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T. Mark Zabriskie

L-pipecolic acid (L-PA), a six carbon imino acid, is an intermediate of lysine degradation in various organisms including bacteria, yeast, fungi and mammals. In primates, the oxidation of L-PA by the enzyme L-pipecolate oxidase (L-PO) is the rate determining step of L-lysine degradation in the central nervous system. This thesis describes efforts to probe the L-PO mechanism by using different heteroatom substrate analogs as alternative substrates and inactivators of the enzyme.

Thirteen heterocyclic analogs of L-PA containing nitrogen, oxygen and sulfur have been synthesized and tested as inhibitors and alternate substrates of L-PO. The oxygen analog, (*R*, *S*)-1,3-oxazine-4-carboxylic acid is neither an inhibitor nor a substrate of the enzyme, while nitrogen analogs such as (*S*)-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid and (*S*)-hexahydropyrimidine-4-carboxylic acid proved to be weak substrates and inhibitors. Sulfur analogs have proved to be the best inhibitors and alternate substrates of L-PO. These include the mechanism-based inactivator and excellent substrate (*S*)-1,3-thiazane-4-carboxylic acid. (*S*)-1,4-Thiazane-4-carboxylic acid is also an excellent substrate with a higher binding affinity for L-PO than the natural substrate.

The turnover product of (*S*)-1,3-thiazane-4-carboxylic has been characterized by derivatization, HPLC analysis and mass spectrometry to be homocysteine. The turnover product of (*S*)-1,4-thiazane-2-carboxylic acid was also characterized and shown to be L-α-amino-4-thioadipate-δ-semialdehyde.

Mechanistic Probes and Inhibitors of L-Pipecolate Oxidase

by

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APPROVED:

Major Professor, representing Pharmacy

Dean of College of Pharmacy

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ABBREVIATIONS

α-AAS	α-aminoadipic acid
α-ASA	α-aminoadipate-δ-semialdehyde
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CI	chemical ionization
CNS	central nervous system
ddH_2O	double deionized water
DMF	dimethyl formamide
EGTA	ethylene glycol-bis(β-aminoethylether) *N*, *N*, *N'*, *N'*-tetraacetic acid
EI	electron impact
FAB	fast atom bombardment
FAD	flavin adenine dinucleotide
GABA	γ-aminobutyric acid
HEPES	*N*-[2-hydroxyethyl]piperazine-*N'*-2-ethanesulfonic acid
HCl	hydrogen chloride
HPLC	high performance liquid chromatography
HRCIMS	high resolution chemical ionization mass spectrometry
HREIMS	high resolution electron impact mass spectrometry
HRFABMS	high resolution fast atom bombardment mass spectrometry
IC_{50}	concentration at 50% inhibition
IR	infrared spectroscopy
KBr	potassium bromide
KCl	potassium chloride
K_i	inhibition constant
K_M	Michaelis constant
L-PA	L-pipecolic acid

ABBREVIATIONS (Cont.)

L-PO	L-pipecolic acid oxidase
LREIMS	low resolution electron impact mass spectrometry
LRFABMS	low resolution fast atom bombardment mass spectrometry
MAO	monoamine oxidase
m-CPBA	*m*-chloroperoxybenzoic acid
MOPS	3-[*N*-morpholino]propane sulfonic acid
mp	melting point
MS	mass spectrometry
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NMR	nuclear magnetic resonance spectroscopy
nOe	nuclear Overhauser effect
Δ^1-P2C	Δ^1-piperideine-2-carboxylic acid
Δ^1-P6C	Δ^1-piperideine-6-carboxylic acid
pI	isoelectric pH value
PTZ	pentylenetetrazole
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLC	thin layer chromatography
Tris	Tri(hydroxymethyl)aminomethane
UV	ultraviolet spectrum spectroscopy
V_{max}	maximal velocity

MECHANISTIC PROBES AND INHIBITORS OF L-PIPECOLATE OXIDASE

CHAPTER 1

BACKGROUND AND INTRODUCTION

L-PIPECOLATE OXIDATION AND LYSINE DEGRADATION

L-Pipecolic acid (Figure 1.1) is a nonprotein amino acid and is the higher homolog of L-proline. It has a widespread occurrence in Nature and is a minor product of L-lysine degradation in bacteria,[1] fungi,[2] yeasts,[3] plants[4] and mammals.[5] Pipecolic acid oxidation was subjected to extensive studies as early as the 1960s. By studying the metabolism of L-pipecolic acid (L-PA) in certain *Pseudomonas* species which can survive on L-PA as a sole source of carbon and nitrogen, Rodwell and co-workers showed that L-PA goes through a flavin-dependent dehydrogenation to Δ^1-piperideine-6-carboxylic acid (Δ^1-P6C), which spontaneously hydrates to form L-α-aminoadipate-δ-semialdehyde.[6] Later it was demonstrated that L-PA oxidation is also a central step of lysine metabolism in animals such as rats,[7] guinea pigs,[8] turkeys [9] and human beings.[10]

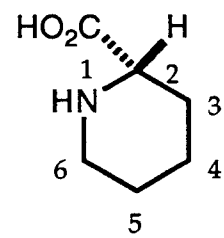
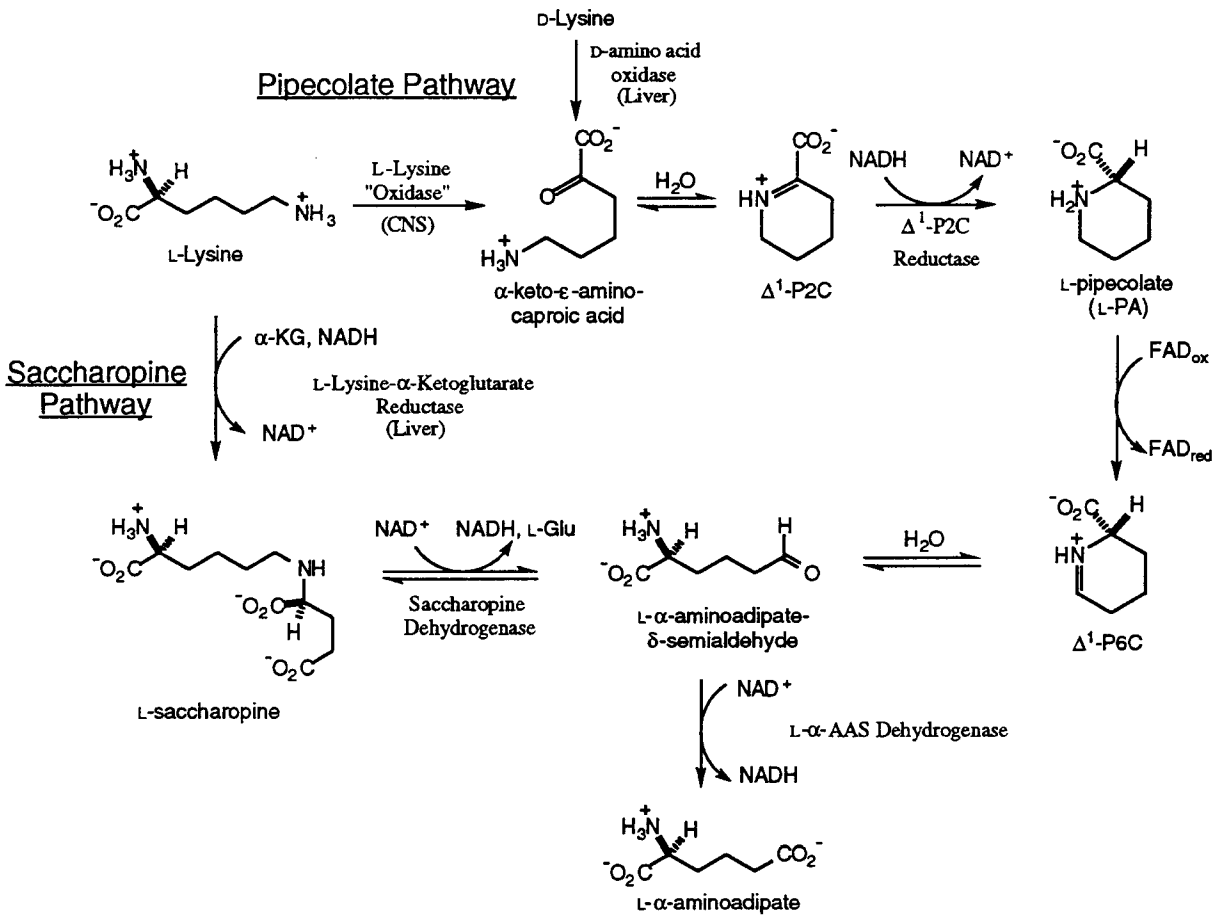


Fig.1.1 Structure of L-Pipecolic Acid (L-PA)

Degradation of lysine in the mammalian system takes many diversified routes,[10] although the major pathway is believed to be via L-saccharopine (ε–*N*-(L-glutar-2-yl)-L-lysine).[11] The L-saccharopine pathway (Scheme 1.1) involves the sequential action of L-lysine ketoglutarate reductase and sacchropine dehydrogenase to form L-α-aminoadipate-δ-semialdehyde. Although this is the major pathway in liver and other tissues, it is not believed to be very active in the brain based on the negligible activities of lysine ketoglutarate reductase and saccharopine dehydrogenase in this tissue.[5,12] In the central nervous system (CNS), L-lysine is mainly metabolized via the L-pipecolate pathway (Scheme 1.1).[13] In this pathway, the initial step involves oxidative deamination of lysine to form α-keto-ε-aminocaproic acid. The following step is spontaneous cyclization to Δ^1-piperideine-2-carboxylate (Δ^1-P2C), which is stereospecifically reduced to L-pipecolic acid. The subsequent step involves the oxidation of L-PA to Δ^1-P6C, which spontaneously hydrates to give L-α-aminoadipate-δ-semialdehyde, the first common intermediate for both pathways. The semialdehyde is further oxidized to L-α-aminoadipic acid by L-α-aminoadipate oxidoreductase.[14] The pipecolate pathway is also found in animal liver, but the precursor to pipecolate is D-lysine instead of the L-isomer.[15]

The type of enzyme catalyzing the oxidation of L-PA has been found to be species dependent. In primates, including humans, L-PA is oxidized by a flavin-dependent peroxisomal oxidase which requires molecular oxygen and generates hydrogen peroxide.[16] In lower mammals, a mitochondrial dehydrogenase coupled to the electron transfer chain is responsible for the L-PA oxidizing activity.[17] In both systems, L-pipecolic acid oxidation is suggested to be the rate-limiting step in the formation of L-α-aminoadipic acid.[12]



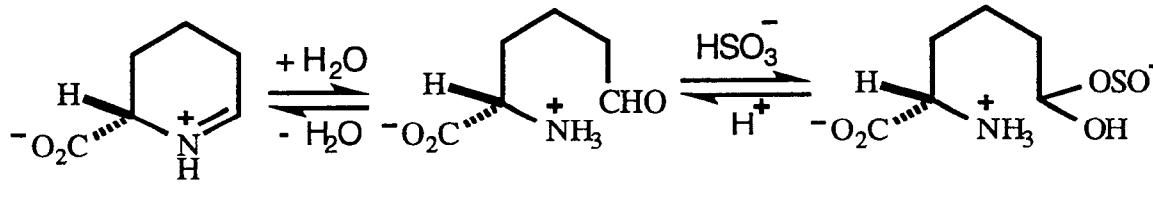
Scheme 1.1 Saccharopine and Pipecolate Pathways of Lysine Degradation

PROPERTIES AND ASSAYS OF L-PIPECOLATE OXIDASE

L-Pipecolate oxidase (L-PO: EC 1.5.37) is a membrane-associated peroxisomal enzyme requiring molecular oxygen and generating hydrogen peroxide and was first isolated from monkey liver by Mihalik's group.[18] The enzyme, a yellow monomer, has a molecular weight of 46 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a high pI of 8.9, characteristic of peroxisomal enzymes. The enzyme contains a covalently bound flavin cofactor with absorption maxima at 457 and 383 nm. L-PO exhibits a K_M of 3.7 mM and a V_{max} of 2.1 mmol•min^{-1}•mg^{-1} for L-

pipecolic acid as substrate. In the same study, benzoic acid was found to be a competitive inhibitor of L-PO with a K_i of 0.75 mM. By using HPLC to compare the derivatized reaction product to an authentic sample, Rao and Chang have identified L-α-aminoadipate-δ-semialdehyde as the isolable product of this enzyme reaction.[19]

Because of the production of hydrogen peroxide, L-PO activity can be measured by a horseradish peroxidase-linked assay, which couples hydrogen peroxide production from the oxidase reaction to the oxidation of the dye *o*-dianisidine catalyzed by horseradish peroxidase.[18] The oxidation is monitored by measuring the absorption change at 460 nm on a UV/vis spectrophotometer. A more sensitive direct assay method was developed by Rao and Chang, which requires a minimum of 16 μg of protein compared to the 100 μg needed for the spectrophotometric assay.[20] In the radioassay, the L-α-amino-[2,3,4,5,6-^{3}H]-adipate-δ-semialdehyde, formed as a direct reaction product of L-[2,3,4,5,6-^{3}H]-pipecolic acid oxidation, is trapped by sodium bisulfite (Scheme 1.2). The adduct can be separated from the substrate by cation exchange chromatography. After interaction with sodium bisulfite, the turnover product has a net negative charge and does not bind to the cation exchange resin.



Δ^1-Piperideine-6-Carboxylic Acid

L-α-Aminoadipate-δ-Semialdehyde

Bisulfite Adduct

Scheme 1.2 Bisulfite Adduct Formation in L-PO Radio Assay

PHARMACOLOGICAL AND PHYSIOLOGICAL ROLE OF L-PIPECOLIC ACID

Because of the possible physiological role of L-PA in the central nervous system (CNS), its role in this tissue has been subjected to intensive study in mammalian systems. L-PA is one of the three cyclic secondary amino acids present in the brain, along with proline and 4-hydroxyproline.[21] It was reported that the regional distribution of L-PA in the dog brain ranged between values of 1 to 10 nmol/g.[22] A similar range of concentration of L-PA has also been reported in the brain of mice[17] and rats.[23] In normal humans, L-PA concentration in plasma is 12 ± 5.6 μM at birth and decreases during the first several months of life to 2.1 ± 1.6 μM where it remains through adulthood.[24]

During recent years, evidence has accumulated suggesting a possible relationship between L-PA and γ-aminobutyric acid (GABA) receptors in cortical and hippocampal neurons in the central nervous system.[25] Neurophysiological studies in the rat suggested that L-PA is a putative neurotransmitter which might modulate GABAergic transmission in the CNS.[26] This influence was attributed to the inhibition of neuronal GABA uptake and/or enhancment of its release through the pre-synaptic $GABA_A$ receptor.[25]

Because the central $GABA_A$ receptor is the site of action for many CNS-active drugs, the anticonvulsant activity of L-PA was investigated. It was reported that intraperitoneal injection of L-PA significantly increased clonic and tonic latencies in a dose-dependent manner against 90 mg/kg pentylenetetrazole (PTZ) induced seizures.[27] Furthermore, L-PA potentiates the suppressing effects of hexobarbitol on electrically- and chemically-induced convulsions, suggesting it could be a useful anticonvulsant agent through modifying central GABA-mediated conduction.[28,29] It has been reported that administration of L-PA protected mice against bicuculline- and picrotoxin-induced convulsions.[30] In this study, it was found that intrathecal injections of L-PA produced a

dose-related increase in the latency of the onset of these convulsions as well as a decrease in their duration.[30]

The physiological significance of L-PA and the pipecolate pathway of lysine degradation was not fully appreciated until the observation of L-PA accumulation in the brain, serum and urine of patients with peroxisomal disorders, such as Zellweger syndrome,[31] hyperlysinemia,[32] and hyperpipecolatemia.[33] These patients typically have severe neurological dysfunction and profound mental retardation which are associated with the loss of most peroxisomal metabolic functions.[33] The L-PA concentration has been found to be as high as 200 μmol/g in the patient's brain tissues and 31.9 ± 10.7 μM in the plasma, compared to the normal value of 2.1 μM.[34]

An oral loading test with L-pipecolic acid indicated that the accumulation of pipecolic acid was due to an impairment in pipecolic acid degradation.[35] Further research found that L-PO was deficient in the liver of Zellweger patients.[31] It was speculated that in hyperlysinemia patients, the normal L-saccharapine pathway for L-lysine degradation is blocked and the increased levels of L-PA are due to an overload of this secondary pathway.[32]

L-PA is also found excreted in the urine of premature and very young infants, but not in older normal infants and normal adults.[32] The formation of L-PA from L-lysine could be detected in mouse brain as early as the 17th day of gestation.[36] A slight increase in formation occurs up to the first day after birth; however, at 90 days, the concentration is back to perinatal values.[36] Also, the uptake of L-PA in the one-day-old mouse brain shows a different pattern than that in the adult.[36] These data suggest that L-PA may play a role in the development of CNS.

Because knowledge of L-PA metabolism is important to understand these lysine degradation disorders and because L-PO may play an important role in the development of potential therapeutic agents, the area has been subject to extensive studies.[18,37,38] The focus of this thesis project is on designing and synthesizing alternative substrates and

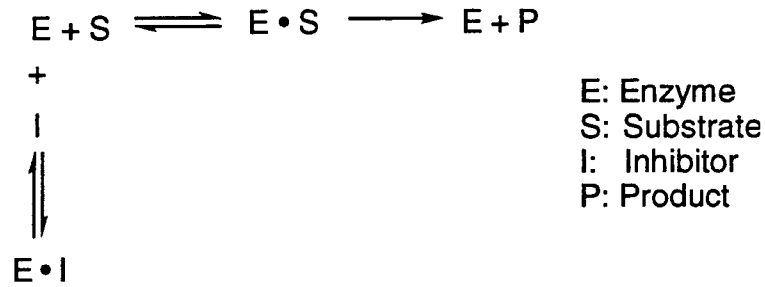
inhibitors of L-PO, specifically mechanism-based inactivators, to probe the enzyme mechanism.

ALTERNATE SUBSTRATES & INHIBITORS IN ENZYMOLOGY STUDIES

In recent years, enzyme alternate substrates and inhibitors not only have made significant contributions to the understanding of enzymatic mechanisms, but also have provided an increasing number of patented therapeutic agents for the treatment of diseases. These substrates and inhibitors act on the target enzymes by influencing the binding of a natural substrate and its turnover. In basic research, enzyme alternate substrates and inhibitors are commonly used to probe the chemical and conformational nature of a substrate-binding site as part of an effort to elucidate the enzyme's catalytic mechanism. For example, a covalent binding mechanism-based inactivator may be used to identify active site amino acid residues which could potentially be involved in the catalytic mechanism of the enzyme.[39] Reversible, competitive inhibitors may be used to facilitate enzyme purification by using the inhibitor as a ligand for affinity chromatography.[40] In medicine, these inhibitors have potential as drugs by selectively blocking certain metabolic pathways, decreasing the concentration of enzymatic product or altering the concentration of an enzyme's substrate. In fact, about half of the top 20 drugs sold worldwide are enzyme inhibitors .[41]

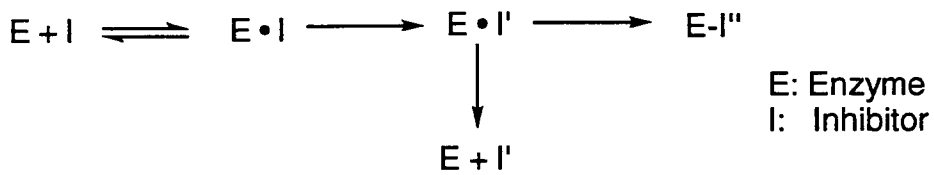
All the inhibitors studied in this project fall into either the competitive inhibitor or mechanism-based inactivator category. A competitive inhibitor is a substrate that directly competes with the natural substrate for an enzymatic-binding site. Usually, such an inhibitor structurally resembles the natural substrate to the extent that it specifically binds to the active site but differs from it so as to be unreactive. Competitive inhibitors usually follow the reaction model in Scheme 1.3. Because the enzyme-inhibitor complex is

catalytically inactive, a competitive inhibitor acts by reducing the concentration of free enzyme available for natural substrate binding and turnover.



Scheme 1.3 General Reaction Model of Competitive Inhibition

Mechanism-based inactivators are typically unreactive compounds which are only converted into highly reactive species when subjected to the normal catalytic mechanism of the target enzyme. Mechanism-based inactivation usually follows the model in Scheme 1.4.



Scheme 1.4: General Reaction Model of Mechanism-Based Inactivator

As a relatively newly discovered type of enzyme inhibitor, mechanism-based inactivators were not subjected to intensive study until the 1970s. They have proven to be very useful in the study of enzyme mechanisms and in the design of highly specific, low-toxicity drugs.[41] Criteria have been developed for the evaluation of a possible mechanism-based inactivator.[41] More often than not, only a few of these criteria are tested when a potential mechanism-based inactivator is studied. However, to truly characterize an

inactivator as falling into this class, most or all of the criteria should be satisfied. These criteria are:

1. A time-dependent loss of enzyme activity (usually, but not necessarily, pseudo-first order with respect to enzyme) is observed.
2. The rate of inactivation is proportional to the concentration of inhibitor at low inhibitor concentration, but independent at high concentration (saturation kinetics).
3. The rate of inactivation is slower in the presence of a natural substrate or competitive inhibitor than in its absence. That is, the inactivation is protected by a natural substrate and indicates the inactivator is acting at the enzyme active site.
4. Enzyme activity does not return upon dialysis or gel filtration. This means that the inactivation is irreversible.
5. A 1:1 stoichiometry of radioactively labeled inactivator to active site usually results after inactivation followed by dialysis or gel filtration.
6. A catalytic step for conversion of the inactivator to a reactive intermediate can be demonstrated.
7. Inactivation should occur prior to the release of any activated species. The presence of exogenous nucleophiles or radical traps has no effect on the inactivation rate, and following inactivation, a second equal addition of enzyme results in the same rate of inactivation as the first addition.

For this study, mechanism-based inactivators can be used to probe the L-PO mechanism by providing clues to possible reactive intermediates. Also, they represent potential therapeutic agents for treating convulsive disorders. Competitive inhibitors are useful tools to study the electronic properties and steric limitations in the active site of L-PO. With alternate substrates, we can study the mechanism by observing the effect that substrates with altered electronic properties and oxidation potentials have on the rate of the reaction.

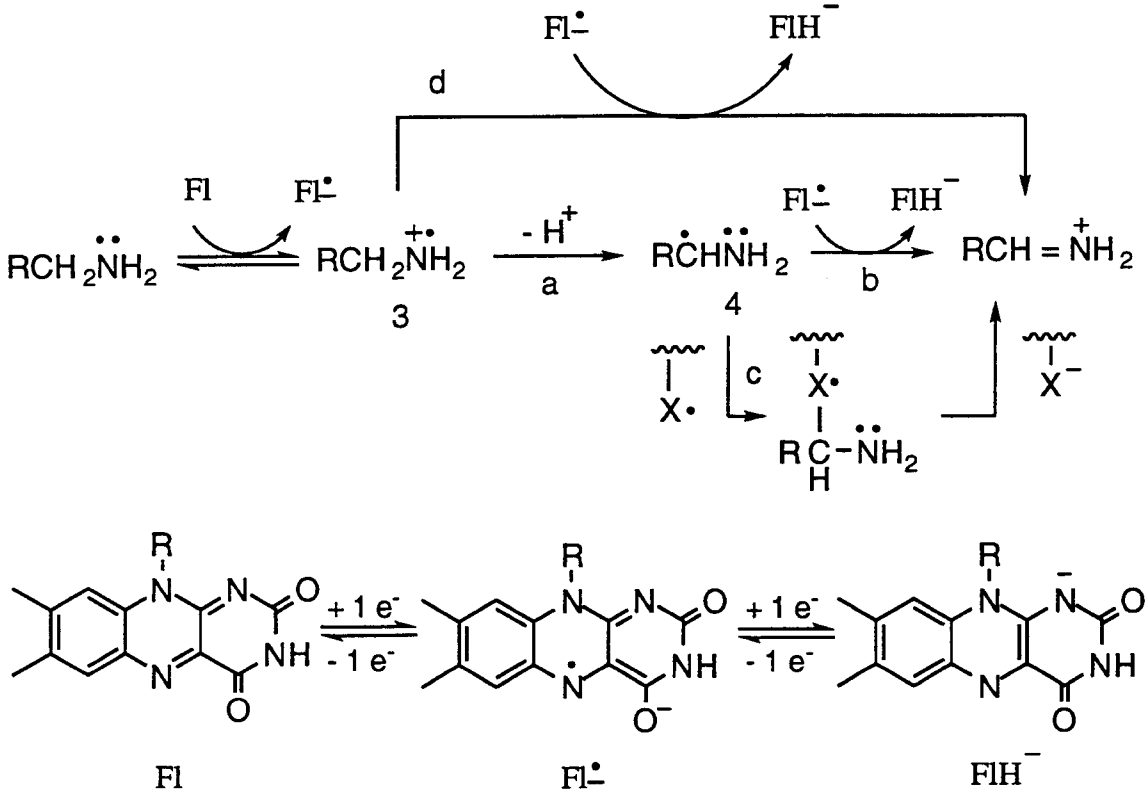
These inhibitors may also find use as potential agents to decrease the level of L-α-aminoadipic acid in the central nervous system. L-α-Aminoadipic acid, a product of lysine metabolism, exists in mammalian brain in micromolar concentration. Its properties as a postsynaptic excitatory amino acid receptor agonist,[42] excitotoxin,[43] and gliotoxin[44] are well documented. It was also reported that the accumulation of L-α-aminoadipic acid could lower the level of kynurenic acid in the CNS.[38] Kynurenic acid is a tryptophan metabolite which serves as one of the brain's defenses against overactivation of excitatory amino acid receptors and pathological consequences, such as excitotoxicity and seizures.[38] Thus, when the level of L-α-aminoadipic acid is decreased by these L-PO inhibitors, kynurenic acid levels should remain unaffected.

MECHANISM MODELS FOR L-PIPECOLIC OXIDASE

The design of mechanism-based inactivators and alternative substrates requires at least some understanding of the enzyme binding site in order to promote the formation of the initial noncovalent enzyme substrate or inhibitor complex. Also, some knowledge of the mechanism of the target enzyme with its normal substrate is important in the design. The amino acid sequence of L-PO has not been published, the active site has not been characterized, and little is known about the L-PO mechanism. To design the target compounds in this study, another well-studied flavoenzyme was used as a model for the possible mechanism of L-PO.

L-PO is a flavin-dependent amine oxidase, belonging to the same family of enzymes as the well-studied monoamine oxidase (EC 1.4.3.4). It is generally accepted that the oxidation reaction catalyzed by monoamine oxidase (MAO) proceeds by a single-electron mechanism (Scheme 1.5).[45] In this mechanism, it is proposed that the first step involves a one-electron transfer from the substrate amino group to the oxidized flavin (Fl)

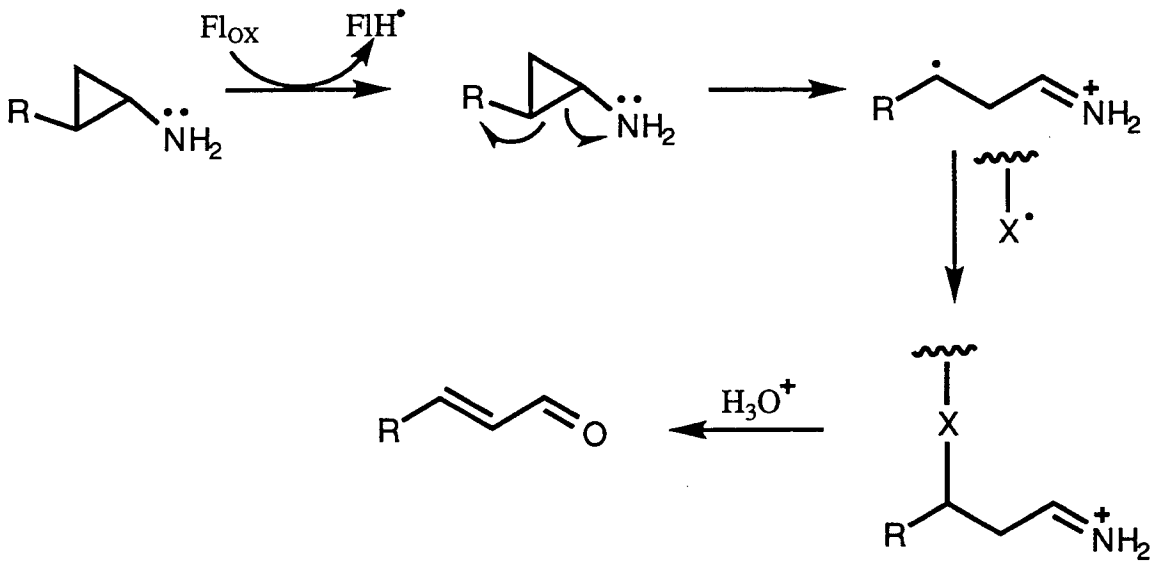
to give the amine radical cation (**3**) and the flavin semiquinone ($Fl^{\bullet}$). Loss of a proton (pathway a) gives the α-amino radical (**4**) which can either transfer a second electron to the flavin semiquinone to give reduced flavin (FlH^-) (pathway b) or undergo a radical combination with an active site radical (pathway c) to give a covalent adduct, which should decompose by β-elimination to give the iminium ion. A potential alternative to proton transfer (pathway a) is hydrogen atom transfer (pathway d), which bypasses the α-amino radical intermediate (**4**).



Scheme 1.5: Single-Electron Transfer Mechanism Proposed for Monoamine Oxidase

In order to detect the formation of radical intermediates, a chemical approach has been used by Silverman. One approach has been to synthesize a variety of cyclopropylamine substrate analogs, which are mechanism-based inactivators of MAO.[46]

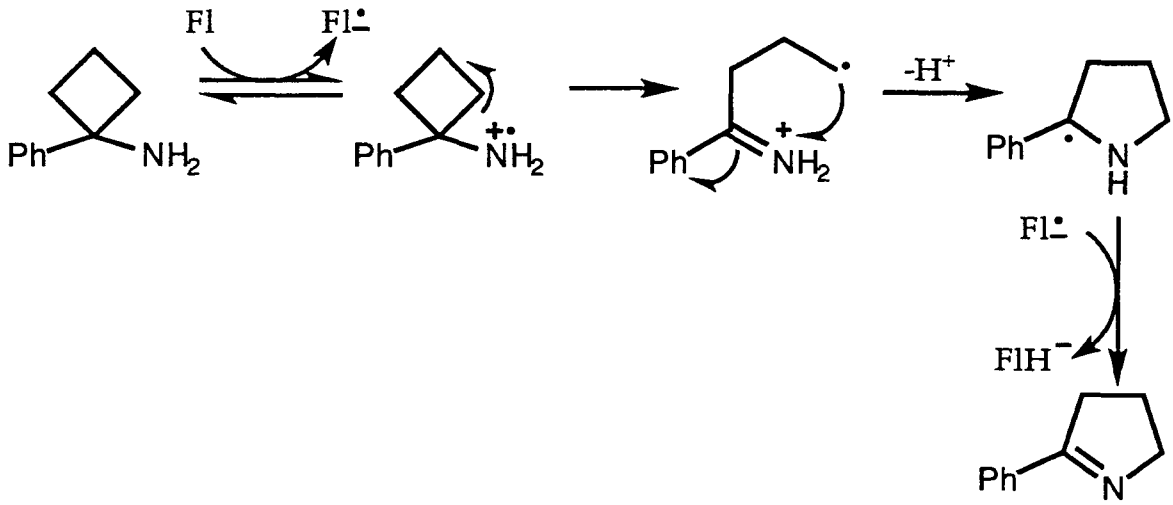
The selection of this group was based on the known chemistry[47] that secondary aminyl radicals could be observed. But when the corresponding cyclopropylaminyl radical was generated by the same method, it could not be observed; instead, the ring-cleaved carbinyl radical was detected. Upon incubation of these compounds with MAO, the enzyme was irreversibly inactivated (Scheme 1.6). The formation of aminyl radical followed by cyclopropyl ring cleavage and attachment to the enzyme were deduced through identification of the stable intermediates. The results were consistent with a one-electron oxidation of the cyclopropyl substrate analogs to the amine radical cation followed by homolytic ring cleavage and attachment to the enzyme.



Scheme 1.6 Proposed Mechanism of Inactivation of MAO by Cyclopropyl Substrates

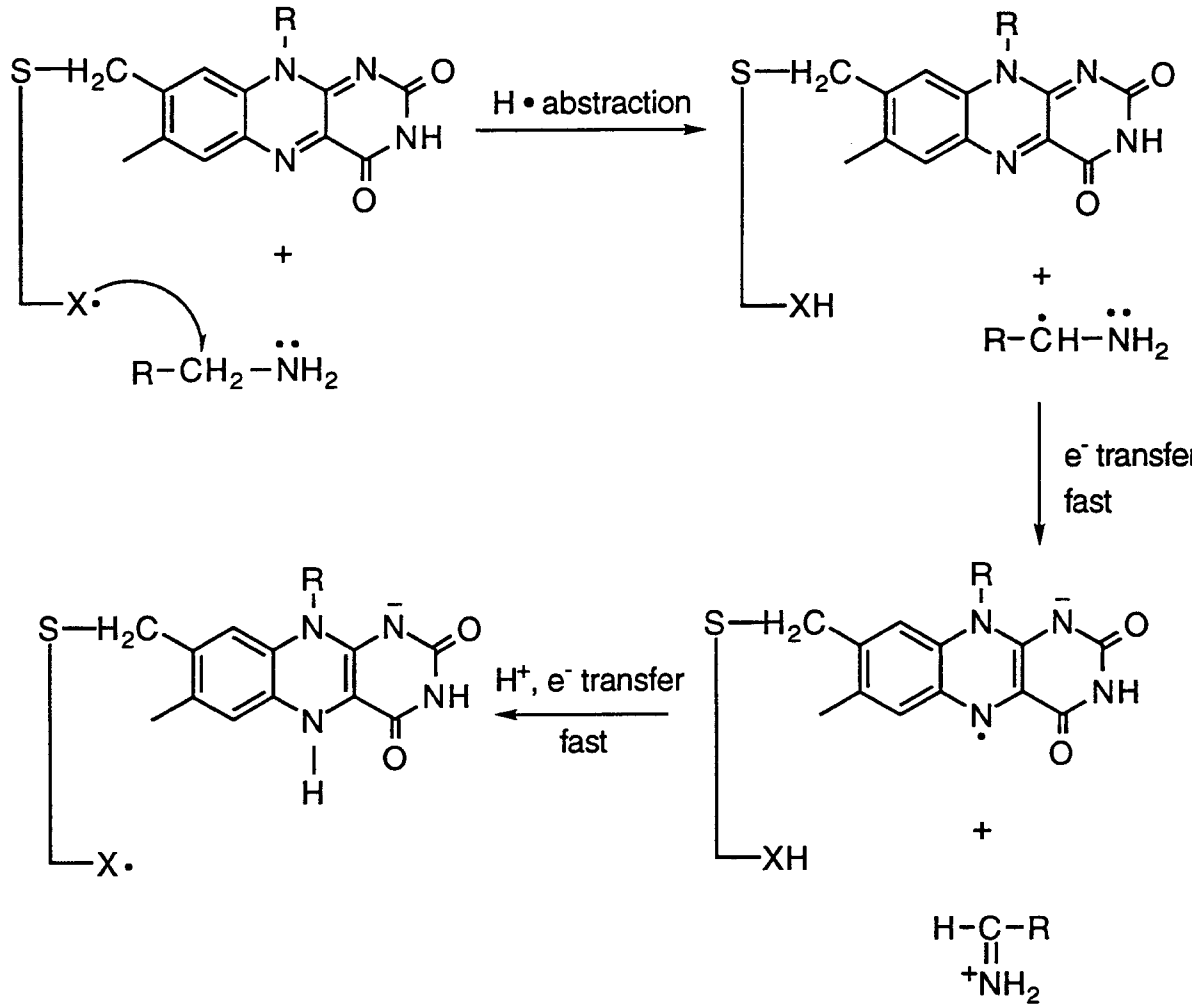
Silverman and coworkers have also studied the inhibition of MAO by 1-phenylcyclobutylamine.[48] The cyclobutylamine would undergo a known one-electron rearrangement which triggers cyclobutyl ring cleavage after generating the initial cyclobutyl amino radical (Scheme 1.7).[49] The observed formation of 2-phenyl-1-pyrroline supports the initial formation of the 1-phenyl-cyclobutylaminyl radical. When

(aminomethyl)cubane was reacted with MAO, the identified turnover products also strongly support the single electron transfer mechanism through electron transfer followed by proton transfer.[50]



Scheme 1.7 Proposed Mechanism of Inactivation of MAO by 1-Phenylcyclobutylamine

Another reasonable mechanism for MAO is the direct hydrogen atom abstraction mechanism proposed by Edmondson (Scheme 1.8).[51] From stopped flow kinetic and kinetic isotope effect studies of substituted benzylamines, it was concluded that the rate-determining step in the reaction was the C-H bond cleavage. These researchers proposed a direct hydrogen abstraction from the α-carbon of the substrate, followed by rapid electron transfer from the aminyl substrate radical to form the flavin semiquinone and the protonated imine product. Although this proposed mechanism is not supported by experimental results, such as spectroscopic evidence for a protein-bound radical, one of the considerations against the single-electron transfer mechanism is the unfavorably large difference in the redox potentials of the amine (approximately +1 V) and the flavin (-0.2 V).[52] The large difference in potentials would seem to make the transfer of electrons on the enzyme from the amine to a flavin energetically unfavorable .



Scheme 1.8: Direct Hydrogen Abstraction Mechanism Proposed for Monoamine Oxidase

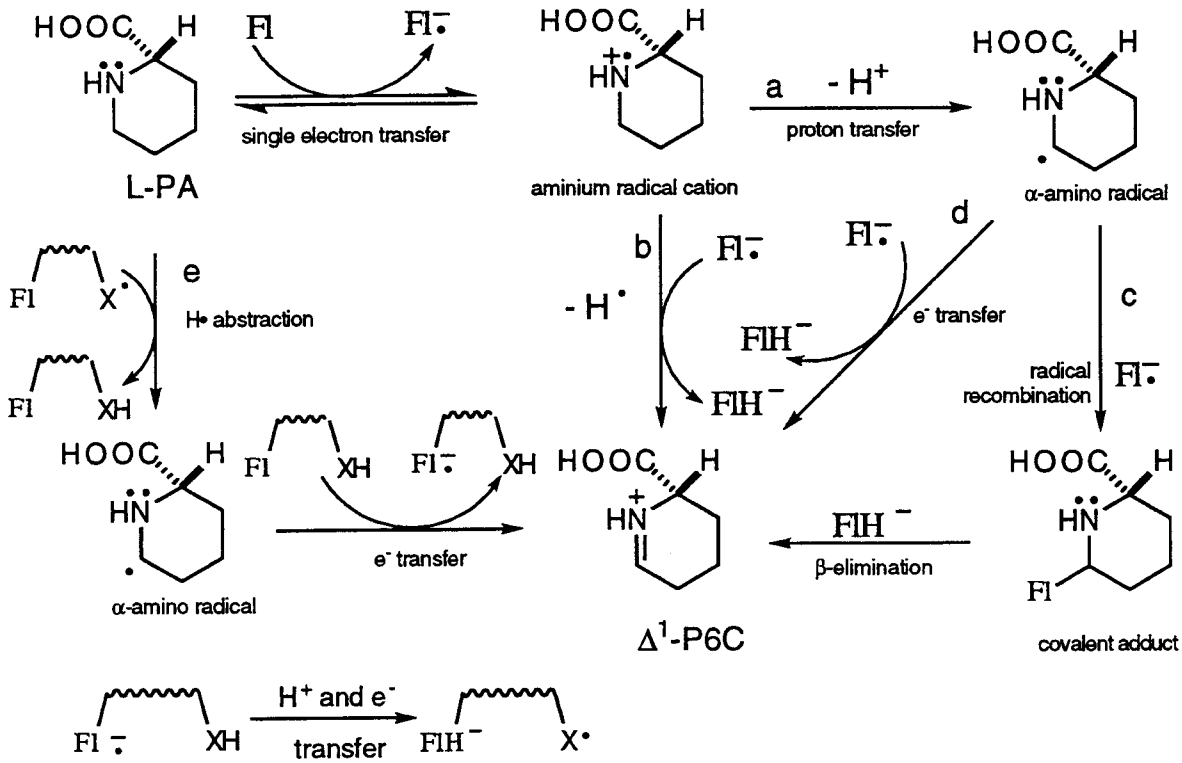
Most important for our studies in both proposed mechanisms, radical intermediates are suggested to be involved. Thus, inhibitors and alternative substrates which can stabilize radical species by various mechanisms may prove to be useful tools for studying L-PO.

CHAPTER 2

SYNTHESIS OF ALTERNATE SUBSTRATES AND INHIBITORS OF L-PIPECOLATE OXIDASE

TARGET COMPOUND DESIGN

Synthesizing and testing substrate analogs is one of the most effective ways to discover alternative substrates and inhibitors of an enzyme, especially when little information about the enzyme mechanism and active site is known.[41] Because of the similarity in structure, these compounds can often fit into the enzyme substrate binding pocket. However, the minor modifications in structure may result in a significant change in noncovalent binding forces, such as electronic, van der Waal's, and hydrophobic interactions. The contribution of these modifications to the overall binding affinity of the analogs may result in blocking of the active site to prevent any further substrate binding and catalysis as well as provide information on the environment of the binding site. Since the active site and amino acid sequence of L-PO have not been characterized, the inhibitor design strategy draws on possible mechanisms of L-PO according to the well-studied model enzyme, monoamine oxidase (MAO). As mentioned in Chapter 1, L-PO and monoamine oxidase are both flavin-dependent amine oxidases. It is very possible that both share a great deal of similarity in the overall chemical mechanism. The possible mechanisms for L-pipecolate oxidase, illustrated in Scheme 2.1, are drawn from the proposed mechanisms for monoamine oxidase.[45,53]



Scheme 2.1 Possible Mechanisms of L-Pipecolate Oxidase

One possible mechanism of L-pipecolate oxidase is a "single-electron transfer" mechanism, analogous to that proposed by Silverman for monoamine oxidase.[45] The initial step proceeds through a one-electron transfer from the amine of a substrate to the flavin, resulting in the nitrogen radical cation and the flavin semiquinone. The second step involves either proton transfer (pathway a) to form an α-amino radical or hydrogen abstraction to form the iminium intermediate Δ^1-piperideine-6-carboxylate (Δ^1-P6C) (pathway b). From the α-amino radical, two routes to Δ^1-P6C are possible. The first (pathway c) involves a radical pair combination with the semiquinone form of the flavin, followed by heterolytic cleavage to the fully reduced flavin and Δ^1-P6C. The other possible route (pathway d) involves a second single electron transfer from the substrate to the cofactor.

Another possibility involves "direct hydrogen abstraction" analogous to the mechanism proposed by Edmondson for monoamine oxidase (pathway e).[51] This route proceeds with a direct hydrogen abstraction from L-PA by an enzyme-bound radical species to form an α-amino radical. Electron transfer to the flavin is next involved to form Δ^1-P6C.

Because radical intermediates are proposed in both mechanisms, the ability to stabilize a radical intermediate may be one important element in the design of potent inactivators and mechanistic probes. Compounds able to stabilize a radical intermediate or which have lower oxidization potentials should serve as good alternate substrates and possible inactivators.

It is well established that heteroatoms, such as oxygen, nitrogen and sulfur have an important role in the stabilization of adjacent carbon-centered radicals.[54] This stabilization is due to the electron donation from the lone-pair electrons of the heteroatom. Moreover, theoretical calculations suggest that sulfur can use an antibonding π-type orbital to accommodate the additional electron to form an α-thioradical with less deviation from planarity, and thus greater stability, than an α-oxyradical.[55] This enhancement can also be explained by the resonance structure shown in Figure 2.1 because sulfur can form one more possible resonance structure than oxygen and nitrogen.[54]

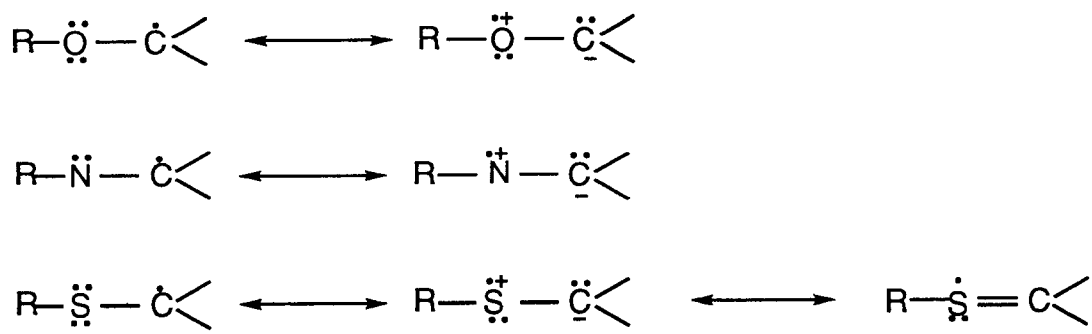


Figure 2.1 Resonance Structures of Carbon-Centered Radicals with Different Heteroatoms

Figure 2.2 illustrates the substrate analogs synthesized as part of this thesis project. Different heteroatoms are introduced either at position 4 or 5 of the piperidine ring. These compounds may be divided into three groups. The first group of compounds has the 3,4,5,6-tetrahydropyrimidine ring structure and includes (*S*)-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid (**1**) and (*S*)-2-amino-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid (**2**). These compounds were not viewed as mechanism-based inactivators or substrates, but rather were hoped to act as reversible inhibitors because the 1,4,5,6-tetrahydropyrimidine, one of the tautomers of the tetrahydropyrimidine structure, is a structural mimic of Δ^1-P6C.[56]

The second group has a saturated heterocyclic ring structure with nitrogen, oxygen or sulfur in position 5. Compounds in this group include (*S*)-hexahydropyrimidine-4-carboxylic acid (**3**), (*R, S*)-1,3-oxazane-4-carboxylic acid (**4**), (*S*)-1,3-thiazane-4-carboxylic acid (**5**), (*R*)-1,3-thiazane-4-carboxylic acid (**6**) and (2*S*, 4*R*)-, (2*S*, 4*S*)- and (2*R*, 4*S*)-2-methyl-1,3-thiazane-4-carboxylic acid (**7**, **8** and **9**, respectively). Because the introduced heteroatoms are at the position α to the proposed radical center, we envisioned that these heteroatoms can stabilize the putative radical intermediate.

The third group of compounds are piperidine derivatives with nitrogen or sulfur at position 4. These compounds include (*S*)-1,4-thiazane-3-carboxylic acid (**10**), (*S*)-1,4-thiazane-3-carboxylic acid 1-oxide (**11**), (*S*)-1,4-thiazane-3-carboxylic acid 1-dioxide (**12**) and (*R, S*)-1,4-piperazine-2-carboxylic acid (**13**). These were selected as interesting compounds to study the effect of polar atoms or groups at position 4 on the conformation of the piperidine ring and on the ability to stabilize a radical β to that atom.[54]

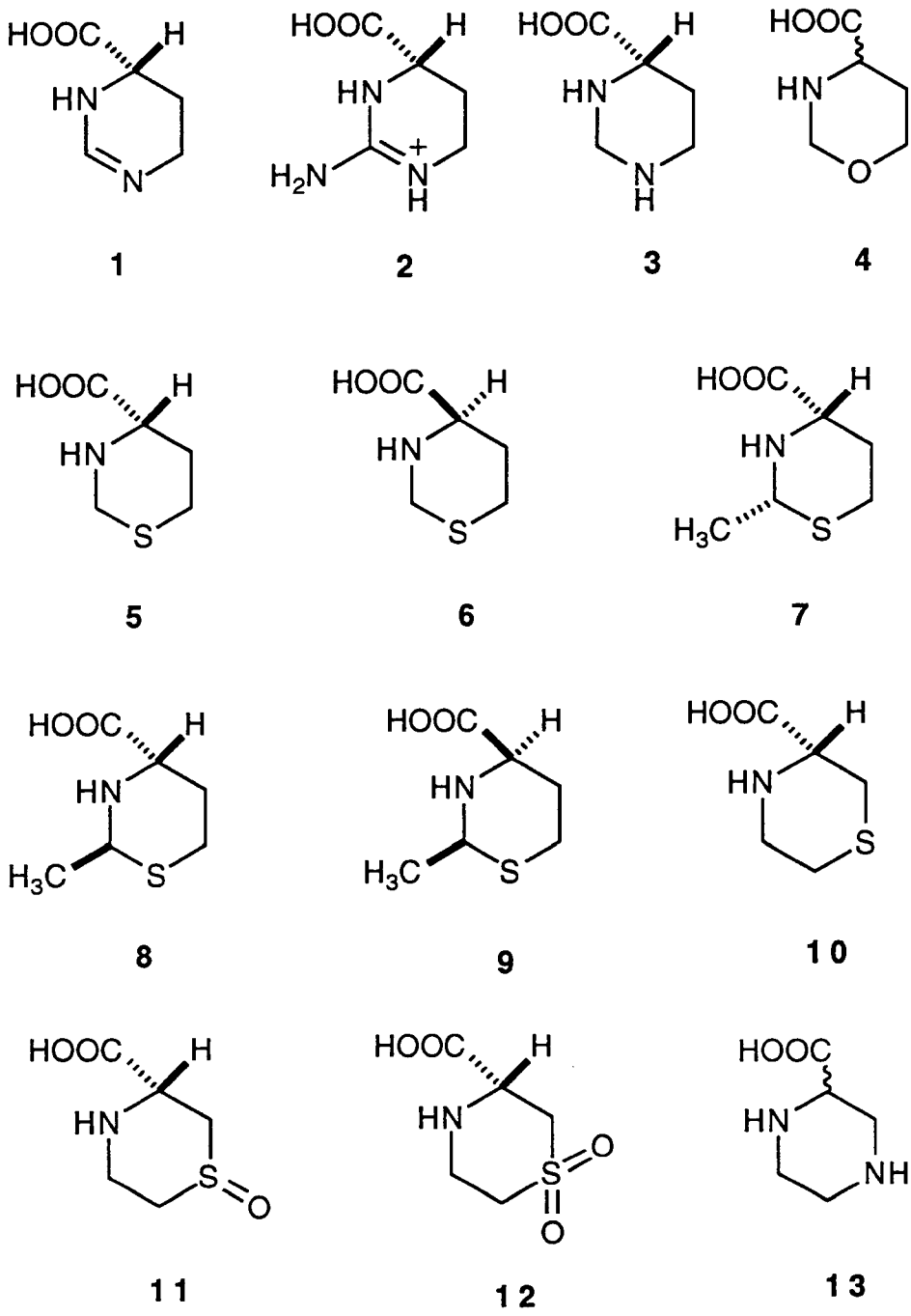
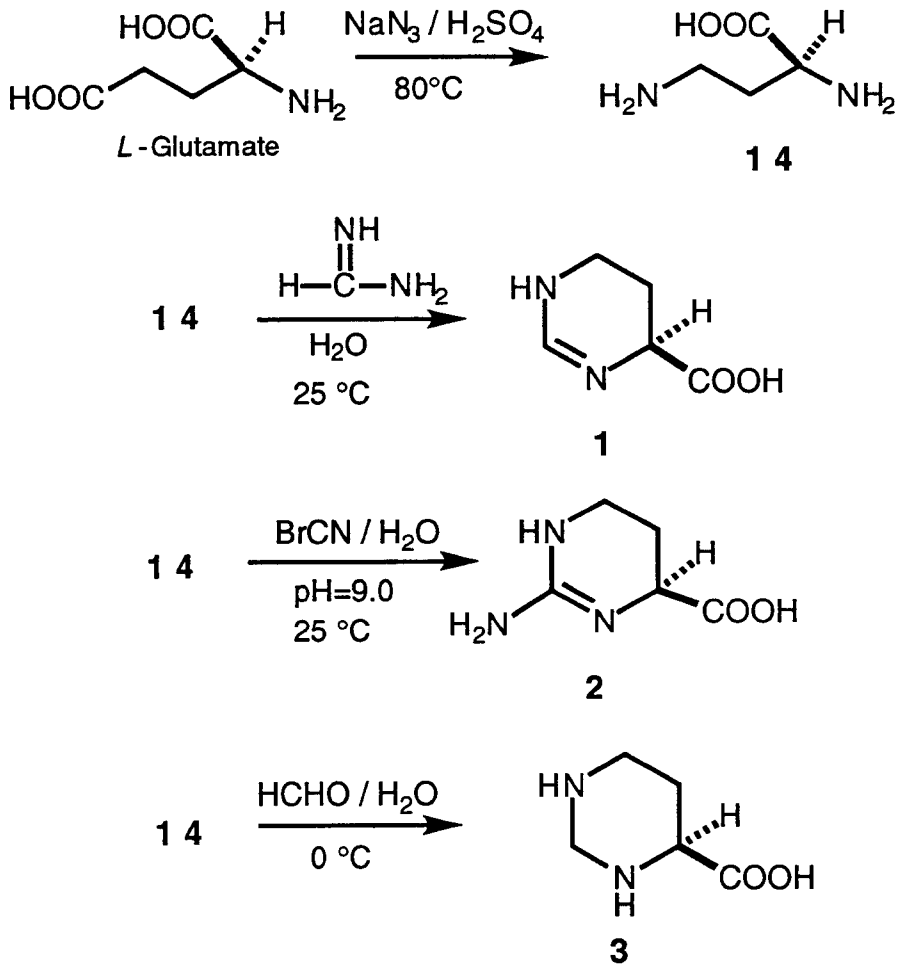


Figure 2.2 Substrate Analogs of L-Pipecolic Acid

Preparation of Tetrahydropyrimidines (1) and (2)

It is known that the condensation of 1,3-diamino compounds with a nitrile group or its equivalent is one of the effective ways to prepare tetrahydropyrimidine derivatives (Scheme 2.2).[56] The required optically active 2,4-diaminobutyric acid (**14**) is available in one step from L-(+)-glutamic acid via a Curtius rearrangement.[57,58] The synthesis of (*S*)-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid (**1**) involves reaction of **14** with a formamidine salt.[59] To synthesize (*S*)-2-amino-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid (**2**), reaction of **14** with cyanogen bromide under basic conditions was adopted.[60] Under these conditions, the amine group is uncharged and capable of nucleophilic attack at the electrophilic carbon atom of the nitrile group.

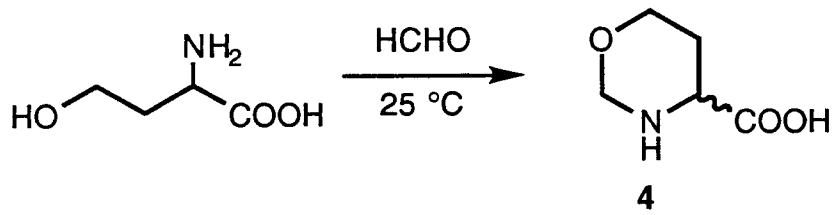


Scheme 2.2. Preparation of Compound **1**, **2** and **3**

Preparation of Pipecolate Analogs with Heteroatoms at Position 5

(*S*)-Hexahydropyrimidine-4-carboxylic acid (**3**) was prepared by the condensation of (*S*)-1,3-diaminobutyric acid (**14**) with formaldehyde (Scheme 2.2).[59] This reaction involves the formation of a Schiff's base intermediate and then nucleophilic attack at the imine carbon by the second amino group completes the cyclic structure.

In a manner similar to the preparation of **3**, (*R, S*)-1,3-oxazine-4-carboxylic acid (**4**) was synthesized through the condensation of D,L-homoserine and formaldehyde (Scheme 2.3). The cyclic product is formed through the reversible formation of an imine (Schiff's base) between the amino group and aldehyde, which is subject to nucleophilic attack from the hydroxy group.[61]



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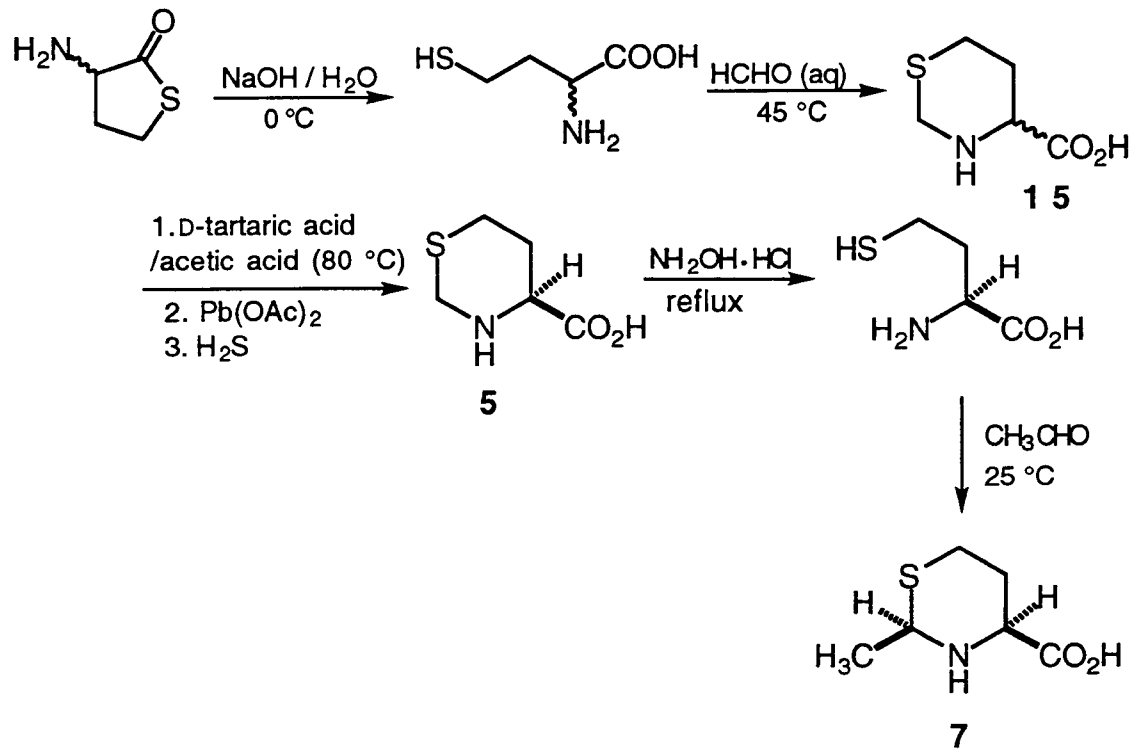
Scheme 2.3 Preparation of 1,3-Oxazine-4-Carboxylic Acid (**4**)

The preparations of 1,3-thiazane-4-carboxylic acid and 2-methyl-1,3-thiazane-4-carboxylic acid derivatives (**5**, **6**, **7**, **8** and **9**) began with D,L-homocysteine thiolactone as starting material (Scheme 2.4). The lactone ring was opened under strongly alkaline condition (4 N NaOH) and the free homocysteine was reacted with formaldehyde to form the racemic 1,3-thiazane-4-carboxylic acid (**15**). An asymmetric transformation was achieved via salt formation with optically active tartaric acid in the presence of salicylaldehyde in acetic acid.[62] Salicylaldehyde, as the catalyst in the asymmetric transformation, accelerates the racemization of the more soluble diastereomeric complex in solution. The optical active tartaric acids only complex with one of enantiomers and the

less soluble diastereomeric salts precipitate from acetic acid. After collecting the salt, excess lead acetate was added to remove the tartate and then hydrogen sulfide was bubbled through the solution to precipitate excess lead acetate. Using D-tartaric acid leads to the conversion of the racemic **15** to (*S*)-1,3-thiazane-4-carboxylic acid (**5**), whereas L-tartaric acid gives rise to (*R*)-1,3-thiazane-4-carboxylic acid (**6**).



Scheme 2.4 Preparation of 5-Thia-L-Pipecolate Analogs **5** and **7**

By treating with hydroxylamine hydrochloride, optically active homocysteine was prepared from either the (*R*) or (*S*)-1,3-thiazane-4-carboxylic acid, which were obtained by the above asymmetric transformation. The (*R*) or (*S*) homocysteine was then condensed with acetaldehyde and the (2*R*, 4*S*) and (2*S*, 4*R*)-2-methyl-1,3-thiazane-4-carboxylic acids (**7** and **9**) were obtained. When the (2*R*, 4*S*) and (2*S*, 4*R*)-2-methyl-1,3-thiazane-4-carboxylic acids (**7** and **9**) were prepared, respectively, it appeared from the

NMR spectra that the cyclization reaction was stereoselective with the carboxyl group oriented *syn* to the methyl group. This stereoselectivity can be explained by the fact that it is energetically most favorable for both groups to be located at the equatorial position of the six member ring. The configuration at C-2 was assigned from the 2D-NOESY data and the known absolute configuration of the carboxylic group at position 4. In the (2*R*, 4*S*) and (2*S*, 4*R*) enantomers, 7 and 9 respectively, a nOe interaction was observed between the protons at position 2 and 4 (Figure 2.3).

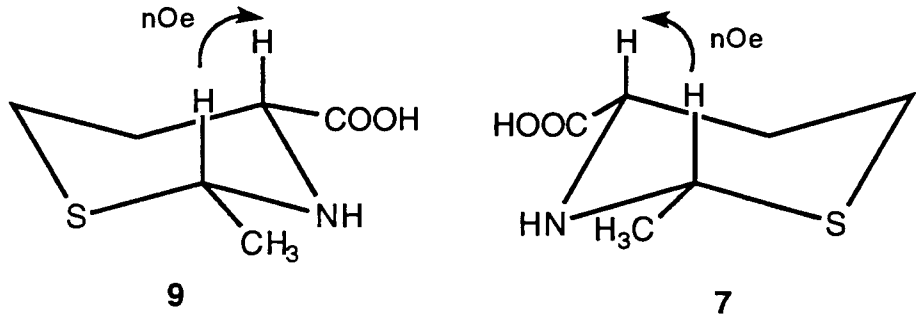
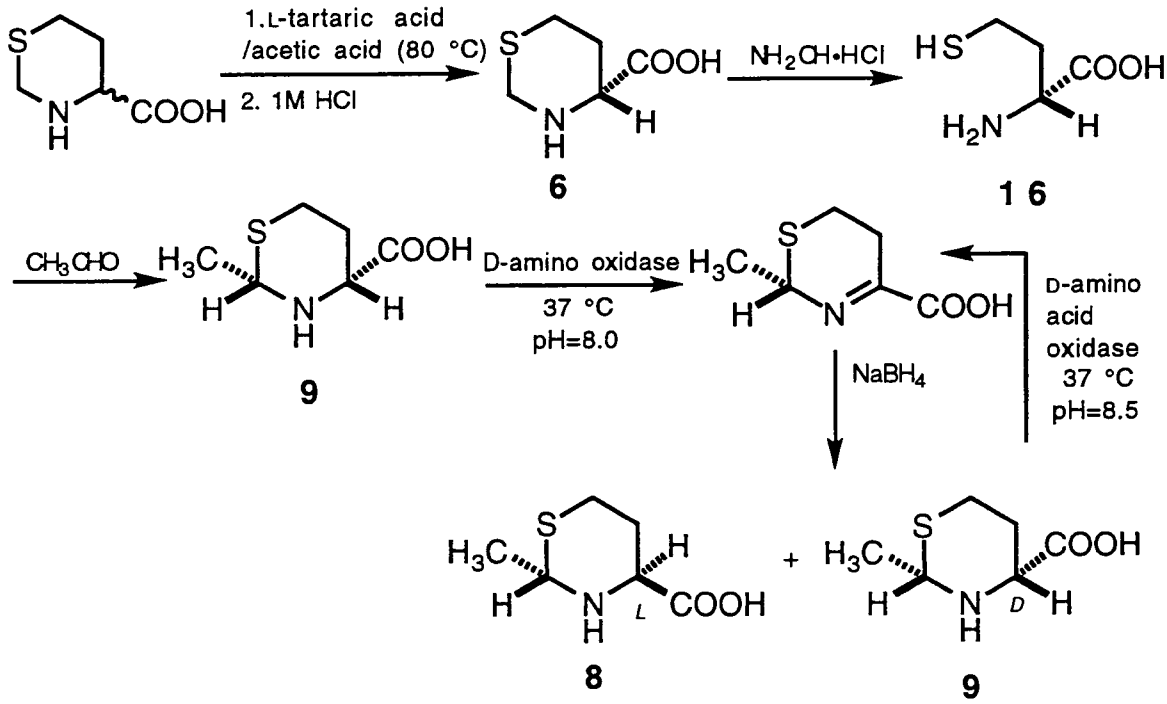


Figure 2.3 Observed nOe Interaction Between H-2 & H-4 in **7** and **9**

Compound **8**, (2*S*, 4*S*)-2-methyl-1,3-thiazane-4-carboxylic acid, was prepared from the (2*S*, 4*R*) diastereomer (**9**) by reaction with D-amino acid oxidase at room temperature followed by reduction with sodium borohydride (Scheme 2.5).[63] Although both (2*S*, 4S) and (2*S*, 4*R*) isomers are obtained after the reduction step, only the (2*S*, 4*R*) diastereomer can be reoxidized by D-amino oxidase. After three oxidation-reduction cycles, the transformation from the (2*S*, 4*R*) isomer to the (2*S*, 4*S*) counterpart was complete as determined by NMR spectroscopy. In the spectra for the (2*S*, 4*S*) diastereoisomer (**8**), no nOe was observed between the protons at positions 2 and 4.



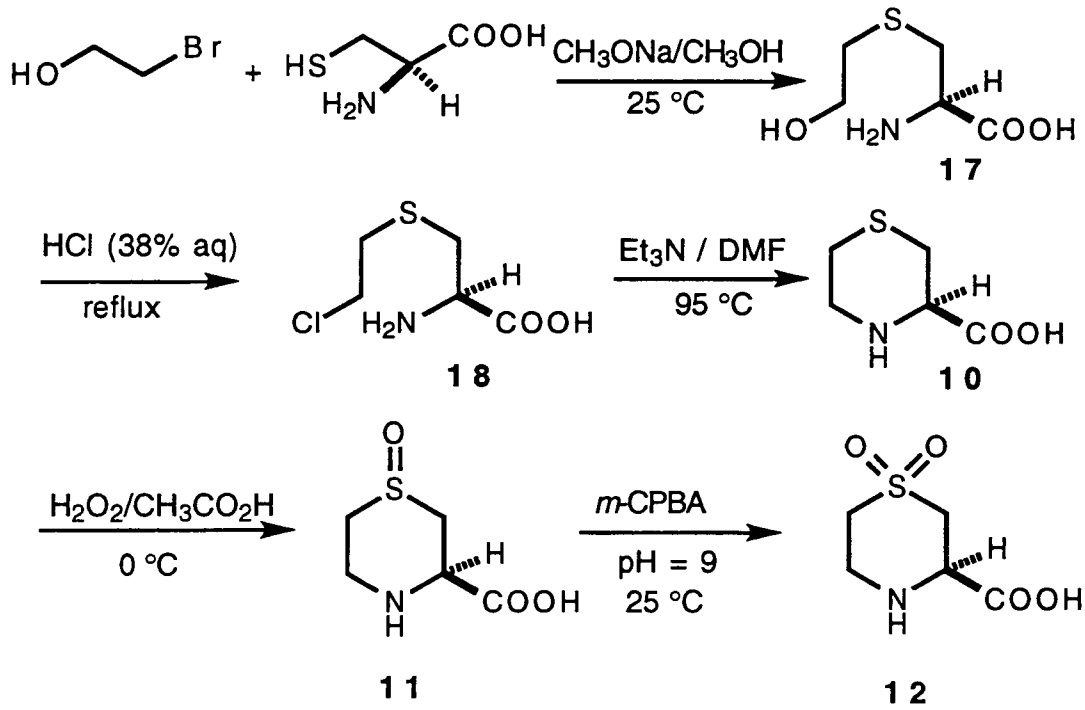
Scheme 2.5 Preparation of 5-Thia-L-Pipecolate Analogs **6**, **8** and **9**

Preparation Of Pipecolate Analogs With Heteroatoms At Position 4

To prepare (*S*)-1,4-thiazane-3-carboxylic acid (**10**), bromoethanol was condensed with L-cysteine under basic conditions to give (*S*)-2-hydroxyethyl-L-cysteine (**17**) (Scheme 2.6). This compound, upon heating in hydrochloric acid (38%), gave (*S*)-2-chloroethyl-L-cysteine hydrochloride (**18**). Finally, the chloride was cyclized in dimethylformamide-triethylamine to yield **10**.[64]

To oxidize (*S*)-1,4-thiazane-3-carboxylic acid (**10**) to the sulfoxide **11**, acid catalysis with hydrogen peroxide was chosen (Scheme 2.6).[64] Sulfur, with its lone pair of electrons, needs an electrophilic oxidizing agent. It is known that hydrogen peroxide is in equilibrium with the protonated peroxide in an acidic medium, which is a stronger electrophile than the conjugate base.[65] However, a nucleophilic attack to the sulfoxide group is necessary to further oxidize the sulfoxide to the sulfone analog **12**. To do this, *m*-chloroperoxybenzoic acid (*m*-CPBA) in a basic medium was chosen (Scheme 2.6).

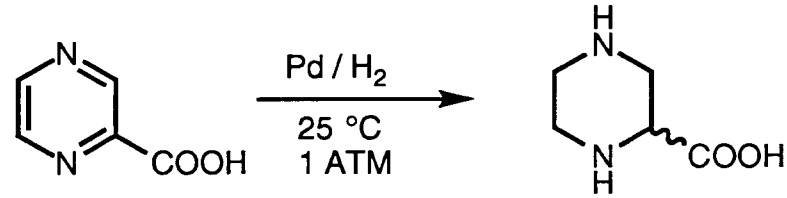
Under basic conditions, *m*-CPBA is almost completely in the anion form, which is a stronger oxidizing form than the neutral molecule.[66]



Scheme 2.6 Preparation 4-Thia-L-Pipecolate Analogs **10**, **11** and **12**

Preparation of (*R*, *S*)-1,4-Piperazine-2-Carboxylic Acid (13)

Catalytic hydrogenation of commercially available 2-pyrazinecarboxylic acid yielded 1,4-piperazine-2-carboxylic acid (**13**) (Scheme 2.7). The reaction proceeds rapidly and cleanly with a palladium catalyst adsorbed on the inert carbon support.



13

Scheme 2.7 Preparation of (*R*, *S*)-1,4-Piperazine-2-Carboxylic Acid (**13**)

MATERIALS AND METHODS

All starting materials and reagents were purchased from commercial sources and were reagent grade or better. All organic solvents were distilled before use and air-sensitive reagents were handled under an atmosphere of dry argon. Anhydrous reactions were done in oven-dried glassware and solvents for reactions were dried and freshly distilled according to Perrin *et al.*[67] Water was double deionized and filtered (0.45 μm) prior to being used. Buffers were prepared with doubly deionized water and the pH was adjusted to the desired value at room temperature with a 4 N NaOH or 4 N HCl solution. All melting points were uncorrected and determined with a Unimelt oil immersion apparatus using open capillary tubes. Optical rotations were measured on a Perkin-Elmer 141 polarimeter with a 10.00 cm (5 mL) cell at ambient temperature. All specific rotations reported were measured at the sodium D line. Infrared spectra (IR) were recorded on a Nicolet 510P-LHS by the potassium bromide plate method. Nuclear magnetic resonance (NMR) spectra were measured on Bruker AC-300 instrument in the specified deuterated solvent. Chemical shifts for ^{1}H-NMR are quoted in ppm and referenced to residual undeuterated solvent. For ^{13}C-NMR, spectra were referenced to internal residual solvent or, for aqueous samples, an external standard of dioxane in D_2O. Mass spectra were obtained in the chemical ionization (CI), electron impact (EI) or fast atom bombardment

(FAB) mode, as specified. Whenever possible, reactions were monitored by thin layer chromatography (TLC). TLC was performed on 0.25 mm aluminum-backed silica gel plates with ultraviolet indicator. Compounds were visualized using ultraviolet light or developed with bromocresol green spray for carboxylic acids and ninhydrin spray for amines. Flash chromatography was performed using silica gel (Merck type 60, 230-400 mesh). Cation-exchange resin was Bio-Rad AG50W-X8 (H^+ form, 100-200 mesh). Anion-exchange resin was Bio-Rad AG1-X8 (acetate form, 100-200 mesh). All ion-exchange resins were regenerated according to the manufacturer's instructions and fully equilibrated and washed before used.

(*S*)-1,2-Diaminobutyric acid (14)

The procedure found in the literature was modified.[57] Sodium azide (5.5 g, 85 mmol) was added in small portions to a mixture of L-(+)-glutamic acid (7.35 g, 50 mmol), 25 mL of concentrated sulfuric acid and 25 mL of chloroform over a period of 6 hours at 45 °C. After overnight stirring, the reaction mixture was poured onto 200 g of ice and the resulting aqueous solution was treated with hot saturated barium hydroxide solution until the pH was 3. Barium sulfate was removed by centrifugation and the aqueous solution was reduced in volume to 100 mL under reduced pressure and applied to a 100 mL Bio-Rad AG50W-X8 (H^+ form, 100-200 mesh) ion-exchange column. The column was washed with 500 mL of water and then eluted with 500 mL of 0.1 N HCl. The volume was reduced to 10 mL under reduced pressure and 100 mL 95% ethanol was added to crystallize the product. Yield: 6.19 g, white needles (65%): mp 198-210 °C dec.; $[\alpha]_D$ +14.7° (*c* 2.15, H_2O) [lit.:[68] m.p. 204 °C; $[\alpha]_D$ +15.1° (*c* 3.82, H_2O)]; IR (KBr) 3051, 1986, 1740, 1616, 1475, 1194 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 3.80 (1H, t, *J*=6.7 Hz, C<u>H</u>COOH), 2.98 (2H, dd, *J*=15.6, 7.1 Hz, $NH_2C\underline{H}_2$), 2.26 (2H, m, $NH_2CH_2C\underline{H}_2$); ^{13}C-NMR (75 MHz, D_2O) δ 175.2 ($CH_3\underline{C}OOH$), 54.5 (<u>C</u>HCOOH),

38.9 ($NH_2\underline{C}H_2$), 30.4 ($NHCH_2\underline{C}H_2$); HREIMS: *m/z* 119.0821 (M^+) [Calcd for $C_{14}H_{10}N_2O_2$: 119.0821].

(*S*)-1,4,5,6-Tetrahydropyrimidine-4-carboxylic acid (1)

A 10 mL solution of (*S*)-1,4-diaminobutyric acid dihydrochloride (**14**) (250 mg, 1.31 mmol) was applied to a 15 mL Bio-Rad AG50W-X8 (H^+ form, 100-200 mesh) cation exchange column. After washing with 60 mL of deionized water, the column was eluted with 60 mL of 0.5 N ammonium hydroxide. The ninhydrin positive fractions was collected and dried down. Part of the residue (150 mg 1.25 mmol) was dissolved with 50 mL water and placed in a 100 ℃ oil bath with formamidine hydrochloride (156 mg, 1.5 mmol) for 3 hours. The mixture was cooled to room temperature and water was removed under reduced pressure. The residue was crystalized from water ethanol and 130 mg white needles was obtained (yield 81.3%): mp 252-253 ℃ dec.; $[\alpha]_D$ +187° (*c* 0.86, H_2O); IR (KBr) 3389, 3154, 1680, 1616, 1496 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 7.98 (1H, s, C<u>H</u>=NH), 4.01 (1H, t, *J*=5.6 Hz, C<u>H</u>COOH), 3.48 (1H, m, NHC<u>H</u>$_2$CH$_2$), 3.34 (1H, m, NHC<u>H</u>$_2$CH$_2$), 2.17 (2H, m, NHCH$_2$C<u>H</u>$_2$); ^{13}C-NMR (75 MHz, D_2O) δ 178.4 (<u>C</u>OOH), 153.1 (<u>C</u>H=NH), 54.8 (<u>C</u>HCOOH), 39.2 (NH<u>C</u>H$_2$CH$_2$), 24.0 (NHCH$_2$<u>C</u>H$_2$); HRFABMS: *m/z* 129.0665 (MH^+) [Calcd for $C_5H_9N_2O_2$: 129.0664]

(*S*)-2-Amino-3,4,5,6-tetrahydropyrimidine-4-carboxy acid (2)

A 50 mL aqueous solution of (*S*)-1,4-diaminobutyric acid (**14**) (0.525 g, 2.75 mmol) was pH adjusted to 9.0 with 1 N sodium hydroxide solution and a 5 M cyanogen bromide acetonitrile solution (0.66 mL, 3.3 mmol) was added. After stirring at room temperature for 5 hours, the mixture was washed three times with ethyl acetate, then reduced to a volume of 10 mL and loaded onto a 100 mL Bio-Rad AG50W-X8 (H^+ form, 100-200 mesh) cation exchange column. The column was washed with 500 mL deionized

water and eluted with 350 mL 0.1 N HCl. The solvent was removed under reduced pressure and the residue was crystallized from water and isopropanol at -20 °C. Yield: 130 mg white needles (36% yield): mp 216 °C; $[\alpha]_D$ +120° (*c* 0.72, H_2O) [lit:[57] mp 211 °C; $[\alpha]_D$ +144° (*c* 1.08, H_2O)]; IR (KBr) 3431, 3325, 1662, 1604, 1562 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 4.39 (1H, t, *J*=10.2 Hz, C<u>H</u>COOH), 3.41 (2H, dt, *J*=2.4, 9.4 Hz, NHC<u>H</u>$_2$CH$_2$), 2.52 (1H, m, NHCH$_2$C<u>H</u>$_2$), 2.09 (1H, m, NHCH$_2$C<u>H</u>$_2$); ^{13}C-NMR (75 MHz, D_2O) δ 180.6 (<u>C</u>OOH), 163.0 (NH$_2$<u>C</u>H) 53.8 (<u>C</u>HCOOH), 41.4 (NH<u>C</u>H$_2$CH$_2$), 25.9 (CH$_2$<u>C</u>H$_2$CH); HRFABMS *m/z* 144.0773 (MH^+) [Calcd for $C_5H_{10}N_3O_2$: 144.0773]

(*S*)-Hexahydropyrimidine-4-carboxy acid (3)

To a 17 mL solution of (*S*)-1,4-diaminobutyric acid (**14**) (199.5 mg, 1.69 mmol) at 0 °C, 37 % aqueous formaldehyde (0.15 mL, 1.86 mmol) was added, dropwise, over half an hour and stirred at 0 °C for another half hour. After drying down the solution, the residual oil was crystallized from methanol and dichloromethane. Yield: 120 mg (55%): mp 191-192 °C; $[\alpha]_D$ +33.1° (*c* 0.21, H_2O); IR (KBr) 3271, 3127, 2955, 1635, 1585 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 4.48 (1H, d, *J*=12.0 Hz, NHC<u>H</u>$_2$NH), 4.05 (1H, d, *J*=12.0 Hz, NHC<u>H</u>$_2$NH), 3.62 (1H, dd, *J*=12.3 Hz, C<u>H</u>COOH), 3.41 (1H, d, *J*=13.0 Hz, NHC<u>H</u>$_2$), 3.11 (1H, m, NHC<u>H</u>$_2$), 2.15 (1H, d, *J*=14.6 Hz, CHC<u>H</u>$_2$CH$_2$), 1.76 (1H, d, *J*=12.3 Hz, CHC<u>H</u>$_2$CH$_2$); ^{13}C-NMR (75 MHz, D_2O) δ 178.9 (<u>C</u>OOH), 60.8 (NH<u>C</u>HNH), 59.1 (<u>C</u>HCOOH), 44.8 (CH$_2$<u>C</u>H$_2$NH), 28.9 (<u>C</u>H$_2$CH$_2$NH); HREIMS: *m/z* 129.0664 (M^+) [Calcd for $C_5H_9N_2O$: 129.0664]

(*R, S*)-1,3-Thiazane-4-carboxylic acid (15)

The procedure found in the literature was modified.[62] A solution of D,L-1,3-homocysteine thiolactone hydrochloride (1.0 g, 6.51 mmol) and sodium hydroxide (1.65 g, 41.25 mmol) in 8 mL of deionized water was stirred at 0 °C for 30 minutes before the

pH was adjusted to 6 with 6 N HCl. Formaldehyde (0.57 mL of 37% aqueous solution, 7.81 mmol) was added and the reaction was stirred at room temperature for 5 hours. The solution was concentrated and 20 mL of acetic acid was added to extract the residue. The filtrate was collected and dried down. After acetic acid was used to extract the residue again, the crude product from the acetic acid filtrate was recrystallized from water and isopropyl alcohol. Yield: 670 mg (70%): mp 213-214 °C; [lit:[62] mp 218-219 °C without crystallization from water and isopropyl alcohol]; IR (KBr) 3364, 2704, 1738, 1635, 1564, 1431, 1377, 1290, 1250, 1205 cm^{-1}; ^{1}H-NMR (300 MHz, CD_3COOD) δ 4.49 (2H, dd, *J*=13.0 Hz, $C\underline{H}_2NH$), 4.05 (1H, d, *J*=10.4 Hz, $C\underline{H}COOH$), 3.04 (1H, t, *J*=13.7, 11.5 Hz, $SC\underline{H}_2CH_2$), 2.88 (1H, d, *J*=13.7 Hz, $SC\underline{H}_2CH_2$), 2.71 (1H, d, *J*=13.7 $SCH_2C\underline{H}_2$), 2.27 (1H, dd, *J*=13.7, 11.5 Hz, $SCH_2C\underline{H}_2$); ^{13}C-NMR (75 MHz, CD_3COOD) δ 177.9 ($\underline{C}OOH$), 63.0 ($S\underline{C}H_2NH$), 50.7 ($COOH\underline{C}H$), 33.2 ($S\underline{C}H_2CH_2$), 31.0 ($SCH_2\underline{C}H_2$); HRCIMS: *m/z* 148.0432 (MH^+) [Calcd for $C_5H_{10}NO_2S$: 148.0432]

(*S*)-1,3-Thiazane-4-carboxylic acid (5)

The procedure found in the literature was modified.[62] To a mixture of D,L-1,3-thiazane-4-carboxylic acid (**15**) (1.44 g, 9.8 mmol) and D-tartaric acid (1.47 g, 9.8 mmol) in 8 mL of acetic acid at 80 °C was added salicylaldehyde (1.2 g, 9.8 mmol). After stirring the mixture for 6 hours and then one addition hour at room temperature, the resulting product was collected by filtration, washed first with 2 mL of acetic acid and then thoroughly with diethyl ether and dried. The residue was dissolved in 100 mL of water and 3 g of lead acetate were added within 10 minutes with stirring at room temperature. After the precipitate was filtered, the filtrate was saturated with hydrogen sulfide gas. The precipitate was removed and the filtrate was dried down *in vacuo*. The residue was crystallized from water and isopropyl alcohol. Yield: 278 mg (38.6%): mp 218-219 °C; $[\alpha]_D$ -12.2° (*c* 0.86, 1 N HCl) [lit:[62] $[\alpha]_D$ -12.5° (*c* 1.00, 1 N HCl); mp 275-278 °C]; IR (KBr) 3437, 2919, 1736, 1631, 1431 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 4.34 (2H, q,

J=13.2 Hz, $SC\underline{H}_2NH_2$), 4.11 (1H, dd, *J*=11.8, 2.8 Hz, C<u>H</u>COOH), 3.00 (1H, d, *J*=14.0 Hz, $SC\underline{H}_2CH_2$), 2.84 (1H, d, *J*=14.0 Hz, $SC\underline{H}_2CH_2$), 2.58 (1H, d, *J*=13.9 Hz, $SCH_2C\underline{H}_2$), 2.10 (1H, m, $SCH_2C\underline{H}_2$); ^{13}C-NMR (75 MHz D_2O) δ 173.4 (<u>C</u>OOH), 59.9 ($S\underline{C}H_2NH$), 47.8 (<u>C</u>HCOOH), 29.8 ($S\underline{C}H_2CH_2$), 28.0 ($SCH_2\underline{C}H_2$); HREIMS: *m/z* 147.0354 (M^+) [Calcd for $C_5H_9NO_2S$: 147.0354]; Analysis: C, 40.65; H, 6.11; N, 9.28 % [Calcd for $C_5H_9NO_2S$: C, 40.81; H, 6.17; N, 9.52 %]

(*R*)-1,3-Thiazane-4-carboxylic acid (6)

The literature procedure was modified.[62] To a mixture of D,L-1,3-thiazane-4-carboxylic acid (**15**) (123 mg, 0.745 mmol) and L-tartaric acid (126 mg, 0.745 mmol) in 2 mL of acetic acid at 80 °C was added salicyaldehyde (10.9 mg, 0.745 mmol). After stirring the mixture for 6 hours and then 1 hour at room temperature, the resulting product was collected by filtration and washed with 2 mL of acetic acid and a large amount of diethyl ether. The resulting product was crystallized from 1 N HCl and isopropyl alcohol. Yield: 42 mg (38%): mp 223-224 °C dec.; $[\alpha]_D$ +12.9° (*c* 0.856, 1 N HCl) [lit:[62] $[\alpha]_D$ +12.5°(*c* 1.00, 1 N HCl), mp 275-277 °C]; IR (KBr) 3412, 1738, 1635, 1552 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 4.43 (2H, dd, *J*=13.2 Hz, $SC\underline{H}_2NH$), 3.75 (1H, d, *J*=10.3 Hz, C<u>H</u>COOH), 3.03 (1H, t, *J*=11.6, 13.4 Hz, $SC\underline{H}_2CH_2$), 2.86 (1H, *J*=14.6 Hz $SC\underline{H}_2CH_2$), 2.61 (1H, d, *J*=14.6 Hz, $SCH_2C\underline{H}_2$), 2.10 (1H, m, $SCH_2C\underline{H}_2$); ^{13}C-NMR (75 MHz, D_2O) δ 175.6 (<u>C</u>OOH), 61.8 ($S\underline{C}H_2NH$), 47.6 (<u>C</u>HCOOH), 30.5 ($S\underline{C}H_2CH_2$), 28.4 ($SCH_2\underline{C}H_2$); HREIMS: *m/z* 147.0350 (M^+) [Calcd for $C_5H_9NO_2S$: 147.0354]

(*S*)-Homocysteine (16)

The literature procedure was modified.[62] To 3 mL of freshly distilled ethanol solution of hydroxylamine hydrochloride (11.72 mg, 0.168 mmol), (*S*)-1,3-thiazane-4-carboxylic acid (**5**) (124 mg, 0.84 mmol) in 5 mL of ethanol solution was added under

reflux. Then, five portions of freshly distilled triethylamine (50 μL, 0.168 mmol) and four portions of the ethanol solution of hydroxylamine hydrochloride (11.7 mg, 0.168 mmol) were alternately added to the reaction mixture at 10 minute intervals to keep the pH of the solution at 8. After refluxing the mixture for another hour, 0.1 mL of triethylamine was added and then the mixture was cooled to room temperature. The precipitate was collected by filtration, washed with a small amount of ethanol and dried. Yield: 60 mg (52.8%): mp 242-244 °C dec.; $[\alpha]_D$ + 25.7° (*c* 0.752, 1 N HCl) [lit:[62] mp 245-247 °C; $[\alpha]_D$ +26.8° (*c* 1.00, 1 N HCl)]; IR (KBr) 2936, 2093, 1581, 1448, 1417, 1321 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 3.92 (1H, t, *J*=6.8 Hz, C<u>H</u>COOH), 2.67 (2H, m, HSC<u>H</u>$_2$), 2.17 (2H, dt, *J*=14.6, 6.4 Hz, HSCH$_2$C<u>H</u>$_2$); ^{13}C-NMR (75 MHz, D_2O) δ 176.6 (<u>C</u>OOH), 56.0 (<u>C</u>HNH$_2$), 37.0 (HS<u>C</u>H$_2$), 22.2 (HSCH$_2$<u>C</u>H$_2$); HRFABMS: *m/z* 136.0432 (MH^+) [Calcd for $C_4H_{10}NO_2S$: 134.0432]

(2*R*, 4*S*)-2-Methyl-1,3-thiazane-4-carboxylic acid (7)

To a stirred solution of (*S*)-homocysteine (**16**) (2.23 g, 16.5 mmol) in water (60 mL), acetaldehyde (0.798 g, 18.14 mmol) was added, dropwise, over 1 hour and the mixture was stirred for 4 hours at room temperature. The precipitate was filtered and 20 mL of acetic acid was used to extract the solid. The filtrate was collected and evaporated to dryness. The residue was crystallized from hot water and ethanol. Yield: 50 mg (19%): mp 198-199 °C; $[\alpha]_D$ -3.5° (*c* 0.43, 0.5 N HCl); IR (KBr) 2910, 2770, 1747, 1549 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 4.53 (1H, q, *J*=6.6 Hz, CH$_3$C<u>H</u>S), 3.68 (1H, dd, *J*=13.8, 2.8 Hz, C<u>H</u>COOH), 3.15 (1H, dt, *J*=14.1, 2.6 Hz, CH$_2$C<u>H</u>$_2$S), 2.91 (1H, m, CH$_2$C<u>H</u>$_2$S), 2.63 (1H, qd, *J*=14.8, 2.8 Hz, C<u>H</u>$_2$CH$_2$S), 1.98 (1H, m, C<u>H</u>$_2$CH$_2$S), 1.65 (3H, d, *J*=6.6 Hz, C<u>H</u>$_3$CH); ^{13}C-NMR (75 MHz, D_2O) δ 173.3 (<u>C</u>OOH), 61.0 (CH$_3$<u>C</u>HS), 58.5 (<u>C</u>HCOOH), 29.3 (CH$_2$<u>C</u>H$_2$S), 29.1 (<u>C</u>H$_2$CH$_2$S), 20.6 (<u>C</u>H$_3$CHS); HREIMS: *m/z* 161.0511 (M^+) [Calcd for $C_6H_{11}NO_2S$: 161.0511]

(2*S*, 4*R*)-2-Methyl-1,3-thiazane-4-carboxylic acid (8)

To a 40 mL freshly distilled ethanol containing with (*R*)-1,3-thiazane-4-carboxylic acid (**6**) (6.53 g, 44.4 mmol) was added 70 mL of freshly distilled ethanol solution with hydroxylamine hydrochloride (0.57 g, 8.88 mmol). After refluxing the mixture in a 100 °C oil bath, five portions of freshly distilled triethylamine (5.7 mL, 44.4 mmol) and four portions of the ethanol solution of hydroxylamine hydrochloride (2.3 g, 35.5 mmol) were added to the mixture at 10 minutes interval to keep the pH at 8. The reaction was refluxed for one addition hour before being cooled down to the room temperature. The precipitate was collected by filtration, washed with a small amount ethanol and dried. Then, it was redissolved with 100 mL of water and acetaldehyde (2.47 mL, 44.4 mmol) was added, dropwise, over a half hour period. The mixture was stirred for four hours at room temperature and concentrated to dryness under reduced pressure. The residue was crystallized from water and isopropanol at 4 °C. Yield: 80 mg white needles (11.7%): mp 197-198 °C; $[\alpha]_D$ +14.5° (*c* 0.77, 0.5 N HCl); IR (KBr) 2801, 1579, 1390, 1329, 1246, 1199 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 4.61 (1H, m, $CH_3C\underline{H}S$), 4.08 (1H, d, *J*=12.8 Hz, $C\underline{H}COOH$), 3.15 (1H, t, *J*=13.1, 12.0 Hz, $CH_2C\underline{H}_2S$), 2.88 (1H, m, $CH_2C\underline{H}_2S$), 2.65 (1H, d, *J*=13.6 Hz, $C\underline{H}_2CH_2S$), 2.01 (1H, m, $C\underline{H}_2CH_2S$), 1.59 (3H, dd, *J*=6.2 Hz, $C\underline{H}_3CH$); ^{13}C-NMR (75 MHz, D_2O) δ 173.0 (<u>C</u>OOH), 60.8 ($CH_3\underline{C}HS$), 58.5 (<u>C</u>HCOOH), 29.2 ($CH_2\underline{C}H_2S$), 29.0 ($CH_2\underline{C}H_2S$), 20.6 ($\underline{C}H_3CH$); HRCIMS: *m/z* 161.05111 (M^+) [Calcd for $C_{12}H_{11}NO_2S$: 161.0511]

(2*S*, 4*S*)-2-Methyl-1,3-thiazane-4-carboxylic acid (9)

To a solution of (2*S*, 4*R*)-2-methyl-1,3-thiazane-4-carboxylic acid (**8**) (160 mg, 0.93 mmol) in 4 mL of 0.5 M pH 8.0 potassium phosphate buffer, there was added 5 units of porcine kidney D-amino acid oxidase suspension. After 45 minutes, sodium borohydride (9.6 mg, 2.38 mmol) was added followed by catalase (150 unit) 15 minutes

later. The same cycle was repeated two more times after the catalase addition. The mixture was adjusted to pH 3.4 and centrifuged at 10,000 x *g* for 15 minutes to remove the precipitated protein. The filtrate was applied to a 30 mL Bio-Rad AG50W-X8 (H^+ form, 100-200 mesh) ion exchange column. After thoroughly washing with water, the column was eluted with 1 N NH_4OH. The ninhydrin positive fractions were collected, pooled and concentrated. The residue was crystallized from water and isopropanol at room temperature. Yield: 80 mg (53.3%): mp 194-195 °C; $[\alpha]_D$ +6.5° (*c* 0.74, 0.5 N HCl); IR (KBr) 2772, 2467, 1747, 1547, 1404, 1298, 1278, 1257 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 4.65 (1H, q, *J*=5.9 Hz $CH_3C\underline{H}S$), 4.14 (1H, d, *J*=12.8 Hz, $C\underline{H}COOH$), 3.25 (1H, t, *J*=13.3 Hz $CH_2C\underline{H}_2S$), 2.99 (1H, dd, *J*=13.8, 2.5 Hz, $CH_2C\underline{H}_2S$), 2.76 (1H, d, *J*=14.8 Hz, $C\underline{H}_2CH_2S$), 2.14 (1H, m, $C\underline{H}_2CH_2S$), 1.70 (3H, d, *J*=5.5 Hz, $C\underline{H}_3CH$); ^{13}C-NMR (75 MHz, D_2O) δ 173.2 ($\underline{C}OOH$), 60.8 ($CH_3\underline{C}HS$), 58.5 ($\underline{C}HCOOH$), 29.2 ($CH_2\underline{C}H_2S$), 29.0 ($\underline{C}H_2CH_2S$), 20.6 ($\underline{C}H_3CH$); HREIMS: *m/z* 161.0511 (M^+) [Calcd for $C_6H_{11}NO_2S$: 161.0511]

1,3-Oxazane-4-carboxylic acid (4)

To a stirred solution of D,L-homoserine (222.5 mg, 1.868 mmol) in 5 mL of water at room temperature, 37% aqueous formaldehyde solution (0.15 mL, 1.868 mmol) was added, dropwise, over a half hour period. The solution continued to stir for one hour at room temperature before being concentrated to dryness. The residue was crystallized from water and isopropyl alcohol. Yield: 268 mg (92%): mp 204 °C; IR (KBr) 3391, 3289, 2991, 2845, 1593 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 4.71 (1H, d, *J*=10.5 Hz, $HNC\underline{H}_2O$), 4.33 (1H, d, *J*=10.5 Hz $HNC\underline{H}_2O$), 4.20 (1H, d, *J*=8.6 Hz, $C\underline{H}COOH$), 3.86 (1H, t, *J*=10.3 Hz, $OC\underline{H}_2CH_2$), 3.50 (1H, dd, *J*=11.4, 3.0 Hz, $OC\underline{H}_2CH_2$), 1.94 (1H, d, *J*=12.6 Hz, $OCH_2C\underline{H}_2$), 1.79 (1H, m, $OCH_2C\underline{H}_2$); ^{13}C-NMR (75 MHz, D_2O) δ 182.3 ($\underline{C}OOH$), 79.7 ($NH\underline{C}H_2O$), 69.3 ($\underline{C}HCOOH$), 59.9 ($CH_2\underline{C}H_2O$), 32.9 ($\underline{C}H_2CH_2O$); HREIMS: *m/z* 130.0504 (M^+) [Calcd for $C_5H_8NO_3$: 130.0504]

(*S*)-2-Hydroxyethyl-L-cysteine (17)

The literature procedure was modified.[64] To a solution of sodium hydroxide (254 mg, 6.35 mmol) in 2.5 mL of water, L-cysteine (769 mg, 6.35 mmol) was added. Bromoethanol (952.5 mg. 7.62 mmol) was then added cautiously in small portions over a period of one hour after which 3 mL of 95% ethanol was added to the solution. The mixture was stirred at room temperature overnight before being dried down. The residue was redissolved with 10 mL of water and washed with 10 mL ethyl acetate three times. The aqueous layer was dried *in vacuo* and the crude product was crystallized from water and ethanol. Yield: 842 mg (80%): mp 187-188 °C; $[\alpha]_D$ -52.5° (*c* 1.38, H_2O) [lit.:[64] mp 189-190 °C; $[\alpha]_D$ -53.3° (*c* 2.00, H_2O)]; ^{1}H-NMR (300 MHz, D_2O) δ 3.95 (1H, t, *J*=5.4 Hz, C<u>H</u>COOH), 3.78 (2H, t, *J*=5.9 Hz, HOC<u>H</u>$_2$CH$_2$), 3.18 (2H, m, HOCH$_2$C<u>H</u>$_2$), 2.79 (2H, t, *J*=5.7 Hz, C<u>H</u>$_2$CHCOOH); ^{13}C-NMR (75 MHz, D_2O) δ 175.2 (<u>C</u>OOH), 62.5 (<u>C</u>HCOOH), 56.1 (HO<u>C</u>H$_2$CH$_2$), 36.1 (HOCH$_2$<u>C</u>H$_2$), 34.6 (<u>C</u>H$_2$CHCOOH); LREIMS: *m/z* 165 (M^+) [Calcd for $C_5H_{11}NO_3S$: 165.0460]

(*S*)-2-Chloroethyl-L-cysteine hydrochloride (18)

The literature procedure was modified.[64] A solution of 842 mg (5.1 mmol) of (*S*)-2-hydroxyethyl-L-cysteine (**17**) in 20 mL of 38% hydrochloric acid was heated for 7 hours at 95 °C and then concentrated to a white solid *in vacuo*. The crude product was crystallized from isopropyl alcohol. Yield: 700 mg white needles (75%): mp 183-184 °C [lit:[64] 181.5-182°C]; $[\alpha]_D$ -6.6° (*c* 0.65, H_2O); IR (KBr) 2901, 1736, 1593, 1437, 1406, 1352, 1300, 1228 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 4.36 (1H, t, *J*=6.3 Hz, C<u>H</u>COOH), 3.80 (2H, t, *J*=6.8 Hz, ClC<u>H</u>$_2$CH$_2$), 3.33 (2H, m, ClCH$_2$C<u>H</u>$_2$), 3.03 (2H, t, *J*=6.3 Hz, C<u>H</u>$_2$CHCOOH); ^{13}C-NMR (75 MHz, D_2O) δ 172.7 (CH<u>C</u>OOH), 54.8 (<u>C</u>HCOOH), 45.9 (Cl<u>C</u>H$_2$CH$_2$), 36.4 (CH$_2$S<u>C</u>H$_2$), 33.8 (<u>C</u>H$_2$SCH$_2$); LRFABMS: *m/z* 183 $(M+H)^+$ [Calcd for $C_5H_{10}NO_2SCl$: 183.0121]

(*S*)-1,4-Thiazane-3-carboxylic acid (10)

The literature procedure was modified.[64] To a solution of 660 mg (3.0 mmol) of (*S*)-2-chloroethyl-L-cysteine hydrochloride (**18**) in 15 mL of freshly distilled dimethylformamide, there was added 5.4 mL (39 mmol) of freshly distilled triethylamine. A white solid precipitated. The mixture continued to stir in a 95 °C oil bath for 2.5 hours. The precipitate was collected and redissolved in 20 mL of water before being applied to a 20 mL column of Bio-Rad AG50W-X8 (H^+ form, 100-200 mesh) cation exchange resin, which was then washed with 200 mL of water and finally eluted with 75 mL of 1 N ammonium hydroxide. The nihydrin positive fractions were concentrated and crystallized from isopropyl alcohol and ethyl acetate. Yield: 270 mg white needles (61%): mp 261-262 °C; $[\alpha]_D$ -53.0° (*c* 0.1, H_2O) [lit:[64] mp 262-263 °C, $[\alpha]_D$ -54° (*c* 1.6, H_2O)]; IR (KBr) 3072, 2885, 2278, 1606, 1469 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 3.90 (1H, dd, *J*=10.1, 3.3 Hz, C<u>H</u>COOH), 3.77 (1H, td, *J*=13.2 3.2 Hz, $CH_2C\underline{H}_2NH$), 3.38 (1H, m, $CH_2C\underline{H}_2NH$), 3.17 (1H, m, $CHC\underline{H}_2S$), 3.06-2.94 (2H, m, $C\underline{H}_2CH_2NH$, $CHC\underline{H}_2S$), 2.84 (1H, m, $C\underline{H}_2CH_2NH$); ^{13}C-NMR (75 MHz, D_2O) δ 174.3 (<u>C</u>OOH), 61.3 (<u>C</u>HCOOH), 47.3 ($CH_2\underline{C}H_2NH$), 29.5 ($\underline{C}H_2S$), 25.8 ($\underline{C}H_2CH_2NH$); HREIMS: *m/z* 147.0353 (M^+) [Calcd for $C_5H_9NO_2S$: 147.0354]

(*S*)-1,4-Thiazane-3-carboxylic acid 1-oxide (11)

The literature procedure was modified.[64] To a suspension of (*S*)-1,4-thiazane-3-carboxylic acid (**10**) (143.2 mg, 0.873 mmol) in 10 mL of acetic acid, 30% hydrogen peroxide (0.11 mL, 0.96 mmol) was added in 0.04 mL portions over a period of two hours with continuous stirring at 25 °C. The solution was allowed to stand at room temperature for one half hour and was then concentrated to an oil. The residue was crystallized from water and acetone. Yield; 65 mg white needles (46%): mp 249 °C; $[\alpha]_D$ +18.1° (*c* 1.03, H_2O) [lit:[64] mp 252 °C from H_2O and ethanol, $[\alpha]_D$ +19.0° (*c* 1.0, H_2O)];

IR (KBr) 3451, 1641, 1392, 1292, 1086 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 4.35 (1H, d, *J*=12.9 Hz, C<u>H</u>COOH), 3.81-3.61 (3H, m, C<u>H</u>$_2$S(O)C<u>H</u>$_2$CH,), 3.34 (1H, d, *J*=15.3 Hz, C<u>H</u>$_2$S(O)CH$_2$), 3.19 (2H, m, C<u>H</u>$_2$NH); ^{13}C-NMR (75 MHz, D_2O) δ 173.7 (<u>C</u>OOH), 50.6 (<u>C</u>HCOOH), 46.2 (S<u>C</u>H$_2$), 42.4 (<u>C</u>H$_2$S), 36.4 (<u>C</u>H$_2$NH); HREIMS: *m/z* 163.0304 (M^+) [Calcd for $C_5H_9NO_3S$: 163.0304]

(*S*)-1,4-Thiazane-3-carboxylic acid 1-dioxide (12)

To a stirred solution of (*S*)-1,4-thiazane-3-carboxyliclic acid 1-oxide (**11**) (254 mg, 1.56 mmol) in 15 mL of 1 N $NaHCO_3$ at pH = 10, *m*-chloroperoxybenzoic acid (455 mg of 60% reagent, 1.56 mmol) was added by small portions over a two-hour period. After stirring the solution half hour at room temperature, adjusting the pH of the solution to 4 and filtering the precipitate, the solution was applied to a 10 mL Bio-Rad AG50W-X8 (H^+ form, 200-400 mesh) cation exchange column, which was then washed thoroughly with water and finally eluted with 0.5 N ammonium hydroxide. The ninhydrin positive fractions were collected, pooled, dried down and the product was crystallized from water and acetone. Yield: 75 mg (26.9%): mp 220-221 °C; $[\alpha]_D$ -2.6° (*c* 0.192, 1 N HCl); IR (KBr) 3364, 2995, 1716, 1398 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 3.67 (1H, dd, *J*=9.1, 2.5 Hz, C<u>H</u>COOH), 3.51 (2H, m, C<u>H</u>$_2$NH), 3.23 (4H, m, C<u>H</u>$_2$S(O)$_2$C<u>H</u>$_2$); ^{13}C-NMR (75 MHz, D_2O) δ 61.3 (<u>C</u>HCOOH), 56.6 (<u>C</u>H$_2$S), 53.1 (<u>C</u>H$_2$S), 44.5 (<u>C</u>H$_2$NH); HRFABMS: *m/z* 178.0174 $(M-H)^+$ [Calcd for $C_5H_8NO_4S$: 178.0174]

1,4-Piperazine-2-carboxylic acid (13)

To a suspension of 2-pyrazinecarboxylic acid (1 g, 8.1 mmol) in 40 mL of acetic acid, 5% palladium on carbon (50 mg) was added and the solution was stirred under a hydrogen balloon atmosphere (balloon pressure) overnight at room temperature. After decolorizing with charcoal three times, the solution was applied to a 20 mL Bio-Rad

AG50W-X8 (H^+ form, 100-200 mesh) cation exchange column, which was washed with 80 mL water and then eluted with 1 N ammonium hydroxide. The ninhydrin positive fractions were collected, pooled, dried down and the product was crystallized with 1 N HCl and isopropyl alcohol at room temperature. Yield: 10 mg (10 %): mp 249-250 °C; IR (KBr) 3026, 2812, 1757, 1633, 1533, 1435, 1388, 1319 cm^{-1}; ^{1}H-NMR (300 MHz, D_2O) δ 4.41 (1H, dd, *J*=11.1, 4.0 Hz, C<u>H</u>COOH), 4.00 (1H, d, *J*=14.0 Hz, C<u>H</u>$_2$CHCOOH), 3.81-3.69 (2H, m, C<u>H</u>$_2$CHCOOH, C<u>H</u>$_2$NH), 3.57-3.40 (3H, m, NHC<u>H</u>$_2$C<u>H</u>$_2$NH); ^{13}C-NMR (75 MHz, D_2O) δ 169.8 (<u>C</u>OOH), 55.6 (<u>C</u>HCOOH), 44.3 (<u>C</u>H$_2$NH), 42.2 (<u>C</u>H$_2$NH), 41.9 (<u>C</u>H$_2$NH); HREIMS: *m/z* 130.0742 (M^+) [Calcd for $C_5H_{10}N_2O_2$: 130.0742]

CHAPTER 3

KINETIC AND MECHANISTIC STUDIES OF ALTERNATE SUBSTRATES AND INHIBITORS OF L-PIPECOLATE OXIDASE

One of the goals of this project was to gain insight into the chemical mechanism of L-pipecolate oxidase. Towards this goal, we have synthesized a variety of substrate analogs and studied the effect of each compound on the reaction catalyzed by L-PO. This chapter is largely concerned with the kinetic analysis of the reaction of L-PO with L-pipecolate analogs because kinetic measurements of enzymatically catalyzed reactions are among the most powerful techniques for elucidating the catalytic mechanism of an enzyme. It is widely accepted that the rate of the enzymatic reaction and how this rate changes in response to different conditions is intimately related to the path followed by the reaction and is therefore indicative of its reaction mechanism. Through kinetic data, we can better understand the details of the various steps in the reaction process and the sequence in which they occur. To conduct such studies, the enzyme must first be isolated and purified.

ENZYME ISOLATION AND ASSAY

L-PO was isolated from Rhesus monkey liver following the method of Mihalik *et al.*[18] The isolation is quite straightforward and makes use of some unique properties of L-PO. First, the monkey liver was finely minced and homogenized. Through centrifugation, the membrane bound L-PO can be separated from soluble proteins. Because the protein is associated with the membrane rather than the soluble fraction, centrifugation results in a 2.5-fold purification. After using detergent to disrupt the membrane and release L-PO into the solution, a heat treatment step was next employed to take advantage of the enzyme's

heat-stability and another 2.5-fold purification was obtained. L-PO is more basic than most proteins with a pI of 8.9. The basic nature of the protein enabled a 13-fold purification by anion exchange chromatography on CM-cellulose resin in a single batch step. The high affinity of L-pipecolic oxidase for phenyl-sepharose, a hydrophobic resin, makes possible another 11-fold purification in a single step. This tight binding to phenyl-sepharose is due to the highly hydrophobic nature of this enzyme. The protein was judged to be about 90% pure as determined by SDS-polyacrylamide gel electrophoresis after this step.

Two enzyme assay methods were used in this study and were described earlier in Chapter 1. The first is a spectrophotometric assay which couples hydrogen peroxide production from the oxidase reaction to the oxidation of *o*-dianisidine catalyzed by horseradish peroxidase.[18] The second assay is more sensitive through the measurement of the radiolabeled reaction product, L-α-aminoadipate-δ-semialdehyde.[20] In this assay, sodium bisulfite is used to trap the semialdehyde. The bisulfite adduct has a net negative charge and is unretained on an AG50W-X8 cation exchange column, whereas the positively charged L-PA is bound. The detailed isolation and assay procedures can be found in the experimental section at the end of this chapter.

KINETIC STUDIES OF SUBSTRATE ANALOGS

Each of the thirteen compounds discussed in Chapter 2 was evaluated as an inhibitor or alternate substrate of L-pipecolate oxidase. These compounds were usually tested as an inhibitor first and then as a substrate. In the inhibition studies, 1 to 5 mM of the L-PA analog was incubated in the enzyme solution along with 3.5 mM L-PA (K_M of the natural substrate). The activity of L-PO was monitored by spectrophotometric or radio-assay. At the same time, a sample without analog was manipulated as a control. The degree of inhibition was determined by comparing the enzyme activity in the test and control samples. If a compound was found to decrease the enzyme activity in the inhibition

experiment, a thorough analysis was carried out to determine the type of inhibition mechanism.

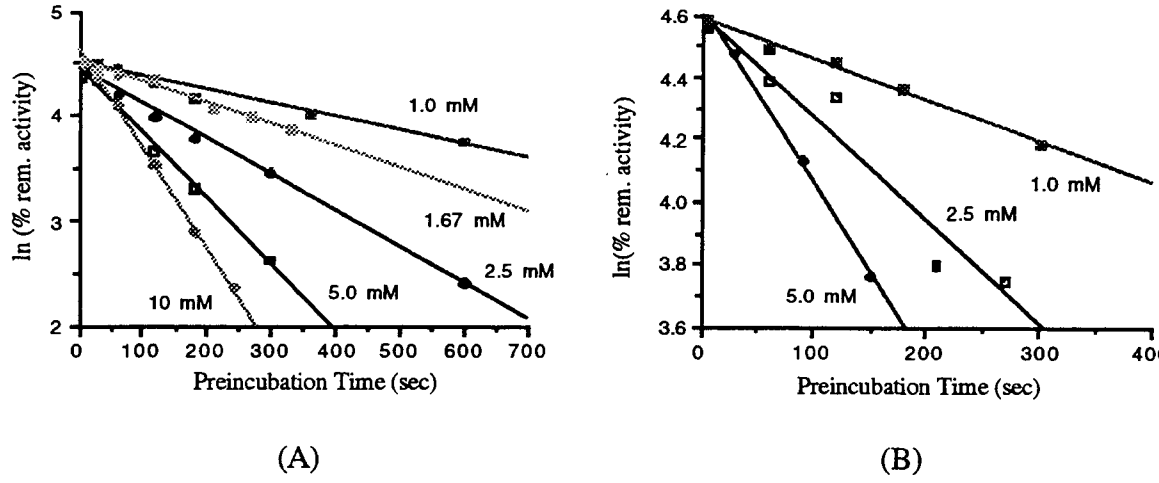
To test compounds as alternate substrates, 1 to 5 mM of the L-PA analog was incubated with L-PO and the generation of H_2O_2 was monitored using the spectrophotometric assay. If a compound was found to serve as a substrate for the enzyme, the Michaelis constant (K_M) and maximum velocity of the reaction (V_{max}) were determined. In the simplest cases, K_M can better be thought as an apparent dissociation constant for all enzyme bound species. K_M is numerically equal to the substrate concentration at which the reaction velocity has attained half of its maximum value. Therefore, an enzyme with a high K_M requires a higher substrate concentration to achieve a given reaction velocity than an enzyme with a low K_M. The maximum velocity, V_{max}, is the velocity when each enzyme active site is occupied by the substrate. The ratio V_{max}/K_M is an apparent second-order rate constant that relates reaction rate to the concentration of free enzyme. The higher this ratio, the more specific an enzyme is for a particular substrate.[69] Several types of plots have been formulated for the determination of K_M and V_{max} from kinetic data. Among them, the Lineweaver-Burk plot (double-reciprocal plot), plotting $1/V$ vs $1/[S]$, is the most commonly used and will be used here. All spectrophotometric assays for the kinetic studies were run in duplicate and at the same time whenever possible.

Characterization of (*S*)-1,3-Thiazane-4-Carboxylic Acid (5) as a Mechanism-Based Inhibitor

When first tested as inhibitor and substrate of L-PO, (*S*)-1,3-thiazane-4-carboxylic acid (**5**) was thought to be only a substrate. However, during extended assays, non-linear progress curves were observed in the spectrophotometric assays which indicated time-dependent inhibition. Therefore, the criteria for mechanism-based inhibitors were used to evaluate this compound.[41]

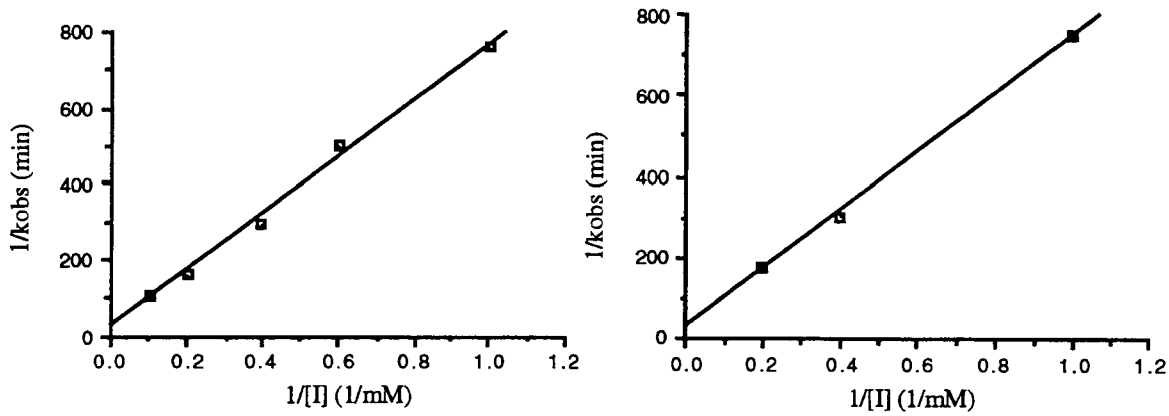
Time- and concentration-dependent inhibition

L-PO was preincubated with various concentrations of compound **5** and the enzyme activity was measured by spectrophotometery or radioactivity assays to determine the remaining activity and the level of inhibition. A time- and concentration-dependent inhibition of L-PO was observed (Figure 3.1). This means that at longer preincubation time or at higher inhibitor concentration, the lower will be the remaining activity of the enzyme. This suggests that after a rapid equilibrium between L-PO and **5**, forming a simple complex, there is a slow reaction that converts the compound to the form that actually inactivates the enzyme. This results in a time-dependent loss of L-PO activity. Moreover, the rate of inactivation is proportional to the amount of (*S*)-1,3-thiazane-4-carboxylic acid (**5**) until all L-PO molecules are saturated. The K_I and k_{inact} were determined to be 43 mM and 0.06 min^{-1} respectively by the Kitz and Wilson method (Figure 3.2) using the spectrophotometric assay data.[70] These results were confirmed by those from the radioassay. Using the radioassay, the same pattern of time- and concentration-dependent loss of activity was found and the K_I and k_{inact} were determined to be 36 mM and 0.03 min^{-1} respectively. The intercept point of the y-axis in the Kitz and Wilson plot (Figure 3.2) is greater than zero, which indicates that saturation kinetics are followed and that specific binding occurs prior to inactivation.[41]



(A) (B)

Figure 3.1 Time- and Concentration-Dependent Inhibition of L-PO by (*S*)-1,3-Thiazane-4-Carboxylic Acid (**5**).
(A: Spectrophotometric Assay; B: Radioassay)



(A) (B)

Figure 3.2 Kitz and Wilson Plot of (*S*)-1,3-Thiazane-4-Carboxylic Acid.
(A: Spectrophotometric Assay; B: Radioassay)

Substrate protection

Substrate protection can be used to demonstrate that an inactivator and L-PA bind to the same site on the enzyme. If (*S*)-1,3-thiazane-4-carboxylic acid (**5**) is a mechanism-based inactivator, it will act as a modified substrate for L-PO and bind to the active site. Therefore, addition of substrate or a competitive inhibitor will slow down the inactivation

of enzyme in proportion to its concentration. The 1.5 and 3 hour preincubation studies at varied concentration of L-PA shows that the natural substrate can protect the inhibition of L-PO by compound **5** (Figure 3.3). These data prove that both (*S*)-1,3-thiazane-4-carboxylic acid and L-pipecolate have the same binding site on the enzyme.

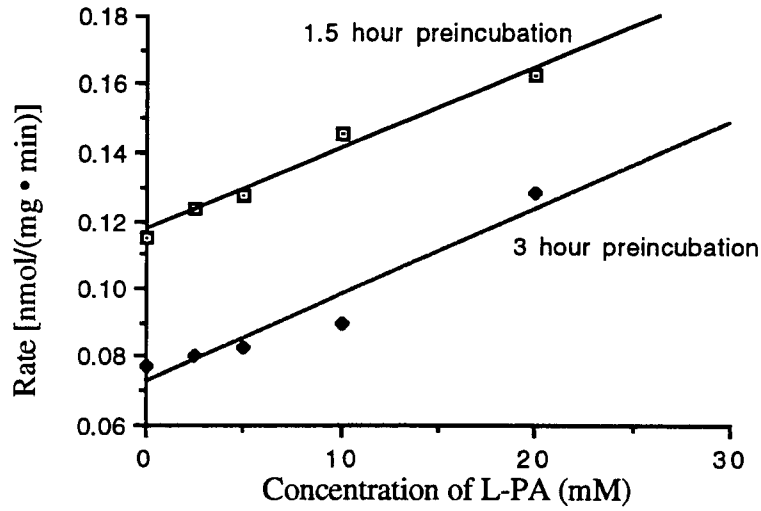


Figure 3.3 Substrate Protection of (*S*)-1,3-Thiazane-4-Carboxylic Acid (**5**) Inhibition by various concentrations of L-PA.

Irreversibility of L-PO inhibition

In most cases, mechanism-based inactivation results in the formation of covalent adducts with the enzyme. Consequently, dialysis does not restore enzyme activity. In this study, following inactivation of L-PO, the preincubation mixture was dialyzed exhaustively in order to remove excess and reversibly bound compound **5**. A non-inhibited control was carried through the same operation for comparison of L-PO activity. The results (Table 3.1) show there is only 12% recovery after seven hours dialysis and no increase after 18 and 27 hours dialysis. Therefore, the inactivation of L-PO by compound **5** is irreversible.

Table 3.1 Irreversibility of L-PO Inhibition by **5**.

Dialysis Time (Hours)	Recover Activity (%)
7	12%
18	14%
27	13%

Test for inactivation prior to the release of an activated species

In this experiment, the inactivation of L-PO by 15 mM (*S*)-1,3-thiazane-4-carboxylic acid (**5**) was continuously monitored for 30 minutes at which time no activity remained. A fresh aliquot of enzyme was added and the rate of inactivation was monitored. If the inhibition is the result of indiscriminant inactivation by a released species, this reactive species will increase in the solution and the inactivation rate of the second aliquot of enzyme will be greater than that of the first one. For the inactivation of L-PO by **5**, k_{obs} (inactivation rate constant) was 2.55×10^{-3} min^{-1} for the first aliquot and 2.40×10^{-3} min^{-1} for the second aliquot. Thus, the results indicate that the inactivation occurs prior to release of an activated species.

Substrate kinetic parameters (K_M and V_{max}) for (S)-1,3-thiazane-4-carboxylic acid (5)

When **5** was assayed as a substrate using the horseradish peroxidase-coupled assay, hydrogen peroxide was produced, indicating that (*S*)-1,3-thiazane-4-carboxylic acid (**5**) was turned over by L-PO. Figure 3.4 shows that the turnover of **5** by L-PO follows Michaelis-Menten kinetics. From the Linewear-Burk plot (Figure 3.4, *insert*), the apparent K_M was determined to be 0.93 ± 0.16 mM with a V_{max} of 0.09 ± 0.01 μmol • min^{-1} • mg^{-1}.

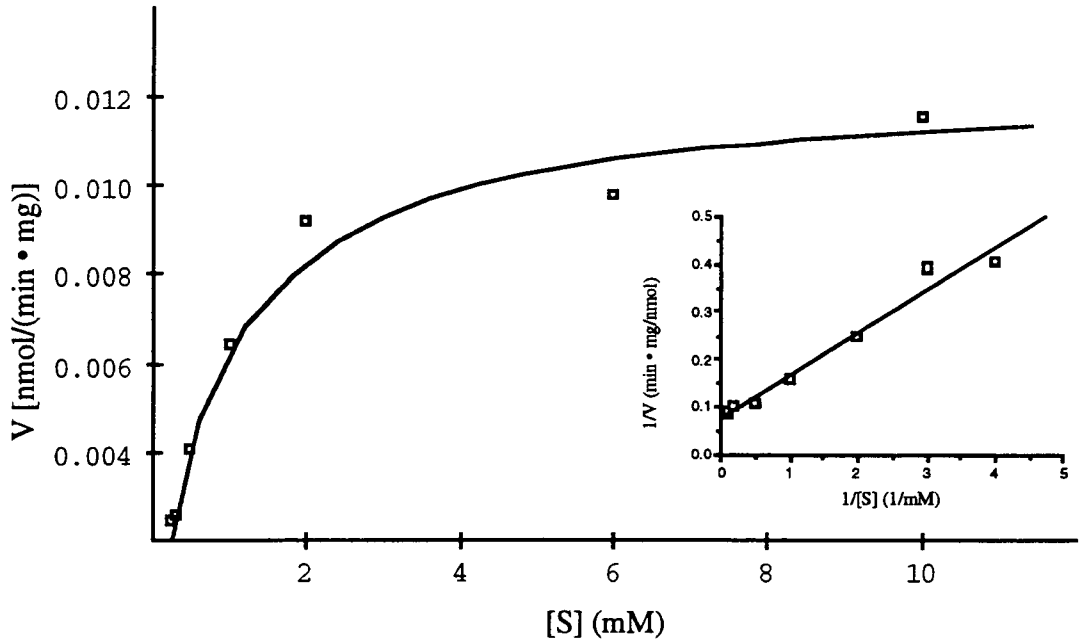


Figure 3.4 Activity of L-Pipecolate Oxidase with (*S*)-1,3-Thiazane-4-Carboxylic Acid (**5**) as Substrate. *Insert*: Linewear-Burk plot used to estimate K_M and V_{max}.

Taken together, these results indicate that (*S*)-1,3-thiazane-4-carboxylic acid (**5**) is a mechanism-based inactivator of L-PO.

When (*R*)-1,3-thiazane-4-carboxylic acid (**6**) was tested as inhibitor and substrate of L-PO, it was found that this compound was neither an inhibitor nor a substrate. Preincubation studies also failed to produce inactivation of L-PO. This indicates that the inactivation of L-PO by 1,3-thiazane-4-carboxylic acid is stereospecific.

Kinetic Study of (*S*)-1,4-Thiazane-3-Carboxylic Acid (10) and the Sulfur Oxidized Analogs (11 and 12)

When testing the (*S*)-1,4-thiazane-3-carboxylic acid (**10**) as an inhibitor of L-pipecolate oxidase, a higher reaction rate was observed when 1 and 3 mM **10** were present in the assay. This indicated that the compound is not a inhibitor but possibly a very good substrate. It was later confirmed with the substrate test. From the plot of the dependency of reaction velocity on the concentration of **10** (Figure 3.5), it was found that

the L-PO catalyzed oxidation of compound **10** followed the Michaelis-Menten model and has K_M of 0.42 ± 0.08 mM and V_{max} of 0.11 ± 0.01 μmol•min^{-1}•mg^{-1}. The V_{max}/K_M is 0.2 mL•min^{-1} •mg^{-1} for this compound which is almost four times greater than that with L-PA as substrate. The V_{max}/K_M provides a measure of catalytic efficiency and specificity of an enzyme for a substrate. Thus, it seems that (*S*)-1,4-thiazane-3-carboxylic acid (**10**) is a more efficient substrate for L-PO than the natural substrate.

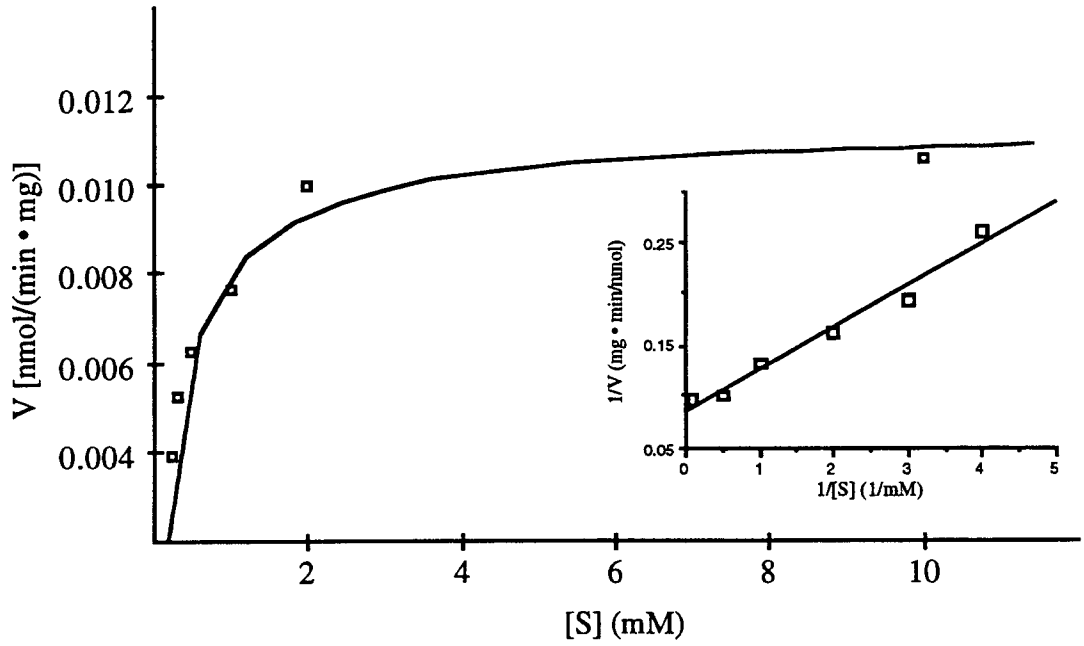


Figure 3.5 Activity of L-PO as a Function of (*S*)-1,4-Thiazane-3-Carboxylic Acid (**10**) Concentration. *Insert*: Lineweaver-Burk Plot of the Data Used to Determination K_M & V_{max}.

When testing (*S*)-1,4-thiazane-3-carboxylic acid 1-oxide (**11**) as an inhibitor, it was found that L-PO was inhibited by 3 and 5 mM **11**. However, there was no indication of turnover in the substrate test. Further kinetic tests were done in order to determine the type of inhibition and the inhibition parameters. The Lineweaver-Burk plot (Figure 3.6) shows that the inhibition is competitive and the K_i was determined to be 1.9 ± 0.3 mM.

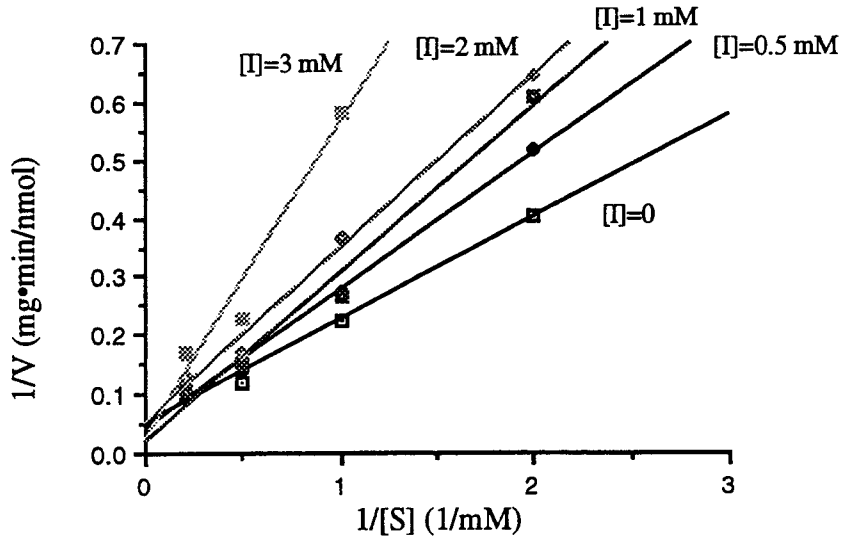


Figure 3.6 Inhibition Study of (*S*)-1,4-Thiazane-3-Carboxylic Acid 1-Oxide (**11**).

The sulfone analog, (*S*)-1,4-thiazane-3-carboxylic acid 1-dioxide (**12**), was also tested as an inhibitor of L-PO at 1 and 5 mM. Only weak inhibition was observed at 5 mM and no turnover by the enzyme was detected. This indicated that the compound was a weak inhibitor and not a substrate at all. The IC_{50} of **12** was determined to be 8.1 ± 0.1 mM (Figure 3.7).

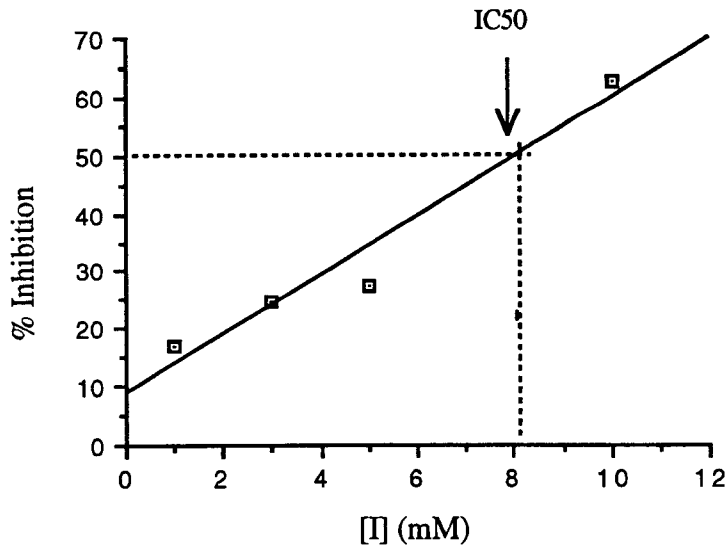


Figure 3.7 Inhibition Study of (*S*)-1,4-Thiazane-3-Carboxylic Acid 1-Dioxide (**12**).

The results of (*S*)-1,4-thiazane-3-carboxylic acid (**10**) and its sulfoxide (**11**) and sulfone analogs (**12**) reveal that **10** is an excellent substrate, its sulfoxide analog **11** is just a moderate competitive inhibitor, and the sulfone analog **12** is only a weak competitive inhibitor. Thus, a polar functionality at position 4 of the pipecolate ring or the change in ring conformation that may result from oxidation of sulfur has a great effect on binding to L-PO.

Kinetic Studies of Tetrahydro and Hexahydro Ring Compounds

One purpose of synthesizing the (*S*)-2-amino-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid (**2**) was to test the effect of the basic guanidine group on binding to the GABA receptor. However, these experiments have not yet been conducted. Inhibition and substrate tests with L-PO were carried out and demonstrated that this compound is neither an inhibitor nor a substrate of the enzyme.

One of the tautomers of (*S*)-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid (**1**) is a structural mimic of Δ^1-piperideine-6-carboxylic acid, which is the turnover product of L-PA. Therefore, this compound was hoped to be a potent competitive inhibitor of L-PO. When tested with L-PO, **1** proved to be a weak competitive inhibitor and, as expected, was not turned over by the enzyme. The K_i for **1**, determined from the Lineweaver-Burk plot shown in Figure 3.8, is 6.5 ± 0.2 mM.

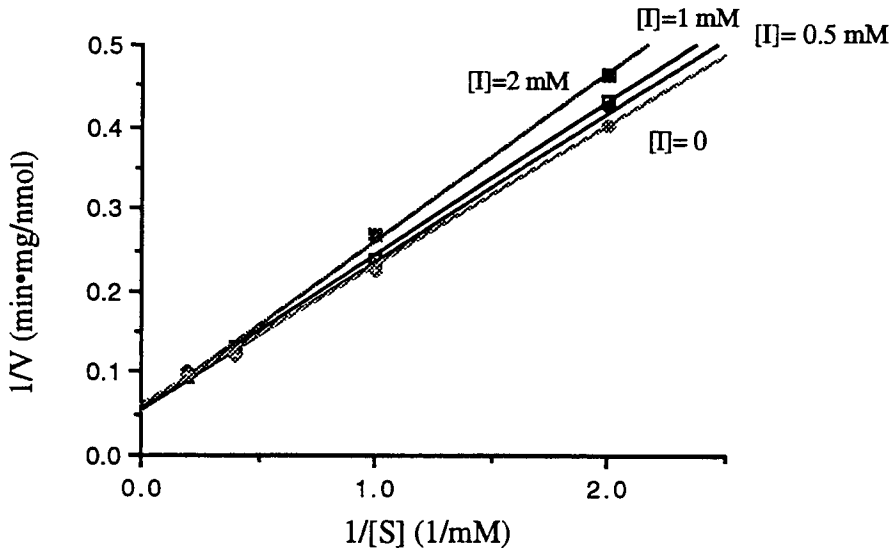


Figure 3.8 K_i Determination for (*S*)-3,4,5,6-Tetrahydropyrimidine-4-Carboxylic Acid (**1**)

Initial evaluation of (*S*)-hexahydropyrimidine-4-carboxylic acid (**3**) as a substrate of L-PO showed that this compound was turned over at concentrations of 1 and 5 mM. Further characterization shows that the K_M is 10.3 $\pm$ 2.1 mM and the V_{max} is 0.10 $\pm$ 0.01 μmol•min^{-1}•mg^{-1} (Figure 3.9). Because the K_M for **3** is three times greater than the K_M of L-PA, it is classified as a poor substrate.

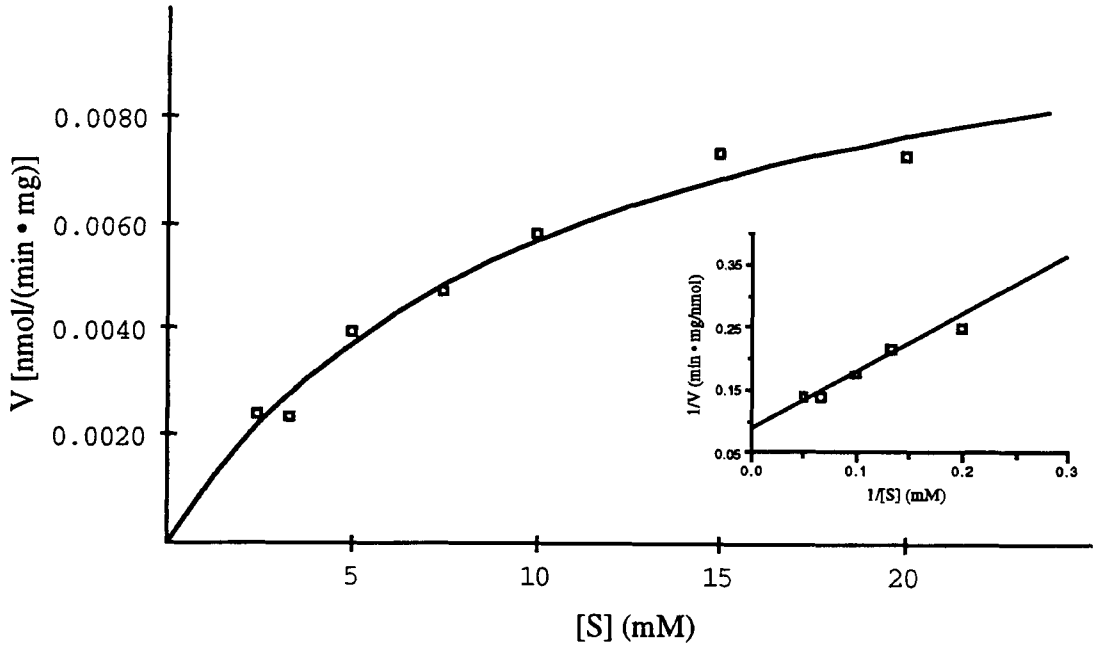


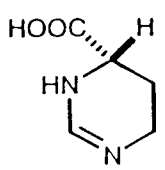
Figure 3.9: Activity of L-PO as a Function of (*S*)-Hexahydropyrimidine-4-Carboxylic Acid (**3**) Concentration. *Insert*: Lineweaver-Burk Plot of the Data Used to Estimate K_M & V_{max}

For the evaluation of (*R, S*)-1,3-oxazane-4-carboxylic acid (**4**), the inhibitor and substrate tests were done at 1 mM, 3 mM and 5 mM. All the results indicate that **4** is neither a substrate nor an inhibitor of L-PO. Because of this, no further attempt was made to synthesize the (*S*) enantiomer from optically active homoserine.

The inhibition test of (*R, S*)-1,4-piperazine-2-carboxylic acid (**13**) revealed weak inhibition at high concentrations (over 5 mM). The substrate analysis showed that **13** is not turned over by L-PO. As a result, no attempt was made to separate the (*R*) and (*S*) enantiomers.

Table 3.1 provide a summary of the evaluation of the natural substrate, L-PA and the substrate analogs discussed in this thesis as inhibitors and substrates of L-pipecolate oxidase.

Table 3.2 Summary of the Kinetic Data of Substrate Analogs



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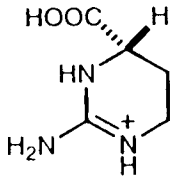

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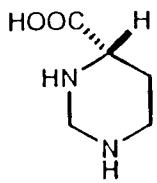

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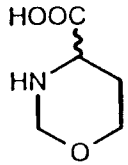

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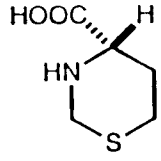

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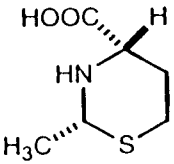

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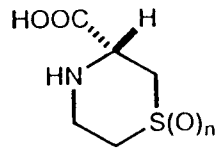
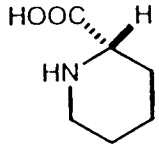


n=0, **10**
n=1, **11**
n=2, **12**



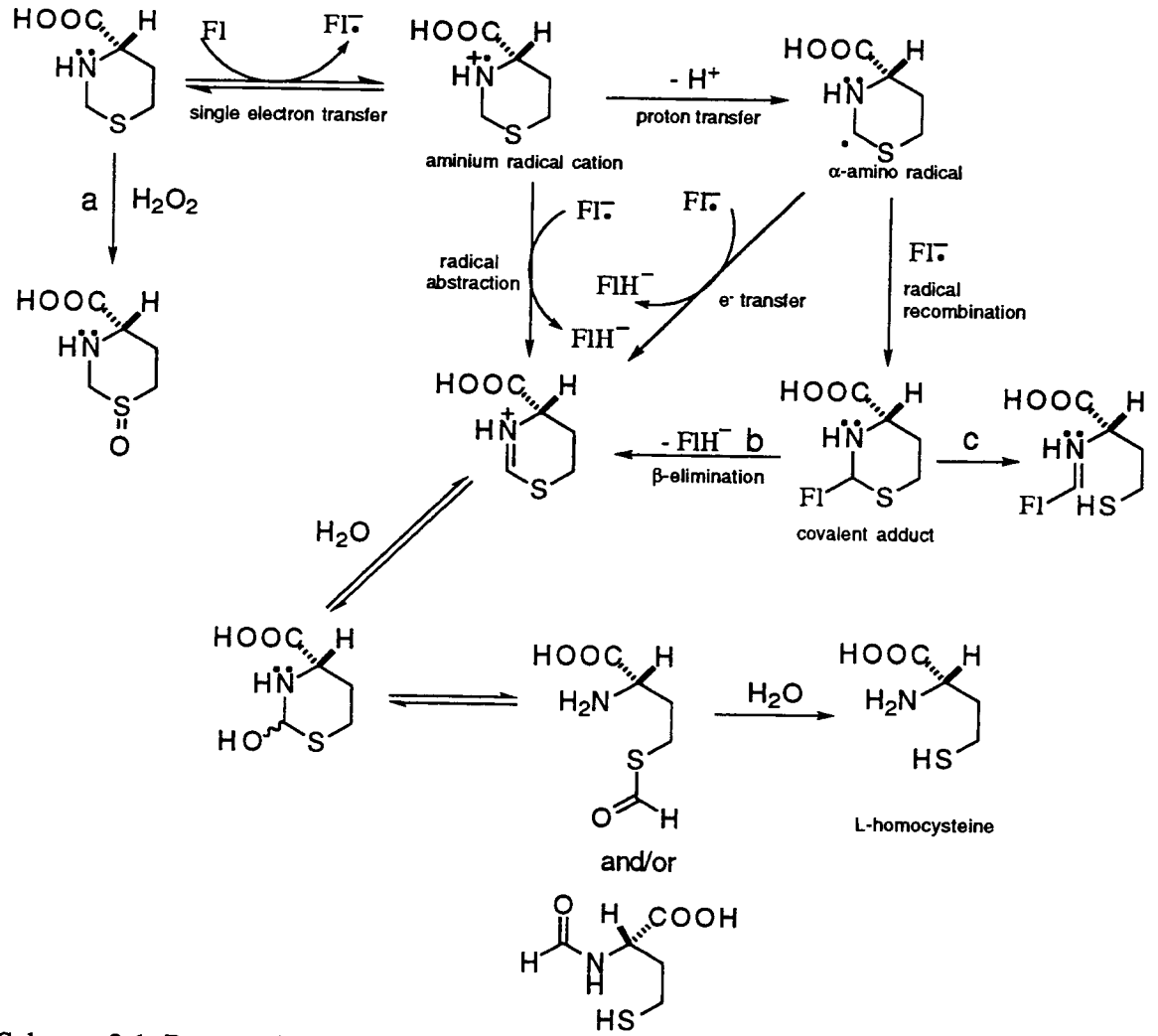
14

Compounds	K_m (mM)	V_{max} ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	K_i (mM)	Comment
(*S*)-3,4,5,6-Tetrahydropyrimidine-4-Carboxylic Acid (**1**)	—	—	6.5 ± 0.2	Competitive Inhibitor
(*S*)-2-Amino-4-carboxy-3,4,5,6-Tetrahydropyrimidine (**2**)	—	—	—	—
(*S*)-Hexahydropyrimidine-4-Carboxylic Acid (**3**)	10.3 ± 2.1	0.10 ± 0.01	—	Substrate
(3*S*)-1,3-Oxazane-4-Carboxylic Acid (**4**)	—	—	—	—
(*S*)-1,3-Thiazane-4-Carboxylic Acid (**5**)	0.93 ± 0.16	0.09 ± 0.01	—	Substrate/ Mech. Based Inhibitor
(2*R*, 4*S*)-2-Methyl-1,3-Thiazane-4-Carboxylic Acid (**7**)	—	—	0.31 ± 0.02	Competitive Inhibitor
(3*S*)-1,4-Thiazane-3-Carboxylic Acid (**10**)	0.42 ± 0.08	0.11 ±0.01	—	Substrate
(3*S*)-1-Oxo-1,4-Thiazane-3-Carboxylic Acid (**11**)	—	—	1.89 ± 0.27	Competitive Inhibitor
(3*S*)-1,1-Dioxo-1,4-Thiazane-3-Carboxylic acid (**12**)	—	—	8.08 ± 0.13	IC_{50}
L-Pipecolic Acid (**14**)	3.5	0.17	—	Natural Substrate

CHARACTERIZATION OF THE ALTERNATE SUBSTRATE TURNOVER PRODUCTS

Characterization of the Turnover Product of (*S*)-1,3-Thiazane-4-Carboxylic Acid (5)

Because the kinetic studies showed that (*S*)-1,3-thiazane-4-carboxylic acid (**5**) is a mechanism-based inactivator of L-PO, further characterization of the turnover product is necessary in order to elucidate possible inactivation mechanisms. Possible inhibition mechanisms for **5** are drawn in Scheme 3.1 according to the proposed mechanism of L-PO (Scheme 2.1). There are several possible pathways for inactivation and product formation. The first possibility is the oxidation of the 1,3-thiazane ring to the sulfoxide analog by the hydrogen peroxide generated by the reoxidation of reduced flavin (pathway a). The other possibility is that after electron and proton transfer, an enzyme-1,3-thiazane adduct is formed. If elimination takes place between the carbon sulfur bond in the thiazane molecule (pathway b), the enzyme will be inactivated. If elimination takes place between the flavin moiety and the thiazane molecule (pathway c), 2,3–dehydro-1,3-thiazane-4-carboxylic acid will be released and hydrolyzed to the 2-hydroxy-1,3-thiazane which is unstable. Spontaneous ring-opening would give either *N*-formyl or *S*-formyl L-homocysteine. The *S*-formyl group should rapidly hydrolyze yielding L-homocysteine and formic acid.



Scheme 3.1 Proposed Mechanisms for the Reaction of (*S*)-1,3-Thiazane-4-Carboxylic Acid (**5**) with L-Pipecolate Oxidase.

To examine the existence of pathway a, an excess amount of catalase, which catalyzes the reduction of hydrogen peroxide to water, was added to the assay solution. There was no change in the enzyme activity when compared to the control without catalase. This confirmed that pathway a is not a dominant pathway of inactivation.

To obtain basic information about the turnover product, *p*-dimethylaminobenzaldehyde was added to the assay solution after incubating the enzyme with (*S*)-1,3-thiazane-4-carboxylic acid (**5**) at 37 ℃ for one and a half hours. A new absorption maximum was seen at 480 nm in the UV spectrum, which indicated that there

was a free amino group in at least one of the turnover products and this free amino group reacted with *p*-dimethylaminobenzaldehyde.[71] When two control samples, containing only (*S*)-1,3-thiazane-4-carboxylic acid (**5**) and L-PA respectively, were reacted with *p*-dimethylaminobenzaldededehyde under the same conditions, neither sample produced a product absorbing at 480 nm.

In order to further characterize the turnover product, phenylisothiocyanate (PITC) was chosen as a derivatizing reagent. After incubation of the (*S*)-1,3-thiazane-4-carboxylic acid (**5**) with enzyme and derivitization, the solution was analyzed by reverse-phase (C_{18}) HPLC. A new peak was identified which had the same retention time as authentic homocysteine-phenylthiohydantoin which was synthesized as a standard (Figure 3.10). This proves that one of the turnover products is homocysteine. This finding was confirmed by mass spectrometry. The mass of the isolated turnover product was measured to be 252.0391 which is the same as that of the homocysteine phenylthiohydantoin standard (252.0391). Moreover, both the isolated turnover product and the standard compound have very similar ion patterns in the mass spectrum.

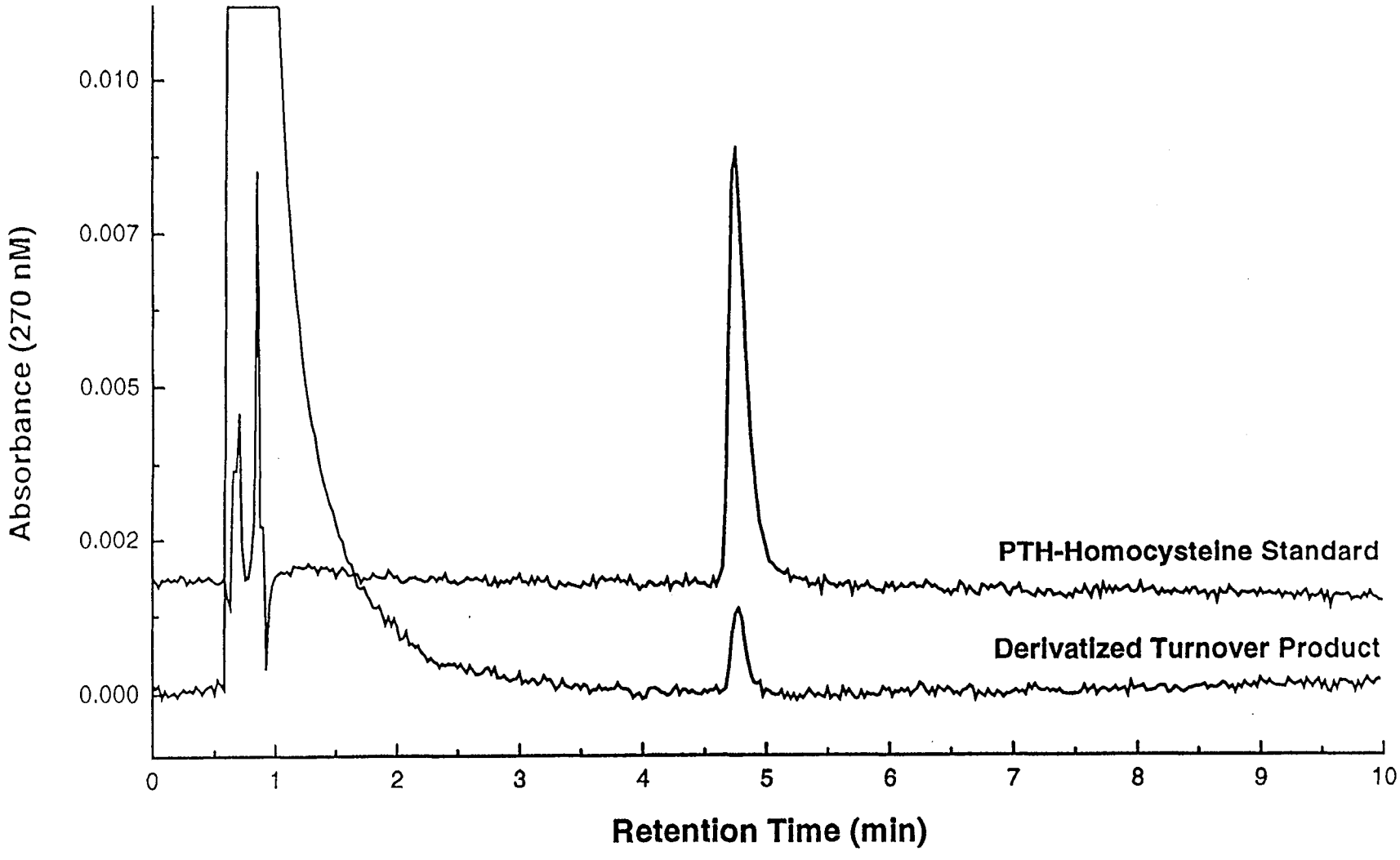
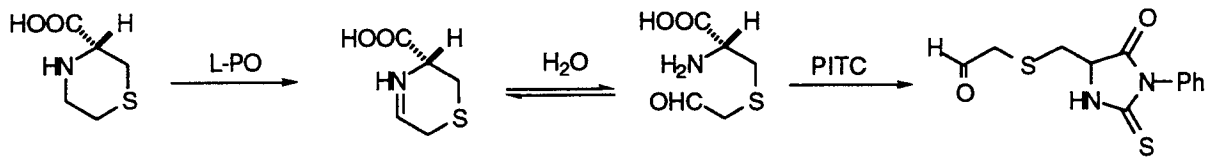


Figure 3.10 HPLC analysis of turnover product of **5**

Characterization of the Turnover Product of (*S*)-1,4-Thiazane-3-Carboxylic Acid (10)

The possible turnover mechanism for the (*S*)-1,4-thiazane-3-carboxylic acid (**10**) is shown in Scheme 3.2. Obviously, it is less complicated than that of 1,3-thiazane. After derivatizing with phenylisothiocyanate, the solution was analyzed on a C_{18} reverse phase HPLC column. Two control samples, one without **10** and one without L-PO were manipulated in the same way and analyzed under the same conditions. A new peak was found when compared to the controls. The mass of this peak is 279, which is the same mass as L-α-amino-4-thioadipate-δ-semialdehyde-phenylthiohydantoin. A standard was not prepared for this metabolite.



Scheme 3.2 Mechanism of Turnover and Product Characterization for (*S*)-1,4-Thiazane-3-Carboxylic Acid (**10**)

DISCUSSION

Thirteen substrate analogs of L-pipecolic acid were synthesized and tested as inhibitors or substrates of L-PO. The results of kinetic analyses indicated quite different binding behavior of these analogs with different functional groups. Because one aspect of the L-PO mechanism is the probable formation of a radical intermediate in some stage of the reaction, the well-developed theories about organic radical species and the proposed enzyme mechanism can be used to interpret the above kinetic data. Forming a radical is a highly unfavorable thermodynamic process in solution; the oxidation potential for a typical amine is about +1.5 V and the reduction potential for free flavin is -0.25 V.[45] As a result,

any structural feature that can stabilize a radical intermediate should lower the required energy and favor the reaction.

A set of stabilization energies for different substitute groups was reported by Ohno (Table 3.2).[72] From Table 3.2, it is seen that the stabilization energies between $-OCH_3$ and $-CH_3$ are small. As a result, the resonance participation from an oxygen atom is not great. However, sulfur shows a very large stabilization energy indicating lower activation energies and implying the prominent participation of the sulfur atom in the stabilization of the radical center at the α-position. A similar conclusion was reached when studying hydrogen atom abstraction to form a radical.[73] This can be attributed to the electron sharing and the electron transfer resonance structure.

Table 3.2: Stabilization Energies (SE) for Carbon-Centered Radicals with Different Functional Groups

Function Group	Stabilization Energy (kcal/mol)
$-CH_3$	4.6
$-OCH_3$	6.4
$-SCH_3$	12.1

The large degree of stabilization of a carbon-centered radical by sulfur but not by oxygen could be a reasonable explanation for the turnover of (*S*)-1,3-thiazane-4-carboxylic acid (**5**) by the enzyme, whereas (*R*, *S*)-1,3-oxazine-4-carboxylic acid (**4**) is neither a substrate nor an inhibitor.

E.S.R. spectroscopy has been employed to study carbon-centered radicals with sulfur, sulfinyl- and sulfonyl- at the β-position by comparing the hyperfine splitting ratio of the β proton and α proton.[74] It was found that the thio group could delocalize 22% of the spin density from the β-carbon radical while the sulfinyl- could only delocalize 6% of

the unpaired spin, and the sulfonyl group was wholly ineffective at removing spin density. This may partially explain why (*S*)-1,4-thiazane-3-carboxylic acid (**10**) is an excellent substrate, the sulfoxide analog **11** is just a moderate competitive inhibitor, and the sulfone analog **12** is a weak competitive inhibitor. In the same study,[74] it was also found that a thio group is a more effective acceptor of spin density than alkyl and alkoxy substitutes. This provides further evidence why (*S*)-1,4-thiazane-3-carboxylic acid (**10**) is more effectively turnover than the natural substrate, L-pipecolic acid.

MATERIALS AND METHODS

Materials: All reagents were purchased from Sigma or Fisher. Monkey liver was obtained from Oregon Regional Primate Research Center. Water was double-deionized (NANOpure, Barnstead) and filtered (0.45 mm). Buffers were prepared with double deionized water and the pH was adjusted to the desired value at room temperature with 4 N NaOH or 4 N HCl solutions. Spectrophotometric studies were performed using a Hitachi U2000 spectrophotometer. D,L-[2,3,4,5,6-^{3}H]-Pipecolic acid was custom-synthesized by Amersham. The resulting D,L-[2,3,4,5,6-^{3}H]-pipecolic acid (D,L-[^{3}H]-PA) had a specific activity of 6200 mCi/mmol and was separated from its radiolytic degradation products and converted to the homochiral L-form by the method of Mihalik and Rhead.[16] L-[^{3}H]-Pipecolic acid was stored at -86 ℃ at a concentration of 10 mM and a specific activity of 500 μCi/μmol. Radioactivity was determined on a Beckman LS 6000SC or a Beckman LS 6800 liquid scintillation counter. HPLC analyses were carried out on a Beckman Gold System using a 4.6 x 100 mm (3 μm) reverse phase (C_{18}) Microsorb-MV column from Rainin. Kinetic parameters were calculated using the Enzyme Kinetics software package from Trinity Software. Plots were constructed with either Enzyme Kinetics or Cricket Graph 1.3 software package from Cricket Software.

L-Pipecolate Oxidase Purification

Homogenization and Solubilization Frozen Rhesus monkey liver (100 g) was finely minced and then homogenized in a Waring blender in 300 mL of 250 mM sucrose containing 1 mM MOPS, pH 7.4 and 0.1 mM EGTA with protease inhibitors (0.1 mM benzamidine, 0.4 mg/mL leupeptin and 0.7 mg/mL pepstatin). This homogenate was centrifuged at 24,000 x *g* for 10 minutes. The supernatant was discarded, and the pellet was re-suspended in another 250 mL of the same buffer, then homogenized and centrifuged as above. The residue pellet was re-suspended in 150 mL of 100 mM KCl, 50 mM HEPES, pH 8.0, 1 mM EGTA and 0.1% CHAPS buffer. The pellet suspension was magnetically stirred for 30 minutes, followed by 30 minutes centrifugation at 27,000 x *g*. The supernatant was decanted. The residual pellet was then re-suspended in 100 mL of the same buffer and stirred again for 30 minutes. After a repeat centrifugation as above, both supernatants were combined.

Heat Treatment: The combined supernatants were rapidly brought to 45 °C and held at this temperature for 10 minutes. Then, the solution was rapidly cooled to 4°C in ice and centrifuged at 27,000 x *g* for 30 minutes. The supernatant was retained.

CM-Cellulose Batch Chromatography: Whatman CM-cellulose cation exchange resin (75 g) was equilibrated with 5 mM KCl, 5 mM HEPES, pH 7.8 and 0.1 mM EGTA after it was treated with 0.5 M KCl, 5 mM HEPES, pH 7.8. The supernatant from the previous step was adjusted to pH 7.65 with 1 M HEPES, pH 6.8 and then diluted ten-fold with deionized water at 4 °C. This solution was stirred for 30 minutes with 125 mL of CM-cellulose resin. The suspension was transferred to a Buchner funnel lined with Whatman #1 filter paper. The resin was separated from the supernant and then washed with about 600 mL equilibration buffer. An enzyme-enriched fraction was eluted with 150 mM KCl, 5 mM HEPES, pH 7.8 and 0.1 mM EGTA.

Phenyl-Sepharose Chromatography: The active fraction from the prior step was adjusted to 0.5 M $(NH_4)_2SO_4$, 25 mM KCl, 12.5 mM HEPES, pH7.8 and 0.25 mM

EGTA and applied to a 10 mL column of Toso-Haas TSK-GEL Phenyl Sepharose 5-PW resin at 4 °C. The column was washed with 30 mL of 0.5 M $(NH_4)_2SO_4$, 25 mM KCl, 12.5 mM HEPES, pH 7.8, 0.25 mM EGTA at a flow rate of 1.0 mL/min. A stepwise elution was carried out using consecutive 13 mL volumes of 0.25 $(NH_4)_2SO_4$, 0.1 M $(NH_4)_2SO_4$ and loading buffer (without ammonium sulfate) at a flow rate of 1.5 mL/min. The enzyme activity was located in the 0.1 M ammonium sulfate fractions.

Enzyme Assay of L-Pipecolic Oxidase Activity

Spectrophotometric Assay. In a 500 µL optical glass spectroscopy cuvette, containing 40 mM Tris, 80 mM KCl, 0.8 mM EGTA, pH 8.5 buffer with 0.32 mM *o*-dianisidine (5 µL of 10 mg/mL stock solution) and 1.8 unit of horseradish peroxidase (5 µL of 3.6 unit/10 µL stock solution), different amounts of the enzyme solution and/or L-pipecolic acid (20 mg/mL stock solution) were mixed along with the test compound. The absorption change at 460 nm was monitored and the reaction was kept at 37 °C. In most assays, enough enzyme was added to provide at least 0.015 absorbance units change per second.

Radioassay. An indicated amount of enzyme solution was added to 10 mM $NaHSO_3$, 225 µM L-[2,3,4,5,6-^{3}H]-pipecolic acid, 60 mM Tris buffer (pH 8.5) and adjusted to a final volume of 200 µL. The preparation was incubated in a 37 °C water bath for 1 hour. The reaction was stopped by adding 25 µL of 1 N HCl and samples were immediately applied to a 2.5 mL Bio-Rad AG50W-X8 (H^+, 100-200 mesh) cation exchange column, which was equilibrated with deionized distilled water. Then the column was eluted with deionized water at gravity flow rate. The elutant was mixted with scintillation cocktail and counted on a liquid scintillation counter. Two parallel blanks omitting either enzyme or L-[2,3,4,5,6-^{3}H]-pipecolic acid were run for each sample.

Testing Compounds as Inhibitors and/or Substrates of L-Pipecolate Oxidase

Testing Compounds as Inhibitors : Assays were conducted at 37 °C in 40 mM Tris, 80 mM KCl, 0.8 mM EGTA, pH 8.5 buffer containing 3.5 mM L-PA, 0.32 mM *o*-dianisidine and 1.8 units of horseradish peroxidase and the indicated concentration of L-PA analog. The reaction was initiated by the addition of L-PO.

Testing Compounds as Alternate Substrates: Assays were conducted at 37 °C in 40 mM Tris, 80 mM KCl, 0.8 mM EGTA, pH 8.5 buffer containing L-PO, 0.32 mM *o*-dianisidine and 1.8 unit of horseradish peroxidase. Reactions were initiated by adding the test substrate.

Evaluation of (*S*)-1,3-Thiazane-4-Carboxylic Acid (5) as a Mechanism-Based Inactivator of L-Pipecolate Oxidase

Time- and Concentration-Dependent Inhibition: Purified L-PO was incubated with the 1 to 10 mM of **5** in 40 mM Tris, 80 mM KCl and 0.8 mM EGTA, pH 8.5 buffer in a total volume of 250 µL at 25 °C in the dark. At the times indicated, 25 µl aliquots were removed and diluted with 475 µL 37 °C assay cocktail. In the radioassay, the total volume of incubation solution was 90 µL. Each time, 11 µL of aliquot were removed and diluted with 389 µL of radioassay cocktail with 1 hour incubation before assay.

Substrate Protection from Inhibition: L-PO was incubated with 5 mM **5** in 80 mM Tris, 160 mM KCl, 1.6 mM EGTA, pH 8.5 buffer with the indicated concentration of L-PA in a total volume of 100 µL at 25 °C in the dark. After 1.5 hr and 3 hr, 25 µL aliquot of solution were removed and the activity was assayed.

Reversibility of Inhibition: After incubating L-PO in 250 µL of 40 mM Tris, 80 mM KCl, 0.8 mM EGTA pH 8.5 buffer with 20 mM **5** for 4 hours in a total volume of 500 µL at 25 °C, the solution was dialyzed against 600 mL buffer at 4 °C. Several buffer changes were made during the 28 hour dialysis. 50 µL of reaction solution were removed

at 7 hours, 18 hours and 27 hours to assay for recovered enzyme activity. A noninactivated control was carried through the same procedure.

Test for Release of Active Species: L-PO was incubated with 15 mM **5** in 250 μL of 40 mM Tris, 80 mM KCl, 0.8 mM EGTA, pH 8.5 buffer containing 0.64 mM *o*-dianisidine and 3.6 unit of horseradish peroxidase in a 0.5 mL optical glass spectroscopy cuvette at 37 °C. Enzyme activity was continuously monitored by following the increased absorption at 460 nm. After 30 minutes, a fresh aliquot of enzyme was added to the solution and enzyme activity was monitored for another 30 minutes.

Characterization of the Enzyme Turnover Product of (*S*)-1,3-Thiazane-4-Carboxylic Acid (5)

After incubating L-PO in 40 mM Tris, 80 mM KCl, 0.8 mM EGTA pH 8.5 buffer containing 10 mM substrate in a volume of 50 μL in the dark overnight, 50 μL of pyridine was added to terminate the reaction. Then, 1 μl of phenylisothiocyanate was added and the mixture was incubated at 37 °C for 20 minutes. After adjusting the pH of the solution to 1.5 with 1 N HCl, the solution was centrifuged at 9000 x *g* and the precipitate was collected. The precipitate was dissolved in a minimum amount of 1 N HCl and underwent analysis using reverse phase HPLC in a solvent system of 0.1% trifluoroacetic acid and acetonitrile. A no-enzyme control sample and a no-substrate control sample were treated under the same conditions.

Characterization of the Enzyme Turnover Product of (*S*)-1,4-Thiazane-3-Carboxylic Acid (10)

After incubating L-PO in 40 mM Tris, 80 mM KCl, 0.8 mM EGTA pH 8.5 buffer containing 10 mM **10** in a total volume of 25 μL in the dark overnight at room temperature, 25 μL of pyridine was added to terminate the reaction. Then, 2 μL of phenylisothiocyanate was added and the reaction was incubated at 37 °C for 30 minutes. After adding 50 μL of 1 N HCl, the solution was centrifugated at 9000 x *g*. The

supernatant was subjected to reverse-phase HPLC analysis in a solvent system of 0.1% trifluroacetic acid and acetonitrile. A no-enzyme control sample and a no substrate control sample were treated under the same condition.

CHAPTER 4

CONCLUSIONS

L-Pipecolic acid (L-PA) is an intermediate of lysine degradation in various organisms. There are two primary pathways for lysine degradation in mammalian systems. In the central nervous system (CNS), the major pathway is the pipecolate pathway in which the first step is oxidation of L-PA by a peroxisomal oxidase, L-pipecolate oxidase (L-PO). In this pathway, the L-PA oxidation is the rate-limiting step. As a result, studying the inhibition of L-PO through the effects of various synthetic inhibitors, especially mechanism-based inactivators, is a good approach to studying the L-PO mechanism and lysine degradation in the CNS. In this thesis, thirteen substrate analogs have been synthesized and tested for their ability to affect L-PO which has been isolated from Rhesus monkey liver.

The kinetic analyses of these substrates were done using spectrophotometric and radioassays. It was found that (*S*)-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid (**1**) is a competitive inhibitor with apparent K_i of 7.3 mM. (*S*)-3,4,5,6-Hexahydropyrimidine-4-carboxylic acid (**3**) proved to be a weak substrate with apparent K_M of 10.2 mM. All testing data indicate that the (*S*)-1,3-thiazane-4-carboxylic acid (**5**) is a mechanism-based inactivator, with apparent K_i and k_{inact} of 36 mM and 0.06 min^{-1} respectively. As a substrate, apparent K_M and V_{max} for **5** were determined to be 0.93 mM and 0.1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively. It was found that the inhibition of L-PO by (*S*)-1,3-thiazane-4-carboxylic acid (**5**) was time- and concentration-dependent. The inhibition is irreversible and the enzyme is protected by natural substrate. It was also shown that the inactivation by **5** occurs prior to release of a highly reactive species. It was shown that (*R*)-1,3-thiazane-4-carboxylic acid (**6**) is neither a substrate nor an inhibitor, which indicates that the inhibition is stereospecific. The testing shows that (*S*)-1,4-thiazane-3-

carboxylic acid (**10**) is an excellent substrate with apparent K_M of 0.42 mM and V_{max} of 0.1 μmol•min^{-1}•mg^{-1}. Its high V_{max}/K_M ratio (0.2 mL•min^{-1}•mg^{-1}) indicates that **10** is a better substrate than the natural substrate. The sulfoxide derivative of **10**, (*S*)-1,4-thiazane-3-carboxylic acid 1-oxide (**11**) is just a moderate competitive inhibitor with apparent K_i of 2.7 mM. The sulfone derivative, (*S*)-1,4-thiazane-3-carboxylic acid 1-dioxide (**12**), is only a weak competitive inhibitor with an IC_{50} of 8.1 mM. The studies also indicated that (*S*)-2-amino-3,4,5,6-tatrahydropyrimidine-4-carboxylic acid (**2**), (*R,S*)-1,3-oxazine-4-carboxylic acid (**4**) and (*R, S*)-1,4-piperazine-2-carboxylic acid (**13**) served as neither substrates nor inhibitors.

Further characterization of the turnover products for (*S*)-1,3-thiazane-4-carboxylic acid (**5**) and (*S*)-1,4-thiazane-3-carboxylic acid (**10**) was performed. After reacting the substrate analogs with L-PO, the mixture was treated with phenylisothiocyanate and analyzed by reverse phase HPLC. It was found that homocysteine was one of the turnover products of (*S*)-1,3-thiazane-4-carboxylic acid (**5**). L-α-Amino-4-thioadipate-δ-semialdehyde was shown to be the turnover product of (*S*)-1,4-thiazane-3-carboxylic acid (**10**).

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