implanted into the left ventricle through a stab wound at the apex. Dogs were allowed to recover from surgery a minimum of two weeks before use in a study. Animals were conditioned to the test laboratory and trained to lie quietly for 4-h periods. This conditioning was necessary to obtain stable, reproducible results from day to day. Dogs were fasted for 18 h before an experiment, and gross behavioral observations of animals were made throughout each study. Drugs or placebo (lactose) were administered in 000 gelatin capsules.

Acknowledgment. We thank Drs. William B. Lacefield and Patrick J. Murphy for their interest and helpful discussions and Della Nation for typing the manuscript.

Registry No. **6**, 21394-91-2; **7**, 36725-27-6; **8**, 21905-78-2; **9**, 72934-84-0; **10**, 5461-50-7; **11**, 19155-24-9; **12**, 100643-97-8; **13**, 20200-86-6; **14**, 103240-29-5; **15**, 103240-30-8; **15** (acid), 100644-06-2; **16**, 70386-01-5; **17**, 101345-97-5; **18**, 100644-05-1; **19**, 103240-31-9; **20**, 100643-96-7; **21**, 100644-04-0; **22**, 100644-03-9; **23**, 100644-02-8; **24**, 100644-01-7; **25**, 100643-98-9; **26**, 100644-00-6; **27**, 103240-32-0; **28**, 103240-33-1; **29**, 103240-34-2; **30**, 103240-35-3; **31**, 71008-63-4; **32**, 101345-95-3; **33**, 101345-98-6; **34**, 70386-06-0; **35**, 101345-96-4; **36**, 103240-36-4; **37**, 103240-37-5; CH_3I, 74-88-4; 3-$(CH_3)_2CHCONHNHC_6H_4CH_3$, 54381-25-8; $C_6H_5NHNHCOCH(CH_3)(CH_2)_2CH_3$, 103240-38-6; H_2NNH_2, 302-01-2; H_3CCH_2COCl, 79-03-8; $N(CH_3)_3$·HCl, 506-59-2; HCHO, 50-00-0; KCN, 151-50-8; acetic anhydride, 108-24-7; succinic anhydride, 108-30-5; 1,3-dihydro-2*H*-indol-2-one, 59-48-3; 1,3-dihydro-3-methyl-2*H*-indol-2-one, 1504-06-9; 1,3-dihydro-3-ethyl-2*H*-indol-2-one, 15379-45-0; 1,3-dihydro-3,3,7-trimethyl-2*H*-indol-2-one, 19501-89-4; 1,3-dihydro-3-ethyl-3-methyl-2*H*-indol-2-one, 36797-37-2; 1,3-dihydro-3,3-diethyl-2*H*-indol-2-one, 53204-33-4; 3,4-dihydro-2(1*H*)-quinolinone, 553-03-7; 3,4-dihydro-4,4-dimethyl-2(1*H*)-quinolinone, 76693-04-0; 3,4-dihydro-1-methyl-2(1*H*)-quinolinone, 826-72-2; 1,3,4,5-tetrahydro-2*H*-1-benzazepin-2-one, 4424-80-0; 1,3-dihydro-3,3,6-trimethyl-2*H*-indol-2-one, 103240-43-3; 1,3-dihydro-3-methyl-3-propyl-2*H*-indol-2-one, 103240-44-4; 1,3-dihydro-3,3-dimethyl-2*H*-indol-2-one, 92367-59-4; 3,4-dihydro-3-methyl-2(1*H*)-quinolinone, 31883-79-1; 2,3-dihydro-*N*,*N*,*N*,-β,1,3,3-heptamethyl-γ,2-dioxo-1*H*-indole-5-propanaminium iodide, 10324-40-0; 2,3-dihydro-*N*,*N*,β,1,3,3-hexamethyl-γ,2-dioxo-1*H*-indole-5-propanamine, 103240-39-7; 1-acetyl-γ-oxo-1,2,3,4-tetrahydroquinoline-6-butanoic acid, 10324-41-1; 1-acetyl-1,2,3,4-tetrahydroquinoline, 4169-19-1; γ-oxo-1,2,3,4-tetrahydroquinoline-6-butanoic acid, 103240-42-2.

Inactivation of γ-Aminobutyric Acid Aminotransferase by (*S*,*E*)-4-Amino-5-fluoropent-2-enoic Acid and Effect on the Enzyme of (*E*)-3-(1-Aminocyclopropyl)-2-propenoic Acid

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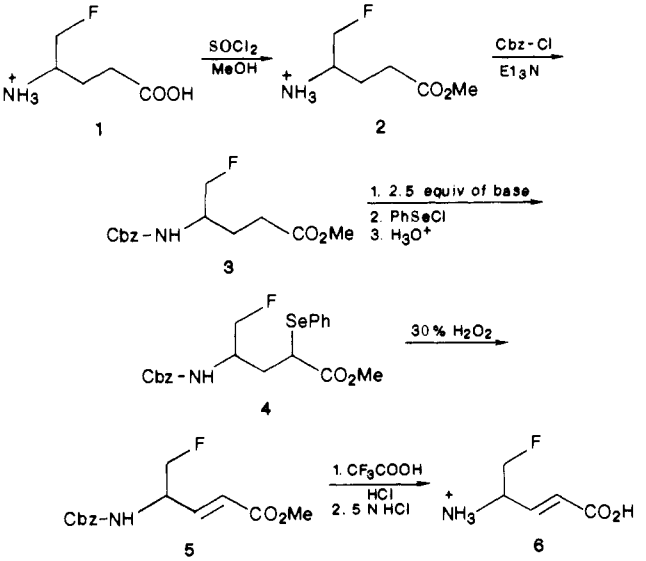
(*S*,*E*)-4-Amino-5-fluoropent-2-enoic acid (**6**) is synthesized in six steps starting from the known γ-aminobutyric acid aminotransferase (γ-Abu-T) inactivator, (*S*)-4-amino-5-fluoropentanoic acid (**1**). Compound **6** is a mechanism-based inactivator of γ-Abu-T: time-dependent inactivation is saturatable and protected by substrate; thiols do not protect the enzyme from inactivation; no enzyme activity returns upon dialysis. This compound (**6**) binds 50 times more tightly to γ-Abu-T than does the saturated analogue (**1**). No transamination of **6** occurs prior to inactivation. However, five molecules of **6** are required to inactivate the enzyme with concomitant release of five fluoride ions. Therefore, four molecules are being converted to product for each inactivation event. (*E*)-3-(1-Aminocyclopropyl)-2-propenoic acid is synthesized in seven steps from 1-aminocyclopropanecarboxylic acid. It is prepared as a cyclopropyl derivative of the proposed intermediate in the inactivation of γ-Abu-T by **6**. The cyclopropyl derivative, however, is a noncompetitive inhibitor and does not inactivate the enzyme. This study shows the usefulness and hazards of incorporation of a trans double bond into potential γ-Abu-T inactivators.

γ-Aminobutyric acid aminotransferase (γ-Abu-T)[1] is an important target in the design of anticonvulsant drugs.[2] Compounds that cross the blood–brain barrier and specifically inactivate this enzyme produce an increase in the concentration of an inhibitory neurotransmitter, γ-Abu, in the brain. This can result in an anticonvulsant effect.[3] Currently, 4-aminohex-5-enoic acid (vigabatrin), a mechanism-based inactivator[4-7] of γ-Abu-T, is in the latter phases of clinical trials and has been shown to be a potent drug for the treatment of epilepsy[8] and, to a lesser extent, tardive dyskinesia.[9] Previously, (*S*)-4-amino-5-fluoropentanoic acid was synthesized in our laboratories.[10] It

Scheme I. Synthetic Route to **6**



(1) Abbreviations used throughout this paper include the following: γ-Abu-T, γ-aminobutyric acid aminotransferase; THF, tetrahydrofuran; PLP, pyridoxal 5′-phosphate.
(2) Mandel, P.; DeFeudis, F. V. *Advances in Experimental Medicine and Biology: GABA Biochemistry and CNS Functions*; Plenum: New York, 1979; Vol. 123.
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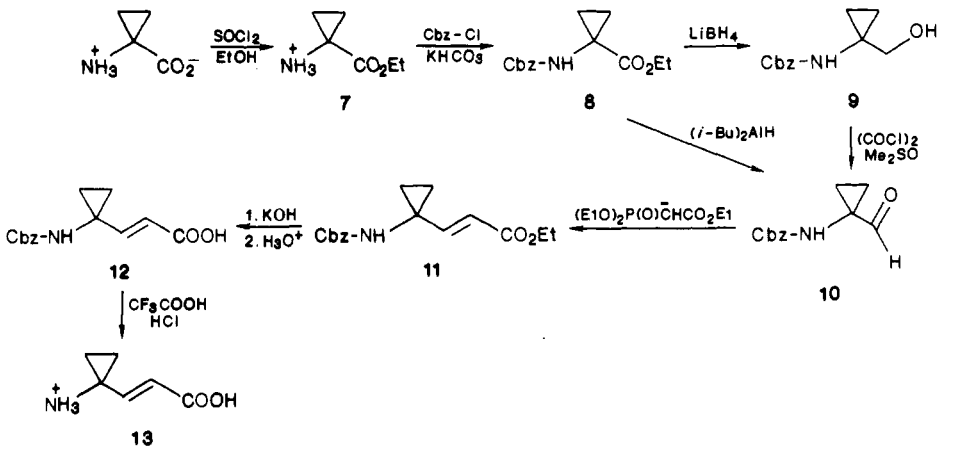
0022-2623/86/1829-1840$01.50/0

Scheme II. Synthetic Route to 13



was shown to be a mechanism-based inactivator of γ-Abu-T,[11] and its mechanism of inactivation was investigated.[12] Furthermore, it was shown to be a potent in vivo inactivator with reasonable specificity for γ-Abu-T;[13,14] however, in one test, it did not show an anticonvulsant effect.

Sometimes the incorporation of a double bond into the backbone of enzyme inactivators and substrates increases their binding to the target enzyme. (*E*)-2,4-Diamino-2-(fluoromethyl)pent-3-enoic acid was shown to be a more potent inactivator of ornithine decarboxylase than the saturated compound, 2-(fluoromethyl)ornithine;[15] (*E*)-1,4-diaminobut-2-ene binds 10^4 times better to ornithine decarboxylase than does its saturated analogue, putrescine.[16] Since (*E*)-4-aminobut-2-enoic acid is almost twice as active a substrate for γ-Abu-T as is γ-Abu,[17] (*S,E*)-4-amino-5-fluoropent-2-enoic acid (Scheme I, **6**) was synthesized and its inactivation of γ-Abu-T investigated. These results are reported here. Furthermore, the cyclopropyl analogue of a possible intermediate in the inactivation of γ-Abu-T by **6** was synthesized. The synthesis and effect of (*E*)-3-(1-aminocyclopropyl)-2-propenoic acid (Scheme II, **13**) on γ-Abu-T also are reported here.

Results and Discussion

Synthesis of (*S,E*)-4-Amino-5-fluoropent-2-enoic Acid (6). The synthesis of (*S,E*)-4-amino-5-fluoropent-2-enoic acid was carried out in six steps from (*S*)-4-amino-5-fluoropentanoic acid (**1**).[10] The synthetic route is outlined in Scheme I. The stereochemistry of the double bond was determined to be *E* on the basis of the olefinic trans coupling constants in the NMR spectra of **5** (15 Hz) and **6** (16 Hz). It is assumed that the chirality of C-4 in **1** remains intact because none of the steps in the synthesis involve reactions at that carbon (e.g., fluoride is not lost); furthermore, the optical rotation of **6** indicates that it is chiral.

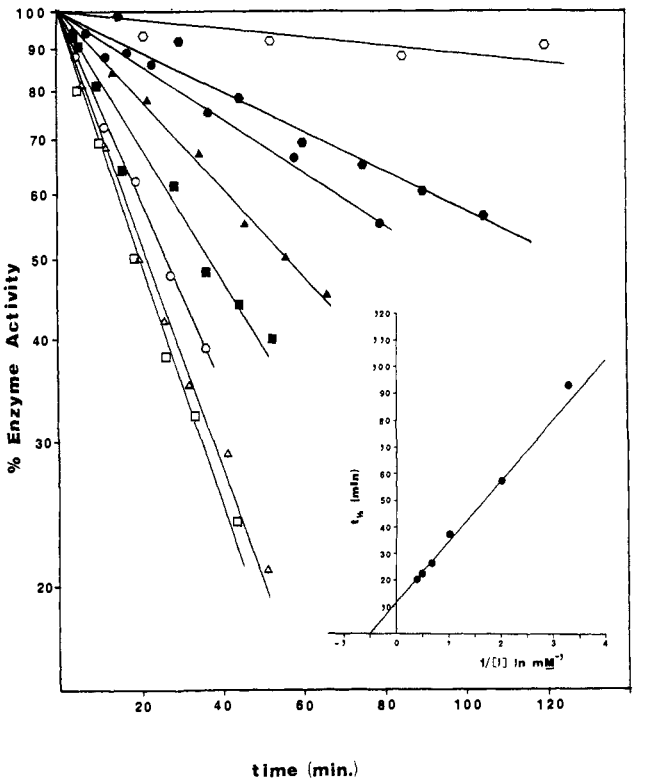


Figure 1. Time-dependent inactivation of γ-Abu-T by **6**. The following concentrations of **6** were used: 2.5 mM (□), 2.0 mM (△), 1.5 mM (○), 0.98 mM (■), 0.49 mM (▲), 0.28 mM (●), no inactivator (○); 0.28 mM **6** + 0.66 mM γ-Abu (●). Insert is a Kitz and Wilson plot[19] of the data.

Synthesis of (*E*)-3-(1-Aminocyclopropyl)-2-propenoic Acid (13). This compound was prepared from commercially available 1-aminocyclopropanecarboxylic acid in seven steps with an overall yield of 45%. Two methods were used to convert ethyl *N*-(benzyloxycarbonyl)-1-aminocyclopropanecarboxylate (**8**) into the corresponding aldehyde (**10**). Diisobutylaluminum hydride reduction generally gave the aldehyde in 30–35% yield along with 40–45% of the corresponding alcohol (**9**). Alternatively, the ester could be reduced with lithium borohydride directly to the alcohol (**9**) in an 88% yield after chromatography, and then the alcohol could be oxidized to the aldehyde (**10**) by the method of Omura and Swern[18]

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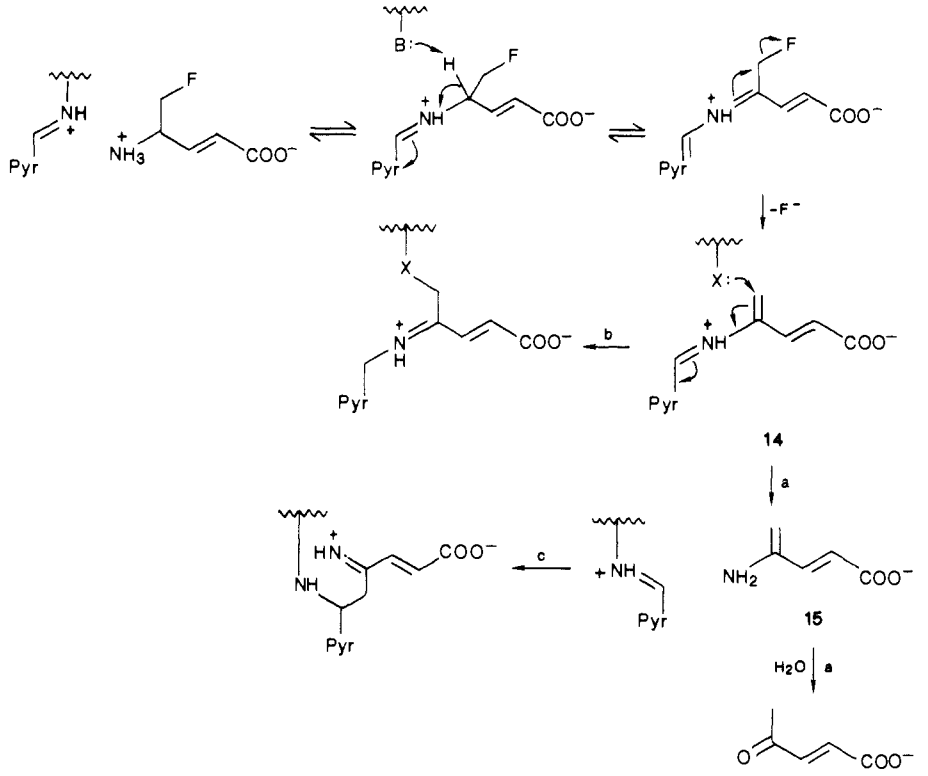
Scheme III. Possible Pathways for Inactivation of γ-Abu-T by **6**[a]



[a] Pyr represents the pyridoxal 5′-phosphate ring.

in an 81% recrystallized yield. During the conversion of the aldehyde to ethyl (*E*)-3-[1-[(benzyloxycarbonyl)-amino]cyclopropyl]-2-propenoate (**11**), varying amounts of the corresponding carboxylic acid (**12**) also were obtained. These products could be separated by extraction, and then, following ester saponification of **11**, the carboxylic acid product (**12**) could be combined with that obtained in the previous step for conversion to **13**.

Enzymology with 6. Compound **6** was a potent time-dependent inactivator of pig brain γ-Abu-T. The reaction could not be monitored conveniently under the usual conditions (pH 8.5, 25 °C) because saturation occurred at all concentrations of **6** high enough to obtain pseudo-first-order rates of inactivation. At pH 6.5 and 0 °C, time-dependent pseudo-first-order inactivation was measured that exhibited saturation kinetics; γ-Abu protected the enzyme from inactivation (Figure 1). No enzyme activity returned upon extensive dialysis. Since the inactivations were routinely run in the presence of β-mercaptoethanol, a mechanism involving release of a reactive species into solution prior to inactivation can be excluded. The K_I and k_{inact} values were determined by the method of Kitz and Wilson[19] (Figure 1, inset) to be 2.0 mM and 0.061 min^{-1}, respectively. Since it is difficult to relate these values obtained at the low pH and temperature values to those reported for other inactivators, a control inactivation of γ-Abu-T at pH 6.5 and 0 °C was carried out with the known inactivator (*S*)-4-amino-5-fluoropentanoic acid (**1**).[12] The values for K_I and k_{inact} for **1** under these conditions are 105 mM and 0.31 min^{-1}, respectively; the corresponding values for **1** at pH 8.5 and 25 °C[11] are 0.395 mM and 0.50 min^{-1}. It is apparent, then, that lowering the pH and temperature has a drastic effect on the K_I values. A comparison of **6** with **1** indicates that the incorporation of the trans double bond into **1** increases the binding by a factor of 52.5 at the expense of a rate decrease by a factor of 5.1. However, as described below, inactivation by **6** requires the turnover of five inactivator molecules, whereas only one molecule of **1** is required for inactivation.[12] Therefore, the factor of 5 in the rate may be related to the fact that with **1**, inactivation occurs with every turnover, but with **6**, inactivation occurs only once in five turnovers. The decrease in the inactivation rate constant for **6** compared with **1** is in contrast to the rate effect seen when the V_{max} of γ-Abu is compared to that of (*E*)-4-aminobut-2-enoic acid. In our hands, the K_m and V_{max} values are 1.1 mM and 0.14 μmol min^{-1} m^{-1} for γ-Abu and 1.3 mM and 0.35 μmol min^{-1} mg^{-1} for (*E*)-4-aminobut-2-enoic acid. Therefore, the double bond increases the substrate rate but decreases the inactivation rate.

It was determined by two different methods that no transamination of **6** occurs prior to inactivation of the enzyme. In the first experiment, no radioactive glutamate was generated when the inactivation was carried out in the presence of radioactive α-ketoglutarate. Transamination of **6** would produce pyridoxamine phosphate, which would convert the α-ketoglutarate to glutamate. This result was confirmed by the observation that complete enzyme inactivation occurred even in the absence of α-ketoglutarate.

Inactivation of each molecule of γ-Abu-T, however, requires more than one molecule of **6**. It was shown by two different experiments that five molecules of inactivator are turned over for each enzyme active site inactivated; that is the partition ratio is 4. In the first experiment, the number of fluoride ions released per enzyme active site inactivated was determined by measuring the fluoride ion concentration with a fluoride ion electrode and comparing this value to the enzyme concentration. In six runs with five different enzyme concentrations, an average of 5.2 ± 0.4 fluoride ions per active site were released during inactivation. In the second experiment, titration of the enzyme with **6** showed that 5.0 equiv of **6** was required for complete irreversible inactivation (Figure 2). Release of

(19) Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245–3249.

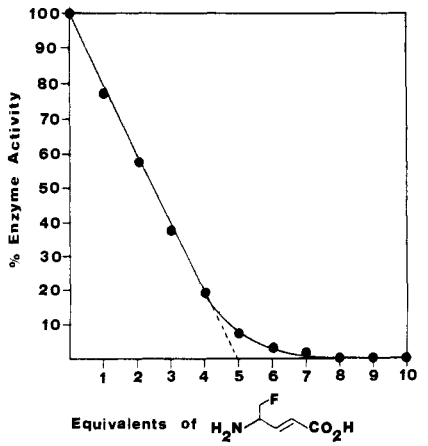
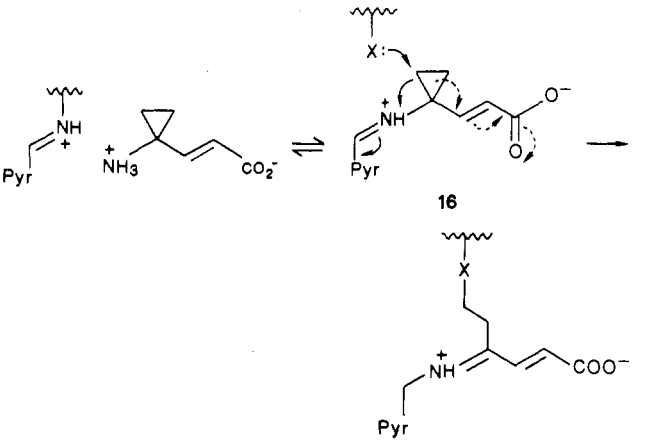


Figure 2. Number of equivalents of **6** required for complete inactivation. See Experimental Section for details.

fluoride ion results from an elimination reaction (Scheme III, pathway a), which does not affect the oxidation state of the cofactor and, therefore, α-ketoglutarate need not be present. These results are in contrast with those reported for **1**;[12] in this case, neither transamination nor product formation was observed. The binding to the enzyme of **6**, apparently, differs from that of **1** and, as a result, may orient the molecule in such a way as to affect k_{cat}/k_{inact}, the partition ratio.

A mechanism for inactivation of γ-Abu-T by **1** previously was proposed.[12] The corresponding inactivation mechanism for **6** is shown in Scheme III (pathway b). Metzler and co-workers[20,21] have proposed an alternative mechanism for the inactivation of PLP-dependent enzymes by substituted α-amino acids. This mechanism, as applied to **6**, is shown in Scheme III (pathway c). By either mechanism, PLP-bound 4-aminopenta-2,4-dienoic acid (Scheme III, **14**) would be involved. Since **15** is a primary enamine, it would not be predicted to be a stable compound. However, it was thought that its cyclopropyl derivative (**13**) would be. Furthermore, on the basis of a report by Danishefsky,[22] this compound has the potential to be a potent mechanism-based inactivator of γ-Abu-T and to shed light on the inactivation mechanism for **6**. Danishefsky and co-workers have found that certain doubly activated cyclopropanes, in which two electron-withdrawing groups are geminally substituted on the cyclopropyl ring, are quite electrophilic; nucleophilic reactions at room temperature are common. Compound **13** is only singly activated until it forms a Schiff base to the PLP, at which time it becomes a doubly activated cyclopropane (Scheme IV, **16**). Intermediate **16**, then, would be the cyclopropyl analogue of the intermediate proposed (Scheme III, **14**) during inactivation of γ-Abu-T by **6**. Inactivation by **13**, therefore, would be evidence for pathway b (Scheme III); competitive inhibition without inactivation could be evidence in support of pathway c (Scheme III). Compound **13** produced no time-dependent inactivation of γ-Abu-T, even at a concentration of 100 mM for 17 h. Lineweaver–Burk analysis, however, showed that **13** was a noncompetitive inhibitor of γ-Abu-T with a K_i value of 170 mM. Since it is known that both the *R* and *S* isomers of 4-aminohex-5-ynoic acid are inactivators for rat brain and bacterial γ-Abu-T,[23] it suggests that the

Scheme IV. Hypothetical Mechanism for Inactivation of γ-Abu-T by **13**[a]



[a] Pyr represents the pyridoxal 5′-phosphate ring.

cyclopropyl ring built into the C-4 position (**13**) should not hinder binding to the active site. However, the double bond may impart additional geometric constraints that prohibit competitive binding of **13**. In this case, geminal disubstitution at C-4 (i.e., the cyclopropyl ring) may interfere with the active-site geometry. Therefore, support for neither pathway b nor c was obtained by these results.

Conclusions. We have found that **6** is a mechanism-based inactivator of γ-Abu-T. The trans double bond appears to have an important effect in binding since the K_I is over 50 times lower than that for the corresponding saturated compound. The increased binding, however, is compensated for by an increase in a side reaction, namely, elimination of fluoride without inactivation, a reaction that occurs 4 times more often than does inactivation. A related analogue in which a cyclopropyl ring is built into the C-4 position of the 4-aminobut-2-enoic acid backbone does not bind competitively. Consequently, incorporation of a double bond into the molecule has important consequences regarding the binding of γ-Abu analogues to γ-Abu-T.

Experimental Section

General Procedures. Proton NMR spectra were recorded on either a Varian EM 360-A or a Varian EM 390-A spectrometer. Chemical shifts are reported as δ values in parts per million relative to tetramethylsilane as an internal standard. Melting points were determined by use of a Fisher-Johns melting point apparatus and are uncorrected. Elemental combustion analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. High-resolution mass spectral determinations were carried out at the Midwest Center for Mass Spectrometry, an NSF Regional Instrumentation Facility (Grant CHE-8211164). Enzyme activity was measured, as described under Enzymes and Assays, spectrophotometrically on either a Gilford 222 or a Perkin-Elmer Lambda 1 spectrophotometer with a constant-temperature cuvette holder. Radioactivity was measured in a Beckman LS-3100 scintillation counter with use of 10 mL of either 3a70B (Research Products International) or Ready Solve MP (Beckman) scintillation fluid. [U-^{14}C]Toluene (4.7×10^5 dpm/mL), obtained from New England Nuclear, was used as an internal standard. Protein concentrations were determined either by the method of Lowry et al.[24] or with BCA Protein Assay Reagent from Pierce Chemical Co., Rockford, IL, with comparable results, using bovine serum albumin as a standard. Fluoride ion concentrations were determined with an Orion Model 96-09 combination fluoride ion electrode attached to an Orion Model 601 pH meter and calibrated with sodium fluoride standard solution (Orion 94-09-06). Dowex 50 × 8 ion-

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exchange resin was supplied by Bio-Rad Laboratories. Silica gel columns for flash chromatography utilized E. Merck silica gel 60 (230–400-mesh ASTM). Analytical thin-layer chromatography (TLC) was conducted on E. Merck silica gel 60F-254 precoated TLC plates.

Reagents. Toluene was distilled under argon from sodium metal. Ethyl acetate was distilled from anhydrous potassium carbonate. Tetrahydrofuran (THF) was distilled under nitrogen from sodium metal with sodium benzophenone ketyl as an indicator. All other reagents and solvents were reagent grade, unless otherwise noted. All nonaqueous reactions were carried out under an argon atmosphere. The in-house distilled water was further purified by passage through a deionizer (Continental Water Conditioning Corp.) before use. Potassium pyrophosphate, α-ketoglutaric acid, β-mercaptoethanol, NADP, glycerol, and 1-aminocyclopropanecarboxylic acid were purchased from Sigma Chemical Co. and used without further purification. γ-Abu also was purchased from Sigma but was recrystallized from water/ethanol before use. [U-^{14}C]-α-Ketoglutaric acid (sp act. 240 mCi/mmol) was purchased from ICN Biomedicals, Inc.

Methyl (*S*)-4-Amino-5-fluoropentanoate Hydrochloride (2). Thionyl chloride (1.2 mL, 16.8 mmol) was added dropwise to a solution of (*S*)-4-amino-5-fluoropentanoic acid (1)[10] (1.6 g, 9.3 mmol) in methanol (8 mL). The solution was stirred at room temperature for 10 min and then at reflux for 2 h. After removal of the solvent, the resulting white paste was dried over P_2O_5 in vacuo for 12 h, giving **2** as a white paste (1.72 g, 99%): NMR (D_2O) δ 1.88 (q, 2 H), 2.44 (t, 2 H), 3.51 (m, 1 H), 3.56 (s, 3 H), 4.50 (s, HDO), 4.51 (dm, 2 H, J_{HF} = 48 Hz).

Methyl 4-[(Benzyloxycarbonyl)amino]-5-fluoropentanoate (3). The crude **2** (1.72 g, 9.25 mmol) was stirred in chloroform (75 mL), and benzyl chloroformate (1.33 mL, 9.4 mmol) was added. After the mixture had cooled in an ice bath, triethylamine (2.61 mL, 18.8 mmol) was added dropwise, and then the clear pale yellow solution was stirred at room temperature for 12 h. The solvent was evaporated, and the residue was dissolved in ethyl acetate (100 mL) and washed with 0.5 N HCl (20 mL), water (10 mL), 1 N $NaHCO_3$ (2 × 15 mL), and water (15 mL). After drying ($MgSO_4$), the solvent was evaporated to give **3** as a colorless oil (2.33 g, 87%): NMR ($CDCl_3$) δ 1.83 (m, 2 H), 2.40 (m, 2 H), 3.62 (s, 3 H), 4.03 (br m, 1 H), 4.38 (dd, 2 H, J_{HF} = 47 Hz), 4.98 (br s, 1 H), 5.06 (s, 2 H), 7.33 (s, 5 H).

Methyl 4-[(Benzyloxycarbonyl)amino]-5-fluoro-2-(phenylselenyl)pentanoate (4). *n*-Butyllithium (2.2 M in *n*-hexane, 5.7 mL, 12.5 mmol) was added dropwise to a solution of hexamethyldisilazane (2.7 mL, 12.7 mmol) and tetramethylethylenediamine (1.9 mL, 12.7 mmol) in THF (30 mL) at −78 °C under nitrogen. After 30 min at −78 °C, a solution of **3** (1.42 g, 5 mmol) in THF (25 mL) was added dropwise over 5 min. The clear yellow solution was stirred for 1 h at −78 °C, and then a solution of phenylselenyl chloride (0.96 g, 5 mmol) in THF (15 mL) was added rapidly. A clear, colorless solution resulted within 2 min; after 10 min, the solution was allowed to warm to −20 to −30 °C and was quenched with 1 N HCl (60 mL) and water (20 mL). The solution was extracted with ether (2 × 50 mL), and the extracts were washed with water (2 × 10 mL), 1 N HCl (20 mL), 1 N $NaHCO_3$ (10 mL), and water (20 mL). After drying ($MgSO_4$), the solvent was evaporated to give a yellow oil (2.1 g), which was chromatographed on silica gel (80 g). The diphenyl diselenide was eluted with *n*-hexane, and then the product was eluted with 30% ethyl acetate–*n*-hexane in the 120–400-mL fractions. Evaporation of the solvent gave **4** as a pale yellow oil (1.4 g, 64%): NMR ($CDCl_3$) δ 2.03 (m, 2 H), 3.53 (s, 3 H), 3.61 (m, 2 H), 4.33 (dm, 2 H, J_{HF} = 47 Hz), 4.86 (br s, 1 H), 5.02 (s, 2 H), 7.28 (s, 5 H), 7.15–7.67 (m, 5 H).

(*E*)-Methyl 4-[(Benzyloxycarbonyl)amino]-5-fluoro-2-pentenoate (5). A solution of **4** (1.4 g, 3.2 mmol) in acetone (25 mL) containing hydrogen peroxide (4.2 mL of a 30% solution) was stirred at 0 °C for 1 h. The colorless solution was warmed to room temperature, and the solvents were evaporated. The residue was diluted with water (50 mL) and 1 N $NaHCO_3$ (20 mL), and then it was extracted with ethyl acetate (2 × 25 mL). The combined organic extracts were washed with water (10 mL), 1 N $NaHCO_3$ (15 mL), and water (10 mL), dried ($MgSO_4$), and evaporated to a yellow oil (0.88 g), which was chromatographed on silica gel (36 g). After removal of yellow impurities with 200 mL of *n*-hexane, the product was eluted with 25% ethyl acetate–*n*-hexane in the 75–250-mL fractions. Evaporation of the solvent gave **5** as a pale yellow viscous oil (0.69 g, 76%): NMR ($CDCl_3$) δ 3.70 (s, 3 H), 4.44 (dm, 2 H, J_{HF} = 47 Hz), 4.75 (m, 1 H), 5.08 (s, 2 H), 5.53 (br m, 1 H), 6.02 (dd, 1 H, J = 15 Hz and 2 Hz), 6.87 (dd, 1 H, J = 15 Hz and 5 Hz), 7.28 (s, 5 H).

(*E*)-4-Amino-5-fluoro-2-pentenoic Acid Hydrochloride (6). A solution of **5** (0.69 g, 2.44 mmol) in trifluoroacetic acid (25 mL) was stirred at room temperature for 10 min, and then concentrated HCl (5 mL) was added dropwise (effervescence). After 3 h, the yellow-orange solution was diluted with water (16 mL) and was stirred for 16 h. The solvents were evaporated in vacuo, and the residue was dissolved in 2 N HCl (30 mL) and extracted with ether (4 × 15 mL). The colorless aqueous layer was evaporated in vacuo to give a pale yellow residue, which was dissolved in 5 N HCl (25 mL) and stirred for 48 h at room temperature. The water was evaporated in vacuo, and the resulting sticky solid was crystallized from acetic acid–ethyl acetate to give the product as white flakes. Recrystallization from methanol–ethyl acetate gave **6** as colorless needles (240 mg, 58%): mp 155–158 °C dec; NMR (D_2O) δ 3.98 (m, 1 H), 4.48 (dm, 2 H, J_{HF} = 48 Hz), 4.50 (s, HDO), 5.93 (dd, 1 H, J = 16 and 1 Hz), 6.50 (dd, 1 H, J = 16 and 6 Hz); $[\alpha]^{23}_D$ −23.9° (*c* 1.4, H_2O). Anal. ($C_5H_9ClFNO_2$) C, H, Cl, F, N.

Ethyl 2-Aminocyclopropanecarboxylate Hydrochloride (7). A suspension of 1-aminocyclopropanecarboxylic acid (950 mg, 9.4 mmol) in absolute ethanol (30 mL) was cooled to 0 °C, and thionyl chloride (2.06 mL, 28 mmol) was added dropwise over 20 min. The cold bath was removed, and the homogeneous solution was heated at reflux for 2 h. The solvent was removed in vacuo, the resulting residue was dissolved in water (50 mL), and the aqueous solution was extracted with ethyl acetate (2 × 15 mL) and then evaporated in vacuo to a white solid (1.46 g, 94%): mp 108–110 °C; TLC in 1-butanol/acetic acid/water (12:3:5 v/v/v) showed three ninhydrin-positive spots: two very minor spots with R_f 0.21 and 0.24 and a major spot corresponding to **7** with R_f 0.50; NMR (D_2O) δ 1.03 (t, 3 H), 1.23–1.28 (m, 2 H), 1.32–1.37 (m, 2 H), 4.02 (q, 2 H), 4.50 (s, HDO).

Ethyl 1-[(Benzyloxycarbonyl)amino]cyclopropanecarboxylate (8). The method of Ito et al.[25] was modified as follows: A suspension of **7** (1.46 g, 8.8 mmol) in ethyl acetate (70 mL) was cooled to 0 °C, and a solution of potassium bicarbonate (7.24 g, 72 mmol) in water (50 mL) was added slowly. Benzyl chloroformate (1.7 mL, 12 mmol) was then added dropwise to the cold solution. The cold bath was removed, and the mixture was stirred at room temperature for 16 h. The organic phase was washed successively with 1 N HCl (50 mL), saturated aq $NaHCO_3$ (50 mL), and brine (50 mL), then dried ($MgSO_4$), filtered, and concentrated in vacuo to a white solid. Chromatography on silica gel, eluting with 30% ethyl acetate in *n*-hexane, gave 2.13 g (92%) of **8** as white crystals, which was recrystallized from ethyl acetate–*n*-hexane to give white needles: mp 57–59 °C; NMR ($CDCl_3$) δ 1.02–1.18 (overlapping t and m, 5 H), 1.38–1.52 (m, 2 H), 4.02 (q, 2 H), 5.04 (s, 2 H), 6.27 (br s, 1 H), 7.25 (s, 5 H); exact mass calcd for $C_{14}H_{17}NO_4$ 263.1158, found 263.1164 (2.4 ppm).

1-[(Benzyloxycarbonyl)amino]cyclopropanecarboxaldehyde (10). Method A: Via 1-[(Benzyloxycarbonyl)amino]-1-(2-hydroxymethyl)cyclopropane (9). A solution of lithium borohydride in THF[26] (1 M, 20 mL, 20 mmol) was added slowly via syringe at room temperature to **8** (3.45 g, 9.3 mmol). The resulting mixture was stirred at room temperature for 21 h, and then it was cooled in an ice bath and 50% aqueous acetic acid was added to destroy excess lithium borohydride. The quenched reaction mixture was diluted with water and ether. The ether layer was washed with saturated aqueous $NaHCO_3$ (50 mL) and brine (50 mL), then dried ($MgSO_4$), filtered, and evaporated in vacuo to yield a yellow oil, which solidified on standing. Chromatography on silica gel eluting with *n*-hexane containing 10–60% ethyl acetate gave 1.82 g (88%) of **9** as a white solid: mp 78 °C; NMR ($CDCl_3$) δ 0.74 (s, 4 H), 3.52 (s, 2 H), 3.96 (br s, 1 H), 5.03 (s, 2 H), 6.04 (br s, 1 H), 7.29 (s, 5 H).

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Alcohol **9** (1.09 g, 4.9 mmol) was oxidized by the method of Omura and Swern[18] (oxalyl chloride/Me_2SO) to yield 1.09 g (100%) of **10** as a slightly yellow solid. The solid was recrystallized from ethyl acetate–*n*-hexane to give 870 mg (81%) of **10** as colorless crystals: mp 70–72 °C; NMR ($CDCl_3$) δ 1.24–1.30 (m, 2 H), 1.37–1.43 (m, 2 H), 5.06 (s, 2 H), 6.07 (br s, 1 H), 7.29, (s, 5 H), 9.05 (s, 1 H); exact mass calcd for $C_{12}H_{13}NO_3$ 219.0896, found 219.0891 (−2.1 ppm).

Method B. The method of Rich et al.[27] was modified as follows: A solution of **8** (1.84 g, 7.0 mmol) in dry toluene (40 mL) was cooled to −78 °C, and a solution of diisobutylaluminum hydride (DIBAH) (1 M in hexane, 16 mL, 16 mmol) was added dropwise over 45 min. The reaction mixture was subsequently stirred at −78 °C for 20 min. Excess DIBAH was destroyed at −78 °C by the slow addition of 3 mL of methanol, and then the reaction mixture was warmed to room temperature and diluted with ether and 1:1 (v/v) 2 N HCl/brine. The organic phase was washed successively with 1:1 (v/v) 2 N HCl/brine (3 × 50 mL), saturated aqueous $NaHCO_3$ (50 mL), and brine (50 mL), and then it was dried ($MgSO_4$), filtered, and concentrated in vacuo to give 1.63 g of an off-white solid. The solid was chromatographed on silica gel with 10–30% (v/v) ethyl acetate in *n*-hexane as eluant to yield two white solids, 524 mg (34%) of **10** and 700 mg (45%) of **9**. The aldehyde was recrystallized from *n*-hexane–ether to give **10** as colorless crystals, mp 70–71 °C.

Ethyl (*E*)-3-[1-(Benzyloxycarbonyl)amino]cyclopropyl]-2-propenoate (11). THF (5 mL) was added to sodium hydride (50% dispersion in mineral oil; 126 mg, 2.6 mmol), which was washed with either *n*-hexane or THF (3 × 3 mL). After the mixture cooled in an ice bath, triethyl phosphonoacetate (0.33 mL, 1.7 mmol) was added slowly, and the resulting mixture was stirred at room temperature for 50 min. A solution of **10** (369 mg, 1.68 mmol) in THF (8 mL) was added, and the resulting mixture was stirred at room temperature for 16 h. Excess sodium hydride was destroyed by the addition of ethanol (1 equiv). The quenched reaction mixture was diluted with water and ether, and the organic phase was washed successively with water (50 mL), saturated aqueous $NaHCO_3$ (2 × 30 mL), and brine (50 mL) and then dried ($MgSO_4$). After concentration in vacuo, **11** was obtained as a white solid (311 mg, 64%). Recrystallization from ether gave white crystals: mp 103–104 °C; NMR ($CDCl_3$) δ 1.09–1.32 (t overlapping m, 7 H), 4.10 (q, 2 H), 5.02 (s, 2 H), 5.76 (d, overlapping br s, 2 H, J = 15 Hz), 6.44 (d, 1 H, J = 15 Hz), 7.25 (s, 5 H); IR (KBr) 3330 (s), 1719 (s), 1694 (s), 1685 (w, sh), 1647 (m), 1517 (s) cm^{-1}.

The aqueous washes from the workup were acidified with concentrated HCl and extracted with ether (3 × 30 mL). The combined ether extracts were washed successively with water (2 × 30 mL) and brine (40 mL) and then dried ($MgSO_4$), filtered, and concentrated in vacuo to give **12** (139 mg, 32%) as a white solid. Recrystallization from ethyl acetate–*n*-hexane gave fine white needles: mp 188–189 °C; NMR ($CDCl_3$/acetone-d_6) δ 1.15–1.33 (m, 4 h), 5.06 (s, 2 H), 5.77 (d, 1 H, J = 16 Hz), 6.52 (d, 1 H, J = 16 Hz), 7.29 (s, 5 H); IR (KBr) 3430 (br), 3320 (m), 1695 (s), 1679 (s), 1642 (w), 1524 (m), 1261 (m) cm^{-1}. High-resolution chemical-ionization mass spectral data (M + 1 peak): calcd for $C_{16}H_{20}NO_4$, 290.1393; found, 290.1392 (0.3 ppm).

(*E*)-3-[1-[(Benzyloxycarbonyl)amino]cyclopropyl]-2-propenoic Acid (12). To a solution of **11** (218 mg, 0.75 mmol) in ethanol (25 mL) was added 1 N potassium hydroxide (20 mL). The resulting solution was stirred at room temperature for 12 h and then diluted with water (100 mL). The aqueous solution was extracted with methylene chloride (2 × 20 mL) and ethyl acetate (20 mL) and then acidified with concentrated HCl. The white solid that formed was extracted from the aqueous phase with methylene chloride (3 × 20 mL). The combined organic extracts were dried ($MgSO_4$), filtered, and concentrated in vacuo to give **12** (152 mg, 78%) as a white solid. Analytical data were the same as described above.

3-(1-Aminocyclopropyl)-2-propenoic Acid Hydrochloride (13). The N-protected amino acid **12** (606 mg, 2.3 mmol) was dissolved in freshly distilled trifluoroacetic acid (23 mL), and concentrated HCl (4.6 mL) was added dropwise at room temperature (effervescence). The resulting solution was stirred at room temperature for 7.5 h. The reaction mixture was diluted with water and extracted with methylene chloride (3 × 15 mL) and ethyl acetate (20 mL). The aqueous phase was evaporated in vacuo to yield **13** (373 mg, 99%) as a flaky white solid. Recrystallization from ethanol–ethyl acetate gave the product as white crystals: mp 195–200 °C dec; TLC on silica gel (1-butanol/acetic acid/water, 12:3:5) showed one ninhydrin-positive spot, R_f 0.32; NMR (D_2O) δ 1.19–1.34 (dm, 4 H), 4.50 (s, HDO), 5.78 (d, 1 H, J = 16 Hz), 6.55 (d, 1 H, J = 16 Hz); IR (KBr) 3430 (br), 2980 (br), 1696 (s), 1659 (s), 1393 (s), 1248 (s). Anal. ($C_6H_{10}ClNO_2$) C, H, Cl, N.

Part of the product (240 mg) was converted to its free base form by elution from a column (1.5 × 14.5 cm) of Dowex 50 (H^+ form) with 2 N aqueous ammonia. Ninhydrin-positive fractions were combined and lyophilized to give 160 mg of off-white crystals: mp 168–171 °C dec; NMR (D_2O) δ 1.00–1.51 (dm, 4 H), 4.50 (s, HDO), 5.65 (d, 1 H, J = 16 Hz), 6.08 (d, 1 H, J = 16 Hz). Recrystallization of the free amino acid was unsuccessful.

Enzymes and Assays. Pig brain γ-Abu-T, Gabase, and succinic semialdehyde dehydrogenase were obtained and assayed as described previously.[12,28]

Time-Dependent Inactivation of γ-Abu-T by 6 and 1. Purified γ-Abu-T (0.066 unit) was incubated at 0 °C (ice/water bath) in 50 mM potassium phosphate buffer, pH 6.5, containing 5 mM β-mercaptoethanol, 1 mM α-ketoglutarate, and varying concentrations of **6** or **1**. A sample containing no inactivator served as the control. At timed intervals, 25 μL was withdrawn and assayed for γ-Abu-T activity[12] by the spectrophotometric method at 25 °C. The logarithm of the percent of activity remaining was plotted vs. time for each concentration of inactivator. A secondary plot[19] of $t_{1/2}$ vs. $[I]^{-1}$ was also prepared to determine the K_I and k_{inact} values.

Extent of Transamination of 6 during Inactivation. γ-Abu-T (0.52 μM) in 50 mM potassium pyrophosphate buffer, pH 8.5, containing 5 mM β-mercaptoethanol, 0.5 mM **6**, and 5 mM [U-^{14}C]-α-ketoglutarate (sp act. 1.0 mCi/mmol) in a total volume of 200 μL was incubated at 25 °C for 195 min, at which time no enzyme activity remained. Concurrently, two control samples were run under identical conditions except that one contained no enzyme and the other contained no **6**. Each sample was quenched with 25 μL of 20% trichloroacetic acid, and the [U-^{14}C]glutamate formed was isolated and counted as previously described.[28]

Release of Fluoride Ions during Inactivation. Six different amounts of γ-Abu-T (59 μg, 47.2 μg, 35.4 μg, 23.6 μg, and 11.8 μg (twice)) were incubated for 17.5 h with **6** (0.54 mM) in 50 mM potassium pyrophosphate buffer, pH 8.5, containing 0.25 mM β-mercaptoethanol and 1.3 mM α-ketoglutarate. A control containing no enzyme also was run. The concentration of fluoride ions in a 100-μL aliquot of each sample was measured with a fluoride ion electrode, and the ratio of fluoride ion concentration to concentration of enzyme active sites was determined.

Number of Equivalents of 6 Required for Complete Inactivation. γ-Abu-T (0.56 μM) was incubated at 25 °C in 50 mM potassium pyrophosphate buffer, pH 8.5, containing 5 mM β-mercaptoethanol, 1.4 mM α-ketoglutarate, and 0–10 equiv (relative to enzyme active sites) of **6**. A control containing no inactivator also was run. After 4 days, the samples were assayed for enzyme activity and the percent activity of each sample, relative to the control, was plotted vs. the number of equivalents of inactivator.

Inhibition of γ-Abu-T by 13. Aliquots of γ-Abu-T (0.08 unit) in 585 μL of 50 mM potassium pyrophosphate buffer, pH 8.5, containing 5 mM α-ketoglutarate, 1 mM NADP, 60 mM potassium chloride, excess succinic semialdehyde dehydrogenase, and varying concentrations of γ-Abu and **13** were incubated at 25 °C. Initial rates, which were linear for at least 5 min, were measured spectrophotometrically.[12] Kinetic constants were determined by Lineweaver–Burk plots[29] with use of at least five different concentrations of γ-Abu. A least-squares linear regression analysis

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was used to obtain the best fit curve to the data. In all cases, the correlation coefficient was greater than 0.99.

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Registry No. 1, 74936-17-7; 2, 98212-71-6; 3, 98212-80-7; 4, 98212-94-3; 5, 98212-95-4; 6, 103500-21-6; 6 (free base), 42303-42-4; 7, 85452-41-1; 8, 103500-22-7; 9, 103500-23-8; 10, 103500-24-9; 11, 103500-25-0; 12, 103500-26-1; 13, 103500-27-2; 13 (free base), 98212-93-2; MeOH, 67-56-1; $PhCH_2OCOCl$, 501-53-1; PhSeCl, 5707-04-0; EtOH, 64-17-5; 1-aminocyclopropanecarboxylic acid, 22059-21-8; triethyl phosphonoacetate, 867-13-0; γ-aminobutyric acid aminotransferase, 9037-67-6.

New Effective Gonadotropin Releasing Hormone Antagonists with Minimal Potency for Histamine Release in Vitro

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In order to minimize the adverse effect of histamine release in the rat of some gonadotropin releasing hormone (GnRH) antagonists, such as [Ac-D2Nal[1],D4FPhe[2],DTrp[3],DArg[6]]-GnRH, new structures with modifications at positions 1, 2, 3, 5, 6, 7, and 10 were synthesized and tested in several biological systems. In vitro: the affinity for the pituitary GnRH receptor was measured as was the ability of the analogues to inhibit GnRH-stimulated release of luteinizing hormone (LH) by dispersed anterior pituitary cells in culture and to release histamine from rat mast cells. In vivo: inhibition of ovulation in the cycling rat was determined after subcutaneous (sc) injection of the peptides at noon on the day of proestrus; the duration of action of the peptides was evaluated by measuring LH levels in the castrated male rat after sc injection of some selected analogues. [Ac-D2Nal[1],D4ClPhe[2],D3Pal[3],Arg[5],D-4-*p*-methoxybenzoyl-2-aminobutyric acid[6],DAla[10]]-GnRH was found to be one of the most potent analogues of this series, causing a 100% inhibition of ovulation at 5 μg/kg or less. Release of histamine was observed at doses 10–25 times that required for [Ac-D2Nal[1],D4FPhe[2],DTrp[3],DArg[6]]-GnRH. Thus, introduction of arginine in position 5 with a hydrophobic amino acid in position 6 is compatible with high potency in several biological systems and results in compounds with lowered potency to release histamine compared to homologous peptides with tyrosine in position 5 and D-arginine in position 6.

It is now well-recognized that the decapeptide gonadotropin-releasing hormone (GnRH) plays a fundamental stimulatory role in the control of reproductive functions. Furthermore, the inhibitory effects of chronic administration of GnRH agonists or of acute administration of GnRH antagonists on reproductive functions have been demonstrated and recently summarized.[1] Because of the fact that these effects are desired for the management of several pathological, steroid-dependent conditions, or as a new means of contraception, potent analogues were developed.[2,3] Whereas superagonists seem to act by desensitization of the pituitary, a process that may take several weeks of treatment, antagonists inhibit LH within 10 min after the first injection of the peptides. In order to be effective, however, the antagonists have to occupy the GnRH receptor at all times, thus emphasizing the need for analogues with high affinity for the receptor and resistance to both degradation and elimination. New antagonists of GnRH have been reported recently that had modifications at positions 1, 2, 3, 6, 7, and 10,[4,5] the most potent of which, in an antiovulatory assay (AOA), had a basic D-amino acid in position 6.[6-8] Subcutaneous (sc) injection of one such analogue, [Ac-D2Nal[1],D4FPhe[2],DTrp[3],DArg[6]]-GnRH ($ED_{100} \leq 5$ μg/kg in the AOA), however, induced transient edema of the face and extremities in the rat;[9] this effect, which was maximal 3–5 h after peptide administration and which had subsided by 24 h, was not observed in mice, rabbits, and rhesus monkeys. Later studies indicated that this peptide and others with similar structural features such as a basic amino acid in position 6 and a hydrophobic N-terminus did release histamine from rat mast cells.[10] Interestingly, analogues such as the superagonists characterized by a hydrophobic D-amino acid in position 6 and the des-Gly[10],Pro[9]-NHEt substitution, or [Ac-Δ[3]Pro[1],D4FPhe[2],DTrp[3,6]]-GnRH (ED_{100} = 25 μg/kg in the AOA), another potent GnRH antagonist, did not produce the in vivo edematous effects and were considerably less

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