Anticonvulsant Activity of New GABA Prodrugs

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Abstract: 4-(3,4-Dihydro-2,4-dioxo-2*H*-1,3-benzoxazin-3-yl)-butyric acid (**7**) and its ethyl (**6**), two potential y-aminobutyric acid (GABA) prodrugs, were synthesized and studied to determine their stability in aqueous buffer and their susceptibility to undergo enzymatic hydrolysis *in vitro* (mouse plasma). Both compounds were fairly stable in aqueous media, ($t_{1/2}$ = 68.2 h and 25.7 h, respectively). The 3,4-dihydro-2,4-dioxo-2 H-I,3-benzoxazine ring underwent enzymatic hydrolysis ($t_{1/2}$ =5.8 h) in compound **7**, whereas in compound **6** it seemed not to be opened by mouse plasma esterases within the observation lime (3 h). Both compounds were tested for their central nervous system activity by using both anticonvulsive and behavioral tests. The anticonvulsive study was performed using the convulsive agent pentetrazole (PTX) and bicuculline. The anticonvulsive study indicated that compound both compounds **6** and **7** (10, 20 and 40 mg/kg, i.p.), injected 60 min before PTX (75 mg/kg, i.p.) or bicuculline (10 μg/intracerebroventricular (i.c.v.)/mouse) induced a dose-dependent and significant reduction of the convulsive activity of PTZ and bicuculline whereas it was ineffective if injected immediately before the convulsive agent.

Both compounds **6** and **7** (10, 20 and 40 mg/kg, i.p.) did not significantly modify animal behavior or the nociceptive threshold of the animals. However, in PTZ- and bicuculline- treated mice, compound **7** showed significant activity, compared to compound **6**, because it was active at relatively low doses. The behavior elements considered were locomotor activity, motor coordination, catalepsy, behavior and antinociception. The results of the behavoral study indicate that these new GABA mimetic drugs did not modify the animal behavior. Our data indicate that these new GABA mimetic drug possesses good anticonvulsive activity without altering the animalbehavior and their ability to block bicuculline-induced convulsions suggests that they could be a $GABA_A$ mimetic drug. Furthermore, since these compounds are able to act after systemic administration, our data suggest that these new GABA mimetic drug cross the blood–brain barrier.

Key Words: γ-Aminobutyric acid (GABA), anticonvulsant, systemic administration.

INTRODUCTION

γ-Aminobutyric acid (GABA) is a neurotransmitter which may be useful in the treatment of Huntington's disease [I], epilepsy [2] and Parkinson's disease [3]. Unfortunately, peripheral adminstration of GABA is not effective because under normal conditions it crosses the "blood brain barrier" (BBB) very poorly, presumably as a result of its low lipophilicity [4]. The brain penetration index (BPI) for GABA was found to be 1% regardless of the mode of administration and the dose [5].

Among the different strategies [6] used to increase the brain uptake of GABA, the prodrug approach is one of the most promising. Different GABA prodrugs bave been reported in the literature, such as methyl ester hydrochloride [7], various aliphatic, lipid and steroid esters [5, 8], some glycerol lipid derivatives [9-11], isonicotinoyl and nicotinoyl arnides [12], pivaloyl and benzoyl amides [13], and a dihidropyridine derivative [14].

In this paper we describe the synthesis, the chemical and enzymatic hydrolysis, and the pharmacological evaluation of 4-(3,4-dihydro-2,4-dioxo-2 *H*-1,3-benzoxazin-3-yl)-butyric acid (**7**) and of its ethyl ester **6** (Scheme **1**) as new GABA prodrugs. The choice of 3,4-dihydro2,4-dioxo-2 *H*-1,3-benzoxazine as pro-moiey to obtain GABA prodrugs was based on the following considerations: this structure should notably enhance the lipophilicity of the parent drug by masking its amino group and the hydrolytic opening of the N-substituted 3,4-dihydro2,4-dioxo-2 *H*-1,3-benzoxazine ring should generate [15,16], by chemical and enzymatic hydrolysis, the corresponding *N*-GABA salicylic amide (**5** or **9**) which, like other analogous *N*-GABA amide prodrugs [12,13], should possess a good susceptibility to be converted back to GABA by brain amidases. According to these considerations, we expected a considerable GABA release in cerebral tissue after intraperitoneal administration of compounds **6** and **7** and consequently a good anticonvulsant activity.

Therefore, this study was carried out to better understand the pharmacological profile of new GABA derivative drugs by performing a behavioral study and using a non specific (PTZ) and a specific convulsant agent, such as bicuculline (a well known $GABA_A$ receptor antagonist), to verify whether the possible anticonvulsant activity was related to the involvement of a specific GABA receptor. Furthermore, a comparative study was performed with pentobarbital (a clinically effective anticonvulsant agent) and muscimol (a well known $GABA_A$ receptor agonist) in order to assess the relative potency of the GABA derivatives drugs.

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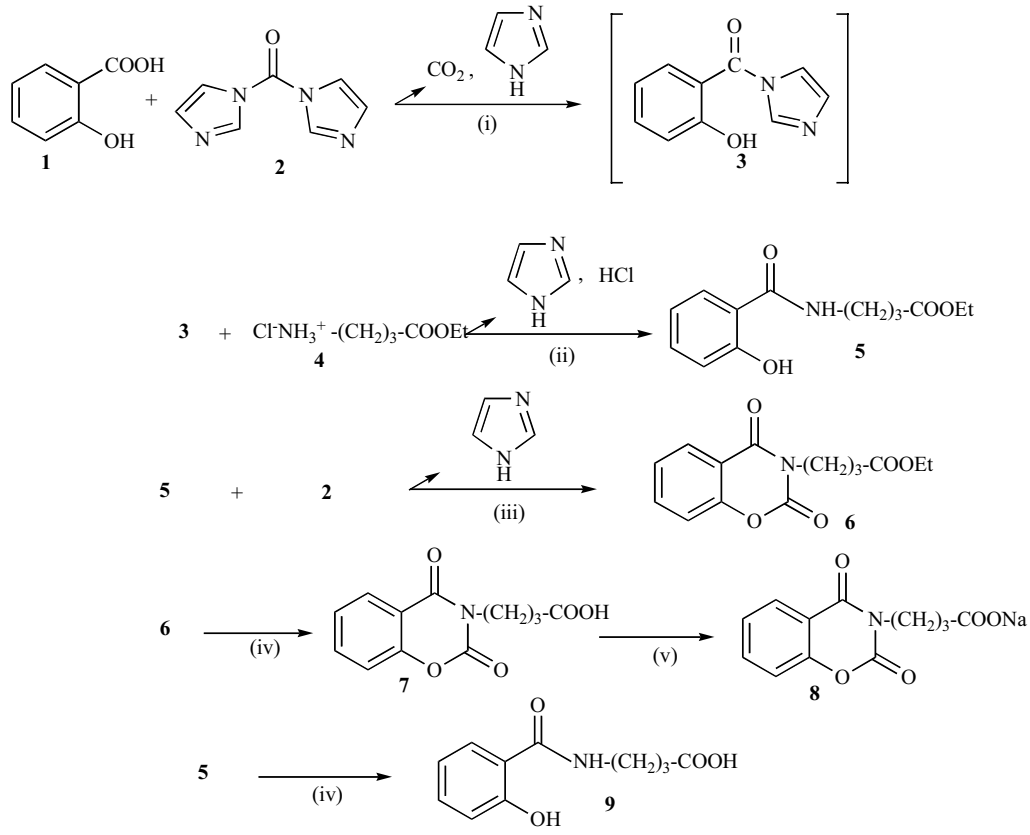


Scheme 1.

MATERIALS AND METHODS

Chemistry

Apparatus

M.p.'s were determined on a Kofler hot-stage microscope and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker WM 250 and/or on a Bruker AMX 500. Chemical shifts are reported in parts per million (δ) relative to the residual solvent signal ($CDCl_3$: δ_H 7.27, d_C 77.0; CD_3OD: bH 3.34, δ_C 49.0; D_2O: δ_H 4.60). Elemental analyses were performed on a Carlo Erba mod 1108 elemental analyzer and were within ±0.4% of theoretical values. FT-IR spectra (KBr pellets) were recorded on a Bruker IFS-48 spectrometer. MS data were obtained with a Fisons TRIO-2000 apparatus, by electron impact at 70 eV. The HPLC consisted of a Waters model 600 pump with a model 486 UV-VIS detector, an automatic sample injection module (Wisp model 712), a Waters C_{18} μ Bondapack 4.6 mm x 30 cm reverse phase collumn, and a NEC Power Mate SX computer.

The progress of all reactions was monitored by TLC on Whatman K_6F glass plates. The TLC plates were developed using diethyl ether/hexane (7:3) (synthesis of **6**) or $CHCl_3$/CH_3OH (9: 1) (synthesis of **7**), and were viewed under UV light (254 nm) or using I_2 vapors. For CC, Merck silica gel (70-230 mesh) was used.

Chemicals

Salicylic acid (**1**) was obtained from Carlo Erba (Italy), 1,1-carbonyldiimidazole (**2**) and ethyl 4-aminobutyrate hydrochloride (**4**) were purchased from Aldrich Chimica (Italy). LC grade CH_3OH and H_2O were used in HPLC procedures and were obtained from Carlo Erba. All other chemicals were of reagent grade.

Synthesis of ethyl 4-(3,4-dihydro-2,4-dioxo-2 H-1,3-benzoxazin-3-yl)butyrate (6)

Salicylic acid (**1**, 4.1 g, 30 mmol) was dissolved in 100 ml of dry ($CaCl_2$) alcohol-free $CHCl_3$. 1,1-Carbonyldiimidazole (**2**, 4.8 g, 30 mmol) was added gradually and the solution mixture was stirred for 20 min at room temperature until effervescence ceased (Scheme **1**). The expected [17] intermediate imidazolide **3** was not isolated, but to the reaction mixture ethyl 4-aminobutyrate hydrochloride (**4**, 7.7 g, 46 mmol) was added. The solution mixture was stirred for 12 h at room temperature, giving the correspondent salicylamide **5**: Rf = 0.56. ^{1}H NMR: 1.12 (3 H, t, J = 6.5 Hz, -0-CH_2-CH_3), 1.93 (2 H, quintet, J = 5.9 Hz, 3-CH_2), 2.39 (2 H, t, J = 5.9 Hz, 2-CH_2), 3.42 (2 H, m, 4-CH_2), 4.06 (2 H, q, J = 6.5 Hz, -0-CH_2-CH_3), 6.77 (1 H, t, J = 8.3 Hz, H-5'), 6.91 (1 H, d, J = 8.3 Hz, H-5'), 7.04 (1 H, broad, NH), 7.29 (1 H, t, J = 8.3 Hz, H4'), 7.34 (1 H, d, J = 8.3 Hz, H-6'). ^{13}C NMR: 14.1 (0-CH_2-CH_3), 24.0 (3-CH_2), 32.2 (2-CH_2), 39.5 (4-CH_2), 60.8 (0-CH_2-CH_3), 114.4 (C-1), 118.5 (2C, C-3' and C-5') 125.9 (C-6'), 133.8 (C4'), 161.6 (C-2'), 170.4 (CONH), 173.3 (COOEt).

$C_{13}H_{16}NO_4$

The chemical characterization of **5** was effected isolating it from a little amount of the reaction mixture, that was

chromatographed on a silica gel column, eluting with a mixture of diethyl ether/hexane (6:4), as well as some polymeric byproducts (Rf = 0.4 and less, spectral data not reported) and traces of the desired compound **6** (Rf = 0.72). To complete the cyclization, a further amount of 1-1-carbonyldiimidazole (4.8 g, 30 mmol) was added gradually. After 48 h all the salicylamide **5** had been converted into **6**: the reaction mixture was evaporated in vacuo, and the residue was extracted three times with diethyl ether (3 x 50 ml). The organic solution was washed with a saturated aqueous solution of $NaHCO_3$, dried on anh. Na_2S04 and chromatographed on a silica gel column, eluting with a mixture of diethyl ether/hexane (6: 4), to afford 2.8 g (10 mmol) of the required product, yield 33%, m.p. 79-80 °C (n-hexane). ^1H NMR ($CDCl_3$): 1.14 (t, 3H, -0-CH_2-CH_3, J = 6.5), 1.96 (3-CH_2, 2 H, quintet, J = 6.5), 2.44 (2-CH_2, 2 H, t, J = 6.5), 4.01 (-0-CH_2-CH_3 and 4-CH_2, 4H, m), 7.20 (H-8', 1 H, d, J = 7.5), 7.30 (H-6', I H;t, J = 7.5), 7.61 (H-7'. 1 H, t, J = 7.5), 8.02 (H-5', 1 H, d, J = 7.5). ^{13}C NMR ($CDCl_3$): 14.1 (-0-CH_2-CH_3), 22.7 (3-CH_2), 31.4 (2-CH_2), 41.7 (4-CH_2), 60.4 (-0CH_2-CH_3), 114.0 (C-4a), 116.3 (C-8), 125.3 (C-5), 127.9 (C-6), 136.0 (C-7), 147.9 (C-8a), 152.4 (C4'), 160.5 (C-2'), 172.5 (-COOEt). EI-MS spectrum: m/z 277 (M+, 8.2%), 232 (M^+- -OC_2H_5, 22.9%), 204 (M^+- -COC_2H_5, 12.8%), 120 ($C_7H_4O_2^-$, 100%).

$C_{I4}H_{15}NO_5$

Synthesis of 4-(3,4-dihydro-2,4-dioxo-2 H-1,3-benzoxazin-3-yl)-butyric acid 7 and its Sodium Salt 8

Compound **6** (2.0 g, 7.1 mmol) was suspended in 0.5 N HCl (100 ml) and stirred at 80 °C for 1 h (Scheme **1**). The reaction mixture became clear. After cooling, the desired product precipitated as white crystals. They were filtered, washed with 0.5 N HCl and dried to afford 1.5 g (6 mmol) of 4(3,4-dihydro-2,4-dioxo-2 *H-* 1,3-benzoxazin-3-yl)-butyric acid (**7**), yield 84%, m.p. 130-131 °C. ^1H NMR (CD_3OD): 2.02 (3-CH_2, 2H, quintet, J=6.5), 2.35 (2-CH2, 2H, t, J=6.5), 4.06 (4-CH_2, 2H, t, J=6.5), 7.43 (H-8', 1 H, d, J=7.5), 7.54 (H-6', 1 H, t, J=7.5), 7.92 (H-7'), 1 H, t, J=7.5), 8.10 (H-5', 1 H, d, J=7.5). ^{13}C NMR (CD_3OD): 23.7 (3-CH_2), 32.2 (2-CH_2), 42.5 (4-CH_2), 115.6 (C-4a), 117.5 (C-8), 127.8 (C-5), 128.5 (C-6), 137.5 (C-7), 149.5 (C-8a), 153.9 (C-4'), 162.3 (C-2'), 175.0 (COOEt). FT-IR (cm-1): 1667, 1730 and 1767 (three carbonyl groups), 3273 (broad, -COOH).

$C_{13}H_{16}NO_4$

Compound **8** (sodium salt of **7**) was prepared dissolving **7** in two parts of acetone, cooling the solution in an ice bath, and adding dropwise an aqueous solution (one part) containing an equimolar amount of $NaHCO_3$. The obtained solution was stirred 2 h at O°C and 1 h at room temperature. Acetone was carefully evaporated in vacuo (bath temperature: 30 °C) and the aqueous solution was iced and lyophilized to afford the required salt, yield 94%.

Water Solubility and Lipophilicity of Compounds 6- 7

The lipophilicity indices (Log K) of compounds **6** and **7** were deterrnined by the reverse-phase HPLC method as previously described [18].

Since compounds **6** and **7** showed poor water stability, their water solubility could not be experimentally measured. So, we calculated this parameter using the theoretical method described in previous paper [19-21] for determining water solubility of poorly stable prodrugs. The water solubility was calculated based on eq. 1:

$$\log Sw = -\log P - 0.01\ MP + 1.05 \quad (1)$$

where Sw, MP and LogP represent the water solubility, m.p. and n-octanol/water partition coefficient, respectively, of the compounds under examination. Log P of derivatives **6** and **7** was calculated, as previously reported [18], by an HPLC method recommended for compounds unstable in solution using eq. 2:

$$\text{Log P} = \text{Log K'} + \log K \quad (2)$$

Log K' was deterrnined from the elution times of standards of known log P (indomethacin and naproxen).

Chemical and Enzymatic Hydrolysis

To elucidate the chemical and enzymatic hydrolysis process of **6** and **7**, we prepared the N-salicylamide of GABA (**9**, Scheme **1**) by acidic hydrolysis of **5** in 0.5 N HCl, as previously described for the synthesis of **7** from **6**. The chemical stability of compounds **5**-**7** and **9** was determined in isotonic phosphate buffer pH 7.4 (μ = 0.5) at 37 °C (compounds concentration: about 10^{-4} M) by following the disappearance of their peaks with the HPLC method described below. Enzymatic hydrolysis of compounds **5**-**7** and **9** was determined in mouse plasma. Mouse plasma fractions (1.5 ml) were diluted with 0.37 ml of isotonic phosphate buffer, pH 7.4 (80% plasma). Plasma samples were thermostated at 37$\pm$0.2 °C during the experiments. The reactions were started by adding 70 µl of a stock solution of each compound (10^{-2} M in CH_3OH) to 1.87 ml of prethermostated plasma. Aliquots (300 µl) were withdrawn at intervals and deproteinized by mixing with 600 µl of 0.01 N HCl in CH_3OH. After centrifugation at 5000 g for 5 min, 50 µl of the clear supernatant was chromatographed as described below. Pseudo-first-order rate constants for the chemical and enzymatic hydrolysis were deterrnined from the slopes of linear plots of the logarithm of residual compound against lime.

HPLC Analysis

Compounds **5**-**7** and **9** were deterrnined by HPLC using a mobile phase consisting of CH_3OH and 0.1 M CH_3COOH (1:1). The chromatography was run at room temperature at flow rate of 1.2 ml/min and the column effluent was monitored continuously at 250 nm. Under these conditions, compounds **5, 6, 7,** and **9** showed the following retention times: **5**: 10.62 min; **6**: 11.59 min; **7**: 5.65 min; **9**: 5.10 min. Quantification of the compounds was performed by measuring the peak areas in relation to those of the standards chromatographed under the same conditions.

PHARMACOLOGYCAL BIOASSAYS

Animals

Male Swiss mice, weighing 20 to 25 g, were supplied by Charles River (Italy). Animal care and use was according to the directions of the Council of the European Communities (1986). The animals were housed in colony cages (10 mice each) under conditions of standard light (light on from 7.00

A.M. to 7.00 P.M.), temperature (22±1°C) and room humidity (60%±10%) for at least 1 week before the experimental sessions. Food and water were available ad libitum.

Locomotor Activity

The animals were placed in the activity cage for at least a 30-min period for acclimatization before receiving an injection of drugs. Temperature, sound and light conditions were not altered during the course of the experiments. Locomotor activity of the mice was recorded in an activity cage (Basile, Milan, Catalogue No 7400; length, 35 cm; width, 23 cm and height, 20 cm) that recorded spontaneous coordinate activity of mice. The Perspex cage had a transparent lid, which allowed the animals to be observed. The floor was made of stainless steel bars (3 mm diameter), spaced 11 mm apart. The sessions in the activity cage lasted for 2 h [22].

Motor Coordination

Motor coordination of the mice was evaluated using a rotarod apparatus (Ugo Basile, Italy), consisting of a bar with a diameter of 3.0 cm, subdivided into five compartments by a disk that was 24 cm in diameter. The bar rotated at a constant speed of 16 rpm [23]. The integrity of motor coordination was assessed on the basis of the endurance time of the animals on the rotating rod. One day before the test, the animals were trained twice. On the day of the test, only mice that were able to stay balanced on the rotating rod for between 70 and 120 s (cut-off time) were selected [24].

Behavior

Rearing (RE): body inclined vertically, forequarters raised; Grooming (GR): mouth or paw on body; Social response test (SRT): social behavior between the animals; Crossing (CR): locomotor activity; Smelling (SM): nose against wall or ceiling and not moving; Washing Face (WF): forepaws on head; Scratching (SC): hind foot raised against body; Bar holding (BH): fore- and hindpaws on the bars of the cage's metal top, body suspended, were performed, as previously reported [25]. Mice were observed in a transparent perspex cage (25×15×15 cm) and the frequency of all behaviors (number of times a particular behavioral attribute was performed, at several time intervals) were recorded manually by one observer who did not know the treatment administered to the animals. The test was conducted for a period of 120 min after injection of GABA prodrugs and each mouse was observed in the following sequences: 10–20 min post-injection (PI), 40–50 min PI, 70–80 min PI and 100–120 min PI.

To check the social responsiveness of the treated mouse, each session contained a SRT. An untreated mouse was placed in the experimental cage together with the drug-treated mouse for 5 min. The presence or absence of common social behavior was recorded (sniffing, play or aggression).

Catalepsy

The presence of a state of catalepsy was detected using the abnormal posture test [26]. In mice, catalepsy was quantitatively estimated by placing the forepaws of the animals on the horizontal rod that was mounted 3 cm above the floor of the experimental box; the test was regarded as positive if the animal remained in this position for at least 45 s. Latency to step down was recorded before and at intervals (20, 40, 80 and 120 min) after drug treatment. A maximum cut-off time of 45 s was used. Cataleptic responses were calculated as a percentage of the maximum possible response (%MPR) defined as (R−B)/(45−B)×100%. B is the mean baseline latency, R is the post-treatment response latency and 45 is the cut-off time [27].

Nociceptive Assays

The nociceptive assays performed were the hot plate (HP) and the tail flick test (TF). The HP test was performed as previously described [28]. Briefly, the HP (Socrel Mod. DS-37, Ugo Basile, Italy, 25 cm×25 cm) was set at a plate temperature of 55±0.5°C to give a latency of 17–20 s in control animals. The time of hind paw licking was recorded and measurements were terminated if the licking exceeded the cut-off time (60 s). The TF test was performed as previously described [29]. Briefly, the TF latency was obtained using a TF unit (Socrel Mod DS-20, Ugo Basile, Italy). The animals were gently immobilized, using a glove, and the radiant heat was focused on a blackened spot 1–2 cm from the tip of the tail. Beam intensity was adjusted to give a tail flick latency of 2–3 s in control animals. Measurements were terminated if the latency exceeded the cut-off time (10 s), to avoid tissue damage. In all of the experiments, mice were tested twice (at 60 and 30 min) before drug administration in the baseline latency determination and 30 min after drug administration.

Convulsant Activity Assessment

The method was that described previously [30]. Mice were placed singly in a transparent perspex cage (25×15×15 cm) for 30 min to acclimatize to their new environment prior to the administration of drugs. The animals were observed for 30 min following the administration of PTZ (75 mg/kg,i.p.) or bicuculline (10 μg/intracerebroventricular (i.c.v./mouse). The time taken for the onset and the number and duration of tonic, clonic and tonic–clonic convulsions and the numbers of animals dead were noted.

Animals that did not convulse or die within 30 min were recorded as not convulsing. Both the control and test experiments were performed in a quiet room with an ambient temperature of 25±2°C, between 7.00 A.M. and 7.00 P.M. each day to avoid behavioral changes resulting from circadian rhythm.

Compounds **5** and **6** were dissolved in a 40% solution of polyethylene glycol 400 (PEG 400); compounds **7** and **9** were administered as sodium salt dissolved in NaCI 0.9%. Control groups were treated with the corresponding vehicles. One hour later, PTZ or bicuculline werw administered intraperitoneally. The animals were observed for 1 h, recording the latency time until convulsions occurred, the number of generalized convulsive seizures, and the number of mice dying within the experimental lime.

Experimental Procedure

On the day of testing, all drugs used in the experimental sessions were dissolved in saline, for intraperitoneal (i.p.) administration, and in distilled water for i.c.v. injection. The

i.c.v. injection was performed according to the method described by Haley and McCormick (1957) [31]. At the end of the experimental session, the injection site was verified in randomly chosen animals (at least six animals per group) using 1% methylene blue and examining the distribution of the dye in the cerebrum. Compounds 5,6,7,9 were administered at doses of 10, 20 and 40 mg/kg, i.p. immediately- and 60 min before distilled water (10 μl/i.c.v./mouse) or PTZ or bicuculline (Sigma, St. Louis, MO, USA). The doses were calculated as the weight of the base.

Statistical Analysis

All data (expressed as mean±S.E.M.) were analyzed using analysis of variance (ANOVA) [32] and Dunnett's procedure for multiple comparisons with a single control group. When the analysis was restricted to two means, Student's *t*-test (two-tailed) was used. The Fisher exact test was used to analyze the rotarod data. Significance was assumed at a 5% level.

RESULTS

Synthesis of GABA Prodrugs

Synthesis of **6** was carried out using a straightforward procedure at room temperature, depicted in Scheme **1**.

Salicylic acid (**1**) was dissolved in dry ($CaCl_2$) alcohol-free chloroform. 1,1-Carbonyldiimidazole (**2**) was added gradually and the solution mixture was stirred at room temperature until effervescence ceased. The expected [17] intermediate imidazolide **3** was not isolated, but to the reaction mixture ethyl 4-aminobutyrate hydrochloride (**4**) was added. The solution mixture was stirred for 12 h at room temperature, giving the correspondent salicylamide **5** as well as some polymeric by-products and traces of the desired compound **6**. To complete the cyc1ization, an other amount of **2** was added gradually. After 48 h all the salicylamide **5** had been converted in **6**. The reaction mixture was evaporated in vacuo and the residue was extracted with diethyl ether: **6** was purified from the organic solution by chromatography on a silica gel column and recrystallized from n-hexane.

It is notable that the use of 1,1-carbonyldiimidazole had two results: the first amount activated the carboxylic moiety of salicylic acid and the second c1osed the heterocyc1ic ring. Thus it was not necessary to isolate the salicylamide **5**, because it was transformed into **6** in the same reaction batch. The use of ethyl 4-aminobutyrate as hydrochloride salt was necessary because the correspondent free base cyc1icizes very easily into 2-pyrrolidone since it possesses and electrophilic carbon which is prompt1y attacked by the nuc1eophilic NH_2.

The synthesis of **7** was effected by an acidic hydrolysis of the carboxyl terminal ethyl ester of **6**, because the 3,4-dihydro-2,4-dioxo-2*H*-l,3-benzoxazine ring does not stand up to basic conditions [15]. For the same reason the preparation of **8** (sodium salt of 7) was effected in very mild conditions.

Lipophilicity and Water Solubility of Compounds 6 and 7

The lipophilic indices of compounds **6** and **7** were 0.630 and 0.195, respectively. As expected, ester **6** was more lipophilic compared with the acid compound **7**. Since compounds **6** and **7** showed poor water stability, we could not measure their water solubility experimentally. So, we calculated compounds **6** and **7** water solubility using a theoretical method reported in a previous paper [18]. Although the lipophilicity of ester **6** was higher than that of compound **7** both compounds showed similar water solubility (**6**: 194 μg/ml; **7**: 147 μg/ml). These results suggest that the increase of lipophilicity of ester **6** was not associated with a decrease of its water solubility compared to derivative **7**. Similar findings have been reported by other authors in designing prodrugs for dermal delivery [20].

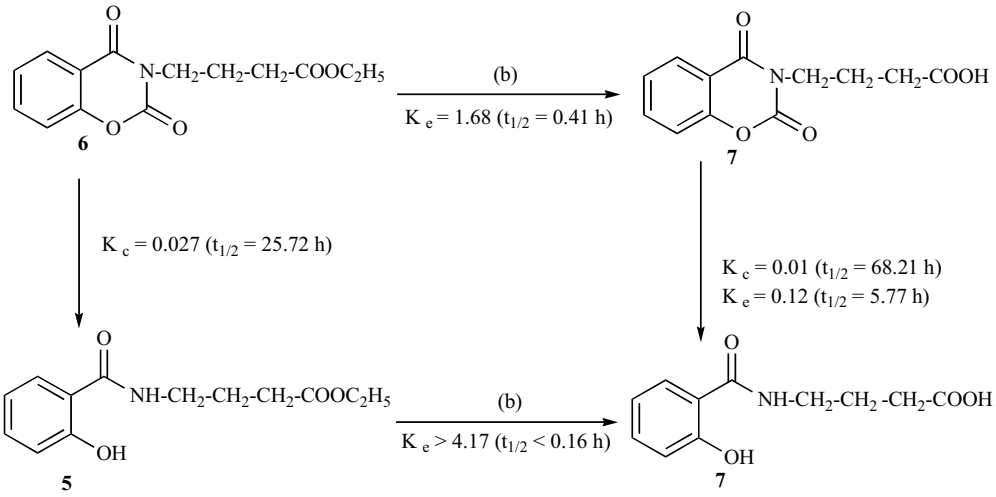
Chemical and Enzymatic Hydrolysis

The results obtained in chemical and enzymatic degradation studies are depicted in Scheme **2**. As regards chemical hydrolysis in phosphate buffer (pH 7.4), compounds **6** and **7** led to the corresponding N-salicylamides **5** and **9** ($t_{1/2}$ = 25.7 h and 68.2 h, respectively), while these last compounds did not show any appreciable amide hydrolysis during the observation period (48 h). No hydrolysis of the carboxyl terminal ester linkage was observed for compounds **5** and **6**. The hydrolytic opening mechanisms of the N-(un)substituted-3,4-dihydro-2,4-dioxo-2 *H*-1,3-benzoxazine ring in aqueous solution was described in detail by Kahns *et al*. [16]. Among the different possible degradation pathways, water attack at the carbamate carbonyl moiety was found to be the only one involved, leading to the quantitative formation of the correspondent N-salicylamide and to the rapid loss of carbon dioxide. We postulate as similar mechanism of 3,4-dihydro-2,4-dioxo-2 *H*1,3-benzoxazine ring cleavage for compounds **6** and **7**.

As regards enzymatic hydrolysis, the carboxyl terminal ester bond was rapidly attacked by plasma esterases, as shown by the rapid transformation of **6** into **7** and of **5** into **9** ($t_{1/2}$ = 0.4 h for compound **6** and $t_{1/2}$ < 0.16 h for compound **5**). Compound **7** was converted into the corresponding N-salicylamide **9** ($t_{1/2}$ = 5.8 h) and this last compound was stable in mouse plasma within the observation period (3 h).

The 3,4-dihydro-2,4-dioxo-2 *H*-1,3-benzoxazine ring underwent enzymatic hydrolysis ($t_{1/2}$ = 5.8 h) in compound **7**, whereas in compound **6** it seemed to stand up to mouse plasma esterases. Since compound **5** was rapidly hydrolyzed into **9** in mouse plasma, it is possible that **5** would not even be observed during the ring hydrolysis of 6 since the ester hydrolysis was so much faster than the ring hydrolysis reaction.

Kahns *et al*. [15] found that the hydrolysis rate of the N-unsubstituted-3,4-dihydro-2,4-dioxo-2 H-1,3-benzoxazine ring and of its N-methyl derivative was only slight1y catalyzed by human plasma, whereas they found for the unsubstituted compound a higher hydrolysis rate in rabbit plasma [16]. The higher enzymatic catalysis in mouse plasma that we found for 3,4-dihydro-2,4-dioxo-2H-l,3benzoxazine ring cleavage in compound **7** could be attributed to the higher enzymatic activity of mouse plasma compared to human and rabbit plasma, as reported by other authors in different prodrug stability evaluations [21].



Scheme 2.

Pharmacologycal Activity

Anticonvulsant Activity vs PTZ

The Pharmacological results are reported in the Table **1**. It can be noted that ethyl ester **6** showed a slight anticonvulsant activity at 20 and 10 mg/kg (at higher doses it was not soluble enough in the hydro-glycolic mixture used). Even at these lower doses, the compound seemed to be somewhat effective in reducing the number of generalized seizures and the death rate compared to the control group ($p < 0.05$). On the contrary, acid 7, administered as sodium salt 8, showed a very interesting dose-dependent anticonvulsant activity. So, at 40 mg/kg dose, it completely abolished the generalized convulsive seizures and notably increased the time of onset of convulsions ($p < 0.05$ compared to the control). Besides, none of the animals died within the experimental rime or later. In order to assess the effect of 3,4-dihydro-2,4-dioxo-2 *H*-l,3-benzoxazine ring opening on the anticonvulsant activity of **6** and **7** derivatives **5** and **9** were also administered to mice at the same doses as compounds **6** and **7**. Compounds **5** and **9** (the latter administered as sodium salt) did not induced significant anticonvulsant activity when compared to the control group.

Table 1. Anticonvulsant Activity vs PTZ

Treatment		Latency Time (s)	Mean Number of Generalized Convulsive Seizures	No.of Dead Animals/ no. of Treated Animals
Control		20.0±2.0	4.0±0.7	8/12
(NaCI 0.9% + PTZ)				
Control		20.0 ±3.0	4.0 ±1.2	8/12
(PEG 400 40% + PTZ)				
6[a]	20 mg/kg	24.5 ±6.6	2.5±0.6	4/12
	10 mg/kg	20.0±1.5	3.3±0.8	6/12
7[b]	40 mg/kg	74.0±13.2	0	0/12
	20 mg/kg	57.0±6.8	1.1±0.3	2/12
	10 mg/kg	38.5±7.1	3.5 ±1.1	4/12
5[a]	20 mg/kg	20.5 ±1.6	4.0±0.2	12/12
5	10 mg/kg	20.2±1.9	4.0±0.3	9/12
9[b]	40 mg/kg	20.8±1.2	4.0±0.1	10/12
9	20 mg/kg	21.0±1.5	4.0±0.1	12/12
9	10 mg/kg	20.5±2.1	4.0±0.2	11/12

[a] It was not possible to test at higher doses owing to solubility difficulties;

[b] adminislered as sodium salt.

Table 2. Anticonvulsant Activity *vs* Bicuculline

Treatment		Latency Time (s)	Mean number of Generalized Convulsive Seizures	No.of Dead Animals/ no. of Treated Animals
Control		20.0±2.0	4.0±0.7	8/12
(NaCI 0.9% + PTZ)				
Control		20.0 ±3.0	4.0 ±1.2	8/12
(PEG 400 40% + PTZ)				
6[a]	20 mg/kg	26.7 ±2.3	3.1±1.2	7/12
	10 mg/kg	20.0±2.8	3.7±2.8	8/12
7[b]	40 mg/kg	83.4±10.5	0	0/12
	20 mg/kg	68.6±8.8	1.0±0.2	2/12
	10 mg/kg	55.9±4.3	1.5 ±1.7	2/12
5[a]	20 mg/kg	20.7 ±1.5	4.0±0.7	12/12
5	10 mg/kg	20.4±1.7	4.0±0.5	12/12
9[b]	40 mg/kg	20.2±1.8	4.0±0.4	12/12
9	20 mg/kg	20.4±1.1	4.0±0.8	12/12
9	10 mg/kg	20.9±1.6	4.0±0.6	12/12
Muscimol 2 mg/kg		58.0±7.0	1.0±0.3	2/12
Muscimol 5 mg/kg		72.3±9.1	1.0+0.6	1/12
Muscimol 10 mg/kg		92.2±5.0	0	0/12
Pentobarbital 10 mg/kg		85.0+8.6	0	0/12

[a] It was not possible to test at higher doses owing to solubility difficulties;

[b] adminislered as sodium salt.

Anticonvulsant Activity vs Bicuculline

A convulsant activity was seen shortly after administration of bicuculline (10 μg/i.c.v./mouse), with mice showing a period of hyperactivity followed by clonic activity of the hind limbs, generalized myoclonus, loss of righting reflex, circling activity, barrel rolling, tonic flexion and an extension of limbs. Respiratory arrest and death occurred within 10 s of tonic convulsion.

The anticonvulsant activity of compounds **5**,**6**,**7**,**9** are reported in Table **2**. Compound **7** pretreatment (10, 20 and 40 mg/kg/i.p.), 60 min before bicuculline administration (10 μg/i.c.v./mouse), dose-dependently reduced the clonic, the tonic and the tonic–clonic phases of the seizure response as well as the number of animals that died. Significant reduction ($F_{(5,84)}$=7.49, P<0.01) of all phases of the seizure response occurred following administration of 20 and 40 mg/kg/i.p. of compounds. However, no significant anticonvulsant activity was observed after administration of the lower dose (10 mg/kg/i.p.). Compound **7** effectively reduced the number of animals convulsing and delayed the onset of bicuculline seizures. Furthermore, compound **7** did not produce significant protection against bicuculline seizures if injected immediately before the convulsant agent (data not shown).

The compound **7** anticonvulsant activity was comparable to that of muscimol (2, 5 and 10 mg/kg/i.p.) whereas the GABA mimetic drug was less active when compared to pentobarbital, which, at dose of 10 mg/kg/i.p., completely abolished bicuculline-induced seizures.

Finally, compounds **5**,**6** and **9** resulted inactive *vs* bicuculline

Behavioral Study

Compounds 5,6,7,9 (10, 20 and 40 mg/kg/i.p.) did not modify the locomotor activity of mice ($F_{(4,29)}$=0.74, n.s.) (Table **3**) as well as motor coordination ($F_{(3,56)}$=1,72, n.s.) (Table **3**) and behavior ($F_{(3,56)}$=0.2, n.s.) (Table **4**). Also, the GABA prodrug did not modify the nociceptive threshold of

Table 3. Behavioral Study. The Effects of Compounds 5,6,7,9 on Locomotor Activity, Motor Coordination, Catalepsy and Nociceptive Threshold in Mice. Results are Mean±SEM (*n*=10).

Treatment		Locomotor Activity (Counts/2 h)	Motor Coordination (Endurance Time/s)	Catalepsy (% of Maximum Possible Response)	Nociceptive Threshold (Hot Plate) Latency/s	Nociceptive Threshold (TailFlick) Latency/s
Control		5670±250	80±7.9	0	17.3±1.9	2.5±0.2
(NaCI 0.9% + PTZ)						
Control		5433 ±310	85 ±9.3	0	18.2±2.2	2.3±0.7
(PEG 400 40% + PTZ)						
6[a]	20 mg/kg	5533±287	82±8.3	0	19.7±2.5	2.2±0.9
	10 mg/kg	5598±295	85±8.9	0	17.5±1.1	2.7±0.5
7[b]	40 mg/kg	5677±278	79±9.1	0	18.3±1.7	2.5±0.9
	20 mg/kg	5565±259	88±8.4	0	19.9±1.4	2.2±0.6
	10 mg/kg	5522±297	84±7.7	0	19.7±1.1	2.4±0.2
5[a]	20 mg/kg	5652±247	86±7.9	0	18.2±1.2	2.5±0.5
5	10 mg/kg	5680±282	78±9.2	0	18.9±1.5	2.2±0.8
9[b]	40 mg/kg	5621±291	89±9.1	0	17.5±1.3	2.2±0.4
9	20 mg/kg	5690±262	77±9.8	0	20.0±2.5	2.5±0.8
9	10 mg/kg	5657±289	83±9.5	0	19.7±2.1	2.3±0.2

the animals (hot plate: $F_{(3,51)}$=0.19, n.s.; tail flick: $F_{(3,51)}$= 2.84, n.s.) (Table **3**) or induce catalepsy ($F_{(3.36)}$=0.2, n.s.) (Table **3**).

DISCUSSION

In the present review, we have considered the pharmacological profile of new GABA mimetic drugs by performing both a behavioral and an anticonvulsant study using a specific $GABA_A$ receptor antagonist, such as bicuculline. A behavioral study for the new anticonvulsant agents was performed because it is important that new anticonvulsants have few behavioral side effects and have good anticonvulsant activity. Although other GABA prodrugs have been reported to possess anticonvulsant activity, few and controversial experiments have been performed to evaluate their side effects (8-10).

The results of our experiments indicate that compound **7** dose-dependently reduces both bicuculline- and PTZ-induced convulsions without altering animal behavior. The ability of compound **7** to reduce the convulsant effect of bicuculline suggests that this new GABA mimetic drug may act through the involvement of $GABA_A$ receptors. This hypothesis may be supported by the comparative study performed with muscimol, a well known $GABA_A$ receptor agonist, whose anticonvulsant activity is comparable to compound **7**.

Compounds **5** and **9** (the latter administered as sodium salt) showed only a slight anticonvulsant activity which was not significant1y different from the control ($p > 0.05$, data not shown).

This last finding suggest that the presence of the 3,4-dihydro-2,4-dioxo-2 *H*1,3-benzoxazine ring was an essential requisite far effectiveness.

Since ester **6** was rapidly ($t_{1/2}$ = 0.4 h) converted to acid **7** in mouse plasma, we did not expect such different anticonvulsant activity between them. The lower pharmacological response of ester **6** could probably be due to its slow absorption after intraperitoneal administration, owing to its high lipophilicity and low dissolution rate. An alternative explanation of this experimental evidence could be the different tissue distribution of this compound in the lipophilic districts of the body. Furthermore, the high lipophilicity of ester **6** could provoke its partial precipitation at the intraperitoneal site of injection in the animal and hence the free ester concentration in the blood at a given time which was available to penetrate the BBB was lower than expected.

As regards **7**, since it is stable enough in mouse plasma ($t_{1/2}$ = 5.8 h) to circulate intact in the blood for a long period of time and to interact with the BBB, we think that it should permeate this barrier owing to his higher lipophilicity compared to GABA. In the cerebral tissue it should be converted to amide 9 by brain esterases, and then, like other analogous *N*-GABA-amide prodrugs [12, 13], generate free GABA in suitable concentrations to produce anticonvulsant effects.

It is generally believed that GABA is the major inhibitory neurotransmitter in the brain and the concept that the im-

Table 4. Effect of Compounds 5,6,7 9 (20 mg/kg/ip) on the Stereotyped Behavior of Mice

	RE	GR	SRT	CR	SM	WF	SC	BH
10-20 min PI								
Control	89 ± 9.0	59 ± 7.0	51 ± 6.0	47 ± 8.0	85 ± 9.0	46 ± 7.0	41 ± 6.0	44 ± 5.0
6 (20 mg/kg)	87 ± 8.0	62 ± 7.0	49 ± 9.0	50 ± 9.0	84 ± 4.0	44 ± 8.0	40 ± 9.0	45 ± 7.0
7 (20 mg/kg)	82 ± 5.0	59 ± 6.0	50 ± 5.0	55 ± 4.0	83 ± 6.0	50 ± 5.0	42 ± 3.0	43 ± 4.0
5 (20 mg/kg)	92 ± 4.0	60 ± 5.0	52 ± 3.0	46 ± 5.0	82 ± 7.0	49 ± 3.0	38 ± 6.0	41 ± 3.0
9 (20 mg/kg)	72 ± 4.0	58 ± 5.0	50 ± 3.0	48 ± 5.0	86 ± 7.0	48 ± 3.0	39 ± 7.0	38 ± 7.0
40-50 min PI								
Control	65 ± 8.0	48 ± 9.0	47 ± 6.0	39 ± 5.0	76 ± 9.0	55 ± 6.0	43 ± 5.0	37 ± 5.0
6 (20 mg/kg)	67 ± 4.0	51 ± 9.0	50 ± 9.0	35 ± 7.0	77 ± 5.0	59 ± 4.0	45 ± 9.0	36 ± 8.0
7 (20 mg/kg)	69 ± 6.0	49 ± 8.0	51 ± 9.0	40 ± 6.0	75 ± 7.0	51 ± 5.0	39 ± 3.0	38 ± 2.0
5 (20 mg/kg)	70 ± 4.0	47 ± 2.0	48 ± 4.0	41 ± 6.0	79 ± 3.0	55 ± 3.0	42 ± 2.0	41 ± 3.0
9 (20 mg/kg)	62 ± 7.0	50 ± 6.0	50 ± 2.0	36 ± 4.0	72 ± 6.0	49 ± 3.0	38 ± 6.0	39 ± 3.0
70-80 min PI								
Control	70 ± 9.0	50 ± 7.0	49 ± 6.0	42 ± 7.0	67 ± 8.0	59 ± 9.0	45 ± 8.0	43 ± 7.0
6 (20 mg/kg)	69 ± 3.0	55 ± 9.0	47 ± 9.0	41 ± 8.0	68 ± 9.0	64 ± 6.0	41 ± 5.0	40 ± 7.0
7 (20 mg/kg)	75 ± 3.0	53 ± 5.0	50 ± 4.0	36 ± 3.0	70 ± 4.0	57 ± 3.0	42 ± 4.0	44 ± 4.0
5 (20 mg/kg)	72 ± 5.0	51 ± 4.0	48 ± 7.0	35 ± 5.0	66 ± 4.0	60 ± 2.0	41 ± 3.0	39 ± 5.0
9 (20 mg/kg)	65 ± 4.0	52 ± 5.0	51 ± 3.0	36 ± 5.0	69 ± 7.0	59 ± 3.0	38 ± 6.0	38 ± 3.0
100-120 min PI								
Control	68 ± 9.0	55 ± 7.0	50 ± 8.0	45 ± 6.0	65 ± 7.0	60 ± 8.0	47 ± 7.0	45 ± 6.0
6 (20 mg/kg)	70 ± 3.0	49 ± 9.0	49 ± 9.0	46 ± 8.0	66 ± 9.0	57 ± 8.0	52 ± 9.0	47 ± 5.0
7 (20 mg/kg)	63 ± 5.0	55 ± 5.0	47 ± 5.0	50 ± 9.0	65 ± 5.0	62 ± 9.0	44 ± 6.0	46 ± 9.0
5 (20 mg/kg)	73 ± 3.0	49 ± 4.0	51 ± 3.0	44 ± 5.0	64 ± 4.0	58 ± 6.0	51 ± 5.0	44 ± 4.0
9 (20 mg/kg)	72 ± 4.0	50 ± 3.0	50 ± 5.0	48 ± 7.0	62 ± 5.0	59 ± 6.0	48 ± 3.0	48 ± 3.0

Compounds **5**,**6**,**7**,**9** did not modify significantly the stereotyped behavior of mice. Results are mean ± SEM (n = 6). The abbreviations are as follows: RE = Rearing; GR = Grooming; SRT = Social response test; CR = Crossing; SM = Smelling; WF = Washing Face; SC = Scratching; BH = Bar holding; PI = post injection.

pairment of GABA-mediated neurotransmission is associated with convulsion is now widely accepted. Inhibitory effects of GABA are mediated, in part, by the $GABA_A$ receptors, which are associated with epilepsy, among other things.

Furthermore, although compound **7** was less active than pentobarbital (a clinically effective anticonvulsant agent), the probable involvement of the $GABA_A$ receptor in the effects of compound **7** cannot be excluded because barbiturates increase chloride flux through chloride channels at the $GABA_A$ receptor site to enhance GABAergic inhibition. Given the above evidence, since compound **7** possesses anticonvulsant activity comparable to that of muscimol rather than pentobarbital, the possibility that compound **7** has a mechanism of action similar to that of muscimol rather than pentobarbital should be considered. However, whatever the mechanism may be, at present, it is of interest that while muscimol and pentobarbital *per se* (at the doses used) significantly modify animal behavior, compound **7** does not. Therefore, our results indicate that this new GABA mimetic drug shows good anticonvulsant activity without causing significant behavioral side effects. Furthermore, the ability of compound **7** to act after systemic administration indicates that this new GABA mimetic drug crosses the BBB as previously reported. The good pharmacological response of compound **7** may be related to its stability in plasma, thus allowing it to circulate intact in the blood for a long period of time and to interact with the BBB.

We think that compound **7** should permeate this barrier due to its higher lipophilicity compared to GABA. In the cerebral tissue, it should lead to the formation of an amide by

the action of brain esterases, and then, like other analogous N-GABA-amide prodrugs (12,13), generate free GABA in suitable concentrations to produce anticonvulsant effects. This hypothesis was supported by experiments performed during a fluorine microscopic study (data not shown). compound **7** is a fluorescent molecule and the presence of fluorescence in areas of the central nervous system indicates the presence of compound **7**. Our previous data indicate that mice treated with compound **7** had marked fluorescence in several nuclei of the brain and in the cerebellum when compared to the control animal (saline-treated mice), thus indicating the presence of compound **7**.

In conclusion, our data indicate that this new GABA prodrug may possess all the requirements of ideal prodrugs, as it did not modify animal behavior and was able to exert significant anticonvulsant activity after systemic injection. Also, we are planning to further assess the BBB transport of compound **7** using an *in vitro* model (which consists of primary cultures of bovine brain endothelial cells) and to study its enzymatic hydrolysis in mouse brain homogenates.

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