Stereochemical Course of the Oxidation of L-Pipecolic Acid by the Flavoenzyme L-Pipecolate Oxidase

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L-Pipecolic acid (L-PA, **1**) is a lysine metabolite found in numerous microbial, plant, and animal species. In addition to its occurrence as the free amino acid, L-PA is also a key structural element in several clinically important natural products including the immunosuppressant FK-506[1] and the glycosidase inhibitor swainsonine.[2] Although L-PA is found in most mammalian tissues, the major route of L-lysine degradation is through L-saccharopine (**2**) in a process resembling the reversal of lysine biosynthesis in yeast and fungi (Scheme 1, path A).[3,4] In mammalian brain, the enzyme activities for the saccharopine pathway are not detectable and L-lysine is predominantly catabolized via L-PA (Scheme 1, path B).[5] Pipecolic acid is also formed in other tissues, notably liver and kidney, but D-lysine appears to be the specific precursor.[6,7]

The oxidative degradation of L-PA begins with formation of the imine Δ^1-piperideine-6-carboxylate (Δ^1-P6C, **3**). In some bacteria and nonprimates, this activity has been assigned to a flavin-dependent dehydrogenase associated with the electron transport process; whereas, in primates, the oxidation is catalyzed by a peroxisomal oxidase.[8,9] The saccharopine and pipecolate paths converge at the common intermediate L-α-aminoadipate-δ-semialdehyde (**4**), which is further oxidized to L-α-aminoadipate (**5**).

The specific formation of L-PA from L-lysine in the CNS,[5,10] together with the identification of a specific degradative system,[9] indicates that **1** might occupy a particular neurological role. A variety of evidence suggests that **1** functions as a neuromodulator, interacting with γ-aminobutyric acid (GABA) receptor complexes to potentiate GABAergic inhibitory neurotransmission.[11] Several clinically useful antiepileptic drugs, including phenobarbital and diazepam, act by affecting the GABA-activated chloride ion channels.[12] In contrast to the possible neuroprotective action of L-PA, L-α-aminoadipate (**5**) is toxic to cultured cerebellar cells,[13] affects L-glutamate transport,[14] and lowers glutathione levels.[15]

To better define the neurological function of lysine metabolites in the CNS and explore strategies for developing new

Scheme 1
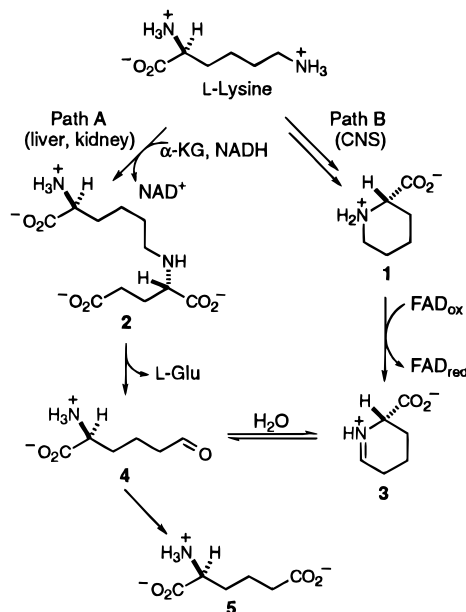
anticonvulsants, we have started a program to develop specific inhibitors of primate L-pipecolate oxidase (L-PO; EC 1.5.3.7).[16,17] The enzyme has been purified and characterized from Rhesus monkey liver and shown to be a peroxisomal, membrane-associated, 46 kDa monomer possessing a covalent FAD cofactor.[18] L-PO requires molecular oxygen and generates H_2O_2 in addition to the imine **3**. Specific inhibitors of L-PO would elevate synaptic levels of **1** and its associated neurological effects while simultaneously lowering amounts of the neurotoxic α-aminoadipate (**5**). Identification of which hydrogen from C-6 of L-PA is removed during the oxidation will aid in the design of the most selective inactivators of L-PO. Herein, we describe the preparation of stereospecifically deuterated L-{6(R)-^{2}H}- and L-{6(S)-^{2}H}pipecolic acids and present results from kinetics and product characterization experiments demonstrating that the *pro*-6(R) hydrogen of L-PA is stereospecifically removed by Rhesus monkey liver L-PO.
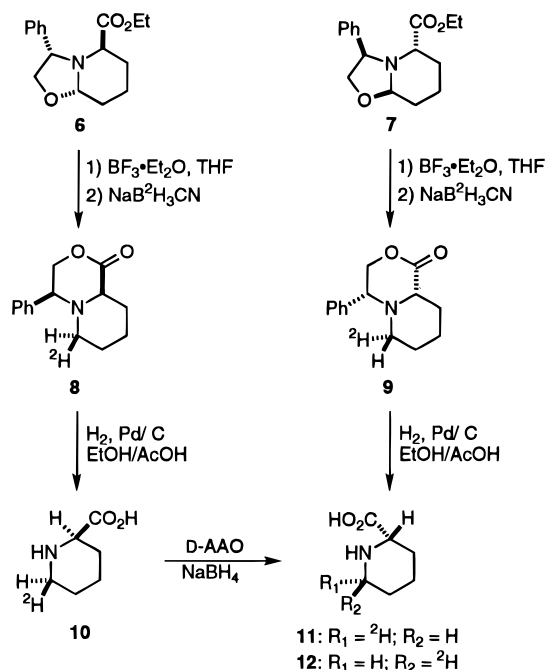
L-Pipecolic acids deuterated at the *pro*-6(S) and *pro*-6(R) positions were prepared by a modified procedure reported for the synthesis of 6-alkyl L-PA derivatives.[19] Briefly, the key intermediates required, phenyloxazolidinopiperidines **6** and **7**, were prepared in two steps from (S)- and (R)-phenylglycinol, respectively. Treatment of **6** or **7** with boron trifluoride etherate in THF followed by reduction with NaB^{2}H$_3$CN yielded the deuterated lactones **8** and **9** (Scheme 2). Deprotection by catalytic hydrogenolysis and purification using cation exchange chromatography afforded the enantiomers D-{6(R)-^{2}H}pipecolic acid (**10**) and L-{6(S)-^{2}H}pipecolic acid (**11**) in greater than 95% ee. Compound **10** was converted to L-{6(R)-^{2}H}PA (**12**) using a combined enzymatic and chemical procedure employing D-amino acid oxidase (D-AAO) and NaBH$_4$.[20] The conversion of **10** to **12** was monitored by chiral HPLC and continued until the configuration at C-2 was $\geq$95% S.[21]

The first insight into the stereochemical course of the oxidation came from kinetics studies evaluating the effect of deuterium position at C-6 on the reaction rate. Rhesus monkey

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Scheme 2

Figure 1. Michaelis−Menten plot (A) and Lineweaver−Burk transformation (B) of the initial velocity data for the L-PO-catalyzed oxidation of **1**, **11**, and **12**. Assays were conducted 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 20 μL L-PO solution, 320 μM o-dianisidine, 1.8 units of horseradish peroxidase, and various concentrations of **1** (■), **11** (△) and **12** (●). Reactions were initiated with the addition of substrate and followed spectrophotometrically by monitoring the increase in absorbance at 460 nm. Each point represents the average of duplicate measurements.

liver L-PO was isolated according to the literature procedure and judged to be at least 90% homogenous by SDS-PAGE.[18] Enzyme activity was monitored spectrophotometrically using a dye-linked assay coupling the production of H_2O_2 to the horseradish peroxidase-catalyzed oxidation of o-dianisidine.[22] Figure 1 shows plots of the initial velocity data for the rate of the L-PO-catalyzed oxidation of **1**, **11**, and **12** as a function of substrate concentration. The kinetic parameters and isotope effects calculated from these data are given in Table 1.[23] Placement of deuterium in the *pro*-6(*S*) position (**11**) had no detectable effect on the reaction rate, while a primary kinetic isotope effect on V_{max}, $^DV = 3.5$, was observed for the oxidation of **12**. This provides evidence for the stereospecific removal of the *pro*-6(*R*) hydrogen of pipecolic acid and indicates that C−H bond cleavage is at least partially rate-determining.

Confirmation that the *pro*-6(*R*) hydrogen is removed from L-PA was obtained from mass spectrometric analysis of the derivatized and isolated oxidation products. Compounds **1**, **11**, and **12** were individually incubated with L-PO for 14 h, and the resulting α-aminoadipate-δ-semialdehydes were derivatized as the phenylthiohydantoins (PTH) by treatment with phenyl isothiocyanate.[24] The PTH derivatives were purified using C_{18} reverse phase HPLC and analyzed by GC-MS. Inspection of the mass spectrum of the undeuterated control revealed a molecular ion at *m/z* 260. Parent ions for the derivatized oxidation products of **11** and **12** were seen at *m/z* 261 and 260, respectively, verifying the findings of the kinetics experiments.

Table 1. Apparent Kinetic Deuterium Isotope Effects for the Oxidation of L-Pipecolic Acid by Rhesus Monkey Liver L-Pipecolate Oxidase[23]

substrate	K_m^{app} (mM)	V_{max}^{app} (nmol min^{-1} mg^{-1})	V_{max}/K_m	^DV	$^D(V/K)$
1	6.0 ± 0.9	113 ± 7	18.9 ± 3.2		
11	6.0 ± 0.8	114 ± 6	19.1 ± 2.6	1.0 ± 0.1	1.0 ± 0.2
12	6.3 ± 1.5	32.2 ± 4.2	5.1 ± 1.4	3.5 ± 0.5	3.7 ± 1.2

In summary, the data described above provide conclusive evidence that pipecolate oxidase stereospecifically removes the *pro*-6(*R*) hydrogen of L-pipecolic acid. This is vital information for developing the most selective inactivators of this enzyme which may lead to a better understanding of lysine metabolism in the CNS.

Supporting Information Available: Information on the preparation and characterization of **11** and **12**, including spectral and chromatographic data, (6 pages). See any current masthead page for ordering and Internet access instructions.

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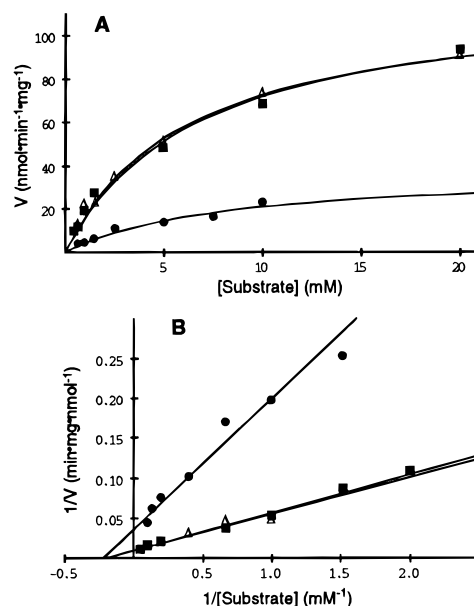(24) Heinrikson, R. L.; Meredith, S. C. *Anal. Biochem.* **1984**, *136*, 65.