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IRREVERSIBLE INHIBITION OF GABA-T BY HALOGENATED ANALOGUES OF β-ALANINE

DANIEL SCHIRLIN†, SYLVIE BALTZER, JEAN-GEORGES HEYDT and MICHEL J. JUNG

Merrell Dow Research Institute, Strasbourg Center, 16 rue d'Ankara, 67084 Strasbourg Cedex, France

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β-Difluoromethyl-β-alanine (3-amino-4,4-difluorobutanoic acid) is a potent *in vitro* and *in vivo* inhibitor of GABA-T. The rate of inhibition of GABA-T is concentration- and time-dependent. The inactivation is active-site directed. No reactive species escapes from the active site before reacting with the enzyme. The inhibition is irreversible and stereospecific. The use of β-^{2}H-β-difluoromethyl-β-alanine results in a marked primary isotope effect *in vitro* and *in vivo*. The use of differently substituted dihalogeno derivatives of β-alanine suggests that the rate of inhibition is dependent on the nature and position of the leaving group. The mechanism of inhibition is discussed on the basis of spectral changes.

KEY WORDS: 4-Aminobutyrate metabolism, irreversible inhibition, 3-amino-4,4-difluorobutanoic acid, 3-amino-4-chloro-4-fluorobutanoic acid, 3-amino-2,4-difluorobutanoic acid.

INTRODUCTION

Agents which enhance GABAergic neurotransmission have potential beneficial effects in diseases such as epilepsy, tardive dyskinesia and possibly Huntington's Chorea[1]. The most widely used of these agents, γ-vinyl GABA (or vigabatrin), an enzyme-activated inhibitor of the main metabolic pathway of GABA (GABA-T or GABA transaminase)[2], undergoes multicenter clinical trials both in Europe and United States for the treatment of epilepsy[3]. A number of other GABA-T inhibitors have been described (for review see reference 4). We and others[5,6] have published on the inhibitory activity of mono, di or trihalomethyl derivatives of GABA and β-alanine. We wish to report here additional studies on the influence of position and nature of the halogen(s) and some mechanistic aspects of the inhibition of GABA-T by analogues of β-alanine.

EXPERIMENTAL

Materials

4-Amino butyric acid, α-ketoglutarate, L-glutamate were purchased from SIGMA. All buffer reagents were obtained from E. Merck, Darmstadt. 1-^{14}C Glutamate was a NEN product.

†Correspondence.

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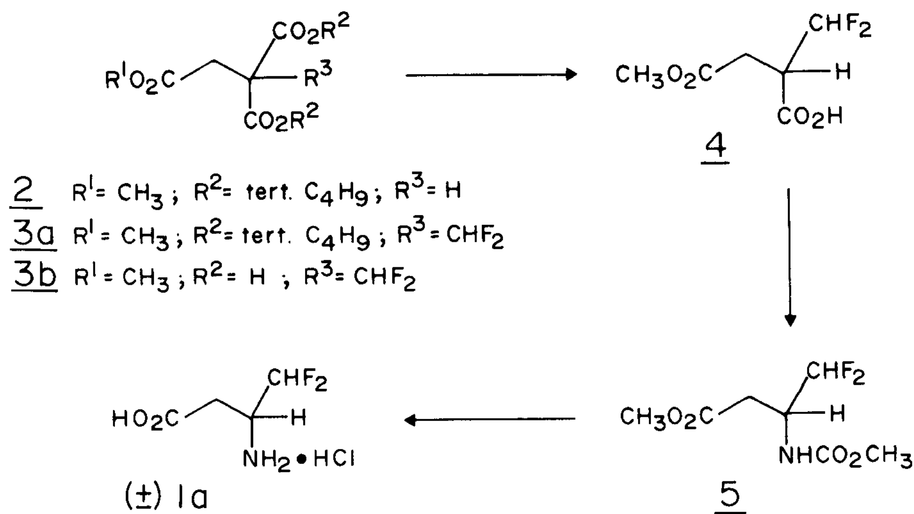

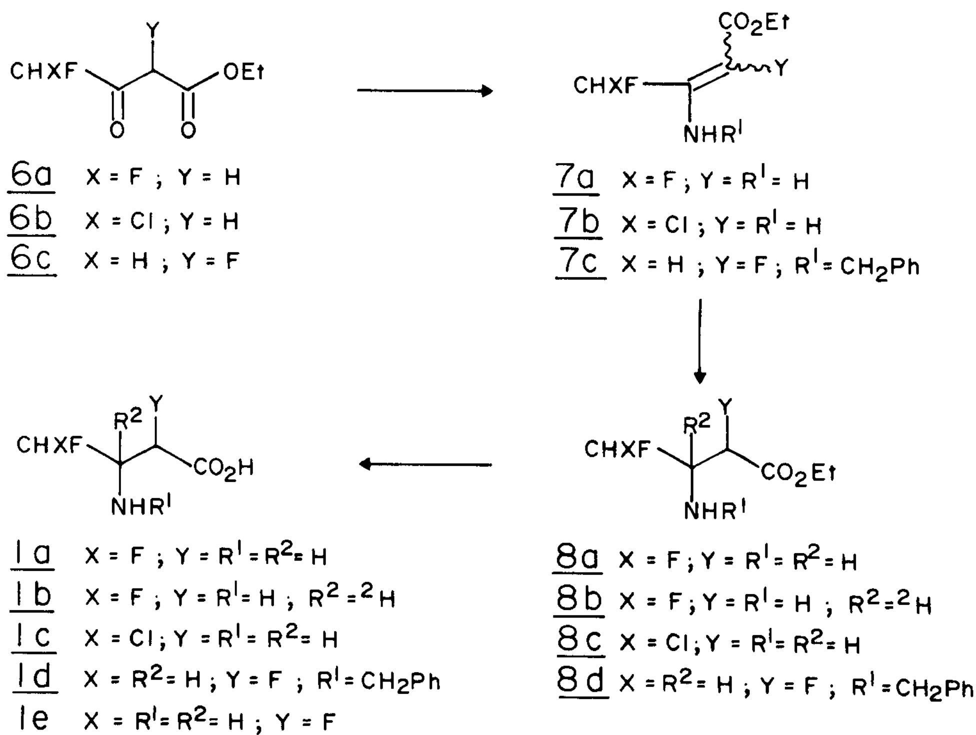

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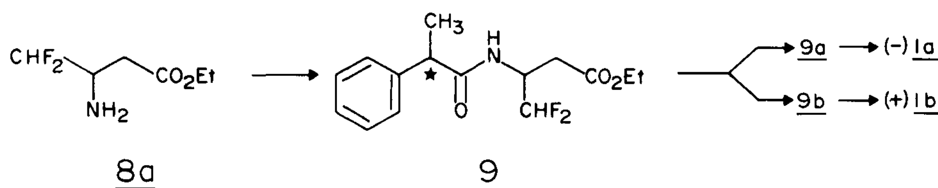
Chemistry

Our two synthetic approaches to (±) β-difluoromethyl-β-alanine **1a** are outlined in Schemes I and II. The first pathway (Scheme I) relies on the Curtius rearrangement of acid **4** a strategy we used to prepare various α-difluoromethylated amines[7]. Our second approach (Scheme II) is based on the reduction of the enamine **7a**, prepared by condensation of ethyl 4,4-difluoro-3-oxo-butyrate **6a**[8], with ammonium acetate, to the amine **8a**, using sodium cyanoborohydride[9]. The same sequence of reactions has been used to synthesize 3-amino-4-chloro-4-fluorobutyric acid **1c**, 3-amino-2,4-difluorobutyric acid **1e** and 3-amino-3-^{2}H-4,4-difluorobutyric acid **1b** (using $NaBD_3CN$, scheme II). Finally the (+) and (−) enantiomers of β-difluoromethyl-β-alanine **1a** were obtained by resolution of the diastereomeric amides **9** by HPLC followed by hydrolysis under acidic conditions (Scheme III).

SCHEME III



Synthesis

Melting points were determined with a Büchi apparatus and are uncorrected. ^{1}H n.m.r. spectra were obtained with a Varian T60 and a Brucker 200 MHz spectrometers, using tetramethyl silane ($CDCl_3$) or TPS (D_2O) as internal standards. TLC were carried out on precoated TLC plates with silica gel 60F-254 (Merck). For column chromatography, silica gel 60 (Merck) was used.

2-(Tert-butoxycarbonyl)butanedioic acid, 1-tert-butyl-4-methyl ester (**2**). Di-tert-butyl malonate (21.60 g, 0.1 mol) was added at room temperature, under nitrogen, to a suspension of sodium hydride (0.11 mol, 4.95 g of a 55% dispersion in oil, washed three times with pentane) in tetrahydrofuran (280 ml). The mixture was stirred for 1 h at room temperature. A solution of methyl α-bromoacetate (15.30 g, 0.1 mol) in tetrahydrofuran (20 ml) was then added dropwise over a period of 15 min. Stirring was continued for 48 h at room temperature. The mixture was hydrolysed and extracted two times with diethyl ether (500 ml). The organic phase was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was distilled to give 18.7 g (65%) of **2** as a colorless oil; bp 78–80°C (0.05 mm Hg): ^{1}H n.m.r. ($CDCl_3$) δ 1.47 (18 H, *s*, tert-butyl), 2.80 (2 H, d, J_{HH} = 7 Hz, CH_2), 3.60 (1 H, *t*, J_{HH} = 7 Hz, –CH), 3.67 (3 H, *s*, CH_3). (Found: C, 58.11; H, 8.11. $C_{14}H_{24}O_6$ requires C, 58.32; H, 8.39%).

2-(Tert-butoxycarbonyl)-2-difluoromethyl butanedioic acid, 1-tert-butyl 4-methyl ester (**3a**). Compound **2** (27.73 g, 0.096 mol) was added, at room temperature, under nitrogen, to a suspension of sodium hydride (0.105 mol, 5.60 g of a 45% dispersion in

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oil, washed three times with pentane) in tetrahydrofuran (200 ml). After the mixture had been stirred for 1 h, a stream of chlorodifluoromethane (freon 22) was rapidly bubbled through the carbanion solution, maintained at 45°C. Upon saturation and completion of the reaction, the mixture was quenched with water and extracted with diethyl ether (3 × 150 ml). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo* to give 21.80 g (67%) of pure **3a** as a white solid (crystallized from pentane): mp 49–50°C; ^{1}H n.m.r. ($CDCl_3$) δ 1.48 (18 H, *s*, tert-butyl), 3.00 (2 H, *s*, $-CH_2-$), 3.60 (3 H, *s*, $-OCH_3$), 6.30 (1 H, *t*, J_{HF} = 54 Hz, $-CHF_2$). (Found: C, 53.70; H, 7.00. $C_{15}H_{24}F_2O_6$ requires C, 53.25; H, 7.15%).

2-Difluoromethyl butanedioic acid, 4-methyl ester (**4**). A solution of **3a** (2.21 g, 6.5 mmol) in trifluoroacetic acid (10 ml) was stirred at 0°C for 0.5 h and then at room temperature for 1 h. The solvent was evaporated under reduced pressure to yield crude **3b**, used in the next step without further purification. Crude **3b** was dissolved in glacial acetic acid (20 ml) and heated at 100°C for 15 h. The acetic acid was then evaporated under reduced pressure to yield an oil, purified by distillation under high vacuo to afford 1.60 g (90%) of **4**: bp 95°C (0.6 mm Hg, Kugelrohr); ^{1}H n.m.r. ($CDCl_3$) δ 2.65–3.68 (3 H, *m*, CH_2–CH), 3.70 (3 H, *s*, OCH_3), 6.18 (1 H, d of *t*, J_{HF} = 54 Hz, J_{HH} = 3 Hz, CHF_2). (Found: C, 38.93; H, 4.49. $C_6H_8F_2O_4$ requires C, 39.57; H, 4.43%).

4,4-Difluoro-3-(methoxycarbonylamino)butanoic acid, methyl ester (**5**). A solution of **4** (12 g, 66 mmol) in thionyl chloride (60 ml) was heated at reflux for 3 h. The excess thionyl chloride was evaporated *in vacuo* to give the acyl chloride derived from acid **4**; ^{1}H n.m.r. ($CDCl_3$) δ 6.25 (1 H, d of *t*, CHF_2, J_{HH} = 3 Hz, J_{HF} = 54 Hz).

The crude acyl chloride was dissolved in acetone (60 ml). The solution was cooled to 0°C and sodium azide (4.68 g, 72 mmol) in water (8 ml) was added dropwise over a period of 15 min. After stirring for 0.5 h at 0°C and 1 h at room temperature, the reaction mixture was extracted with diethyl ether (3 × 50 ml). The organic layers were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo at 20°C to yield the acyl azide; IR (film): 2140, 1740 cm^{-1}.

The crude acyl azide was dissolved in anhydrous methanol (30 ml) and the solution heated at reflux temperature for 14 h. The residue obtained after evaporation of the solvent *in vacuo* was purified by chromatography on silica gel (MPLC, ethyl acetate/cyclohexane 4:6) and crystallized from ethyl acetate/pentane to yield 6.30 g (61%) of pure **5**: mp 61°C; ^{1}H n.m.r. ($CDCl_3$) δ 2.67 (2 H, d, J_{HH} = 6 Hz, CH_2), 3.68 (6H, *s*, 2–OCH_3), 3.96–4.80 (1 H, *m*, CH), 5.50 (1 H, *bd*, NH), 5.90 (1 H, d of *t*, J_{HF} = 56 Hz, J_{HH} = 3 Hz, CHF_2). (Found: C, 39.85; H, 5.33; N, 6.59. $C_7H_{11}F_2NO_4$ requires C, 39.81; H, 5.25; N, 6.63%).

(±)-3-Amino-4,4-difluorobutanoic acid, hydrochloride [(±) **1a**]. A mixture of **5** (6.30 g, 29.8 mmol), conc. hydrochloric acid (80 ml) and acetic acid (20 ml) was heated at reflux temperature for 48 h. Evaporation of the solvent *in vacuo* and treatment of the residual oil with water and active charcoal afforded after filtration, removal of the solvent *in vacuo*, and recrystallization from isopropanol/diethyl ether, 4.25 g (81%) of pure (±) **1a**: mp 150°C; ^{1}H n.m.r. (D_2O) δ 2.90 (H_2^A, dd, $J_{H_2^AH_3}$ = 9.6 Hz, $J_{H_2^AH_2^B}$ = 17 Hz) and 3.00 (H_2^B, $J_{H_2^BH_3}$ = 4.6 Hz, $J_{H_2^BH_2^A}$ = 17 Hz) (2 H, $-CH_2$), 4.10

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(1 H, *m*, –CH), 6.12 (1 H, d*t*, J_{H_4F} = 53 Hz, $J_{H_4H_3}$ = 4 Hz, CHF_2). (Found: C, 27.19; H, 4.50; N, 8.06. $C_4H_8ClF_2NO_2$ requires C, 27.36; H, 4.59; N, 7.98%).

3-Amino-4,4-difluoro-2-butenoic acid, ethyl ester (**7a**). Ammonium acetate (62 g, 0.805 mol) was added at room temperature to a solution of ethyl 4,4-difluoroacetoacetate **6a**[8] (7.4 g, 0.044 mol) in anhydrous methanol (120 ml), and the mixture was stirred for 40 h at that temperature. The mixture was then poured into a solution of sodium bicarbonate (5%, 200 ml) and extracted with diethyl ether (2 × 150 ml). The combined organic layers were dried over $MgSO_4$. Filtration and evaporation of the solvent under reduced pressure afforded 4.7 g (65%) of **7a** as an oil; ^1H n.m.r. ($CDCl_3$) δ 1.27 (3 H, *t*, J_{HH} = 7 Hz, $-CH_3$), 4.13 (2 H, *q*, J_{HH} = 7 Hz, OCH_2-), 4.83 (1 H, *bs*, =CH). 5.97 (1 H, *t*, J_{HF} = 55 Hz, CHF_2), 6.25 (2 H, very broad signal, NH_2).

3-Amino-4,4-difluorobutanoic acid, ethyl ester (**8a**). Bromophenol blue (0.005 g) was added to a solution of **7a** (3.10 g, 18.8 mmol) in anhydrous methanol (30 ml) at room temperature and the solution ajusted to pH 3.5–4.0 with a 2 N HCl/CH_3OH solution. Sodium cyanoborohydride (1.83 g, 28.2 mmol) was added portionwise with stirring. The mixture was maintained at pH 3.5–4.0 (by addition of 2 N HCl/CH_3OH solution). Stirring was continued for 3 h at room temperature after completion of the addition of the reducing agent. The mixture was then poured into NaOH (15 ml, 0.1 M). The solution was saturated with NaCl and extracted with diethyl ether (3 × 20 ml). The combined organic layers were dried over $MgSO_4$, filtered and evaporated *in vacuo* to yield 2.88 g (70%) of **8a**; ^1H n.m.r. ($CDCl_3$) δ 1.20 (3 H, *t*, J_{HH} = 7 Hz, $-CH_3$), 1.60 (2 H, *s*, NH_2), 2.40 (H_2^A, dd, $J_{H_2^AH_3}$ = 9.2 Hz, $J_{H_2^AH_2^B}$ = 16 Hz) and 2.60 (H_2^B, dd, $J_{H_2^BH_3}$ = 2.4 Hz, $J_{H_2^BH_2^A}$ = 16 Hz) (2H, $-CH_2-CO$), 3.40 (1 H, *m*, –CH), 4.10 (2 H, *q*, J_{HH} = 7 Hz, $-OCH_2$), 5.70 (1 H, d*t*, J_{HF} = 54 Hz, $J_{H_4H_3}$ = 5 Hz, CHF_2).

(±)-3-Amino-4,4-difluorobutanoic acid, hydrochloride (**1a**). A mixture of **8a** (3.34 g, 20 mmol) in 1 N HCl (20 ml) was heated at 100°C for 3 h. The solvent was evaporated *in vacuo*, and the residue was crystallized from isopropanol/diethyl ether to afford 2.80 g (80%) of (±) **1a**.

3-Amino-3-[²H]-4,4-difluorobutanoic acid, hydrochloride (**1b**). Sodium cyanoborodeuteride (0.251 g, 3.75 mmol) was added with stirring at room temperature to a mixture of 3-amino-4,4-difluoro-2-butenoic acid, ethyl ester **7a** (0.420 g, 2.54 mmol) and bromophenol blue (0.005 g) in anhydrous methanol (15 ml). The pH of the solution was maintained around 3.5–4.0 by addition of 2 N HCl/methanol solution. After stirring for 5 h at room temperature, the solution was poured into sodium hydroxide (10 ml, 0.1 M). The aqueous layer was saturated with sodium chloride and extracted with diethyl ether (3 × 20 ml). The combined organic phases were washed with water and then extracted with 3 N HCl (2 × 10 ml). The acidic aqueous phase was heated at 100°C for 3 h. The solvent was then evaporated *in vacuo* to yield a white solid, recyrstallized from isopropanol/diethyl ether 0.350 g (80%), mp 152°C; TLC R_f: 0.46 (silica gel Merck F-254, EtOH/NH_4OH 80/20, ninhydrin). Mass spectrum, ^{19}F and ^1H n.m.r. analysis showed that the compound was deuterated to the extent of

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70% in the 3 position (modest deuterium incorporation due probably to partial hydrolysis of $NaBD_3CN$).

By reduction of the enamine in deuterated methanol in the presence of DCl, bis-deuterated **1a** (in position 3 and 2, as expected from the mechanism of reduction[9]) was obtained with 98% deuterium labelling in position 3.

Resolution of racemic 3-amino-4,4-difluorobutanoic acid, hydrochloride. A solution of amino-ester **8a** (0.720 g, 4.32 mmol) in acetonitrile (5 ml) was added at 0°C to a mixture of R(–)-α-phenylpropionic acid[12] (0.648 g, 4.32 mmol) and dicyclohexylcarbodiimide (0.890 g, 4.32 mmol) in acetonitrile (50 ml). Stirring was continued for 48 h at room temperature. The solvent was then evaporated *in vacuo*, and the residue taken up in diethyl ether. Filtration and evaporation of the solvent under reduced pressure afforded an oil. The mixture of diastereomeric amides **9** was purified by column chromatography (MPLC, silica gel, ethyl acetate/cyclohexane 2:8) to give 0.800 g (62%) of a 1:1 mixture of **9a** and **9b**. The diastereomeric amides were separated by high pressure liquid chromatography on silica gel[13] (room temperature, ethyl acetate/hexane 2:8).

First eluted diastereomer (**9a**). Crystallized from diethyl ether/pentane: mp 59–60°C. $[\alpha]_D^{25}$ = – 5.9⁰ ($CHCl_3$, c = 1): ^{1}H n.m.r. ($CDCl_3$) δ 1.17 (3H, t, J_{HH} = 7 Hz, CH_3CH_2O), 1.51 (3 H, d, J_{HH} = 7 Hz, CH_3CHPh), 2.53 (2 H, d, J_{HH} = 6 Hz, CH_2CHCHF_2), 3.57 (1 H, *q*, J_{HH} = 7 Hz, CH_3CHPh), 4.02 (2 H, *q*, J_{HH} = 7 Hz, OCH_2CH_3), 4.23–4.95 (1 H, *m*, $CH-CHF_2$), 5.93 (1 H, d of *t*, J_{HF} = 56 Hz, J_{HH} = 3 Hz, CHF_2), 6.14 (1 H, *bd*, J_{HH} = 8 Hz, NH), 7.32 (5 H, *s*, phenyl). Found: C, 60.10; H, 6.47; N, 4.50. $C_{15}H_{19}F_2NO_3$ requires C, 60.19; H, 6.40; N, 4.68%).

Second eluted diastereomer (**9b**). Crystallized from diethyl ether/pentane: mp 57–58°C. $[\alpha]_D^{25}$ = – 9.2° ($CHCl_3$, *c* = 1); ^{1}H n.m.r. ($CDCl_3$) δ 1.20 (3 H, *t*, J_{HH} = 7 Hz, CH_3CH_2O), 1.50 (3 H, d, J_{HH} = 7 Hz, CH_3CHPh), 2.57 (2 H, d, J_{HH} = 6 Hz, CH_2CHCHF_2), 3.60 (1 H, *q*, J_{HH} = 7 Hz, $PhCHCH_3$), 4.08 (2 H, *q*, J_{HH} = 7 Hz, CH_3CH_2O), 4.25–4.92 (1 H, *m*, CH–CHF_2), 5.82 (1 H, d of *t*, J_{HF} = 56 Hz, J_{HH} = 3 Hz, CHF_2), 6.10 (1 H, *bd*, J_{HH} = 8 Hz, NH), 7.27 (5 H, *s*, phenyl). (Found: C, 59.96; H, 6.46; N, 4.46. $C_{15}H_{19}F_2NO_3$ requires C, 60.19; H, 6.40; N, 4.68%).

(–)-3-Amino-4,4-difluorobutanoic acid, hydrochloride [(–) **1a**]. A solution of the amide **9a** (0.195 g, 0.65 mmol) in a mixture of hydrochloric (6 M) and acetic acids (40:1, 20 ml) was heated at 100°C for 15 h. The solvent was then evaporated *in vacuo*. The residue was taken up in water and washed with diethyl ether (3 × 50 ml). The aqueous layer was concentrated *in vacuo* to afford 0.080 g (70%) of (–) **1a** as a white solid which was recrystallized from ethanol/diethyl ether to give slightly hygroscopic white crystals mp 144°C. $[\alpha]_D^{25}$ = – 7.1° (H_2O, *c* = 1). ^{1}H n.m.r. was identical to that for (±) **1a**. (Found: C, 26.90; H, 4.42; N, 7.70. $C_4H_8ClF_2NO_2$ requires C, 27.36; H, 4.59; N, 7.98%).

(+)-3-Amino-4,4-difluorobutanoic acid, hydrochloride [(+) **1a**]. The same hydrolysis procedure for **9a** was used to hydrolyse **9b** (0.195 g, 0.65 mmol) to obtain (+) **1a** as a white solid. This was then recrystallized from ethanol/diethyl ether to give 0.080 g (70%) of pure (+) **1a**: mp 132°C. $[\alpha]_D^{25}$ = + 6.9⁰ (H_2O, *c* = 1). ^{1}H n.m.r. was

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identical to that for (±) **1a**. (Found: C, 27.11; H, 4.33; N, 7.92. $C_4H_8ClF_2NO_2$ requires C, 27.36; H, 4.59; N, 7.98%).

Ethyl-4-chloro-4-fluoro acetoacetate (**6b**). A mixture of ethyl chlorofluoroacetate[11] (28.10 g, 0.2 mol) and sodium hydride (0.1 mol, 5.33 g of a 45% dispersion in oil, washed three times with pentane), in anhydrous diethyl ether (150 ml) was heated, under nitrogen, at 45°C for 1 h. Ethyl acetate (8.80 g, 0.1 mol) was then added and the mixture was heated at 45°C for 15 h. The crude mixture was then poured onto crushed ice 50 g) and concentrated H_2SO_4 (5 ml) and extracted with diethyl ether (3 × 100 ml). The combined organic phases were washed with water, dried over $MgSO_4$, filtered and evaporated at atmospheric pressure to yield a yellow oil. Fractional distillation afforded 8.60 g (47%) of pure **6b** as a colorless oil (bp 88–90°C/15 mm Hg).

3-Amino-4-chloro-4-fluoro-2-butenoic acid, ethyl ester (**7b**). The procedure described for the synthesis of **7a** (from **6a**) was used to prepare **7b** from **6b**. 1.350 g (74%) of **7b** was obtained. ^{1}H n.m.r. ($CDCl_3$) δ 1.25 (3 H, *t*, J_{HH} = 7 Hz, $-CH_3$), 4.11 (2 H, *q*, J_{HH} = 7 Hz, OCH_2–), 4.82 (1 H, *bs*, =CH), 5.93–6.67 (*bs*) and 6.35 (d, J_{HF} = 48 Hz) (3 H).

3-Amino-4-chloro-4-fluorobutanoic acid, hydrochloride (**1c**). The enamine **7b** was reduced to **8c** and hydrolyzed to **1c** using procedures described for the preparation of **8a** and (±) **1a** respectively. Starting from 1.350 g (7.4 mmol) of **7b**, 0.650 g (46%) of pure **1c** was obtained. Recrystallized from isopropanol/diethyl ether gave a 7:3 mixture of the two diastereoisomeric forms, mp 148°C as seen by ^{1}H n.m.r. (Ma: major and mi: minor). ^{1}H n.m.r. (200 MHz, D_2O) δ 2.84 (H_2^B-Ma, dd, $J_{H_2^AH_2^B}$ = 17.8 Hz, $J_{H_2^BH_3}$ = 8.4 Hz), 2.92 (H_2^B-mi, $J_{H_2^AH_2^B}$ = 18.1 Hz, $J_{H_2^BH_3}$ = 7.3 Hz), 2.99 (H_2^A-Ma, dd, $J_{H_2^AH_2^B}$ = 17.8 Hz, $J_{H_2^AH_3}$ = 5.4 Hz) and 3.05 (H_2^A-mi, dd, $J_{H_2^AH_2^B}$ = 18.1 Hz, $J_{H_2^AH_3}$ = 5.1 Hz) (2 H, CH_2), 4.20 (mi) and 4.35 (Ma) (1 H, *m*, –CH), 6.68 (H_4-mi, dd, J_{H_4F} = 48.3 Hz, $J_{H_4H_3}$ = 3.7 Hz) and 6.70 (H_4-Ma, dd J_{H_4F} = 47.2 Hz, $J_{H_4H_3}$ = 2.6 Hz) (1 H, C*H*ClF). (Found: C, 24.90; H, 4.34; N, 7.27. $C_4H_8Cl_2FNO_2$ requires C, 25.02; H, 4.20; N, 7.29%).

2,4-Difluoro-3-phenylmethylamino-2-butenoic acid, ethyl ester (**7c**). A mixture of 2,4-difluoro-3-oxobutanoic acid, ethyl ester (1.280 g, 7.7 mmol), phenylmethylamine[10] (0.824 g, 7.7 mmol) *p*-toluene sulfonic acid (0.005 g) and benzene (50 ml) was heated at reflux for 20 h in a flask fitted with a Dean Stark apparatus. The solvent was then evaporated *in vacuo* to yield an oil purified by distillation under reduced pressure to give 1.330 g (68%) of pure **7c**, bp 175°C/0.075 mm Hg (Kugelrohr apparatus). ^{1}H n.m.r. showed a 1:1 mixture of cis and trans isomers. ^{1}H n.m.r. ($CDCl_3$) δ 1.30 (3 H, *t*, J_{HH} = 7 Hz, CH_3), 4.22 (*q*, J_{HH} = 7 Hz, OCH_2–), 4.40 (AB, J_{AB} = 16 Hz, ν_{AB} = 15.5 Hz, –CH_2Ph) and 4.50 (*bs*, NH) (5 H), 5.13 (dd, J_{HF_4} = 47 Hz, J_{HF_2} = 4 Hz, CH_2F *trans*) and 5.47 (dd, J_{HF_4} = 47 Hz, J_{HF_2} = 2 Hz, CH_2F *cis*) (2 H), 7.20 (5 H, *s*, phenyl).

2,4-Difluoro-3-phenylmethylaminobutanoic acid, ethyl ester (**8d**). A procedure similar to that used to reduce **7a** to **8a** was utilised. 1.240 g (54%) of pure **8d** was obtained

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starting from 2.265 g (8.9 mmol) of **7c**. ^{1}H n.m.r. ($CDCl_3$) δ 1.25 and 1.26 (3 H, 2 *t*, J_{HH} = 7 Hz, CH_3), 1.67 (1 H, *bs*, NH), 2.90–4.00 (3 H, *m*, $-CH-N-CH_2$), 4.22 and 4.23 (2 H, 2 *q*, O–CH_2), 4.47 (2 H, d*m*, J_{HF} = 48 Hz, CH_2F), 5.00 (1 H, d*m*, J_{HF} = 48 Hz, –*CH*F–), 7.27 (5 H, *s*, phenyl).

3-Amino-2,4-difluorobutanoic acid (**1e**). A solution of **8d** (1.240 g, 4.8 mmol) in hydrochloric acid (30 ml, IM) was heated at 100°C for 4 h. The solvent was removed *in vacuo* to leave **1d** hydrochloride as a white solid when recrystallized from ethanol/diethyl ether (1.010 g, 80%). ^{1}H n.m.r. (D_2O) δ 3.70–4.70 (3 H, *m*, *CH*–N–CH_2–Ph), 4.97 (2 H, d*m*, J_{HF} = 46 Hz, CH_2F) and 5.50 (1 H, d*m*, J_{HF} = 46 Hz, –CHF–), 7.47 (5 H, *s*, phenyl).

A mixture of **1d** (1.010 g, 3.84 mmol) and 5% Pd/C (type H, 0.150 g) in glacial acetic acid (30 ml) was shaken under hydrogen (60 psi) in a Parr hydrogenator for 16 h at room temperature. Filtration of the catalyst, and removal of the solvent *in vacuo* yielded a colorless oil.

A first crop of pure **1e** (0.185 g, 34%) mp 188°C was isolated by crystallization from H_2O/ethanol. A second crop (0.190 g, 36%) was obtained by passing the mother liquors through an ion exchange column (Dowex 50, H^+; eluted with water). ^{1}H n.m.r. (D_2O) δ 3.55–4.30 (1 H, *m*, H-3), 4.75 (2 H, d*m*, J_{HF} = 46 Hz, H-4), 5.05 (1 H, d*m*, J_{HF} = 46 Hz, H-2). (Found: c, 34.50; H, 4.82; N, 10.03. $C_4H_7F_2NO_2$ requires C, 34.54; H, 5.07; N, 10.07%).

Biochemistry

Enzyme preparation. GABA-T was purified from pig brain following the method described by Fowler and John for rabbit brain[14]. After the DEAE column an enzyme with a specific activity of 172 μM/mg prot./h was obtained, and after the CM-cellulose column, the specific activity of the enzyme was 770 μM/mg prot./h i.e. a purification of 1800 fold over the starting homogenate. This preparation showed by isoelectric focussing on acrylamide two closely associated bands which may be the two subunits of the enzyme. The specific activity obtained is comparable to that reported by Fowler and John for the pure enzyme[14].

Enzyme assay. Enzyme activity was determined by measuring the formation of glutamate by HPLC with *o*-phthaldialdehyde derivatisation post-column. The conditions of incubation were: pyrophosphate 0.1 M pH 8.5, mercaptoethanol 3 mM, α-ketoglutarate 5 mM, GABA 3 mM. Incubation was carried out at 37°C and the reaction was started by addition of enzyme. At the appropriate time, aliquots were withdrawn (200 μl) and quenched with 10 μl $HClO_4$ 4 N. After dilution (1:100) in the eluant buffer (CH_3CN–H_2O; 15:85 v/v) buffered at pH 3.0 and containing 500 mg/l octane sulfonic acid, the samples were injected on a Lichrosorb RP 18 column.

In vivo assays. Swiss Albino mice (20–25 g) were given the drug either orally or i.p. At the appropriate times after injection, the animals were decapitated and the brain was split sagitally. One half served for enzyme determinations, the other half for GABA measurements. Both methods have been described previously[15].

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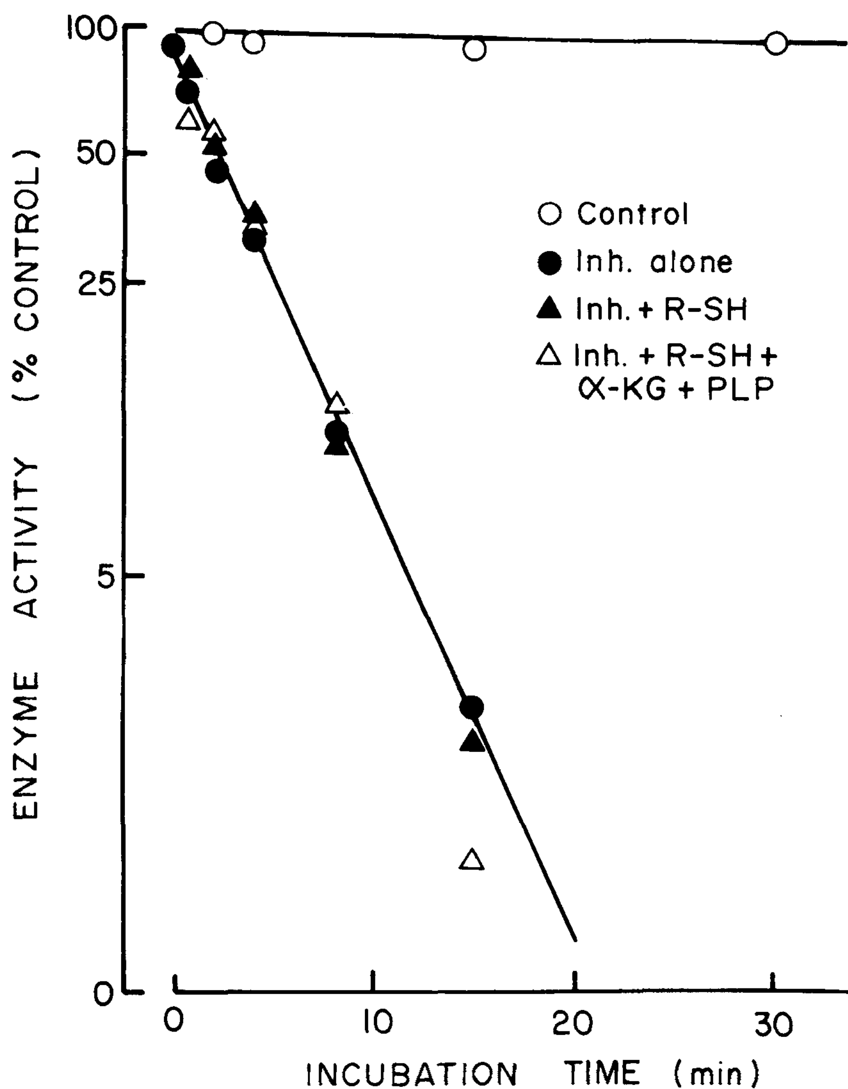

FIGURE 1 Characteristics of the inhibition of GABA-T by compound (±) **1a**. Nine units of purified GABA-T were incubated with 4 mM **1a** in a total volume of 100 μl of potassium pyrophosphate 100 mM, pH 8.6 containing different constituents as indicated in the Figure. Aliquots were withdrawn at different time intervals and assayed for remaining enzyme activity as described under Methods.

RESULTS

Characterisation of the type of inhibition of GABA-T by (±)-β-difluoromethyl-β-alanine (**1a**). GABA-T was incubated with (±)-β-difluoromethyl-β-alanine **1a** at a concentration of 4 mM under different conditions: buffer alone, buffer plus mercaptoethanol, α-ketoglutarate and/or pyridoxal phosphate in addition to the other con-

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TABLE I
Kinetic parameters of GABA-T inhibition by halogenated derivatives of β-alanine. Purified GABA-T was incubated with the compounds (±) **1a**, **1c** and **1d** at concentrations ranging from 0.1 to 10 mM. Enzyme activity measurement and data treatment were carried out as described in Figures 1 and 2 respectively

Compound	K_i (mM)	τ_{50} (min)
(±) **1a**	1.4	2.5
(±) **1c**	10.0	2.0
(±) **1e**	5.1	1.2

stituents. In all cases there was a time dependent decrease of enzyme activity (Figure 1). The rate of decrease of enzyme activity was independent of the various additives. Enzyme activity could not be restored by dialysis.

Influence of the nature and position of the halogen atoms on inhibition. Purifed GABA-T was incubated with compounds (±) **1a**, **1c** and **1e** at various concentrations. Remaining enzyme activity was measured after different time intervals. All three

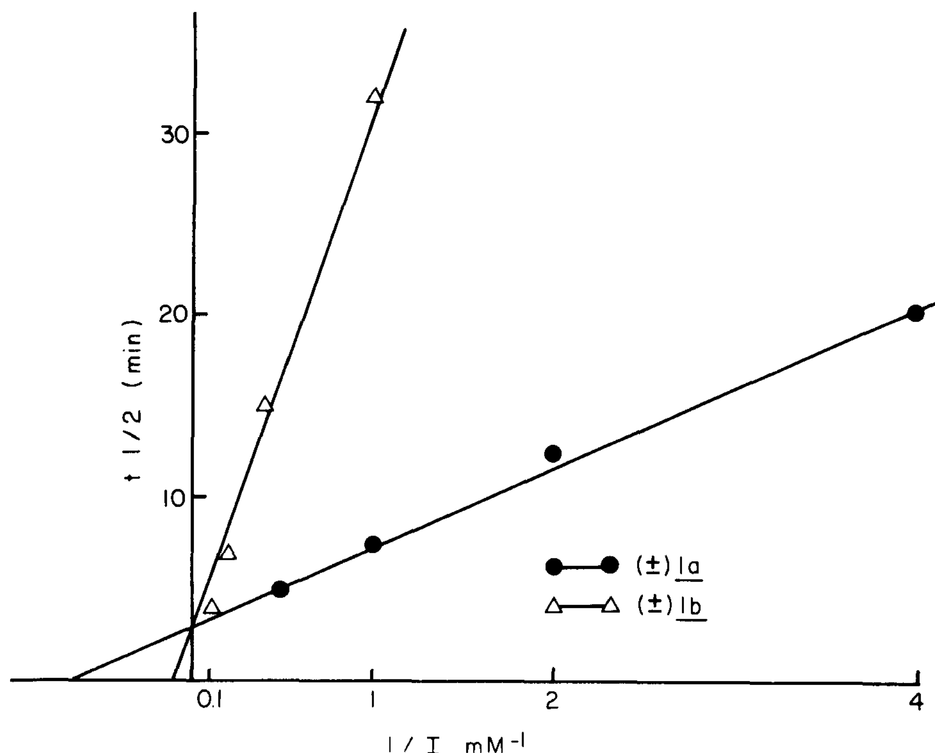

FIGURE 2 Primary deuterium isotope effect on the inhibition of GABA-T by compound (±) **1a** versus (±) **1b**. The conditions of incubation and assay were as described in Figure 1 except that the concentration of inhibitors was varied as indicated. From the regression lines of log (enzyme activity) versus time, $t_{\frac{1}{2}}$ was graphically determined and plotted as a function of $1/I$. The intercept with the x-axis give $-1/K_I$, the intercept with y axis gives τ_{50} i.e. the half-life of enzyme activity at saturating concentrations of inhibitor.

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compounds produced a time-dependent decrease of GABA-T activity. Kinetic constants were determined by plotting $t_{\frac{1}{2}}$ at a given concentration as a function of the reciprocal of inhibitor concentration[16]. These data are reported in Table I.

Deuterium isotope effect on inhibition of GABA-T by 3-amino-3-deutero-4,4-difluoro butyric acid (**1b**). Compounds (±) **1a** and **1b** were compared for their effectiveness in inhibiting GABA-T *in vitro*. The results are shown in Figure 2. The deuterated compound is a very poor inhibitor of GABA-T and concentrations from 2 to 10 mM had to be used to see a rapid time-dependent effect.

As can be seen from Figure 2, the minimum half life (i.e. at saturating concentrations of inhibitor) was not affected by deuterium substitution, however, the K_i was increased tremendously. At a concentration of 1 mM, the $t_{\frac{1}{2}}$ was increased from 7 min to 32 min by deuterium substitution, i.e. an isotope effect of ca. 4.5.

Stereochemistry of the inhibition of GABA-T by (+) and (−)-3-amino-4,4-difluoro butyric acid (**1a**). As can be seen in Figure 3, 1 mM of racemic 3-amino-4,4-difluorobutyric acid (±) **1a** inhibits GABA-T at the same rate as 0.5 mM of the (−) enantiomer (−) **1a** . The (+) enantiomer (+) **1a**, at this concentration has no effect on GABA-T activity.

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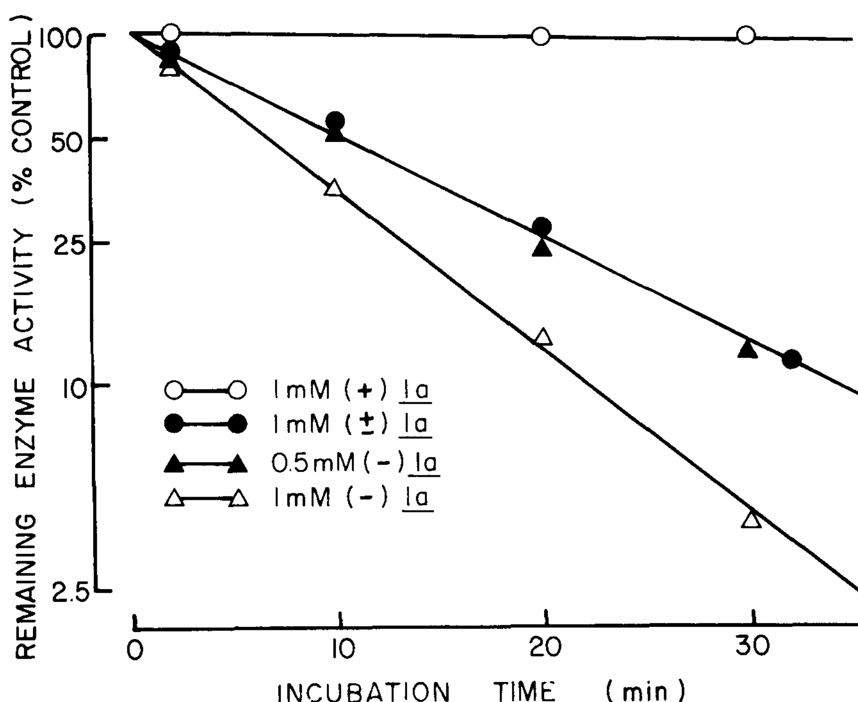

FIGURE 3 Stereoselectivity of the inhibition of GABA-T by enantiomers of compound **1a**. Conditions of incubation and assay were as in Figures 1 and 2. The preparation and properties of (+) and (−) **1a** are described in the experimental part.

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Spectral changes during GABA-T inhibition. The UV-visible absorption spectrum of purified GABA-T between 500 and 300 nm was recorded during ongoing inhibition by compounds (±) **1a, 1c** and **1e**. The absorption changes are qualitatively similar. With time the absorption band at 415 nm due to the pyridoxal Schiff base decreases while the absorption at 330 nm increases. As a representative example, the changes observed during the inhibition by 3-amino-4-chloro-4-fluorobutyric acid **1c** (2.5 mM) are shown in Figure 4.

In vivo biochemistry. The effects of a single administration of compound (±) **1a** and **1e** have been reported previously[5]. Compound **1c**, when given i.p. had no effect on brain GABA-T activity or brain GABA levels up to a dose of 25 mg/kg. The effects of (±) **1a** and **1b** on brain GABA metabolism were compared after subchronic administration (Figure 5). As for the *in vitro* study, introduction of deuterium into position 3 of the inhibitor produces a marked isotope effect; the dose of 2.5 mg/kg of the protiocompound produced a greater inhibition of GABA-T and accumulation of brain GABA than the dose of 10 mg/kg of the deuterocompound. This is an interesting example of a deuterium isotope effect *in vivo*.

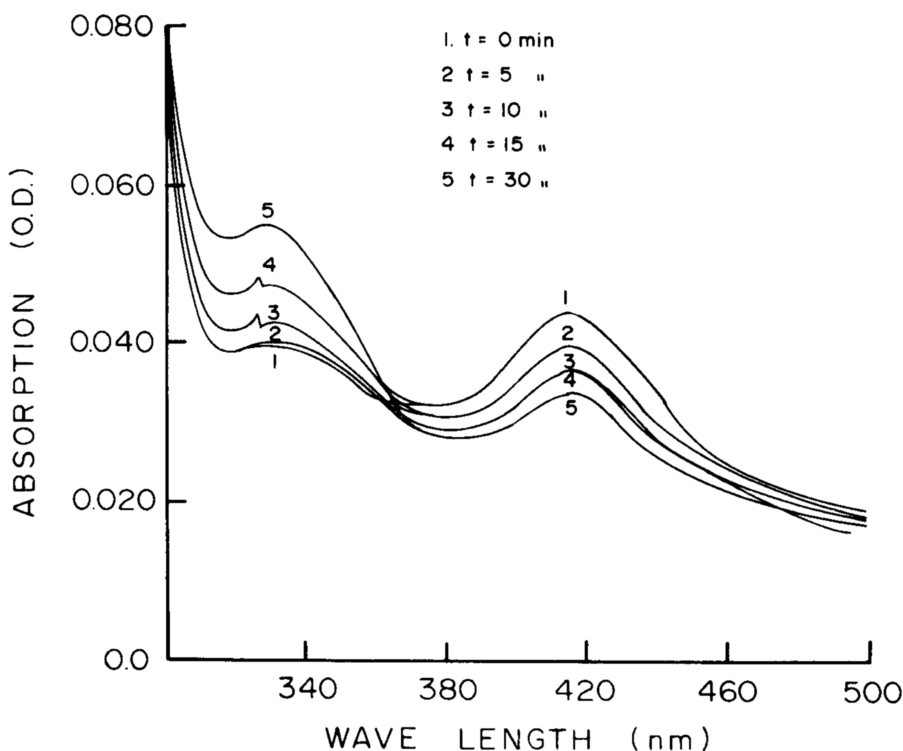

FIGURE 4 Spectroscopic changes during inhibition of GABA-T by compound **1c**. One hundred and thirty μg of GABA-T with a specific activity of 170 μmole/mg prot./h were incubated in a total volume of 0.5 ml with 2.5 mM **1d** in quartz UV micro-cuvettes. The cells were thermostated at 31°C and spectra recorded at fixed intervals on a DU 7 spectrophotometer. At the end of the 30 min incubation, the enzyme had lost 50% of its activity which agrees with an equivalent decrease of the absorption at 415 nm.

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BRAIN GABA METABOLISM AFTER THE 5th DAILY DOSE OF DFM-β-Ala (1a) AND THE DEUTERIO ANALOGUE (1b)

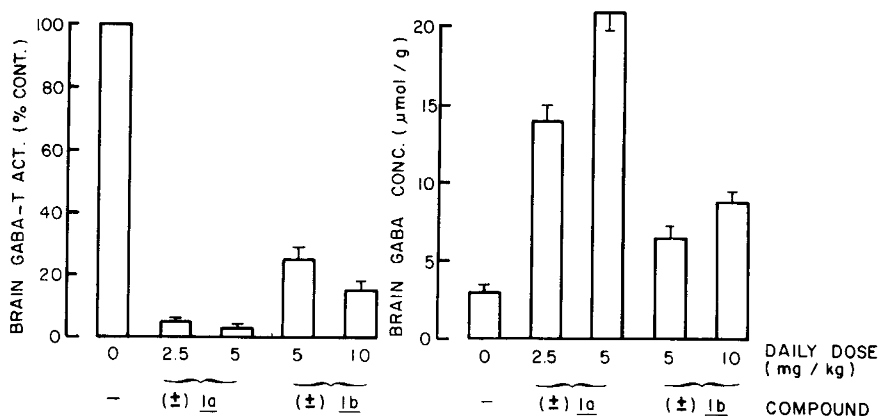

FIGURE 5 Comparison of the *in vivo* effects of compounds **1a** and **1b** on brain GABA metabolism in mice. Drugs were given by the i.p. route as aqueous solutions once daily for five consecutive days. Twenty four hours after the last administration, the animals were killed by decapitation. The brains were split and rapidly frozen in liquid nitrogen and analyzed for GABA content and GABA-T activity as described. Each value is the mean ± SEM of five animals.

DISCUSSION

β-Difluoromethyl-β-alanine **1a** was, in our hands, the most potent *in vivo* GABA-T inhibitor yet described. As appears from the dose response curves on brain GABA metabolism[5], there was about a 100 fold increase in potency over γ-vinyl GABA. This was not predictable from the data on inhibition of purified GABA-T *in vitro* (Table I). In the present paper, we have attempted to further characterize the mode of interaction of fluorinated GABA-T inhibitors with purified enzyme.

The rate of inhibition of GABA-T by racemic (±) **1a** is concentration- and time-dependent, but is not influenced by the various conditions described in Figure 1. This finding suggests that the inactivation is active-site directed and that no reactive species escapes from the active site before reacting with the enzyme. Moreover as no activity is restored by dialysis, the inhibition is irreversible.

The inactivation of GABA-T by **1a** is also stereospecific, at least at the concentrations used here[17], as only the (−) enantiomer of **1a** is able to inhibit the enzyme, the (+) enantiomer being inactive (Figure 3). The use of a β-deuterium labelled inhibitor **1b** results in a marked primary isotopic effect *in vitro* (~4.5) (Figure 2) and even more interestingly *in vivo* (Figure 5). The apparent dissociation constant K_i is tremendously increased while the inactivation rate constant is not affected. This suggests that the elongation step of the C_3–H bond is part of the recognition step but as the inactivation rate constant is not affected, the hydrogen abstraction is not rate determining. We have already reported and discussed a similar finding for the inhibition of ODC by α-difluoromethylputrescine and its α-deuterated analogue[18].

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The rate of inhibition is dependent on the nature and position of the leaving group X (Table I): these two factors affect strongly the acidity of the β-hydrogen of compounds **1a, 1c, 1e** as well as the pK_a of their amino and carboxylic acid functions.

Changes in those two parameters will in turn affect the different steps leading to enzyme inactivation: the recognition step (steric and electronic effects); the ease of formation of the intermediate Schiff base; the ease of abstraction of the β-hydrogen atom; the rate of elimination of the halide ion: (a) a chloride ion being expected to be a better leaving group than a fluoride ion[19], (b) the lability of a fluoride ion is decreased by increasing the numbers of fluorine atoms attached to the halogen bearing carbon, in a β elimination reaction, withE_{1cb} character[19] (formation of a negative charge in β-position to the leaving group by abstraction of a β-hydrogen atom).

Two important additional remarks can be made: (1) the nature of the leaving group plays an important role on the rate of inactivation but the difference in the inhibition constants observed between **1c** (**1c** is a 7/3 mixture of diastereomers, see experimental section) and **1e** is rather small ($K_i^{Cl}/K_i^{F} = 7$) as compared to those observed by Silvermann[20] between 4-amino-5-fluoro and 4-amino-5-chloropentanoic acids ($K_i^{Cl}/K_i^{F} = 33$) for the same enzyme. (2) the position of the leaving group is essential, and the inhibition observed for **1e** occurs probably through elimination of the 4-fluorine atom, as elimination of an internal leaving group (eg. fluorine 2) in GABA analogues did not produce inactivation of GABA-T[21].

The spectral changes observed during GABA-T inhibition by **1a, 1c** and **1e** could be explained by two different hypothetical mechanisms (Scheme IV): (a) Transamination with formation of 4-halo-3-ketobutyric acid and pyridoxamine (PMP, absorption at 330 nm) followed by recombination of the haloketone and inactivation. This mechanism was suggested for the inhibition of GABA-T in the pyridoxamine form with 5-fluorolevulinic acid ($K_i \sim 20.2$ mM at 25°C, $t_{\frac{1}{2}} = 0.6$ min)[22] (Scheme IV, pathway **a**). (b) Formation of the Schiff base and elimination of a halide ion with formation of an enamine E_1. This enamine could *either* be directly alkylated by a nucleophilic residue of the enzyme active site[5] (Scheme V, pathway **c**) *or* alternatively, E_1 could undergo a transimination with formation of an enamine E_2. The nucleophilic methylene carbon atom of compound E_2 could then attack the internal Schiff's base to form an adduct, covalently bound to the enzyme (absorption at 330 nm)[23] (Scheme IV, pathway **b**).

Although we cannot definitely rule out mechanism **a**, it is unlikely to occur due to the discrepancy in K_i values beteen fluoroketones and fluoroamines (compare 5-fluorolevulinate[22] to 4-amino-5-fluoropentanoic acid[20]). Even stronger evidence against this mechanism is provided by the experiment described in Figure 1 where the presence of a nucleophile (mercaptoethanol) rules out the release of a reactive species. In addition, α-ketoglutarate should compete with the fluoroketone for the PMP holoenzyme and slow down the rate of inhibition. To decide between mechanisms **b** and **c** would require identification of the fate of PLP and/or of a residue covalently modified at the active site. This was not attempted due to the availability of only low amounts of purified enzyme.

Finally the unexpected high *in vivo* activity of the β alanine analogues **1a** and **1e** could be explained by a greater blood brain barrier permeability as compared to the fluorinated GABA analogues[5]. Development of these promising GABA-T inhibitors was stopped due to unexplained delayed toxicity after chronic treatment in mice.

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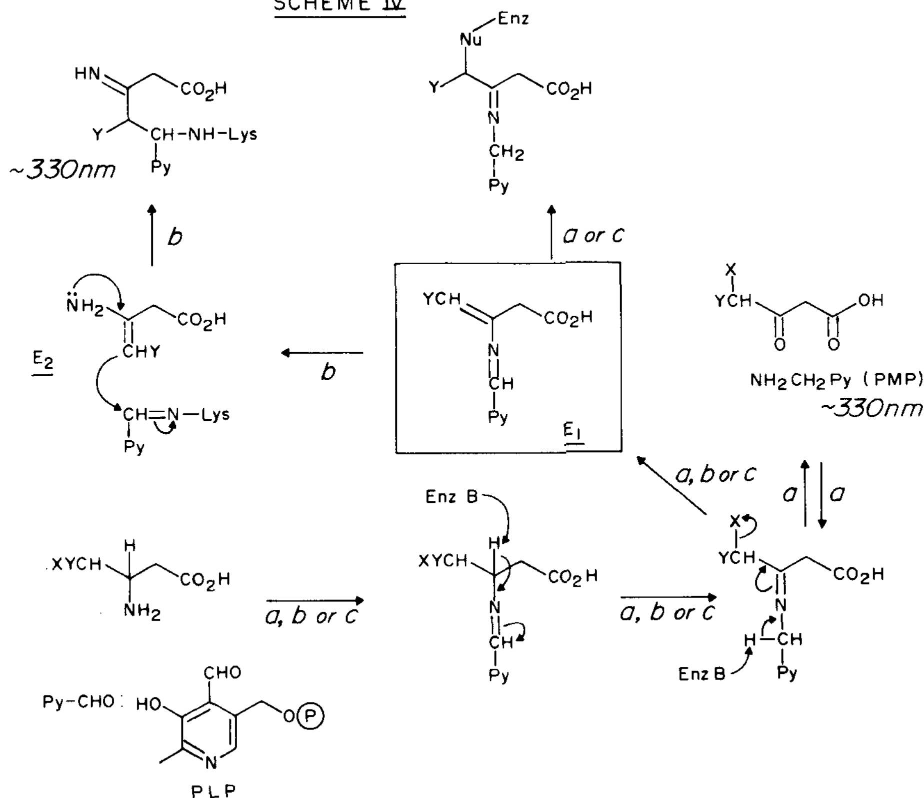

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