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Synthesis of Deuterium and Tritium labelled (*RS*)-2-Amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)-propionic Acid (ATPA), a selective Kainic Acid Receptor Agonist

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SUMMARY

(*RS*)-2-Amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid (ATPA) is an excitatory amino acid receptor agonist showing selectivity for the kainic acid receptor subtype GluR5. As part of the pharmacological characterization of GluR5 receptors, we now report the synthesis of [^{3}H]ATPA based on a four-step synthesis using 5-*tert*-butyl-3-methoxy-4-isoxazolylcarbaldehyde as the starting material. Using this synthetic procedure deuterium and tritium labelled ATPA have been prepared. [^{3}H]ATPA, with a specific activity of 17 Ci/mmol, was obtained with a radiochemical purity of 98.9%. Attempts to demonstrate specific binding of [^{3}H]ATPA under conventional rat brain membrane receptor binding conditions were unsuccessful.

Key words: Excitatory amino acid receptors, ATPA, kainic acid, GluR5, tritium labelling.

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INTRODUCTION

(*S*)-Glutamic acid (Glu) is the major excitatory amino acid (EAA) in the central nervous system and exerts its action by interacting with ionotropic as well as metabotropic EAA receptors (1–3). In light of the apparent involvement of these receptors in the etiology of neurodegenerative disorders such as Alzheimer's disease and stroke both classes of EAA receptors are pharmacologically and therapeutically interesting (4,5). Based on pharmacological and structural criteria, the ionotropic EAA receptors are subdivided into *N*-methyl-D-aspartic acid (NMDA), (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA), and kainic acid receptors all of which are named after the standard agonists (Figure 1) (1–3,6).

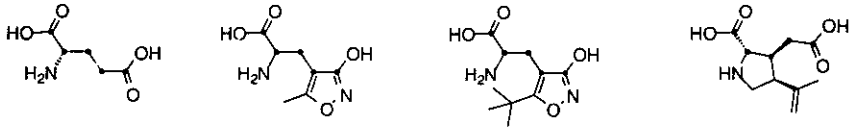


Figure 1. Structures of the endogenous excitatory amino acid, (*S*)-glutamic acid, and some key AMPA and kainic acid receptor agonists.

Molecular biological studies on EAA receptors have identified four AMPA preferring subunits, GluR1–GluR4, which are activated by AMPA as well as kainic acid (7–9). Furthermore, two groups of kainic acid preferring subunits, KA1 and KA2 (10,11) and GluR5–GluR7 (12–14), have been identified showing high and low affinity for [^{3}H]kainic acid, respectively. At present, [^{3}H]kainic acid is the only radioligand available for studies of kainic acid receptor subunits, but due to the lack of subunit selectivity and due to low affinity of [^{3}H]kainic acid for GluR5–GluR7, high affinity radioligands showing a high degree of selectivity are required for detailed receptor characterization.

The AMPA analogue, (*RS*)-2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid (ATPA) (15) (Figure 1) has been tested in receptor binding and in spinal and cortical neurons and found to be an AMPA receptor agonist markedly less potent than AMPA (16,17). In contrast to AMPA, ATPA, which contains a lipophilic *tert*-butyl substituent, is pharmacologically active after systemic administration in mice (18,19). However, when tested at recombinant receptors, ATPA was recently found to be a potent and subtype selective agonist at

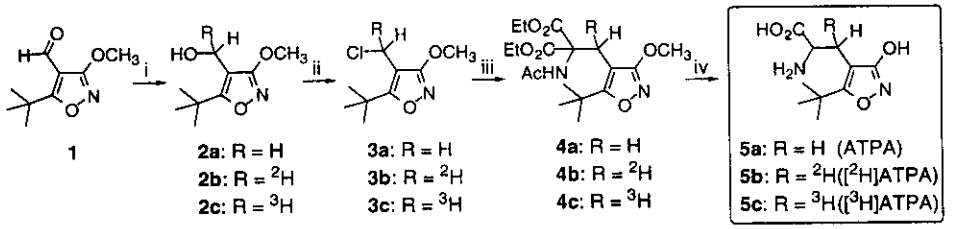
recombinant GluR5 receptors with nanomolar affinity in [^{3}H]kainic acid binding assays and only a weak inhibitor of [^{3}H]AMPA in binding studies using cloned GluR1–GluR4 receptor subunits (20). The agonist activities of ATPA reside exclusively in the *S*-enantiomer whereas (*R*)-ATPA is a relatively weak AMPA receptor antagonist (T. B. Stensbøl *et al.*, to be published).

In order to investigate the usefulness of [^{3}H]ATPA as a radioligand, we now report a new synthesis of ATPA and, based on this new procedure, the syntheses of [^{2}H]ATPA and [^{3}H]ATPA. Furthermore, this new radioligand has been examined in conventional receptor binding assays using rat brain membrane preparations.

RESULTS AND DISCUSSION

Chemistry

The syntheses of ATPA, [^{2}H]ATPA, and [^{3}H]ATPA are shown in Scheme 1. At first, a straight forward four-step synthesis of ATPA was developed starting out from 5-*tert*-butyl-3-methoxy-4-isoxazolylcarbaldehyde (**1**) (21). Reduction of the aldehyde afforded the corresponding alcohol (**2a**) which was easily converted into the chloride (**3a**) using thionyl chloride. Based on **3a**, a Sørensen synthesis gave the diethyl acetamidomalonate derivative (**4a**), which was deprotected to ATPA (**5a**) using hydrobromic acid in water.



Scheme 1. Syntheses of ATPA, [^{2}H]ATPA, and [^{3}H]ATPA. i: $NaBH_4$ (a), $NaB[^2H]_4$ (b), $NaB[^3H]_4$ (c); ii: $SOCl_2$; iii: diethyl acetamidomalonate, NaH; iv: 48% HBr/H_2O.

This sequence was then used for the incorporation of deuterium to give [^{2}H]ATPA (**5b**) in an overall yield of 17%. According to ^{1}H NMR and ^{13}C NMR (DEPT) spectra of the deuterated alcohol (**2b**), the reduction of aldehyde **1** using sodium boro[^{2}H]$_4$hydride resulted in incorporation of one deuterium atom.

Treatment of **2b** with thionyl chloride gave **3b** without detectable loss of deuterium. Likewise, neither the nucleophilic displacement of the chloro atom of **3b** to give the acetamidomalonate derivative (**4b**) nor the final deprotection of **4b** resulted in significant loss of deuterium. Compounds **2a–5a** co-eluted on TLC with the deuterated analogues **2b–5b**, respectively, and had very similar IR-spectra.

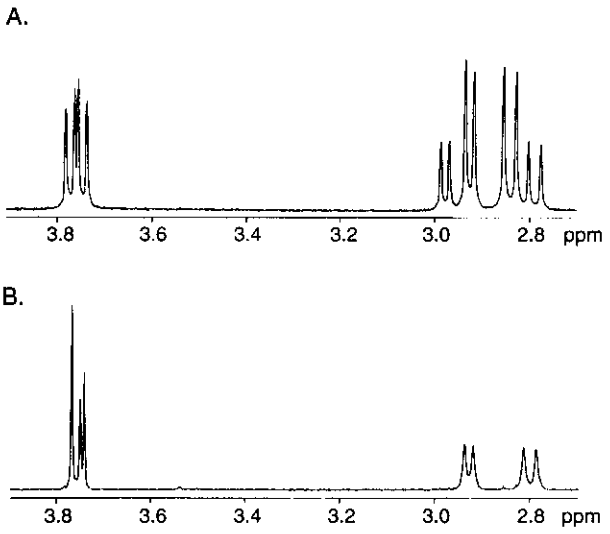


Figure 2. ^{1}H NMR spectra (relevant sections) of ATPA (**5a**) (**A**) and [^{2}H]ATPA (**5b**) (**B**).

Due to the presence of a deuterium atom, the ^{1}H NMR spectrum of [^{2}H]ATPA (**5b**) was clearly different from that of ATPA (**5a**) (Figure 2). As a consequence of the presence of two chiral centers in **5b** it was possible from the ^{1}H NMR spectrum to identify signals arising from two diastereomeric pairs of enantiotropic protons with coupling constants of $^{3}J_{HH}$ = 5.7 and 7.7 Hz, respectively (Figure 2B). In the ^{13}C NMR (DEPT) spectrum of compound **5b** a triplet ($^{1}J_{CD}$ = 19.8 Hz) corresponding to a CHD-group appeared, whereas no signal arising from a CH_2-group could be detected. Thus, the incorporated deuterium was essentially intact in the final deuterated product [^{2}H]ATPA.

In the reduction of **1** with sodium boro[$^{3}H]_4$hydride to give the tritiated alcohol (**2c**) only one tritiated compound was formed according to TLC analyses. Using thionyl chloride, **2c** then was converted into **3c** with a radiochemical purity of 98%, and **3c** was subsequently transformed, in a small scale reaction, into tritiated protected ATPA, compound **4c**, which was purified by TLC. Deprotection

of **4c** in 48% hydrobromic acid generated a mixture of four major tritiated components. This mixture was subjected to reverse phase HPLC purification to give [^{3}H]ATPA (**5c**) with a radiochemical purity of 98.9% and with a specific activity of 17 Ci/mmol.

Receptor Binding

Attempts were made to demonstrate specific binding of [^{3}H]ATPA to rat brain membranes. A membrane preparation, routinely used for the characterization of EAA receptors was used (22). Using standard and slightly modified conditions corresponding to those used for the characterization of [^{3}H]AMPA binding (30 mM Tris-HCl, 2.5 mM $CaCl_2$, 100 mM KSCN; incubation for 10 to 240 min at 0–4 °C or at 20 °C) (23), [^{3}H]kainic acid binding (50 mM Tris-HCl; incubation for 10 to 240 min at 0–4 °C) (24), or [^{3}H](*RS*)-4-(3-phosphonopropyl)-2-piperazinylcarboxylic acid ([^{3}H]CPP) binding (30 mM Tris-HCl, 2.5 mM $CaCl_2$; incubation for 10 to 240 min at 0–4 °C or for 60 min at 20 °C) (25) and concentrations of [^{3}H]ATPA ranging from 0.1 nM to 100 nM no significant specific binding was demonstrated (100 μM ATPA or 1 mM Glu were used to correct for nonspecific binding). Neither did binding experiments using conditions described for the identification of $CaCl_2$ dependent [^{3}H]Glu binding (very low protein concentration; incubation 2 to 60 min at 37 °C) (26) demonstrate any specific binding (100 μM ATPA or 1 mM Glu were used to correct for nonspecific binding). The lack of specific binding of [^{3}H]ATPA, under the conditions described above, may reflect a low density of [^{3}H]ATPA sensitive binding sites in the rat brain homogenates used, since the expression level of GluR5 receptor subunits in the adult rat brain generally is low as compared to AMPA preferring subunits (12).

EXPERIMENTAL

General procedures

Melting points were determined in capillary tubes and are uncorrected. Infrared spectra were obtained on a Perkin-Elmer grating infrared spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC-200 F spectrometer at 200 MHz and 50 MHz, respectively. For ^{13}C NMR spectra $CDCl_3$ was used as internal standard, unless otherwise stated. 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. Analytical TLC and column chromatography (CC) were carried out using silica gel F_{254} plates (Merck) and silica gel (Woelm, 0.063–0.200 mm), respectively.

5-*tert*-Butyl-4-hydroxymethyl-3-methoxyisoxazole (2a)

To an ice cooled solution of 5-*tert*-butyl-3-methoxy-4-isoxazolylcarbaldehyde (**1**) (21) (1.00 g, 5.5 mmol) in isopropanol (30 ml) was added $NaBH_4$ (77 mg, 2.0 mmol). The reaction mixture was stirred for 30 min at 0 °C. After addition of acetone (30 ml) and hydrochloric acid (30 ml, 1 M) the mixture was concentrated to approximately 30 ml followed by extraction with ethyl acetate (2 × 30 ml). The combined organic phases were dried ($MgSO_4$) and evaporated to give a pale yellow oil, which upon ball-tube distillation (0.1 mm Hg, 150–160 °C) gave **2a** (0.60 g, 59%) as a colourless oil. ^{1}H NMR ($CDCl_3$, TMS) δ 1.40 (s, 9 H), 2.7 (br s, 1 H), 4.00 (s, 3 H), 4.52 (s, 2 H). ^{13}C NMR ($CDCl_3$) δ 28.4 (C[$\underline{C}H_3$]$_3$), 34.2 ($\underline{C}$[CH_3]$_3$), 52.2 (CH_2), 56.7 (OCH_3), 103.5 (C-4), 171.0, 177.5 (C-3, C-5). Anal. ($C_9H_{15}NO_3$) C, H, N.

(*RS*)-5-*tert*-Butyl-4-hydroxy[^{2}H]methyl-3-methoxyisoxazole (2b)

Compound **2b** was prepared as described for the synthesis of **2a**, using sodium boro[^{2}H]$_4$hydride (130 mg, 3.1 mmol) for the reduction of compound **1** (1.5 g, 8.2 mmol). Ball-tube distillation of crude **2b** (0.1 mm Hg, 150–160 °C) afforded compound **2b** (0.98 g, 64%) as a colourless oil. ^{1}H NMR ($CDCl_3$, TMS) δ 1.39 (s, 9 H), 2.35 (br s, 1 H), 3.96 (s, 3 H), 4.49 (br s, 1 H). ^{13}C NMR ($CDCl_3$) δ 28.4 (C[$\underline{C}H_3$]$_3$), 34.2 ($\underline{C}$[CH_3]$_3$), 52.2 (t, J = 22.0 Hz, CHD), 56.6 (OCH_3), 102.7 (C-4), 171.0, 177.5 (C-3, C-5).

(*RS*)-5-*tert*-Butyl-4-hydroxy[^{3}H]methyl-3-methoxyisoxazole (2c)

To an ice-cold isopropanol solution (2 ml) of **1** (12.2 mg) was added sodium boro[^{3}H]$_4$hydride (5 Ci at 63 Ci/mmol). The reaction mixture was stirred at 0 °C for 1.5 h and then stopped by addition of glacial acetic acid (50 µl). The crude reaction was evaporated to dryness and the residue dissolved in toluene–ethyl

acetate (6:4, 2 ml). This solution was purified on a short silica gel column (500 mg, Amprep, Amersham Pharmacia Biotech, RPN1916) eluting in toluene–ethyl acetate (6:4, 5 × 2 ml). Compound **2c** eluted in the first 6 ml of eluent. Yield: 758 mCi.

5-*tert*-Butyl-4-chloromethyl-3-methoxyisoxazole (3a)

A solution of compound **2a** (70 mg, 0.38 mmol) in thionyl chloride (1 ml) was heated to reflux for 25 min and then evaporated. The residue was subjected to ball-tube distillation (0.1 mm Hg, 110 °C) to give **3a** (45 mg, 58%) as a colourless oil. ^{1}H NMR ($CDCl_3$, TMS) δ 1.41 (s, 9 H), 4.00 (s, 3 H), 4.45 (s, 2 H). ^{13}C NMR ($CDCl_3$) δ 28.2 (C[$\underline{C}H_3$]$_3$), 33.4 (CH_2), 34.4 ($\underline{C}$[CH_3]$_3$), 56.8 (OCH_3), 100.9 (C-4), 170.4, 177.9 (C-3, C-5). Anal. ($C_9H_{14}NO_2Cl$) C, H; N: calcd, 6.90; found, 6.38.

(*RS*)-5-*tert*-Butyl-4-chloro[^{2}H]methyl-3-methoxyisoxazole (3b)

Compound **3b** was prepared from compound **2b** (0.80 g, 4.3 mmol) in analogy with the synthesis described for **3a**. Ball-tube distillation (0.1 mm Hg, 110–120 °C) afforded compound **3b** (0.57 g, 65%) as a colourless oil.^{1}H NMR ($CDCl_3$, TMS) δ 1.40 (s, 9 H), 4.00 (s, 3 H), 4.43 (br s, 1 H). ^{13}C NMR ($CDCl_3$) δ 28.1 (C[$\underline{C}H_3$]$_3$), 33.2 (t, J = 23.5 Hz, CHD), 34.4 ($\underline{C}$[CH_3]$_3$), 56.8 (OCH_3), 100.8 (C-4), 170.4, 177.8 (C-3, C-5).

(*RS*)-5-*tert*-Butyl-4-chloro[^{3}H]methyl-3-methoxyisoxazole (3c)

2c (758 mCi) was dissolved in thionyl chloride (1 ml) and heated at 70 °C for 1 h. The solution was evaporated to dryness successively from toluene, water, and ethanol. The crude material was purified on a silica gel column (500 mg, Amprep) eluting in toluene (3 × 2 ml) and toluene–ethyl acetate (95:5, 4 × 2 ml) and toluene–ethyl acetate (9:1, 4 × 2 ml). Fractions 2–4 containing **3c** as the major tritiated component were combined and evaporated. TLC analysis eluting in toluene–ethyl acetate [2:1] showed that **3c** had a radiochemical purity of 98%. Yield: 430 mCi.

Ethyl 2-acetamido-2-ethoxycarbonyl-3-(5-*tert*-butyl-3-methoxy-4-isoxazolyl)propionate (4a)

Sodium hydride (24 mg, 80%, 0.80 mmol) was added to a solution of diethyl acetamidomalonate (160 mg, 0.74 mmol) in *N*,*N*-dimethylformamide (DMF) (5 ml)

at room temperature during a period of 1 min. After stirring for 5 min, a solution of **3a** (120 mg, 0.59 mmol) in DMF (5 ml) was added and the resulting mixture stirred for 6 h at 100 °C. After cooling and addition of glacial acetic acid (1 ml), the reaction mixture was evaporated to dryness. Upon addition of water (10 ml), the mixture was extracted with dichloromethane (3 × 10 ml). The combined organic phases were dried ($MgSO_4$) and evaporated to an oil, which after CC (toluene–ethyl acetate [2:1]) and recrystallization (ethyl acetate–light petroleum) gave **4a** (140 mg, 62%): mp 104.3–104.8 °C. Litt: 104–105 °C (15). ^{1}H NMR ($CDCl_3$, TMS) δ 1.27 (t, J = 7.1 Hz, 6 H), 1.32 (s, 9 H), 1.97 (s, 3 H), 3.51 (s, 2 H), 3.89 (s, 3 H), 4.1–4.4 (m, 4 H), 6.88 (br s, 1 H). ^{13}C NMR ($CDCl_3$) δ 13.7 ($\underline{C}H_3CH_2$), 22.9 ($\underline{C}H_3CO$), 25.8 ($\underline{C}H_2C_{isoxazole}$), 28.7 ($C[\underline{C}H_3]_3$), 34.3 ($\underline{C}[CH_3]_3$), 56.7 ($OCH_3$), 62.3 ($CH_3\underline{C}H_2$), 65.1 ($\underline{C}CH_2C_{isoxazole}$), 97.0 (C-4), 167.5, 168.9, 171.3, 177.4 (C-3, C-5, COO, $CH_3\underline{C}O$).

Ethyl (*RS*)-2-acetamido-2-ethoxycarbonyl-3-(5-*tert*-butyl-3-methoxy-4-isoxazolyl)-[3-^{2}H]propionate (4b)

Treatment of compound **3b** (90 mg, 0.44 mmol) as described above for **3a** afforded compound **4b** (110 mg, 65%): mp 104.7–105.0 °C. ^{1}H NMR ($CDCl_3$, TMS) δ 1.27 (t, J = 7.1 Hz, 6 H), 1.32 (s, 9 H), 1.98 (s, 3 H), 3.49 (br s, 1 H), 3.89 (s, 3 H), 4.1–4.4 (m, 4 H), 6.70 (br s, 1 H). ^{13}C NMR (200 MHz, $CDCl_3$) δ 13.6 ($\underline{C}H_3CH_2$), 22.8 ($\underline{C}H_3CO$), 25.4 (t, J = 19.6 Hz, $\underline{C}HDC_{isoxazole}$), 28.6 ($C[\underline{C}H_3]_3$), 34.3 ($\underline{C}[CH_3]_3$), 56.6 ($OCH_3$), 62.2 ($CH_3\underline{C}H_2$), 65.0 ($\underline{C}CHDC_{isoxazole}$), 96.9 (C-4), 167.5, 168.9, 171.3, 177.3 (C-3, C-5, COO, $CH_3\underline{C}O$).

Ethyl (*RS*)-2-acetamido-2-ethoxycarbonyl-3-(5-*tert*-butyl-3-methoxy-4-isoxazolyl)-[3-^{3}H]propionate (4c)

To a stirred solution of diethyl acetamidomalonate (110 mg, 0.51 mmol) in DMF (2 ml) was added sodium hydride (16 mg, 80%, 0.53 mmol). This mixture was allowed to stir at room temperature for 5 min. An aliquot of this solution (50 µl) was added to a solution of compound **3c** (150 mCi) in DMF (1 ml). The reaction mixture was heated at 100 °C for 2.5 h and then terminated by addition of glacial acetic acid (25 µl). The reaction mixture was evaporated to dryness under vacuum and the residue re-dissolved in toluene. Purification of the crude product by TLC eluting in toluene–ethyl acetate [1:1] afforded compound **4c**. Yield 35 mCi.

(*RS*)-2-Amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid (ATPA, 5a)

A solution of compound **4a** (70 mg, 0.18 mmol) in aqueous hydrobromic acid (2 ml, 48%) was refluxed at 140 °C for 25 min. After evaporation and re-evaporation twice from water, the residue was subjected to ion-exchange chromatography (IRA-400) using 1 M acetic acid as an eluent. Evaporation of appropriate fractions followed by recrystallization (water) afforded compound **5a** (17 mg, 41%) as the zwitterion: mp 235.6–236.4 °C (dec). Litt: 246–247 °C (dec) (15). ^{1}H NMR (D_2O, 1,4-dioxane) δ 1.29 (s, 9 H), 2.94 (dd, J = 15.6 and 7.7 Hz, 1 H), 3.04 (dd, J = 15.6 and J = 5.6 Hz, 1 H), 3.87 (dd, J = 7.7 and J = 5.6 Hz, 1 H). ^{13}C NMR (dissolved in D_2O as a hydrobromide, 1,4-dioxane) δ 24.2 ($\underline{C}H_2C_{isoxazole}$), 29.0 ($C[\underline{C}H_3]_3$), 35.4 ($\underline{C}[CH_3]_3$), 53.6 (CH), 96.1 (C-4), 171.7, 172.1, 179.3 (C-3, C-5, COO).

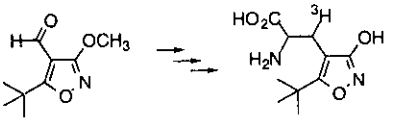
rac-2-Amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)-[3-^{2}H]propionic acid ([^{2}H]ATPA, 5b)

Treatment of compound **4b** (130 mg, 0.34 mmol) in analogy with the method described above for the synthesis of **5a** gave **5b** (48 mg, 62%): mp 241.5–242.0 °C (dec). ^{1}H NMR (D_2O, 1,4-dioxane) δ 1.29 (s, 9 H), 2.90 (br d, J = 7.7 Hz, 1/2 H), 3.23 (br d, J = 5.7 Hz, 1/2 H), 3.86 (d, J = 7.7 Hz, 1/2 H), 3.87 (d, J = 5.7 Hz, 1/2 H). ^{13}C NMR (dissolved in D_2O as a hydrobromide, 1,4-dioxane) δ 24.0 (t, J = 19.8 Hz, $\underline{C}HDC_{isoxazole}$), 29.0 ($C[\underline{C}H_3]_3$), 35.4 ($\underline{C}[CH_3]_3$), 53.6 (CH), 96.0 (C-4), 171.6, 172.1, 179.3 (C-3, C-5, COO).

rac-2-Amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)-[3-^{3}H]propionic acid ([^{3}H]ATPA, 5c)

Compound **4c** was dissolved in 48% hydrobromic acid (2 ml) and heated at 140 °C for 30 min. The reaction mixture was evaporated and re-evaporated several times from water and purified by reverse phase HPLC on a Sperisorb ODS column eluting in methanol–water–trifluoroacetic acid [25:75:0.1] to give [^{3}H]ATPA (**5c**), 6 mCi. The radiochemical purity of [^{3}H]ATPA was determined by reverse phase HPLC on a Vydac C18 column using an acetonitrile–water–trifluoroacetic acid gradient system and found to be 98.9%. Mass spectral analysis showed that [^{3}H]ATPA had a specific activity of 17 Ci/mmol and was consistent with nonlabelled ATPA (**5a**).

Tritiation of the GluR5 agonist ATPA



1 [^{3}H]ATPA

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