Eur J Med Chem (1995) 30, 245–251

Short communication

β–Carbolines as inverse agonistic benzodiazepine receptor ligands. 2. Synthesis and *in vitro* and *in vivo* binding of some new 6-amino- and 6-fluoro-β-carboline-3-carboxylates

G Settimj[1], MR Del Giudice[1], R Ferretti[1], V Cotichini[1], G Bruni[2], MR Romeo[2]

[1]*Istituto Superiore di Sanità, Laboratorio di Chimica del Farmaco, Viale Regina Elena, 299, 00161 Rome;*
[2]*Istituto di Farmacologia, Università di Siena, Via delle Scotte, 6, 53100 Siena, Italy*

(Received 24 May 1994; accepted 7 November 1994)

Summary — Six new 6-fluoro-β-carboline-3-carboxylates (**3a–f**) with their related 6-amino analogues (**2a–f**) are described and their *in vitro* and *in vivo* capabilities to bind to rat cerebral cortex 'benzodiazepine receptors' checked by radioreceptor assay (RRA). For some of the derivatives, the tests were also accomplished in the absence and presence of 10 μM GABA, whereby an inverse agonistic activity resulted. Their IC_{50} for [^{3}H]flunitrazepam displacement were in the 10^{-9}–10^{-12} molar range. The same compounds, with the exception of the hydroxylated compounds **2e**, **2f**, **3e** and **3f**, crossed the blood–brain barrier in the rat, generally giving rise to higher concentrations in the brain (ng/g) than in the plasma (ng/ml). The synthetic pathway preferred here allows a rapid fluorine incorporation in this moiety and an easy isolation of the fluorinated compounds.

inverse agonist / β-carboline

Introduction

For some years now the interest of our laboratory has been directed to central nervous system (CNS) agents with a particular stress on new benzodiazepines and, in the last few years, on new β-carboline derivatives [1, 2]. The well-established binding strength of some β–carboline-3-carboxylates [3, 4] to brain 'benzodiazepine receptors' and the inverse agonist activity [5, 6] of some of them, prompted us to prepare new derivatives of this moiety, which could antagonize the so-called benzodiazepine activity on the CNS *in vivo*.

Because of the great interest in β-carbolines since the late 1970s and the wide variety of chemical and pharmacological studies carried out on the large number of new derivatives synthesized, we focused our attention on both the preparation and biological study of some 6-fluorinated-β-carboline-3-carboxylates. These derivatives are previously unknown, with the unique exception of ethyl 6-fluoro-β-carboline-3-carboxylate **3b**, which was reported in two Schering patents [7, 8], but no data were given.

Of the possible replacements for hydrogen in C-H bonds, fluorine offers the unique advantages of effecting a marked change in electronic-density distribution and related properties and a minimal change in molecular size or shape [9].

In the same way that electron-withdrawing groups at position 3 of a β-carboline enhance the ability of the indole N_9-H to interact with a hydrogen bond donor site on the receptor *via* polarization of the indole N_9-H bond [10, 11], the introduction of a second electron-withdrawing group, such as ^{19}F nuclide at the symmetrical (relative to the indole N_9-H) position 6 of the β-carboline moiety, could further affect the polarization of the group and thereby enhance efficacy.

The thrust of the present work was to find new very active (at low nanomolecular-range concentrations) fluorine compounds, which could be utilized as inverse benzodiazepine agonists.

Chemistry

In a previous paper [1], some 5-, 6- and 7-amino derivatives of β-carboline-3-carboxylic acid methylester (β-CCM) and β-carboline-3-carboxylic acid ethylester (β-CCE) were prepared and described. In this paper, we report the synthesis of the new 6-fluoro-β-carboline-3-carboxylates **3a–f** starting from their appropriate 6-nitro-β-carbolines **1a–f**, which were reduced by iron and hydrochloric acid to the corresponding 6-amino derivatives **2a–f**.

Diazotization of **2a–f** in the presence of lithium tetrafluoroborate led to the isolation of the 6-diazonium fluoroborates, the heating of which at 160°C and reduced pressure afforded the target fluoro compounds **3a–f** (fig 1). In order to introduce fluorine into the β-carboline moiety, we preferred to follow the more rapid Schiemann procedure [12–14], rather than its photochemical modification more recently introduced by Kirk [9, 15], since the described yields are in most cases comparable and the β-carboline moiety endures the drastic temperature conditions required to decompose diazonium fluoroborates. Whatever the mechanism for this intramolecular decompositon, in order to reduce the side reactions (*eg*, splitting of ester groups by boron trifluoride evolution, formation of coupling and tar products, *etc*), the diazonium fluoborate was previously diluted in dry silica gel, heated under reduced pressure at 160°C, then stratified on a chromatographic column to isolate the so-formed fluorocarbolines.

Compounds **1a**, **1b**, **2a** and **2b** have been described previously [1]. Nevertheless, as the easier reduction of the nitro compounds **1** with iron/hydrochloric acid reported here led to higher yields of the amino derivates **2** than the previously published procedure, the new yields of **2a** and **2b** are also reported in this paper.

The hydroxylated substituents **e** and **f** (see fig 1) were introduced to enhance the solubility in water, and also to facilitate a ^{1}H- and ^{13}C-NMR study of the influence of the hydroxy groups on the dynamic and conformational properties of these β-carbolines in aqueous media (Delfini *et al*, manuscript in preparation) in relation to their biological activity.

Biological test results

The 50% inhibition concentration (IC_{50}) and the apparent inhibition constants (K_i) are presented in the table I. The IC_{50} values for [^{3}H]flunitrazepam (FNZ) displacement from brain cortex receptors of the amino compounds **2a–d** and the fluorinated derivatives **3c–f** are in the same nanomolar range of diazepam (DAZ) [16]. They are also similar to the described values [17] of methyl-(β-CCM), ethyl-(β-CCE), *n*-propyl-(β-PrCC) and *n*-butyl (β-CCB) esters; β-CCB was recently reported to be an endogenous benzodiazepine binding inhibitor from brain with high affinity (K_i = 3–4 nM) [18, 19]. In our series, however, the 6-fluoro analogues of β-CCM **3a** and β-CCE **3b** show a particularly high affinity, especially the latter which is in the 10^{-12} molar range.

The data obtained in another series of *in vitro* experiments performed for some of the active compounds described above and the reference compounds DAZ and β-CCE in the absence and presence of 10 μM GABA are presented in table II. The IC_{50} value for FNZ displacement for the most active compound **3b** is still in the 10^{-12} molar range, thus confirming its very high affinity with respect to DAZ and β-CCE. The GABA ratio is the lowest of all the compounds tested including β-CCE.

The mean values of concentration in plasma and brain of the compounds ± SE in *in vivo* experiments on Wistar rats are summarized in table III (see *Experimental protocols*).

Discussion

The results obtained (tables I–III) allow some conclusions to be drawn. First, in both the unsubstituted [17] and the fluorinated series of β-carboline-3-carboxylates, the ethylesters are the most active compounds; the affinities of the remaining compounds decrease from methyl to butyl esters. In our series, however, the 6-fluoro analogues of β-CCM **3a** and β-CCE **3b** show outstanding affinities, which cannot be completely accounted for by the electronic effect of the fluorine atom. They could be explained on the basis of lipophilic or, better, steric effects. Indeed, only molecular shape and size differences appear to be meaningful for a reasonable answer to the observed affinities of **3a** and **3b** as compared with those of their fluorinated analogues **3c** and **3d** (table I).

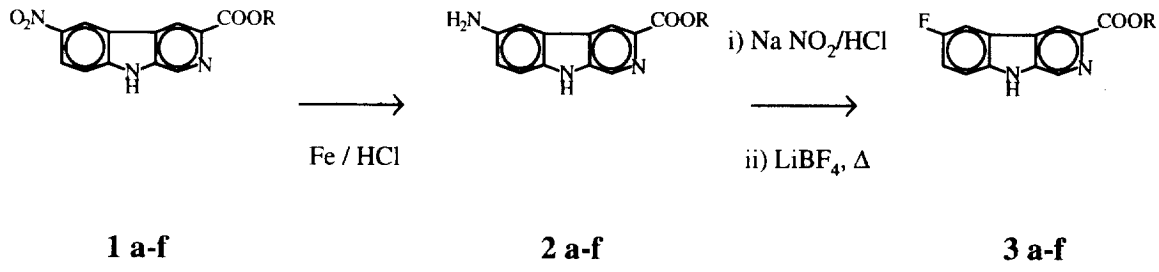

Fig 1. Synthetic pathway to compounds **2** and **3**. **a**: R = -CH_3; **b**: R = -CH_2CH_3; **c**: R = -$CH_2CH_2CH_3$; **d**: R = -$CH_2CH_2CH_2CH_3$; **e**: R = -CH_2CH_2OH; and **f**: R = -$CH_2CH(OH)CH_2OH$.

Table I. Inhibition of [^{3}H]flunitrazepam binding.

Compound	$IC_{50} \pm SE$ (*M*)	K_i (*M*)
2a	$2.7 \pm 0.39 \times 10^{-9}$	2.0×10^{-9}
2b	$3.9 \pm 0.35 \times 10^{-9}$	2.9×10^{-9}
2c	$7.02 \pm 2.02 \times 10^{-9}$	5.17×10^{-9}
2d	$4.39 \pm 1.38 \times 10^{-9}$	3.23×10^{-9}
2e	NA	NA
2f	NA	NA
3a	$1.6 \pm 0.68 \times 10^{-10}$	1.2×10^{-10}
3b	$6.2 \pm 1.57 \times 10^{-12}$	4.6×10^{-12}
3c	$3.68 \pm 1.60 \times 10^{-9}$	2.71×10^{-9}
3d	$7.08 \pm 1.02 \times 10^{-9}$	5.21×10^{-9}
3e	$1.1 \pm 0.26 \times 10^{-9}$	7.9×10^{-9}
3f	$2.1 \pm 0.46 \times 10^{-9}$	1.5×10^{-9}
DAZ	$4.4 \pm 0.54 \times 10^{-9}$	3.2×10^{-9}

NA = not active; SE = standard error.

Table II. Inhibition of [^{3}H]flunitrazepam binding with and without GABA.

Compound	$IC_{50} \pm SE$ (*M*)	*With GABA* $IC_{50} \pm SE$ (*M*)	*GABA ratio*
2a	$1.49 \pm 0.17 \times 10^{-9}$	$2.31 \pm 0.21 \times 10^{-9}$	0.645
2b	$1.86 \pm 0.21 \times 10^{-9}$	$2.08 \pm 0.52 \times 10^{-9}$	0.894
3a	$7.21 \pm 1.74 \times 10^{-10}$	$2.02 \pm 0.34 \times 10^{-9}$	0.357
3b	$5.05 \pm 0.78 \times 10^{-12}$	$5.05 \pm 0.64 \times 10^{-11}$	0.100
β-CCE	$1.65 \pm 0.39 \times 10^{-9}$	$7.26 \pm 1.21 \times 10^{-9}$	0.227
DAZ	$3.15 \pm 1.32 \times 10^{-9}$	$2.06 \pm 0.18 \times 10^{-9}$	1.530

Table III. Mean values of concentration in rat plasma and brain of the test compounds (± SE) 1 h later 5 mg/kg oral administration.

Compound	*Plasma (ng/ml)* ± *SE*	*Brain (ng/g)* ± *SE*
2a	40.7 ± 10.22	47.7 ± 9.10
2b	46.5 ± 10.66	35.5 ± 6.79
2c	42.2 ± 10.46	48.41 ± 10.25
2d	41.6 ± 12.63	42.60 ± 11.85
3a	0.3 ± 0.02	0.8 ± 0.03
3b	0.01 ± 0.004	0.03 ± 0.007
3c	0.14 ± 0.031	0.32 ± 0.05
3d	0.22 ± 0.06	0.58 ± 0.09
3e	0.09 ± 0.02	–
3f	8.7 ± 1.44	–

As regards the influence of hydroxy groups, a preliminary ^{1}H- and ^{13}C-NMR study of dynamic and conformational properties of **2a**, **2b** and **2e** (Delfini *et al*, manuscript in preparation) indicates that, while **2a** and **2b** satisfy the common features of planar topography required for high affinity binding [9, 10], the hydroxy group distorts the planarity of **2e** through an intramolecular hydrogen bond between the hydroxyl and the N-2 of β-carboline. The same distorted planarity is present in **3e** for the same reason. Nevertheless, an NMR study of the interactions between **2e** and **3e** with human albumin to seek similarities between the indole binding site on albumin and the benzodiazepine receptor in the brain (Delfini *et al*, manuscript in preparation), has revealed that **2e** does not interact whereas **3e** does. This behavior agrees with the observed displacement potency *in vitro* of **3e** and **3f** (table I).

In conclusion, the observed lack of binding capability for **2e** and **2f** could be the effect of both the OH group and the general low lipophilicity of these molecules. The enhancement of receptor affinity induced by fluorine in the 6 position is evidenced by the generally higher concentration in brain (ng/g) than in plasma (ng/ml) for compounds **3a–d**. Their corresponding amino analogues **2** resulted in the same concentrations. The data in table III suggests, however, that the rates of absorption (or bioavailabilities) of the fluorinated compounds **3a–d** are much lower than those of the amino compounds **2a–d**. This fact is in agreement with the higher water solubility of **2a–d**.

Finally, the data in table II highlight the so-called 'inverse agonistic' activity of the β-carboline derivatives **2** and **3** towards DAZ. The outstanding inverse agonist activity of **3b**, in comparison with the well-known β-CCE, is clearly evidenced.

Conclusion

The values found for the above-described β-carboline derivatives prompt further investigation into their pharmacological profile, whose results will be published elsewhere.

Experimental protocols

Chemistry

Melting points were determined in open capillaries in a Büchi apparatus and are uncorrected. Microanalyses were performed by the microanalytical section of our laboratory. The analytical results for pure compounds, indicated by the symbols of the elements (C, H, N) were within ±0.4% of the theoretical values and are not reported in this paper. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian XL 300 or a Bruker AM 500 spectrometer using DMSO-d_6, TFA or D_2O as further specified.

and TMS as an internal standard; all values are reported in ppm (δ), standard abbreviations are used (s, bs, d, m, q, t; at = apparent triplet; dd = doublet of doublets; td = triplet of doublets; ud = unresolved doublet; udd = unresolved doublet of doublets). Only ^{13}C chemical shifts of 5-, 6-, 7- and 8-C, with their J_{CF} coupling constants (Hz) of the fluorinated compounds **3a–f** are given here in order to confirm fluorine incorporation. Mass spectra were recorded on a Hewlett-Packard 5989A quadrupole mass spectrometer (70 eV; MS source 250°C; MS analyzer 100°C) connected to HP 1050 HPLC system through an HP 59980 B Particle/Beam LC/MS interface operating with a 40–60 psi helium inlet pressure and desolvation chamber at 40°C. The compounds were introduced by either direct inlet probe or HPLC injection of methanolic solutions; principal *m/z* peaks, with their relative intensities in brackets are reported here. The purity of compounds was checked by tlc on silica-gel plates with ethyl acetate/methanol 85:15 (v/v) and/or by an HPLC system consisting of Waters 501 + 600 pumps, a Perkin-Elmer LC-235 diode array detector, a Waters 745 integrator, and an RP 18 5μ column; the mobile phase (isocratic) was a 1:1 mixture of 0.5% aqueous ammonium acetate (adjusted to pH 6 with acetic acid) and methanol/acetonitrile 1:1 mixture. Detection wavelengths were 240 nm for the amino compounds **2** and 270 nm for the fluoro derivatives **3**. For the GABA ratio determinations, the ethyl β-carboline-3-carboxylate (β-CCE) was prepared according to literature [17].

6-Nitro-β-carboline-3-carboxylic acid

A suspension of methyl 6-nitro-β-carboline-3-carboxylate **1a** (10.1 g) [1] in aqueous 2% sodium hydroxide (1000 ml) was heated at 60°C in a water bath while rotating in a Büchi rotavapor for 3.5 h, after which time almost complete solution occurred. The red solution obtained was filtered until warm through fluted filter paper, made neutral with glacial acetic acid (35 ml; the mixture turned yellow) and the resulting precipitate was collected, washed with water and dried at 60°C under reduced pressure. Yield: 8.65 g (90.3%), yellow powder (fig 1, formula **1**, R = H). For analytical purposes it was crystallized from dimethylsulfoxide/methanol, mp 312–313°C. Anal $C_{12}H_7N_3O_4$ (C, H, N). ^{1}H-NMR (TFA): 7.97 (1H, d, 8-H); 8.73 (1H, dd, 7-H); 9.43 (3H, broad, 1-H, 4-H, 5-H). MS: 258 (M^+ + 1, 4); 257 (M^+, 3); 240 (M^+ – 17, 2); 214 (13); 213 ($M^+ - CO_2$, 66); 197 (5); 183 (20); 167 (100); 155 (30); 140 (37); 113 (6); 63 (5); 44 (CO_2, 9).

n-Propyl-6-nitro-β-carboline-3-carboxylate ***1c***

A mixture of 6-nitro-β-carboline-3-carboxylic acid (5 g), *n*-propyl alcohol (200 ml) and conc sulfuric acid (d = 1.84, 15 ml) was refluxed under stirring for 72 h. The resulting mixture was then poured onto an ice-cooled solution of sodium carbonate (40 g) in water (100 ml). The yellowish precipitate obtained was collected, washed with water and dried at reduced pressure at 50°C. The crude product (4.7 g, 81%) was crystallized from hot (90°C) dimethylformamide (250 ml) in fine needles, mp 316–317°C (dec). Anal $C_{15}H_{13}N_3O_4$ (C, H, N). ^{1}H-NMR (DMSO-d_6): 1.15 (3H, t, -CH_3); 1.92 (2H, m, -CH_2-CH_3); 4.43 (2H, t, $COOCH_2$-); 7.94 (1H, d, 8-H); 8.58 (1H, dd, 7-H); 9.21 (1H, s, 4-H); 9.31 (1H, s, 1-H); 9.65 (1H, d, 5-H); 12.8 (1H, broad, N_9-H). MS: 300 (M^+ + 1, 29); 299 (M^+, 4); 283 (3); 270 ($M^+ - C_2H_5$, 12); 269 (13); 241(8); 240 (M^+ – C_3H_7O, 8); 214 (19); 213 ($M^+ - COOC_3H_7 + H^+$, 100); 212 (18); 197 (4); 183 (38); 182 (12); 167 (18); 166 (21); 155 (9); 140 (8); 139 (12); 113 (4); 88 (34).

n-Butyl-6-nitro-β-carboline-3-carboxylate ***1d***

This ester was prepared in a similar manner by allowing a mixture of 6-nitro-β-carboline-3-carboxylic acid (3.5 g), *n*-butyl alcohol (250 ml) and conc sulfuric acid (10 ml) to react under vigorous stirring at no more than 80°C for 72 h. The mixture was then poured onto an ice-cooled solution of sodium carbonate (28 g) in water. The crude product obtained (2.1 g, 49.3%) was then crystallized from hot (90°C) dimethylformamide (50 ml) in fine whitish needles mp 317–318°C (dec). Anal $C_{16}H_{15}N_3O_4$ (C, H, N). ^{1}H-NMR (DMSO-d_6): 0.94 (3H, t, -CH_3); 1.45 (2H, m, -CH_2CH_3); 1.74 (2H, m, $COOCH_2$-CH_2-); 4.32 (2H, at, -$COOCH_2$-); 7.75 (1H, d, 8-H); 8.39 (1H, dd, 7-H); 9.02 (1H, s, 4-H); 9.08 (1H, s, 1-H); 9.43 (1H, d, 5-H); 12.6 (1H, broad, N_9-H). MS: 314 (M^+ + 1, 8); 313 (M^+, 3); 283 (4); 269 (4); 241 (12); 240 ($M^+ - C_4H_9O$, 11); 214 (15); 213 ($M^+ - COOC_4H_9 + H^+$, 100); 212 (20); 197 (4); 183 (14); 167 (16); 166 (19); 139 (10); 113 (2); 88 (3).

2-Hydroxyethyl-6-nitro-β-carboline-3-carboxylate ***1e***

A mixture of 6-nitro-β-carboline-3-carboxylic acid (1.5 g) ethylene glycol (60 ml) and conc sulfuric acid (d = 1.84, 4 ml) was heated under stirring at no more than 70°C for 48 h, in which time complete dissolution occurred. The solution was then poured onto an ice-cooled solution of sodium carbonate (16 g) in water (50 ml). The resulting gelatinous brownish precipitate was collected, washed with water and dried at reduced pressure at 50°C. The crude product was crystallized by dissolution in dimethylsulfoxide (40 ml) at 90°C and addition of hot water (15 ml). Yield 1.2 g (68.6%), yellow needles, mp 288–289°C (dec). Anal $C_{14}H_{11}N_3O_5$ (C, H, N). ^{1}H-NMR (DMSO-d_6): 3.88 (2H, m, CH_2OH); 4.45 (2H, m, -$COOCH_2$); 5.00 (1H, broad, OH); 7.70 (1H, d, 8-H); 8.27 (1H, dd, 7-H); 8.88 (2H, s, 1-H and 4-H); 9.15 (1H, d, 5-H); 12.45 (1H, bs, N_9-H). MS: 302 (M^+ + 1, 5); 301 (M^+, 1); 284 (M^+ – 17, 2); 257 ($M^+ - C_2H_4O$, 3); 240 ($M^+ - OC_2H_4OH$, 14); 239 (12); 226 (4); 214 (14); 213 ($M^+ - COOCH_2CH_2OH + H^+$, 100); 212 (68); 193 (9); 183 (10); 167 (43); 155 (10); 140 (13); 113 (3); 45 (7); 44 (4).

2,3-Dihydroxypropyl-6-nitro-β-carboline-3-carboxylate ***1f***

This ester was prepared analogously by allowing a mixture of 6-nitro-β-carboline-3-carboxylic acid (3.9 g), anhydrous glycerol (200 ml) and conc sulfuric acid (20 ml) to react for 48 h at no more than 70°C, and pouring the resulting solution onto an ice-cooled solution of sodium carbonate (80 g) in water (200 ml). The dried crude product obtained was crystallized by dissolution in dimethylsulfoxide (50 ml) at 90°C and addition of hot water until turbidity occurred (about 40 ml). Yield 4.8 g (95.6%), yellow needles, mp 294°C (dec). Anal $C_{15}H_{13}N_3O_6$ (C, H, N). ^{1}H-NMR (DMSO-d_6): 3.62 (2H, d, CH_2OH); 3.98 (1H, m, -$CHOH$); 4.43 (4H, m, -$COOCH_2$ + 2OH); 7.73 (1H, d, 8-H); 8.40 (1H, dd, 7-H); 9.00 (2H, s, 1H and 4-H); 9.27 (1H, d, 5-H); 12.50 (1H, bs, N_9-H). MS: 332 (M^+ + 1, 1); 258 (332-$C_3H_6O_2$, 5); 240 ($M^+ - OC_3H_7O_2$, 12); 213 ($M^+ - COOC_3H_7O_2 + H^+$, 98); 197 (6); 183 (23); 167 (100); 155 (30); 140 (40); 113 (7); 75 (5); 74 (5); 61 (29); 44 (41); 43 (63).

6-Amino-β-carboline-3-carboxylic acid esters ***2a–f****. General procedure*

To a warm solution (50°C) of each of the nitro compounds **1a–f** (1 g) in 85% formic acid (20 ml), 6 N hydrochloric acid (20 ml) and iron powder (1 g) were added and the resulting suspension allowed to react under stirring for about 10 min. The warm suspension was then filtered through a G-4 sintered glass funnel, and the undissolved material rinsed with small volumes of warm water. The insoluble matter on the filter, if any, was then collected and submitted to a repeated reduction with iron/hydrochloric acid and filtered as described. The hydrochloric solutions collected were then allowed to cool at

room temperature and kept overnight in a refrigerator. The crystals obtained were filtered, rinsed with a small volume ice-cooled 6 N hydrochloric acid, and dried under reduced pressure over phosphoric anhydride at room temperature. Concentration to about 1/3 volume of the mother liquor under reduced pressure at no more than 50°C gave further product. For analytical purposes, the so-obtained products were purified by dissolution in the smallest possible volume of warm water and addition of about the same volume conc hydrochloric acid.

Methyl-6-amino-β-carboline-3-carboxylate dihydrochloride **2a**. This compound was obtained in 77.6% yield instead of the described 60% resulting from another synthetic route [1].

Ethyl-6-amino-β-carboline-3-carboxylate dihydrochloride **2b**. This compound was obtained in 78.3% yield instead of the reported 60% [1].

n-Propyl-6-amino-β-carboline-3-carboxylate dihydrochloride **2c**. This compound was obtained in 75.1% yield, mp > 320°C. Anal $C_{15}H_{15}N_3O_2 \cdot 2HCl \cdot H_2O$ (C, H, N, Cl). ^{1}H-NMR (DMSO-d_6): 1.13 (3H, t, CH_3); 1.95 (2H, m, -CH_2-CH_3); 4.53 (2H, m, $COOCH_2$-); 7.84 (1H, dd, 7-H); 7.93 (1H, d, 8-H); 8.42 (1H, d, 5-H); 9.04 (1H, s, 4-H); 9.11 (1H, s, 1-H); 13.2 (1H, s, N_9-H). MS: 270 (M^+ of the free base + 1, 61); 269 (M^+, 39); 210 ($M^+ - OC_3H_7$, 27); 199 (5); 183 ($M^+ - COOC_3H_7$ + H^+, 100); 155 (25); 128 (9); 65 (22); 63 (12).

n-Butyl-6-amino-β-carboline-3-carboxylate dihydrochloride **2d**. This compound was obtained in 72.5% yield, mp 315–316°C (dec). Anal $C_{16}H_{17}N_3O_2 \cdot 2HCl \cdot H_2O$ (C, H, N, Cl). ^{1}H-NMR (DMSO-d_6): 0.94 (3H, t, CH_3); 1.47 and 1.77 (4H, two m, -CH_2-CH_2-); 4.42 (2H, m, COO-CH_2-); 7.80 (1H, udd, 7-H); 7.91 (1H, d, 8-H); 8.60 (1H, ud, 5-H); 9.23 (1H, s, 4-H); 9.30 (1H, s, 1-H); 13.40 (1H, s, N_9-H). MS: 284 (M^+ of the free base + H^+, 58); 283 (M^+, 33); 210 ($M^+ - OC_4H_9$, 25); 199 (5); 183 ($M^+ - COOC_4H_9$ + H^+, 100); 155 (25); 128 (8); 65 (15); 63 (8).

2-Hydroxyethyl-6-amino-β-carboline-3-carboxylate dihydrochloride **2e**. This was obtained in 87.7% yield, mp > 320°C (dec). Anal $C_{14}H_{13}N_3O_3 \cdot 2HCl \cdot 1.5H_2O$ (C, H, N). ^{1}H-NMR (D_2O): 3.98 (2H, m, CH_2OH); 4.49 (2H, m, -$COOCH_2$); 7.52 (1H, d, 8-H), 7.58 (1H, dd, 7-H); 8.04 (1H, d, 5-H); 8.60 (1H, s, 4-H); 8.69 (1H, s, 1-H). MS: 271 (M^+ of the free base, 35); 210 ($M^+ - C_2H_5O_2$, 14); 183 (210 – CO + H^+, 100); 155 (22); 128 (8); 91 (14); 36 (HCl, 31).

2,3-Dihydroxypropyl-6-amino-β-carboline-3-carboxylate dihydrochloride **2f**. This compound was obtained in 88.5% yield, mp > 320°C (dec). Anal $C_{15}H_{15}N_3O_4 \cdot 2HCl \cdot H_2O$ (C, H, N). ^{1}H-NMR (D_2O): 3.71 and 3.75 (2H, two q, CH_2OH); 4.14 (1H, m, -CHOH); 4.43 and 4.52 (2H, two q, -$COOCH_2$-); 7.64 (2H, d, 7-H and 8-H); 8.15 (1H, d, 5-H); 8.75 (1H, s, 4-H), 8.82 (1H, s, 1-H). MS 301 (M^+ of the free base, 4); 267 (2); 227 ($M^+ - C_3H_7O_2$ + H^+, 3); 210 ($M^+ - C_3H_7O_3$, 6); 183 (210 – CO + H^+, 31); 182 (18); 155 (8); 133 (3); 91 (5); 61 (27); 57 (10); 44 (67); 43 (100); 36 (HCl, 45).

6-Fluoro-β-carboline-3-carboxylic acid esters **3a–f**. *General procedure*

Each compound **2a–f** (1 g) was dissolved or suspended in water (50 ml) and the mixture was cooled under stirring at 0–5°C in a ice-bath. Freshly prepared 10% aqueous sodium nitrite (2 ml) was then added dropwise followed by a few drops conc hydrochloric acid, and the mixture was allowed to react at the same temperature for 30 min. This sodium nitrite addition was repeated once more in the same conditions. The mixture was allowed to react 30 min further, and then filtered through a sintered glass funnel from undissolved material at 5°C. Powdered lithium borotetrafluoride (1 g) was then added to the clear solution and the mixture stirred for 30 min at 0–5°C. The precipitate of diazonium fluoroborate obtained was collected on a sintered glass funnel, rinsed with ice-cooled 5% aqueous lithium fluoroborate solution, and then with methanol, diethylether, and finally dried under reduced pressure. To the resulting solid (1.0–1.1 g) silica-gel Merck for column chromatography (3–4 g) was added and the mixture finely ground in a mortar to give a homogeneous light-brown powder. This was heated in a oil bath under reduced pressure at 160–170°C for no more than 5 min to give a dark-greenish freely flowing powder, which was poured onto a previously prepared silica-gel column (60 g, h = 42 cm, id 2.5 cm). Elutions were performed with 5% methanol (v/v) in ethyl acetate for **3a** and **3b**, with 10% isopropanol in toluene for **3c** and **3d**, and with 10% methanol in ethyl acetate for **3e** and **3f**. Fractions of about 10–12 ml volume were collected. Thin-layer chromatography on silica-gel plates with ethyl acetate/methanol 85–15 (v/v) allowed the fractions containing the fluorinated compounds to be separated (**3a**, $R_f \sim 0.4$; **3b**, $R_f \sim 0.45$; **3c**, $R_f \sim 0.5$; **3d**, $R_f \sim 0.55$; **3e**, $R_f \sim 0.25$; **3f**, $R_f \sim 0.2$). Evaporation of the solvent led to the isolation of almost pure compounds, which were further purified by crystallization from hot (90°C) *n*-butanol or toluene (for **3c** and **3d**).

It was possible to isolate the fluorinated compounds **3a–f** with satisfactory purity by direct extraction with methanol of the solid resulting from the heating at 160°C.

Methyl-6-fluoro-β-carboline-3-carboxylate **3a**. This compound resulted in 25% yield, mp 262–263°C (dec). Anal $C_{13}H_9FN_2O_2$ (C, H, N). ^{1}H-NMR (DMSO-d_6): 3.91 (3H, s, $COOCH_3$), 7.47 (1H, td, 7-H); 7.68 (1H, dd, 8-H); 8.28 (1H, dd, 5-H); 8.95 (1H, s, 4-H); 8.98 (1H, s, 1-H); 12.00 (1H, s, N_9-H). ^{13}C-NMR (DMSO-d_6): 107.65 (5-C, J_{CF} = 24.40); 113.66 (8-C, J_{CF} = 9.40); 116.85 (7-C, J_{CF} = 25.85); 158.48 (6-C, J_{CF} = 235.24). MS: 244 (M^+, 31); 226 (2); 213 ($M^+ - OCH_3$, 5); 186 ($M^+ - COOCH_3$ + H^+, 100); 185 (36); 158 (24); 131 (5); 107 (4); 92 (7).

Ethyl-6-fluoro-β-carboline-3-carboxylate **3b**. This compound was obtained in 25.4% yield, mp 296–298°C. Anal $C_{14}H_{11}FN_2O_2$ (C, H, N). ^{1}H-NMR (DMSO-d_6): 1.40 (3H, t, CH_2-CH_3); 4.40 (2H, q, CH_2-CH_3); 7.46 (1H, td, 7-H); 7.67 (1H, dd, 8-H); 8.28 (1H, dd, 5-H); 8.94 (1H, s, 4-H); 8.98 (1H, s, 1-H); 12.12 (1H, s, N_9-H). ^{13}C-NMR (DMSO-d_6): 107.67 (5-C, J_{CF} = 24.45); 113.61 (8-C, J_{CF} = 9.00); 116.85 (7-C, J_{CF} = 26.10); 156.93 (6-C, J_{CF} = 238.50). MS: 258 (M^+, 16); 214 ($M^+ - OC_2H_5$ + H^+, 7); 186 ($M^+ - COOC_2H_5$ + H^+, 100); 158 (20); 113 (7); 111 (7); 99 (12); 97 (12); 85 (20); 71 (26); 69 (16).

n-Propyl-6-fluoro-β-carboline-3-carboxylate **3c**. This compound was obtained in 31.8% yield, mp 267–268°C. Anal $C_{15}H_{13}FN_2O_2$ (C, H, N). ^{1}H-NMR (DMSO-d_6): 0.98 (3H, t, -CH_3); 1.76 (2H, m, -CH_2-CH_3); 4.27 (2H, t, -$COOCH_2$-); 7.45 (1H, td, 7-H); 7.66 (1H, dd, 8-H); 8.29 (1H, dd, 5-H); 8.94 (1H, s, 4-H); 8.98 (1H, s, 1-H); 12.17 (1H, broad, N_9-H). ^{13}C-NMR (DMSO-d_6): 107.74 (5-C, J_{CF} = 23.85); 113.64 (8-C, J_{CF} = 9.10); 116.89 (7-C, J_{CF} = 25.45); 156.97 (6-C, J_{CF} = 233.55). MS: 273 (M^+ + H^+, 34); 272 (M^+, 11); 213 ($M^+ - OC_3H_7$, 18); 186 ($M^+ - COOC_3H_7$ + H^+, 100); 158 (25); 131(4).

n-Butyl-6-fluoro-β-carboline-3-carboxylate ***3d***. This compound was obtained in 29.0% yield, mp 253–254°C. Anal $C_{16}H_{15}FN_2O_2$ (C, H, N). ^{1}H-NMR (DMSO-d_6): 0.94 (3H, t, -CH_3); 1.44 (2H, m, -CH_2-CH_3); 1.73 (2H, m, -$COOCH_2$-CH_2-); 4.31 (2H, t, -$COOCH_2$-); 7.45 (1H, td, 7-H); 7.65 (1H, td, 8-H); 8.30 (1H, dd, 5-H); 8.94 (1H, s, 4-H); 8.98 (1H, s, 1-H); 12.11 (1H, s, N_9-H). ^{13}C-NMR (DMSO-d_6): 107.66 (5-C, J_{CF} = 23.85); 113.59 (8-C, J_{CF} = 9.10); 116.82 (7-C, J_{CF} = 25.90); 156.96 (6-C, J_{CF} = 233.10). MS: 287 (M^+ + H^+, 30); 286 (M^+, 7); 213 (M^+ – OC_4H_9, 22); 186 (M^+ – $COOC_4H_9$ + H^+, 100); 158 (24); 131 (4).

2-Hydroxyethyl-6-fluoro-β-carboline-3-carboxylate ***3e***. This compound was obtained in 12.5% yield, mp 280–282°C (dec). Anal $C_{14}H_{11}FN_2O_3$•H_2O (C, H, N). ^{1}H-NMR (DMSO-d_6): 3.78 (2H, m, -CH_2OH); 4.37 (2H, m, -$COOCH_2$-); 4.93 (1H, broad, OH); 7.47 (1H, td, 7-H); 7.69 (1H, dd, 8-H); 8.26 (1H, dd, 5-H); 8.97 (1H, s, 4-H); 8.99 (1H, s, 1-H); 12.09 (1H, s, N_9-H). ^{13}C-NMR (DMSO-d_6): 107.83 (5-C, J_{CF} = 23.72); 113.96 (8-C, J_{CF} = 9.22); 117.15 (7-C, J_{CF} = 25.71); 157.26 (6-C, J_{CF} = 233.41). MS: 274 (M^+, 15); 244 (M^+ – HCHO, 2); 214 (9); 213 (M^+ – $C_2H_5O_2$, 14); 186 (M^+ – $COOCH_2$ – CH_2OH + H^+, 100); 185 (50); 158 (23); 131 (4); 107 (2); 92 (4); 61 (5); 60 (25).

2,3-Dihydroxypropyl-6-fluoro-β-carboline-3-carboxylate ***3f***. This compound was prepared in 12.3% yield, mp 254–255°C. Anal $C_{15}H_{13}FN_2O_4$•$1/2H_2O$ (C, H, N). ^{1}H-NMR (DMSO-d_6): 3.47 and 3.66 (2H, two bs, -CH_2OH); 4.02 (1H, broad, -CHOH); 4.40 and 4.52 (2H, two dd, -$COOCH_2$-); 4.86 (1H, broad, -CH_2OH); 5.15 (1H, ud, -CH-OH); 7.62 (1H, td, 7-H); 7.83 (1H, dd, 8-H); 8.42 (1H, dd, 5-H); 9.12 (1H, s, 4-H); 9.15 (1H, s, 1-H); 12.2 (1H, s, N_9-H). ^{13}C-NMR (DMSO-d_6): 107.50 (5-C, J_{CF} = 24.27); 113.63 (8-C, J_{CF} = 8.93); 116.23 (7-C, J_{CF} = 25.78); 156.93 (6-C, J_{CF} = 234.79). MS: 304 (M^+, 3); 286 (M^+ – 18, 3); 244 (M^+ – $C_2H_4O_2$, 29); 231 (13); 213 (M^+ – $C_3H_7O_3$, 17); 186 (M^+ – $COOCH_2CH(OH)CH_2OH$ + H^+, 100); 163 (5); 129 (6); 97 (5); 61 (88); 60 (98).

Pharmacology

Materials and methods

In most experiments (see table I) the synaptosomal membranes of rat cerebral cortex were prepared according to Mohler and Okada [20]. Cerebral cortex of rats was homogenized in 22 volumes of 0.32 M sucrose at 0°C. The homogenate was centrifuged for 10 min at 1000 *g* at 4°C and the supernatant was recentrifuged for 10 min at 20 000 *g*. The pellet was suspended in 22 volumes of 25 mM of sodium phosphate buffer (pH 7.4) and centrifuged. Finally, the pellet was suspended in 10 volumes of the same buffer and used in the binding assay.

In some experiments (see table II), in order to determine the influence of endogenous GABA on the binding, the homogenization was performed with 10 volumes of the buffer containing protease inhibitors [21] and the 20 000 *g* pellet was frozen and washed using a procedure previously described for removing endogenous GABA from rat cerebral cortex [22].

Binding determination (RRA)

In most experiments the receptor binding assay was performed as follows: 500 µl of the receptor preparation was incubated for 20 min at room temperature with 100 µl [^{3}H]flunitrazepam 1.6 x 10^{-9} M (79.6 Ci/mmol) (NEN) and 100 µl of the test compounds (dissolved in 5% aq DMSO) in 25 mM sodium phosphate buffer pH 7.4 (total vol 1 ml). In the experiments without endogenous GABA, 700 µl of the receptor preparation were used instead. The incubations were stopped by adding 4 ml of cold buffer followed by rapid filtration through glass-fiber filter disks (Whatman GF/B). The samples were subsequently washed 3 times with 4.5 ml of the same buffer and placed into scintillation vials; 10 ml Filter-Count (Packard) liquid scintillation cocktail was then added to each vial and counting was carried out by a scintillation spectrometer (Packard Tri-Carb 300 C).

Nonspecific binding was defined as non-displaceable binding in the presence of 10^{-4} M DAZ, and specific binding as the difference between total and nonspecific binding.

Blank experiments were carried out to determine the effect of the solvent (5% aq DMSO) on the binding.

The concentration of the test compounds that inhibited [^{3}H]flunitrazepam binding by 50% (IC_{50}) was determined by log-probit analysis with six concentrations of the displacers, each performed in duplicate (table I) or triplicate (table II). The IC_{50} values obtained were used to calculate apparent inhibition constants (K_i) by the method of Cheng and Prusoff [23], by the following equation: $K_i = IC_{50} / (1 + S/K_D)$ were S represents the concentration of the ligand used and K_D is its receptor dissociation constant, obtained by Scatchard analysis [24]. The K_D value for [^{3}H]flunitrazepam was 2.8 x 10^{-9} M. The IC_{50} determinations for GABA ratio values were carried out in the absence and presence of 10 µM GABA.

Blood–brain barrier translocation

Male albino Wistar rats (body weight 180–200 g) were used. The test compounds suspended in 5% gum arabic were orally administered at the dose of 5 mg/kg. The animals were sacrificed by decapitation 1 h after the dose and plasma and brain were collected.

The homogenate of the brain was diluted 1:4, and the plasma was diluted with an equal volume of saline (0.9% sodium chloride solution) and the proteins were precipitated by keeping them at 65°C for 45 min. After a 10 min centrifugation at 4000 *g*, the supernatant was separated and stored at –20°C for the assay. The assay was performed as follows: 500 µl of the receptor preparation was incubated for 20 min at room temperature with 100 µl of [^{3}H]flunitrazepam 1.6 x 10^{-9} M (79.6 Ci/mmol) (NEN) and 100 µl plasma or brain preparation in 25 mM sodium phosphate buffer pH 4.7 (total volume 1 ml). The incubation was then stopped by addition of 4 ml cold buffer and work-up continued according to the above procedure described under *Binding determination*.

Acknowledgments

We thank L Turchetto for mass spectra, R Piergallini for microanalyses and F Ponti for technical assistance. This paper has been supported in part by a grant (91.01776.PF72) of the Consiglio Nazionale delle Ricerche.

References

1 Settimj G, Del Giudice MR, Ferretti R, Gatta F (1988) *J Heterocycl Chem* 25, 1391–1397

2 Del Giudice MR, Gatta F, Settimj G (1990) *J Heterocycl Chem* 27, 967–973

3 Braestrup C, Rodbard D, Pert CB (1979) *Nature (Lond)* 277, 315–317
4 Braestrup C, Schmiechen R, Neef G, Nielsen M, Petersen EN (1982) *Science* 216, 1241–1243
5 Cain M, Weber RW, Guzman F *et al* (1982) *J Med Chem* 25, 1081–1091
6 Polc P, Bonetti EP, Schattner R, Haefely W (1982) *Naunyn Schmiedeberg's Arch Pharmacol* 321, 260–264
7 Schering AG (1981) Jpn Kokai Tokhyo Koho 81-43, 283; *Chem Abstr* (1981) 95: P115508a
8 Biere H, Schering AG (1984) Ger Offen DE 3, 240, 511 (3.5.1984); *Chem Abstr* (1984) 101, P72711j
9 Kirk KL, Cohen LA (1973) *J Am Chem Soc* 95, 4619–4624
10 Allen MS, Hagen TJ, Trudell ML, Codding PW, Skolnick P, Cook JM (1988) *J Med Chem* 31, 1854–1861
11 Trudell ML, Lifer SL, Tan YC *et al* (1990) *J Med Chem* 33, 2412–2420
12 Roe A (1949) *Organic Reactions*, Vol 5, J Wiley, New York, USA, 193–228
13 Mueller E (1962) *Methoden der Organischen Chemie*, Vol 5/3, Thieme Verlag, Stuttgart, Germany, 213–245
14 Suschitzky H (1965) *In: Advances in Fluorine Chemistry*, Vol 4, Butterworths, London, UK, 1–30
15 Kirk KL, Nagai W, Cohen LA (1973) *J Am Chem Soc* 95, 8389–8392
16 Stephens DN, Kehr W, Schneider HH, Schmiechen R (1984) *Neurosci Lett* 47, 333–338
17 Lippke KP, Schunack WG, Wenning W, Mueller WE (1983) *J Med Chem* 26, 499–503
18 Peña C, Medina JH, Novas ML, Paladini AC, De Robertis E (1986) *Proc Natl Acad Sci USA* 83, 4952–4956
19 Novas ML, Wolfman C, Medina JH, De Robertis E (1988) *Pharmacol Biochem Behav* 30, 331–336
20 Mohler H, Okada T (1977) *Science* 198, 849–851
21 Martini C, Lucacchini A, Ronca G, Hrelia S, Rossi CA (1982) *J Neurochem* 38, 15–19
22 Martini C, Rigacci T, Lucacchini A (1983) *J Neurochem* 41, 1183–1185
23 Cheng YC, Prusoff WH (1973) *Biochem Pharmacol* 22, 3099–3108
24 Scatchard G (1949) *Ann NY Acad Sci* 51, 660-672