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Analogues of the low-efficacy partial $GABA_A$ agonist 4-PIOL. Syntheses and *in vitro* pharmacological studies

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Summary — 4-PIOL (3-hydroxy-5-(4-piperidyl)isoxazole) is a low-efficacy $GABA_A$ agonist showing a dominating $GABA_A$ antagonist profile. Three dihydro analogues of 4-PIOL were synthesized, including (*RS*)-3-hydroxy-5-(4-piperidyl)-2-isoxazoline (**1**). The synthesis of **1** was based on a regioselective 1,3-dipolar cyclo-addition reaction between 1-benzyloxycarbonyl-4-vinylpiperidine (**7**) and bromonitrile oxide, prepared *in situ* from dibromoformoxime. Furthermore, the spiro analogues of **1** 3-hydroxy-1-oxa-2,8-diazaspiro[4.5]dec-2-ene (**2**) and (*RS*)-3-hydroxy-1-oxa-2,7-diazaspiro[4.5]dec-2-ene (**3**) were synthesized regiospecifically *via* cycloaddition of bromonitrile oxide to the *N*-benzyloxycarbonyl-protected forms of 4-methylenepiperidine (**11**) and 3-methylenepiperidine (**15**), respectively. In contrast to 4-PIOL, none of the new compounds **1–3** showed detectable effects on the binding of ^{3}H-$GABA_A$ or the subunit-selective $GABA_A$ agonist, ^{3}H-THIP, to $GABA_A$ receptor sites, and they did not significantly affect the muscimol-stimulated binding of ^{3}H-diazepam to the benzodiazepine site of the $GABA_A$ receptor complex.

Résumé — Analogues du 4-PIOL agonist partiel du $GABA_A$ de faible efficacité. Synthèses et études pharmacologiques *in vitro*. *Le 4-PIOL (3-hydroxy-5(4-pipéridyl)isoxazole) est un agoniste gabaergique de basse efficacité, montrant un profil d'antagoniste gabaergique dominant. Trois analogues dihydrogénés du 4-PIOL ont été synthétisés, incluant la (*RS*)-3-hydroxy-5-(4-pipéridyl)-2-isoxazoline (**1**). La synthèse de **1** est basée sur une réaction de cycloaddition 1,3-dipolaire régiosélective entre la 1-benzyloxycarbonyl-4-vinylpipéridine (**7**) et l'oxyde de bromonitrile, préparé* in situ *à partir de la dibromoformoxime. De plus, les analogues spiraniques de **1**, les 3-hydroxy-1-oxa-2,8-diazaspiro[4,5-]déc-2-ène (**2**) et (*RS*)-3-hydroxy-1-oxa-2,7-diazaspiro[4,5]déc-2-ène (**3**) ont été synthétisés de façon régiospécifique par cycloaddition de l'oxyde de bromonitrile aux formes protégées par des N-benzyloxycarbonyles de la 4-méthylènepipéridine (**11**) et de la 3-méthylènepipéridine (**15**) respectivement. Contrairement au 4-PIOL, aucun des nouveaux composés **1–3** n'a montré d'effets décelables sur la liaison du ^{3}H-GABA ou la sous-unité sélective de l'agoniste du $GABA_A$, ^{3}H-THIP, aux sites du récepteur $GABA_A$ et ils n'ont pas affecté de façon significative la liaison stimulée par le muscimol du ^{3}H-diazépam au site benzodiazépinique du complexe récepteur du $GABA_A$.*

$GABA_A$ receptors / partial $GABA_A$ agonists / THIP analogues / 4-PIOL analogues / 3-hydroxy-2-isoxazolines / 3-hydroxy-2-isoxazoline spiro compounds / cycloaddition reactions / dibromoformoxime / bromonitrile oxide

Introduction

Dysfunctions of the central 4-aminobutanoic acid (GABA) neurotransmitter system(s) have been associated with certain neurological disorders such as Huntington's chorea [1], epilepsy [2, 3], and tardive dyskinesia [4, 5]. In Huntington's chorea there is a marked loss of GABA neurones, whereas no significant changes in numbers and binding characteristics of $GABA_A$ receptors could be detected [6]. Never-

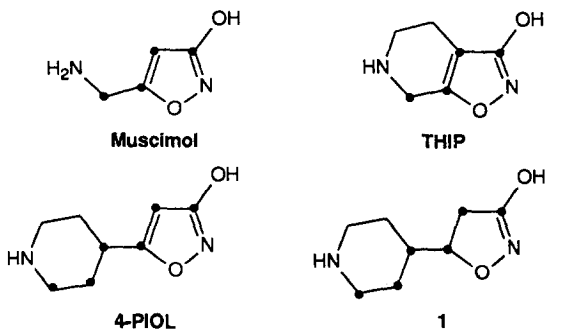

Fig 1.

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theless, replacement therapies using the specific $GABA_A$ agonists muscimol [7] or 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-3-ol (THIP) [8] (fig 1) did not significantly ameliorate the symptoms of choreic patients. Similarly, THIP only marginally improved the symptoms of patients suffering from epilepsy [9] or tardive dyskinesia [10].

In spite of these largely negative clinical results, $GABA_A$ receptor stimulating drugs may have clinical interest in the diseases concerned. The lack of marked clinical effects of muscimol or THIP may reflect rapid desensitization of the target $GABA_A$ receptors [11] as a result of prolonged activation by these very potent GABA agonists showing full $GABA_A$ agonist efficacy [12, 13]. If this is the case, low-efficacy $GABA_A$ agonists may have therapeutic interest [14]. Furthermore, low-efficacy $GABA_A$ agonists expressing a dominating $GABA_A$ antagonist profile may have clinical interest in Alzheimer's disease, where central cholinergic neurones are in a state of progressive degeneration [15]. Since cholinergic neurones in the brain are under inhibitory GABAergic control *via* $GABA_A$ receptors [16], non-convulsant $GABA_A$ antagonists, which in addition show marginal $GABA_A$ agonist effects, are expected to reduce this inhibition and thus stimulate acetylcholine release. In principle, compounds showing this pharmacological profile have therapeutic interest as drugs capable of improving learning and memory in Alzheimer patients without causing convulsions.

The non-fused THIP analogue 5-(4-piperidyl)isoxazol-3-ol (4-PIOL) (fig 1) has been shown to act as a relatively weak $GABA_A$ agonist on cat spinal neurones [17] but to antagonize muscimol-stimulated benzodiazepine binding in rat cortical membranes [18]. Single-cell pharmacological studies have demonstrated that 4-PIOL is acting as a low-efficacy $GABA_A$ agonist showing a dominating $GABA_A$ antagonist profile on spinal as well as supraspinal neurones in culture [19].

In this paper we describe the syntheses and *in vitro* pharmacological studies of (*RS*)-3-hydroxy-5-(4-piperidyl)-2-isoxazoline (**1**), a dihydro analogue of 4-PIOL (fig 1), and the spiro analogues 3-hydroxy-1-oxa-2,8-diazaspiro[4,5]dec-2-ene (**2**) and (*RS*)-3-hydroxy-1-oxa-2,7-diazaspiro[4.5]dec-2-ene (**3**) (fig 2).

Chemistry

The synthesis of the target compound **1** was based on a 1,3-dipolar cycloaddition reaction between 1-benzyloxycarbonyl-4-vinyl-piperidine (**7**) and bromonitrile oxide, generated *in situ* from dibromoformoxime (scheme 1). The monosubstituted dipolarophile **7** was synthesized from the *N*-protected isonipecotic acid methyl ester **5**, which was prepared

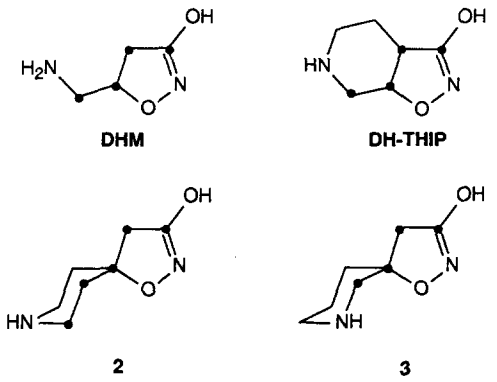


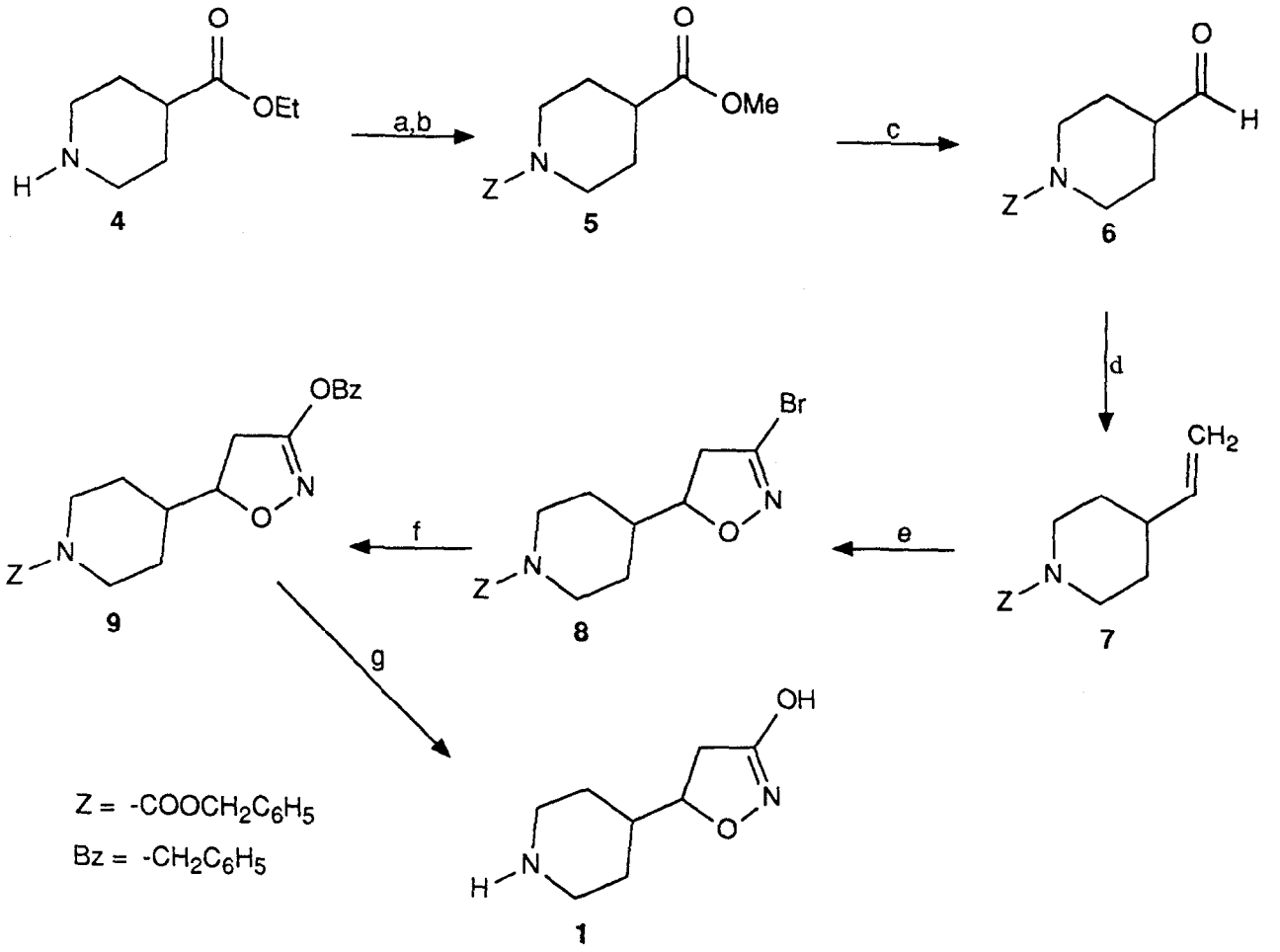
Fig 2.

in 2 steps from **4**. A DIBAH reduction of crude **5** gave **6**, which was converted into **7** by a Wittig reaction. In the subsequent cycloaddition reaction **7** was transformed regioselectively into the 3-bromo-2-isoxazoline **8**. Treatment of **8** with a DMSO suspension of lithium benzylate [20] gave **9**, and the deprotection of **9** to give zwitterionic **1** was carried out under conditions which did not cause significant hydrogenolysis of the 2-isoxazoline ring.

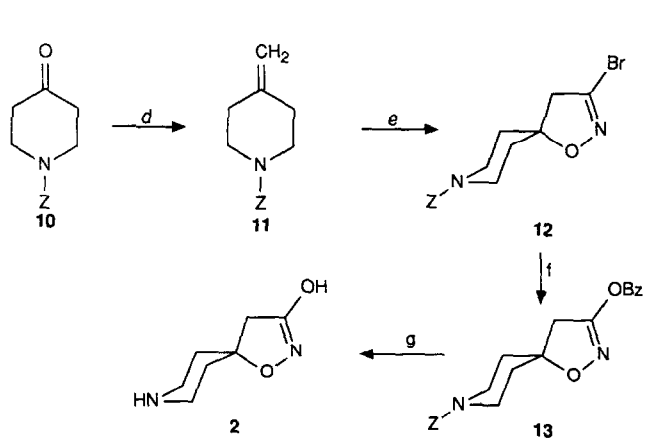
The zwitterionic spiro compounds **2** (scheme 2) and **3** (scheme 3) were synthesized analogously by treatment of the disubstituted dipolarophiles **11** and **15**, respectively, with bromonitrile oxide.

All of the 3 cycloaddition reactions described were performed by using a 2-fold excess of the bromonitrile oxide precursor, dibromoformoxime [21]. The reaction was accomplished in an ethyl acetate suspension of sodium bicarbonate, and by this slow *in situ* generation of the 1,3-dipole, the desired reaction with the dipolarophile was facilitated at the expense of the competing dimerization of the 1,3-dipole [22]. Under these reaction conditions the 5-substituted 3-bromo-2-isoxazolines **8** (92%), **12** (87%), and **16** (81%) were synthesized in high yields as indicated.

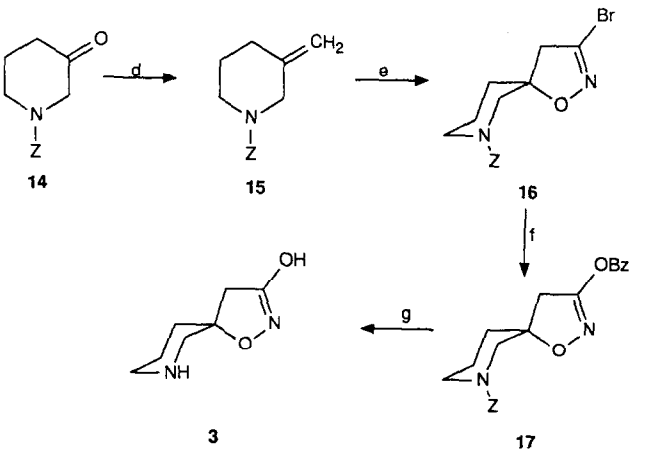
The regioselectivity of the 1,3-dipolar cycloadditions described was established by ^1H NMR spectroscopy. Thus, in no case did 90 MHz ^1H NMR analyses of the crude cycloaddition reaction products disclose the formation of detectable amounts of the unwanted regioisomers containing piperidine substituents in the 4-position of the 2-isoxazoline rings. The following ^1H NMR spectroscopic evidence allowed unequivocal structure determinations of the cycloaddition reaction products: in compound **8** the H-5 proton of the 2-isoxazoline ring resonated as a multiplet centered at δ 4.50 in agreement with previously reported general findings [20], whereas the H-4 protons of **12** and **16** resonated as a singlet (δ 2.89)



Scheme 1. a: 2 N NaOH, $ClCOOCH_2C_6H_5$; b: CH_2N_2, Ether; c: DIBAH, –60°C; d: $Ph_3P{=}CH_2$, THF; e: $Br_2C{=}NOH$, $NaHCO_3$; f: BzOLi/DMSO; g: H_2, 5% Pt/C.



Scheme 2. d, e, f, g, Z and Bz, see scheme 1.



Scheme 3. d, e, f, g, Z and Bz, see scheme 1.

and as a collapsed AB system (δ 2.92), respectively. It is worth pointing out that the ^{1}H NMR spectrum of compound **2** showed the same pattern as that of the 3-bromoisoxazoline **12**, while, on the contrary, the isoxazoline protons of zwitterion **3** resonated as doublets (J_{gem} = 16.3 Hz).

The structures of all new compounds described were established on the basis of ^{1}H NMR spectroscopic analyses, and these structural assignments were supported by elemental analyses.

In vitro pharmacology

The compounds **1–3** were tested *in vitro* as inhibitors of the binding of ^{3}H-GABA and the subunit-selective [23] $GABA_A$ agonist ^{3}H-THIP [12, 24] to $GABA_A$ receptor sites in rat brain synaptic membranes. In order to detect a potential $GABA_A$ antagonist affinity of compounds **1–3**, their effects on the muscimol-stimulated binding of ^{3}H-diazepam to benzodiazepine receptor sites were tested. However, none of the test compounds showed significant effects in these binding assays representing different sites of the postsynaptic $GABA_A$ receptor complex [14] (table I).

Results and discussion

A number of previous papers describe the successful use of 1,3-dipolar cycloaddition chemistry for the synthesis of heterocyclic GABAergic [20, 25], cholinergic [26, 27], and adrenergic [28] compounds. Using this type of heterocyclic chemistry, we have now synthesized the zwitterionic 3-hydroxy-2-isoxazoline derivatives **1–3**, which are structurally related to the $GABA_A$ receptor ligand 4-PIOL (figs 1, 2).

4-PIOL is a $GABA_A$ antagonist, which, in addition, shows weak and low-efficacy $GABA_A$ agonist effects [17–19]. This pharmacological profile of 4-PIOL is, at least theoretically, of therapeutic interest in certain diseases, notably Alzheimer's disease.

As an attempt to shed further light on the relationship between structure and $GABA_A$ agonist efficacy of heterocyclic GABA analogues the compounds **1–3** were synthesized and tested. Compound **1** is a dihydro analogue of 4-PIOL, whereas **2** is a lower homologue of **1** of restricted conformation. The design of **3** did, however, serve a dual purpose. Not only is **3** a lower homologue of **2**, it also is a conformationally restricted analogue of the very potent $GABA_A$ agonist dihydromuscimol (DHM) [29, 30] (fig 2). The dihydro analogue of THIP, DH-THIP (fig 2), has previously been shown to be completely devoid of affinity for $GABA_A$ receptor sites [31], and **3** has now been shown also to be inactive as an inhibitor of the binding of ^{3}H-GABA or ^{3}H-THIP (table I). On the basis of these observations it may be concluded that neither DH-THIP nor **3** reflect the active conformation(s) of DHM (fig 2).

Similarly, **1** and **2** do not inhibit detectably the $GABA_A$ receptor binding of ^{3}H-GABA or ^{3}H-THIP, and, accordingly, the muscimol-stimulated binding of ^{3}H-diazepam to the $GABA_A$ receptor-associated benzodiazepine site (table I). Since the conformational flexibility of the piperidine rings of 4-PIOL and **1** is very similar, it may be assumed that the planar structure of the isoxazole ring of 4-PIOL is essential for its interaction with the $GABA_A$ receptor.

In agreement with the very strict structural requirements for full agonist activity at the $GABA_A$ receptor [14, 18, 32], the present studies seem to indicate that compounds showing a pharmacological profile similar to that of 4-PIOL also have to meet strict structural requirements. Further synthetic, theoretical, and molecular pharmacological studies on this subject are in progress.

Table I. Effects of GABA and some heterocyclic GABA analogues on $GABA_A$ synaptic sites.

	Effects in vitro on		
	^{3}H-GABA		*^{3}H-THIP*
	Receptor binding		*Muscimol-stimulated ^{3}H-Diazepam binding*
Compound	*(IC_{50}, μM)*		*(ED_{50}, μM)*
GABA	0.03	0.06	–
THIP	0.1	0.04	–
4-PIOL	6	10	100
1	> 100	> 100	> 1000
2	> 100	> 100	> 1000
3	> 100	> 100	> 1000

Experimental protocols

Materials and methods

Melting points are corrected and were determined in capillary tubes. Elemental analyses of the crystalline compounds were performed at the Chemical Laboratory II, University of Copenhagen.

The 60 MHz ^{1}H NMR spectra were recorded on a Varian 360L spectrometer. The 90 MHz ^{1}H NMR spectra were recorded on a JEOL FX 90Q spectrometer. Me_4Si was used as an internal standard except for the compounds dissolved in D_2O, where sodium 3-(trimethylsilyl)-propanesulfonate was used. TLC analyses were performed on silica gel 60 F_{254} precoated on aluminium sheets (Merck); compounds were visualized on the TLC plates by UV_{254} light and by spraying with a potassium permanganate solution. Column chromatography separations were performed on silica gel (Merck, 60H). Liquid derivatives were characterized by the oven temperature for Kugelrohr distillations. Ethyl isonipecotate, 4-piperidone monohydrate

hydrochloride, 1-benzyl-3-piperidone hydrochloride hydrate were purchased from Aldrich. All evaporations were performed under vacuum on a rotary evaporator at ≈ 15 mmHg.

*Methyl 1-benzyloxycarbonyl-4-piperidinecarboxylate (**5**)*
To a solution of ethyl isonipecotate (**4**) (3.1 ml, 20 mmol) in NaOH (75 ml, 2 N), 4 ml (28 mmol) of benzylchloroformate in NaOH (10 ml, 2 N) were added in one portion. The reaction mixture was stirred for 1 h and then extracted with diethyl ether (4 x 20 ml). The aqueous phase was acidified (pH = 2) by addition of 4 N HCl and extracted with ethyl acetate (3 x 30 ml). The pooled and dried ($MgSO_4$) organic extracts were concentrated and the crude intermediate carboxylic acid was treated with ethereal diazomethane. Excess of reagent was destroyed with glacial acetic acid and the residue was purified through column chromatography (eluent: toluene–ethyl acetate 2:1). Compound **5** was isolated as a TLC pure colourless oil (4.55 g, 82% from **4**) and was used without further purification. ^{1}H NMR (60 MHz, $CDCl_3$): δ 7.46 (5H, s), 5.18 (2H, s), 4.37–4.16 (1H, m), 4.14–3.92 (1H, m), 3.71 (3H, s), 3.25–2.66 (2H, m), 2.64–2.25 (1H, m), 2.14–1.65 (4H, m).

*1-Benzyloxycarbonyl-4-piperidinecarbaldehyde (**6**)*
DIBAH (32 ml, 1.6 M solution in hexane) was added dropwise over a period of 30 min to a stirred solution of crude **5** (4.43 g, 16 mmol) in toluene with cooling to –60°C. The reaction mixture was vigorously stirred for 1 h, then HCl (80 ml, 2 N) was cautiously added. The cooling bath was removed and the slurry was allowed to warm at 0°C. After filtration, the organic layer was separated and the aqueous phase was extracted with diethyl ether (3 x 25 ml). The combined organic phases were dried ($MgSO_4$) and concentrated under vacuum. The crude reaction mixture was column chromatographed (eluent: toluene–ethyl acetate 9:1) to give **6** as a light yellow oil (2.69 g, 68%). Anal ($C_{14}H_{17}NO_3$): C, 69.98; H, 6.93; N, 5.69; Found: C, 69.38; H, 6.59; N, 5.27. ^{1}H NMR (60 MHz, $CDCl_3$): δ 9.78 (1H, s), 7.42 (5H, s), 5.17 (2H, s), 4.33–4.08 (1H, m), 4.06–3.85 (1H, m), 3.29–2.74 (2H, m), 2.70–2.16 (1H, m), 2.11–1.33 (4H, m).

*1-Benzyloxycarbonyl-4-vinylpiperidine (**7**)*
Methyltriphenylphosphonium bromide (2.72 g, 7.6 mmol) was suspended in THF (25 ml). Butyl lithium (4.75 ml, 1.6 M solution in hexane) was slowly added at 0°C under an inert atmosphere using a syringe. The suspension was stirred for 1 h and the resulting orange-red solution was added dropwise at room temperature to a stirred solution of the aldehyde **6** (0.94 g, 3.8 mmol) in THF (25 ml). Stirring was maintained overnight. To the solution 15 ml of water were added and the crude reaction mixture was extracted with diethyl ether (4 x 15 ml). The combined organic extracts were dried ($MgSO_4$), concentrated under vacuum and the residue was purified by column chromatography (eluent: toluene–ethyl acetate 20:1) to give **7** (0.49 g, 52.5%); bp = 190–195°C/1 mmHg as a colourless oil. Anal ($C_{15}H_{19}NO_3$): C, 73.42; H, 7.80; N, 5.73; Found: C, 73.23; H, 7.91; N, 5.58. ^{1}H NMR (90 MHz, $CDCl_3$): δ 7.32 (5H, s), 5.72 (1H, ddd, J = 9.6, 6.4, 17.6 Hz), 5.11 (2H, s), 5.08–4.61 (2H, m), 4.16–3.69 (2H, m), 2.90–2.43 (2H, m), 2.25–1.85 (1H, m), 1.82–1.03 (4H, m).

*(RS)-3-Bromo-5-(1-benzyloxycarbonyl-4-piperidyl)-2-isoxazoline (**8**)*
A suspension of dibromoformoxime [21] (0.66 g, 3.26 mmol), olefin **7** (0.40 g, 1.63 mmol) and sodium bicarbonate (1.4 g, 16.6 mmol) in ethyl acetate (30 ml) was stirred at room temperature until gas evolution had ceased (≈ 36 h). The slurry was then treated with water (20 ml) and extracted with ethyl acetate (3 x 15 ml). The combined organic layers were dried ($MgSO_4$), concentrated under vacuum and the residue was purified by column chromatography (toluene-ethyl acetate 4:1) yielding **8** as a colourless oil (0.55 g, 92%). Anal ($C_{16}H_{19}N_2O_3Br$): C, 52.31; H, 5.21; N, 7.66; Br, 21.75; Found: C, 51.80; H, 5.27; N, 7.31; Br, 21.20. ^{1}H NMR (90 MHz, $CDCl_3$): δ 7.35 (5H, s), 5.12 (2H, s), 4.64–4.16 (3H, m), 3.10 (1H, dd, J = 10.4 Hz, J_{gem} = 17.4 Hz), 3.01 (1H, dd, J = 9.1 Hz, J_{gem} = 17.4 Hz), 2.95–2.49 (2H, m), 2.00–1.04 (5H, m).

*(RS)-3-Benzyloxy-5-(1-benzyloxycarbonyl-4-piperidyl)-2-isoxazoline (**9**)*
To a solution of benzyl alcohol (3.3 ml) in anhydrous DMSO (12 ml) butyl lithium (2.8 ml, 1.6 M in hexane) was added. The suspension was stirred at room temperature for 20 min, then poured into a solution of **8** (0.53 g, 1.44 mmol) in DMSO (10 ml). The dark yellow mixture was stirred for 3 h and after addition of water (30 ml), extracted with diethyl ether (4 x 20 ml). The combined organic extracts were washed with water (3 x 15 ml) and dried ($MgSO_4$). The filtered reaction mixture was concentrated at 40°C/10 mmHg and the distilled at 100°C/1 mmHg to remove excess of benzyl alcohol. The residue was column chromatographed (toluene–ethyl acetate 9:1) to yield derivative **9** (0.43 g, 75.5%) as a TLC pure oil. Anal ($C_{23}H_{26}N_2O_4$): C, 70.01; H, 6.64; N, 7.13; Found: C, 70.39; H, 6.99; N, 6.71. ^{1}H NMR (60 MHz, $CDCl_3$): δ 7.37 (5H, s), 7.34 (5H, s), 5.10 (4H, s), 4.47–3.95 (3H, m), 3.05–2.45 (4H, m), 1.95–1.05 (5H, m).

*(RS)-3-Hydroxy-5-(4-piperidyl)-2-isoxazoline (**1**)*
A solution of 0.43 g (1.09 mmol) of **9** in methanol (20 ml) was submitted to catalytic hydrogenation at atmospheric pressure over 5% Pd/C (90 mg). The disappearance of the starting material was followed by TLC. Removal of the catalyst and evaporation of the solvent yielded crude **1** (0.143 g, 77%) as an oil which was crystallized from absolute ethanol (colourless prisms, mp = 170–171°C. Anal ($C_8H_{14}N_2O_2$): C, 56.41; H, 8.28; N, 16.51. Found: C, 56.15; H, 8.49; N, 16.15. ^{1}H NMR (90 MHz): δ 4.27 (1H, m), 3.43 (2H, m), 3.05–2.85 (2H, m), 2.76 (1H, dd, J = 9.0 Hz, J_{gem} = 16.1 Hz), 2.51 (1H, dd, J = 8.2 Hz, J_{gem} = 16.1 Hz), 2.06–1.90 (1H, m), 1.88–1.72 (2H, m), 1.58–1.32 (2H, m).

*1-Benzyloxycarbonyl-4-piperidone (**10**)*
10 was prepared in 90% yield from commercial 4-piperidone hydrochloride monohydrate following a known procedure [33] (bp = 165–170°C/2 mmHg, lit [34]: mp = 37–38°C). ^{1}H NMR (60 MHz, $CDCl_3$): δ 7.45 (5H, s), 5.23 (2H, s), 3.80 (4H, t, J = 6 Hz), 2.43 (4H, t, J = 6 Hz).

*1-Benzyloxycarbonyl-3-piperidone (**14**)*
To a solution of 1-benzyl-3-piperidone hydrochloride hydrate (11.29 g, 50 mmol) in water (100 ml) solid potassium carbonate (13.82 g, 0.1 mol) was added portionwise under vigorous stirring. The free amine was extracted with dichloromethane (4 x 20 ml) and the combined extracts were dried ($MgSO_4$) and were immediately reacted with excess benzyl chloroformate (21.3 ml, 0.15 mol) at room temperature. The reaction mixture was stirred for 3 h, washed with 4 N HCl (3 x 20 ml) and dried ($MgSO_4$). The residue was column chromatographed (eluent: toluene–ethyl acetate 5:1) to yield **14** as an oil (8.96 g, 77%). IR and ^{1}H NMR data were identical to those previously published for **14** [34].

*1-Benzyloxycarbonyl-4-methylenepiperidine (**11**) and 1-benzyloxy-carbonyl-3-methylenepiperidine (**15**)*

The piperidine substituted alkenes **11** and **15** were synthesized following the same protocol as described above for **7**. Compound **11** (yield 4.51 g, 52% from **10**) was column chromatographed (toluene–ethyl acetate 15:1) and distilled (bp = 200–205°C/0.3 mmHg) to give a colourless oil. Anal ($C_{14}H_{17}NO_2$): C, 72.68; H, 7.41; N, 6.08; Found: C, 72.42; H, 7.43; N, 5.74. ^{1}H NMR (60 MHz, $CDCl_3$): δ 7.43 (5H, s), 5.20 (2H, s), 4.80 (2H, broad s), 3.68–3.36 (4H, m), 2.44–2.05 (4H, m). Compound **15** (yield 2.08 g, 24% from **14**) was column chromatographed (toluene–ethyl acetate 10:1) and distilled (bp = 205–210°C/0.6 mmHg) to give a colourless oil. Anal ($C_{14}H_{17}NO_2$): C, 72.68; H, 7.41; N, 6.08; Found: C, 72.88; H, 7.43; N, 5.92. ^{1}H NMR (60 MHz, $CDCl_3$): δ 7.31 (5H, s), 5.07 (2H, s), 4.73 (2H, m), 3.89 (2H, s), 3.61–3.22 (2H, m), 2.38–2.04 (2H, m), 1.82–1.44 (2H, m).

*8-Benzyloxycarbonyl-3-bromo-1-oxa-2,8-diazaspiro[4.5]dec-2-ene (**12**) and (RS)-7-benzyloxycarbonyl-3-bromo-1-oxa-2,7-diazaspiro[4.5]dec-2-ene (**16**)*

1.88 g (8.14 mmol) of the dipolarophiles **11** or **15** were reacted with dibromoformoxime (3.3 g, 16.28 mmol) under the same conditions as previously reported for compound **8**. After the usual workup, the crude cycloaddition mixtures underwent column chromatography purification. Compound **12** (eluent: toluene–ethyl acetate 5:1) was obtained as a colourless oil, which solidified and was recrystallized from 2-propanol to give **12** (2.13 g, 74%) as colourless crystals, mp = 94–95°C. Anal ($C_{15}H_{17}N_2O_3Br$): C, 50.99; H, 4.85; N, 7.96; Br, 22.61; Found: C, 51.02; H, 5.02; N, 7.87; Br, 22.70. ^{1}H NMR (60 MHz, $CDCl_3$): δ 7.30 (5H, s), 5.09 (2H, s), 4.05–3.20 (4H, m), 2.89 (2H, s), 1.95–1.57 (4H, m). Compound **16** (eluent: toluene–ethyl acetate 5:1) was obtained as a colourless oil (2.32 g, 80.8%). Anal ($C_{15}H_{17}N_2O_3Br$): C, 50.99; H, 4.85; N, 7.96; Br, 22.61; Found: C, 51.42; H, 5.01; N, 7.75; Br, 22.48. ^{1}H NMR (90 MHz, $CDCl_3$): δ 7.35 (5H, s), 5.13 (2H, s), 3.88–3.54 (2H, m), 3.44–3.07 (2H, m), 2.92 (2H, broad s), 2.09–1.50 (4H, m).

*8-Benzyloxycarbonyl-3-benzyloxy-1-oxa-2,8-diazaspiro[4.5]-dec-2-ene (**13**) and (RS)-7-benzyloxycarbonyl-3-benzyloxy-1-oxa-2,7-diazaspiro[4.5]dec-2-ene (**17**)*

1.50 g (4.25 mmol) of the 3-bromo-2-isoxazolines **12** or **16** in DMSO (20 ml) were reacted with a lithium benzylate suspension (13.22 mmol) in DMSO (30 ml) according to the procedure described for **9**. After the reaction workup, the crude mixtures were purified by column chromatography. Compound **13** (eluent: toluene–ethyl acetate 3:1) was isolated as a crystalline compound which was recrystallized from 2-propanol as colourless needles (1.28 g, 79%), mp = 81–82°C. Anal ($C_{22}H_{24}N_2O_4$): C, 69.44; H, 6.36; N, 7.39. Found: C, 69.64; H, 6.42; N, 7.38. ^{1}H NMR (90 MHz, $CDCl_3$): δ 7.37 (5H, s), 7.35 (5H, s), 5.13 (4H, s), 3.98–3.62 (2H, m), 3.59–3.21 (2H, m), 2.77 (2H, s), 2.12–1.31 (2H, m). Compound **17** (eluent: toluene–ethyl acetate 7:1) was isolated as a colourless oil (1.19 g, 74%). Anal ($C_{22}H_{24}N_2O_4$): C, 69.44; H, 6.36; N, 7.39. Found: C, 69.21; H, 6.44; N, 7.17. ^{1}H NMR (90 MHz, $CDCl_3$): δ 7.35 (5H, s), 7.32 (5H, s), 5.12 (2H, s), 5.11 (2H, s), 3.66 (2H, m), 3.32–3.02 (2H, m), 2.74 (2H, broad s), 1.84 (4H, m).

*3-Hydroxy-1-oxa-2,8-diazaspiro[4.5]dec-2-ene (**2**) and (RS)-3-hydroxy-1-oxa-2,7-diazaspiro[4.5]dec-2-ene (**3**)*

The intermediates **13** or **17** (0.75 g, 1.97 mmol) were dissolved in methanol (35 ml) and hydrogenated over 5% Pd-C (140 mg) at atmospheric pressure. After disappearance of the starting material (TLC control), the catalyst was filtered off and the solvent evaporated. Compound **2** was isolated as an oil (0.225 g, 72%) which was crystallized from methanol (prisms mp = 202–204°C, dec). Anal ($C_7H_{12}N_2O_2$, $1/4H_2O$): C, 52.28; H, 7.83; N, 17.42; Found: C, 52.35; H, 7.68; N, 17.10. ^{1}H NMR (90 MHz, D_2O): δ 3.16 (4H, m), 2.54 (2H, s), 2.05–1.77 (4H, m). Compound **3** was obtained as an oil which solidified after column chromatography purification (eluent: 2-propanol containing 2% aqueous ammonia (25%)). Yield: 0.24 g (78%). mp = 180–182.5°C (from methanol–ether). Anal ($C_7H_{12}N_2O_2$, H_2O): C, 48.30; H, 8.10; N, 16.09; Found: C, 48.32; H, 7.96; N, 15.69. ^{1}H NMR (90 MHz, D_2O): δ 3.38–3.17 (2H, m), 2.96–2.79 (2H, m), 2.64 (1H, d, J_{gem} = 16.3 Hz), 2.40 (1H, d, J_{gem} = 16.3 Hz), 1.98–1.67 (4H, m).

Inihibition of $GABA_A$ receptor binding

The ^{3}H-GABA binding assay was performed with rat brain synaptic membranes as previously described in detail [24]. Aliquots of synaptic membranes (0.8–1.2 mg of protein) were incubated in triplicate at 2°C in 2 ml of Tris–citrate buffer (pH 7.1) containing 5 nM ^{3}H-GABA. Test substances were added in various concentrations. The samples were incubated for 15 min at 2°C, followed by centrifugation. The pellets were rinsed twice with 5 ml portions of cold water and suspended in water (0.4 ml). The IC_{50} values were estimated by measuring the inhibition of at least 4 different concentrations. Non-specific binding in the presence of 1 mM GABA was subtracted.

^{3}H-THIP receptor binding experiments were performed by incubating membranes suspended in Na^+-free 50 mM–Tris-citrate (pH 7.1 with 5 nM ^{3}H-THIP alone or together with 1 mM-THIP to estimate non-displaceable binding. Generally, ≈ 1 mg of protein in an assay volume of 2 ml was incubated for 15 min at 0–4°C followed by centrifugation at 48 000 *g* for 10 min (Sorvall, rotor SM-34). The supernatants were discarded and the pellets twice rinsed superficially with 5-ml portions of cold water. Rinsed pellets were suspended in water (0.5 ml) and the radioactivity in 0.4 ml of suspension was counted (Packard Tri-carb) in 3 ml of Ria Luma (Lumac, Basel).

Stimulation of diazepam binding

The enhancement by GABA agonists of the ^{3}H-diazepam binding to membranes from rat cerebella was studied at 0°C and in the absence of chloride or at 30°C and in the presence of 150 mM sodium chloride. An earlier method [35] was modified, as described elsewhere in detail [36]. To aliquots of membranes in 0.1 M Tris–citrate buffer (pH 7.1), optionally containing sodium chloride in a concentration of 150 mM, was added the GABA agonist and the membranes were incubated in triplicate with 0.8 nM ^{3}H-diazepam for 30 min at 30°C. After incubation, the samples were diluted with ice-cold buffer and filtered immediately through Whatman GF/C glass-fibre filters. The filters were washed with 10 ml of buffer, and the radioactivity was measured by conventional scintillation counting methods. All binding values were calculated as specific binding, which is total binding minus binding in the presence of 3 μm diazepam.

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