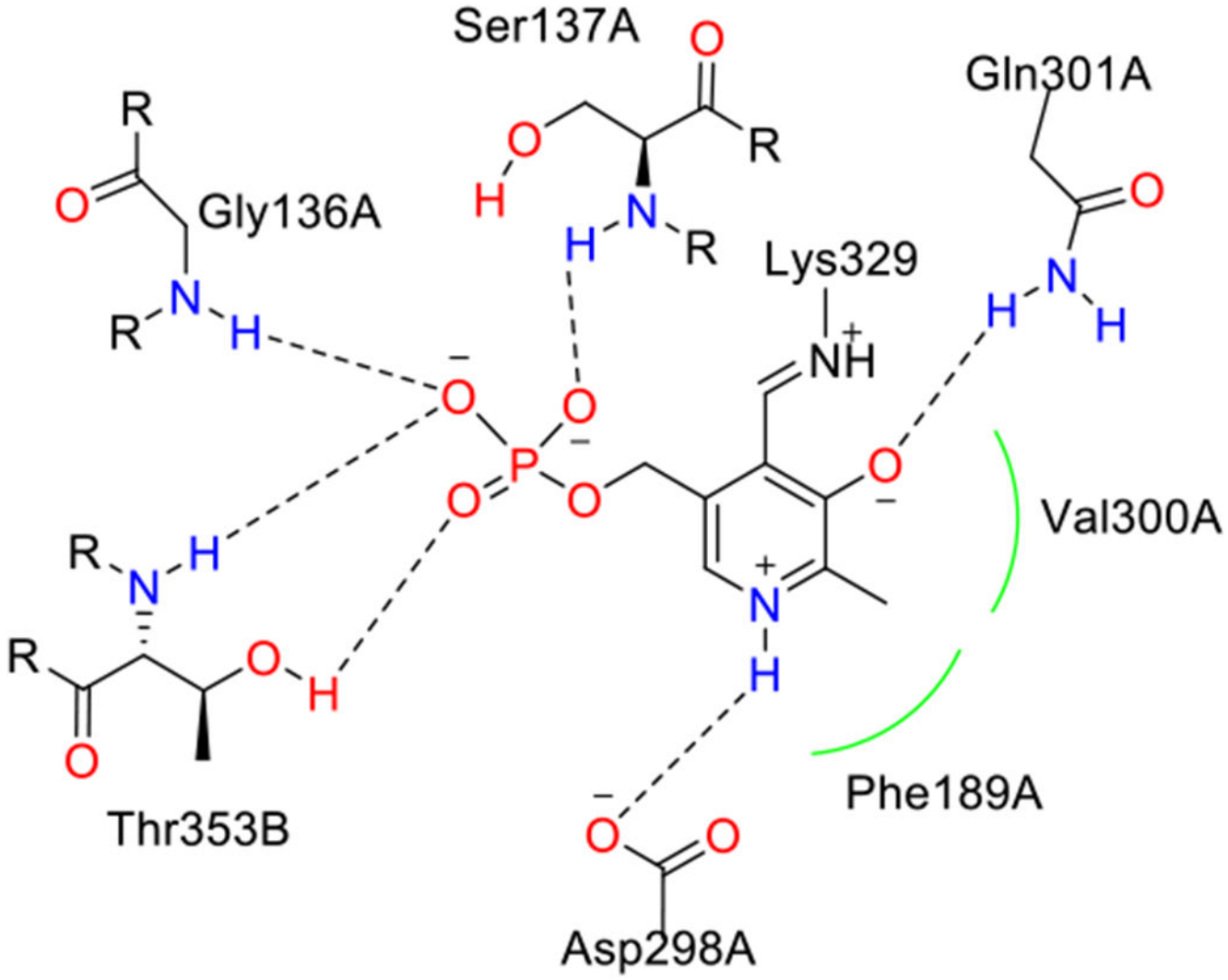


Figure 3.
Active site of GABA-AT. Shown are the residues that interact with PLP. Green lines show hydrophobic interactions. Dashed lines indicate hydrogen bonds. The A and B letters indicate the two subunits of GABA-AT.

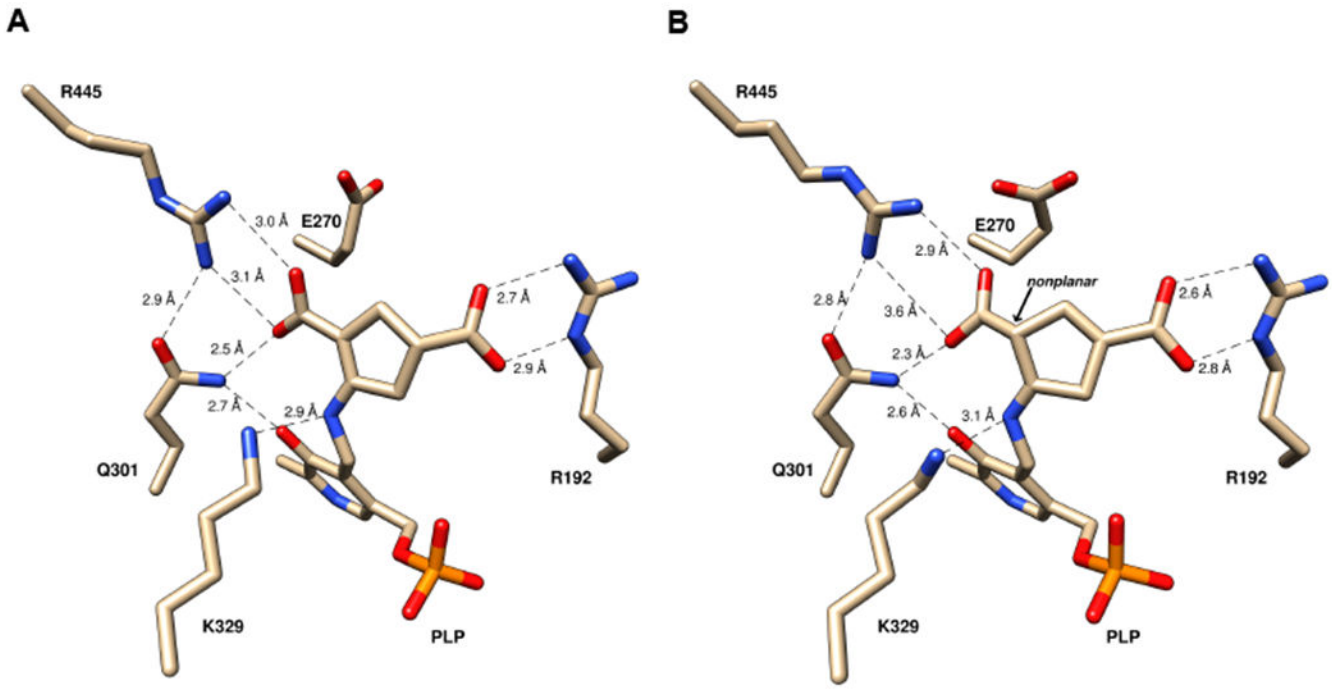


Figure 4.

X-ray crystal structure of GABA-AT inactivated by (A) (1*S*,3*S*)-3-amino-4-difluoromethylenyl-1-cyclopentanoic acid (**42**) (PDB code: 4Y0D) and (B) (*S*)-3-amino-4-(difluoromethylenyl)cyclopent-1-ene-1-carboxylic acid (**48**) (PDB code: 6B6G). Carbon is in gold, nitrogen in blue, oxygen in red, and phosphorus in orange. Figure 4A was adapted with permission from Reference 81. Copyright 2015 American Chemical Society. Figure 4B was adapted with permission from Reference 82. Copyright 2018 American Chemical Society.

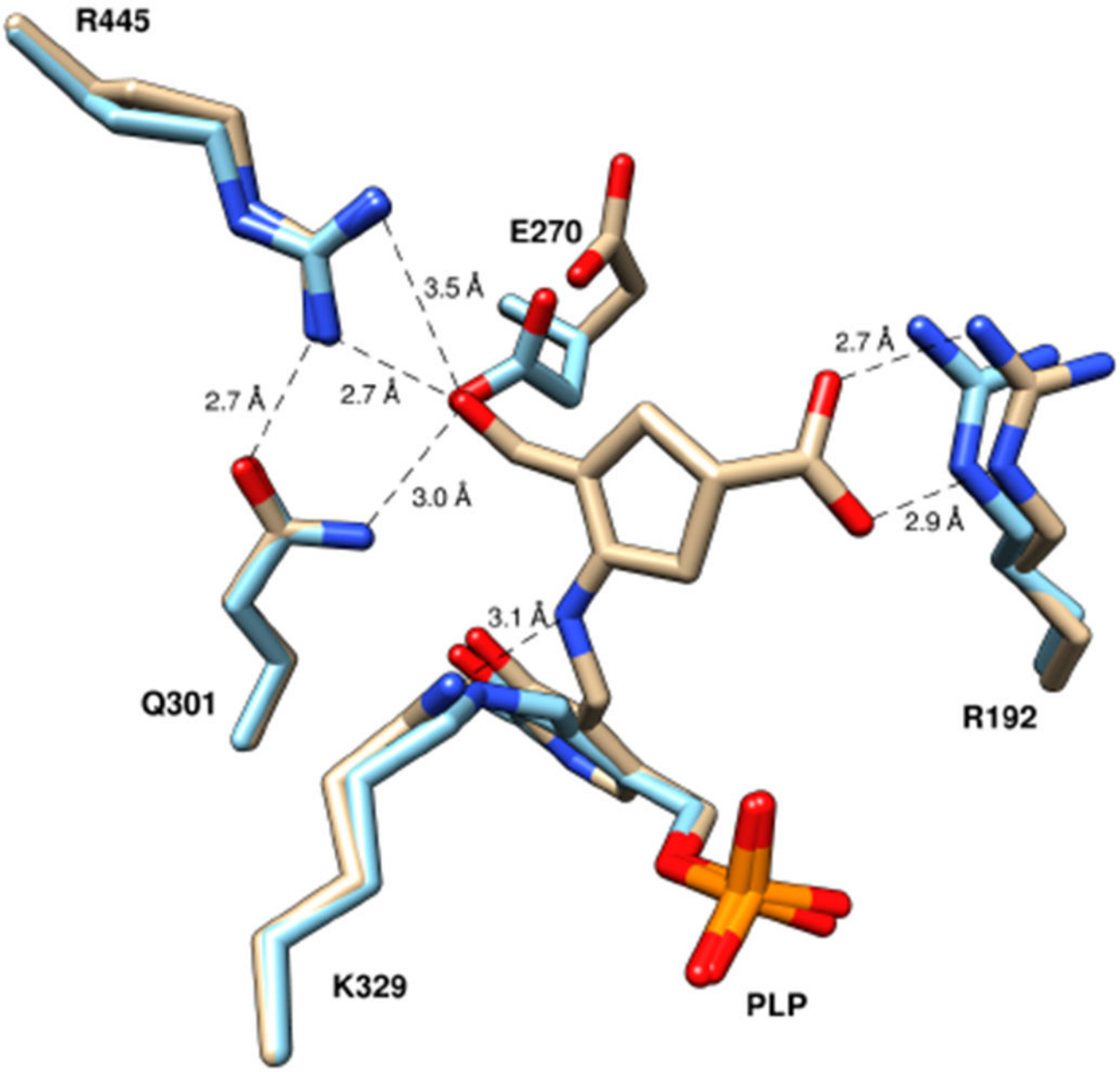


Figure 5.

Superimposition of the X-ray crystal structures of **51**-inactivated GABA-AT (carbon in gold, nitrogen in blue, oxygen in red, and phosphorus in orange) and native GABA-AT (carbon in cyan) monomers (**52** gave similar structures) (PDB code: 4ZSW). Adapted with permission from Reference 89. Copyright 2015 American Chemical Society.

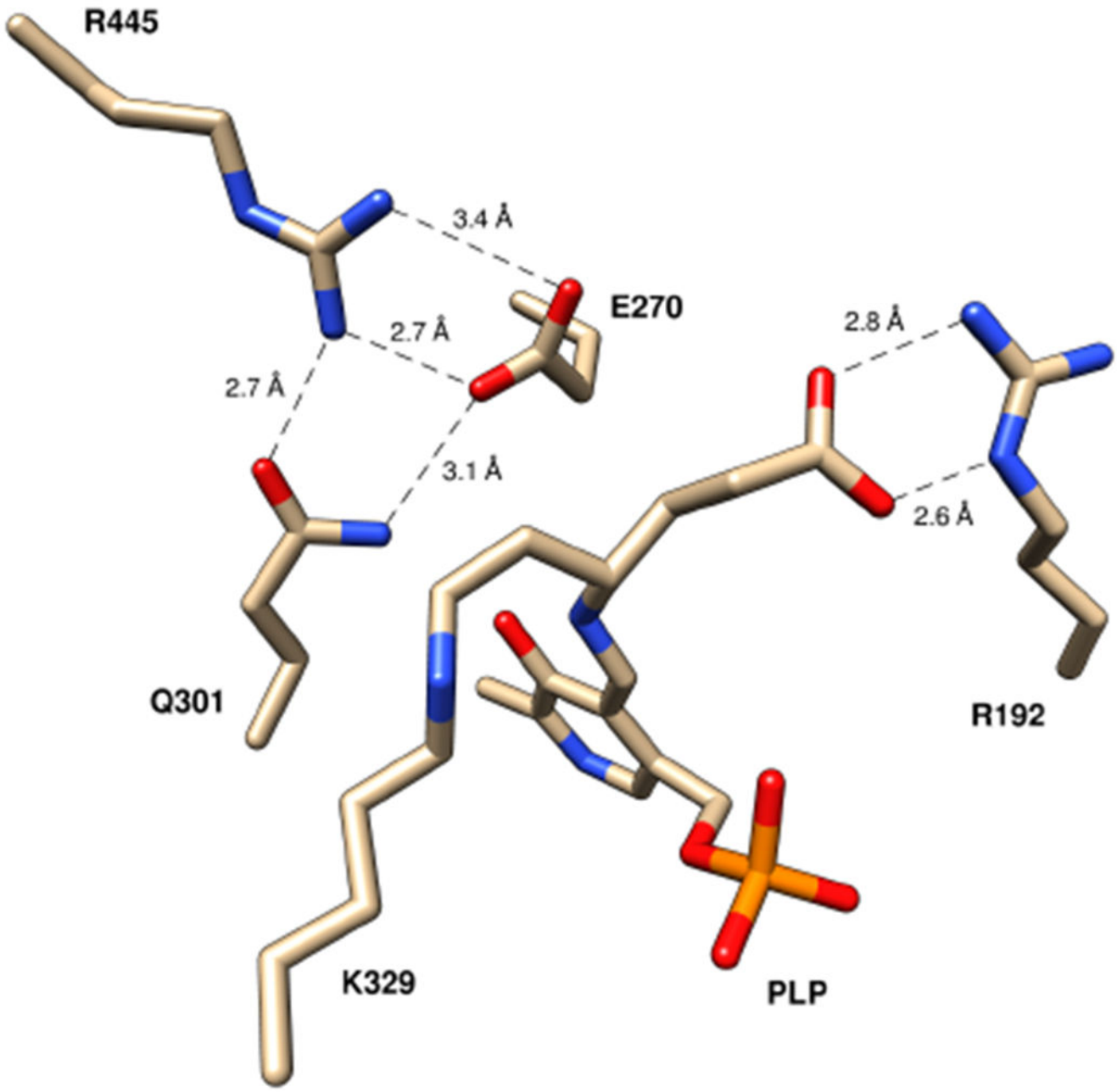


Figure 6.

Crystal structure at 2.3 Å resolution of vigabatrin (**62**) bound to GABA-AT (carbon in gold, nitrogen in blue, oxygen in red, and phosphorus in orange; PDB code: 1OHW). Adapted from the PDB code with permission from Reference 66. Copyright 2004 The American Society of Biochemistry and Molecular Biology, Inc.

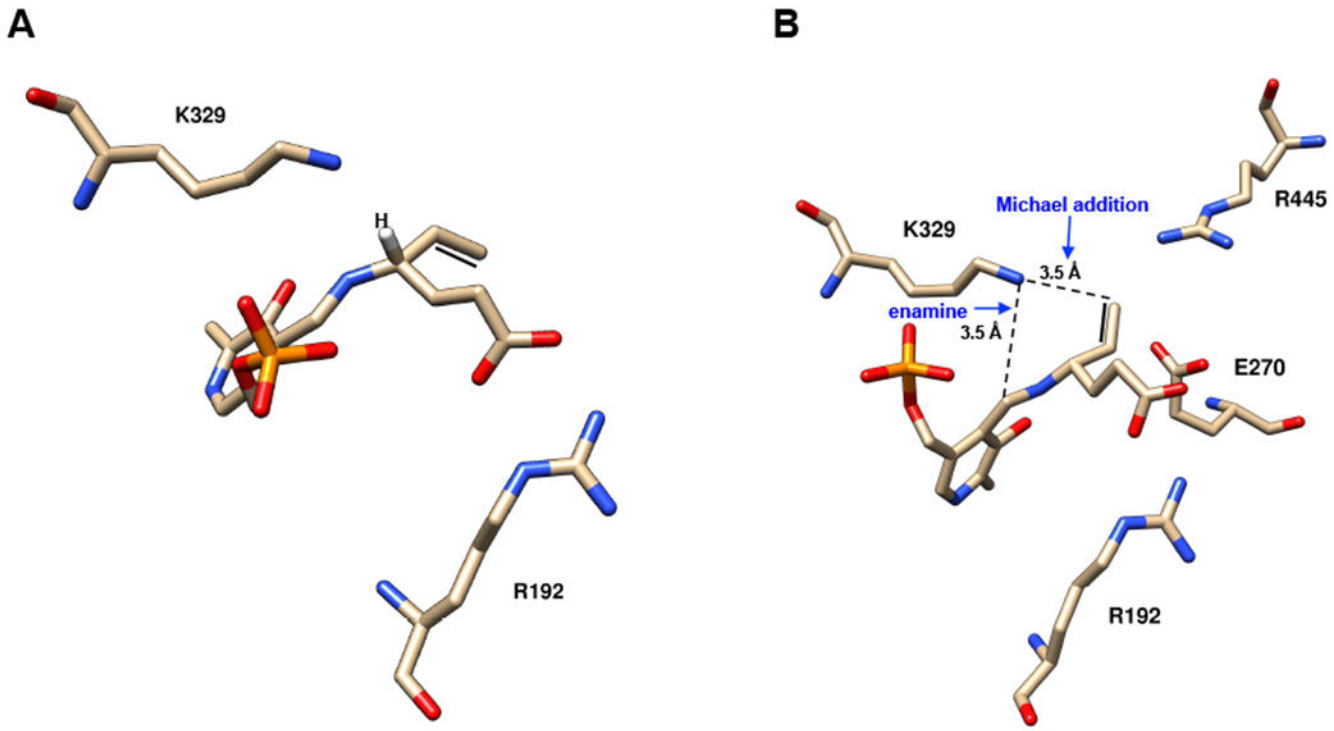


Figure 7.

(A) Energy minimized molecular model of PLP-bound vigabatrin (**62**) in GABA-AT. Only two relevant residues are shown. (B) Molecular model of PLP-bound **62** with vinyl group rotated 180°. Adapted with permission from Reference 130. Copyright 2002 American Chemical Society.

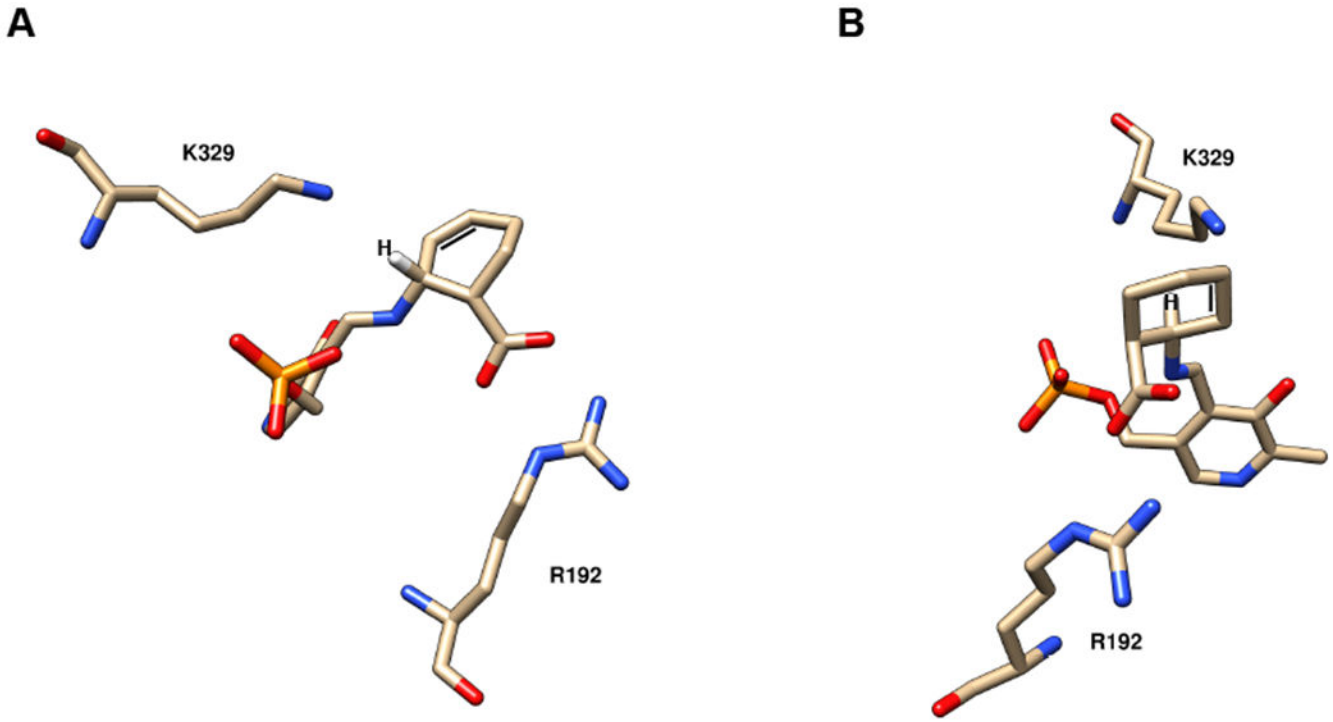


Figure 8.

Molecular model of two low-energy conformations of **108** with alkene faced away from Lys329 (A) and where Lys329 can undergo nucleophilic attack on the alkene (B). Adapted with permission from Reference 130. Copyright 2002 American Chemical Society.

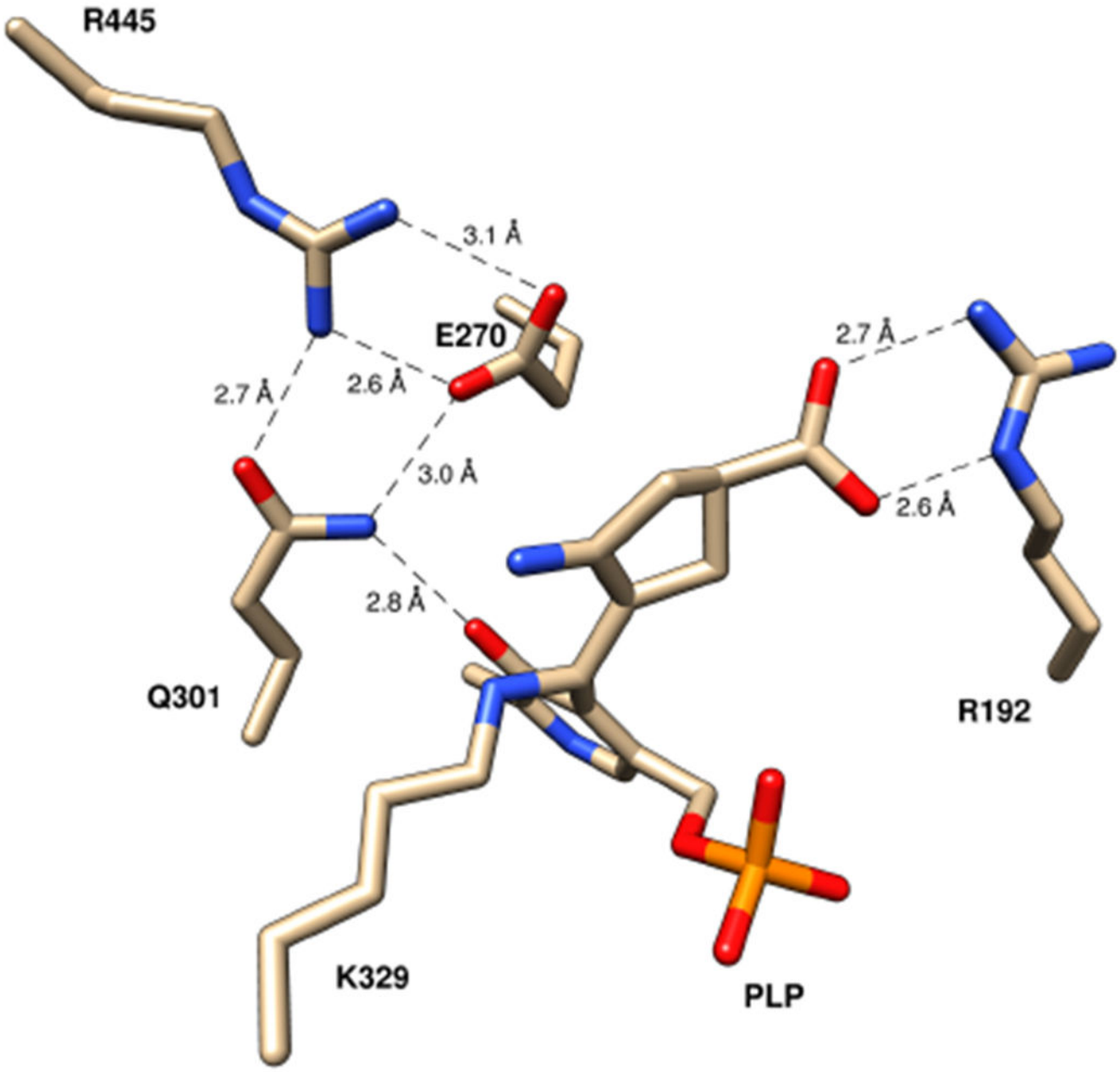


Figure 9.

GABA-AT inactivated by **123** (X = F, Y = H) with a $2F_o$-F_c map (contour level 1.2 σ). No PDB code was deposited, but we obtained coordinates from the authors; a similar structure was obtained for the inactivation of ornithine aminotransferase by **123** (PDB code: SVWO).[146] Carbon is in gold, nitrogen in blue, oxygen in red, and phosphorus in orange. Adapted with permission from Reference 147. Copyright 2004 American Chemical Society.

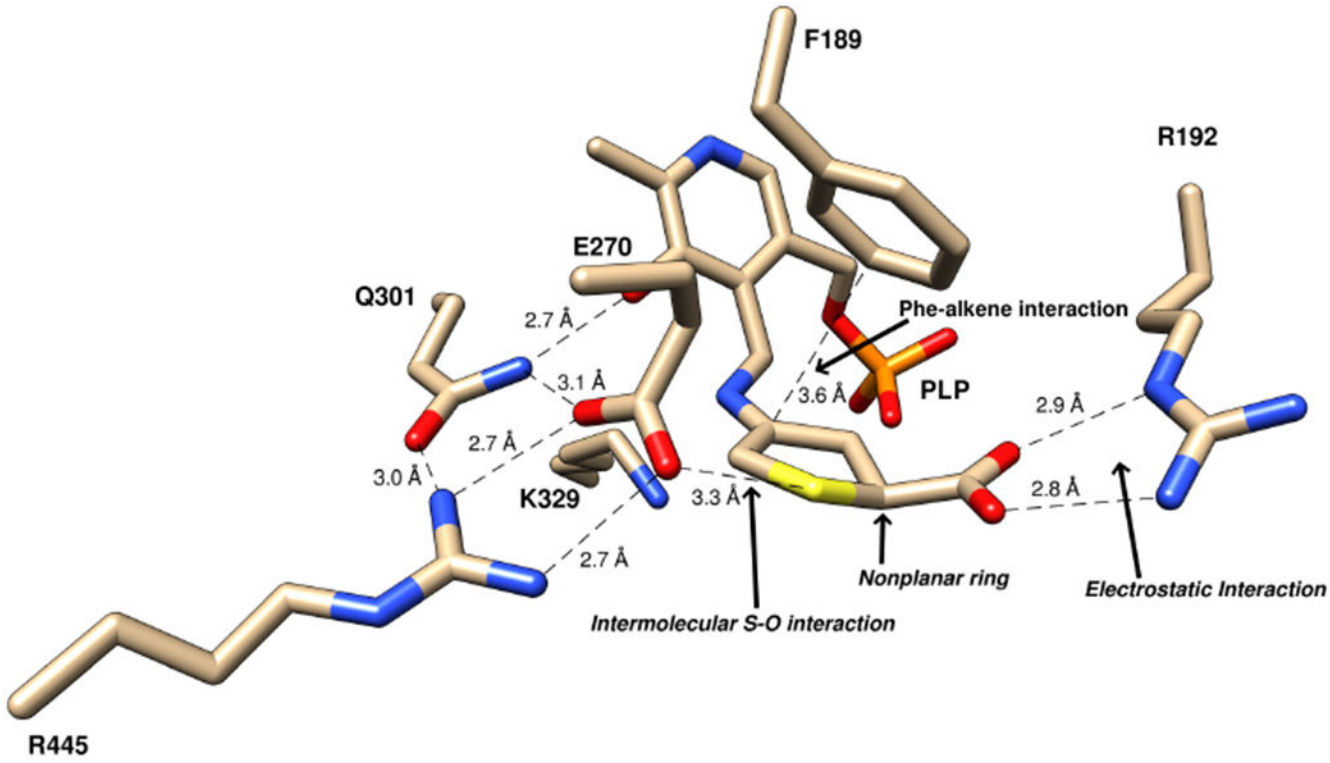
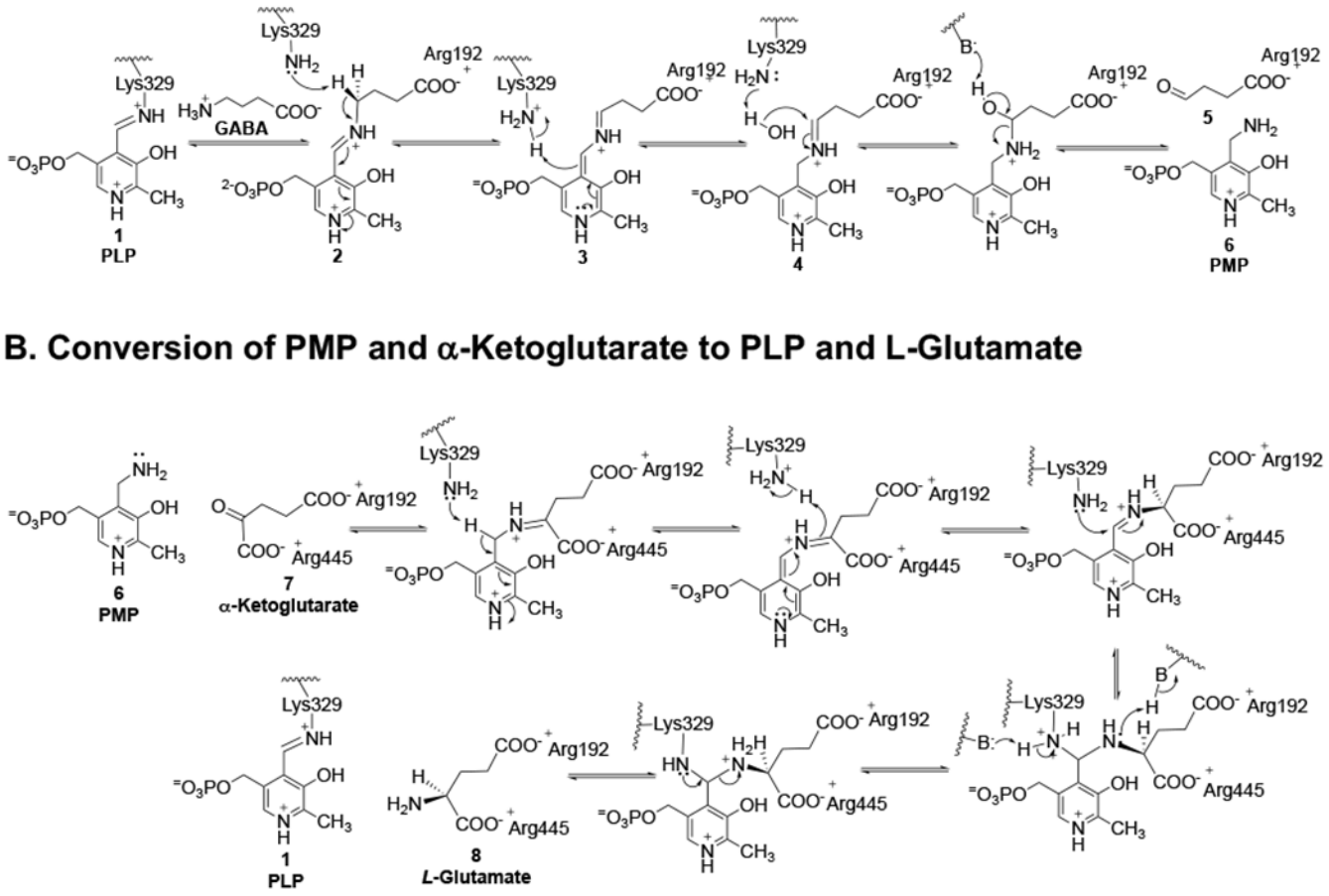
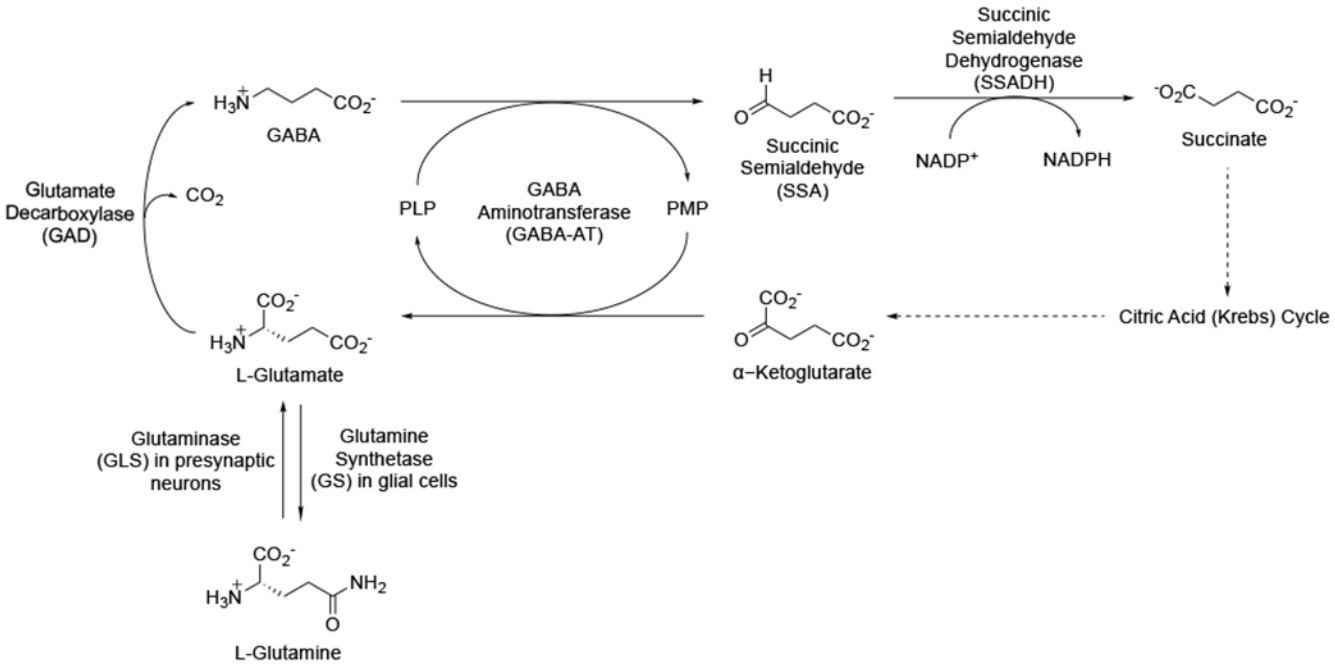


Figure 10.

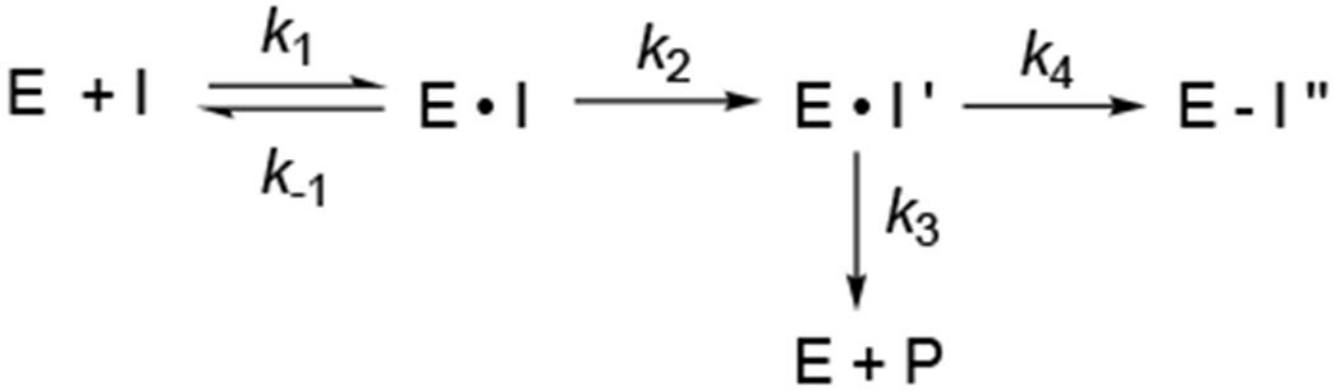
X-ray crystal structure of GABA-AT inactivated by (2*S*,4*S*)-4-aminotetrahydrothiophene-2-carboxylic acid hydrochloride (**130**) (PDB code: 4Y0I). Interactions that are not hydrogen bonds are indicated by arrows. Carbon is in gold, nitrogen in blue, oxygen in red, sulfur in yellow, and phosphorus in orange. Adapted with permission from Reference 153. Copyright 2015 American Chemical Society.



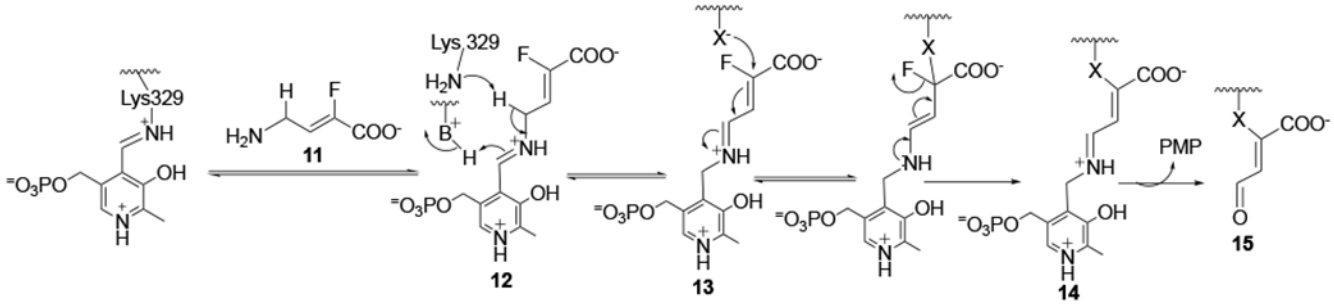
Scheme 1.
Two Half Reactions Catalyzed by GABA-AT



Scheme 2.
GABA Shunt

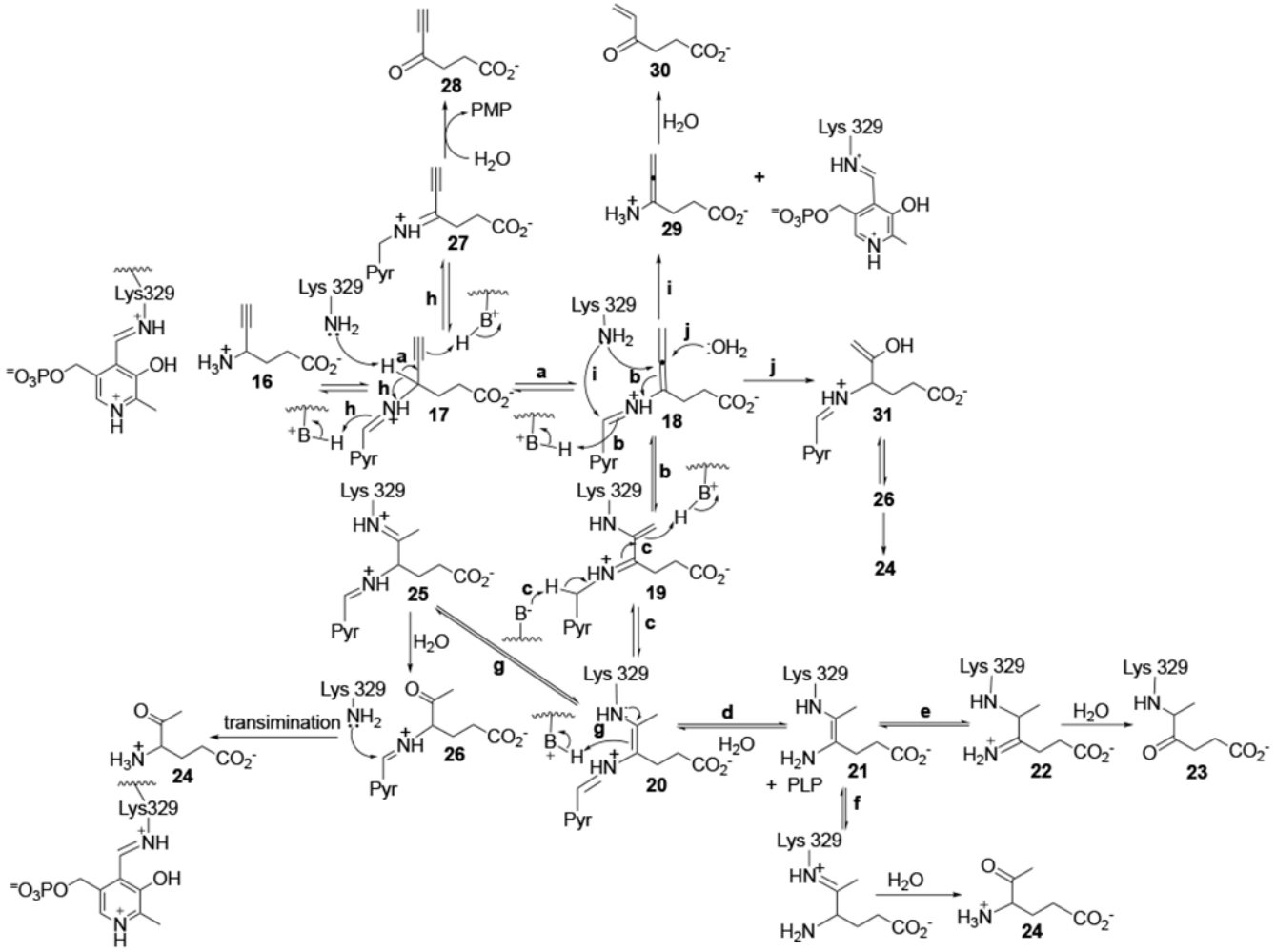


Scheme 3.
Simplified kinetic scheme for mechanism-based enzyme inactivation



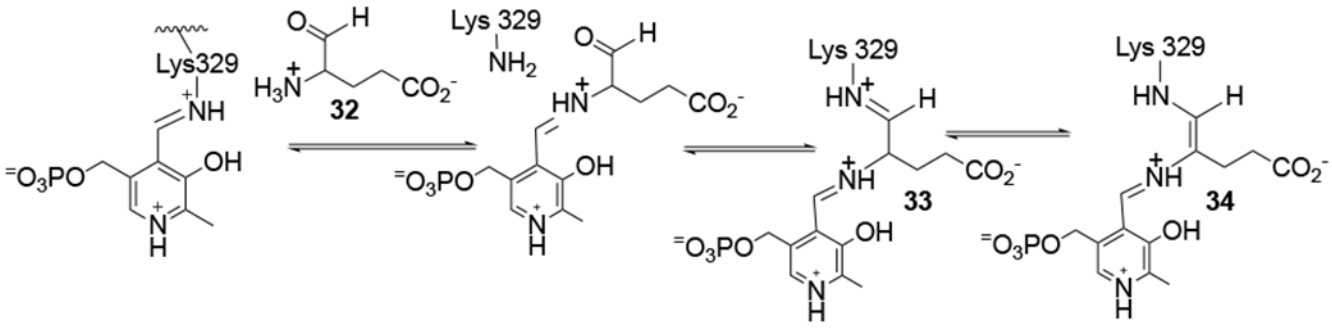
Scheme 4.

Mechanism of inactivation of GABA-AT by (*Z*)-4-amino-2-fluorobut-2-enoic acid (**11**)



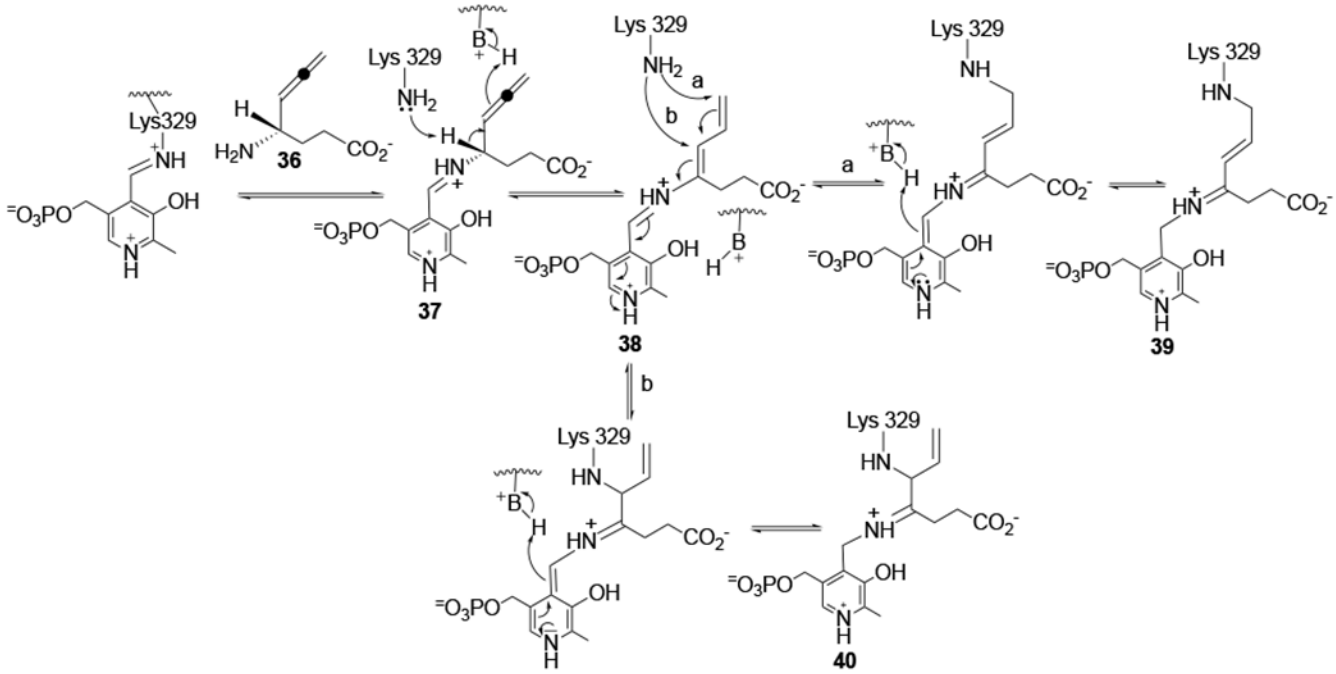
Scheme 5.

Mechanisms of inactivation of GABA-AT by 4-aminohex-5-ynoic acid (**16**). Pyr stands for the pyridine ring of PLP or PMP. Because of the complexity of this scheme not all of the tautomerization mechanisms are drawn out.

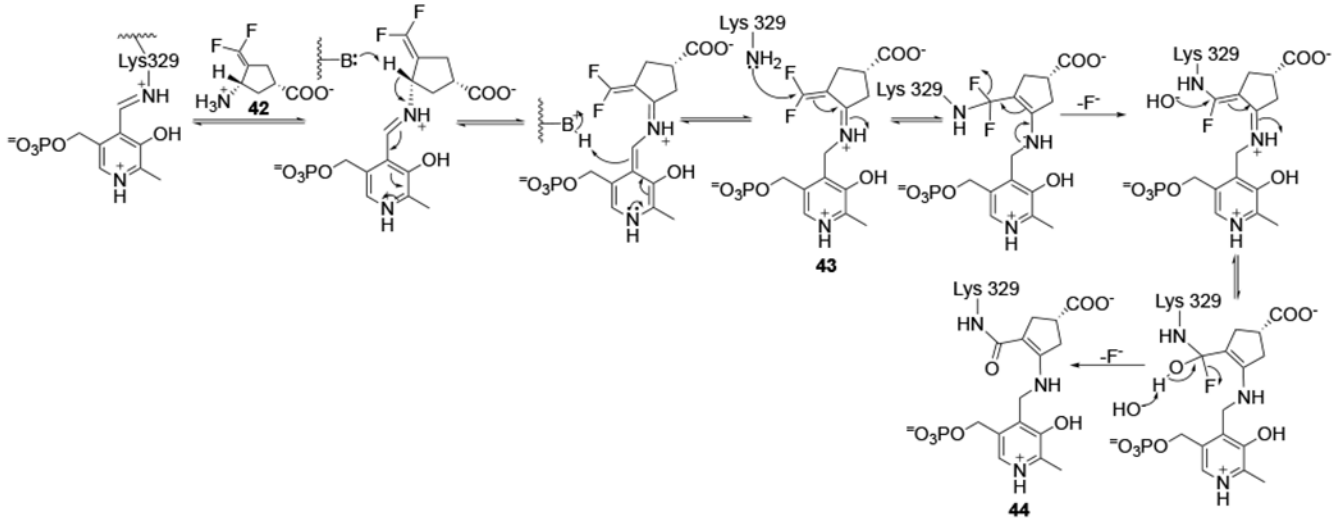


Scheme 6.

Hypothetical mechanism of inactivation of GABA-AT by 4-amino-5-oxopentanoic acid (**32**)

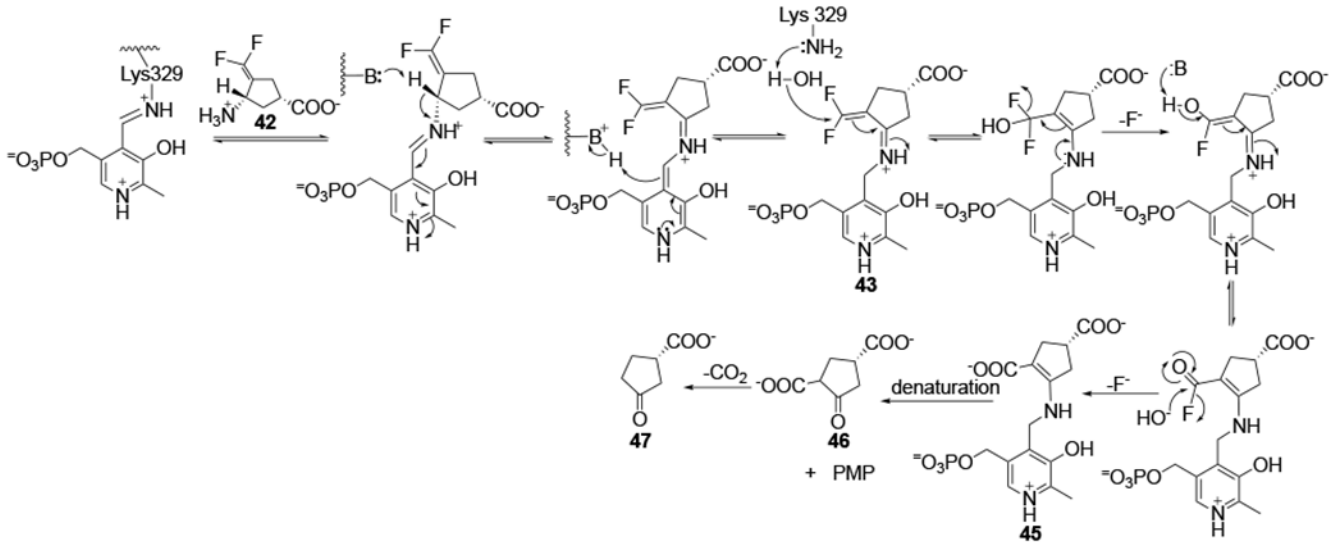


Scheme 7.
Potential mechanisms of inactivation of GABA-AT by **36**



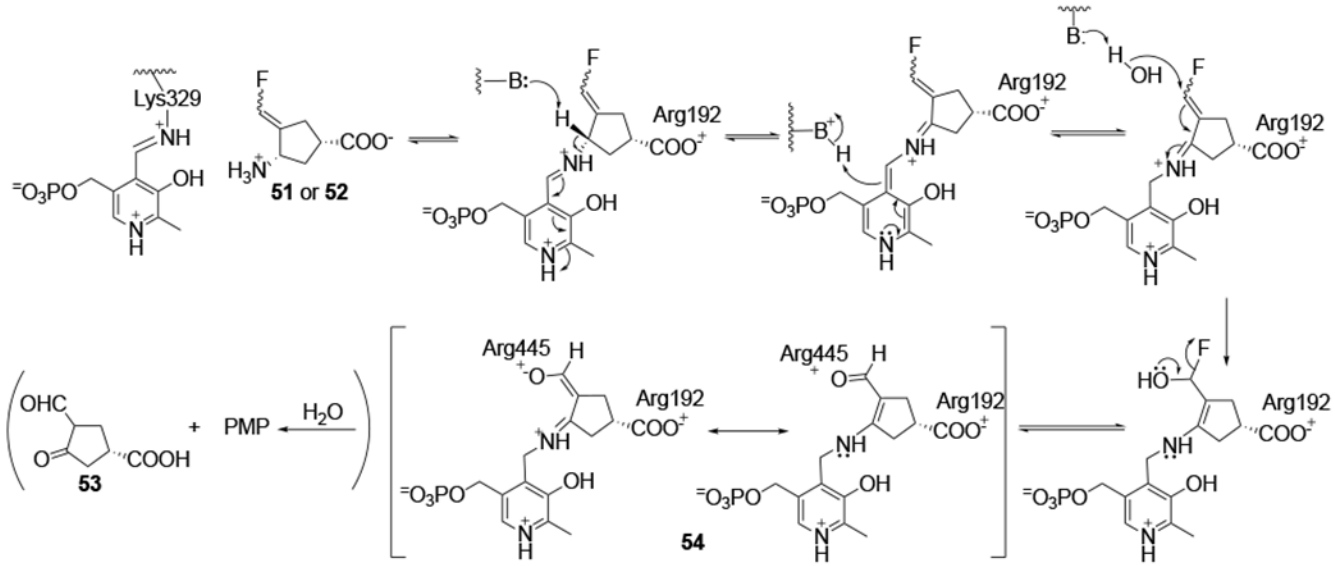
Scheme 8.

Hypothetical Michael addition inactivation mechanism for (1*S*,3*S*)-3-amino-4-difluoromethylenyl-1-cydopentanoic acid (**42**)



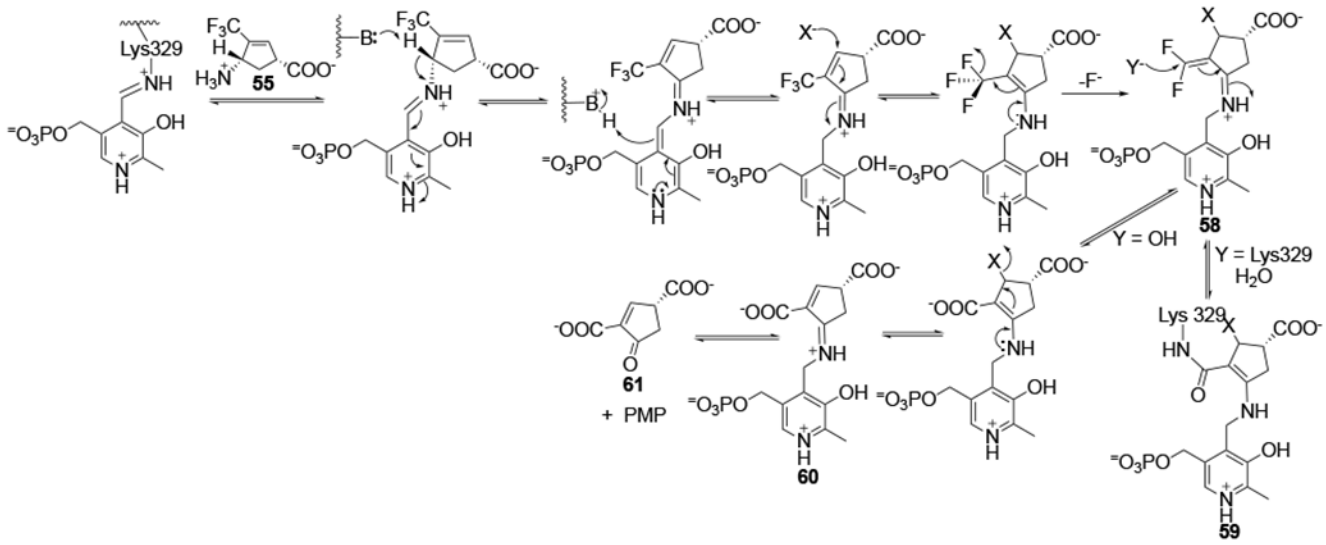
Scheme 9.

Mechanism for GABA-AT catalyzed hydrolysis of (1*S*,3*S*)-3-amino-4-difluoromethylenyl-1-cyclopentanoic acid (**42**)



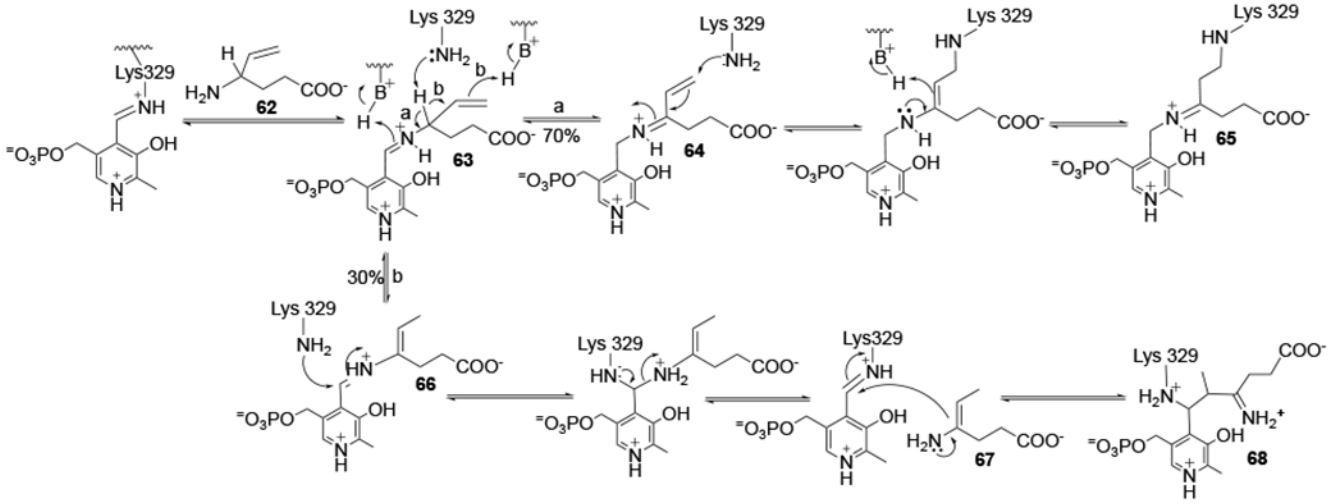
Scheme 10.

Mechanism of inactivation of GABA-AT by (*E*)-(**51**)- or (*Z*)-(1*S*,3*S*)-3-amino-4-fluoromethylenyl-1-cyclopentanoic acid (**52**)



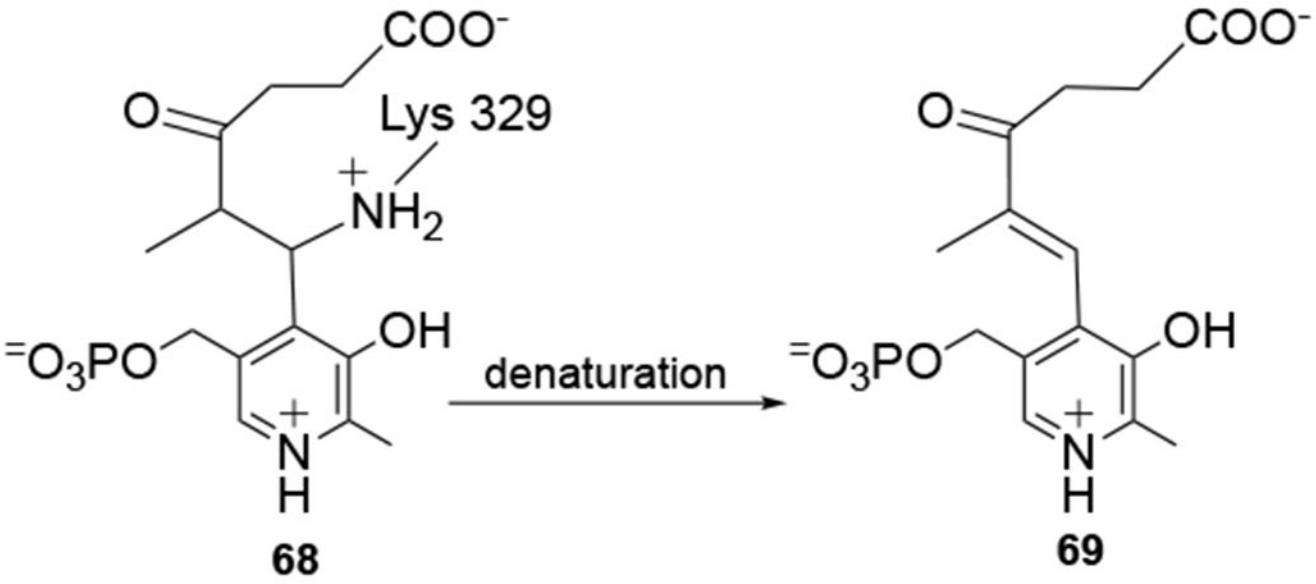
Scheme 11.

Hypothetical inactivation and turnover mechanisms for (1*R*,4*S*)-4-amino-3-trifluoromethylcyclopent-2-enecarboxylic acid (**55**)

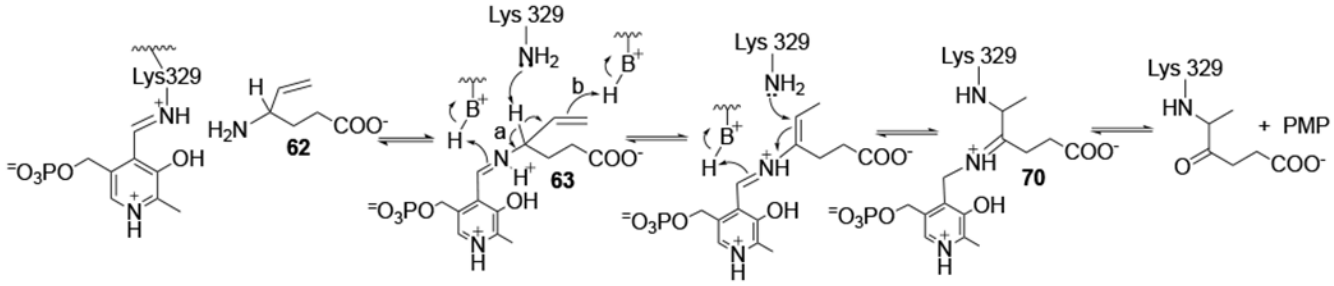


Scheme 12.

Mechanisms of inactivation of GABA-AT by 4-aminohex-5-enoic acid (**62**)

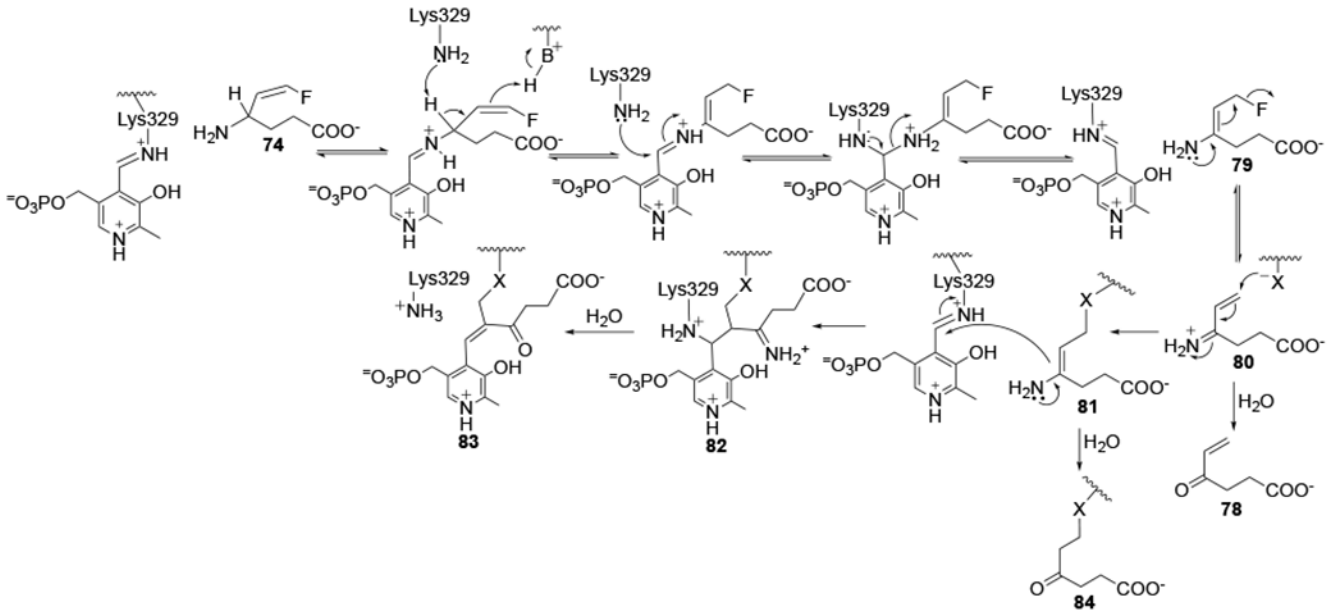


Scheme 13.
Evidence for the enamine pathway (Scheme 12, pathway b)



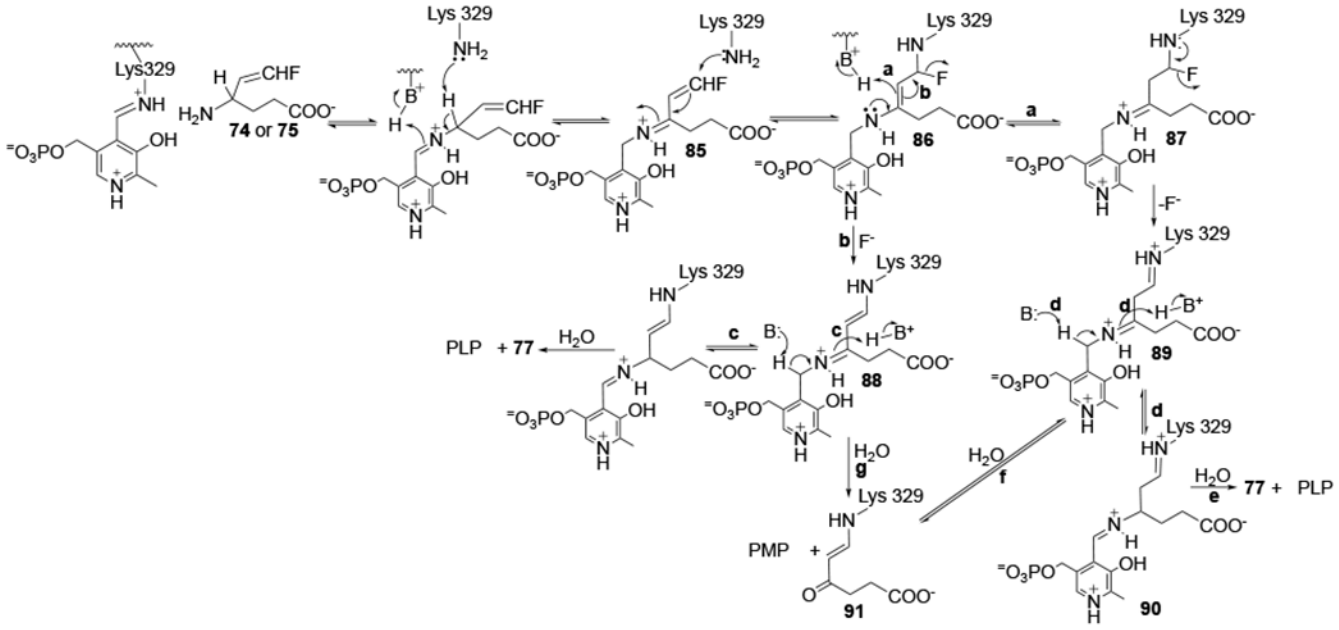
Scheme 14.

Incorrect mechanism of inactivation of GABA-AT by vigabatrin (**62**)[96]

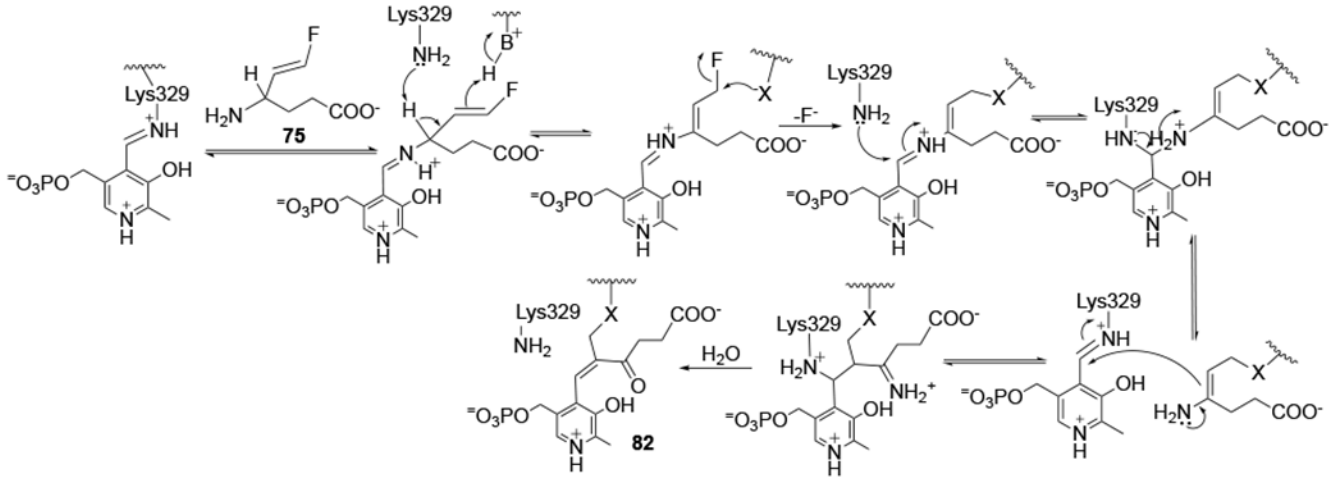


Scheme 15.

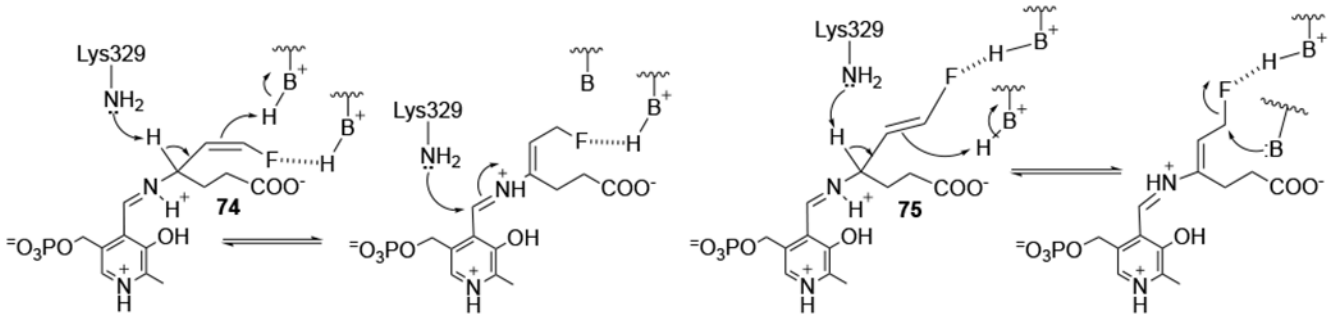
Major pathways for inactivation and metabolite formation by **74**



Scheme 16.
Minor inactivation pathways for **74**

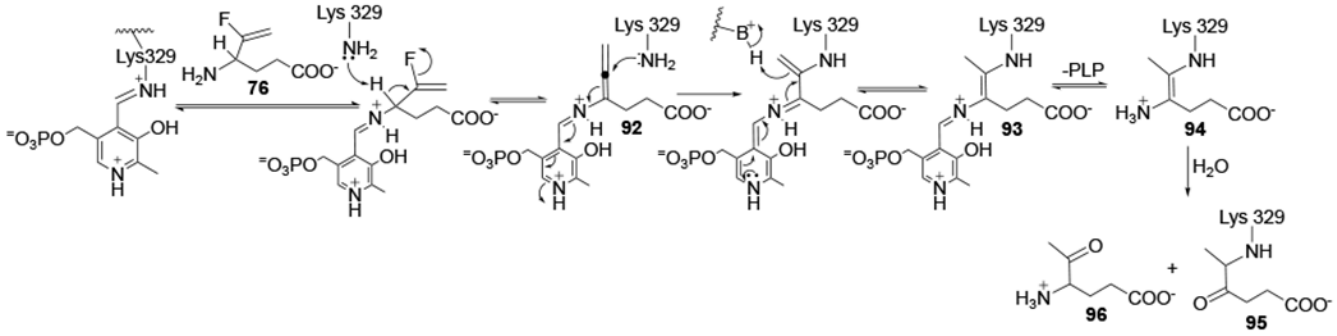


Scheme 17.
Inactivation mechanism for **75**

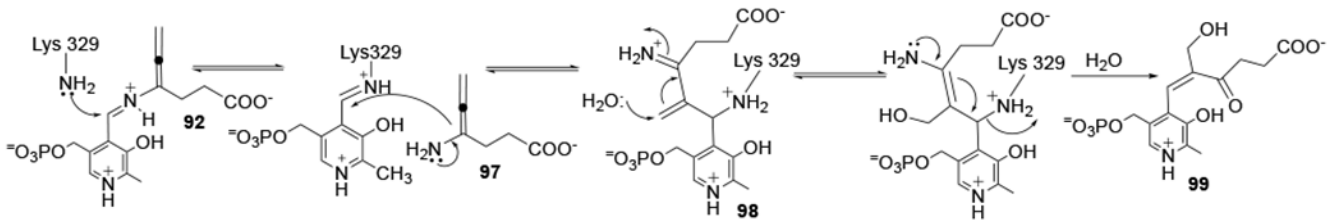


Scheme 18.

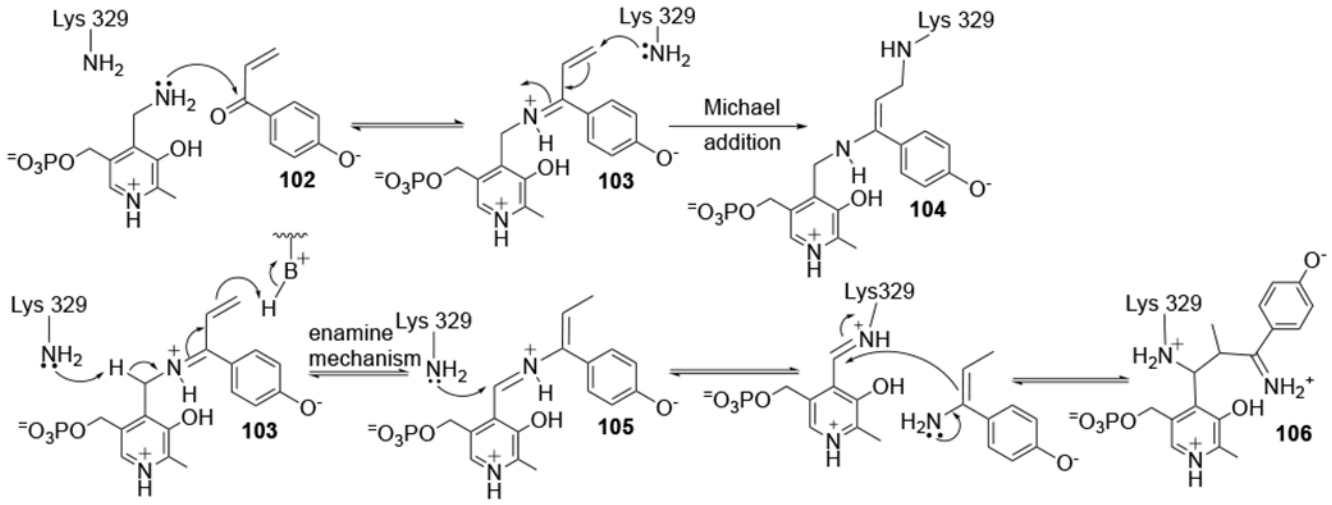
Rationalization for the different inactivation mechanisms of **74** and **75**



Scheme 19.
Inactivation mechanism 1 for **76**

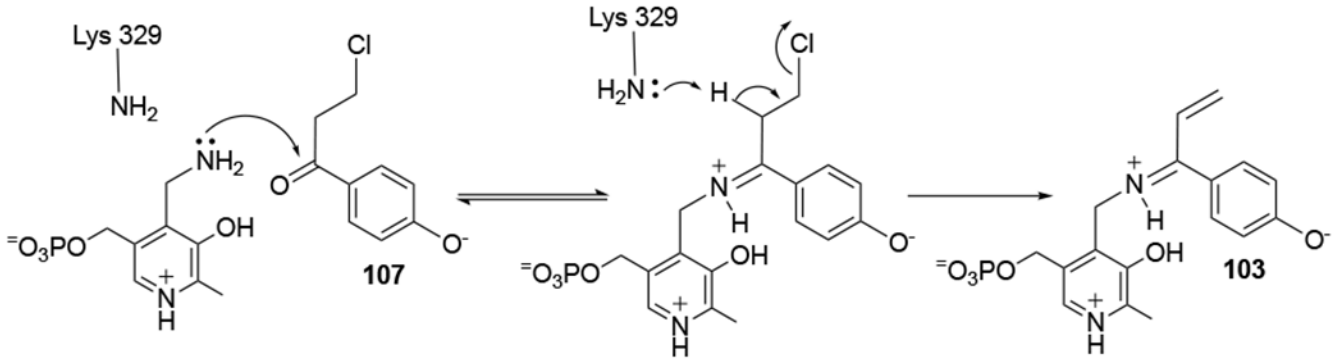


Scheme 20.
Third inactivation mechanism for **76**



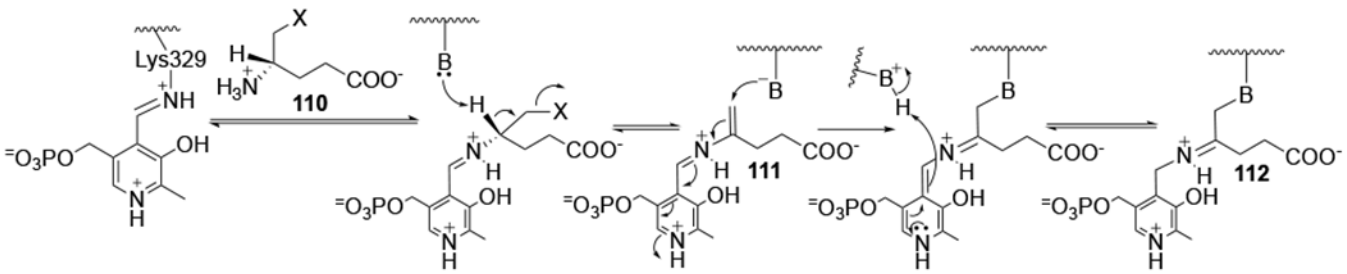
Scheme 21.

Proposed mechanisms of inactivation of GABA-AT by 4-acryloylphenol



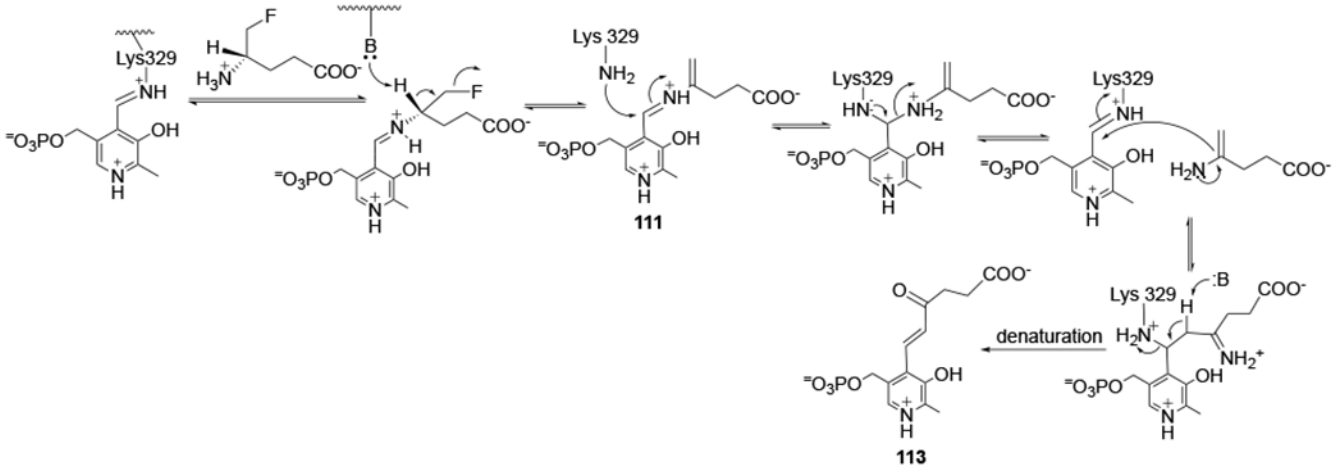
Scheme 22.

Proposed inactivation mechanism for 3-chloro-1-(4-hydroxyphenyl)propan-1-one (**107**)



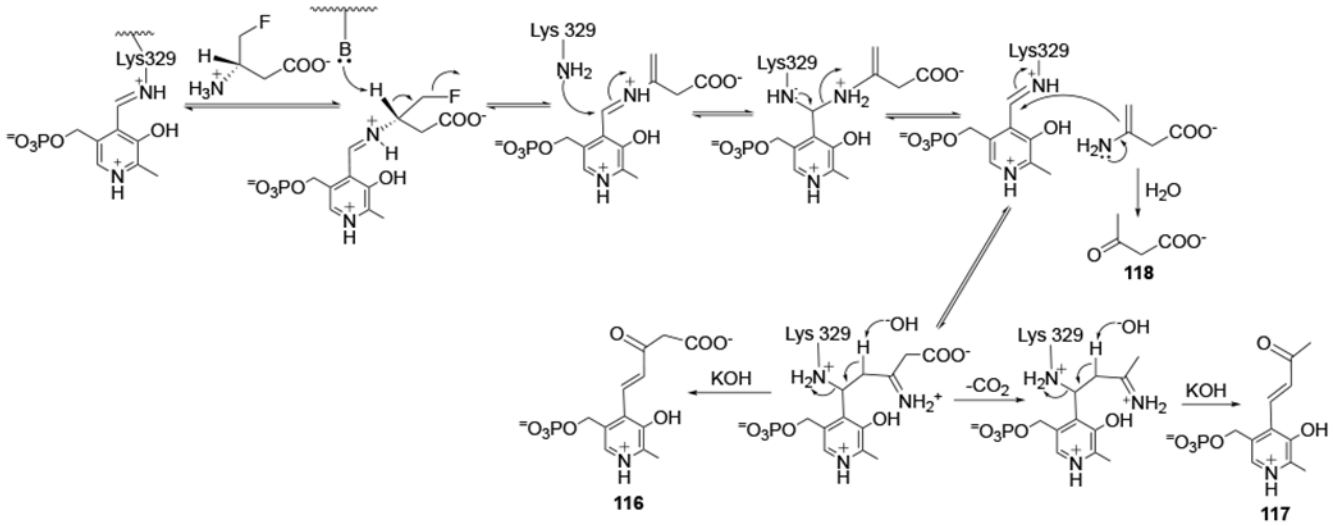
Scheme 23.

Initially proposed inactivation mechanism for (*S*)-4-amino-5-pentanoic acids



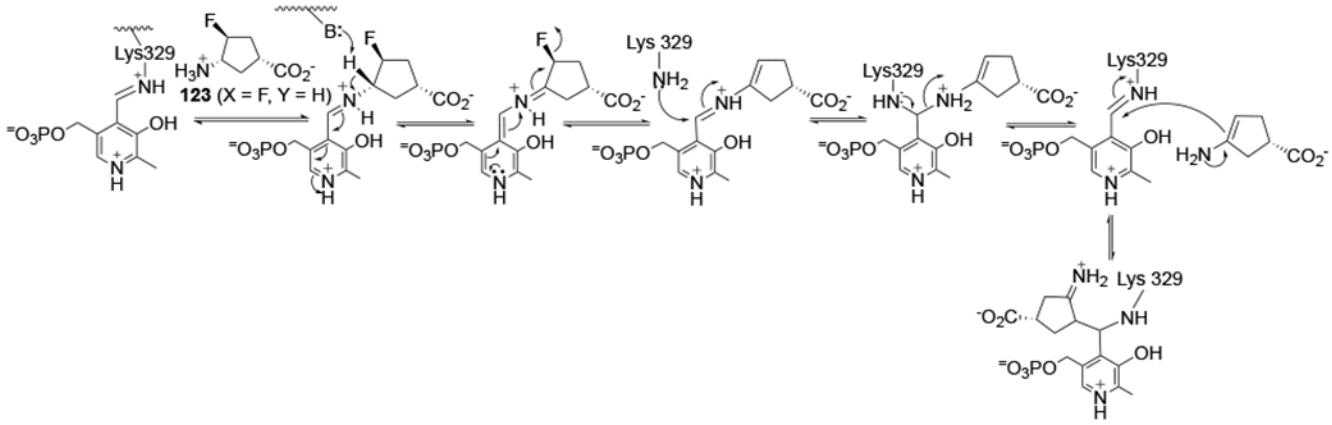
Scheme 24.

Enamine inactivation mechanism for 4-amino-5-halopentanoic acids



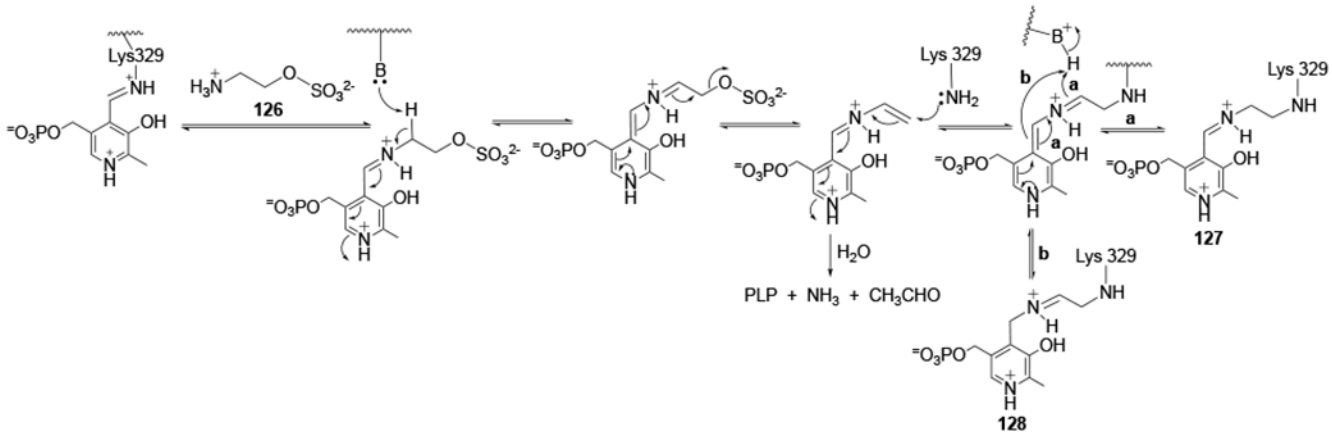
Scheme 25.

Enamine mechanism for inactivation of GABA-AT by **114** (n = 1)



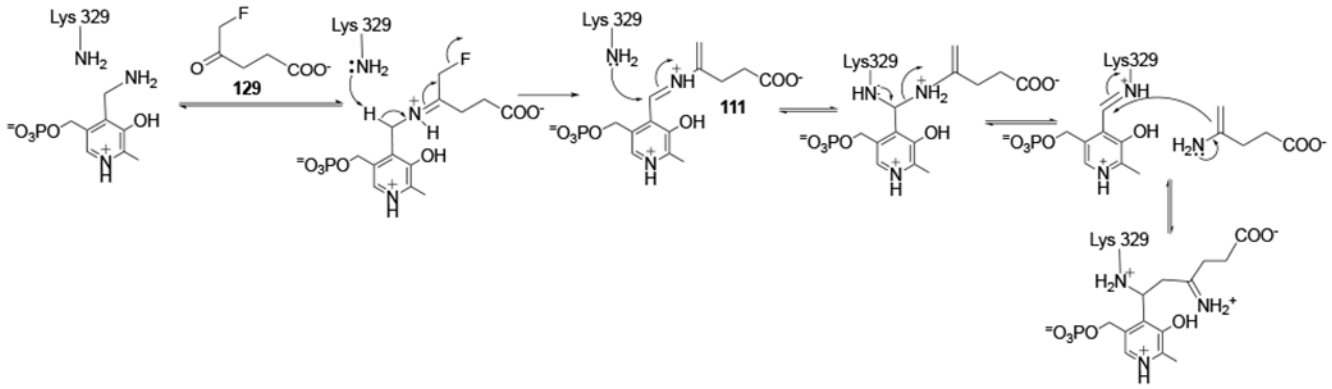
Scheme 26.

Enamine inactivation mechanism for **123** (X = F, Y = H)



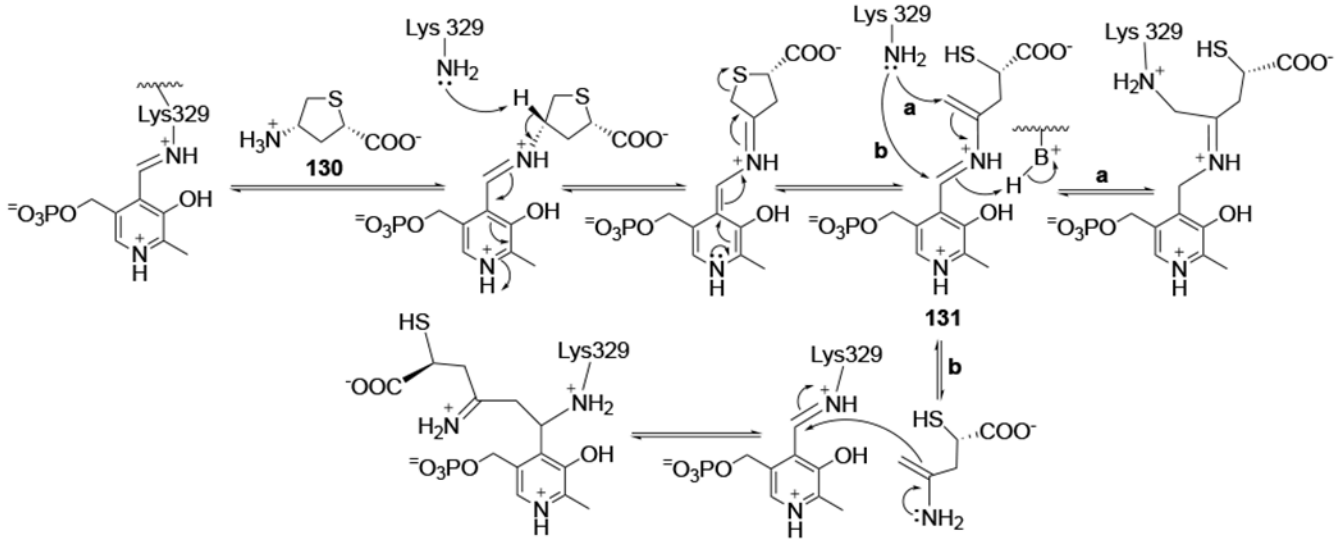
Scheme 27.

Proposed inactivation mechanism for ethanolamine *O*-sulfate (**126**)



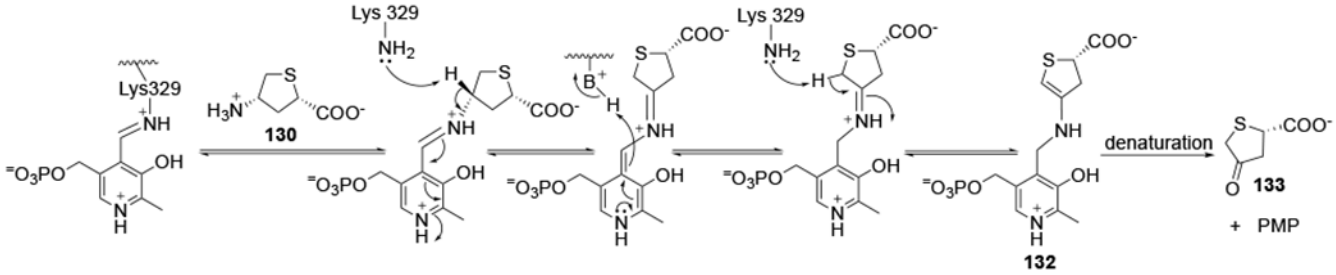
Scheme 28.

Potential mechanism of inactivation of GABA-AT by 5-fluorolevulinate (**129**)



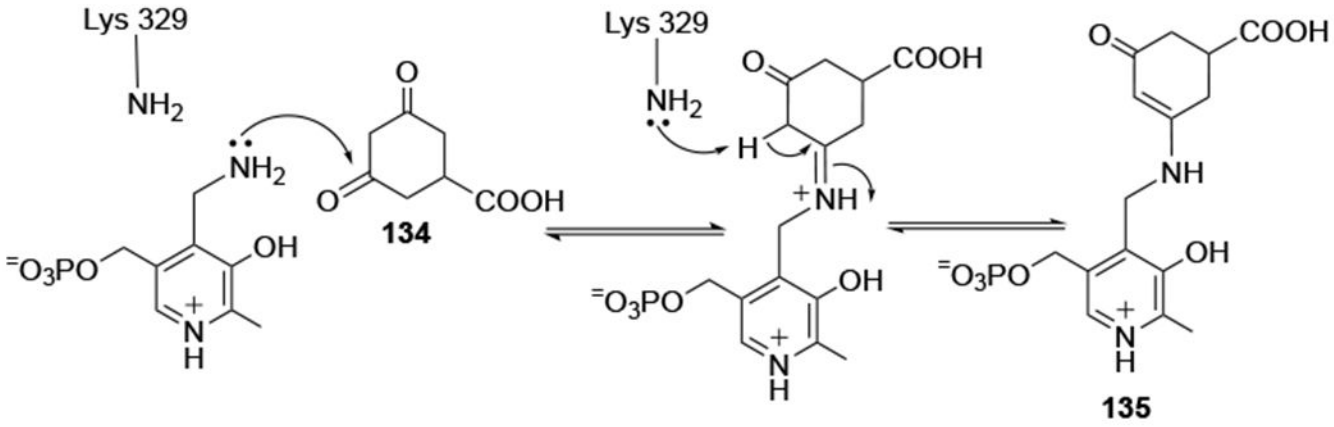
Scheme 29.

Initially hypothesized inactivation mechanisms for (2*S*,4*S*)- and (2*R*,4*S*)-4-aminotetrahydrothiophene-2-carboxylic acid hydrochloride (**130**)



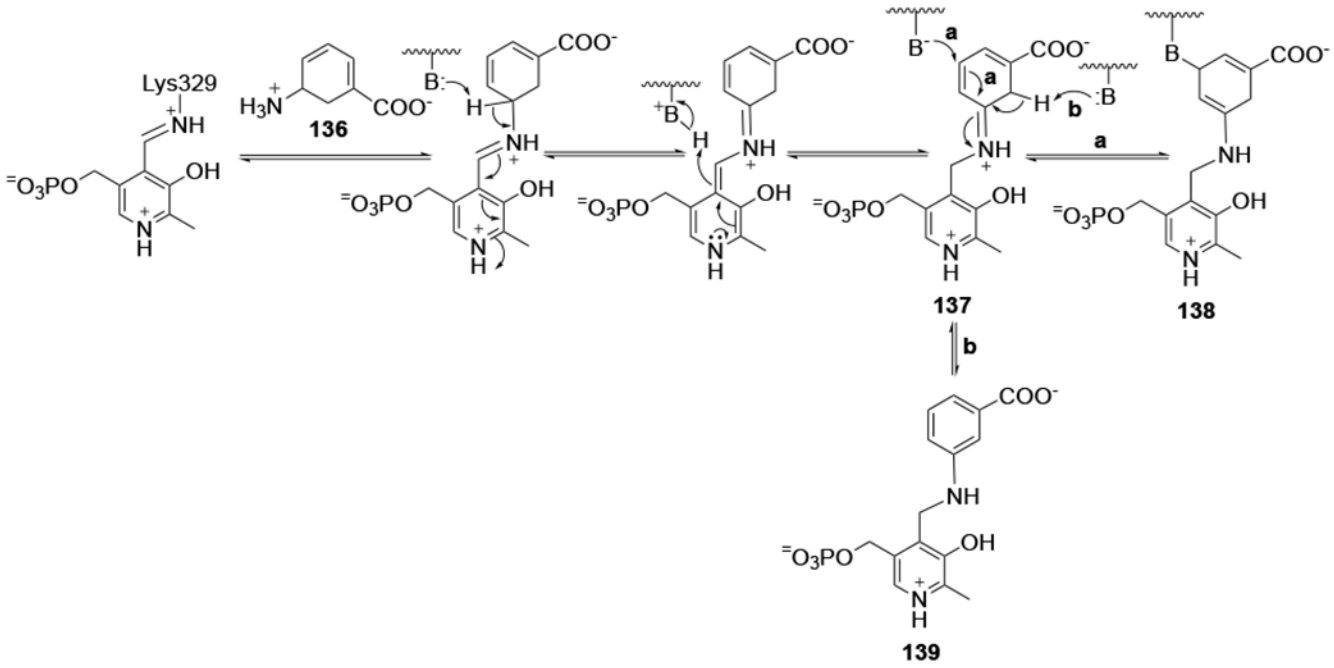
Scheme 30.

Mechanism of inactivation of GABA-AT by (2*S*,4*S*)-4-aminotetrahydrothiophene-2-carboxylic acid hydrochloride (**130**)



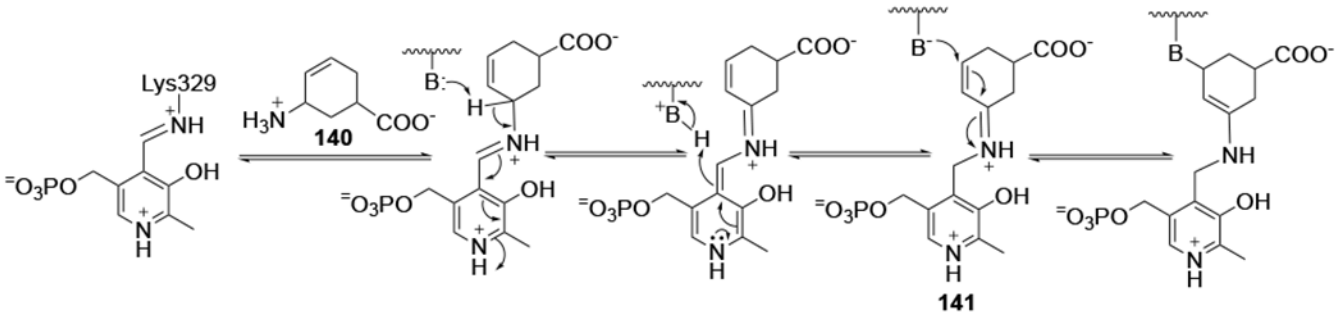
Scheme 31.

Inactivation of the PMP form of GABA-AT by 3,5-dioxocyclohexanecarboxylic acid (**134**)

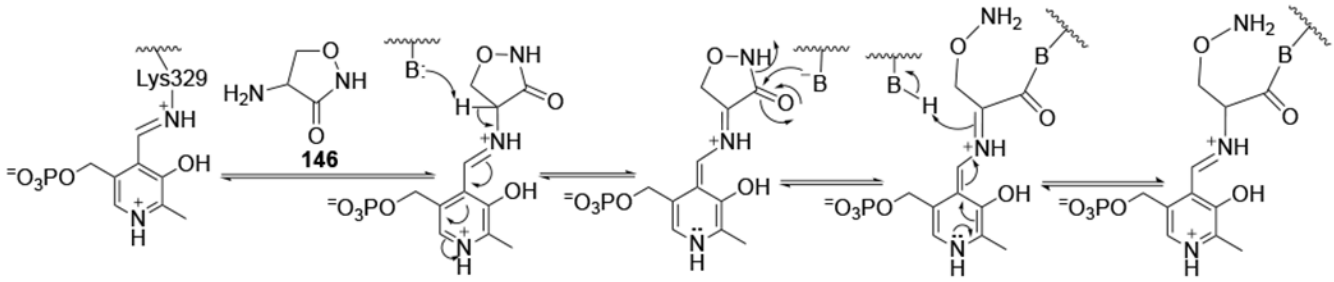


Scheme 32.

Two potential inactivation mechanisms initially proposed for gabaculine (**136**)

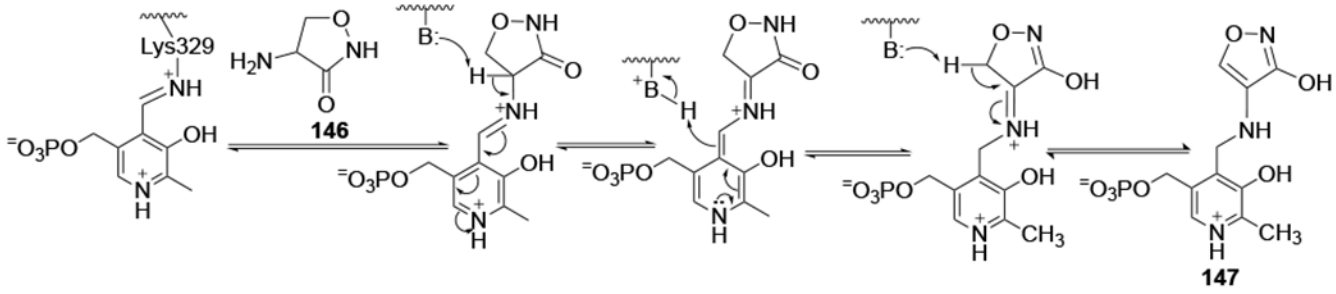


Scheme 33.
Potential Michael addition inactivation mechanism for 1,2-dehydrogabaculine



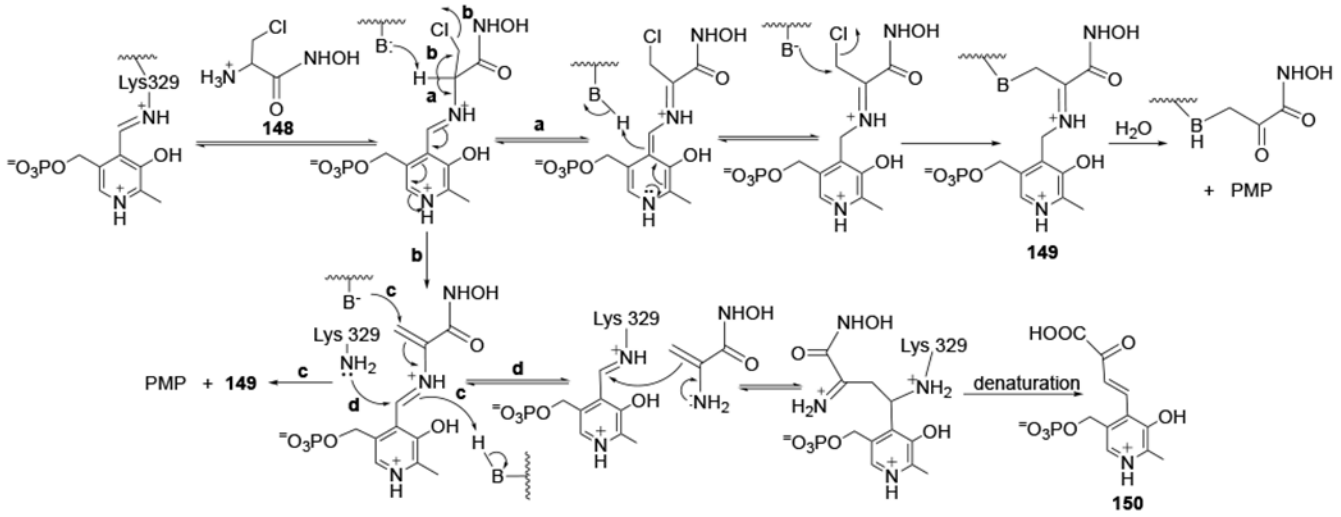
Scheme 34.

Initial incorrect proposal for the inactivation mechanism of cycloserine



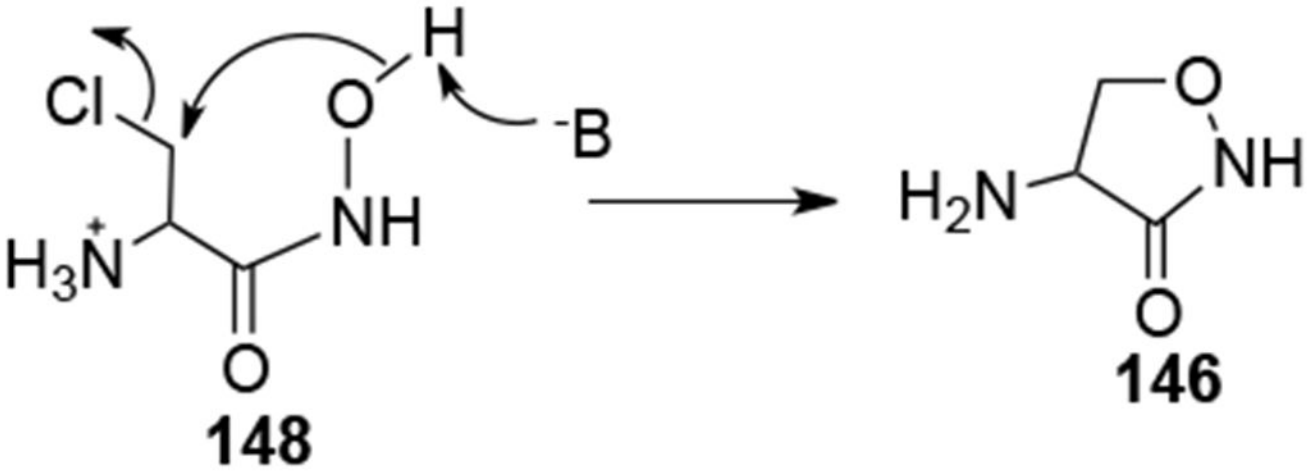
Scheme 35.

Aromatization inactivation mechanism for L-cycloserine (**146**)

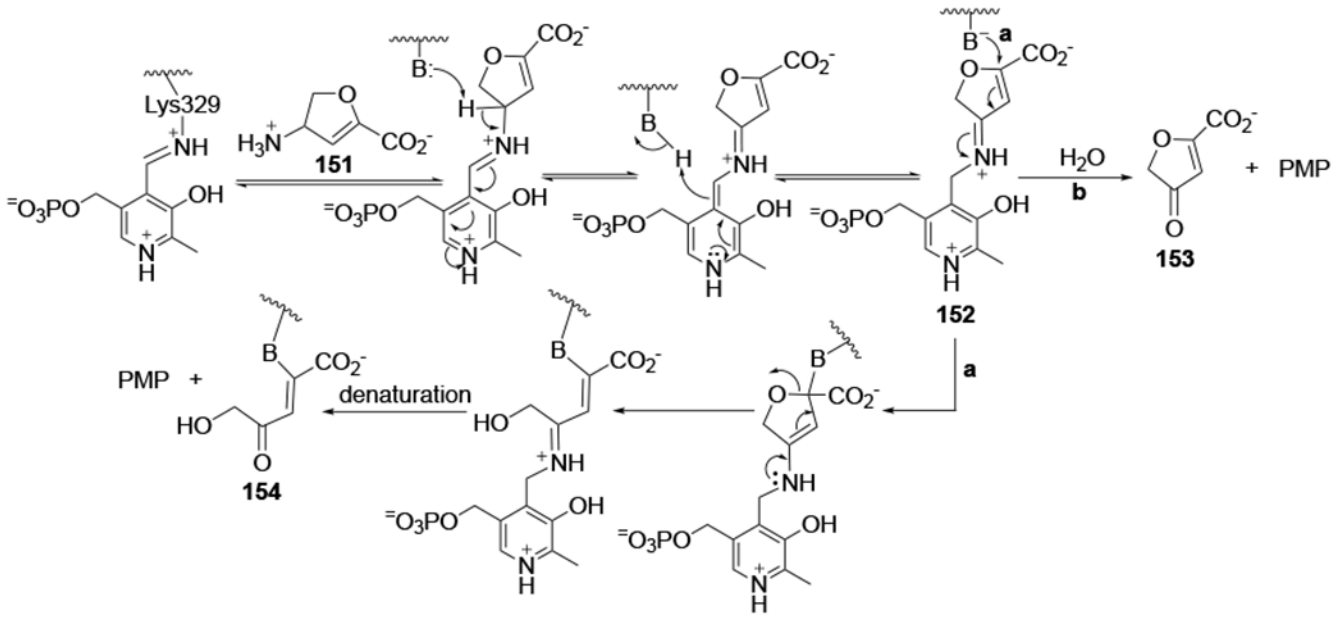


Scheme 36.

Potential inactivation mechanisms for L-3-chloroalanine hydroxamate (**148**)

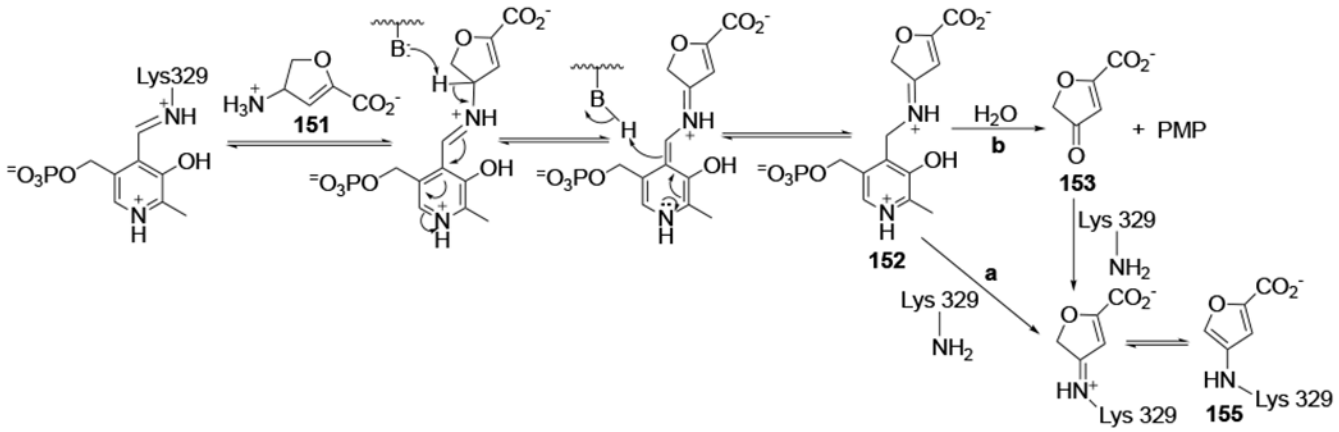


Scheme 37.
Buffer-catalyzed cyclization of L-3-chloroalanine hydroxamate (**148**) to cycloserine (**146**)



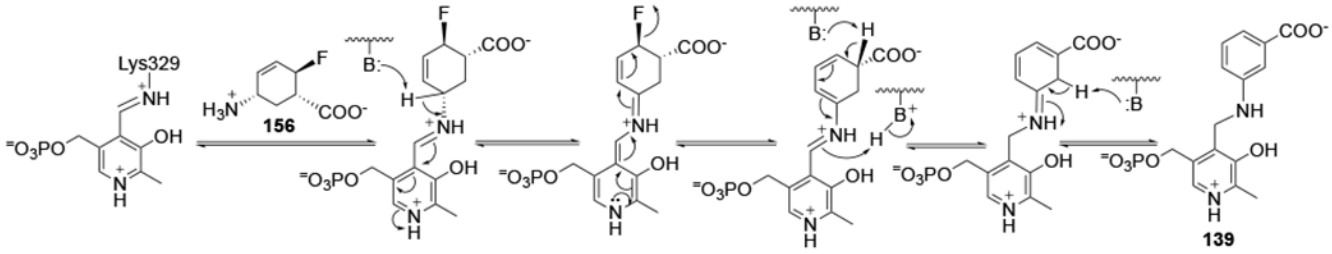
Scheme 38.

Two initially proposed inactivation mechanisms for (*S*)-4-amino-4,5-dihydrofuran-2-arboxylic acid (**151**)



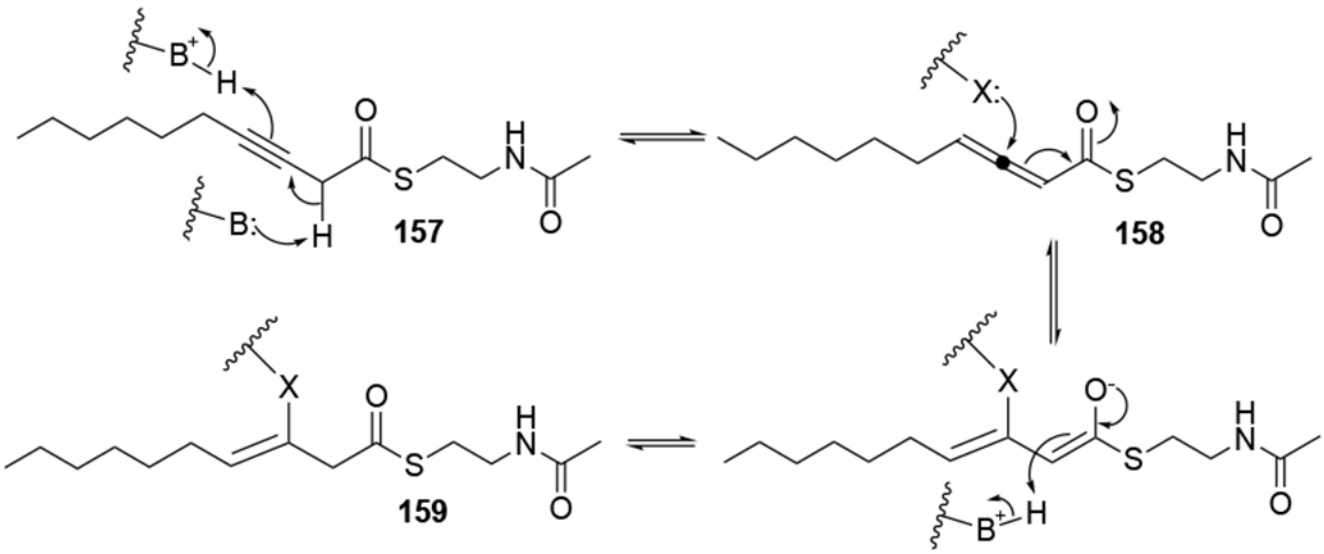
Scheme 39.

Possible mechanism for inactivation of GABA-AT by **151**



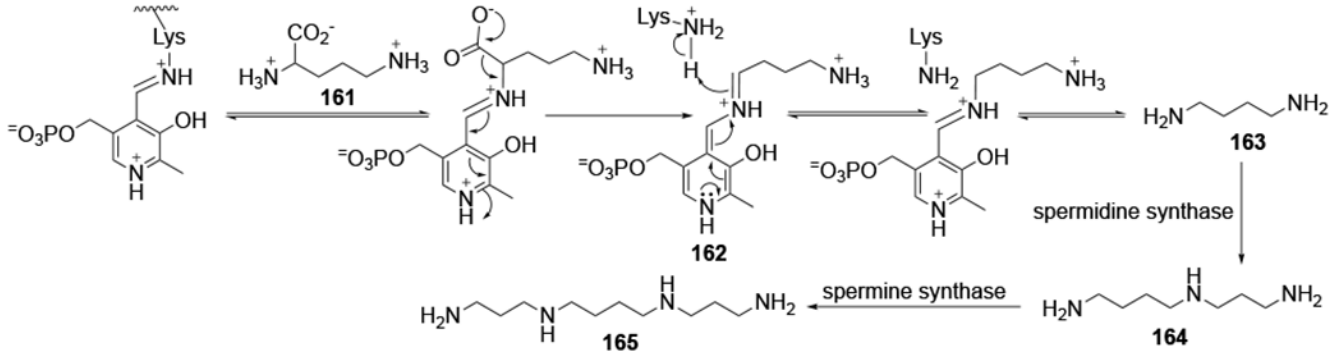
Scheme 40.

Mechanism of inactivation of GABA-AT by with (1*S*,2*R*,5*S*)-5-amino-2-fluorocyclohex-3-enecarboxylic acid (**156**)



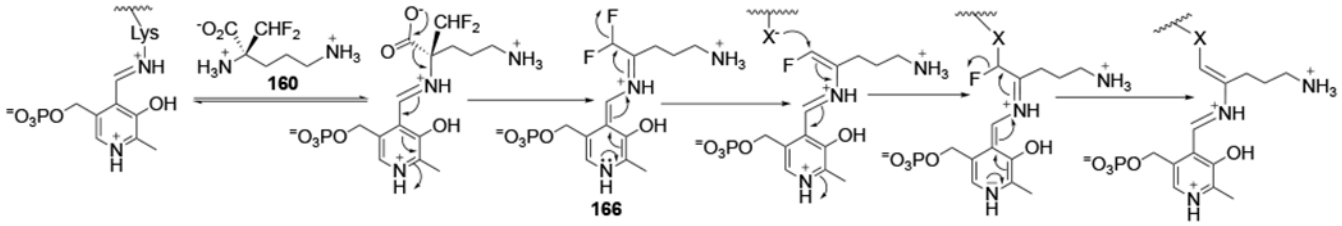
Scheme 41.

Proposed mechanism for the inactivation of β-hydroxydecanoyl thioester dehydrase by 3-decynoyl-*N*-acetylcysteamine (**157**)



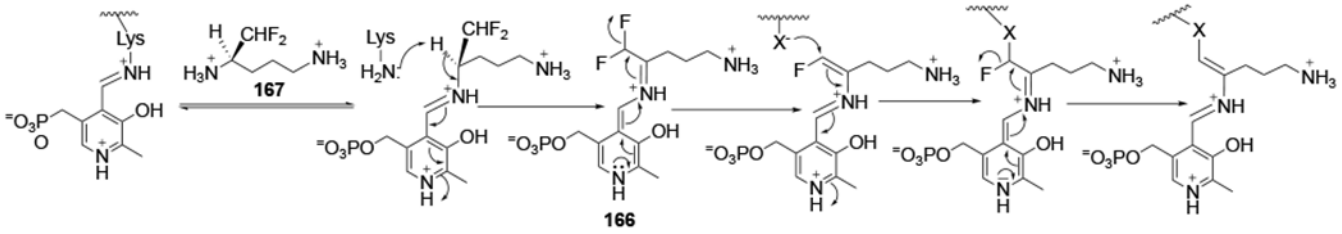
Scheme 42.

Ornithine decarboxylase and the polyamine biosynthetic pathway



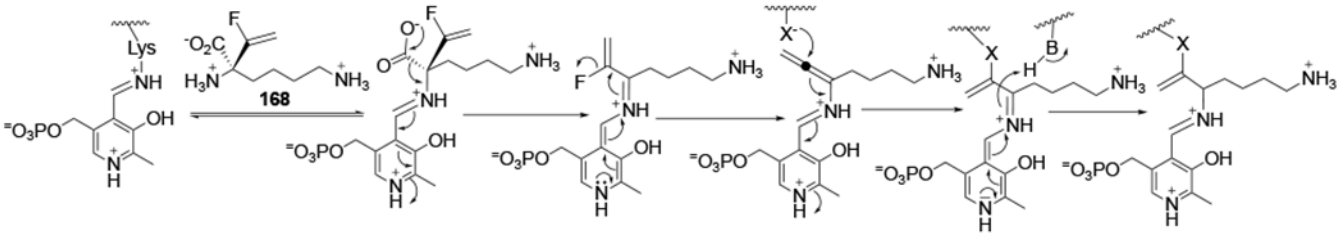
Scheme 43.

Proposed mechanism of inactivation of ornithine decarboxylase by eflornithine (**160**)



Scheme 44.

Proposed mechanism of inactivation of ornithine decarboxylase by α-difluoromethylputrescine (**167**)



Scheme 45.

A proposed mechanism of inactivation of lysine decarboxylase by α-(1'-fluoro)vinyllysine (**168**)