



STRUCTURAL ANALOGUES OF ZAPA AS GABA_A AGONISTS

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(Received 18 June 1996; accepted 31 July 1996)

Abstract—Structural analogues of ZAPA, Z-3-[(aminoiminomethyl)thio]prop-2-enoic acid, an isothiourenium analogue of GABA, are potent GABA_A agonists as seen in the isolated guinea-pig ileum and in the facilitation of [³H]diazepam binding to rat brain membranes. Compounds with guanidino or amidine groups replacing the amino functionality of GABA were also found to be active. The highest activity was displayed by the isothiourenium salts in which the conformational flexibility of the molecule is restricted by a Z-substituted carbon–carbon double bond. A series of bis-isothiourenium compounds was prepared from aliphatic α,ω -bis-thioureas as mixtures of *E* and *Z* adducts. Maximum GABA_A agonist activity for this series was found with a C₆–C₈ carbon chain, and the results were consistent with an interaction at the GABA_A receptor with only one end of the molecule, rather than the more potent effect expected of a molecule bridging two active sites. GABA_A antagonist/partial agonist activity was observed on the guinea-pig isolated ileum for a number of different analogue types, with the most potent being bis-isothiourenium derivatives. None of the substituted derivatives of ZAPA was as active as ZAPA itself, and maximum GABA_A activity was found in the n-pentyl and n-hexyl analogues. © 1997 Elsevier Science Ltd

ZAPA, Z-3-[(aminoiminomethyl)thio]prop-2-enoic acid, is an isothiourenium analogue of the inhibitory neurotransmitter GABA, of restricted conformation due to the presence of a *cis* double bond. ZAPA is a selective agonist for low affinity GABA_A receptors which are modulated by benzodiazepines (Allan *et al.*, 1986b). In addition, it is a substrate for the neuronal GABA transport system, and thus could act in GABA replacement therapy (Allan *et al.*, 1991). However, ZAPA does not cross the blood–brain barrier, therefore a suitable prodrug would have to be developed before a clinically useful central nervous system agent could emerge from compounds related to ZAPA. Nevertheless, the inability of ZAPA to cross the blood–brain barrier is advantageous, as ZAPA has a potent GABA-agonist action in nematodes and is regarded as an important lead compound for the design of novel anthelmintics (Holden-Dye and Walker, 1988). The present study was undertaken in

order to explore various structural analogues of ZAPA for their activity on:

1. GABA_A receptors in the guinea pig ileum;
2. low affinity GABA_A receptors as assessed by their ability to stimulate the binding of diazepam to rat brain membranes;
3. GABA uptake in rat brain slices; and
4. GABA transamination by extracts of rat brain mitochondria.

Most successful structural manipulations of the GABA molecule (Krogsgaard-Larsen, 1981; Johnston *et al.*, 1979) have been confined to the carboxyl end of the molecule, or to additions or alterations to the carbon chain (Allan *et al.*, 1986a), as exemplified by compounds such as muscimol, 3-amino-propanesulphonic acid and *trans*-4-aminocrotonic acid (Allan and Johnston, 1983). Apart from simple incorporation into a ring system, as in THIP and 4-PIOL (Krogsgaard-Larsen *et al.*, 1994), manipulations of the amino group of GABA generally lead to marked decreases in activity (Breckenridge *et al.*, 1981) unless the amino function is incorporated in a charge-delocalized system, such as in guanidino,

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isothioureyl, imidazolyl and pyridazinyl GABA derivatives (Breckenridge *et al.*, 1981; Iversen *et al.*, 1979; Wermuth *et al.*, 1987), the latter group giving rise to important new GABA_A antagonists. Our initial investigations into replacement of the amino group of GABA with an isothiuronium group led to the discovery of ZAPA (Fig. 1), a very potent and selective agonist for low affinity GABA_A receptors (Allan *et al.*, 1986b). We report here the synthesis of some structural analogues of ZAPA and a structure–activity profile on various GABA-related assays. The structure–activity profile of these compounds led to the findings that low affinity GABA_A receptors are able to accommodate relatively large carbon chains, such as that in the n-hexyl substituted ZAPA derivative (Compound 5). The bis isothiuronium compounds (40–44) were developed by analogy with the bis quaternary ammonium cholinergic drugs such as hexamethonium and decamethonium. If two agonist molecules are involved in the activation of each GABA–receptor–ionophore complex (Sakmann *et al.*, 1983), then these “dimeric” analogues may have shown a much higher affinity for the receptors than the simple n-alkyl isothiuronium derivatives.

EXPERIMENTAL

Pharmacology

GABA_A receptors in the guinea pig isolated ileum. Isolated segments of ileum (2–3 cm) from guinea-pigs (250–300 g) of either sex were set up in a modified Krebs–bicarbonate solution in 25 ml organ baths at 32°C in the same manner as described previously (Ong and Kerr, 1983; Allan *et al.*, 1986a, 1986b). Possible GABA antagonist activity was tested by adding the compound to the organ bath 5 min prior to addition of concentrations of GABA that produced the normal GABA concentration–response curve. All compounds were dissolved in distilled water and screened at 500 μM, and EC₅₀s were determined using log-probit analysis of at least four experiments.

Facilitation of diazepam binding to rat brain membranes. The preparation of the well washed synaptosomal rat brain membranes and the procedure used to assay the activity of the GABA analogues on [³H]diazepam binding have been described previously (Burch *et al.*, 1983; Allan *et al.*, 1986b). Essentially, 50 μl aliquots of [³H]diazepam (0.5–0.8 nM) were incubated at 20°C for 15 min in 1.45 ml Tris–HCl buffer (50 mM Tris Base, pH 7.4) with 200 μl of compound at various concentrations, water or 100 μM diazepam. Membranes were collected by rapid filtration on GF/B glass fibre filters which were soaked for 1 h in 1 ml H₂O, after which scintillant was added and the radioactivity counted. All compounds were screened at 100 μM, then EC₅₀s determined as described above, from at least three experiments, using 500 μM and 100 μM concentrations of each compound as determinants of the maximum response.

Inhibition of GABA uptake in rat brain slices. The pro-

cedure used to assay the inhibition of [³H]GABA (2.5 nM) uptake into rat brain cortical slices was modified as described previously (Iversen and Johnston, 1971; Allan *et al.*, 1986b). Aliquots of rat cortical slices (100 μl) were preincubated at 25°C for 15 min with inhibitors (500 μM for screening) in 10 ml of freshly oxygenated phosphate medium. Fifty microlitres of [³H]GABA was then added and the incubation continued for a further 10 min, after which the slices were collected by rapid filtration on GF/C glass fibre filters and the radioactivity counted as described above. IC₅₀ values were determined from at least three experiments.

Inhibition of GABA transamination. Transamination of [¹⁴C]GABA in the presence of 1 mM compound was carried out as described previously, using extracts of rat brain mitochondria (Beart *et al.*, 1972).

Chemical synthesis

¹H Nuclear magnetic resonance (NMR) spectra were obtained on a 90 MHz JEOL FX90Q FT instrument, and chemical shift (δ) values are given for tetramethylsilane as external reference. Microanalyses were determined by the Australian Microanalytical Service, Melbourne. Low resolution mass spectral data refer to chemical ionization with methane as the reagent gas on a TSQ46 Finnigan/MAT mass spectrometer.

The following are new compounds except where indicated by a specific reference to a literature preparation.

Derivatives of ZAPA (1–20, 36). These were prepared by either of two methods, based on the addition of thiourea to propionic acid (Kataev *et al.*, 1969).

- **Method A:** The alkyl substituted thiourea (1 mmol) was dissolved in glacial acetic acid (5 ml) to which was added hydrogen bromide (0.1 ml of 50% solution in acetic acid); this was stirred while propionic acid (1 mmol) was added at room temperature. The solution was stirred at room temperature for 1 h, then the solvent was allowed to evaporate overnight in a desiccator over potassium hydroxide under vacuum. The oily residue was stirred with ethyl acetate at room temperature and the resulting solution allowed to stand at 0°C over a period of several days to crystallize. The crystalline material was filtered off, washed with ethyl acetate and dried.
- **Method B:** This method was essentially the same as for Method A except that 1 M aqueous hydrochloric acid was used as the solvent in order to afford hydrochlorides.

(*Z*)-*N*-Methyl-3-[(aminoiminomethyl)thio]propenoic acid hydrochloride (1). Colourless solid m.p. 140–143°C (d); ¹H-NMR [D₂O]: 3.50 (3H, s), 6.78 (1H, d, *J* 10 Hz), 8.03 (1H, d, *J* 10 Hz).

(*Z*)-*N*-Ethyl-3-[(aminoiminomethyl)thio]propenoic acid hydrochloride (2). Colourless solid m.p. 165–170°C (d). Anal. (C₆H₁₁N₂O₂SCl) C, H, N. ¹H-NMR [D₂O]: 1.70 (3H, t, *J* 7 Hz), 3.89 (2H, q, *J* 7 Hz), 6.73 (1H, d, *J* 10 Hz), 8.00 (1H, d, *J* 10 Hz).

(*Z*)-*N*-Propyl-3-[(aminoiminomethyl)thio]propenoic acid hydrochloride (3). Colourless plates m.p. 116–119°C (d). Anal. (C₇H₁₃N₂O₂SCl) C, H, N. ¹H-NMR [D₂O]: 7.92 (1H, d, *J* 10 Hz), 6.69 (1H, d, *J* 10 Hz), 3.80 (2H, t, *J* 7 Hz), 2.13 (2H, sextet, *J* 7 Hz), 1.39 (3H, t, *J* 7 Hz). Mass spectrum: *m/z* 189 (M⁺, 1%), 171 (18), 131 (10), 119 (14), 103 (100), 87 (17).

(*Z*)-*N*-Hexyl-3-[(aminoiminomethyl)thio]propenoic acid hydrochloride (5). This was obtained as a gum which would not crystallize (isolated as a mixture with 15% of the *E*-

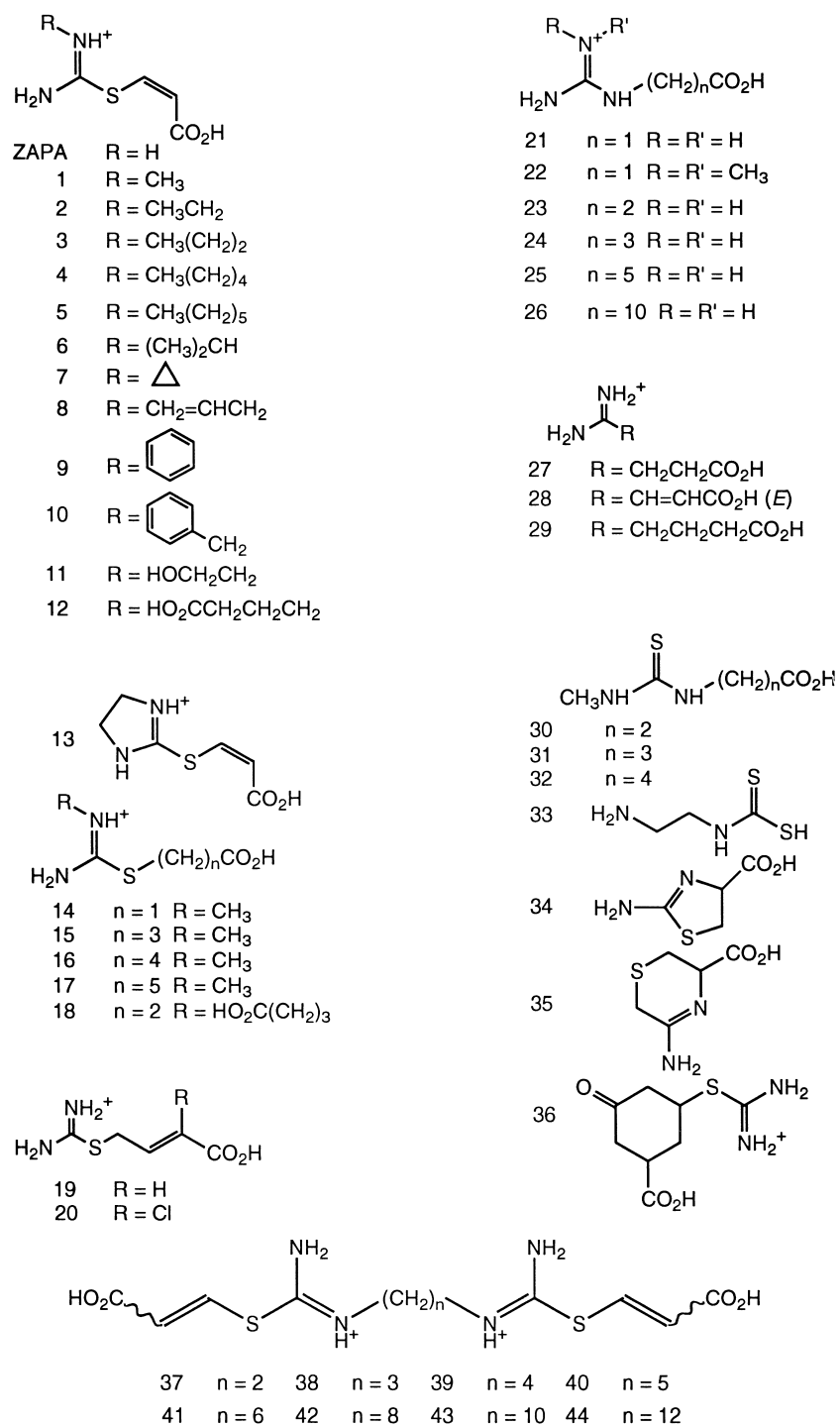


Fig. 1. Structure of ZAPA and some structural analogues.

isomer). ¹H-NMR [D₂O]: 8.10 (1H, d, *J* 16 Hz), 6.80 (1H, d, *J* 10 Hz), 3.87 (2H, t, *J* 7 Hz), 1.80 (8H, m), 1.30 (3H, t, *J* 6 Hz). Mass spectrum: *m/z* 231 (1%), 213 (9), 189 (12), 175 (11), 161 (100), 145 (60), 127 (86), 105 (42), 87 (32).

(*Z*)-*N*-Isopropyl-3-[(aminoiminomethyl)thio]propenoic acid hydrochloride (**6**). Colourless solid m.p. 140–146°C (d). ¹H-NMR [D₂O]: 1.72 (6H, d, *J* 7 Hz), 4.30 (1H, septet, *J* 7 Hz), 6.78 (1H, d, *J* 10 Hz), 8.03 (1H, d, *J* 10 Hz).

(*Z*)-*N*-Cyclopropyl-3-[(aminoiminomethyl)thio]propenoic acid hydrochloride (**7**). m.p. 158–160°C (d). Anal. (C₇H₁₁N₂O₂SCl) C, H, N. ¹H-NMR [D₂O]: 7.87 (1H, d, *J* 10 Hz), 6.69 (1H, d, *J* 10 Hz), 3.19 (1H, m), 1.34 (4H, m). Mass spectrum: *m/z* 187 (2%), 169 (60), 141 (14), 129 (28), 117 (28), 101 (100), 87 (29).

N-Allyl-3-[(aminoiminomethyl)thio]propenoic acid hydrochloride (**8**). This was obtained as a 1:1 mixture of *E* and *Z* isomers m.p. 160–165°C (d). ¹H-NMR [D₂O]: 8.30 (1H, d, *J* 10 Hz), 7.24 (1H, d, *J* 10 Hz), 8.20 (1H, d, *J* 16 Hz), 6.80 (1H, d, *J* 16 Hz), 6.31 (1H, m), 5.76 (1H, m), 4.49 (1H, d, *J* 5 Hz). Mass spectrum: *m/z* 187 (12%), 145 (6), 117 (24), 111(12), 105 (41), 87 (14), 83 (100).

N-Phenyl-3-[(aminoiminomethyl)thio]propenoic acid hydrobromide (**9**). This was obtained as a crystalline 1:1 mixture of *E* and *Z* isomers. ¹H-NMR [D₂O]: 6.89 (1H, d, *J* 16 Hz), 8.30 (1H, d, *J* 16 Hz), 6.80 (1H, d, *J* 10 Hz), 8.07 (1H, d, *J* 10 Hz), 7.96 (5H, m). Mass spectrum: *m/z* 223 (1%), 205 (18), 165 (11), 153 (22), 137 (100), 105 (28), 87 (33), 83 (23), 81 (25).

(*E*)-*N*-Benzyl-3-[(aminoiminomethyl)thio]propenoic acid hydrochloride (**10**). This was obtained as a white solid m.p. 175–178°C (d). Anal. (C₁₁H₁₃N₂O₂SCl) C, H, N. ¹H-NMR [D₂O]: 8.35 (1H, d, *J* 16 Hz), 8.00 (5H, s), 6.88 (1H, d, *J* 16 Hz), 5.20 (2H, s). Mass spectrum: *m/z* 237 (1%), 219 (3), 195 (5), 167 (22), 151 (12), 133 (10), 105 (20) 91 (100), 87 (15).

(*Z*)-*N*-(4-Carboxypropyl)-3-[(aminoiminomethyl)thio]propenoic acid (**12**). This was obtained as a gum. ¹H-NMR [D₂O]: 2.53 (2H, quintet, *J* 7 Hz), 3.08 (2H, t, *J* 7 Hz), 4.02 (2H, t, *J* 7 Hz), 6.90 (1H, d, *J* 10 Hz), 8.10 (1H, d, *J* 10 Hz).

(*Z*)-*N,N*-Ethylene-3-[(aminoiminomethyl)thio]propenoic acid hydrochloride (**13**). This was obtained as a white crystalline solid m.p. 153–155°C (d). Anal. (C₆H₉N₂O₂SCl.H₂O) C, H, N. ¹H-NMR [D₂O]: 7.87 (1H, d, *J* 10 Hz), 6.78 (1H, d, *J* 10 Hz), 4.45 (4H, s). Mass spectrum: *m/z* 173 (3%), 155 (19), 103 (15), 87 (100).

Utilizing the method of Kataev *et al.* (1969), the following isothiuronium compounds were prepared from either the corresponding acrylic acids or ω -haloacids using either thio-urea or *N*-methylthiourea.

N-Methyl-2-[(aminoiminomethyl)thio]acetic acid hydrochloride (**14**). This obtained as pale yellow needles m.p. 240–242°C. ¹H-NMR [D₂O]: 3.76 (3H, s), 4.77 (2H, s).

N-Methyl-4-[(aminoiminomethyl)thio]butanoic acid hydrobromide (**15**). This isolated as colourless crystals m.p. 123–124°C. ¹H-NMR [D₂O]: 2.42 (2H, quintet, *J* 7 Hz), 2.98 (2H, t, *J* 7 Hz), 3.43 (3H, s), 3.59 (2H, t, *J* 7 Hz). Mass spectrum: *m/z* 177 (8%), 131 (11), 119 (28), 103 (26), 91 (100), 87 (88), 83 (25), 81 (25).

N-Methyl-5-[(aminoiminomethyl)thio]pentanoic acid hydrobromide (**16**). This had m.p. 120–121°C. ¹H-NMR [D₂O]: 2.15 (4H, m), 2.85 (2H, t, *J* 7 Hz), 3.42 (3H, s), 3.56 (2H, t, *J* 7 Hz). Mass spectrum: *m/z* 191 (53%), 117 (100), 101 (90), 91 (94), 83 (93), 81 (92).

N-Methyl-6-[(aminoiminomethyl)thio]hexanoic acid hydrobromide (**17**). This had m.p. 88–93°C. ¹H-NMR [D₂O]:

2.08 (6H, m), 2.92 (2H, t, *J* 6 Hz), 3.52 (3H, s), 3.76 (2H, t, *J* 6 Hz). Mass spectrum: *m/z* 205 (27%), 131 (100), 115 (14), 97 (18), 91 (39).

(*E*)-4-[(Aminoiminomethyl)thio]-2-butenic acid hydrobromide (**19**). This had m.p. 139–141°C. ¹H-NMR [D₂O]: 4.39 (2H, dd, *J* 6, 1 Hz), 6.55 (1H, dt, *J* 16, 1 Hz), 7.39 (1H, dt, *J* 16, 6 Hz).

(*Z*)-4-[(Aminoiminomethyl)thio]-2-chloro-2-butenic acid hydrobromide (**20**). This was obtained from 4-bromo-2-chloro-2-butenic acid (Allan *et al.*, 1980) as colourless crystals m.p. 176–178°C. Anal. (C₅H₈N₂O₂SCl Br) C, H, N. ¹H-NMR [D₂O]: 4.53 (2H, d, *J* 8 Hz), 7.63 (1H, t, *J* 8 Hz). Mass spectrum: *m/z* 129 (8%), 117 (52) 105 (15), 101 (93), 77 (100).

3-[(Aminoiminomethyl)thio]-5-carboxycyclohexanone hydrobromide (**36**). This was prepared from 3-bromo-5-carboxycyclohexanone and thiourea by method A and isolated as colourless needles m.p. 206–207°C (d). Anal. (C₈H₁₃N₂O₃SBr) C, H, N. ¹H-NMR [D₂O]: 4.51 (1H, bs), 3.36 (1H, m), 2.80–2.50 (6H, m). Mass spectrum: *m/z* 181 (3%), 169 (10), 141 (53), 105 (20), 95 (14), 77 (100).

N-(3-Carboxypropyl)-4-[(aminoiminomethyl)thio]propenoic acid hydrochloride (**18**). This isolated as a white solid m.p. 135–136°C (d). Anal. (C₈H₁₅N₂O₄SCl) C, H, N. ¹H-NMR [D₂O]: 3.86 (2H, t, *J* 7 Hz), 3.79 (2H, t, *J* 7 Hz), 3.26 (2H, t, *J* 7 Hz), 2.92 (2H, t, *J* 7 Hz), 2.38 (2H, quintet, *J* 7 Hz). Mass spectrum: *m/z* 217 (15%), 199 (23), 145 (56), 128 (26), 111 (67), 73 (100).

Guanidines **21–26** were prepared by standard procedures involving the addition of amino acids to *S*-methylisothiurea or to *O*-methylisourea. Compounds **30–32** were prepared by amine addition to methyl isothiocyanate, and literature procedures were used for the preparation of compounds **33** (Frahn and Mills, 1964), **34** (Semenenko *et al.*, 1975) and **35** (Goodman *et al.*, 1958).

E-3-(Aminoiminomethyl)propenoic acid hydrochloride (**28**) (Wiley and Daniels, 1965). This was prepared from *Z*-cyanopropenoic acid (Sauers and Cotter, 1961) using the same procedure as that described by McElvain and Schroeder (1949) for the preparation of the saturated analogue, and was obtained as a buff coloured solid after recrystallization from ethanol/ether. ¹H-NMR. (1H, d, *J* 16 Hz); (1H, d, *J* 16 Hz).

3-(Aminoiminomethyl)propenoic acid hydrochloride (**27**) (McElvain and Schroeder, 1949). This was prepared from **28** by hydrogenation in ethanol over palladium on charcoal (10%) catalyst until uptake of hydrogen ceased.

Compounds **37–44** were prepared by Method A from the corresponding bis-thioureas (Takagi *et al.*, 1959) using concentrated hydrochloric acid instead of hydrogen bromide. These compounds were obtained as mixtures of *E* and *Z* isomers and could not be purified further. In most cases, freeze-drying was an important step for obtaining a solid product. The ¹H-NMR spectra indicated that the materials prepared were quite clean, and the ratio of the *E* to *Z* isomers was obtained by comparing the integration of the signals at δ 6.35 (d, *J* 16 Hz) and 7.80 (d, *J* 16 Hz) to those at δ 6.35 (d, *J* 10 Hz) and 7.60 (d, *J* 10 Hz). Other resonances occurred at approximately δ 1.5 for non-terminal, and at approx. δ 3.4 for terminal, methylene groups in the correct ratios.

1,2-Bis[(aminoiminomethyl)thio]propenoic acid ethane dihydrochloride (**37**). This was obtained as a hygroscopic white solid *E:Z* ratio 1:1.

1,3-Bis[(aminoiminomethyl)thio]propenoic acid propane

dihydrochloride (**38**). This was obtained as a hygroscopic white solid *E:Z* ratio 1:3.

1,4-Bis([aminoiminomethyl]thio)propenoic acid)butane dihydrochloride (**39**). This was obtained as a white solid *E:Z* ratio 1:2.

1,5-Bis([aminoiminomethyl]thio)propenoic acid)pentane dihydrochloride (**40**). This was obtained as a white solid *E:Z* ratio 1:5.

1,6-Bis([aminoiminomethyl]thio)propenoic acid)hexane dihydrochloride (**41**). The white solid obtained from Method A was dissolved in water, filtered, then freeze-dried to give the final product as a white powder, *E:Z* ratio 1:4.

1,8-Bis([aminoiminomethyl]thio)propenoic acid)octane dihydrochloride (**42**). This was obtained as a white powder *E:Z* ratio 2:3.

1,10-Bis([aminoiminomethyl]thio)propenoic acid)decane dihydrochloride (**43**). This was obtained as a white powder *E:Z* ratio 1:1.

1,12-Bis([aminoiminomethyl]thio)propenoic acid)dodecane dihydrochloride (**44**). The white solid obtained from Method A was dissolved in water, filtered, and then freeze-dried to give the final product as a white powder, *E:Z* ratio 1:2.

RESULTS

Effects on GABA_A receptors in the guinea-pig ileum

The GABA agonist activity of the compounds in Fig. 1 was confined mainly to the compounds for which EC₅₀ values are listed in Table 1. Compounds **1–7**, **11**, **12**, **21**, **23** and **33** had parallel concentration–response curves for the transient contraction of the

guinea-pig isolated ileum similar to those shown for GABA and ZAPA in Fig. 2. Peak activity was exhibited by the n-hexyl and n-pentyl N-substituted derivatives **5** and **4**, respectively, which were 7–8 times less potent than ZAPA. Activity was observed to increase when the n-propyl group of **3** was replaced by a 2-hydroxyethyl group as in compound **11** (EC₅₀ 14 μM). All compounds that produced transient contractions in the guinea-pig isolated ileum could be antagonized by 16 μM bicuculline, consistent with these compounds acting at GABA_A receptors.

Of the guanidino derivatives (**21–26**), only the *n* = 1 and 2 compounds (**21** and **23**, respectively) showed appreciable activity, with peak activity residing in the *n* = 2 compound (**23**, 28 times less active than ZAPA), which was of similar potency to the corresponding isothiuronium analogue which is 21 times less active than ZAPA; EC₅₀ = 13.4 ± 1.8 μM. Compound **21** showed some activity and was 100 times less potent than ZAPA (Table 1), in contrast to its isothiuronium analogue, which was not significantly active at 500 μM.

Within the imidate series of compounds (**27–29**), only the saturated C-4 analogue **27** showed substantial activity, being 75 times less potent than ZAPA (EC₅₀ = 47 ± 3 μM). Compounds **28** and **29** had very weak activity, with EC₅₀s of 1182 ± 263 μM for **29**, and approximately 300 μM for **28**. Low solubility as

Table 1. *In vitro* activity of GABA, ZAPA and some ZAPA analogues

	Contraction of guinea-pig ileum EC ₅₀ (μM) ^a	Facilitation of [³ H] diazepam binding EC ₅₀ (μM) ^b	GABA uptake IC ₅₀ (μM) ^c	GABA transamination IC ₅₀ (μM) ^d
GABA	2.21 ± 0.13	0.46 ± 0.06	7.22 ± 0.46 ^f	NT ^g
ZAPA	0.63 ± 0.07	0.19 ± 0.03	74 ± 8	NS ^e
1	525 ± 125	> 100	NS ^e	NS
2	67 ± 5	11 ± 1	NS	NS
3	20 ± 3	1.18 ± 0.17	> 500	> 1000
4	5.2 ± 0.5	0.25 ± 0.04	NS	NS
5	4.8 ± 0.3	0.19 ± 0.03	NS	NS
6	37 ± 3	4.9 ± 0.8	NS	NS
7	25 ± 3	5.38 ± 0.65	NS	NS
11	14.0 ± 0.6	28.4 ± 5.4	NS	NS
12	24 ± 2	36 ± 5	NS	NS
19	NS ^e	18 ± 1	NS	≥ 1000
21	60 ± 4	NS ^e	NS	NS
23	18.2 ± 1.4	25 ± 2	≥ 500	NS
33	128 ± 9	NS	NS	NS

^aFor contraction of the guinea-pig ileum, compounds were screened at 500 μM and EC₅₀ values for active compounds were determined using log-probit analysis using at least four different drug concentrations in at least four experiments.

^bFor potentiation of [³H]diazepam binding, compounds were screened at 100 μM and EC₅₀ values determined using at least four different drug concentrations in at least three experiments.

^cFor inhibition of GABA uptake, compounds were screened at 500 μM and IC₅₀ values determined from at least three experiments.

^dFor inhibition of GABA transaminase, compounds were screened at 1000 μM.

^eNS, not significant at the screening concentration.

^fIC₅₀ for nipecotic acid.

^gNT, not tested.

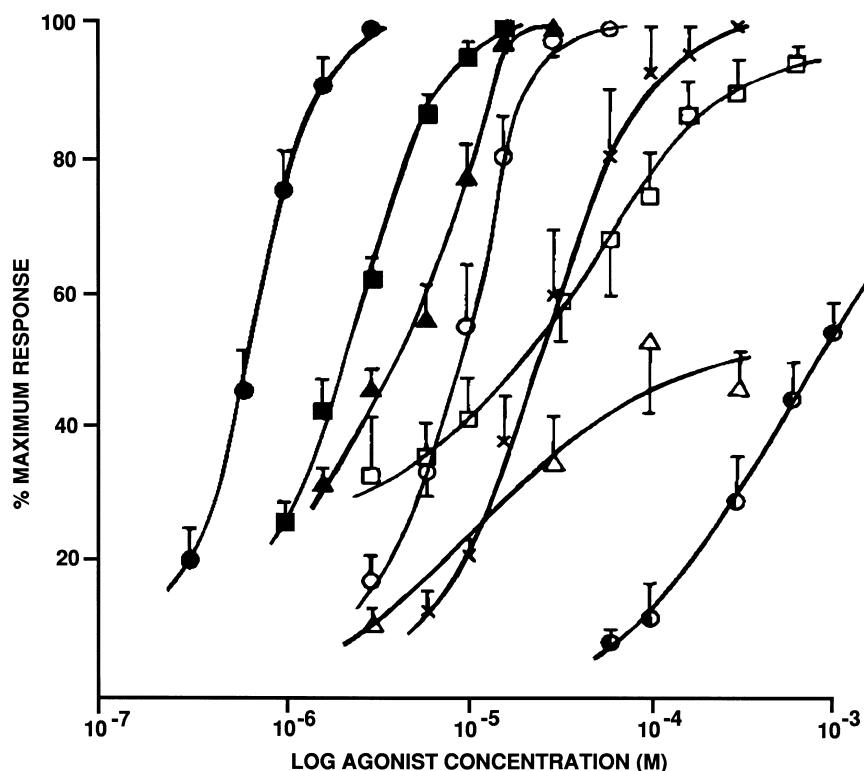


Fig. 2. Concentration–response curves for the contraction of the isolated guinea-pig ileum by ZAPA (●), GABA (■), and compounds 39 (●), 40 (×), 41 (○), 42 (▲), 43 (□), 44 (△). Curves are drawn as lines of best fit for means \pm SE mean from at least four experiments.

well as possible decomposition presenting problems in the analysis of the activity of 28.

The bis isothiuronium analogues (37–44) were the final compounds of the series shown in Fig. 1 to exhibit GABA-like activity on the guinea-pig isolated ileum. The only compounds to have parallel concentration–response curves with GABA and ZAPA were the C5, C6 and C8 compounds (40, 41 and 42, respectively), with the concentration–response curve for the C8 deviating from parallel at lower concentrations (Fig. 2). For the C4, C10 and C12 analogues (39, 43 and 44, respectively) the concentration–response curves did not follow the characteristic shape observed for the other compounds. Compound 39 was considerably weaker, compounds 43 and 44 had decreased slopes, and compounds 39 and 44 at the maximum concentration of 1 mM did not produce the same maximum contraction as that observed with GABA. EC_{50} s, as calculated by log-probit analysis for at least $n = 5$ experiments, are 3.9 ± 0.4 , 8.1 ± 0.7 , 20.1 ± 2 and $22.6 \pm 2.4 \mu\text{M}$ for 42, 41, 43 and 40, respectively. EC_{50} s for compounds 39 and 44 that would relate to their

GABA-like activity could not be assigned in the normal way. However, they produced contractions of half the maximal response achieved by GABA at concentrations of approx. 600 and 100 μM , respectively. Compounds 37 and 38 did not produce significant contractions at concentrations of 500 μM .

GABA_A receptor antagonist activity was observed for the following: compounds 5, 8 and 9, which could only be obtained as mixtures of *E* and *Z* isomers; the *E* isomer of 10; and compounds 15, 16, 17, 19, 22, 25, 26, 39, 41–44. Compound 40 was not tested due to insufficient sample. The bis compounds 39 and 41–44 were the most potent. Compound 39, at a concentration of 60 μM , reduced the 100 μM GABA response by 50%, while the other bis compounds 41–44 totally blocked the 100 μM GABA response when administered to the organ bath at a concentration of 60 μM . The remaining antagonist compounds reduced the 100 μM GABA response to 70–80% when added to the bath at a concentration of 300 μM , and thus were considerably weaker than the bis compounds or bicuculline, which totally blocked the 100 μM GABA

response at a concentration of 16 μM . None of these compounds showed activity as antagonists of acetylcholine-induced contractions of the ileum.

Effects on the binding of [^3H]diazepam

Generally, the activity of the compounds for the facilitation of [^3H]diazepam binding to rat brain membranes paralleled their activity profile for transient contractions of the guinea-pig ileum, with the exception of compounds **19**, **21** and **33**. Compound **19** had no significant agonist activity on the guinea-pig ileum, yet displayed moderate ability to augment the binding of [^3H]diazepam, being 95 times less active than ZAPA (Table 1). Compounds **21** and **33** were active on the guinea-pig isolated ileum but inactive on [^3H]diazepam binding at 100 μM .

Of the bis compounds **37–44**, all but **37** showed some ability to interact with the benzodiazepine site of GABA. However, at higher concentrations (100 μM), the facilitation of [^3H]diazepam binding decreased and direct inhibition of binding occurred. This complex activity observed with the bis compounds is illustrated in Fig. 3, and may reflect partial agonist actions of varying efficacy.

Effects on GABA uptake and transamination in rat brain

Practically all the compounds shown in Fig. 1 had little or no activity on the inhibition of [^3H]GABA uptake into rat brain slices or on the enzyme degradation of [^{14}C]GABA in rat brain mitochondrial preparations. Some significant, but weak, activity on GABA uptake ($\text{EC}_{50}\text{s} > 500 \mu\text{M}$) was seen by compounds **3** (38% inhibition at 500 μM), **23** (59%), **25** (52%), **27** (39%) and **28** (30%). Compounds **3** (25% inhibition at 1 mM), **19** (48%) and **20** (58%) showed weak ability to inhibit the transamination of GABA. Compounds **37–44** were not tested on these assays.

DISCUSSION

Replacement of the amino functionality of GABA with the planar isothiuronium or guanidino groups leads to active GABA_A receptor agonists. In the series of simple ω -guanidino carboxylic acids, maximum activity resides in compound **23**, which has a 2 carbon chain between the zwitterionic groups (EC_{50} 18 μM on the guinea-pig isolated ileum). A similar result applies

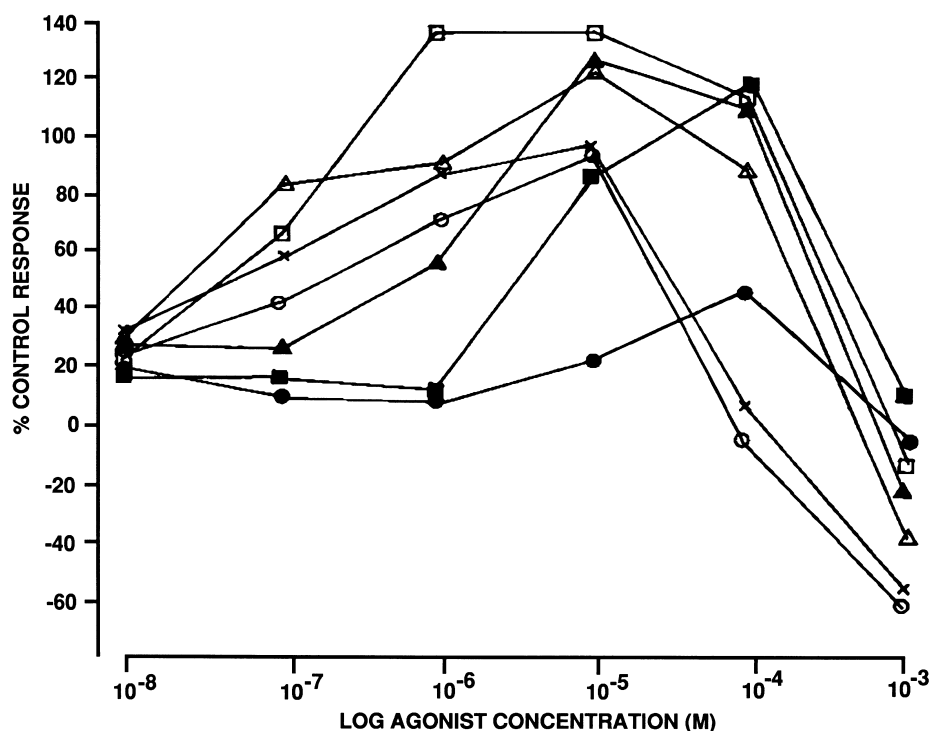


Fig. 3. Effects on [^3H]diazepam binding of compounds **38–44**. **38** (●), **39** (■), **40** (▲), **41** (○), **42** (□), **43** (Δ), **44** (×). The points are means from three to five experiments with error bars omitted for clarity.

to the saturated isothiuronium series where 3-[(aminoiminomethyl)thio]propanoic acid is slightly more potent [EC_{50} 13 μ M; Allan *et al.* (1986b)]. This relationship between chain length and activity is carried through to the imidate series where, from the available compounds, the analogue with a similar chain length (**27**) possesses considerable activity (EC_{50} 47 μ M). Considering the compounds with one carbon less, the guanidino derivative (**21**) has moderate activity in contrast to the corresponding isothiuronium compound which gave no significant contraction of the guinea-pig isolated ileum at 500 μ M.

While incorporation of a conformationally restricting double bond into a two carbon chain results in unstable guanidino derivatives, unsaturated imidates such as **28** (Wiley and Daniels, 1965) and a much wider variety of unsaturated isothiuronium analogues can be prepared. The affinity of guanidino, isothiuronium and imidate replacement groups is probably related to their planar nature and the delocalization of the positive charge. These two factors have previously been the basis for the development of other GABA receptor agents (Allan *et al.*, 1986b; Wermuth *et al.*, 1987). Within the isothiuronium analogues, those in the *Z* conformation were by far the most active as GABA_A agonists, while those in the *E* conformation tended to exhibit antagonist activity. The low activity of *N*-methyl ZAPA (**1**) is surprising. Table 1 shows that, for agonist activity of the *N*-substituted ZAPA derivatives, activity increases dramatically up to the hexyl compound. Attempted preparation of higher homologues resulted in impure mixtures of *E* and *Z* isomers which were difficult to characterize and were not investigated further. The unfavourable effect of short chain substituents is also evident in the series of bis compounds (**37–44**), where no appreciable activity is observed with ethylene and propylene linkages. As the carbon chain of the bis compounds increases to $n=8$, maximum agonist activity is achieved and the EC_{50} for the contraction of the guinea-pig isolated ileum is similar to that of the *n*-hexyl compound (**5**). It is probable that activation of the receptor by the bis compounds involves interaction of only one of the two "ZAPA" moieties. As the chain length increases further to C10–12, agonist activity decreases and antagonist activity becomes evident.

Partial agonist/antagonist activity on the guinea-pig ileum preparation was seen in three groups of compounds: the bis analogues; *E* analogues of ZAPA with bulky substituents; and some analogues with chains of three or more carbons linking the carboxyl

group to the amino replacement group. These results indicate that, in designing GABA antagonists, two factors can contribute to activity; firstly, an increased distance between the zwitterionic groups, and secondly, a bulky substituent such as an aromatic group on the cationic end of the molecule. Partial agonists of GABA_A receptors, particularly those of relatively low efficacy, show promise as therapeutic agents (Krogsgaard-Larsen *et al.*, 1994).

Acknowledgements—We are grateful to the National Health and Medical Research Council of Australia for financial support of this work and Ms Ann McGregor for her assistance in preparing this manuscript.

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