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# INHIBITION OF [$^3$H]GABA BINDING TO RAT BRAIN SYNAPTIC MEMBRANES BY BICUCULLINE RELATED ALKALOIDS

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**Abstract**—The binding of 45 bicuculline related phthalideisoquinoline alkaloids to the $GABA_A$ receptor was studied using rat brain synaptic membranes prepared both in Tris–HCl and in Tyrode buffers. The $IC_{50}$ values determined in Tyrode for phthalideisoquinolines are lower (by about one order of magnitude) than and correlate well ($r^2 = 0.95$) with the $IC_{50}$ data obtained by [$^3$H]GABA displacement in Tris–HCl. Applying Tyrode, the activities of GABA agonists relative to Tris–HCl are decreased. It can be recognized that activities in receptor binding are dependent on the conformations phthalideisoquinolines prefer in solution. On the basis of systematic alterations in the phthalideisoquinoline molecule the main structural elements involved in the binding of phthalideisoquinoline alkaloids appear to be identical with those of GABA agonists, suggesting that the same binding conformation of the $GABA_A$ receptor may be implicated for both agonists and antagonists. The opposite shift in relative potencies of agonists and antagonists may be the consequence of an alteration in the "ionic status" rather than that in the conformation of the $GABA_A$ receptor.

In the early 1970s bicuculline was introduced as a useful agent for critical tests of the γ-aminobutyric acid (GABA) mediated inhibition [1]. Bicuculline has been strongly criticized [2–4] as a GABA antagonist for being neither potent nor reliable enough. Some of the shortcomings might be due to the instability of bicuculline in aqueous solutions [5], since experiments performed with the more stable and soluble quaternary salt form of bicuculline gave more consistent results [6, 7]. Although bicuculline differentiates bicuculline-sensitive and insensitive GABA receptors [8–11], one is left with the impression that a better antagonist for the GABA receptor would be desirable [9].

Attempts have been made using molecular models to explain the GABA antagonist action of bicuculline in terms of a stereochemical match with GABA [12–15]. Unfortunately, there is some confusion concerning the structural prerequisites of antagonist activity, i.e. (i) the necessity of an atomic sequence present in bicuculline and isosteric with GABA [1, 14, 15], (ii) the importance of relative configuration [12–16], since published formulae of bicuculline [12, 13] represent either its diastereomer (adlumidine) [18] or the disastereomers are not distinguished at all [14] and (iii) the necessity of cationic nitrogen (protonated or quaternary forms) in the interaction with the GABA receptor [15–17, 19, 20].

Binding experiments with [$^3$H]GABA [21, 22] and [$^3$H]-methiodide of (+)-bicuculline [17] show that GABA and (+)-bicuculline mutually displace each other at their binding sites; (+)-bicuculline also displaces the GABA analogue [$^3$H]muscimol [23]. In addition, the *in vitro* binding of optical isomers of bicuculline is enantiospecific in favour of (+)-bicuculline (erythro, 1*S*,9*R* configuration). The *threo* epimer (+)-adlumidine (1*S*,9*S* configuration) was reported to have the same affinity as (+)-bicuculline for receptor binding [24]. This finding is not supported by neurophysiological studies; all *threo* forms of bicuculline and related alkaloids tested have been proved iontophoretically inactive [14], which suggests that the specificity of bicuculline as antagonist of GABA-mediated central inhibition is connected with the erythro relative configuration. Additionally, it has been demonstrated by preliminary pharmacological experiments [25, 26] that only the compounds with erythro relative configuration manifested convulsive activities.

The above conflicting reports have led us to extend investigations on the inhibition of [$^3$H]GABA binding to a series of phthalideisoquinoline (PIQ) diastereomer pairs, secophthalideisoquinolines and other structurally related derivatives. The activities of the compounds in GABA receptor binding have been measured applying both Tris–HCl and glucose-free Tyrode (pH 7.1) buffers. The reason for such an alteration is the increased potency of bicuculline relative to GABA [27] in the latter bicarbonate-buffered $Na^+$ ion containing physiological buffer. In the presence of $Na^+$ ions, however, [$^3$H]GABA can be the subject of uptake [27], or binding to specific uptake sites [28]. In order to inhibit the binding of [$^3$H]GABA to uptake sites the specific uptake inhibitor guvacine (Fig. 1h) was used in $10^{-4}$ M concentration. At this concentration, guvacine does not interfere with receptor binding [29].

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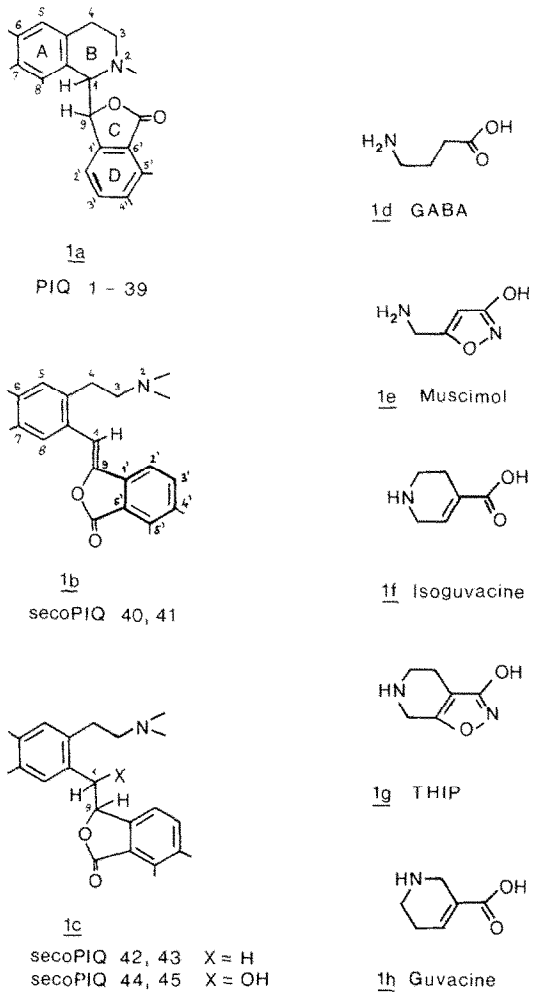


Fig. 1. General formulae and numbering system of PIQs, secoPIQs and compounds structurally related to GABA. For detailed substitution pattern and stereochemistry, consult Table 1.

## MATERIALS AND METHODS

*Materials.* The PIQ alkaloids investigated can be divided into two classes; one possessing the complete tetracyclic nucleus (PIQ) and the other, commonly referred to as secophthalide-isoquinolines (secoPIQs) characterized by an open ring B (Figs. 1a–c). PIQ alkaloids contain two asymmetric centers at C(1) and C(9) so they exist as two diastereomeric pairs of enantiomers, i.e. (+)-erythro (1*S*, 9*R*), (−)-erythro (1*R*, 9*S*), (+)-threo (1*S*, 9*S*) and (−)-threo (1*R*, 9*R*) stereoisomers (for the complete listing of PIQ alkaloids see Ref. 30). Synthesis of the phthalideisoquinoline compound lacking substituents in rings A and D, that of 6,7-desmethoxy-cordrastine and *N*-desmethyl-tetradeshydro-cordrastine has been described in Ref. 31. Narcotines and lactones from narcotines were obtained according to Refs. 32 and 33, respectively. Aobamidine, *N*-methylhydrastine, lactones from bicuculline and β-hydrastine were prepared according to Ref. 34. Quaternarization of cordrastine II, bicuculline and adlumidine was performed with methyliodide in boiling benzene. Isoguvacine (Fig. 1f), THIP (Fig. 1g) and guvacine (Fig. 1h) were kindly supplied by Prof. Krogsgaard-Larsen. GABA (Fig. 1d) and muscimol (Fig. 1e) were obtained from Sigma. [$^3$H]GABA (29.1 Ci/mmol) was purchased from New England Nuclear.

*Preparation of synaptic membranes.* Crude synaptic membranes were prepared according to Ref. 35 with modification [36, 37]. In short, rat brains (male, Wistar) were homogenized in 15 vol. ice-cold 0.32 M sucrose solution with Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min, the pellet discarded and the supernatant centrifuged at 20,000 *g* for 10 min. The pellet was resuspended in distilled water and after 2 hr centrifuged at 8000 *g* for 20 min. The supernatant together with the upper layer of the pellet was resuspended and pelleted 3 times at 45,000 *g* for 20 min in 0.05 M Tris–HCl (pH 7.1) or in Tyrode (pH 7.1). After the third centrifugation the pellet was frozen for at least 18 hr at −20°, then thawed and centrifuged. This freezing-thawing-washing cycle performed in the appropriate buffer was repeated 3 times. The synaptic membranes prepared in Tris–HCl (TRIS) or in Tyrode (TYR) and being possibly free from endogenous GABA or other inhibitory substances [38, 39] were freeze-dried [40].

*Binding assays.* Prior to binding experiments, the lyophilized synaptic membranes (40 mg wet tissue weight/ml) were reconstituted in the appropriate buffer (TRIS or TYR assay). After 10 min of incubation at 4°, the binding equilibrium (with 3 nM [$^3$H]GABA) was reached [41] without apparent decomposition [5] of PIQs. Cold filtrations [24, 42] were made in triplicate. Washing was performed with 8 × 2.5 ml ice-cold buffer. Non-specific binding determined in the presence of 0.1 mM GABA represented less than 15% of the total [$^3$H]GABA bound. About 40% of specific [$^3$H]GABA bound (displaceable by $10^{-4}$ M GABA) was found guvacine sensitive under TYR assay conditions. PIQs were dissolved in ethanol. The ethanol content in the incubation mixture amounted to 4.5% v/v. In order to verify that the $GABA_A$ receptor [11] is labelled, standards [43] such as GABA, muscimol, THIP and isoguvacine were tested in addition to PIQ derivatives. Bound radioactivities were determined by placing the filters immediately after filtration into liquid scintillation vials. Counting was performed with a Packard TRI-CARB spectrometer (counting efficiency 37%). $IC_{50}$ values (the concentrations of PIQ derivatives which displace 50% of [$^3$H]GABA bound to receptor sites) were determined from 3 to 9 concentrations of the displacer. Standard deviations for the individual displacements at a given concentration are less than ± 10% of the mean. Considering the individual scatter, estimated errors for $IC_{50}$ values are less than ± 20%.

*$pK_A$ determination.* To determine $pK_A$ values, the HCl salts of PIQs were dissolved in $CO_2$-free, twice glass-distilled water. Concentrations ($c_{salt}$) were about $10^{-4}$ M. pH values were obtained on a RADELKIS OP-211/1 pH meter with an accuracy of ±0.02 pH. Measurements were carried out in the presence of 0.01 M NaCl at 25° in an inert gas atmos-

phere. $pK_A$ values were determined using the formula given for salts of strong acid with weak base [44], i.e. $pH = 0.5\ (pK_A - \log c_{salt})$. The agreement of our $pK_A$ values with those published so far is varying from perfect for narcotine [45] to a difference of 0.4 unit for bicuculline [16].

*N.m.r. measurements.* N.m.r. data were collected on a Bruker WP 200 MHz Supercon FT spectrometer in $CDCl_3$ solution. The chemical shift values ($\delta$) are in parts per million from internal $Me_4Si$. Nuclear Overhauser enhancement (n.O.e.) experiments were carried out according to Ref. 46 in which n.O.e. data of different C(8) unsubstituted PIQs are reported.

## RESULTS

Binding affinities of PIQ derivatives and GABA agonists have been determined from [$^3$H]GABA displacement experiments performed under TRIS and TYR assay conditions. Results are collected in Table 1. As can be seen, $IC_{50}$ values of PIQs are more than 10 times lower in the TYR assay, while GABA analogues are 5 times more potent in the TRIS assay. These shifts in affinity agree well with that observed for (+)-bicuculline relative to GABA by other workers [20, 27]. The $IC_{50}$ values measured in the two kinds of assay show significant correlations ($r^2 = 0.95$, Fig. 2).

GABA analogues show relative potencies characteristic of the $GABA_A$ receptor [43]. Some of the PIQ derivatives [(+)- and (−)-bicuculline methochloride, (+)-adlumidine, (−)-α-narcotine, (+)- and (−)-α-hydrastine] were tested earlier [24]. With the exception of (+)-adlumidine mentioned before, our data from the TRIS assay and the published values obtained from experiments performed also in TRIS buffer are in fair agreement.

### *Effect of substituents in rings A and D*

None of the PIQ compounds investigated was proved to be more active than (+)-bicuculline. In accordance with pharmacological studies [25, 26], progressive substitution of the PIQ skeleton (Nos. 1, 2, 3 in Table 1) increase the affinity. Comparing different substitutions of isoquinoline and phthalide rings, our findings that the affinity decreases more sensitively on substitution of methoxyl groups for methylenedioxy in the phthalide ring (cf. Nos. 32 vs 26 and 21 in Table 1) are supported by electrophysiological measurements [14]. It is important to note, however, that the presence of an additional methoxyl at C(8) strengthens the influence of replacing 6,7-methylenedioxy by methoxyl groups (cf. Nos. 16 ~ 11 and 14 ~ 9 in Table 1).

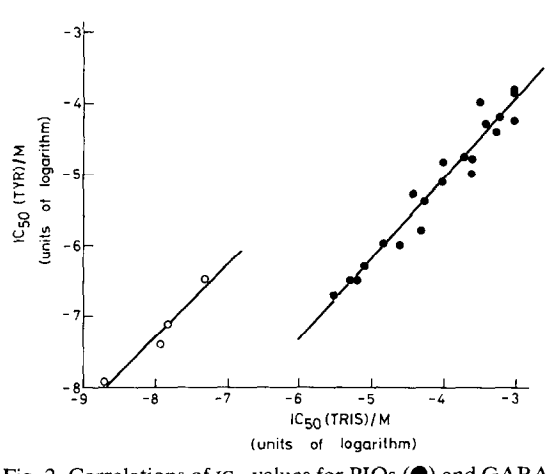


Fig. 2. Correlations of $IC_{50}$ values for PIQs (●) and GABA analogues (○) determined by displacement of [$^3$H]GABA under TRIS and TYR assay conditions. Slopes are 1.01 and 1.13 for GABA analogues and PIQs, respectively; $r^2 = 0.95$ for both sets of data.

### *The role of the chiral centres*

Several compounds show enantioselectivity in the [$^3$H]GABA displacing activity. Enantioselectivities can be observed either directly (Nos. 9, 10; 12, 13; 14, 15; 17, 18; 35, 36) or comparing the racemic compound with one of its enantiomers (Nos. 28, 29; 31, 32). The degree of enantioselectivity depends on the affinity of the dextro-rotatory optical isomer. Since all (−)-antipodes are poor displacers of [$^3$H]GABA, the (+)-compounds of higher potency are characterized by greater enantioselectivity (Table 2). The data clearly indicate in both erythro and threo series that *S* absolute configuration of the C(1) centre is preferred by the receptor.

The (+)-sign of optical rotation associated with the *S* absolute configuration of C(1) is in itself an insufficient stereochemical criterion for bicuculline analogues. (Note that salt formation may change the sign of optical rotation, while leaving the configuration of the chiral centres unchanged.) The GABA receptor exerts different affinities towards erythro and threo PIQs. The binding of erythro isomers is more favoured that can be represented by the ratio of $IC_{50}$ values of C(9) epimers, defined as diastereoselectivity. This quotient expresses the preference by the GABA receptor between diastereomers with identical sign of optical rotation. Diastereoselectivity data for epimer and racemic PIQ compounds are summarized in Table 3.

It is worth mentioning that the lack of chirality at C(1) as in the case of the compound containing aromatic B ring results in intermediate potency between erythro and threo analogues (No. 8 vs Nos. 4 and 7 in Table 1).

### *The nitrogen moiety*

The character of nitrogen atom is an additional structural property which might modify the affinity of bicuculline related compounds towards the GABA receptor. The appearance of positive charge at the nitrogen has influenced the activities of quaternary *N*-metho-salts of (+)-bicuculline and (+)-adlumidine in opposite manner (cf. Nos. 34 and 39 vs 31 and 35 respectively in Table 1). According to $pK_A$ values, 3–10% of *N*-methyl PIQs and about 30% of *N*-nor analogues are protonated at the pH of the assays. The degree of protonation seemingly does not influence the activity of *N*-nor and *N*-methyl erythro PIQs (Nos. 3, 4; 21, 22; 26, 27; 32, 33 in Table 1) in contrast to secondary and tertiary threo compounds. The threo *N*-nor PIQs are clearly less active than their *N*-methyl analogues (Nos. 6, 7; 29, 30; 37, 38 in Table 1).

Table 1. [$^3$H]GABA displacing activities of bicuculline related compounds (Nos. 1–45), GABA and its analogues (Nos. 46–49) under TRIS and TYR assay conditions and p$K_A$ values for *N*-nor and *N*-methyl PIQ derivatives

| No. | Compounds | Relative configuration | N (2) | C (6) | C (7) | C (8) | C (4′) | C (5′) | p$K_A$ | $IC_{50}$ [$\mu$M] assay TRIS | TYR |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Phthalideisoquinolines | | | | | | | | | | | |
| 1. | (±)-Phthalidisoquinoline (PIQ) | erythro | $CH_3$ | H | H | H | H | H | | >60* | 83 |
| 2 | (±)-6,7-Desmethoxy-cordrastine | erythro | $CH_3$ | H | H | H | $OCH_3$ | $OCH_3$ | | >20* | 20 |
| 3 | (±)-Cordrastine II | erythro | $CH_3$ | $OCH_3$ | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | 5.9 | 27 | 1,1 |
| 4 | (±)-Norcordrastine II | erythro | H | $OCH_3$ | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | 6.7 | 30 | |
| 5 | (±)-Cordrastine II methiodide | erythro | $(CH_3)_2$ | $OCH_3$ | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | | 320 | |
| 6 | (±)-Cordrastine I | threo | $CH_3$ | $OCH_3$ | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | 5.5 | 250 | 17 |
| 7 | (±)-Norcordrastine I | threo | H | $OCH_3$ | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | 6.8 | 600 | |
| 8 | *N*-desmethyl-tetradeshydro-cordrastine | | — | $OCH_3$ | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | | >5* | 4.9 |
| 9 | (+)-Pentamethoxy-PIQ II | erythro | $CH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | 6.5 | 260 | 11 |
| 10 | (−)-Pentamethoxy-PIQ II | erythro | $CH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | | ~1000 | 56 |
| 11 | (±)-Pentamethoxy-PIQ II | erythro | $CH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | | 310 | |
| 12 | (+)-Pentamethoxy-PIQ I | threo | $CH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | | 410 | 51 |
| 13 | (−)-Pentamethoxy-PIQ I | threo | $CH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | 6.0 | ~1000 | 190 |
| 14 | (+)-α-Narcotine | erythro | $CH_3$ | $OCH_2O$ | | $OCH_3$ | $OCH_3$ | $OCH_3$ | | 54 | 4.3 |
| 15 | (−)-α-Narcotine | erythro | $CH_3$ | $OCH_2O$ | | $OCH_3$ | $OCH_3$ | $OCH_3$ | | 560 | 39 |
| 16 | (±)-α-Narcotine | erythro | $CH_3$ | $OCH_2O$ | | $OCH_3$ | $OCH_3$ | $OCH_3$ | 6.2 | 66 | |
| 17 | (+)-β-Narcotine | threo | $CH_3$ | $OCH_2O$ | | $OCH_3$ | $OCH_3$ | $OCH_3$ | | 100 | 15 |
| 18 | (−)-β-Narcotine | threo | $CH_3$ | $OCH_2O$ | | $OCH_3$ | $OCH_3$ | $OCH_3$ | 5.8 | ~1000 | 160 |
| 19 | (±)-β-Narcotine | threo | $CH_3$ | $OCH_2O$ | | $OCH_3$ | $OCH_3$ | $OCH_3$ | | 120 | |
| 20 | (−)-β-Hydrastine | erythro | $CH_3$ | $OCH_2O$ | | H | $OCH_3$ | $OCH_3$ | | ~1000 | |
| 21 | (±)-β-Hydrastine | erythro | $CH_3$ | $OCH_2O$ | | H | $OCH_3$ | $OCH_3$ | 6.3 | 50 | 1.6 |
| 22 | (±)-β-Norhydrastine | erythro | H | $OCH_2O$ | | H | $OCH_3$ | $OCH_3$ | 6.6 | 53 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 23 | (+)-α-Hydrastine | threo | $CH_3$ | $OCH_2O$ | | H | $OCH_3$ | $OCH_3$ | | >1000 | |
| 24 | (±)-α-Hydrastine | threo | $CH_3$ | $OCH_2O$ | | H | $OCH_3$ | $OCH_3$ | 6.0 | >1000 | 110 |
| 25 | (±)-α-Norhydrastine | threo | H | $OCH_2O$ | | H | $OCH_3$ | $OCH_3$ | 6.7 | >1000 | |
| 26 | (±)-Corlumine | erythro | $CH_3$ | $OCH_3$ | $OCH_3$ | H | $OCH_2O$ | | 6.2 | 8.1 | 0.46 |
| 27 | (±)-Norcorlumine | erythro | H | $OCH_3$ | $OCH_3$ | H | $OCH_2O$ | | 6.8 | 8.0 | |
| 28 | (−)-Adlumine | threo | $CH_3$ | $OCH_3$ | $OCH_3$ | H | $OCH_2O$ | | | 930 | |
| 29 | (±)-Adlumine | threo | $CH_3$ | $OCH_3$ | $OCH_3$ | H | $CCH_2O$ | | | 324 | 95 |
| 30 | (±)-Noradlumine | threo | H | $OCH_3$ | $OCH_3$ | H | $OCH_2O$ | | | ~1000 | |
| 31 | (+)-Bicuculline | erythro | $CH_3$ | $OCH_2O$ | | H | $OCH_2O$ | | 6.1 | 3.0 | 0.17 |
| 32 | (±)-Bicuculline | erythro | $CH_3$ | $OCH_2O$ | | H | $OCH_2O$ | | | 5.5 | 0.33 |
| 33 | (±)-Norbicuculline | erythro | H | $OCH_2O$ | | H | $OCH_2O$ | | | 6.3 | 0.35 |
| 34 | (+)-Bicuculline, methiodide of | erythro | $(CH_3)_2$ | $OCH_2O$ | | H | $OCH_2O$ | | | 15 | 1.1 |
| 35 | (+)-Adlumidine | threo | $CH_3$ | $OCH_2O$ | | H | $OCH_2O$ | | | 560 | |
| 36 | (−)-Capnoidine | threo | $CH_3$ | $OCH_2O$ | | H | $OCH_2O$ | | | 680 | |
| 37 | (±)-Adlumidine | threo | $CH_3$ | $OCH_2O$ | | H | $OCH_2O$ | | | 580 | 66 |
| 38 | (±)-Noradlumidine | threo | H | $OCH_2O$ | | H | $OCH_2O$ | | | >1000 | 120 |
| 39 | (+)-Adlumidine, methiodide of | threo | $(CH_3)_2$ | $OCH_2O$ | | H | $OCH_2O$ | | | 38 | 5.4 |
| Secophthalideisoquinolines | | | | | | | | | | | |
| 40 | Aobamidine/enol lactone from bicuculline/ | | $(CH_3)_2$ | $OCH_2O$ | | H | $OCH_2O$ | | | 100 | 7.8 |
| 41 | *N*-methylhydrastine/enol lactone from hydrastine/ | | $(CH_3)_2$ | $OCH_2O$ | | H | $OCH_3$ | $OCH_3$ | | 200 | |
| 42 | Lactone from bicuculline | | $(CH_3)_2$ | $OCH_2O$ | | H | $OCH_2O$ | | | ~1000 | 140 |
| 43 | Lactone from hydrastine | | $(CH_3)_2$ | $OCH_2O$ | | H | $OCH_3$ | $OCH_3$ | | ~1000 | |
| 44 | (+)-1*R*, 9*R* lactone from narcotine | | $(CH_3)(CN)$ | $OCH_2O$ | | $OCH_3$ | $OCH_3$ | $OCH_3$ | | 200 | 18 |
| 45 | (−)-1*S*, 9*S* lactone from narcotine | | $(CH_3)(CN)$ | $OCH_2O$ | | $OCH_3$ | $OCH_3$ | $OCH_3$ | | ~1000 | 130 |
| GABA analogues | | | | | | | | | | | |
| 46 | Muscimol | | | | | | | | | 0.002 | 0.012 |
| 47 | GABA | | | | | | | | | 0.012 | 0.041 |
| 48 | Isoguvacine | | | | | | | | | 0.015 | 0.076 |
| 49 | THIP | | | | | | | | | 0.050 | 0.331 |

* The low solubility of these compounds did not allow the estimation of $IC_{50}$ values.

Table 2. [$^3$H]GABA displacing activities of the (+)-enantiomers of bicuculline related compounds and the corresponding enantioselectivity data (TRIS assay)

| Compound | Configuration absolute | Configuration relative | $IC_{50}$ [$\mu$M] | Enantio-selectivity* |
|---|---|---|---|---|
| (+)-Bicuculline | (1*S*, 9*R*) | erythro | 3 | >100† |
| (+)-$\alpha$-Narcotine | (1*S*, 9*R*) | erythro | 54 | 10 |
| (+)-$\beta$-Narcotine | (1*S*, 9*S*) | threo | 100 | 10 |
| (+)-Adlumine | (1*S*, 9*S*) | threo | 230† | 4† |
| (+)-Pentamethoxy PIQ II | (1*S*, 9*R*) | erythro | 260 | 3.9 |
| (+)-Pentamethoxy PIQ I | (1*S*, 9*S*) | threo | 410 | 2.4 |
| (+)-Adlumidine | (1*S*, 9*S*) | threo | 560 | 1.2 |

* Ratio of $IC_{50}$ values of the respective (−)- and (+)-enantiomers.
† Estimation implying additive contributions of enantiomers in the racemate. (This assumption is not valid for C(8)-methoxyl substituted compounds.)

The fact that the presence of positive charge at the nitrogen (of protonated or quaternary state) does not provide unequivocal interpretation of the activities of erythro and threo PIQs suggests that additional structural properties should also influence the GABA displacing activity of these compounds.

*The GABA sequence*

The investigation of secoPIQ compounds (Nos. 40–45) in Table 1) which do not contain the contiguous GABA sequence was included for testing whether this structural unit is a prerequisite of the alkaloids to possess affinity towards the GABA receptor. Hence, it is surprising enough that not all these compounds have been proved inactive. Aobamidine was found to inhibit the GABA enhancement of [$^3$H]flunitrazepam binding to TRIS membranes (data not shown) suggesting that secoPIQs are antagonists at the GABA receptor.

*Conformation of PIQ derivatives*

According to previous n.m.r. studies [16, 46, 47] it is known that the rotation of the phthalide ring of PIQs is hindered in solution. Hence, the majority of molecules are found in certain, thermodynamically most favourable (preferred) conformations which are different for diastereomers [46, 47]. On the basis of coupling constants $J_{C(1)H-C(9)H}$ available for erythro and threo PIQs [46, 30] dihedral angles can be calculated using the Karplus formula [48]. Comparative n.O.e. experiments [46] have shown that the favoured conformation of *N*-nor and *N*-methyl erythro compounds are identical (Fig. 3a) while they are different for threo derivatives (Figs. 3b, c). The additional C(8)-methoxyl group of narcotines modifies the thermodynamically favoured conformations. The observed enhancements (see Table 4) are larger between C(9)–H and C(1)–H in the narcotine series than in the case of $\alpha$- and $\beta$-hydrastines [46] suggesting that both erythro and threo narcotine type molecules (Figs. 3d and 3e respectively) have an approximately 30° anticlockwise torsion around the C(1)–C(9) bond as compared to C(8)–unsubstituted PIQs.

## DISCUSSION

*Conformation and affinity of PIQs*

The conformational analysis outlined above offers an opportunity to rationalize the different activities

Table 3. Diastereoselectivity data for optically active and racemic bicuculline related compounds

| No. | Diastereomeric pairs of compounds erythro ~ threo | Diastereoselectivity* in [$^3$H]GABA binding assay TRIS | TYR |
|---|---|---|---|
| 1 | (+)-Bicuculline ~ (+)-adlumidine | 190 | — |
| 2 | (±)-Bicuculline ~ (±)-adlumidine | 110† | 200† |
| 3 | (±)-Norbicuculline ~ (±)-noradlumidine | 160† | 360† |
| 4 | (±)-Corlumine ~ (±)-adlumine | 40† | 210† |
| 5 | (±)-Norcorlumine ~ (±)-noradlumine | 130† | — |
| 6 | (±)-$\beta$-Hydrastine ~ (±)-$\alpha$-hydrastine | >20 | 70† |
| 7 | (±)-Cordrastine II ~ (±)-cordrastine I | 9.3† | 16† |
| 8 | (±)-Norcordrastine II ~ (±)-norcordrastine I | 20† | — |
| 9 | (+)-$\alpha$-Narcotine ~ (+)-$\beta$-narcotine | 1.9 | 3.5 |
| 10 | (−)-$\alpha$-Narcotine ~ (−)-$\beta$-narcotine | 1.8 | 4.1 |
| 11 | (+)-Pentamethoxy PIQ II ~ (+)-pentamethoxy PIQ I | 1.6 | 4.6 |
| 12 | (−)-Pentamethoxy PIQ II ~ (−)-pentamethoxy PIQ I | — | 3.4 |
| 13 | (+)-Bicuculline, methiodide of ~ (+)-adlumidine, methiodide of | 2.5 | 4.9 |

* Ratio of $IC_{50}$ values of threo and erythro diastereomers.
† Estimated values for racemic diastereomer pairs implying identical enantioselectivities for the compounds involved.

Table 4. $^1$H NMR chemical shifts ($\delta$) and n.O.e. data for narcotines and nornarcotines

| Compound | Proton irradiated | Proton observed | Area increase (%) |
|---|---|---|---|
| $\alpha$-Nornarcotine | H–C(1) (4.86) | H–C(9) (5.91) | 26.0 |
| | H–C(9) (5.91) | H–C(1) (4.86) | 15.0 |
| | | $OCH_3$–C(8) (4.06) | 1.2 |
| $\alpha$-Narcotine | H–C(1) (4.41) | H–C(9) (5.59) | 35.0 |
| | | $CH_3$–N(2) (2.55) | 4.0 |
| | H–C(9) (5.59) | H–C(1) (4.41) | 27.5 |
| | | H–C(2′) (6.09) | 6.3 |
| | $OCH_3$–C(8) (4.03) | H–C(9) (5.59) | 4.2 |
| $\beta$-Nornarcotine | H–C(1) (4.57) | H–C(9) (5.92) | 31.3 |
| | | H–C(2′) (7.09) | 15.9 |
| | H–C(9) (5.92) | H–C(1) (4.57) | 15.0 |
| | | H–C(2′) (7.09) | 4.5 |
| | | $OCH_3$–C(8) (4.04) | 2.7 |
| $\beta$-Narcotine | H–C(1) (4.20) | H–C(9) (5.52) | 27.0 |
| | | H–C(2′) (6.98) | 11.3 |
| | | $CH_3$–N(2) (2.15) | 3.3 |
| | H–C(9) (5.52) | H–C(1) (4.20) | 19.4 |
| | | H–C(2′) (6.98) | 6.3 |

observed for erythro and threo PIQs. The preferred conformations of PIQs (Fig. 3) can be characterized by two main structural factors: (i) the distance between nitrogen and lactone carbonyl; (ii) the steric hindrance for these moieties to fit the receptor recognition site. The *N*-methyl and *N*-nor erythro PIQs exist in the same preferred conformation, hence, the N–CO distance in these compounds is identical. Moreover, the nitrogen as well as the lactone carbonyl both are placed on the easily accessible side of the molecule. The anticlockwise torsion of the phthalide ring in $\alpha$-narcotine decreases the N–CO distance by approximately 10% (cf. Fig. 3a and 3d). In the case of threo C(8)-unsubstituted *N*-methyl PIQ derivatives, the nitrogen and the lactone carbonyl are in a sterically rather free position; however, the N–CO distance is larger by about 30% relative to the erythro series. In the case of *N*-nor threo compounds the N–CO distance is somewhat smaller (*ca.* 10%) than that of bicuculline, and the nitrogen and carbonyl functions are strongly hindered due to the opposite steric arrangements of the isoquinoline and phthalide rings. As a consequence of the anticlockwise torsion of the phthalide ring in $\beta$-narcotine, the N–CO distance reaches approximately the same value as in bicuculline. $\beta$-Narcotine resembles the *N*-nor threo PIQs concerning the steric hindrance of amine and carbonyl functions (cf. Fig. 3b and 3e).

It is the identity of both conformations and activities of *N*-methyl and *N*-nor erythro compounds that

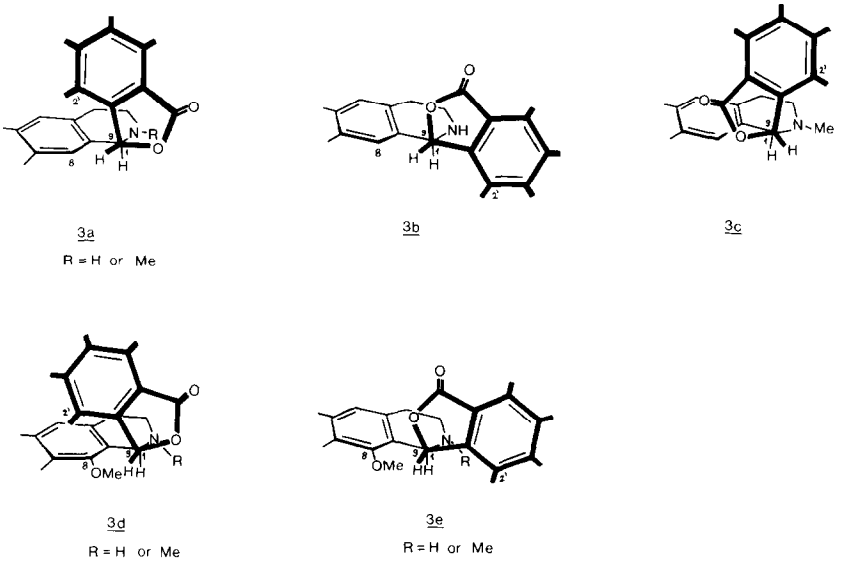


Fig. 3. Favoured conformations represented by Newman projection formulae for erythro and threo series of PIQs in solution: (a) *N*-nor and *N*-methyl erythro compounds; (b) *N*-nor threo compounds; (c) *N*-methyl threo compounds; (d) *N*-nor and *N*-methyl erythro narcotines; (e) *N*-nor and *N*-methyl threo narcotines.

emphasizes most explicitly the relation between these properties. Accordingly, the decrease in affinity of *N*-nor threo PIQs compared to *N*-methyl analogues is parallel with the different conformations of these series and seems to represent the steric control of the binding process. The result is reflected by an increase in the diastereoselectivities of nor-PIQs (Nos. 2, 3; 4, 5; 7, 8 in Table 3). Considering its highest affinity, the distance and accessibility of nitrogen and lactone carbonyl of bicuculline can be regarded as optimal. In the threo series β-narcotines tend to realize the right (bicuculline-like) N–CO distance resulting in a markedly increased affinity of the (+)-enantiomer as compared to (+)-α-hydrastine (Nos. 17 vs 23 in Table 1) which can be rationalized by different preferred conformations of the molecules. Analogously, an explanation may be given for the strongly decreased diastereoselectivity for the bicuculline methiodide and adlumidine methiodide pair of salts; compared with the parent tertiary amines an anticlockwise rotation of the phthalide ring exerted by the additional methyl group at the nitrogen decreases the difference in the N–CO distance for the epimer methiodides. Hence, the conformation is found to be a powerful structural property interpreting the binding affinities of bicuculline related compounds to the GABA receptor.

Considerations on the N–CO distance help to interpret the weak binding activity of secoPIQ compounds. Owing to conjugation in enol lactones (Fig. 1b), the A, C, and D rings have planar structure. With the aminomethyl group freely rotating, the N–CO distance in certain conformers of secoPIQ enol lactones can be similar to the corresponding distance in PIQs. The presence of a C(1)–C(9) single bond in secoPIQ lactones, however, results in a more flexible conformation which affords very low probability for the compound to attain a conformation resembling the PIQ structure. Interestingly enough, enantiomers of the lactones derived from narcotines (Nos. 44, 45 in Table 1) exert stereoselectivity towards the GABA receptor. In other words, the 1*R*, 9*R* lactone has certain affinity while its antipode is inactive. The activity of the (+)-enantiomer can be rationalized by presuming the formation of a hydrogen bond [33] between C(8)-methoxyl and C(1)-hydroxyl substituents which reduce the rotational freedom for the phthalide moiety (C, D rings). It is remarkable that the 1*R*, 9*R*-lactone has the steric attributes to form the conformer matching the 1*S*, 9*R* erythro PIQ structure while the 1*S*, 9*S*-lactone lacks this ability.

*Main structural elements of binding affinity*

On the basis of the structure–activity relationships outlined above, the main structural elements involved in [$^3$H]GABA displacement by bicuculline related GABA antagonists appear to be as follows:

(a) An N–CO distance in the erythro series represented by bicuculline, the most active PIQ alkaloid. An atomic sequence in the PIQ structure which is isosteric with GABA may not be a prerequisite of affinity towards the receptor as indicated by detectable affinities of some secoPIQ compounds clearly devoid of any GABA-analogue structural element. According to MO calculations on the conformation of protonated bicuculline [49] the distance is about 5.6 Å.

(b) Erythro relative and *S* absolute configuration for the C(1) atom next to the nitrogen moiety. Changing this stereochemical orientation either to threo or to *R* configuration [or $sp^2$ character of C(1)] results in less effective binding.

(c) Protonated and unprotonated forms of the PIQs may have identical binding affinities, hence the appearance of the positive charge on nitrogen does not seem to be critical.

The binding of GABA analogues favour the following structural requirements:

(a) The $GABA_A$ receptor prefers an optimal N–CO distance [50] being about 5.4 Å.

(b) The affinity of GABA agonists containing a chiral centre next to the nitrogen atom resides at the *S* absolute configuration [51, 52].

(c) The cationic nitrogen may not be so important since the potent agonist imidazol acetic acid exists with delocalized positive charge [52]. The above comparison demonstrates that PIQs and GABA agonists show remarkably similar structural properties favoured by the binding process which tends to indicate that the two groups of compounds bind to the same receptor site. The only obvious difference is the absence of negative charge in PIQ derivatives which may account for both their lower affinity and antagonistic profile. The localized negative charge was reported important for agonist activity [43, 51–53], while negative charge delocalization might lead to the appearance of antagonistic character [51, 52].

*The receptor conformation*

On the basis of the observed increased relative affinity of [$^3$H]-methiodide of (+)-bicuculline (in the presence of $SCN^-$ ions) a hypothesis was put forward [20] on GABA receptors existing in different conformations for agonists and antagonists. The shift of antagonist affinities relative to agonist activities could then be regarded as the consequence of a shift in the equilibrium between agonist and antagonist receptor conformations. An identical shift of agonist and antagonist affinities to bovine retinal membranes has recently been reported [28] to be brought about by $SCN^-$ ions.

Our TRIS and TYR assay conditions contain different ions. One may regard the TYR assay somewhat nearer to physiological conditions and the lower $IC_{50}$ values obtained for PIQs tend to support this view since they agree better with the concentration range (0.01–0.1 μM) required for central effects as suggested [54] on the basis of *in vivo* doses of bicuculline.

The correlations shown in Fig. 2 express two different meanings. First, they clearly differentiate agonists and antagonists as a consequence of opposite shifts of affinities towards receptors of the TYR assay for the two groups of compounds. Second, with slopes not significantly different from one, the correlations indicate that whatever structure–activity relationship holds for either group of compounds in TRIS assay, it should be valid also in TYR assay. This latter argument together with the remarkable identity of structural requirements favoured by both agonists and antagonists led us to assume (i) that

TRIS and TYR assays cannot be characterized by GABA receptors existing in different conformations, (ii) that both GABA agonists and phthalideisoquinolines bind to the $GABA_A$ receptor exerting one and the same conformation, and (iii) that receptors of the TRIS and TYR assays differ in their "ionic status" which differentiates the binding of ligands bearing or lacking the negative charge, but otherwise no difference is expressed in terms of structural preference by the two receptor types.

Irrespective of the actual mechanism involved, our results point to the importance the more physiological assay conditions may have in [$^3$H]GABA binding studies.

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*Note added in proof*

A graph essentially similar to Fig. 2 has independently been set up recently by G. Maksay and M. K. Jicku, *J. Neurochem.* **43**, 261 (1984).

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