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Synthesis, physicochemical properties, anticonvulsant activities and voltage-sensitive calcium channels affinity of *N*-substituted amides of α-(4-phenylpiperazino)-GABA

Part 3: Search for new anticonvulsant compounds

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This paper describes the synthesis and preliminary anticonvulsant evaluation of some GABA analogues i.e. derivatives of 2-(4-phenylpiperazino)- or 2-(4-benzylpiperidino)-GABA (**5**, **6**), *N*-substituted amides of 2-(4-phenylpiperazino)-4-phthalimidobutyric acid and *N*-substituted amides of 2-(4-phenylpiperazino)-GABA. *N*-Substituted amides of 2-(4-phenylpiperazino)-4-phthalimidobutyric acid (**7**–**11**) were prepared by condensation of the acid with the corresponding derivatives of benzylamine in the presence of different coupling reagents (2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and carbonyldiimidazole (CDI)). *N*-Substituted benzylamides of 2-(4-phenylpiperazino)-4-aminobutyric acid (**12**–**14**) were prepared by hydrazinolysis of amides **9**–**11**. Anticonvulsant activities were determined in mice (for all compounds) and in rats using the subcutaneous metrazol (scMet) and maximal electroshock (MES) screens. The amides (**12**–**14**) showed protection against MES and/or scMet seizures in mice. *N*-(4-Methoxybenzyl)-2-(4-phenylpiperazin-1-yl)-4-aminobutyric amide (**13**) was the most effective and displayed anticonvulsant activity in both tests at doses of 100–300 mg/kg in mice and at 30 mg/kg in the MES screen in rats. The active compounds (**12**–**14**) were tested for their ability to displace [^{3}H]nitrendipine binding sites (voltage-sensitive calcium channel receptors) from rat cortex. Amide **13** was the most active both in pharmacological and biochemical tests. These preliminary results suggest that the anticonvulsant activity of compounds **12**–**14** may be related to their influence on voltage-sensitive calcium channel receptors.

1. Introduction

Various studies of the mechanisms of epilepsy and anticonvulsant agents have revealed both specific neuronal relationships and many of the neurotransmitters and neuromodulators that are considered important in the control of neuronal excitability [1]. The mechanism of action of anticonvulsants is involved in: inhibitory synaptic processes, excitatory transmitters and ionic channels [2, 3]. The neutral amino acid, 4-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain, plays an important role in the etiology and control of epilepsy by mediating the inhibition processes of epilepsy [4, 5]. Recent data suggested that the inhibitory action of GABA mediated by low-affinity receptors could involve a regulation of the activity of voltage-gated calcium channels [6].

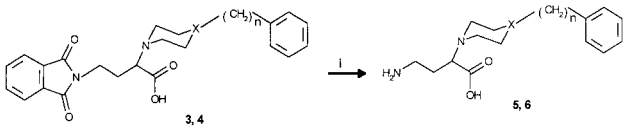
In search for the new anticonvulsants we undertook a study of GABA derivatives. This paper describes the synthesis and preliminary anticonvulsant evaluation of some GABA analogues i.e. derivatives of 2-(4-phenylpiperazino)-GABA, derivatives of *N*-substituted amides of 2-(4-phenylpiperazino)-4-phthalimidobutyric acid and *N*-substituted amides of 2-(4-phenylpiperazino)-GABA. We have previously prepared some derivatives of 4-phthalimidobutyric acid [7]. These compounds displayed protection against MES and subcutaneous metrazol (scMet) induced seizures. For a better and wider definition of structure-activity relationships, derivatives of *N*-substituted amides of 2-(4-phenylpiperazino)-4-phthalimidobutyric acid (**7** and **8**) were synthesized and also examined. These phthalimide series may be considered as analogues of GABA, in which the amino function is converted into an imido group. The choice to address our research towards *N*-substituted amides of 2-(4-phenylpiperazino)-GABA (**12**–**14**) depended on the consideration that several *N*-substituted amides of 2-(4-phenylpiperazino)-4-hydroxybutyric acic exhibit anticonvulsant activity [8]. These compounds could be viewed as analogues of active amide in which the hydroxyl group was replaced by an amino group. The anticonvulsant activity of the newly synthesized compounds was examined in MES, scMet and neurotoxicity screens after i.p. injection into mice. Promising compounds will be administered to rats by the oral route. Recent data have shown that several antiepileptic drugs, i.e. diphenylhydantoin, carbamazepine, barbiturates and benzodiazepines are thought to interact with voltage-gated Ca^{2+} channels [9, 10]. Carbamazepine which is effective in different types of epilepsy has also been suggested to reduce the calcium entry through L-type voltage-sensitive Ca^{2+} channels [10]. Nitrendipine and other dihydropyridine voltage-sensitive calcium channel (VSCC) antagonists as well as derivatives of piperazine (flunarizine, cinnarizine, lidoflazine, dotarizine) possess anticonvulsant and neuroprotective activity [11–13]. Therefore the amides **12**–**14** containing a phenylpiperazine moiety were also evaluated on voltage-sensitive calcium channels affinity *in vitro*.

2. Investigations, results and discussion

2.1. Chemistry

The synthesis of new derivatives of 2-(4-phenylpiperazino)- or 2-(4-benzylpiperidino)-4-aminobutyric acid started from lactones **1** and **2**. 3-(4-Phenylpiperazino)-tetrahydrofuran-2-one (**1**) and 3-(4-benzylpiperidino)-tetrahydrofuran-2-one (**2**), have been previously described and were prepared according to the reported methods [14]. Acids **3** and

Scheme 1



No	**3**, **5**	**4**, **6**
X	N n = 0	−CH− n = 1

Reagents and conditions: **i**, 80% aq. H_2N-NH_2, RT, 2h; then 25% aq HCl, 50 °C, 1 h

4 were obtained by reaction of the appropriate lactone (**1** or **2**) with potassium phthalimide in diethylformamide according to [7]. 2-(4-Phenylpiperazino)-4-aminobutyric acid (**5**) and 2-(4-benzylpiperidino)-4-aminobutyric acid (**6**) were prepared by hydrazinolysis of acids **3** and **4** respectively (Scheme 1).

The *N*-substituted amides of 2-(4-phenylpiperazino)-4-phthalimidobutyric acid (**7**–**11**) were synthesized according to the procedures shown in Scheme 2. The amides **9**–**11** were previously obtained [7] by reaction of acid **3** with derivatives of benzylamine in the presence of benzotriazol-1-yloxy-tris-(dimethylamino)-phosphonium-hexafluorophosphate (BOP) [15] and *N*-methylmorpholine (NMM) in yields of 75–89%. In order to avoid BOP reagent and to the facilitate purification, two synthetic approaches were explored for the preparation of amides **7**–**11**. These methods were based on a reaction of the *N*-protected amino acid with an amino component in the presence of a different coupling reagents.

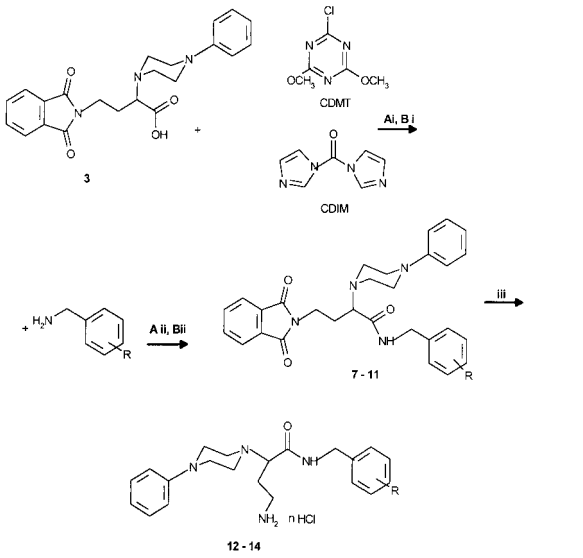
In the first route (method **A**) we used 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) [16], for the amide bond formation. The preparation of amides **7**–**11** using CDMT proceeds as a sequence of two independent steps in a one-pot synthesis. In the first step, acid **3** was activated by treatment with stoichiometric amounts of CDMT and NMM. The reaction was carried out in DMF or dimethylacetamide (DMAC). In the second step, the amide bond was formed after addition of an appriopriate benzylamine derivative directly to the preactivated mixture at −5 °C, followed by reaction at room temperature for 14 h. The yields of compounds **7**–**11** were between 87 and 91%.

In the second procedure (method **B**) we used the carbonyldiimidazole (CDI) method [17], and compounds **7**–**11** were isolated in moderate yields ∼55%. Reaction of *N*-substituted benzylamides of 2-(4-phenylpiperazino)-4-phthalimidobutyric acid **9**–**11** with hydrazine hydrate led to the formation of *N*-substituted benzylamides of 2-(4-phenylpiperazino)-4-aminobutyric acid **12**–**14**. Compounds **12**–**14** were isolated as hydrochloride.

2.2 Pharmacology

Preliminary anticonvulsant evaluation of all the synthesized compounds was provided by the Antiepileptic Drug Development (ADD) Program of the National Institute of Neurological Disorders and Stroke by testing procedures which have been described earlier [18]. For the identification of anticonvulsant activity in mice, test compounds were administered intraperitoneally and challenged by maximal electroshock (MES) and subcutaneous metrazol (scMet) induced seizures [19, 20]. Compounds which are effective in these seizure challenges are regarded to be effective for absence or petit mal (scMet), and generalized tonic clonic or grand mal (MES) epilepsy. Neurotoxicity

Scheme 2



No	**7**	**8**	**9**, **12**	**10**, **13**	**11**, **14**
R	2-Cl	3,4(OCH_3)	H	4-OCH_3	4-F

Reagents and conditions: Activation : **Ai**, DMF or DMAC (dimethylacetamide), NMM (*N*-methylmorpholine), −5 to 0 °C, 3 h; **Bi**, DMF, room temp., 0.5h; coupling: **Aii**, NMM, 0 °C, 1 h; then RT, 16 h; **Bii**, RT, 20 h; **iii**, 80% aq. H_2N-NH_2, EtOH anhydr., RT, 24 h; then EtOH solution HCl gas, 50 °C 2 h; n HCl (for **12** n = 2, for **13** and **14** n = 3).

of the test compounds was determined using the rotorod toxicity test (TOX) [19, 20]. Phase I is a qualitative assay involving a small number of mice (1–4) at dose levels of 30, 100 and 300 mg/kg.

Some compounds (**5**, **7**, **8**, **10**) were evaluated in the threshold tonic extension (TTE) test [21]. The TTE test is a clinically nonselective, electroconvulsive seizure model that identifies compounds that raise seizure threshold as well as those that prevent seizure spread. In addition this test can identify certain compounds that are inactive in the MES and in the scMet test. The test is similar to the MES screen but uses a lower level of electrical current. The lower current makes the TTE test more sensitive but less discriminating than the MES screen. Compounds **5**, **7**, **8** and **10** were advanced to Phase VIa and were evaluated for oral activity in rats by the ADD Program. The results of in the *vivo* tests are summarized in Tables **1**–**3**.

The investigated acids **5** and **6** were inactive in the MES and scMet in mice (Phase 1, class 3 according to ASP classification, not shown). Acid **5** was active in the MES screen in rats at 30 mg/kg (p.o. dose) (1/4, 1/4 animals protected at 0.25 h and 4 h), and at 50 mg/kg (i.p. dose) (2/4, 2/4, 1/4, 1/4 animals protected at 0.25 h, 0.5 h, 1 h and 2 h; Table 2), and showed weak activity in the TTE test (MES screen in mice, Table 3). The investigated *N*-substituted amides of 2-(4-phenylpiperazino)-4-phthalimidobutyric acid (**7** and **8**) were inactive in the MES screens in mice; amide **8** was inactive and amide **7** was marginally active at a dose of 100 mg/kg (1/5 animals protected at 0.5 h) in the scMet screens (Table 1). The *N*-substituted amides of 2-(4-phenylpiperazino)-GABA (**12**–**14**) showed protection against MES and/or scMet seizures in mice (Phase 1, i.p. dose, class 1 acc. to ASP classification). Compound **12** was active at a dose of 30 mg/kg (1/5 animals protected at 0.5 h) and at a dose of 100 mg/kg (2/5 animals protected at 0.5 h) in scMet screen. Compound **14** was active at doses of 100 mg/kg (1/3 animals protected

Table 1: Anticonvulsant screening project (ASP): phase 1. Test results in mice

Compd.	Dose (mg/kg)	Activity					TOX.[c]			ASP class.[d]
		MES[a] (time (h))		ScMet[b] (time (h))			time (h)			
		0.5	4	0.5	1	4	0.5	1	4	
7	30[xx]	0/1	0/1	0/1	–	0/1	0/4	–	0/2	
	100[xx]	0/3	0/3	1/5	–	0/1	0/8	–	0/4	**1**
	300[xx]	0/1	0/1	0/1[1]	–	0/1	0/4	–	0/2	
12	30[xx]	0/1	0/1	1/5	–	0/1	0/4	–	0/2	
	100[xx]	0/3	0/3	2/5	–	0/1	2/8	–	1/4	**1**
	300[xx]	–	–	–	–	–	4/4[2(4)]	–	–	
13	30[x]	0/1	1/1	0/1	–	0/1	0/4	–	0/2	
	100[x]	0/3	2/3	0/2	2/5	0/1	1/8	3/5	1/4	**1**
	300[x]	0/1	–	1/1	–	–	4/4[3]	–	2/2[2(2)]	
14	30[x]	0/1	0/1	0/1	–	0/1	0/4	–	0/2	
	100[xx]	0/3	1/3	0/1	–	0/1	4/8	–	1/4	**1**
	300[xx]	1/1	–	0/1	–	–	4/4[3]	2/3	2/2[2(2)]	

[a] Maximal electroshock test (number of animals protected/number of animals tested); [b] Subcutaneous pentylenetrazole test; [c] Rotorod toxicity (number of animals exhibiting toxicity/number of animals tested); [d]The classifications are as follows; **1**: anticonvulsant activity at 100 mg/kg or less; **2**: anticonvulsant activity at doses greater than 100 mg/kg; **3**: compound inactive at 300 mg/kg, [1] Death following clonic seizure, [2( )] -Death (number of deaths), [3] Unable to grasp rotorod; Form: [x] solution, [xx] suspension.

Table 2: Anticonvulsant screening project (Phase VIa ASP); Test results in rats

Test	Compd.	Time (h)				
		0.25	0.50	1.00	2.00	4.00
MES[a]	**5**[*]	1/4	0/4	0/4	0/4	1/4
	5[**]	2/4	2/4	1/4	1/4	0/4
TOX[c]	**5**[*]	0/4	0/4	0/4	0/4	0/4
	5[**]	0/4	0/4	1/4	0/4	0/4
MES[a]	**13**[*]	1/4	0/4	0/4	0/4	1/4
TOX[c]	**13**[*]	0/4	0/4	0/4	0/4	0/4

[*] p.o. identification (dose 30 mg/kg.), [**] i.p. identification (dose 50 mg/kg); Meaning of other superscripts as for Table **1**.

Table 3: Anticonvulsant screening project; Test result in mice (i.p.; dose 100 mg/kg), Threshold Tonic Extension (TTE) Test

Compd.	Time (h)					
	0.25	0.5	1	2	4	6
5	0/4[a]	0/4	0/4	1/4	2/4	0/4
8	1/4	0/4	0/4	1/4	0/4	
10	1/4	0/4	0/4	1/4	0/4	–

[a] MES confirmation; number of animals protected/number of animals tested.

at 4 h) and 300 mg/kg (1/1 animals protected at 0.5 h) in the MES screen. Compound **13** was the most effective: in the MES screen, protected 1/3 mice (100 mg/kg) at 4 h and 1/1 mice (300 mg/kg) at 0.5 h; in the scMet screen protected 2/5 mice (100 mg/kg) at 1 h and 1/1 mice (300 mg/kg) post drug administration. This compound was active at 30 mg/kg (p.o. dose) in the MES screen in rats 1/4 animals protected at 0.25 h and 4 h. However, the active amides **12**–**14** at dose 100 and 300 mg/kg in mice screen were toxic.

The active compounds **12**–**14** were tested for their ability to displace [^{3}H]nitrendipine (voltage-sensitive calcium channel receptors) from rat cortex. Binding studies presented in Table 4 and the Fig. are in agreement with the

Table 4: [^{3}H] Nitrendipine displacement by the investigated amides 12–14

Compounds	K_i (μM)
Nifedipine	0.001
Carbamazepine	5.5
12	77
13	16.3
14	53

K_i value was calculate according to the Cheng-Prusoff equation [25]
$K_i = IC_{50}/(1 + ([L]/[K_D]))$, where [L] = ligand concentration and K_D = affinity [^{3}H]-nitrendipine

pharmacological results and show that amides **12**–**14** possess affinity to voltage-sensitive calcium channel receptors. K_i values, as measure of affinity, of tested compounds, to [^{3}H]nitrendipine binding sites in rat cerebral cortex are as follows: 16.3 μM for **13**, 53 μM for **14**, and 77 μM for amide **12**. The shape of the curves suggested competitive binding. Amide **13** was the most active both in pharmacological and biochemical tests. These preliminary results suggest that the anticonvulsant activity of compounds **12**–**14** may be related to their influence on voltage-sensitive calcium channel receptors.

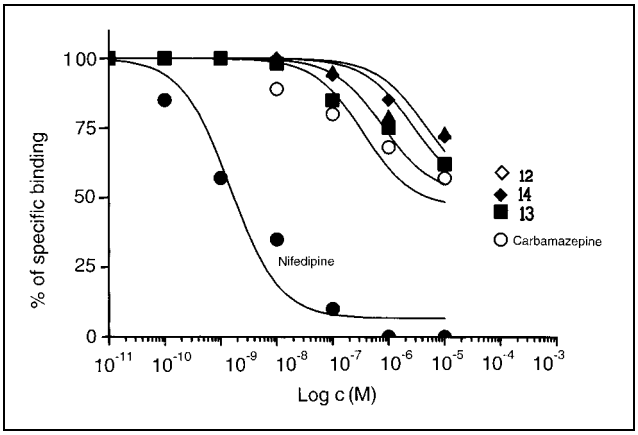


Fig.: Displacement of [^{3}H]-Nitrendipine (Ca^{2+} channel) – by investigated compounds in rat cerebral cortex.

3. Experimental

3.1. Apparatus, source and preparation of the starting materials

Satisfactory elemental analyses ±0.4% of the calculated values were obtained for the new compounds. Uncorected m.p.'s were determined on a Buchi apparatus. The MS at 70 eV were taken with AMD-604 spectrometer. ^{1}H, ^{13}C NMR spectra were recorded on a Gemini spectrometer (200 MHz), chemical shifts are reportes in δ values [ppm] relative to the internal reference of $(CH_3)_4Si$. TLC was conducted on precoated Kieselgel 60 T_{254} plates [Ar. 5554, Merck] using $S_1 = CHCl_3/(CH_3)_2CO$ (1 : 1), $S_2 = MeOH/NH_3$ (25% aq.) (98 : 2), $S_3 = n$-BuOH/CH_3COOH/H_2O (3 : 1 : 1), $S_4 = CHCl_3/CH_3OH/CH_3COOH$ (60 : 10 : 5), $S_5 = CHCl_3$/MeOH/NH_3 (25% aq.) (2 : 2 : 1) as solvent systems. Spots on TLC were detected by their absorption under UV light. CC was performed on silica gel 60 (70–23 mesh ASTM).
2-Chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) from Institut of Organic Chemistry, Technical University of Łódź, Poland. α-Bromo-γ-butyrolactone and carbonyldiimidazole were commercial products (Aldrich). Syntheses of the other starting materials were described previously: 3-(4-phenylpiperazino)-tetrahydrofuran-2-one (**1**), 3-(4-benzylpiperidino)-tetrahydrofuran-2-one (**2**), 2-(4-phenylpiperazino)-4-phthalimidobutyric acid (**3**) and 2-(4-benzylpiperidino)-4-phthalimidobutyric acid (**4**) [7, 14].

3.2. Preparation of acids 5 and 6 (general procedure)

To a solution of 5 mmol of the corresponding 2-(4-substituted)-4-phthalimidobutyric acid in 30 ml absolute ethanol 2.5 ml (50 mmol) of hydrazine hydrate were added. The reaction mixture was stirred for 2 h at room temperature. The alcohol was removed in vacuo, the residue was acidified with 25% HCl at 50 °C for 10 min and the mixture was stirred at RT. for about 2 h. The insoluble phthalylhydrazine was removed by filtration and the filtrate was treated with saturated aqueous $NaHCO_3$. The product was extracted in *n*-BuOH (3 × 50 ml), then the organic layer was separated, dried over Na_2SO_4 and concentrated under reduced pressure to afford the expected acid, that was crystallized in the appropriate solvent.

3.2.1. 2-(4-Phenylpiperazin-1-yl)-4-aminobutyric acid (5)

From **3**. Yield 78 % (from EtOH/H_2O). Mp 247–249 °C. TLC: $R_f = 0.30$ (S_3), $R_f = 0.55$ (S_5). MS (70 eV); *m/z* (%) = 263 (0.1) [M^+], 245.2 (27.12) [M^+-H_2O], 189 (39.7), 175 (21.39), 161 (42.58), 145 (9.26), 132 (98.5), 120 (49.48), 113 (100), 105 (58.37), 100 (5.97), 91 (23.55), 77 (38.9).
$C_{14}H_{21}N_3O_2$ (263.3)

3.2.2. 2-(4-Benzylpiperidin-1-yl)-4-aminobutyric acid (6)

From **4**. Yield 65 % (from 96% EtOH). Mp 233–236 °C. TLC: $R_f = 0.18$ (S_2), $R_f = 0.62$ (S_5). MS (70 eV); m/z (%) = 276 (0.2) [M^+], 258 (3,0) [M^+-H_2O], 232 (8.88), 215 (8.57), 202 (60.77), 189 (15.44), 188 (100), 174 (69.78), 117 (5.95), 110 (5.75), 91 (35.04), 82 (12.47), 56 (25.8).
$C_{16}H_{24}N_2O_2 + 2\ H_2O$ (312.4)

3.3. Synthesis of amides 7–11 (general procedure)

Method A

Activation: To a stirred solution of CDMT (0.88 g, 5 mmol) and 2-(4-phenylpiperazino)-4-phthalimidobutyric acid (1.96 g, 5 mmol) in DMF or DMAC (dimethylacetamide) (20 ml), NMM (*N*-methylmorpholine) (0.56 ml, 5.1 mmol) was added dropwise at such a rate as to keep temperature at −5 to 0 °C, and stirring was continued at 0 °C for 3 h.
Coupling: To the crude solution obtained as described above, a mixture of the appropriate amine (5 mmol) and NMM (0.55 ml, 5 mmol) in DMF or DMAC (5 ml) was added at −5 to 0 °C. Stirring was continued at 0 °C for 1 h, then at RT for 16 h. The resultant clear solution was diluted with water (80–100 ml), the precipitated product was collected, washed with H_2O (50 ml), dried, and then purified by crystallization.

Method B

Carbonyldiimidazole (0.81g, 5 mmol) was added to a solution of compound **3** (1.96 g, 5 mmol) in dry DMF (20 ml). Thirty minutes later, a solution of the appropriate amine (5 mmol) in 5 ml DMF was added and the reaction mixture was stirred at RT overnight. Then, the solution was diluted with water (80–100 ml), the crude product was collected, washed with water (50 ml), dried and purified by recrystallization.

3.3.1. N-(2-Chlorobenzyl)-2-(4-phenylpiperazin-1-yl)-4-phthalimidobutyric amide (7)

Method **A**: yield 87% (from EtOH). Method **B**: yield 52 %. Mp 149.5–150.7 °C. TLC: $R_f = 0.83$ (S_1), $R_f = 0.86$ (S_4). MS (70 eV); *m/z* (%) = 517.1 (0.66) [M^+], 516 (2), 348.3 (100), 201.6 (52.9), 186.4 (1.6), 173 (1.1), 160.1 (7.3), 144 (1.4), 132.2 (6.4), 125.3 (2), 104.4 (3.9), 91.2 (1.3), 77 (2.5). ^{1}H NMR (DMSO-d_6): δ (ppm) = 1.96 (m) CH_2 (3), 2.66 (m) 4H, 3.10 (m) 4H, piperazine, 3.28 (t), 1H (C-2) J = 6.75 Hz, 3.70 (t) CH_2 (4) J = 6.9 Hz, 4.23 (dd) 1H (C-5) ^{3}J = 5.6 Hz, J_{gem} = 15.32 Hz, 4.32 (dd) J = 4.87 Hz, 4.34 (dd) J = 4.97 Hz, 4.43 (dd) 1H (C-5) ^{3}J = 5.7 Hz, $J_{gem.}$ = 15.34 Hz, 6.75 (t) 1H (C-19) J = 7.2 Hz, 6.86 (d) 2H (C-17, -21) J = 8.06 Hz, 7.18 (t) 2H (C-18, -20) J = 7.87Hz, 7.24–7.45 (m) 4H (C7–C10), 7.85 (m) 4H phth., 8.44 (t) N-H J = 5.7 Hz.
$C_{29}H_{29}N_4O_3Cl$ (517.0)

3.3.2. N-(3,4-Dimethoxybenzyl)-2-(4-phenylpiperazin-1-yl)-4-phthalimidobutyric amide (8)

Method **A**: yield 90 % from toluene. Mp 181.1–182.7 °C. TLC: $R_f = 0.78$ (S_1), $R_f = 0.82$ (S_4). MS (70 eV); *m/z* (%) = 542.8 (0.80) [M^+], 542.1 (2.3), 348.3 (100), 201.4 (51.3), 186.4 (1.5), 160.1 (6.6), 151.1 (6.7), 132.2 (6.1), 104 (3.4), 96.3 (2.1), 91.3 (2.6), 77 (2.3). ^{1}H NMR (DMSO-d_6): δ (ppm) = 1.93 (m) CH_2 (3), 2.65 (m) 4H, 3.0 (m) 4H, piperazine, 3.21 (t) 1H (C-2) J = 7.1 Hz, 3.68, 3.70 (s) CH_2 (C-4), 6H (−O−CH_3), 4.11 (dd) 1H (C-5), ^{3}J = 5.45 Hz, $J_{gem.}$ = 14.8 Hz, 4.27 (dd) 1H (C-5), ^{3}J = 6.1 Hz, $J_{gem.}$ = 14.8 Hz, 6.75 (m) 2H, 6.85 (m) 4H (C-19, -17, -21, -7, -8, -11), 7.18 (t) 2H (C-18, -20) J = 7.84 Hz, 7.85 (m) 4H phth., 8.37 (t) N-H, J = 5.7 Hz.
$C_{31}H_{34}N_4O_5$ (542.6)

3.3.3. N-Benzyl-2-(4-phenylpiperazin-1-yl)-4-phthalimidobutyric amide (9)

Yields: Method **A**: 90% Method **B**: 65%

3.3.4. N-(4-Methoxybenzyl)-2-(4-phenylpiperazin-1-yl)-4-phthalimidobutyric amide (10)

Yields: Method **A**: 89% Method **B**: 52%

3.3.5. N-(4-Fluorobenzyl)-2-(4-phenylpiperazin-1-yl)-4-phthalimidobutyric amide (11).

Yields: Method **A**: 91% Method **B**: 59%

3.4. Synthesis of amides 12–14 (general procedure)

To a solution of 5 mmol of the corresponding *N*-substituted amide of 2-(4-phenylpiperazine)-4-phthalimidobutyric acid in 30 ml absolute ethanol 0.5 ml (10 mmol) of hydrazine hydrate were added. The reaction mixture was stirred 24 h at RT, then was acidified with HCl gas solution in EtOH and heated at 50 °C for about 2 h. The insoluble phthalylhydrazine was removed by filtration and the filtrate was evaporated to dryness in vacuo. The residue was purified by CC and crystallization. Amides **12–14** were converted into hydrochloride salts.

3.4.1. N-Benzyl-2-(4-phenylpiperazin-1-yl)-4-aminobutyric amide (12)

From **9**. The product was purrified by CC using S_2 as solvent system. Then the residue was dissolved in anhydrous ethanol and the solution HCl in ethanol was added until the mixture became acidic. The solvent was evaporated, the product was washed with anhydrous ether and dried in vacuo. Yield 52 %. Mp 146 °C (dec.) TLC: $R_f = 0.19$ (S_2), $R_f = 0.91$ (S_5). MS (70 eV); m/z (%) = 352.2 (2.4) [M^+], 334 (1.6), 292 (1.9), 245 (7.5), 234 (7.9), 218 (45.8), 189.4 (100), 161.9 (24.9), 132 (17.3), 120 (9.8), 104.4 (22.9), 91 (9.4), 84 (9.9), 77 (7.2). ^{1}H NMR (DMSO-d_6): δ (ppm) = 2.24 (m) CH_2 (3), 2.84 (m) CH_2 (4), 3.29 (m) 2H, 3.39 (m) 6H piperazine protons, 3.99 (m) CH (2), 4.25 (m) CH_2 (5), 6.68 (t) 1H (C-19) J = 8.1 Hz, 6.98 (d) 2H (CH-17, -21) J = 8.07 Hz, 7.24–7.37 (m) 7H (C-18, -20, C7–C11), 8.05 (s) 3H, NH_3^+, 9.41 (t) N-H, J = 5.7 Hz. ^{13}C NMR δ = 26.13 (C-3), 36.24 (C-4), 43.76 (C-5), 47.06, 50.57 (piperazine carbons), 65.38 (C-2), 117.37 (C-17, -21), 122.1 (C-19), 128.34 (C-7, -11), 129.63 (C-8, -10, -18, -20), 130.29 (C-9), 138.49 (C-6), 149.66 (C-16), 166.80 (C-1).
$C_{21}H_{30}N_4O + 2$ HCl (425.4)

3.4.2. N-(4-Methoxybenzyl)-2-(4-phenylpiperazin-1-yl)-4-aminobutyric amide (13)

From **10.** The product was purified by crystallization from isopropanol, then washed with anh. ether and dried in vacuo. Yield 62%. Mp 163 °C (dec.). TLC: $R_f = 0.26$ (S_2), $R_f = 0.24$ (S_4). MS (70 eV); m/z (%) = 382.1 (2.9) [M^+], 364 (2.1), 264 (4.5), 245 (19.6), 218 (44.3), 189 (100), 161 (18.9), 135.9 (26.1), 132 (33.5), 121 (35.4), 105 (20.8), 83.9 (15.6), 76.9 (19.9). ^{1}H NMR (DMSO-d_6): δ (ppm) = 2.29 (m) CH_2 (3), 2.82 (m) CH_2 (4), 3.37 (m), 3.60 (m) 9H, piperazine protons, CH(2), 3.73 (s) (OCH_3), 4.30 (d), CH_2 (5) J = 5.42 Hz, 6.82-6.92 (m) 3H (C18–C20), 6.99 (d), 2H (C-17, -21), J = 8.14 Hz, 7.27 (m) 4H (C-7, -8, -10, -11), 8.32 (s) NH_3^+, 9.66 (t) N−H. ^{13}C NMR δ = 25.29 (C-3), 35.31 (C-4), 42.11 (C-5), 45.50, 49.36 (piperazine carbons), 55.14 (−OCH_3), 63.71 (C-2), 113.95 (C-8. -10), 116.08 (C-17, -21), 120.30 (C-19), 128.95 (C-7, -11), 129.19 (C-18, -20), 130.02 (C-6), 149.36 (C-16), 158.47 (C-9), 165.65 (C-1).
$C_{22}H_{30}N_4O + 3HCl + H_2O$ (510.0)

3.4.3. N-(4-Fluorobenzyl)-2-(4 phenylpiperazin-1-yl)-4-aminobutyric amide (**14**)

From **11**. The product was purrified by crystallization from isopropanol, then by CC using S_2 as solvent system. Compound **14** was dissolved in anh. ethanol and a solution of HCl in ethanol was added until the mixture became acidic. The solvent was evaporated, the product was washed with anhydrous ether and dried in vacuo. Yield 55 %. Mp 138–140 °C (dec.). TLC: $R_f = 0.13$ (S_2), $R_f = 0.19$ (S_4). MS (70 eV); m/z (%) = 370.1 (2.8) [M^+], 352 (1.2), 264.3 (3.1), 252.5 (8), 245.6 (3.6), 238.5 (5.2), 218.5 (41.9), 189.3 (100), 161.6 (6.3), 132.4 (14.9), 109.6 (6.9), 84 (10), 77 (3.4). ^{1}H NMR (DMSO-d_6): δ (ppm) = 2.19 (m) CH_2 (3), 2.80 (m) CH_2 (4), 3.19, 3.29 (m) 4H, 3.35 (m) 4H pipererazine protons, 3.90 (m) CH(2), 4.32 (d) CH_2 (5) J = 5.8 Hz, 6.86 (t) (C-19) J = 8.2 Hz, 6.96 (d) 2H (C-17, -21) J = 8.16 Hz, 7.11 (t) 2H (CH-18, -20) J = 8.2 Hz, 7.21–7.35 (m) 4H, (C-7, -8, -10, -11), 8.05 NH_3^+, 9.36 (t) N–H, J = 5.6 Hz. ^{13}C NMR δ = 25.95 (C-3), 36.24 (C-4), 42.72 (C-5), 47.05, 50.25 (piperazine carbons), 65.14 (C-2), 115.82, 116.24 (C-8, -10), 116.93 (C-17, -21), 121.38 (C-19), 130.02 (C-18, -20), 130.19, 130.35 (C-7, C-11), 134.84, 134.90 (C-6), 149.93 (C-16), 159.65, 164.48 (C-9), 167.25 (C-1).
$C_{12}H_{31}N_4O + 3\ HCl + 0.5\ H_2O$ (488.9)

3.5. Pharmacology (anticonvulsant assays)

Animal anticonvulsant and neurotoxicity assays were conducted by the Antiepileptic Drug Development (ADD) Program, Epilepsy Branch Neurological Disorders Program, National Institutes of Neurological and Communicative (NINCDS) Disorders and Stroke, Bethesda, Maryland U.S.A. Male albino mice (18–25 g) and male albino rats (100–150 g) were used in the experiment. Compounds were suspended in 0.5% methylcellulose and administered intraperitoneally to mice and orally to rats.

3.5.1. MES-Maximal Electroshock Seizure Test [22]

Maximal electroshock seizures were elicited with a 60 cycle alternating current of 50 mA intensity (5–7 times that necessary to elicit minimal electroshock seizures) delivered for 0.2 s via corneal electrodes. A drop of 0.9% saline was instilled in the eye prior to application of the electrodes in order to prevent the death of the animal. Abolition of the hind limb tonic extension component of the seizure is defined as protection, and results are expressed as number of animals protected/number of animals tested.

3.5.2. Subcutaneous Metrazol Anticonvulsant Screens [19]

The scMet seizure threshold test was performed by administering 85 mg/kg of metrazol as a 0.5% solution in the posterior midline. Protection in this screen was defined as a failure to observe a single episode of clonic spasms of at least 5 s duration during a 30 min period following administration of the test compound.

3.5.3. Neurotoxicity

The rotorod test was used to evaluate neurotoxicity [23]. The animal was placed on a 1 in. diameter knurled plastic rod rotating at 6 rpm. Normal mice can remain on a rod rotating at this speed indefinitely. Neurologic toxicity is defined as the failure of the animal to remain on the rod for 1 min and is expressed as number of animals exhibiting toxicity/number of animals tested.

3.5.4. The Threshold Tonic Extension (TTE) Test [21]

Twenty mice were pretreated intraperitoneally with 100 mg/kg of the test substance. At several time intervals (1/4, 1/2, 1, 2 and 4 h) post treatment with the test compound, four mice at each time point were challenged with 12.5 mA of electrical current for 0.2 s via corneal electrodes. This produced a TTE seizure in the animals. For each time interval results were expressed as a ratio of the number of animals protected over the number tested.

3.6. Binding experiments. [^{3}H]Nitrendipine displacement by the investigated compounds (**12–14**)**: *Membrane preparation and receptor binding assay***

The cerebral cortex of naive rats were used. The tissue was homogenized using a polytron disintegrator (setting 4, 15 s) at 0 °C in 20 volumes 50 mmol/l Tris-HCl buffer, pH 7.6. The homogenate was centrifuged at 0 °C and 1000 × g for 10 min, the supernatant was decanted and recentrifuged at 0 °C and 25000 × g for 30 min. The pellet thus obtained (fraction P2) was reconstituted in Tris-HCl buffer to give a final protein concentration (measured according to Lowry et al. [24]) of approximately 0.8 mg/ml. The incubation mixture (final volume 550 µl) consisted of 450 µl membrane suspension, 50 µl of [^{3}H] Nitrendipine (NEN, specific activity 78.3 Ci/mmol solution was prepared in the dark in concentration 0.8 nM) and 50 µl buffer containing six concentrations of nifedipine (0.01 nM–1 µM), or eight concentrations (0.1 nM–100 µM) of carbamazepine, or investigated compounds (in concentrations 1 nM–100 µM). For measuring unspecific binding, nifedipine in a final concentration of 1 µM was used. The incubation was carried out in duplicate, in a shaking water bath, at 25 °C for 30 min. Addition of the radioligand initiated the incubation, which was terminated by rapid filtration through GF/C Whatman fiberglass filters. The filters were then rinsed twice with 5 ml portions of ice-cold incubation buffer and placed in plastic scintillation minivials. Scintillation fluid (Akwascynt, BioCare) was added (3 ml) and the samples were counted for radioactivity in a Beckman LS 3801 scintillation counter.

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