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EPOXIDE DERIVATIVES OF PIPECOLIC ACID AND PROLINE ARE INHIBITORS OF PIPECOLATE OXIDASE

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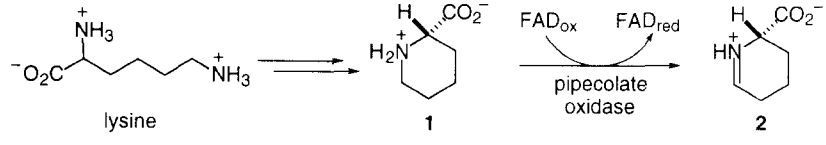
Abstract. The *cis*-4,5-epoxide derivative of L-pipecolic acid (2*S*,4*S*,5*R*-epoxypipecolic acid, *cis*-**3**) was synthesized and found to serve as an excellent substrate for L-pipecolate oxidase (L-PO) and also to cause time-dependent, irreversible inactivation of the enzyme. Data are presented showing this compound is a mechanism-based inhibitor of L-PO, whereas 2*S*,3*R*,4*S*-epoxyproline acts as a reversible inhibitor.

Epilepsy is the second most common neurological disorder after stroke.[1] Although 70–80% of all epileptics are adequately treated by currently available drugs, seizure protection is often accompanied by a diversity of side effects.[2] Consequently, there is a need for improved antiepileptic drugs with greater selectivity and lower toxicity, and the search for new anticonvulsant drugs continues to be an active area of investigation in medicinal chemistry.[3]

L-Pipecolic acid (L-PA, **1**) is a cyclic, nonproteinogenic amino acid that has been shown to be a metabolite of the amino acid lysine.[4,5] L-PA is an intermediate in the major route of L-lysine degradation in the central nervous system (CNS) and is a minor D-lysine metabolite in other tissues.[6] The specific formation of L-PA from L-lysine in the CNS prompted studies to define a neurological role for L-PA. Evidence suggests that L-PA may act as a neuromodulator of the γ-aminobutyric acid (GABA) receptor complex to potentiate GABAergic-inhibitory neurotransmission.[7,8] Administration of L-lysine or L-PA by the intraperitoneal route significantly decreased the onset of clonic and tonic seizures induced by pentylenetetrazol in mice.[9] Intraperitoneal pretreatment with L-PA potentiates the suppressing effects of phenobarbital on electrically and chemically induced convulsions.[10] Based on these observations, specific inhibitors of L-pipecolate oxidase (L-PO; EC 1.5.3.7)), a peroxisomal flavoenzyme catalyzing the oxidation of L-PA to Δ^1-piperideine-6-carboxylate (Δ^1-P6C, **2**) in the first step of L-PA catabolism (Scheme 1), may provide a novel strategy for the treatment of convulsive disorders and better insight into the role of L-lysine and L-PA in the CNS.

Scheme 1: Degradation of Lysine via L-Pipecolic Acid



Rhesus monkey liver L-PO has been purified and characterized as a membrane-associated 46 kDa monomer possessing a covalent flavin cofactor.[11] Previous work to develop mechanism-based inactivators of L-PO suggests that the enzyme shares mechanistic similarities with other flavin-dependent amine oxidases such as monoamine oxidase (MAO).[12,13] The chemical mechanism of these enzymes is believed to proceed either via a

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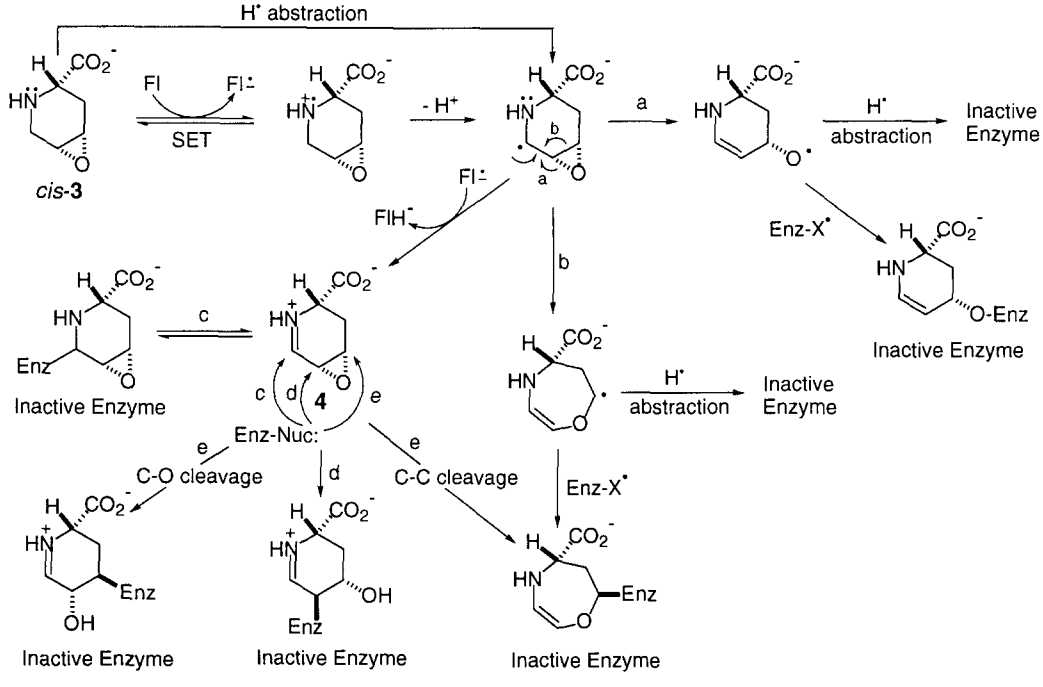
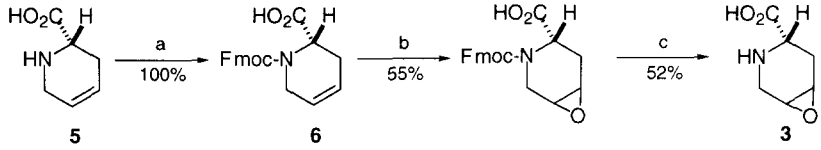
mechanism involving single electron transfer (SET) from the amine to the flavin followed by proton transfer,[14] or by a direct hydrogen abstraction.[15] Central to both proposed mechanisms is the intermediacy of an α-aminyl radical. Such a radical formed at C-6 of the pipecolate skeleton could be relocated via an adjacent epoxide to give a distal carbon- or oxygen-centered radical capable of inactivating the enzyme (Scheme 2). Inactivation could occur by hydrogen abstraction from L-PO or by combination with an active site radical (Enz-X'). An additional route to inactivation would be two-electron oxidation to an electrophilic epoxy iminium species (**4**) which could react with an active site nucleophile in several ways (Scheme 2; pathways c, d, and e). Here we describe that 2*S*,4*S*,5*R*-epoxypipecolic acid (*cis*-**3**) is a time-dependent, irreversible inactivator of Rhesus liver pipecolate oxidase, whereas the 3,4-epoxyproline analog serves as a reversible inhibitor.

Scheme 2: Possible Mechanisms for the Inactivation of L-PO by *cis*-**3**.



The target compound, 4,5-epoxy-L-pipecolic acid (**3**) was synthesized as outlined in Scheme 3. Briefly, 4,5-dehydro-L-pipecolate ($\Delta^{4,5}$-L-PA, **5**) was prepared using a combined enzymatic and chemical method.[12] The amine was protected using *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (FmocONSuc) to give Fmoc-$\Delta^{4,5}$-L-PA (**6**) in theoretical yield.[16] Epoxidation of **6** was achieved using *m*-chloroperoxybenzoic acid (*m*-CPBA) in CH_2Cl_2 and deprotection was carried out with 20% piperidine in THF to give compound **3** in 52% recovered yield after recrystallization from water/acetone. Inspection of the NMR spectra of **3** revealed a single set of signals, indicating only one epoxide diastereomer had been formed. Chiral HPLC analysis also showed the product to consist of a single isomer.[17]

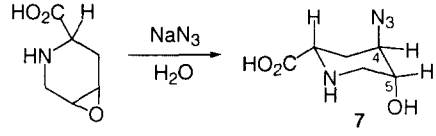
Scheme 3[a]:



[a] (a) FmocONSuc, $NaHCO_3$/NaOH; (b) *m*-CPBA, CH_2Cl_2; (c) 20% piperidine, THF

Because the configuration of the epoxide relative to the carboxyl could not be unambiguously assigned using 1-D and 2-D NMR methods, we resorted to opening the epoxide with NaN_3 in order to obtain a product with more predictable couplings. Hence, racemic **3** was treated with excess aqueous NaN_3 at room temperature for 12 h yielding a single product after purification by cation exchange chromatography. A combination of COSY, HMQC, and NOESY 2-D NMR experiments allowed us to assign the structure of the product as 4-azido-5-hydroxypipecolate (**7**) with the relative configuration shown in Scheme 4.[18] The product of the epoxidation was concluded to be 2*S*,4*S*,5*R*-epoxypipecolic acid (*cis*-**3**). The formation of the epoxide on the same face as the free carboxyl presumably occurs because of a directing effect in the nonpolar solvent.

Scheme 4:



Inhibition studies were conducted using L-PO isolated from frozen Rhesus monkey liver essentially as described by Mihalik et al.[11] The enzyme was judged to be >90% homogeneous by SDS-PAGE and routine enzyme activity measurements were performed using a horseradish peroxidase-coupled, dye-linked spectrophotometric assay.[11]

The initial evaluation of 4,5-epoxypipecolate as an inhibitor of L-PO was conducted using racemic material prepared from D,L-**5**. These studies established that the epoxide derivative was recognized as a substrate for L-PO and caused nonlinear progress curves characteristic of time-dependent inhibition. This was confirmed by preincubation/dilution experiments (data not shown) and provided the impetus to prepare the L-isomer.

The preliminary inhibition studies of L-PO by *cis*-**3** using the peroxidase-coupled assay resulted in observed reaction rates greater than those seen for controls containing only 3.5 mM L-PA. When L-PA was omitted from the assays hydrogen peroxide was still formed, indicating cofactor reoxidation, and clearly establishing *cis*-**3** as a good alternate substrate for L-PO. A plot of the effect of *cis*-4,5-epoxy-L-pipecolate concentration on the rate of the reaction demonstrated saturation kinetics and estimates for the kinetic parameters K_M and k_{cat} of 6.0 mM and 6.3 min^{-1}, respectively, were calculated from a Lineweaver–Burk plot. Typical values for K_M and k_{cat} with L-PA as substrate are 4 mM and 7 min^{-1}, respectively.

A time-dependent loss of L-PO activity was observed during the kinetic experiments with *cis*-**3** just as it was with the racemic material. Figure 1 (panel A) illustrates the nonlinear progress curves resulting from the incubation of various concentrations of *cis*-**3** with L-PO in the presence of 3.5 mM L-PA. Because *cis*-**3** is

such a good substrate and product formation occurs much more often than inactivation (see below) the observed initial velocities parallel the total concentration of L-PA and *cis*-**3**. However, nonlinear regression analysis of the curves indicates the observed rate of inactivation (k_{obs}) is proportional to the concentration of *cis*-**3** in the assay. Figure 1 (panel B) also shows a Kitz and Wilson plot correlating the dependence of the half-life for inactivation ($t_{1/2}$) with the concentration of *cis*-**3**, which was used to calculate values for K_I and k_{inact} of 0.55 mM and 0.05 min^{-1}, respectively.[19] A partition ratio of 130 can be calculated from the ratio k_{cat}/k_{inact}.

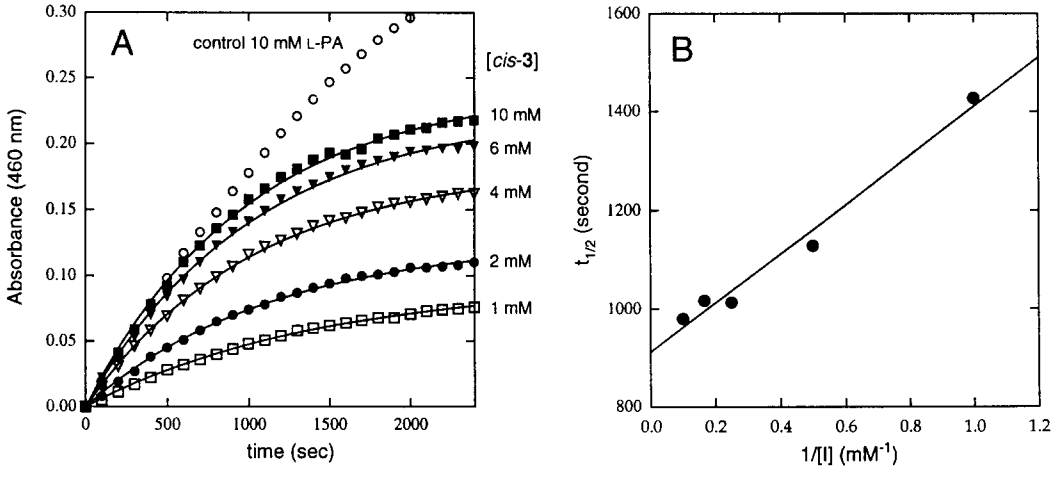


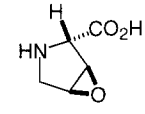
Figure 1. A: Progress curves showing the effect of the concentration of *cis*-**3** on the rate of inactivation of L-PO. Assays were carried out in a total volume of 0.5 mL at 37 °C in 40 mM Tris, 80 mM KCl, 0.8 mM EGTA, pH 8.5 buffer containing 3.5 mM L-PA, 320 μM *o*-dianisidine, 1.8 units horseradish peroxidase, and *cis*-**3** at the following final concentrations: (○) 0 mM with 10 mM L-PA, (■) 10 mM, (▼) 6 mM, (▽) 4 mM, (●) 2 mM, and (□) 1 mM. Reactions were initiated with enzyme. **B**: Kitz and Wilson plot of the dependence of the rate of inactivation of L-PO on the concentration of *cis*-**3**.

To determine if the progressive loss of activity was irreversible, a sample of L-PO was incubated with 10 mM *cis*-**3** at 25 °C for 2 h at which point there was no measurable L-PO activity. After dialyzing the remaining enzyme solution at 25 °C for 9 h, only 6% of the original activity was recovered relative to a control treated in an identical manner. Confirmation that *cis*-**3** acts at the active site of the enzyme came from substrate protection experiments wherein L-PO was incubated with 2 mM *cis*-**3** and various concentrations of L-PA at 25 °C in the dark. At 0.5 h, aliquots were removed and the activity measured. The percent remaining enzyme activity was directly proportional to the L-PA concentration in the assays.

In order to provide further evidence that *cis*-**3** is a mechanism-based inactivator of L-PO, experiments were conducted to test if inactivation occurs prior to release of an activated species from the enzyme. First, the addition of the thiol agents 2-mercaptoethanol, dithiothreitol, and L-cysteine at concentrations of 5 μM had no effect on the inactivation rate of L-PO by *cis*-**3**. Second, a sample of L-PO was incubated with 5 mM *cis*-**3** at 37 °C and the activity monitored until there was no detectable activity. A second aliquot of L-PO was added to the assay mixture resulting in immediate H_2O_2 production which decreased with time at a rate slightly greater

than that observed for the first aliquot. While it is possible that a small portion of inactivation results from a released radical or electrophilic species or the accumulation of a potent reversible inhibitor, these results suggest that the majority of the inactivation occurs at the active site. Evidence for the formation of a covalent adduct was sought using electrospray mass spectrometry but there was no indication of the modified enzyme. However, this analysis will be repeated when greater quantities of the enzyme are available.

The proline analog of **3** was also viewed as a possible inactivator of L-PO and was prepared from commercially available 3,4-dehydro-L-proline in a manner similar to that described above. Analysis of NMR data and modeling studies indicated that the epoxidation occurred on the face *trans* to the proline carboxyl group, establishing the product as 2*S*,3*R*,4*S*-epoxyproline (**8**).[20] Thus, while a directing effect dictates facial selectivity in the epoxidation of 4,5-dehydro-L-pipecolate, steric effects must dominate in the epoxidation of 3,4-dehydro-L-proline. Evaluation of **8** as an inhibitor of L-PO revealed the compound to be a potent reversible inhibitor, exhibiting an IC_{50} of 0.2 mM. There was no evidence for turnover by the enzyme or loss of activity after preincubation. The limited amount of this inhibitor prevented the determination of K_i.



8

In summary, 2*S*,4*S*,5*R*-epoxypipecolic acid (*cis*-**3**) was synthesized by a combined enzymatic and chemical method and shown to serve as an excellent substrate for L-pipecolate oxidase and to act as an irreversible, mechanism-based inactivator of the enzyme. The lower homologue, 2*S*,3*R*,4*S*-epoxyproline (**8**), acts as an excellent reversible inhibitor. Efforts to prepare the other diastereomers of the epoxides and elucidate the mechanism of inactivation are ongoing.

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17. 2*S*,4*S*,5*R*-Epoxypipecolic acid (*cis*-**3**): mp 233–235 °C; $[\alpha]_D$ -107 (*c* 1.0, H_2O); IR (KBr) 3410, 3012, 1628, 1408 cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 3.77 (1H, d, *J* = 14.6 Hz), 3.63 (1 H, dd, *J* = 11.1, 6.1 Hz), 3.60 (1H, dd, *J* = 4.6, 4.5 Hz), 3.54 (2H, m), 2.63 (1H, ddd, *J* = 16.3, 6.1, 4.6 Hz), 2.26 (1H, dd, *J* = 16.3, 11.1 Hz); ^{13}C NMR (100 MHz, D_2O) δ 172.6, 53.2, 49.8, 48.6, 40.4, 23.7; CIHRMS: *m/z* 144.0660 (MH^+) [calcd for $C_6H_{10}NO_3$: 144.0660].
18. 4-Azido-5-hydroxypipecolate (**7**): mp 244–246 °C; IR (KBr) 3427, 2108, 1633, 1593, 1402 cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 3.95 (1H,m), 3.88 (1 H, m), 3.84 (1H, dd, *J* = 9.7, 4.5 Hz), 3.25 (1H, dd, *J* = 13.3, 2.6 Hz), 3.18 (1H, dd, *J* = 13.3, 5.1 Hz), 2.37 (1H, ddd, *J* = 14.8, 9.7, 3.4 Hz), 2.15 (1H, ddd, *J* = 14.8, 5.5, 4.5 Hz); ^{13}C NMR (100 MHz, D_2O) δ 176.5, 67.3, 61.0, 56.9, 47.2, 29.4; FABHRMS: *m/z* 187.0831 (MH^+) [calcd for $C_6H_{11}N_4O_3$: 187.0831].
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20. 2*S*,3*R*,4*S*-Epoxyproline (**8**): mp 224–226 °C; The ee was > 70 % as determined by chiral HPLC; $[\alpha]_D$ -77 (*c* 0.11, H_2O); IR (KBr) 3433, 2978, 1635, 1377 cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 4.39 (1H, s), 4.10 (1 H, d, *J* = 2.9 Hz), 4.00 (1H, dd, *J* = 2.9, 0.8 Hz), 3.65 (1H, d, *J* = 13.1 Hz), 3.51 (1H, dd, *J* = 13.1, 0.8 Hz); ^{13}C NMR (100 MHz, D_2O) δ 171.7, 64.1, 59.1, 56.5, 48.0; CIHRMS: *m/z* 130.0505 (MH^+) [calcd for $C_5H_8NO_3$: 130.0504]. Dihedral angles used to predict coupling constants were estimated from models built using the Chem3D Pro software package (CambridgeSoft Corporation).