ChemInform Abstract: Synthesis, anti-GABA Activity and Preferred Conformation of Bicuculline and Norbicuculline Enantiomers

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Synthesis, anti-GABA activity and preferred conformation of bicuculline and norbicuculline enantiomers

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Summary — Synthesis of *erythro*-(±)-[1*SR*,9*RS*]-norbicuculline and *threo*-(±)-[1*SR*,9*SR*]-noradlumidine from piperonal was performed using Bischler–Napieralski cyclization as a key step. Resolution gave rise to (+)-[1*S*,9*R*]-norbicuculline ([1*S*,9*R*] norBIC) and (–)-[1*R*,9*S*]-norbicuculline ([1*R*,9*S*] norBIC) in >99.5% enantiomeric purity. Bicuculline enantiomers were readily obtained by methylation of the latter products. [1*S*,9*R*]BIC was about 70 times more potent than [1*R*,9*S*] BIC as an inhbitor of $GABA_A$ receptor binding and was about 100 and 900 times more potent than [1*S*,9*R*] norBIC at pH 7.1 and 5.0 respectively. Similarly, [1*S*,9*R*] norBIC was much less potent than [1*S*,9*R*] BIC as an inhibitor of GABA-specific $^{36}Cl^-$ ion flux. The observed increase of about two orders of magnitude in the in vitro biological activity caused by *N*2-CH_3 substitution in [1*S*,9*R*] norBIC was attributed to different conformations for *erythro*- and nor-*erythro*-bicucullines indicated by ^1H nuclear Overhauser enhancements of [1*S*,9*R*] BIC and [1*S*,9*R*] norBIC.

bicuculline enantiomer / norbicuculline enantiomer / Bischler–Napieralski cyclization / ^1H nuclear Overhauser enhancement / [^3H]GABA binding / $^{36}Cl^-$ ion flux

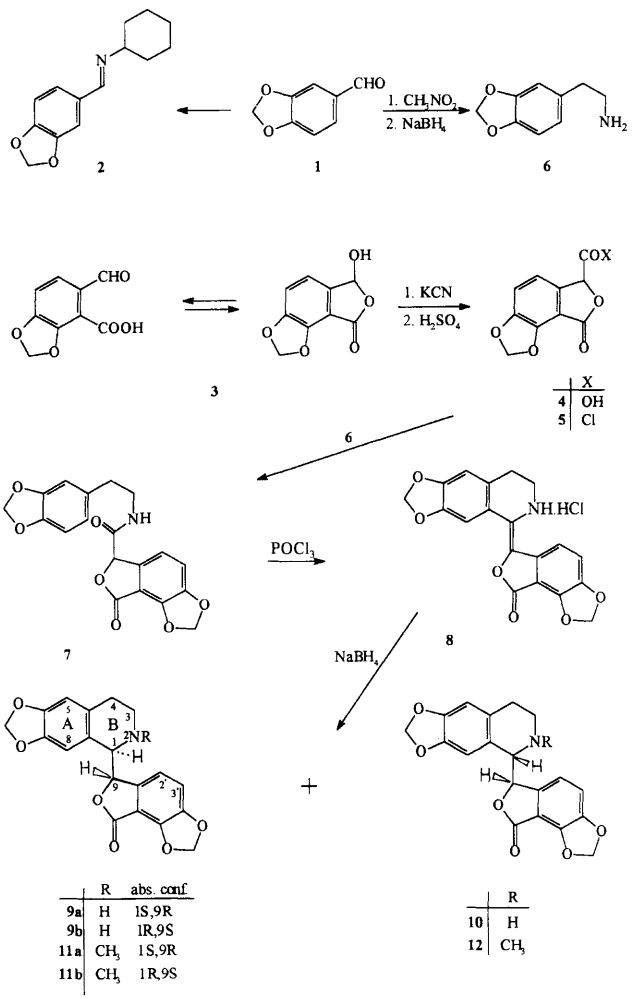
Since the announcement by Curtis et al [1] that the phthalideisoquinoline (PIQ) alkaloid, [1*S*,9*R*] BIC possessed potent inhibitory activity against the depressant action of GABA in the central nervous system, it has been of interest to study the effect of its antipode, [1*R*,9*S*] BIC. The effect of [1*R*,9*S*] BIC, however, has not been unequivocally reported because of the scarcity of this natural product and the confusion caused by the reversal of the sign of optical rotation for quaternary bicuculline derivatives [2]. Based on the available structural data and in vitro biological activity measurements of 39 PIQ derivatives, a good correlation was found [3] between activities and preferred conformations of *erythro* and *threo* PIQs and analogs.

We report herein a synthetic route for bicuculline and norbicuculline enantiomers. The effect of *N*-methyl substitution on $GABA_A$ receptor activity and preferred conformations were evaluated by [^3H] GABA binding, GABA specific $^{36}Cl^-$ ion flux and ^1H nuclear Overhauser enhancement (nOe) measurements respectively.

Chemistry

[1*S*,9*R*] BIC **11a**, first encountered as a constituent of *Dicentra cucullaria* [4] and subsequently found in other species of genera *Rhoeadales* [5, 6], was first totally synthesized by Groenewoud and Robinson [7] as early as 1936. Reissert synthesis of [1*SR*,9*RS*] norBIC has been cited [8] as an unpublished result of Kerekes [8], however, total synthesis of [1*SR*,9*RS*] norBIC **9** has not been described up to now.

As demethylation of PIQ alkaloids results in decomposition of the molecule, a linear approach to [1*SR*,9*RS*] BIC via [1*SR*,9*RS*] norBIC had to be elaborated to meet our demand to produce [1*S*,9*R*] BIC and [1*S*,9*R*] norBIC as well as [1*R*,9*S*]BIC and [1*R*,9*S*] norBIC. For this purpose the Bischler–Napieralski route, applied to the synthesis of (±)-[1*SR*,9*RS*]-α-narcotine [9–11], seemed to be appropriate. The two building blocks needed for Bischler–Napieralski cyclization, ie, carboxylic acid **4** (scheme 1; stereostructures are represented according to [12]) and homopiperonylamine **6** [13], were both prepared from pipe-



Scheme 1.

ronal **1**. According to Ziegler and Fowler [14], **1** gave a Schiff base **2** with cyclohexylamine. This was treated with butyllithium at –78 °C, then with CO_2 in THF/hexane solution to produce carboxylic acid **3** after hydrolysis. Phthalide-3-carboxylic acid **4** was obtained from **3** in two steps; addition of HCN was followed by aqueous sulfuric acid hydrolysis [9]. Compound **4** was then converted to acyl chloride **5** with thionyl chloride, which, without isolation, was reacted with homopiperonylamine **6** obtained from piperonal **1** in two steps via nitrostyrol [13], to supply amide **7**. $POCl_3$ cyclization of **7** led to $\Delta^{1,9}$-norBIC **8** containing a double bond between the two rings ('enamine form') even in the protonated salt form (no C9-H signal could be observed in the ^{1}H-NMR spectra of **8** in D_2O solution). Sodium borohydride reduction of **8** resulted in a diastereomeric mixture of *erythro*-[1*SR*,9*RS*] norBIC **9** and its *threo* epimer (±)-[1*SR*,9*SR*]-noradlumidine ([1*SR*,9*SR*] norADLD, **10**) in a ratio of about 5:1. The relative stereochemistry of the chiral atoms C1 and C9 in the two compounds was deduced from the characteristic chemical shifts of the C2'-H protons (5.90 and 6.93 ppm for the major and minor epimers respectively) and by comparing the different nOe values of protons C1-H, C8-H, C9-H and C2'-H (table I) with other *erythro* and *threo* PIQ alkaloids [15].

After separating the two diastereomeric racemates on the column, the major *erythro* base **9** was resolved in acetone with (–)- and (+)-*O*,*O*-dibenzoyltartaric acid and the enantiomeric purity was checked on a chiral HPLC column (Chiralcel OD) showing a baseline separation of **9a** and **9b**. After repeated recrystallization of the two diastereomeric salt-pairs, [1*S*,9*R*] norBIC **9a** and [1*R*,9*S*] norBIC **9b** were obtained in more than 99.5% optical purity. Eschweiler–Clark or methyl iodide methylation of the optically pure [1*S*,9*R*] norBIC and [1*R*,9*S*] norBIC gave [1*S*,9*R*] BIC and [1*R*,9*S*] BIC **11a** and **11b** respectively, the

Table I. Nuclear Overhauser enhancement data (%) in ^{1}H NMR.

Proton irradiated	*Proton observed*				
	C2'-H	*C1-H*	*C8-H*	*C9-H*	*N2-CH$_3$*
10[a]					
H8	–	4.4	–	15.4	–
H9	2.4	6.3	15.3	–	–
H1	5.6	–	5.5	7.4	–
12[b]					
H8	–	6.8	–	3.5	–
H9	2.2	7.3	4.4	–	4.4
H1	2.0	–	9.8	8.2	6.4[c]
9a[d]					
H8	1.9	3.4	–	15.0	–
H9	2.3	6.4	17.8	–	–
H1	1.0	–	5.4	6.8	–
11a[e]					
H8	–	7.6	–	10.7	–
H9	3.0	8.3	12.0	–	0.8
H1	3.6	–	10.3	8.9	6.4[c]

[a] $J_{C1\text{-}H,C9\text{-}H}$ = 3.2 Hz; $\delta_{C4\text{-}Hax}$ = 2.74 ppm. [b] $J_{C1\text{-}H,C9\text{-}H}$ = 3.3 Hz; $\delta_{C4\text{-}Hax}$ = 2.75 ppm. [c] Share of C3-H is higher in [1*SR*,9*SR*] ADLD than in [1*S*,9*R*] BIC. [d] $J_{C1\text{-}H,C9\text{-}H}$ = 4.0 Hz; $\delta_{C4\text{-}Hax}$ = 2.43 ppm. [e] $J_{C1\text{-}H,C9\text{-}H}$ = 4.1 Hz; $\delta_{C4\text{-}Hax}$ = 2.24 ppm; no nOe was observed between *N*2-CH_3 and C4-H_{ax} protons suggesting the following configuration: *N*2-CH_3 is equatorial (α); lone pair electrons at *N*2 (*N*2-LPE) are axial (β).

former being identical in every respect (mp, TLC, IR, ^{1}H- and ^{13}C-NMR, MS) with the natural sample, thus providing a final proof of the *erythro* assignment for [1*SR*,9*RS*] norBIC (**9**).

The *threo* racemate ([1*SR*,9*SR*] norADLD, **10**) and its methylated product ([1*SR*,9*SR*] ADLD, **12**) were also obtained but not biologically evaluated.

Biological data

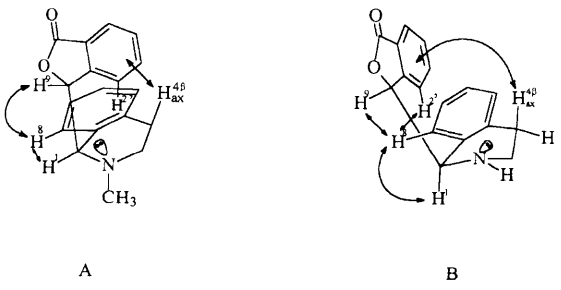
Enantiomers of **9** and **11** were initially evaluated for inhibition of [^{3}H]GABA binding in membranes from the rat cerebral cortex. As shown in table II, $GABA_A$ receptors expressed greater enantioselectivity towards **11a** and **11b** than **9a** and **9b**, a result that is consistent [3] with the higher potency of **11a**. As racemic bicuculline and norbicuculline appeared to have identical activities and so were thought to possess similar conformations [3], we were quite surprised to find that *N*2–H → *N*2–CH_3 substitution in **11a** caused an approximately 100-fold increase in inhibition of $GABA_A$ receptor binding compared with **9a**.

The effect of *N*2–H → *N*2–CH_3 substitution on [^{3}H]-GABA binding was further evaluated with the protonated **9a** and **11a** analogs. By protonation at pH 5.0 [16], the affinity of **11a** increased (K_i = 0.39 μM, K_{GABA}/K_i = 0.11), while that of **9a** decreased (K_i = 340 μM, K_{GABA}/K_i = 0.0001).

Substantial increase in the affinity caused by *N*2-CH_3 substitution of unprotonated (K_{9a}/K_{11a} = 100) and protonated (K_{9a}/K_{11a} = 870) analogs and the differential effect of protonation on the affinities of **11a** ($K_{11a}/K_{11a,H+}$ = 2.3) and **9a** ($K_{9a}/K_{9a,H+}$ = 0.27) suggested that the *erythro* conformations of bicuculline and norbicuculline were different. A comparison of the nOe values and the chemical shift data for **11a** and **9a** (table I) revealed that (i) nOe interactions of C1-H and C8-H in [1*S*,9*R*] BIC were twice as large as in [1*S*,9*R*] norBIC; (ii) smaller $\delta_{C4\text{-}Hax}$ in [1*S*,9*R*] BIC (2.24 ppm vs 2.43 ppm) indicated that C4-H_{ax} is in the shielding zone of the phthalide ring; (iii) the nOe effects of C8-H and both C2'-H and C9-H were larger in [1*S*,9*R*] norBIC than in [1*S*,9*R*] BIC. These nOe interactions are indicated in figure 1. The nOe data are in agreement with a conformational change of [1*S*,9*R*] norBIC which results in an increased distance between the lone-pair electrons (*N*2-LPE) and the carbonyl group (fig 1).

The inhibitory effect of these bicuculline derivatives (**9a**, **9b**, **11a** and **11b**) on bicuculline-sensitive GABA binding and GABA receptor function was compared (table II). Inhibition of the in vitro binding of [^{3}H] GABA is parallel to the inhibition of GABA-mediated influx of $^{36}Cl^-$ ions into the membrane vesicles (40 μM GABA, 6 s, 30 °C) by the same concentrations (46 μM) of the bicuculline derivatives. It is apparent that **9a** was a less potent antagonist of $GABA_A$ function ($^{36}Cl^-$ flux) than **11a**.

The *N*2–H → *N*2–CH_3 substitution is apparently responsible for a substantial improvement of anti-GABA activity of *erythro* PIQ derivatives. The unusually large effect was explained by the conformational change in ring B resulting in different *N*2-LPE-carbonyl distances in nor-*erythro*- and *erythro*-bicuculines.



A B

Fig 1. Different conformations of the bicuculline (**A**) and norbicuculline (**B**) skeleton as indicated by the nOe interactions.

Table II. [^{3}H] GABA binding and GABA-specific $^{36}Cl^-$ ion flux inhibition data in membranes from the rat cerebral cortex.

Compound	*Binding[a] inhibition affinity*			*$^{36}Cl^-$ transport[b] inhibition influx*	
	K_i (μM)	K_{11a}/K_i	K_{GABA}/K_i	*dpm*	*%*
GABA	0.018 ± 0.001	49.4	1.0	383 ± 48	0 ± 7
9a	91.5 ± 9.2	0.01	0.002	274 ± 59	28 ± 8
9b	365 ± 70	0.002	0.0005	360 ± 15	6 ± 4
11a	0.89 ± 0.04	1.0	0.02	5 ± 20	99 ± 5
11b	64.0 ± 6.4	0.014	0.0003	282 ± 37	26 ± 10

[a]Reduced χ_r^2 values were the following: 0.34, 2.81, 1.52, 7.35 and 0.94 for GABA, **9a**, **9b**, **11a** and **11b** respectively; other displacement experiments gave similar results; [b]data ± SEM were from six to ten determinations.

Experimental protocols

The ^1H and ^{13}C NMR spectra were taken on a UXR 400 spectrometer. Mass spectra were run on a MS 902 spectrometer. Infrared spectra were taken on a Nicolet 205 FT-I. HPLC was run on a PU 4000 with a Chiralcel OD column eluted with isopropanol. Analyses indicated by the symbols of the elements were within ±0.4% of theoretical values.

Synthesis

2-Formyl-5,6-methylenedioxybenzoic acid ***3***
Compound **3** was prepared from piperonal **1** in three steps according to Ziegler and Fowler [14] in 63% yield, mp 164–165 °C (ref [14]: mp 165–165.5 °C).

6,7-Methylenedioxyphthalide-3-carboxylic acid ***14***
To the stirred suspension of **3** (5 g, 25.7 mmol) in water (15 mL), cooled to 0–5 °C, a solution of KCN (10 g, 154 mmol) in water (25 mL) was added over 15 min and stirred for an additional 15 min. A mixture of conc HCl aq (17.5 mL) and water (17.5 mL) was added and stirred for 5 h while cooling. The precipitate was filtered off and the reaction mixture was extracted with EtOAc. The combined precipitate and oil, obtained by evaporating the dried EtOAc solution, was refluxed for 2 h in a mixture of conc H_2SO_4 (3.5 mL) and water (15 mL). After cooling, the mixture was extracted with EtOAc and the organic phase was dried and evaporated. On CH_2Cl_2 treatment the residue gave colourless crystals of **4** (4.65 g, 81%) mp 190–192 °C; anal $C_{10}H_6O_6$ (C, H, O); IR (KBr) γ-lactone, 1775 cm^{-1}, C=O, 1720 cm^{-1}; ^1H NMR (DMSO-d_6/$CDCl_3$) δ 5.80 (s, 1H, C3-H), 6.21 (s, 2H, OCH_2O), 7.12 (m, 1H, C5-H), 7.16 (m, 1H,C4-H), 8.80 (s, 1H, COOH).

Amide 7
To a stirred solution of carboxylic acid **4** (4.0 g, 18 mmol) in dry benzene, (40 mL) thionyl chloride (20 mL) was added dropwise at room temperature, then the mixture was refluxed for 2 h. The mixture containing acyl chloride **5** was evaporated in vacuo. The amine **6** [13] (2.9 g, 18 mmol) was dissolved in benzene (80 mL) and 2.5% aq NaOH solution (18 mL) was added with stirring. A solution of acyl chloride **5** in benzene (30 mL) was added and stirred for 1 h. The precipitate formed was filtered off. The benzene solution was dried and evaporated. The residue and the precipitate were combined and recrystallized from EtOAc to yield amide **7** (3.6 g, 54%), mp 165–166 °C; anal $C_{19}H_{15}NO_7$ (C, H, N, O); mass spectrum *m/z* (%) 369 (M^+, 126), 339 (2), 177 (25), 148 (100); IR (KBr) NH, 3280 cm^{-1}, lactone C=O, 1760 cm^{-1}, amid C=O, 1660 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.71 (t, 2H, benzyl-CH_2), 3.46 (m, 2H, NHCH_2), 5.68 (s, 1H, OCH), 5.90 (s, 2H, OCH_2O), 6.20 (s, 2H, OCH_2O), 6.49 (s, 1H, NH), 6.52 (s, 1H, C2-H), 6.68 (s, 1H, C5-H), 7.12 (m, 1H, C3'-H), 7.30 (m, 1H, C2'-H); ^{13}C NMR (DMSO-d_6/$CDCl_3$); 34.65 (benzyl-C), 40.32 (CH_2NH), 78.47 (OCH), 100.50 + 103.25 (OCH_2O), 106.33 (C6'), 107.85 (C5), 108.65 (C2), 114.11 (C2'), 115.89 (C3'), 121.27 (C6), 131.77 (C1), 137.36 (C1'), 144.57, 145.79, 147.27 and 149.41 (C3, C4, C4' and C-5'), 166.07 (C=O).

$\Delta^{1,9}$-Norbicuculline ***8***
Amide **7** (3.50 g, 9.5 mmol), dissolved in freshly distilled $POCl_3$ (35 mL), was stirred at 100 °C for 1 h under argon. The cooled mixture was poured onto ice (300 g), extracted with diethyl ether, the water phase was then neutralized with conc NH_4OH, the yellow precipitate filtered, dissolved in EtOAc and acidified with conc HCl to pH 4. The crystals were filtered to give $\Delta^{1,9}$-norBIC. HCl **8** (2.7 g, 76%) mp 173–174° C (MeOH); anal $C_{19}H_{14}NO_6Cl$ (C, H, N,O, Cl); mass spectrum *m/z* (%) 351 (M – HCl, 85), 322 (100), 294 (11), 177 (91), 36 (HCl) ; IR (KBr) = N^+H_2, 3290 cm^{-1}, lactone C=O, 1720 cm^{-1}; ^1H NMR (D_2O) δ 3.15 (m, 2H, C4-H_2), 3.90 (m, 2H, C3-H_2), 6.12 + 6.22 (s-s, 2-2H, OCH_2O), 6.98 (s, 1H, C8-H), 7.02 (s, 1H, C5-H), 7.11 (d, 1H, C3'-H), 7.42 (d, 1H, C2'-H).

Norbicuculline ***9*** *and noradlumidine* ***10***
Cyclized product **8** (5.4 g 13.9 mmol) was dissolved in a mixture of CH_2Cl_2 (300 mL) and AcOH (6 mL), cooled to 0–5 °C. To the stirred mixture $NaBH_4$ (1.62 g, 42.8 mmol) was added in small portions (~3 h) then stirred for 1 h. Excess reducing agent was then decomposed with acetone (2 mL). The mixture was washed with water, dried ($MgSO_4$), and evaporated in vacuo. Chromatography on 250 g 63-200 mesh Kieselgel 60 (eluent: benzene/acetone, 1:1) gave **9** (2.1 g, 43%) mp 184–185 °C and **10** (0.4 g, 8%) mp 203–204 °C. **9**: Anal $C_{19}H_{15}NO_6$ (C, H, N, O); mass spectrum *m/z* (%) 353 (M^+, 1), 335 (2), 176 (100), 149 (3); IR (KBr) C=O, 1750 cm^{-1}; ^1H NMR ($CDCl_3$) 1.86 (br s, 1H, NH), 2.43 (m, *J* = 15.5 + 8.05 + 5.4 HZ, 1H, C4-H_{ax}), 2.53 (m, *J* = 15.5 + 5.4 + 4.5 Hz, 1H, C4-H_{eq}), 2.73 (m, *J* = 11.6 + 5.4 + 5.4 Hz, 1H, C3-H_{eq}), 2.83 (m, *J* = 11.6 + 8.0 + 4.5 Hz, 1H, C3-H_{ax}), 4.73 (d, *J* = 4 Hz, 1H, C1-H), 5.72 (dd, *J* = 4 + 1 Hz, 1Hz, C9-H), 5.99 + 6.15 (s-s, 2-2H, OCH_2O), 5.90 (d, *J* = 7 + 1 Hz, 1H, C2'-H), 6.60 (s, 1H, C5-H), 6.74 (s, 1H, C8-H), 6.83 (d, *J* = 7 Hz, 1H, C3'-H), see table I for nOe data; ^{13}C NMR ($CDCl_3$) δ 29.72 (C4), 40.65 (C3), 56.76 (C1), 84.62 (C9), 100.91 + 103.15 (OCH_2O), 106.28 (C8), 109.16 (C5), 109.88 (C6'), 113.21 (C2'), 115.29 (C3'), 124.93 (C4a), 130.80 (C8a), 139.78 (C1'), 144.49 (C5'), 146.07 and 146.54 (C6 and C7), 148.95 (C4'), 167.48 (C=O). **10**: Anal $C_{19}H_{15}NO_6$ (C, H, N, O); mass spectrum *m/z* (%) 353 (M^+, 1), 335 (3), 176 (100), 149 (4); IR (KBr) C=O, 1750 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.80 (br s, 1H, NH), 2.55–2.80 (m, 2H, C4-H_2), 2.90–3.20 (m, 2H, C3-H_2), 4.45 (d, *J* = 3.2 Hz, 1H, C1-H), 5.77 (d, *J* = 3.2 Hz, 1H, C9-H), 5.88 + 6.16 (d, 4H, OCH_2O), 6.55 (s, 1H, C5H), 6.70 (s, 1H, C8-H), 6.93 (d, *J* = 7 Hz, 1H, C2'-H), 7.08 (d, *J* = 7 Hz, 1H,C3'H), see table I for nOe data; ^{13}C NMR ($CDCl_3$) δ 29.91 (C4), 41.52 (C3), 58.04 (C1), 84.63 (C9), 100.82 + 103.35 (OCH_2O), 106.21 (C8), 109.11 (C5), 109.78 (C6'), 113.65 (C2'), 114.62 (C3'), 126.26 (C4a), 130.32 (C8a), 140.42 (C1'), 144.85 (C5'), 145.94 and 146.45 (C6 and C7), 149.23 (C4'), 167.24 (C=O).

(+)-[1S,9R]- and (–)-[1R,9S]-Norbicuculline ***9a*** *and* ***9b***
To the solution of **9** (200 mg, 0.6 mmol) in acetone (12 mL), a solution of (–)-*O,O*-dibenzoyltartaric acid (DBTA, 200 mg, 0.6 mmol) in acetone (4 mL) was added. After stirring for 30 min the mixture was left to stand at room temperature for 2–3 h. The tartrate salt was filtered, recrystallized from acetone, then dissolved in water (20 mL) treated with conc NH_4OH (pH 8), and extracted with CH_2Cl_2. Having distilled off the solvent the residue was crystallized from MeOH to produce **9a** (80 mg, 40%) mp 195–197 °C: optical rotation (*c* = 1, $CHCl_3$) $[\alpha]_D^{25}$ = +256°; enantiomeric purity (HPLC) >99–95%. Mother liquor treated with (+)-DBTA (100 mg, 0.3 mmol) similarly gave **9b** (90 mg, 45%): optical rotation (*c* = 1, $CHCl_3$) $[\alpha]_D^{25}$ = –250°; enantiomeric purity (HPLC) >99.5%.

(+)-[1S,9R]- and (–)-[1R,9S]-Bicuculline ***11a*** *and* ***11b***
To **9a** or **9b** (200 mg, 0.58 mmol each) dissolved in HCOOH (2.5 mL), 37% HCHO was added (0.5 mL) and stirred at 100 °C for 15 min, then evaporated in vacuo. The residue was dissolved in 10% HCl (20 mL), neutralized with conc NH_4OH

and extracted with CH_2Cl_2. The organic phase was dried over $MgSO_4$, evaporated and purified on 20 g 63-200 mesh Kieselgel 60 column (eluent: $CHCl_3$/MeOH, 10:1) to yield **11a** identical with an authentic sample (170 mg, 81%), mp 192–193 °C: optical rotation (c = 1, $CHCl_3$) $[\alpha]_D^{25}$ = +126° (ref [6]: $[\alpha]_D^{20}$ = +132.7°, c = 0.49, $CHCl_3$); and **11b** (175 mg, 84%): optical rotation (c = 1, $CHCl_3$) $[\alpha]_D^{25}$ = –124.8° (ref [6]: $[\alpha]_D^{33}$ = –128°, c = 0.27, $CHCl_3$).

*(+)-[1SR,9SR]-Adlumidine **12***

Compound **10** (100 mg, 0.29 mmol) was methylated as above. Purification on 10 g 63-200 mesh Kieselgel 60 column (eluent: $CHCl_3$/MeOH, 20:1) gave **12** (70 mg, 67%) mp 201–202 °C (205 °C in ref [7]; ^{1}H NMR nOe data are summarized in table I.

Inhibition of [^{3}H]GABA binding and GABA-specific $^{36}Cl^-$ ion flux

Cortical membranes in 50 mM Tris-HCl pH 7.1 buffer were incubated in the dark for 40 min with 4 nM [^{3}H] GABA at 4 °C in the presence or absence of test compounds [3]. Stock solutions (2 mM) of PIQ derivatives were freshly prepared in diluted HCl solution (pH 3) and stored in the dark on ice until use. After incubating **9a** and **11a** with membranes for 40 min at 4 °C, relative changes in UV absorbances at λ_{max2} = 326 nm (A_2) and λ_{max1} = 292 nm (A_1), A_2/A_1, indicated less than 5–6% opening of the phthalide ring [17]. Non-specific binding was determined in the presence of 46 μM **11a**. Goodness of fit for one-site ligand analysis [18] of the displacement experiment was expressed as the reduced χ_r^2 value (table II.) Saturation data with 2–250 nM [^{3}H] GABA indicated 0.8 pmol/mg protein density of **11a**-sensitive binding sites.

For GABA-specific $^{36}Cl^-$ transport, the fast kinetic technique [19–21] was applied. In the presence or absence of 46 μM **9a**, **9b**, **11a** or **11b**, a cortical membrane vesicle suspension [21] (0.225 mL) in 10 mM HEPES buffered physiological salt solution (HBSS, pH 7.5) was rapidly mixed with 0.225 mL of HBSS containing 15 μCi/mL $^{36}Cl^-$ ion, 40 μM GABA and incubated for 6 [19, 20] at 30 °C; under the conditions the A_2/A_1 ratio for **9a** and **11a** did not change.

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