SYNTHESIS AND RECEPTOR BINDING OF 5-AMINO[^{3}H]$_2$METHYL-3-ISOTHIAZOLOL ([^{3}H]THIOMUSCIMOL), A SPECIFIC GABA$_A$ AGONIST PHOTOAFFINITY LABEL

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SUMMARY

The synthesis of tritium labelled thiomuscimol (5-amino[^{3}H]$_2$methyl-3-isothiazolol) (**7c**), a specific and high-affinity agonist photoaffinity label for GABA$_A$ receptors, is described. The synthesis of **7c** is based on a procedure for the preparation of 5-amino[^{2}H]$_2$methyl-3-isothiazolol (**7b**). Methyl 3-ethoxyisothiazole-5-carboxylate (**3**), synthesized from 3-hydroxyisothiazole-5-carboxamide (**1**) *via* the corresponding methyl ester (**2**), was reduced with sodium borotritide to give 3-ethoxy-5-hydroxy[^{3}H]$_2$methyl-3-isothiazole (**4c**). 3-Ethoxy-5-phthalimido[^{3}H]$_2$-methylisothiazole (**6c**), synthesized from the 5-chloro[^{3}H]$_2$methyl analogue (**5c**) of (**4c**), was deprotected by treatment with concentrated hydrochloric acid to give **7c** with a specific radioactivity of 29 Ci/mmol. In pilot binding assays, **7c** was shown to bind to membranes from rat forebrain in a saturable manner and with K_D and B_{max} values of 28 ± 6 nM and 50 ± 4 fmol/mg original tissue, respectively.

Key Words: 5-aminomethyl-3-isothiazolol (thiomuscimol), GABA$_A$ agonist, GABA$_A$ receptor, photoaffinity label, deuteration, tritiation, radioligand, receptor binding

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INTRODUCTION

The central inhibitory neurotransmitter, 4-aminobutyric acid (GABA), is involved in the regulation of a variety of physiological processes and in the pathophysiology of certain neurological and psychiatric disorders [1]. The GABA neurotransmission is mediated by the $GABA_A$ and $GABA_B$ receptor families, both of which appear to comprise several receptor subtypes [2,3]. A number of specific $GABA_A$ receptor agonists, including 5-aminomethyl-3-isoxazolol (muscimol) [4], 5-aminomethyl-3-isothiazolol (thiomuscimol) [5], and 4,5,6,7-tetrahydro-isoxazolo[5,4-*c*]pyridin-3-ol (THIP) [6] (Figure 1) have been developed and used to characterize the $GABA_A$ receptors. [^{3}H]THIP [7] and, in particular, [^{3}H]muscimol [8] have been used as radioligands for studies of the GABA recognition site of the $GABA_A$ receptor complex.

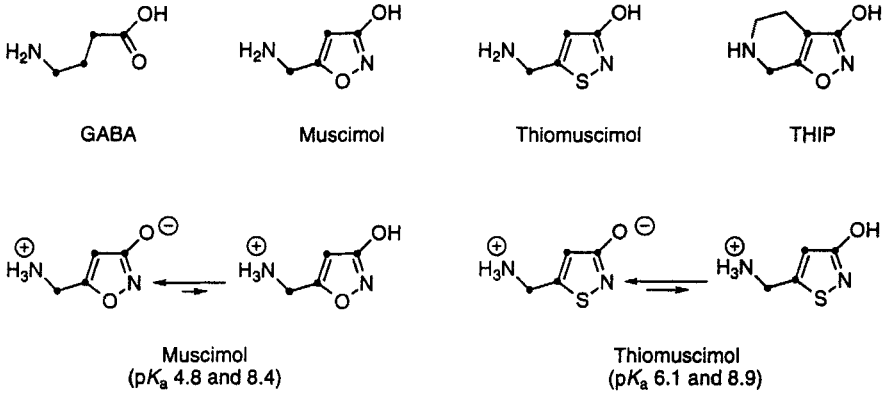


Figure 1. Structures of GABA and the $GABA_A$ agonists muscimol, thiomuscimol and THIP. Muscimol and thiomuscimol are depicted in the zwitterionic as well as the cationic forms.

[^{3}H]Muscimol has been used to photoaffinity label $GABA_A$ receptors [9-11], but this ligand only produces relatively limited irreversible inhibition of $GABA_A$ receptor binding upon UV irradiation. We have recently introduced thiomuscimol as a photolabel of high-affinity $GABA_A$ receptor sites [12]. Under the experimental conditions used, photolabelling experiments using muscimol did not irreversibly incorporate muscimol into rat brain synaptic membranes to any significant extent [12].

In spite of the structural similarity of muscimol [13] and thiomuscimol [14], these two $GABA_A$ agonists have different physico-chemical characteristics. Based on the p*K*a values of thiomuscimol and muscimol, it can be calculated that, under the test conditions (pH 7.1), about 10% of thiomuscimol and 0.5% of muscimol exist in the cationic form containing unionized heterocyclic acid groups (Figure 1). Since the unionized form of the 3-isoxazolol group (λ_{max} < 210 nm) [15], and probably also the 3-isothiazolol group (λ_{max} = 262 nm) [14], are the photochemically most sensitive groups of these compounds, it is understandable that thiomuscimol by irradiation with UV light at a wavelength of 254 nm inactivates $GABA_A$ receptor sites more effectively than muscimol [12].

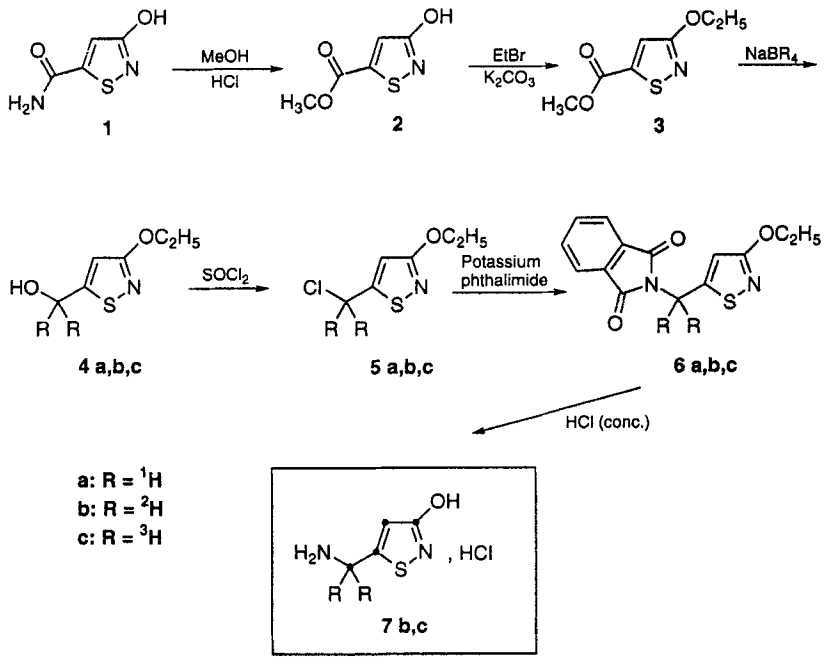
We now describe the synthesis of [^{3}H]thiomuscimol (**7c**) with a specific radioactivity of 29 Ci/mmol. Furthermore, we describe pilot comparative radioligand binding assays of [^{3}H]thiomuscimol (**7c**) and [^{3}H]muscimol showing saturable specific binding of both ligands.

RESULTS

Chemistry

The key step in the synthesis of [^{3}H]thiomuscimol (**7c**) is the treatment of methyl 3-ethoxyisothiazole-5-carboxylate (**3**) with sodium borotritide to give 3-ethoxy-5-hydroxy[^{3}H]$_2$methylisoxazole (**4c**) (Scheme 1). Since the unlabelled forms of the tritiated compounds **4c-6c**, compounds **4a-6a**, respectively, are also new, the syntheses of these synthetic intermediates are described. The procedures for the preparation of the respective deuterated analogues, **4b-6b**, which were used as model experiments for the syntheses of tritiated compounds, are also described. Accordingly, the syntheses of both [^{2}H]thiomuscimol (**7b**) and [^{3}H]thiomuscimol (**7c**) are described.

The starting material, 3-hydroxyisothiazole-5-carboxamide (**1**) [14] was converted into the substrate for tritium incorporation, compound **3**, *via* methyl 3-hydroxyisothiazole-5-carboxylate (**2**). The syntheses of **4a-c** from **3** were carried out under similar reaction conditions using sodium borohydride, sodium borodeuteride, and sodium borotritide, respectively, as reducing agents.



Scheme 1

Compound **4c** was purified by preparative thin-layer chromatography (TLC) to yield 1300 mCi of product. The specific radioactivity of this sample of **4c** was determined to 29.3 Ci/mmol by mass spectroscopy (MS). Treatment of **4a-c** by thionyl chloride gave **5a-c**, respectively. Whereas **5a,b** were synthesized by treatment with refluxing thionyl chloride, attempts to convert **4c** into **5c** under similar reaction conditions were unsuccessful. This latter reaction could only be accomplished in the presence of pyridine. Compounds **6a-c** were synthesized by treatment of **5a-c**, respectively, with potassium phthalimide in *N,N*-dimethylacetamide (DMAA), 250 mCi of **5c** giving 220 mCi of **6c**. The phthalimides **6b** and **6c** (220 mCi) were *O*- and *N*-deprotected by treatment with concentrated hydrochloric acid under reflux for 16 and 48 h, respectively (Scheme 1). After an initial purification of the crude product from the deprotection of **6c** (220 mCi) by preparative paper chromatography (PC), 170 mCi of **7c** was obtained at a purity of 92%. Compound **7c** was further purified by high performance liquid chromatography (HPLC) to give 110 mCi of 98% pure **7c**, as determined by HPLC and TLC analyses. The specific radioactivity of this sample of **7c** was 29 Ci/mmol, as determined by MS.

Receptor Binding

In pilot binding assays, [^{3}H]thiomuscimol (**7c**) was shown to bind to membranes from rat forebrain in a saturable manner (Figure 2). Under identical conditions, the binding of [^{3}H]muscimol was shown also to be saturable. In these preliminary comparative experiments, the capacity of rat synaptic membranes for binding **7c** (B_{max} = 50 ± 4 fmol/mg brain tissue) was shown to be markedly lower than that measured for [^{3}H]muscimol (82 ± 11 fmol/mg brain tissue). In agreement with the relative potencies of thiomuscimol and muscimol as inhibitors of the binding of [^{3}H]GABA to $GABA_A$ receptor sites on rat brain membranes [5], the K_D value for **7c** (28 ± 6 nM) was some five times higher than that measured for [^{3}H]muscimol (5.4 ± 2.8 nM) (Figure 2).

Detailed comparative studies on the binding characteristics, pharmacology, and regional distribution of $GABA_A$ receptor binding sites for **7c** and [^{3}H]muscimol in rat brain will be reported elsewhere.

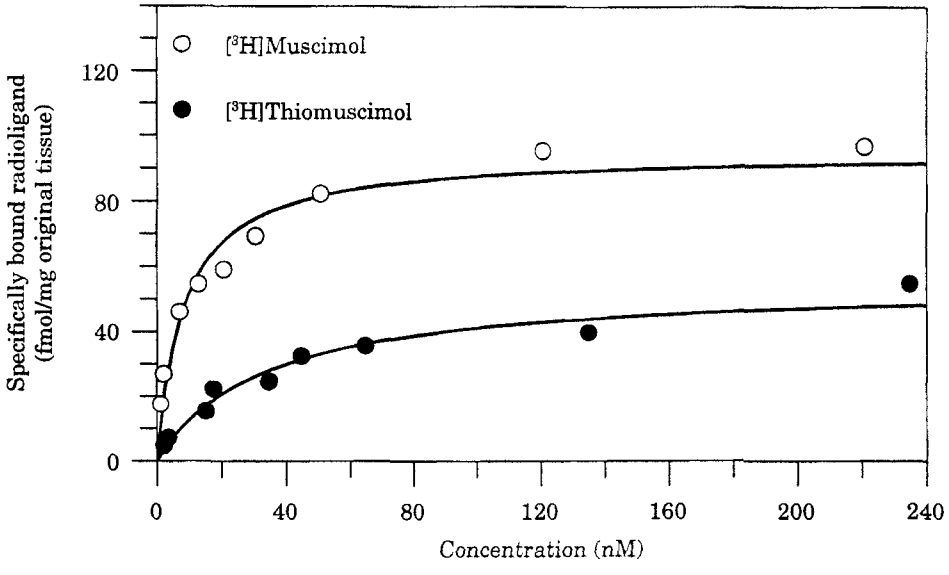


Figure 2. Saturation curves for the binding of **7c** and [^{3}H]muscimol to rat brain synaptic membranes.

DISCUSSION

In previous attempts [16] to radiolabel the specific $GABA_A$ receptor agonist thiomuscimol [5], deuteration model experiments led to [^{2}H]thiomuscimol containing deuterium atoms in the 4-position of the isothiazole ring and in the methylene group. This synthesis of [^{2}H]thiomuscimol was based on catalytic deuteration of an *O*- and *N*-protected derivative of thiomuscimol containing bromine in the 4-position of the ring. Attempts to synthesize [^{3}H]thiomuscimol by catalytic tritiation of the same precursor under identical reaction conditions were, however, unsuccessful (unpublished results).

We now report the synthesis of [^{3}H]thiomuscimol (**7c**), in which tritium has been incorporated exclusively into the methylene group, with a specific radioactivity of 29 Ci/mmol. The intermediate compound, **4c**, was synthesized with a specific radioactivity of 29.3 Ci/mmol by sodium borotritide reduction of **3** (Scheme 1). Thus, conversion of **4c** into the phthalimide derivative, **6c**, *via* the chloromethyl analogue, **5c**, and subsequent deprotection of **6c** by prolonged treatment under reflux with concentrated hydrochloric acid gave **7c** without detectable loss of radioactivity.

The binding of **7c** to rat brain synaptic membranes was saturable (Figure 2). Quite surprisingly, the density of binding sites for **7c** was markedly lower than that measured for the classical $GABA_A$ receptor radioligand, [^{3}H]muscimol [8,11], determined under identical experimental conditions. Thus, only a limited population of $GABA_A$ receptor sites on rat brain membranes seems to be capable of binding **7c**. This observation may reflect that the two compounds interact with different receptor populations. An interpretation of this observation must, however, await the results of more detailed receptor binding studies in progress.

Thiomuscimol has previously been shown to be an effective photoaffinity label of the high-affinity $GABA_A$ receptor sites [12], making **7c** a potentially very useful tool for studies of the $GABA_A$ receptor complex. The availability of **7c** may facilitate the localization and the mapping of the topography of the $GABA_A$ recognition site. Studies along these lines using recombinant $GABA_A$ receptors of different subunit combination are in progress.

EXPERIMENTAL SECTION

Chemistry. General Procedures

Melting points were determined in capillary tubes and are uncorrected. ^{1}H-NMR, ^{13}C-NMR and DEPT spectra were recorded on a Bruker AC-200 F (200 MHz) spectrometer in C^2HCl_3 solutions using TMS as an internal standard or in ^2H_2O solutions using 1,4-dioxane as an internal standard. Column chromatography (CC) was performed on Merck silica gel 60 (0.06-0.200 mm). Analytical TLC was carried out using Merck silica gel 60 F_{254} plates. Elemental analyses were performed by Mr. G. Cornali, Microanalytical Laboratory, LEO Pharmaceutical Products, Denmark, or by Mr. P. Hansen, Department of General and Organic Chemistry, University of Copenhagen, and are within ± 0.4% of the calculated values, unless otherwise stated. Evaporations were performed under vacuum on a rotary evaporator at 15 mmHg. Mass spectrometric experiments were carried out on a Kratos MS-25 instrument equipped with Desorption Chemical Ionization (DCI) capability. Specific radioactivity was determined by comparing peak intensities of the isotopomers in the molecular ion region of the mass spectrum.

Methyl 3-hydroxyisothiazole-5-carboxylate (2)

Acetyl chloride (70 mL; 1.0 mol) was added dropwise to ice-cooled methanol (450 mL). 3-Hydroxyisothiazole-5-carboxamide (**1**) [14] (13.7 g; 95 mmol) was added to the reaction mixture and stirring was continued for 18 h at 50 °C. Upon evaporation, the residue was dissolved in ethyl acetate (200 mL) and isopropanol (40 mL) under heating. The mixture was filtered and the filtrate evaporated to give **2** (10.1 g; 67%). An analytical sample was recrystallized from toluene to give **2**: mp 170-171 °C. ^{1}H-NMR (200 MHz, C^2HCl_3 + DMSO-d_6): δ 3.91 (s, 3H), 7.07 (s, 1H). Anal. ($C_5H_5NO_3S$) C, H, N, S.

Methyl 3-ethoxyisothiazole-5-carboxylate (3)

A mixture of **2** (500 mg; 3.14 mmol), potassium carbonate (820 mg; 5.9 mmol) and *N,N*-dimethylformamide (18 mL) was added ethyl bromide (0.5 mL; 6.7 mmol) and stirring was

continued at room temperature for 20 h. The mixture was filtered and ether (10 mL) and water (50 mL) were added to the filtrate. The organic phase was washed with water (3 x 50 mL), dried ($MgSO_4$) and evaporated to give **3** (350 mg; 60%): mp 43-45 °C. ^{1}H-NMR (200 MHz, C^2HCl_3): δ 1.41 (t, J = 7.1 Hz, 3H), 3.91 (s, 3H), 4.41 (q, J = 7.1 Hz, 2H), 7.09 (s, 1H). Anal. ($C_7H_9NO_3S$) C, H, N, S.

3-Ethoxy-5-hydroxymethylisothiazole (4a)

To an ice-cooled solution of **3** (300 mg; 1.6 mmol) in methanol (10 mL) was added sodium borohydride (240 mg; 6.3 mmol) over a period of 15 min. Stirring was continued at 25 °C for 2.5 h. The solvent was evaporated, and the residue was added water (4 mL) and extracted with dichloromethane (4 x 10 mL). The combined extracts were dried $MgSO_4$ and evaporated. CC [toluene-ethyl acetate (4:1)] gave **4a** as a colorless oil (180 mg; 71%). ^{1}H-NMR (200 MHz, C^2HCl_3): δ 1.37 (t, J = 7.0 Hz, 3H), 4.32 (q, J = 7.0 Hz, 2H), 4.51 (t, J = 6.0 Hz, 1H), 4.83 (d, J = 6.0 Hz, 2H), 6.41 (s, 1H). ^{13}C-NMR (50.3 MHz, C^2HCl_3): δ 14.3, 58.3 (CH_2OH), 64.1, 108.3, 168.8, 169.0. Anal. ($C_6H_9NO_2S$) C, H, N, S.

3-Ethoxy-5-hydroxy[^{2}H]$_2$methylisothiazole (4b)

Methanol (5 mL) was added dropwise over a period of 1 h to a refluxing mixture of **3** (910 mg; 4.9 mmol) and sodium borodeuteride (264 mg; 6.3 mmol) in tetrahydrofuran (THF) (25 mL). Stirring was continued at reflux temperature for 1 h. After cooling to room temperature, 1 M hydrochloric acid (30 mL) was added, the mixture was extracted with ether (4 x 60 mL), and the combined ether phases were dried ($MgSO_4$). The solvent was evaporated, and the crude residue was purified by CC [toluene-ethyl acetate (4:1)] to give the product (520 mg; 66%) as a colorless oil. ^{1}H-NMR (200 MHz, C^2HCl_3): δ 1.36 (t, J = 7.1 Hz, 3H), 4.30 (q, J = 7.1 Hz, 2H), 4.80 (pert. t, 0.6H), 4.90 (br. s, 1H). ^{13}C-NMR (50.3 MHz, C^2HCl_3): δ 14.2, 57.6 (C^2H_2OH, p, J_{C^2H} = 22.2 Hz), 57.9 (C^2HHOH, t, J_{C^2H} = 22.0 Hz), 58.2 (CH_2OH), 64.0, 108.2, 168.7, 168.9.

3-Ethoxy-5-hydroxy[^{3}H]$_2$methylisothiazole (4c)

3 (25 mg; 0.13 mmol) in THF (1 mL) was added *via* syringe to sodium borotridide (60 Ci/mmol; 20 mg) and heated to 55 °C. To the reaction mixture was added dropwise methanol (130 µL) during a period of 1 h, and this mixture was stirred for an additional 2 h. The reaction was cooled to room temperature and quenched with 1 M hydrochloric acid (2 mL). Following solvent evaporation and labile removal with methanol (3 x 2 mL), the product was taken up in ether for assay, and the purity was evaluated [1700 mCi; 80% on TLC: silica gel GF eluting with toluene-ethyl acetate (4:1)]. The solvent was removed under reduced pressure, and the reaction mixture was purified by preparative TLC on a silica gel plate in toluene:ethyl acetate (4:1) and eluted with ethyl acetate to yield 1300 mCi product. Specific activity was determined by MS and found to be 29.3 Ci/mmol.

3-Ethoxy-5-chloromethylisothiazole (5a)

A solution of **4a** (430 mg; 2.7 mmol) in thionyl chloride (3 ml) was refluxed for 1 h followed by evaporation. To the residue was added water (8 mL), and the mixture was extracted with dichloromethane (3 x 15 mL). The combined extracts were dried ($MgSO_4$) and evaporated. CC [toluene-ethyl acetate (8:1)] gave the product as an oil (380 mg; 80%). An analytical sample was ball-tube distilled (150 °C/0.5 mmHg). ^{1}H-NMR (200 MHz, C^2HCl_3) δ 1.38 (t, J = 7.1 Hz, 3H), 4.37 (q, J = 7.1 Hz, 2H), 4.69 (s, 2H), 6.55 (s, 1H). ^{13}C-NMR (50.3 MHz, C^2HCl_3) δ 14.4, 37.3 (CH_2Cl), 64.1, 111.6, 163.4, 168.4. Anal. (C_6H_8NOSCl) C, H, N, S, Cl.

3-Ethoxy-5-chloro[^{2}H]$_2$methylisothiazole (5b)

Compound **5b** was synthesized as described for **5a** by using **4b** (520 mg; 3.2 mmol) and thionyl chloride (4 mL). CC [toluene-ethyl acetate (8:1)] gave the product as an oil (490 mg; 81%). ^{1}H-NMR (200 MHz, C^2HCl_3): δ 1.39 (t, J = 7.1 Hz, 3H), 4.38 (q, J = 7.1 Hz, 2H), 4.69 (pert. s, 0.6H), 6.56 (s, 1H). ^{13}C-NMR (50.3 MHz, C^2HCl_3): δ 14.4, 36.8 (C^2H_2Cl, p, J_{C^2H} = 23.2 Hz), 37.1 (C^2HHCl, t, J_{C^2H} = 23.4 Hz), 37.3 (CH_2Cl), 64.1, 111.6, 163.4, 168.4.

3-Ethoxy-5-chloro[^{3}H]$_2$methylisothiazole (5c)

A solution of **4c** (300 mCi; 0.01 mmol) in ethyl acetate was evaporated to dryness. To this residue was added thionyl chloride (1 mL) and pyridine (100 µL), and the solution was refluxed for 18 h under nitrogen. The solvent was removed under reduced pressure, water (1 mL) was added, and the product was extracted with dichloromethane (3 x 3 mL). The dichloromethane solution was dried over Na_2SO_4, filtered, and assayed (250 mCi).

3-Ethoxy-5-phthalimidomethylisothiazole (6a)

A mixture of **5a** (350 mg; 1.97 mmol) and potassium phthalimide (550 mg; 2.9 mmol) in DMAA (10 mL) was stirred at 60 °C for 2 h. Stirring was continued at 25 °C for 16 h. Water (80 mL) was added, and the mixture was cooled to 4 °C. The precipitate was washed with water (2 x 3 mL) and dried to give the product as colourless crystals (360 mg; 63%). An analytical sample was recrystallized (ethyl acetate-light petroleum) to give **6a**: mp 129-130 °C. ^{1}H-NMR (200 MHz, C^2HCl_3): δ 1.36 (t, J = 7.1 Hz, 3H), 4.34 (q, J = 7.1 Hz, 2H), 4.99 (s, 2H), 6.60 (s, 1H), 7.72-7.77 (m, 2H), 7.86-7.90 (m, 2H). ^{13}C-NMR (50.3 Hz, C^2HCl_3): δ 14.5, 34.0 (CH_2N), 64.1, 112.3, 123.5, 131.7, 134.2, 161.3, 167.2, 168.5. Anal. ($C_{14}H_{12}N_2O_3S$) H, N, S. C: calcd, 58.30; found, 57.53.

3-Ethoxy-5-phthalimido[^{2}H]$_2$methylisothiazole (6b)

Compound **6b** was synthesized as described above for **6a** using **5b** (490 mg; 2.7 mmol) and potassium phthalimide (709 mg; 3.8 mmol) in DMAA (9 mL). The reaction gave the product as colourless crystals (700 mg; 88%): mp 128-130 °C. ^{1}H-NMR (200 MHz, C^2HCl_3): δ 1.36 (t, J = 7.1 Hz, 3H), 4.34 (q, J = 7.1 Hz, 2H), 4.99 (pert. s, 0.6H), 6.60 (s, 1H), 7.72-7.77 (m, 2H), 7.86-7.90 (m, 2H). ^{13}C-NMR (50.3 MHz, C^2HCl_3): δ 14.4, 33.6 (C^2H_2N, p, J_{C^2H} = 19.0 Hz), 33.8 (C^2HHN, t, J_{C^2H} = 19.0 Hz), 34.0 (CH_2N), 64.1, 112.3, 123.5, 131.7, 134.2, 161.3, 167.2, 168.5.

13-Ethoxy-5-phthalimido[^{3}H]$_2$methylisothiazole (6c)

A solution of **5c** (250 mCi; 1.5 mg; 0.0085 mmol) in dichloromethane (1 mL) was evaporated to dryness and dissolved in DMAA (200 µL). Potassium phthalimide (2.4 mg; 0.0127 mmol) was added, and the solution was stirred at 60 °C for 2 h and at 25 °C for a further 20 h. The mixture was cooled to room temperature and water (1 mL) was added. The product was extracted into ethyl acetate, dried over Na_2SO_4, filtered, and assayed (220 mCi).

5-Amino[^{2}H]$_2$methyl-3-isothiazolol ([^{2}H]Thiomuscimol) Hydrochloride (7b)

A mixture of **6b** (100 mg; 0.34 mmol) and concentrated hydrochloric acid (3 mL) was refluxed for 16 h. The solution was cooled to 4 °C and filtered. The filtrate was evaporated and the residue recrystallized (methanol-ether) to give **7b** (34 mg; 61%): mp 164-167°C (decomp.). ^{1}H-NMR (200 MHz, ^2H_2O): δ 4.36 (broad s, 0.3H), 6.60 (s, 1H). ^{13}C-NMR (50.3 MHz, ^2H_2O): δ 36.6 (C^2HHN, t, $J_{C^2_H}$ = 22.0 Hz), 36.8 (CH_2N), 114.6, 157.7, 170.7.

5-Amino[^{3}H]$_2$methyl-3-isothiazolol ([^{3}H]Thiomuscimol) (7c)

A solution of **6c** (220 mCi) in ethyl acetate was taken to dryness, and the residue was taken up into concentrated hydrochloric acid (0.6 mL) and refluxed for 48 h. The solution was cooled and then evaporated. The product was cooled in an ice bath and neutralized with dilute sodium hydrogencarbonate solution. The product was again evaporated to dryness. An initial purification was done by preparative PC on whatman 3 paper run in n-butanol-acetic acid-water (25:4:10) to give 170 mCi at a purity of 92%. Further purification was done *via* HPLC on a Zorbax SCX column run at 1 mL/min with 0.1% triethylammonium acetate pH 4-acetonitrile (55:45). The product was evaporated to dryness, dissolved in ethanol-water (1:1), and assayed to give 110 mCi. The purity of the final compound was found to be 98% when checked by HPLC on a Zorbax SCX column with 10 mM potassium phosphate, pH 3 as mobile phase and by TLC on silica gel eluting with ethyl acetate-isopropanol-ammonium hydroxide (9:7:4). MS of the product indicated a specific radioactivity of 29 Ci/mmol.

Receptor Binding

Brains were obtained from male adult Sprague-Dawley rats. Rat forebrain tissue consisted of cerebral cortex, striatum, and hippocampus. This tissue was homogenized in 15 volumes of ice-cold 30 mM Tris-HCl + 2.5 mM calcium chloride, pH 7.1, then centrifuged at 30,000xg for 15 min. The membrane pellet was washed three times by re-suspension in approximately 15 volumes of buffer, followed by centrifugation. The pellet was suspended in buffer and incubated for 30 min at 37 °C, then washed three more times, each time followed by centrifugation. The final washed pellet was re-suspended in approximately 10 volumes of buffer and frozen at -18 °C for up to 6 months prior to assay.

On the day of the experiment, frozen homogenates were thawed, and the tissue was washed three times by suspension in approximately 50 volumes of ice-cold assay buffer consisting of 50 mM Tris-HCl (pH 7.1). The final pellet was re-suspended in cold assay buffer corresponding to approximately 6.5 mg original tissue per mL and used as source of binding tissue.

Binding was carried out essentially as described previously [17]: Incubation buffer: 50 mM Tris-HCl (pH 7.1). Following incubation for 1 h at 0 - 4 °C in a total volume of 1 mL the samples were filtered through Whatman GF/C filters (pretreated with 0.1% PEI in water for at least 1 h) and washed with 3 times 2 mL ice-cold buffer. Non-specific binding was determined with GABA (100 µM) or muscimol (100 µM) (no difference).

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REFERENCES

1. P. Krogsgaard-Larsen, B. Frølund, F.S. Jørgensen and A. Schousboe — *J. Med. Chem.* **37**:2489-2505 (1994).

2. R.L. Macdonald and R.W. Olsen — *Annu. Rev. Neurosci.* **17**:569-602 (1994).

3. G. Bonanno and M. Raiteri — *Trends Pharmacol. Sci.* **14**:259-261 (1993).

4. P. Krogsgaard-Larsen, G.A.R. Johnston, D.R. Curtis, C.J.A. Game and R.M. McCulloch — *J. Neurochem.* **25**:803-809 (1975).

5. P. Krogsgaard-Larsen, H. Hjeds, D.R. Curtis, D. Lodge and G.A.R. Johnston — *J. Neurochem.* **32**:1717-1724 (1979).

6. P. Krogsgaard-Larsen, G.A.R. Johnston, D. Lodge and D.R. Curtis — *Nature* **268**:53-55 (1977).

7. E. Falch and P. Krogsgaard-Larsen — *J. Neurochem.* **38**:1123-1129 (1982).

8. Y.-J. Wang, P. Salvaterra and E. Roberts — *Biochem. Pharmacol.* **28**:1123-1128 (1979).

9. D. Cavalla and N.H. Neff — *J. Neurochem.* **44**:916-921 (1985).

10. M.H. Bureau and R.W. Olsen — *J. Neurochem.* **61**:1479-1491 (1993).

11. T. Asano, J. Sakakibara and N. Ogasawara — *FEBS Lett.* **151**:277-280 (1983).

12. M. Nielsen, M.-R. Witt, B. Ebert and P. Krogsgaard-Larsen — *Eur. J. Pharmacol. Mol. Pharmacol. Sect.* 1995 (in press).

13. P. Krogsgaard-Larsen and S.B. Christensen — *Acta Chem. Scand.* **B30**:281-282 (1976).

14. J. Lykkeberg and P. Krogsgaard-Larsen — *Acta Chem. Scand.* **B30**:781-785 (1976).

15. H. Göth, A.R. Gagneux, C.H. Eugster and H. Schmid — *Helv. Chim. Acta* **50**:137-142 (1967).

16. P. Jacobsen and P. Krogsgaard-Larsen — *J. Labelled Compd. Radiopharm.* **21**:253-261 (1984).

17. R.M. McKernan, K. Quirk, R. Prince, P.A. Cox, N.P. Gillard, I. Regan and P. Whiting — *Neuron* **7**:667-676 (1991).