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Mechanism of the Stereospecific Irreversible Inhibition of Bacterial Glutamic Acid Decarboxylase by (*R*)-(−)-4-Aminohex-5-ynoic Acid, an Analogue of 4-Aminobutyric Acid†

Michel J. Jung,* Brian W. Metcalf, Bruce Lippert, and Patrick Casara

ABSTRACT: 4-Aminohex-5-ynoic acid inhibits bacterial glutamic acid decarboxylase in a time-dependent irreversible manner. The inhibition is stereospecific and requires the abstraction of the propargylic hydrogen from 4(*R*)-(−)-4-aminohex-5-ynoic acid. This leads to the generation of a reactive alkylating agent in the active site which can react with a nucleophilic residue. At complete inhibition, there is incorporation of one molecule of inhibitor per pyridoxal binding site. If the decarboxylation of glutamate occurs with retention of configuration, the irreversible inhibition of this enzyme by the 4-(*R*) isomer can be rationalized on the basis of reversibility of the protonation step in the normal catalytic mechanism.

We reported previously that (±)-4-aminohex-5-ynoic acid is a suicide enzyme inactivator of GABA-aminotransferase from *Ps. fluorescens* (Jung and Metcalf, 1975) and that this compound decreases brain GABA-aminotransferase activity when administered to rats or mice by a systemic route (Jung et al., 1977). During the in vivo investigations, it was found that there is also a decrease in brain glutamate decarboxylase activity, and the mechanism of the action of the GABA-aminotransferase inhibitor on glutamate decarboxylase from mammalian brain and of *Escherichia coli* was investigated. The present work discloses that this GABA analogue is a catalytic irreversible inhibitor of bacterial glutamate decarboxylase and that the inhibition requires the abstraction of the hydrogen α to the acetylenic group (propargylic hydrogen) from 4(*R*)-4-aminohex-5-ynoic acid. The proposed mechanism of inactivation is based on the reversibility of the protonation step during the normal decarboxylation of L-glutamic acid to GABA and should apply to the inhibition of other α-amino acid decarboxylases.

Materials and Methods

Chemicals. L-Glutamate and (±)-2-methylglutamate were purchased from Sigma Chemical Co. (St. Louis, Mo.). Pyridoxal phosphate, dithiothreitol, and ruthenium dioxide were obtained from Merck (Darmstadt). [1-^{14}C]-DL-Glutamic acid (sp. act. 50 Ci/mol) was bought from New England Nuclear.

Enzyme. Type II glutamate decarboxylase of *E. coli* was obtained from Sigma (sp. act. 2.5 units/mL). This preparation was used as such or in a purer form readily obtained by heating a solution (10 mg/mL) of the commercial enzyme in 0.2 M pyridine hydrochloride, pH 4.5, at 37 °C for 1 h; centrifugation and dialysis at room temperature against 0.1 M pyridine hy-

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[1] Abbreviations used are: GABA-aminotransferase, 4-aminobutyric acid-2-ketoglutarate aminotransferase (EC 2.6.1.19); glutamate decarboxylase, L-glutamic acid carboxylyase (EC 4.1.1.15); GABA, 4-aminobutyric acid; Py CH, the phosphorylated pyridoxilidene moiety; Me_3Si, trimethylsilyl.

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drochloride yield a preparation with a specific activity of 45–50 units/mg, about half the maximum specific activity reported for this enzyme (Strausbauch and Fischer, 1967). One unit is defined as the amount of enzyme which catalyzes the decarboxylation of 1 μmol of L-glutamate/min under the conditions specified below.

Enzyme Activity Measurements. This was done by a radiometric assay based on the release of $^{14}CO_2$ from [1-^{14}C]-L-glutamate (Strausbauch and Fischer, 1967). The closed reaction vessel with the hyamine hydroxide soaked filter paper described previously (Jung et al., 1977) contained 2 mL of assay medium (0.2 M pyridine hydrochloride buffer, pH 4.5, 10^{-4} M pyridoxal phosphate, 2.5 mM L-Glu, 0.25 μCi of [1-^{14}C]-DL-Glu). The incubation was started by the addition of enzyme to the complete assay medium at 37 °C, was allowed to proceed for 20 min, and then was stopped by the addition of 0.5 mL of 40% trichloroacetic acid. The $^{14}CO_2$ trapped on the filter paper was counted in a standard scintillation cocktail.

Synthesis of Labeled (±)-4-Aminohex-5-ynoic Acid

(*a*) *Synthesis of [4-^{2}H]-4-Aminohex-5-ynoic Acid.* The synthesis was the same as for the protium compound (Metcalf and Casara, 1975), except that the propargylamine derivative was replaced by $Me_3SiC{\equiv}CC^2H_2N{=}CHC_6H_5$. The latter compound was obtained from $Me_3SiC{\equiv}CCH_2N{=}CHC_6H_5$ by two sequential treatments with *n*-butyllithium and perdeuterated methanol and contained 6% hydrogen in the propargylic position (estimated by proton magnetic resonance). No propargylic hydrogen could be detected in the derived [4-^{2}H]-4-aminohex-5-ynoic acid, probably due to a kinetic isotope effect during hydrogen abstraction from $Me_3SiC{\equiv}CC^2H_2N{=}CHC_6H_5$ before the addition of methyl acrylate.

(*b*) *Synthesis of [2-^{3}H]-4-Aminohex-5-ynoic Acid.* The synthesis of tritiated inhibitor (sp act. 0.65 Ci/mol) was described earlier (Jung et al., 1977).

Resolution and Determination of the Absolute Configuration of the Two Optical Isomers of (±)-4-Aminohex-5-ynoic Acid. Racemic 4-aminohex-5-ynoic acid was resolved using (+)- and (−)-binaphthylphosphoric acid (Jacques et al., 1971). After four recrystallizations of the derived salts, the free amino acids reached constant rotations: $[\alpha]_D$ −32° (*c* 1.50, H_2O) for the (−) isomer and $[\alpha]_D$ +30° (*c* 1.28, H_2O) for the (+) isomer. The absolute configuration of each isomer could be established by chemical conversion into L- or D-glutamic acid as follows (adapted from Gopal and Gordon, 1971). A suspension of 10 mg of RuO_2 in 2 mL of water was treated at 0 °C with a saturated solution of $NaIO_4$, so that the ruthenium was oxidized to RuO_4. Then, an aqueous solution of one of the isomers (100 mg) was added slowly together with more $NaIO_4$ at a ratio such that the ruthenium tetroxide was continuously regenerated. After complete addition, the dark solution was treated with charcoal and chromatographed on a Dowex 50 resin. Elution with 2 N ammonia yielded after evaporation 40–50 mg of a crystalline material which cochromatographed with glutamic acid in two solvent systems [BuOH–AcOH–H_2O (6:2:2) and NH_4OH–EtOH–H_2O (1:16:3)]. The action of bacterial glutamate decarboxylase on the product derived from the (−) isomer of 4-aminohex-5-ynoic acid yielded only 5–10% GABA (judged by thin-layer chromatography on silica gel in the same solvent systems as above), while 90% of the product remained unchanged (D-glutamate). In contrast, 90–95% of the glutamate derived from the (+) isomer was decarboxylated to GABA.

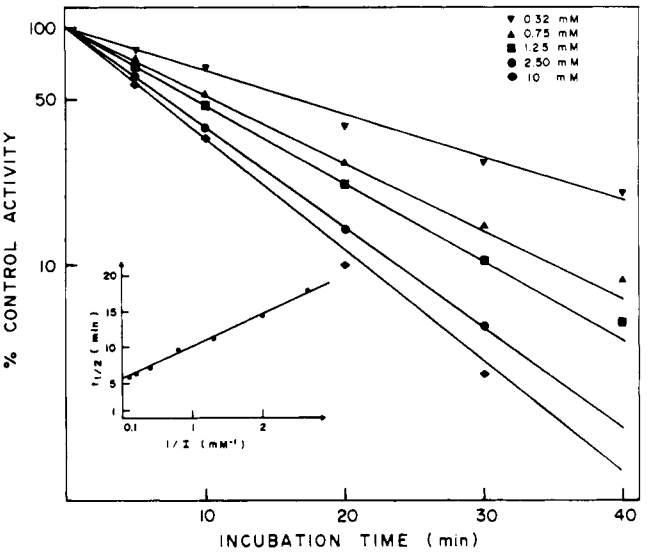


FIGURE 1: Time-dependent loss of glutamate decarboxylase activity upon incubation with (±)-4-aminohex-5-ynoic acid. A stock solution of 6 mg of commercial GAD was made up in 5 mL potassium phosphate buffer (10 mM, pH 6.5) containing 0.2% of bovine serum albumin. For a typical experiment 100 μL of this solution was mixed with 50 μL of pyridine hydrochloride buffer (0.4 M, pH 4.5) and 30 μL of water. At time zero, 20 μL of a stock solution of the inhibitor in water was added. At different times, 20-μL aliquots were assayed for remaining enzyme activity. In a standard assay, no pyridoxal phosphate was present in the incubation.

Spectral Changes during the Inhibition. The spectra were recorded on a Beckman Acta III spectrophotometer using 1-cm light-path semimicro cells (volume 0.5 mL). The temperature of the cell was 30 °C. The buffer used in this study was 0.1 M (glycylglycine hydrochloride), pH 4.4.

Incorporation of Radioactivity and Loss of Enzyme Activity. At time zero, 200 μL of 10^{-2} M [2-^{3}H]-4-aminohex-5-ynoic acid was added to 1800 μL of purified enzyme (0.4 mg of protein/mL) and this mixture was incubated at 37 °C. At different time intervals, 200-μL aliquots were taken and added to 1 mL of a 0.2% solution of albumin (used as carrier), and the proteins were precipitated by immediate addition of 1 mL 40% trichloroacetic acid. The protein precipitate was separated by ultrafiltration and then extensively washed, and the membrane was dried at 50 °C and digested in 2 mL of Protosol. Radioactivity was measured in Instagel using an external standard correction for quenching. Blank values, obtained by omission of enzyme in the incubation medium, did not change with time and were substracted from each value. In a parallel experiment with unlabeled inhibitor, the decrease of enzyme activity was measured under strictly identical conditions.

Results

Time-Dependency and Irreversibility of the Glutamate Decarboxylase Inhibition. Incubation of crude or purified glutamate decarboxylase from *E. coli* with 4-aminohex-5-ynoic acid results in a time-dependent loss of enzyme activity (Figure 1), which follows pseudo-first-order kinetics until inhibition is essentially complete. By plotting $T_{1/2}$ as a function of $1/I$ as described by Kitz and Wilson (1962), it appears that the enzyme can be saturated by the inhibitor, the apparent dissociation constant being 1 mM and the minimal half-life at infinite inhibitor concentration 5.6 min which corresponds to a catalytic constant of 2.05×10^{-3} s^{-1}.

The inhibition is irreversible, since only negligible amounts of enzyme activity can be restored by a 48-h dialysis against several buffer changes (pyridine hydrochloride, 0.1 M, pyri-

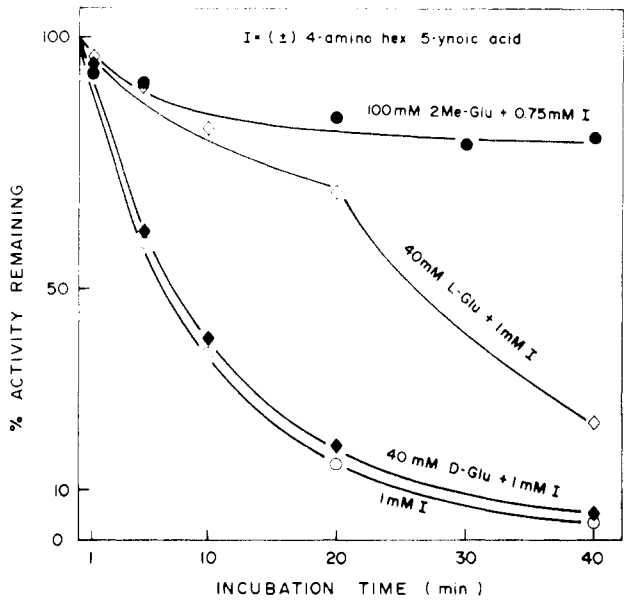


FIGURE 2: Effect of L- or D-glutamate, pyridoxal phosphate, and 2-methylglutamate on the inhibition of glutamate decarboxylase by (±)-4-aminohex-5-ynoic acid. The incubation and assay conditions are as described for Figure 1, except that instead of water the substrate or substrate analogue was added. Twenty microliters of purified glutamate decarboxylase (0.4 mg/mL; sp act. 50 units/mg of protein) was used in the incubations.

TABLE I: Stereospecificity of the Inactivation of Glutamate Decarboxylase by 4-Aminohex-5-ynoic Acid.[a]

4-aminohex-5-ynoic acid		half-life of glutamate decarboxylase (min)
4(*R*)-(−) (mM)	4(*S*)-(+) (mM)	
1.25	0	3.6
1.25	1.25	6.7
1.25	3.75	12
0	3.75	>100

[a] Incubation and assay were carried out as described in Figure 1 using 0.4 unit of purified glutamate decarboxylase in a volume of 0.2 mL for the incubation.

TABLE II: Primary Deuterium Isotope Effect on the Glutamate Decarboxylase Inactivation by 4-Aminohex-5-ynoic Acid.[a]

	4-aminohex-5-ynoic acid	
	4-^{1}H	4-^{2}H
half-life at 0.5 mM inhib (min)	12.2	34
half-life at infinite concn of inhib (min)	5.6	6.7
K_I (mM)	1.0	2

[a] The rate of inactivation of glutamate decarboxylase at five different concentrations of the two isotopic forms was measured in parallel with the same enzyme preparation. The kinetic constants were determined as in Figure 1.

doxal phosphate, 0.1 mM, pH 4.5). When the incubation with 5 mM inhibitor is carried out at pH 6.0 where the enzyme is essentially inactive (Shukuya and Schwert, 1960a), there is no loss of enzyme activity. For the following reasons the inhibition is not due to the accumulation of a product released in the medium by the action of glutamate decarboxylase on the inhibitor [as for instance during the inhibition of yeast alcohol dehydrogenase by allyl alcohol (Rando, 1974)]: there is no lag time before the onset of inhibition, mercaptoethanol at 1 mM concentration does not prevent the loss of enzyme activity, and upon addition of new enzyme to an inhibited incubation the rate of decrease of enzyme activity is strictly identical to the first one.

Effect of Substrate, Substrate Analogues or Coenzyme on the Rate of Inhibition. Bacterial glutamate decarboxylase acts only on L-glutamic acid and has no reported affinity for the D isomer. When the enzyme is incubated with 4-aminohex-5-ynoic acid in the presence of L-Glu (Figure 2), the rate of activity loss is less than in the absence of L-Glu. The protection by L-Glu lasts only for 20 min, at which time all the glutamic acid has been decarboxylated to GABA. D-Glutamate at the same concentration does not protect significantly. The addition of pyridoxal phosphate (10^{-4}–10^{-3} M) seems to enhance the inhibition slightly (not shown). A substrate analogue, 2-methylglutamate (0.1 M), in the presence of pyridoxal phosphate (1 mM) completely protects glutamate decarboxylase against the inhibition by 4-aminohex-5-ynoic acid.

Stereochemistry. 4-Aminohex-5-ynoic acid has been resolved into its optical isomers. Only the (−) isomer inhibits irreversibly glutamate decarboxylase, and it seems that the other isomer protects the enzyme against inhibition, suggesting that glutamate decarboxylase can bind both isomers (Table I). However, the effect of the (+) isomer on glutamate decarboxylation by this enzyme could not be studied, as there was always some contamination (less than 4–5%) by the irreversibly acting isomer. The (−) isomer could be assigned the *R* configuration, as the oxidation of the triple bond by RuO_4 yielded D-glutamate. Using this chemical conversion of the irreversible inhibitor into the substrate, it was also possible to estimate the optical purity of both isomers.

Deuterium Isotope Effect. Replacing the protium compound by [4-^{2}H]-4-aminohex-5-ynoic acid slows down the rate of enzyme inhibition (Table II). In this table the kinetic and binding constants of the two isotopic forms are compared. There is an isotope effect of 2 on the apparent affinity constants and a smaller effect (1.35) on the minimum half-life. Isotope effects on apparent binding constants are more the rule than the exception (Jencks, 1969), especially as the mathematical expression of this type of K_I incorporates the catalytic constant (Jung and Metcalf, 1975). The lesser isotope effect on the kinetic constant has been attributed in a similar situation (Belleau and Moran, 1963) to the elongation during the binding step of the C–H bond which must be broken.

Spectral Change during the Inhibition (Figure 3). The native enzyme shows the absorption at 415 nm due to the pyridoxal Schiff base (Shukuya and Schwert, 1960b). Upon addition of 4-aminohex-5-ynoic acid, there is an immediate decrease of this peak, probably due to the formation of another Schiff base between pyridoxal phosphate and the inhibitor. After this initial drop, there is a slow decrease at 415 nm, accompanied by an increase of absorption at 330 nm which had been previously attributed to the formation of pyridoxamine (Sukhareva et al., 1971). Within the precision of our measurements, there seems to be an isobestic point around 350 nm. The spectrum of the native enzyme, however, does not cross this point, as it represents a different species. Upon dialysis of the completely inhibited enzyme, the residual activity at 415 nm and the shoulder at 330 nm disappear. These spectral changes are similar to those found during the abnormal decarboxylation of 2-methylglutamate by the same enzyme, in the absence of pyridoxal phosphate in the incubation medium (Sukhareva et al., 1971). However, in this latter case, the ac-

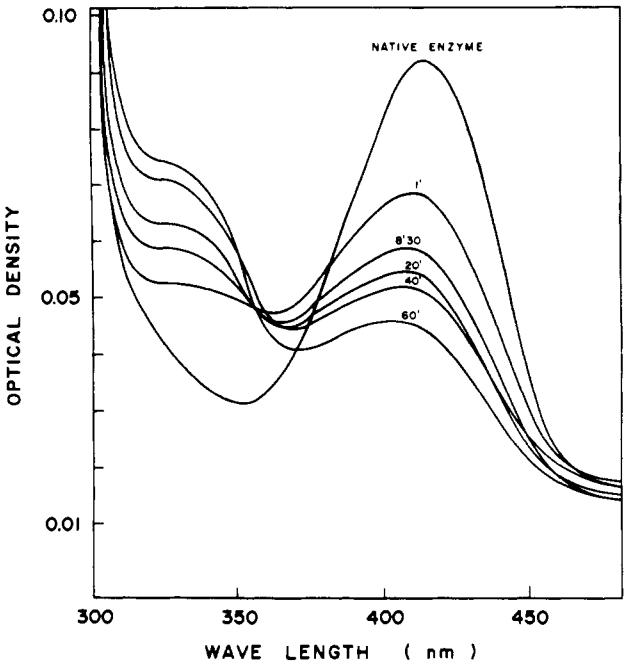


FIGURE 3: Spectral changes of glutamate decarboxylase during incubation with (±)-4-aminohex-5-ynoic acid. The purified enzyme preparation was dialyzed against a 0.1 M glycine–glycine hydrochloride buffer, pH 4.4; final concentration 0.35 mg/mL. The spectra of the native enzyme (450 μL) was recorded before the addition of 50 μL of inhibitor (4×10^{-3} M), and then the spectrum was recorded at the indicated time intervals. The cuvette holder was thermostated at 30 °C.

tivity of the enzyme is restored by the addition of pyridoxal phosphate (Fonda, 1975) while it has been shown above that pyridoxal phosphate neither prevents the inactivation of glutamate decarboxylase by 4-aminohex-5-ynoic acid, nor restores the lost enzyme activity.

Incorporation of Radioactivity. When glutamate decarboxylase is incubated with (±)-[2-^{3}H]-4-aminohex-5-ynoic acid, there is a gradual incorporation of radioactivity into the protein, which levels off when the enzyme is fully inhibited (Figure 4). Therefore, we assume that the loss of enzyme activity is due to the modification of the active site, rather than to the inability to bind pyridoxal phosphate as could be suggested from the spectral changes described above. The amount of radioactivity incorporated in the completely inhibited enzyme corresponds to 0.95 mol of inhibitor per 75 000 g of protein, that is about 1 mol per pyridoxal phosphate binding site (Strausbauch and Fisher, 1967).

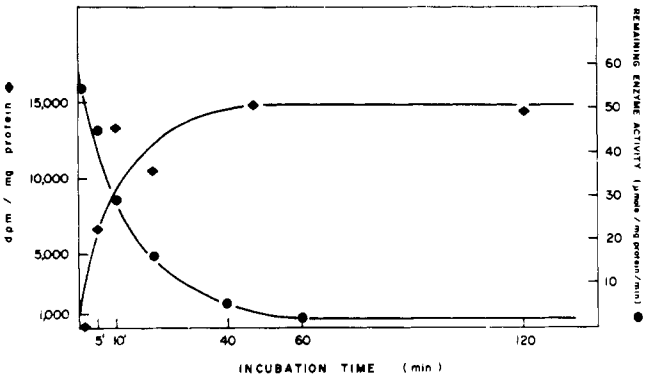


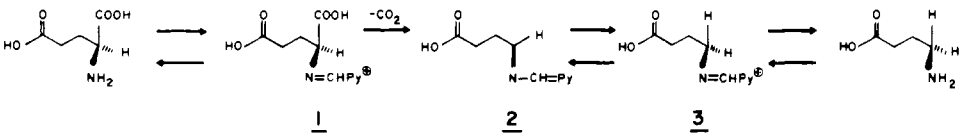
FIGURE 4: Correlation between the loss of enzyme activity and the incorporation of radioactivity upon incubation of bacterial glutamate decarboxylase with (±)-[2-^{3}H]-4-aminohex-5-ynoic acid.

Discussion

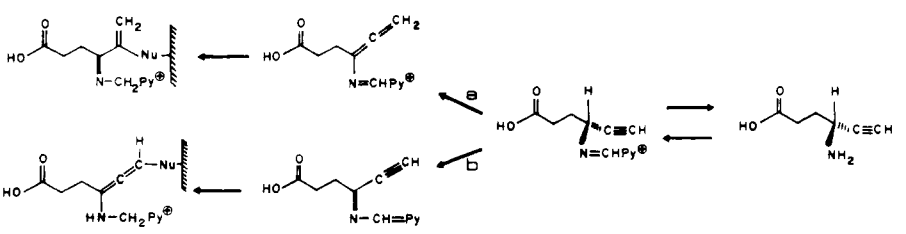
From the described experiments it appears that 4(*R*)-4-aminohex-5-ynoic acid is a time-dependent, irreversible inhibitor of bacterial glutamate decarboxylase. During the inhibition, which requires an active enzyme, there is an abstraction of the propargylic hydrogen of the inhibitor, the pyridoxal holoenzyme is converted to the pyridoxamine form, and there is a stoichiometric incorporation of one molecule of inhibitor per pyridoxal phosphate binding site. All these results suggest that the inhibitor is active-site directed and needs a catalytic activation.

Scheme I represents the accepted mechanism for the decarboxylation of L-2(*S*)-glutamic acid (Boeker and Snell, 1972). Loss of CO_2 from the pyridoxaldimine **1** generates the aldimine **2** which is protonated at the C_4 atom of the GABA skeleton to give the isomeric aldimine **3**, hydrolysis of which releases 4-aminobutyric acid. Mandeles et al. (1954) have reported that glutamate decarboxylase catalyzes a stereospecific hydrogen exchange on GABA to give the same [4-^{2}H]-4-aminobutyric acid obtained by decarboxylation of glutamate in deuterated water, which suggests that the protonation step 2 → 3 is reversible. More recently, it has been impossible to confirm this result (Yamada and O'Leary, 1977). We suggest that if (*R*)-4-aminohex-5-ynoic acid can replace GABA in the active site, the postulated hydrogen abstraction implicit in the reverse reaction (which could be facilitated by the acetylenic group) should lead to the formation of a propargylic anion (Scheme II). This anion on protonation could give either a conjugated allene (pathway a) as previously

SCHEME I



SCHEME II



postulated (Jung and Metcalf, 1975) or an α,β unsaturated Schiff base (pathway b). In either case, an α,β unsaturated imine would be generated which now can be alkylated by a nucleophilic residue in the enzyme active site, leading to irreversible inhibition.

Finally, if the enzyme-catalyzed replacement of the 1-carboxylic group by a hydrogen atom occurs with retention of configuration, as has been found for the decarboxylation of L-tyrosine (Belleau and Burba, 1960), L-histidine (Chang and Snell, 1968), and L-lysine (Leistner and Spenser, 1975), the inhibition of glutamate decarboxylase by the 4(*R*) isomer of 4-aminohex-5-ynoic acid is in agreement with the expected stereospecificity of the hydrogen abstraction. 4-Aminohex-5-enoic acid, another catalytic inhibitor of brain GABA-aminotransferase, which could in principle induce the formation of a vinyl imine in the active site of glutamate decarboxylase by a similar deprotonation, is devoid of time-dependent inhibitory activity on glutamate decarboxylase of bacterial or mammalian origin (Lippert et al., 1977). This is in agreement with the suggestion that the hydrogen abstraction could be facilitated by the acetylenic group. GABA-aminotransferase from *Ps. fluorescens* and mammalian brain as well as pig brain glutamate decarboxylase are inhibited by 4(*S*)-4-aminohex-5-ynoic acid (to be published). The mechanism by which mammalian brain glutamate decarboxylase is inhibited by the 4(*S*) isomer is under investigation. Finally, it might be mentioned that (±)-5-hexyne-1,4-diamine, the α-ethynyl analogue of putrescine, is also a catalytic inhibitor of L-ornithine decarboxylase (EC. 4.1.1.17) (Metcalf et al., 1978), showing that the concept of inhibition of an α-amino acid decarboxylase by a suitably substituted analogue of the product amine can be extended.

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