Biochemical Pharmacology, Vol. 33, No. 11, pp. 1741–1746, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
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STEREOCHEMISTRY OF THE INACTIVATION OF 4-AMINOBUTYRATE: 2-OXOGLUTARATE AMINOTRANSFERASE AND L-GLUTAMATE 1-CARBOXYLASE BY 4-AMINOHEX-5-YNOIC ACID ENANTIOMERS

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(*Received* 8 *August* 1983; *accepted* 28 *November* 1983)

Abstract—Incubation of rat brain or bacterial 4-aminobutyrate aminotransferase (EC 2.6.1.19) with both (*S*)-(+)- and (*R*)-(−)-enantiomers of 4-aminohex-5-ynoic acid results in a time-dependent irreversible loss of enzymatic activity. Rat brain glutamate decarboxylase (EC 4.1.1.15) is inactivated by the (*S*)-(+)-enantiomer while the bacterial glutamate decarboxylase is inactivated by the (*R*)-(−)-enantiomer. In addition, we demonstrate that (*R*)-(−)-4-aminohex-5-ynoic acid is a selective and effective inhibitor of rat brain 4-aminobutyrate aminotransferase *in vivo*.

The recognition of 4-aminobutyric acid as a major inhibitory neurotransmitter in the mammalian central nervous system [1, 2] has stimulated the search for inhibitors of 4-aminobutyrate: 2-oxoglutarate aminotransferase (EC 2.6.1.19, 4-aminobutyrate aminotransferase), the 4-aminobutyric acid catabolizing enzyme [3]. (*R,S*)-4-Aminohex-5-ynoic acid was designed and synthesized as an enzyme-activated irreversible inhibitor of this enzyme [4]. However, although it does inactivate 4-aminobutyrate aminotransferases from mammalian and bacterial origin, it also inactivates L-glutamate decarboxylase (EC 4.1.1.15), the enzyme responsible for the biosynthesis of 4-aminobutyric acid [3, 6]. Previous work with the enantiomers of 4-aminohex-5-ynoic acid showed that (*S*)-(+)-4-aminohex-5-ynoic acid was responsible for the inactivations of bacterial and mammalian 4-aminobutyrate aminotransferase and of mammalian L-glutamate decarboxylase [7] while the (*R*)-(−)-enantiomer was the species inactivating bacterial L-glutamate decarboxylase [8].

Our recent observation that both (*S*)- and (*R*)-enantiomers of 5-hexyne-1,4-diamine, a precursor of 4-aminohex-5-ynoic acid [9], inactivated brain 4-aminobutyrate aminotransferase *in vivo* [10] lead us to reinvestigate the stereoselectivity of the inactivation of 4-aminobutyrate aminotransferases and glutamate decarboxylase by enantiomers of 4-aminohex-5-ynoic acid *in vitro* and *in vivo*.

MATERIALS AND METHODS

Chemical products. D,L-[1-^{14}C] glutamate (specific radioactivity 50 Ci/mol) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Bacterial glutamate decarboxylase (type II from *E. coli*) and 'Gabase' (bacterial 4-aminobutyrate aminotransferase from *Pseudomonas fluorescens*) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). The pure enantiomers of 4-aminohex-5-ynoic acid were obtained according to a published procedure [8]. The rotations were $[\alpha]_D$ + 35.6° (C 1.03, H_2O) and $[\alpha]_D$ − 37.6° (C 1.00, H_2O) for the (*S*)- and the (*R*)-enantiomer respectively. The enantiomeric purity of enantiomers was assessed, after appropriate derivatization, by a gas chromatographic assay (J. Wagner, unpublished results; see also ref. [11]). The enantiomeric excess was higher than 99.5% for both compounds. All other chemical products were of the purest grade commercially available.

Enzyme preparation and activity measurements. Rat brain 4-aminobutyrate aminotransferase was purified following the method of John and Fowler [12] up to the second ammonium sulfate precipitation except that 2-oxoglutarate was omitted from the last dialysis buffer. The resulting preparation had a sp. act. of 8 μmol/hr/mg protein. Succinate semialdehyde dehydrogenase from guinea pig kidney was partially purified by following the method of Pitts *et al.* [13] up to the second ammonium sulfate precipitation.

Rat brain glutamate decarboxylase was partially purified by the method of Lippert *et al.* [14]. The resulting preparation had a sp. act. of 0.6 μmol/h/mg protein.

The commercial preparations of bacterial 4-aminobutyrate aminotransferase and glutamate decarboxylases were used without further purification.

Assays of enzyme activities and determination of time-dependent enzyme inhibition were performed essentially as described previously [5, 7, 8, 14].

Animals. Male rats of the Sprague–Dawley strain (200–220 g body weight) were purchased from Charles River (St. Aubin-lés-Elbeuf, France). Animals had access to standard diet and water *ad libitum* and were kept under a constant 12 hr light/12 hr dark lighting schedule. They were killed by decapitation

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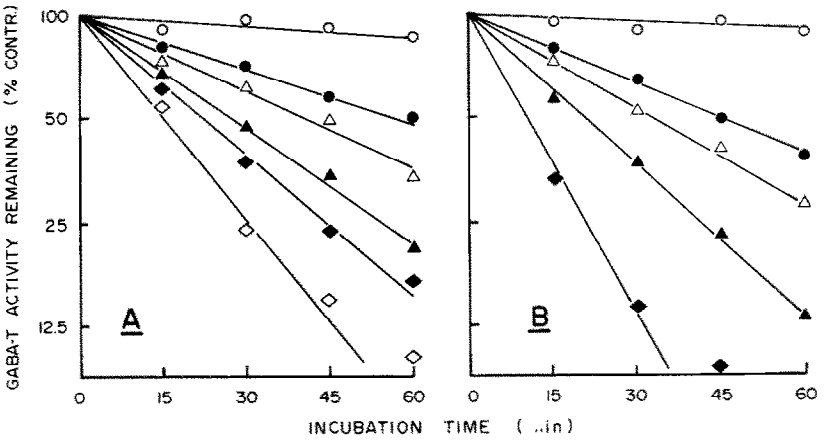

Fig. 1. Time-dependent inhibition of rat brain 4-aminobutyrate aminotransferase activity by the 4-aminohex-5-ynoic acid enantiomers : A. (*S*)-(+)-enantiomer (○, control; ●, 0.005 mM; △, 0.0067 mM; ▲, 0.010 mM; ◆, 0.015 mM; ◇, 0.025 mM.); B. (*R*)-(−)-enantiomer (○, control; ●, 0.10 mM; △, 0.15 mM; ▲, 0.20 mM; ◆, 0.50 mM). Partially purified rat brain 4-aminobutyrate aminotransferase was incubated in 0.1 M pyrophosphate buffer (pH 8.6) containing 1 mM 2-mercaptoethanol and the above-mentioned concentrations of inhibitor, at 20°. At given time-intervals, aliquots were removed and assayed for remaining enzyme activity. (GABA-T = 4-aminobutyrate aminotransferase).

at about the same time of day to minimize effects due to diurnal fluctuations. Drugs, dissolved in 0.9% saline, were injected intraperitoneally. Rats given saline served as controls.

Determinations of activities of brain 4-aminobutyrate aminotransferase and glutamate decarboxylase and of 4-aminobutyric acid concentrations. The assays of brain 4-aminobutyrate aminotransferase and glutamate decarboxylase, and the measurements of whole brain 4-aminobutyric acid concentration were performed according to a described procedure [6].

Data processing. Kinetic constants were calculated by using a least-squares fit of the data points with a Hewlett–Packard 9820 Calculator. *Ex vivo* values of enzyme activities and of 4-aminobutyric acid concentrations were the means ± S.E.M. of five animals. The significance of the differences between controls and treated animals was calculated by Student's *t*-test with the above-described calculator.

RESULTS

Effects of 4-aminohex-5-ynoic acid enantiomers on 4-aminobutyrate aminotransferase activity in vitro. Incubation of the preparation of rat brain 4-aminobutyrate aminotransferase with the (*S*)-(+)- and the (*R*)-(−)-enantiomers of 4-aminohex-5-ynoic acid resulted in a time-dependent loss of enzyme activity which followed pseudo first-order kinetics for approximately two half-lives. Over longer time-periods, the semilogarithmic plots deviated from linearity (Fig. 1). Loss of activity was related to the

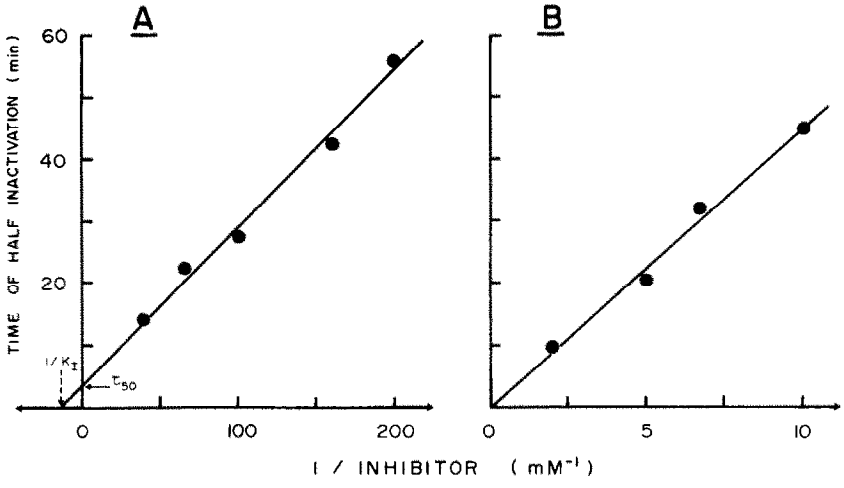

Fig. 2. Effect of concentrations of 4-aminohex-5-ynoic acid enantiomers: A. (*S*)-(+)-enantiomer; B. (*R*)-(−)-enantiomer) on the time of half-inactivation of rat brain 4-aminobutyrate aminotransferase.

Table 1. Effects of preincubation with various ligands on the time of half-inactivation of rat brain 4-aminobutyrate aminotransferase by the 4-aminohex-5-ynoic acid enantiomers. Times of half-inactivation were calculated by using a least-squares fit of the data-points with a Hewlett-Packard 9820 Calculator

Enantiomer	Addition to incubation media	Time of half-inactivation (min)
None	None	> 300
(*S*)-(+)- (0.015 mM)	None	23
id.	4-Aminobutyric acid (1 mM)	120
(*R*)-(−)- (0.20 mM)	None	21
id.	4-Aminobutyric acid (1 mM)	130
id.	4-Aminobutyric acid (1 mM) + 2-oxoglutarate (0.1 mM)	41
id.	Glutamate (5 mM)	170

concentration of inhibitor. By plotting the time of half-inactivation (t 1/2) as a function of the reciprocal of the inhibitor concentration (1/I) according to Kitz and Wilson [15], straight lines were obtained (Fig. 2). In the case of (*S*)-(+)-4-aminohex-5-ynoic acid the line did not pass through the origin but intercepted the positive y axis, demonstrating a saturation-effect which involves the enzyme's active-site in the inhibitory process (Fig. 2A). The kinetic constants for the time-dependent inhibition of rat brain 4-aminobutyrate aminotransferase by the (*S*)-(+)-enantiomer can be extrapolated from Fig. 2A. The apparent dissociation constant (K_I) is 75 μM and the time of half-inactivation extrapolated at infinite concentration of inhibitor (τ_{50}) is 3.4 min. In the case of (*R*)-(−)-4-aminohex-5-ynoic acid, no saturation-effect was observed (Fig. 2B). However, the inhibition of rat brain 4-aminobutyrate aminotransferase by both 4-aminohex-5-ynoic acid enantiomers is active-site directed since the natural substrate 4-aminobutyric acid protected against inactivation (Table 1). Further studies concerning rat brain 4-aminobutyrate aminotransferase inactivation by (*R*)-(−)-4-aminohex-5-ynoic acid showed that the alternative substrate L-glutamate, also protected against inactivation (Table 1). Moreover, the addition of 2-oxoglutarate to enzyme previously-protected by either L-glutamate or 4-aminobutyric acid, partially restored the original rate of inactivation of 4-aminobutyrate aminotransferase by (*R*)-(−)-4-aminohex-5-ynoic acid (Table 1).

Incubation with 0.7 mM of either enantiomer resulted in 93% inactivation of rat brain 4-aminobutyrate aminotransferase after 1 hr. Pro-

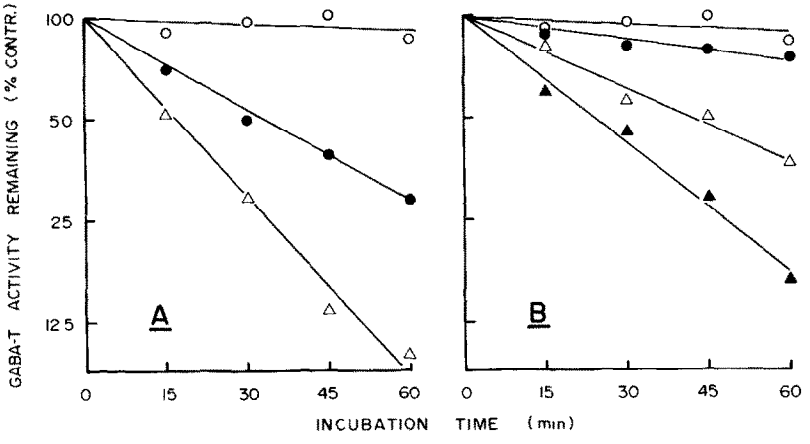

Fig. 3. Time-dependent inhibition of *Pseudomonas fluorescens* 4-aminobutyrate aminotransferase activity by the 4-aminohex-5-ynoic acid enantiomers : A. (*S*)-(+)-enantiomer (○, control; ●, 0.05 mM; △, 0.10 mM); B. (*R*)-(−)-enantiomer (○, control; ●, 0.10 mM; △, 0.50 mM; ▲, 1 mM). Partially purified 4-aminobutyrate aminotransferase of *Pseudomonas fluorescens* was incubated at 20° in 0.1 M pyrophosphate buffer (pH 8.6) containing 3 mM 2-mercaptoethanol and the above-mentioned concentrations of inhibitor. At given time-intervals, aliquots were removed and assayed for remaining enzyme activity. (GABA-T = 4-aminobutyrate aminotransferase).

longed (24 hr) dialysis against two buffer changes (10 mM sodium phosphate, 1 mM EDTA; 0.1 mM pyridoxal phosphate; adjusted to pH 6.8 with acetic acid) did not lead to regeneration of enzyme activity.

The effects of both enantiomers of 4-aminohex-5-ynoic acid were also studied on a commercial 4-aminobutyrate aminotransferase preparation from *Pseudomonas fluorescens*. As shown in Fig. 3, incubation of the bacterial enzyme preparation with each enantiomer also resulted, in both cases, in a time-dependent loss of enzyme activity which followed pseudo first-order kinetics for at least two half lives. However, the (*R*)-(−)-enantiomer was, once again, approx. 10–20 times less potent than the (*S*)-(+)-enantiomer as inactivator of bacterial 4-aminobutyrate aminotransferase.

Effects of 4-aminohex-5-ynoic acid enantiomers on glutamate decarboxylase activity in vitro. Incubation of rat brain glutamate decarboxylase with 1 mM (*S*)-(+)-4-aminohex-5-ynoic acid resulted in a time-dependent loss of enzyme activity while the same concentration of (*R*)-(−)-enantiomer had no effect (Fig. 4). Conversely 1 mM (*R*)-(−)-4-aminohex-5-ynoic acid inactivated bacterial glutamate decarboxylase with a half-life of 4 min while the (*S*)-(+)-enantiomer was without effect (Fig. 5). These results are in agreement with previously published data [7, 8].

Effects of 4-aminohex-5-ynoic acid enantiomers on 4-aminobutyrate aminotransferase and glutamate decarboxylase activities and 4-aminobutyric concentrations in the whole brain of rats in vivo. We investigated the dose-dependent effects of single administration of each enantiomer on the activities of 4-aminobutyrate aminotransferase and glutamate decarboxylase, and on the 4-aminobutyric acid concentrations in the whole brain, 4 hr after intraperitoneal injection to rats. Results given in Table 2 show that both enantiomers caused dose-dependent decreases of 4-aminobutyrate aminotransferase activity in the rat brain, but that the (*S*)-(+)-enantiomer was approx. 4 times more potent than the (*R*)-(−)-enantiomer. However, as distinct from the (*S*)-(+)-enantiomer, the (*R*)-(−)-enantiomer did not affect glutamate decarboxylase activity and, thus, appears to be selective for 4-aminobutyrate aminotransferase. In parallel with the decreases of 4-aminobutyrate aminotransferase activity, brain concentrations of 4-aminobutyric acid increased. However, despite its inhibitory effect on glutamate decarboxylase activity, the (*S*)-(+)-enantiomer caused higher increases of 4-aminobutyric acid concentrations than did the (*R*)-(−)-enantiomer for the same 4-aminobutyrate aminotransferase inhibition.

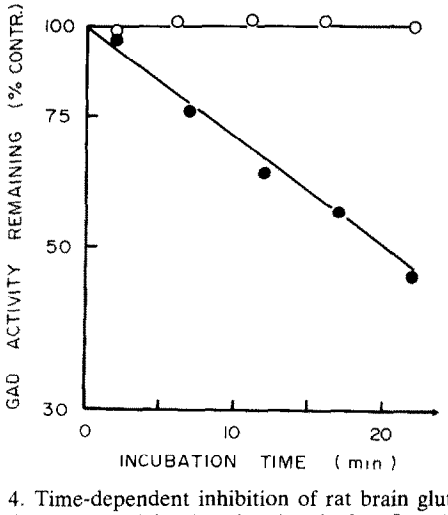

Fig. 4. Time-dependent inhibition of rat brain glutamate decarboxylase activity by the 4-aminohex-5-ynoic acid enantiomers. Partially purified rat brain glutamate decarboxylase was incubated at 37° in 50 mM potassium phosphate buffer (pH 6.5) containing 1 mM mercaptoethanol, 0.1 mM pyridoxal phosphate and 1 mM (*S*)-(+)-enantiomer (●) or 1 mM (*R*)-(−)-enantiomer (○). At given time-intervals, aliquots were removed and assayed for remaining enzyme activity. (GAD = glutamate decarboxylase).

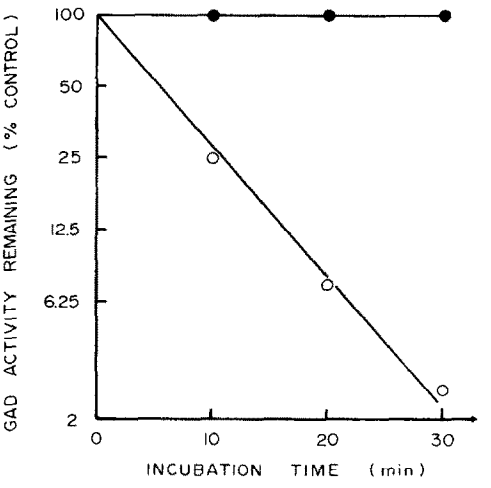

Fig. 5. Time-dependent inhibition of *E. coli* glutamate decarboxylase activity by the 4-aminohex-5-ynoic acid enantiomers. A stock solution of 3 mg of commercial enzyme was made up in 2.5 ml potassium phosphate buffer (10 mM, pH 6.5) containing 0.2% of bovine serum albumin. For a typical experiment 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 10 mM (*S*)-(+)-enantiomer (●) or 10 mM (*R*)-(−)-enantiomer (○) in solution in water was added. At given time-intervals, aliquots were removed and assayed for remaining enzyme activity. (GAD, glutamate decarboxylase).

DISCUSSION

The stereochemistry of proton removal by ω-aminotransferases has been studied recently by several groups [7, 16–19]. It has been reported that the pro-S hydrogen at the prochiral ω-carbon is specifically abstracted from lysine by L-lysine ε-aminotransferase [17], from ornithine by L-ornithine δ-aminotransferase [17, 18] and from 4-aminobutyric acid by 4-aminobutyrate aminotransferase of bacterial [7, 19] or mammalian origin [7], whereas bacterial ω-amino acid: pyruvate aminotransferase removes specifically the 4-pro-R hydrogen of 4-aminobutyric acid [19]. Therefore it could be expected that 4-aminobutyrate aminotransferases would remove, the hydrogen at C-4 of (*S*)-(+)-(4)-

Table 2. Effects of one single intraperitoneal injection of 4-aminohex-5-ynoic acid enantiomers, 4 hr before killing of animals on 4-aminobutyrate aminotransferase and glutamate decarboxylase activities, and 4-aminobutyric acid concentrations in the whole brain of rats. The significance of the differences between control and treated animals was calculated by Student's *t*-test (* $P < 0.001$, † $P < 0.005$)

	4-Aminobutyrate aminotransferase activity (% control)		Glutamate decarboxylate activity (% control)		4-Aminobutyric acid concentration (μmole/g)	
Enantiomer	(*S*)-(+)-	(*R*)-(−)-	(*S*)-(+)-	(*R*)-(−)-	(*S*)-(+)-	(*R*)-(−)-
Dose (mg/kg)						
0	100 ± 2		100 ± 2		2.5 ± 0.1	
25	37 ± 2*	69 ± 1*	67 ± 3*	101 ± 4	6.1 ± 0.1*	2.7 ± 0.1
50	16 ± 2*	50 ± 1*	50 ± 2*	101 ± 1	8.5 ± 1.3*	3.3 ± 0.1†
100	18 ± 3*	34 ± 1*	45 ± 10*	99 ± 1	9.5 ± 1.6*	4.7 ± 0.3*
200	10 ± 6*	18 ± 1*	31 ± 6*	101 ± 2	11.2 ± 0.8*	7.2 ± 0.3*

aminohex-5-ynoic acid but not that of the (*R*)-(−)-enantiomer. Indeed a previous study showed that the (*S*)-(+)-enantiomer inactivated both pig brain and *Pseudomonas fluorescens* 4-aminobutyrate aminotransferases at the same rate as the racemate at a double concentration [7]. In the present study, we show that 10–20 times higher concentrations of the (*R*)-(−)-enantiomer inhibit also in a time-dependent irreversible manner mammalian (rat brain) and bacterial (*Pseudomonas fluorescens*) 4-aminobutyrate aminotransferases. The protection against enzyme inactivation by 4-aminobutyric acid, or by L-glutamate, is explained by the transformation of the holoenzyme from the pyridoxal into the pyridoxamine form which is, then, unable to bind and activate the inhibitor [5]. Conversely the addition of 2-oxoglutarate regenerates the pyridoxal-phosphate holoenzyme which can bind the inhibitor. These data show that inactivation of 4-aminobutyrate aminotransferases by (*R*)-(−)-4-aminohex-5-ynoic acid is active-site directed and catalytic. This is corroborated by the presence of mercaptoethanol (1 mM) in the preincubation medium and by the absence of a lag time before the onset of inhibition which rule out the possibility of inhibition *via* affinity labelling by a diffusible alkylating species [20].

In the first half reaction catalyzed by aminotransferases, the pyridoxal phosphate form of the enzyme binds the amino group of the substrate and forms the coenzyme–substrate Schiff base. Cleavage of the C–α–H bond is followed by protonation at C-4′ of the cofactor during aldimine-ketimine tautomerization. In many examples reviewed by Floss and Vederas [16], this reaction has been demonstrated to proceed with internal proton transfer. This internal proton transfer strongly suggest that deprotonation and protonation are mediated by a single base of the active site, implying that the process must be suprafacial [16, 21]. Tanizawa *et al.* [17] have extended this hypothesis to explain the mechanism of ω-amino acid aminotransferase. On the other hand, racemization of α-amino acids catalyzed by pyridoxal phosphate-dependent racemases could be accomplished by two acid/base groups situated on opposite sides of the coenzyme–substrate complex, or by a single base which first abstracts a proton on one face and then adds it back either on the same or the opposite face [16, 22, 23]. This demands, of course, that the base and the binary complex have undergone relative displacements. In this respect, 4-aminobutyrate aminotransferase may act both as an aminotransferase and as a racemase. In the presence of the natural substrate, the effect of one base in the proton abstraction may be predominant. This same base would be responsible for the proton abstraction from (*S*)-(+)-4-aminohex-5-ynoic acid. Regarding the mechanism of 4-aminobutyrate aminotransferase inactivation by the (*R*)-(−)-enantiomer, a second base, normally unoperative, could abstract the proton at C-4 of the compound if we assume that both the aldimine and the ketimine formed occupy the same position in the active site. This proton abstraction would be facilitated by the ethynyl group which increases the acidity of the α-hydrogen. Alternately, in the hypothesis of a single-base mechanism of racemases [16, 23], the steric hindrance of the ethynyl group of (*R*)-(−)-4-aminohex-5-ynoic acid may induce a misplacement of the coenzyme–substrate complex within the active site allowing the catalytic base to abstract the proton.

In addition, we confirm previous results [7] showing that (*R*)-(−)-4-aminohex-5-ynoic acid does not inactivate mammalian glutamate decarboxylase. This led us to the interesting finding that (*R*)-(−)-4-aminohex-5-ynoic acid is a selective and effective inhibitor of rat brain 4-aminobutyrate transaminase *in vivo* as distinct from the racemic mixture [6] and the (*S*)-(+)-enantiomer which inhibits also glutamate decarboxylase in the brain.

Acknowledgements—The authors gratefully acknowledge the help of Dr. J. Grove for the determination of 4-aminobutyric acid concentrations.

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