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Isothiouronium compounds as γ-aminobutyric acid agonists

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1 Analogues of γ-aminobutyric acid (GABA) incorporating an isothiouronium salt as a replacement for a protonated amino functional group have been investigated for activity on: GABA receptors in the guinea-pig ileum; [^{3}H]-GABA and [^{3}H]-diazepam binding to rat brain membranes; and GABA uptake and transamination.

2 For the homologous series of ω-isothiouronium alkanoic acids, maximum GABA-mimetic activity was found at 3-[(aminoiminomethyl)thio]propanoic acid.

3 Introduction of unsaturation into this compound gave two isomeric conformationally restricted analogues. The *trans* isomer was inactive at GABA receptors while the *cis* compound ((*Z*)-3-[(aminoiminomethyl)thio]prop-2-enoic acid (ZAPA)) was more potent than muscimol and GABA as a GABA agonist with respect to low affinity GABA receptor sites.

4 Both isomers were moderately potent at inhibiting the uptake of [^{3}H]-GABA into rat brain slices.

5 Comparison of possible conformations of the two unsaturated isomers by interactive computer graphics modelling and comparison with muscimol has led to a plausible active conformation of ZAPA, which may be a selective and potent agonist for low affinity GABA binding sites.

Introduction

Analogues of the neurotransmitter γ-aminobutyric acid (GABA) which have limited molecular flexibility have provided considerable information about the active conformations of the molecule involved in receptor activation and cellular uptake (Krogsgaard-Larsen & Falch, 1981; Allan & Johnston, 1983). Conformationally restricted analogues often exhibit enhanced selectivity as well as high potency for interaction at particular active sites.

One way to achieve conformational restriction of the GABA molecule is to incorporate the zwitterionic amino and carboxylic acid groups into unsaturated, carbocyclic or heterocyclic molecules that limit the separation of the polar functional groups of GABA (Allan & Johnston, 1983). Another way to develop selective GABA-mimetic agents is by bioisoteric replacement (Thornber, 1979) of the amino or carboxylic acid functional groups of GABA. Recognition that the acidic hydroxyioxazole of muscimol could be considered as a replacement for the carboxylic acid group of GABA led to discovery of muscimol as a GABA agonist, and further development along these lines led to 4,5,6,7-tetrahydroisoxazole[4,5-c]pyridine-3-ol (THIP) as a selective conformationally restricted GABA agonist (Krogsgaard-Larsen & Falch, 1981).

Replacement of the amino group in GABA agonists has received relatively little attention apart from investigation of the activity of the imino derivatives such as SL 75102 (Kaplan *et al.*, 1980; Desarmenian *et al.*, 1981; Bowery *et al.*, 1982). Indications that amino group replacement could give rise to very potent GABA analogues came from previous work on a number of derivatives. Imidazoleacetic acid is a moderately potent bicuculline-sensitive GABA agonist with little affect on GABA uptake and degradation (McGeer *et al.*, 1961; Godfraind *et al.*, 1973; Bowery & Jones, 1976; Johnston *et al.*, 1978; Nistri & Constanti, 1979) while guanidinoacetic acid and guanidinopropionic acid are potent on GABA receptor assays both *in vivo* and *in vitro* (Purpura, 1960; McGeer *et al.*, 1961; Iversen & Johnston, 1971; Nistri & Constanti, 1979). The analogous isothiouronium compounds 2-[(aminoiioiminomethyl)thio]propionic acid (*2*) (Purpura, 1960) and [(aminoiminomethyl)thio]acetic acid (*1*) (Breckenbridge *et al.*, 1981) have also been shown to have moderate activity on an *in vivo* cerebral cortex preparation and on inhibition of [^{3}H]-GABA binding to membranes from human brain, respectively.

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The pK_a for the proton loss from the protonated isothioronium salts [pK_a 9.8 for S-methyl isothiouronium sulphate (Perrin, 1965)] more closely resembles that of the amino group of GABA [pK_a 10.7 (Krogsgaard-Larsen *et al.*, 1975)] than does the pK_a of the strongly basic guanidino compounds [pK_a 13.4 for *N*-methyl guanidine (Perrin, 1965)]. The isothiouronium group was therefore investigated more thoroughly as a biosteric replacement for the amino group in GABA analogues.

Methods

Preparation of GABA analogues

The isothiouronium compounds were prepared either by reaction of thiourea on the corresponding ω-haloacids (Moore & Rappola, 1947), *trans*-3-chloro-2-propenoic acid (Kataev *et al.*, 1969), or by acid catalysed addition of thiourea to propiolic acid (Kataev *et al.*, 1969) or acrylic acid. The following known compounds were prepared by the above methods: 2-[(aminoiminomethyl)thio]acetic acid hydrobromide (*1*), 3-[(aminoiminomethyl) thio]propanoic acid hydrobromide (*2*) (Behringer & Zillikens, 1951), 4-[(aminoiminomethyl)thio]butanoic acid hydrobromide (3), 5-[(aminoiminomethyl)thio]pentanoic acid hydrobromide (*4*), 6-[(aminoiminomethyl)thio]hexanoic acid hydrobromide (*5*), 11-[(aminoiminomethyl)thio]undecanoic acid hydrobromide (*6*) (Moore & Rappola, 1947); (*Z*)-3-[(aminoiminomethyl)thio]-2-propenoic acid hydrochloride (ZAPA, *7*), *E*-3-[(aminoiminomethyl)thio]-2-propenoic acid hydrochloride (*8*), (*Z*)-3-[(aminoiminomethyl)seleno]-2-propenoic acid hydrochloride (*9*) (Kataev *et al.*, 1969). All compounds had satisfactory physical and spectral properties (melting points, infrared and ^1H nuclear magnetic resonance at 60 MHz).

Guinea-pig isolated ileal preparation

Guinea-pigs of either sex, weighing between 250–350 g, were stunned by a blow to the head and their necks broken. Segments of ileum, 2 cm in length taken 10 cm proximal to the caecum, were quickly removed and placed in a 25 ml organ bath containing modified Krebs-bicarbonate solution (Krantis *et al.*, 1980) of the following composition (mM); Na^+ 151.0, K^+ 4.7, Mg^{2+} 0.6, Ca^{2+} 2.8, Cl^- 143.7, HCO_3^- 16.3, $H_2PO_4^{2-}$ 1.3, SO_4^- 0.6 and glucose 7.7. The solution was maintained at 32°C and gassed with a mixture of 95% O_2 and 5% CO_2.

The segments of ileum were attached to a tissue holder at one end to the bottom of the bath, and the other end attached by cotton thread to an isotonic transducer. Changes in length were recorded by means of a Curken chart recorder. The tissues were allowed to equilibrate in the organ bath for 60 min before drug application. Application of drugs was at intervals of 10–15 min, and antagonists were added at least 3 min before agonists were tested. Drug volumes used were never more than 1% of the bath volume, and solvents other than water were tested on the tissue for any possible reactions. Each experiment was performed at least five times on tissue from at least 3 different animals.

Rat brain membrane preparations

Both washed synaptosomal membranes and Triton-treated membranes were prepared by a modified procedure of that used by Skerritt & Johnston (1982).

Washed synaptosomal membrane preparation (WSM)

Male Sprague-Dawley rats (20–250 g) were stunned by a blow to the back of the neck, decapitated and their brains rapidly removed and placed on ice. The brain tissue was chopped into smaller sections (6–8) with a scalpel blade then homogenized in 8 vol (by tissue weight) of ice-cold 0.32 M sucrose. Homogenizations were performed with a chilled smooth-glass homogenizer with a tight fitting teflon pestle, using 8–10 up and down strokes. This homogenate was centrifuged (1,000 *g* for 10 min, 4°C) and the resulting pellet together with the buffy coat was discarded after the supernatant was carefully decanted. This supernatant was then centrifuged (27,000 *g* for 20 min, at 4°C) and the resulting pellet resuspended in 8 vol (by original tissue weight) of ice-cold distilled water and spun at 48,000 *g* for 20 min (at 4°C). The supernatant was again discarded and the remaining pellet was washed by resuspension in 8 vol of 50 mM ice-cold Tris-citrate buffer (pH 7.1) and centrifugation (31,000–48,000 *g* for 20 min, at 4°C) a total of 8 times. This final pellet, containing synaptosomes, light myelin fragments and large mitochondria, was resuspended in 5 vol (by original tissue weight) Tris citrate buffer (50 mM, pH 7.1, 4°C) and stored at − 20°C for up to 3 months before use.

Triton-treated membranes

WSM were prepared as above and stored in 8 vol (by original tissue weight) Tris-citrate buffer at − 20°C. The membranes were thawed then homogenized and centrifuged (48,000 *g*, 20 min, 4°C). The pellet was resuspended in half the required volume of Tris-citrate buffer and an equal amount of 1% Triton X-100. This suspension was incubated at 37°C for 30 min and recentrifuged as above. The pellet was then washed by the procedure followed for the WSM preparation, a total of 4 times, then stored, at − 20°C, no longer than

14 days before use. Before use in binding assays, the membranes were thawed and refrozen twice.

Binding assays

Potentiation of [^{3}H]-diazepam binding

The procedure used to assess the activity of the compounds on facilitation of [^{3}H]-diazepam binding was a modified form of that described by Skerritt *et al.* (1982a). Frozen WSM preparations were thawed, centrifuged (48,000 *g*, 20 min, 4°C) and resuspended in 5 vol (by original tissue weight) of cold Tris HCl buffer (50 mM, pH 7.4, 4°C). This suspension was recentrifuged under the same conditions and the pellet finally resuspended in Tris HCl buffer at a final protein concentration of 6–8 mg ml^{-1}. Binding assays were performed in 10 ml plastic tubes at room temperature. The incubation mixture contained 0.3 ml membrane preparation, 0.2 ml test compound or water, 0.05 ml radioligand (0.7 nM) and 1.45 ml of 50 mM Tris HCl (pH 7.4). Incubations were in quadruplicate and initiated by addition of the radioligand (0.5–0.8 nM [^{3}H]-diazepam). The incubation was stopped after 15 min by addition of 4 ml of 50 mM Tris HCl buffer and rapid filtration under high vacuum, on Whatman GF/B filters. The filters were washed with 2 × 4 ml Tris HCl buffer then transferred to scintillation vials and soaked for 1 h in 1 ml of water. Scintillation cocktail (8 ml, scintillator 299^{TM}, Packard) was then added and the radioactivity counted after 6–12 h in a LKB liquid scintillation system. Non-specific binding was defined as that remaining in the presence of 10 μM unlabelled diazepam.

All compounds were tested initially at 100 μM and compared with 100 μM GABA, which gave maximum facilitation of [^{3}H]-diazepam binding (186 ± 11% facilitation, $n = 20$). If compounds tested at 100 μM gave greater than 80% facilitation, ED_{50}s were determined, using 100 and 500 μM screening doses for the maximum response. Experiments were performed at least three times.

High affinity GABA binding

The binding assay used to investigate the binding of compounds to the high affinity GABA site was a modification of the procedure described by Skerritt & Johnston (1982). Triton-treated membranes were then thawed and centrifuged (48,000 *g*, 20 min, 4°C) and the pellet resuspended in Tris-citrate buffer (8 vol, per original tissue weight; 50mM, pH 7.1, 4°C). This suspension was recentrifuged as above and the pellet resuspended a further three times. The final pellet was resuspended to a final protein concentration of 1.5–2.0 mg ml^{-1}, which generally corresponded to 4 vol as per original tissue weight.

Assays were performed in 1.5 ml plastic centrifuge tubes (Kartell, 298) on ice, with an incubation mixture of 1 ml. Incubations, in triplicate, were initiated by the addition of the membrane preparation to the incubation mixutre which contained 0.1 ml [^{3}H]-GABA (1 nM); 0.1 ml inhibitor or water; and 0.7 ml Tris-citrate buffer. The incubations were terminated after 5 min by centrifugation at 4°C in Eppendorf 5412 centrifuges (10,000 *g*, 5 min) and the pellets superficially washed 3 times with 1 ml ice-cold water. The pellets were then left in 1 ml water for 24 h and resuspended. Samples (0.9 ml) of the resuspended pellets were transferred to scintillation vials and 2.5 ml scintillant (Aquassure, NEN) was added and the radioactivity counted by liquid scintillation spectrometry in a Packard (model 300CD) liquid scintillation system. Non-specific binding was defined as the amount of radioligand bound in the presence of 1 μM unlabelled GABA.

Inhibition of [^{3}H]-GABA uptake

The procedure used was basically that of Iversen & Neal (1968). Slices of rat cerebral grey matter were preincubated at 25°C for 15 min with inhibitors (5×10^{-4} M) in freshly oxygenated phosphate medium (10 ml). [^{3}H]-GABA (50 μl) was added (final conc. = 2.5×10^{-9} M) and the incubation allowed to continue for a further 10 min. The slices were collected by rapid filtration through Whatman GF/C filter papers (2.5 cm diameter) and washed with ice-cold (10 ml) physiological saline and transferred to scintillation vials. The radioactivity was extracted by soaking the filters in 1 ml of water for 1 h and counted in the same manner as described above.

The more active inhibitors revealed by the preliminary screening procedure, were tested as above over a range of concentrations to determine IC_{50} values as described by Iversen & Johnston (1971). The IC_{50} values quoted are means ± s.e.mean for at least 3 experiments.

Inhibition of GABA-transaminase activity

Inhibition of brain GABA-transaminase activity was tested by investigating the deamination of [^{14}C]-GABA by the methods described by Beart *et al.* (1972).

Analysis of data

All results are given as means ± s.e.mean, where ED_{50} and IC_{50} values were determined by plotting activity as a percentage of control (from at least 3 experiments) on a probability scale against inhibitor or agonist concentrations on a log scale (log probit analysis), through use of a computer programme, which then

gave an estimated error for the fit of the curves (Balcar *et al.*, 1976). Dose-response curves for the guinea-pig ileum are plotted from the mean ± s.e.mean at each concentration for at least $n = 5$ experiments.

Chemicals

[^{3}H]-GABA (60 Ci mmol^{-1}), [^{14}C]-GABA (224 m Ci mmol^{-1}) and [^{3}H]-diazepam (90 Ci mmol^{-1}) were obtained from Amersham. Unlabelled GABA was obtained from Calbiochem Behring, and bicuculline and muscimol were from Sigma. Bicuculline was dissolved in water with 100 µl of 1 M HCl added. Unlabelled diazepam was from Hoffman-La Roche, and dissolved in dimethylsulphoxide at a concentration of 10^{-2} M, then diluted in water to the required concentration.

Computer graphics

Interactive computer graphics modelling of molecular structures was carried out on a DEC PDP11/23 + minicomputer with a NJC Colour Graphics terminal using software previously developed for molecular superimpositions (Andrews & Johnston, 1979b).

Results

Guinea-pig isolated ileum

GABA, muscimol and compounds *2*, the *cis* (or *Z*) isothiouronium compound ZAPA (*7*) and *9* elicited transient dose-dependent contractions of the guinea-pig intestine, which were blocked by 16 µM bicuculline as illustrated for GABA and ZAPA in Figure 1. The maximum contractions observed with these analogues did not differ from those observed with GABA and the dose-response curves (Figure 2) showed a clear parallelism with that of GABA suggesting that these compounds have the same mode of action.

The most potent analogue, ZAPA, was about 3.5 times more active than GABA and 1.5 times more potent than muscimol (ED_{50} 0.99 ± 0.16 µM), while its *trans* (or *E*) isomer *8* was inactive at 500 µM. The saturated derivative *2* was about 6 fold less active than GABA and the selenium containing derivative *9* was of equal potency. All other compounds (*1*, *3*, *4*, *5*, *6*) were inactive at 500 µM. Table 1 shows the ED_{50}s derived for these compounds and for GABA. The ED_{50} for muscimol was 0.99 ± 0.16 µM.

Enhancement of [^{3}H]-diazepam binding to rat brain membranes

The activity profile of the isothiouronium analogues and the seleno compound for the facilitation of [^{3}H]-diazepam binding to rat brain membrane was similar to that observed for their activity in the guinea-pig isolated ileum. Considering the ED_{50} for muscimol was 0.4 µM, the order of potency for the potentiation of [^{3}H]-diazepam binding was: ZAPA > muscimol > GABA > *9* > *2*, and *1*, *3*, *4*, *5*, *6* and *8* were inactive at 100 µM. The ED_{50} s are shown in Table 1 and Figure 3 shows examples of the dose-response curves for ZAPA, GABA, *9* and *2* from which the ED_{50} s were calculated.

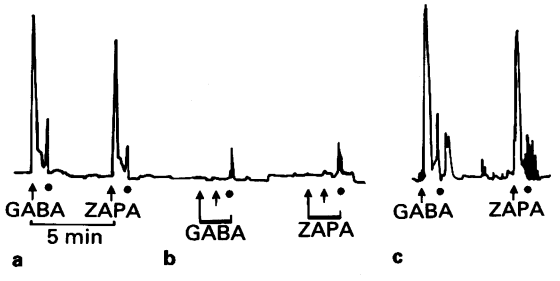


Figure 1 Contractions in the guinea-pig isolated ileum preparation. All records are from a single experiment. The point of injection of the compounds is indicated by ↑ and the washout by ●. (a) Transient contractions evoked by 6 µM GABA and 1.6 µM ZAPA. (b) In the presence of 16 µM bicuculline (———), the responses to GABA (6 µM) and ZAPA (1.6 µM) were antagonized. (c) After 30 min and 5 washouts following the introduction of bicuculline, the contractions evoked by GABA (6 µM) and ZAPA (1.6 µM) returned to control values. Calibration: horizontal bar 5 min.

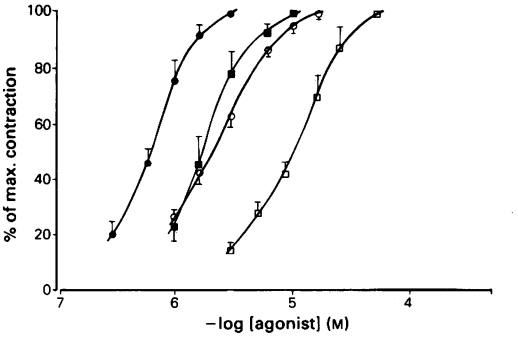
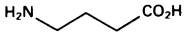
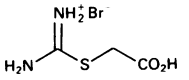
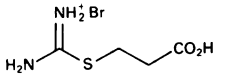
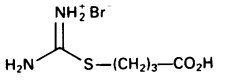
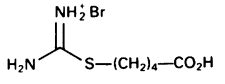
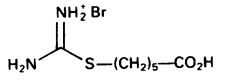
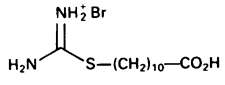
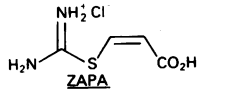
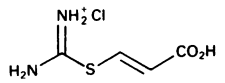
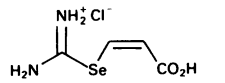


Figure 2 Dose-response curves for the transient contraction of the guinea-pig isolated ileum by ZAPA (●); *9* (■); GABA (○); and *2* (□). Each point is the mean of the percentage of the maximal contraction induced by each of the agonists, and the vertical lines represent the s.e.mean for at least $n = 5$ experiments.

Table 1 Activity of γ-aminobutyric acid (GABA) and isothiouronium compounds on the contraction of the guinea-pig ileum; facilitation of [^{3}H]-diazepam binding; inhibition of [^{3}H]-GABA uptake from rat cortical slices; and inhibition of transamination of [^{14}C]-GABA in rat brain mitochondrial preparations

Compound		*G–P. ileum* ED_{50} (μM)	*Benzo. binding* ED_{50} (μM)	*Uptake* IC_{50} (μM)	*Gaba-T* IC_{50} (μM)
	GABA				
	1	2.21 ± 0.13	0.46 ± 0.06	—	—
	2	NS	NS	NS	NS
	3	13.4 ± 1.8	6.6 ± 0.9	88 ± 22	480 ± 11
	4	NS	NS	NS	NS
	5	NS	NS	NS	NS
	6	NS	NS	NS	NS
 ZAPA	7	0.63 ± 0.07	0.19 ± 0.03	74 ± 8	NS
	8	NS	NS	215 ± 9	NS
	9	2.07 ± 0.27	0.67 ± 0.11	43% Inhib. at 500 μM	*

NS – not significant at screening concentration
*Inconsistent results; see text.

High affinity GABA binding

Only ZAPA, its *trans* isomer *8*, and the saturated derivative *2*, were tested as inhibitors of high affinity GABA binding. The IC_{50}s for these isothiouronium analogues were: ZAPA, 0.032 ± 0.006 μM; *8*, 2.7 ± 0.5 μM; *2*, 0.54 ± 0.16 μM; and GABA, 0.016 ± 0.002 μM.

Inhibition of [^{3}H]-GABA uptake from rat cortical slices

When tested as inhibitors of [^{3}H]-GABA uptake into rat brain slices compounds *2*, ZAPA, *8* and *9* were found to have moderate to weak activity. Table 1 shows the calculated IC_{50} values where the *trans* isomer *8* is weak, but only 2–3 times less active than ZAPA and its saturated analogue *2*.

Inhibition of GABA-transaminase activity in rat brain

With the exception of compounds *2* and *9*, no significant inhibition of GABA-transaminase activity at 1 mM was observed. The saturated derivative *2* was found to be a weak inhibitor with an IC_{50} of 480 ± 11 μM. On testing the selenium analogue *9* as an inhibitor of GABA-transaminase inconsistent results were obtained. At 1 mM, 95% inhibition was generally

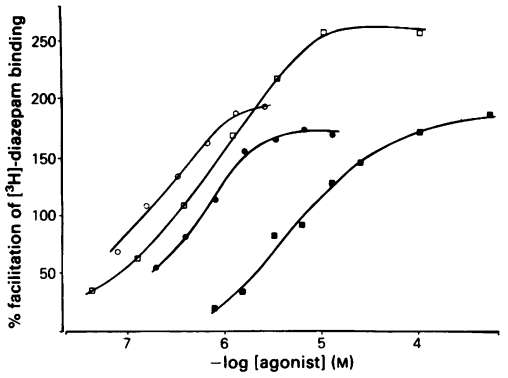


Figure 3 Typical log dose-response curves for the facilitation of [^{3}H]-diazepam binding to rat brain membranes by GABA (□) and the compounds ZAPA (○), *9* (●) and *2* (■).

observed with about 20% inhibition occurring at 0.1 mM. However, no consistent and graded effect was observed when tested between these concentrations.

Discussion

Structure-activity studies on GABA receptor agonists have revealed a number of very active analogues such as muscimol and 3-aminopropanesulphonic acid (3-APS) (Allan & Johnston, 1983). A considerable amount of information about the active conformation of GABA has been deduced from those analogues which are conformationally restricted. For example, examination and superimposition of conformations of GABA, muscimol, THIP and the antagonist bicuculline has led to an understanding of a plausible range of active conformations that act at $GABA_A$-receptors (Andrews & Johnston, 1979a). In contrast, flexible analogues such as 3-APS effectively mimic GABA and can give an idea of the maximum charge separation required at the receptor, but detailed interpretation of the results is limited by the large number of possible active conformations. For the flexible homologous series of ω-amino carboxylic acids, peak GABA-mimetic activity occurs with GABA but β-alanine and δ-aminovaleric acid are active to some extent (Allan & Johnston, 1983).

On comparing the chain length of the ω-isothiouronium carboxylic acids *1–6* with that of GABA, peak activity was expected for the acetic acid derivative *1* which, if the positive charge is resident on the nitrogen of the isothiouronium salt, has the same charge separation as GABA. The fact that compound *2* was the only one of the series to show significant activity on this battery of assays would be consistent with two alternative explanations. Firstly, the receptor may respond to a delocalized positive charge which acts as if it were formally on the carbon of the isothiouronium group rather than on a terminal nitrogen and thus, the conformation of the chain of *2* could match that of GABA. Alternatively, if a charge on the nitrogen is involved in receptor interactions, the conformation of the carbon chain of *2* may be different from that of GABA, and could be related to that of δ-aminovaleric acid which has moderate activity (guinea-pig ileum ED_{50} 105 ± 8 μM; facilitation of [^{3}H]-diazepam binding ED_{50} 30 ± 4 μM; Allan *et al.*, 1985). It is probable that some of the other homologues such as *1* and *3* do have some weak GABA-mimetic activity but that the potency is too low to produce a significant response at the screening concentrations.

It is well known that introduction of π-electrons into the carbon chain of a GABA analogue, although not essential for high potency (Krogsgaard-Larsen *et al.*, 1979), is consistent with retained activity as in *trans*-4-aminocrotonic acid (Johnston *et al.*, 1975) or muscimol (Krogsgaard-Larsen & Falch, 1981), and the potent compounds can be considered as containing *trans* substituted carbon-carbon double bonds. It was not surprising that conformational restriction of the active compound *2*, by introduction of a double bond, should generate two geometric isomers, one of which is more active and the other which is less active than *2*. Three main points on the activity of ZAPA and *8* should be noted:- (a) Introduction of the double bond makes one isomer extremely potent, even more potent than muscimol on the contraction of the guinea-pig ileum and potentiation of benzodiazepam binding to rat brain membranes. (b) This potent isomer has the *cis* configuration of substitutents about the double bond. The correct assignment of structure about the double bond has been confirmed by ^{1}H nuclear magnetic resonance spectroscopy, whereby the coupling constant between the olefinic protons on the *cis* double bond (11 Hz) is smaller than on the *trans* isomer (16 Hz). (c) The pure *trans* isomer *8* is approximately 100 times less active on [^{3}H]-GABA binding while it does not have significant activity on the guinea-pig ileum and potentiation of [^{3}H]-diazepam binding, and is therefore at least 1000 times less active than the *cis* isomer on these two assays. This low activity of the *trans* isomer would argue against considering the isothiouronium group as if the charge were formally on the carbon. If a protonated aminomethyl group ($-CH_2NH_3^+$) could be effectively replaced by a $-SC^+(NH_2)_2$ group, then it would be expected that the *trans* isomer would have comparable activity to *trans*-4-aminocrotonic acid and would be more active than the *cis* isomer.

For interaction with benzodiazepines, the activity of the *cis* isomer (ZAPA) (ED_{50} 0.19 μM) is similar to that

of (RS)-4,5-dihydromuscimol (ED_{50} 0.22 μM (Karobath *et al.*, 1979)) and is therefore one of the most potent GABA agonists yet reported with respect to enhancing the binding of [^{3}H]-diazepam to rat brain membranes, a process known to involve 'low affinity' GABA binding sites with an apparent K_D of 1.8 μM for GABA (Skerritt *et al.*, 1982b). ZAPA may be a relatively selective and potent agonist for 'low affinity' GABA binding sites since it is less potent than GABA in displacing GABA bound to 'high affinity' binding sites. We have previously noted differences in apparent substrate specificity of 'low' and 'high' affinity GABA binding sites (Johnston *et al.*, 1982).

The selenium analogue *9* was as active as GABA in the GABA receptor assays (Table 1) and, considering the appreciable increase in size going from sulphur to selenium this result indicates that steric constraints about this position at the receptor are not severe.

The only compounds active as inhibitors of [^{3}H]-GABA uptake (Table 1) were those with a three carbon chain. The saturated compound *2* and ZAPA were of a similar moderate potency, reinforcing evidence that the stereochemical constraints on GABA uptake and receptor active sites are different. As inhibitors of GABA-transaminase the only notable result was the inconsistent but moderate to potent activity of the selenium compound. The possibility that the inhibition seen was due to the formation of selenium-containing degradation products rather than the actual compound is worthy of further examination.

The marked difference in activity between ZAPA and *8*, for which the only difference in structure is the stereochemistry of the functional groups about the double bond, led to an investigation of possible active conformations which may explain such a marked contrast in potency. Factors which are likely to be important include the degree of delocalization of the charge and the pK_as of the polar groups, the magnitude of the charge separation, and presence or lack of steric hindrance towards the molecule at the active site. Because of instability of these two compounds above pH 8 and the poor solubility of ZAPA, only one approximate pK_a value for *8* has been obtained (pK_a ca. 5.9). However, there is no reason to believe that the pK_a for ZAPA is sufficiently different to explain the difference in activity between the two isomers at pH 7.4. We have therefore looked closest at conformational charge separation and possible steric hinderance by molecular modelling and superimposition using computer graphics.

In modelling the potent agonist ZAPA only two rotations about the bonds to sulphur need to be considered. Such rotations give a large number of conformations available to the molecule within a 20 kcal mol^{-1} energy barrier, and many of these could be reasonably superimposed onto the flexible GABA molecule. When a distance range between charged atoms (calculated from muscimol and *trans*-4-aminocrotonic acid as 5.4 to 5.8 Å) was applied to the molecule the range of low energy conformations was considerably reduced. Matching these conformations of ZAPA with that of muscimol in the 'bicuculline

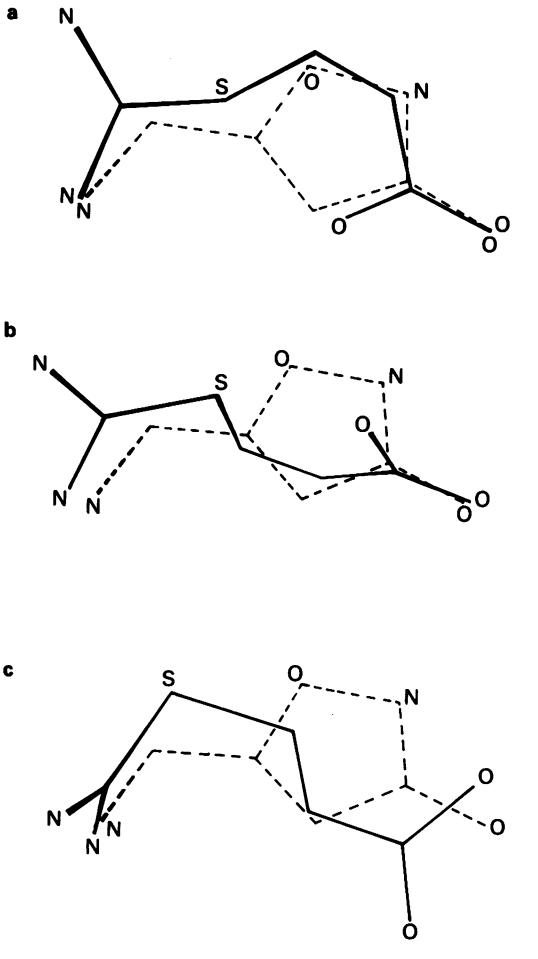


Figure 4 Superimposition of low energy conformations of ZAPA and the *trans* isomer *8* (solid lines) onto muscimol (– – –) in the 'bicuculline conformation'. A better fit was obtained by overlapping the carbon chain of ZAPA onto the O and N of the heterocyclic ring of muscimol as shown in (a) than by overlapping the carbon chain of ZAPA onto the carbon chain of muscimol as illustrated in (b). In contrast to the good fit for ZAPA shown in (a), the comparatively inactive *trans* isomer *8* cannot be well matched onto the muscimol structure, diagram (c) illustrating one of the fits obtained by molecular modelling by computer graphics. Hydrogen atoms have been omitted for clarity.

conformation' (Andrews & Johnston, 1979a) gave a good fit between the polar groups and also between the plane of the π-electron cloud of muscimol and the π-bonding system of ZAPA. It is particularly noteworthy that a good fit is obtained when the carbon-carbon double bond of ZAPA is superimposed onto the N-O atoms of the heterocyclic ring in muscimol as illustrated in Figure 4(a) rather than on the carbons of the 'GABA backbone' of muscimol as shown in Figure 4(b). The conformations illustrated were generated by matching one of the oxygens, one of the nitrogens, and the three carbon chain of ZAPA onto corresponding atoms of muscimol, and the residual after least squares fit was 0.205 for 4(a) and 0.837 for 4(b). An additional point to favour 4(a) over 4(b) is that the space occupied by the lone pairs of electrons on the sulphur more closely overlaps the muscimol molecule in 4(a), while in 4(b) these lone pairs would be in a completely different region. When the *trans* isomer is similarly investigated there are few low energy conformations in which the distance between the charged atoms is in the 5.4 to 5.8 Å range. When these conformations of *8* are compared to muscimol (for example as in Figure 4(c) for which the residual after least squares fit was 1.966) the bulky sulphur atom with its accompanying lone pairs of electrons lies in a new position that is quite distinct from any of the atoms of muscimol and steric hindrance of this sulphur at the GABA receptor may account for the low activity of the *trans* isomer.

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