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SYNTHESIS OF TRITIATED (*RS*)-2-AMINO-2-(3-HYDROXY-5-METHYLISOXAZOL-4-YL)ACETIC ACID (AMAA), A POTENT AND SELECTIVE NMDA AGONIST

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SUMMARY

(*RS*)-2-Amino-2-(3-hydroxy-5-methylisoxazol-4-yl)acetic acid (AMAA) is a potent and selective agonist at the NMDA subgroup of excitatory amino acid receptors. To probe its interaction with these receptors we have developed a synthesis of [^{3}H]AMAA. Bromination of a protected form of AMAA with NBS followed by hydrogenolysis with tritium gas in the presence of Pd/C and subsequent deprotection gave [^{3}H]AMAA with a specific activity of 25 Ci/mmol. Attempts to develop a receptor binding assay based on rat brain homogenates and using [^{3}H]AMAA as radioligand for the NMDA receptors have not been successful.

Keywords: (*RS*)-2-amino-2-(3-hydroxy-5-methylisoxazol-4-yl)acetic acid, AMAA, NMDA agonist, tritium NMR, bromination.

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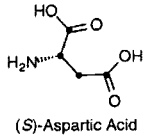
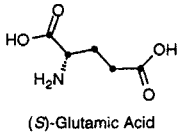
INTRODUCTION

(*S*)-Glutamic acid and (*S*)-aspartic acid are considered to be the major excitatory amino acid (EAA) neurotransmitters in the mammalian central nervous system. Excessive stimulation of the EAA receptors by these amino acids probably is implicated in neurodegenerative diseases such as Huntington's disease, dementia of the Alzheimer type, as well as neuronal degeneration associated with ischemia, hypoxia and hypoglycemia. Hence, there is increasing interest in developing subtype selective agonists and antagonists at EAA receptors both as pharmacological tools to delineate the excitatory and neurotoxic processes, and as potential therapeutic agents [1,2].

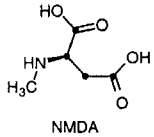
Central ionotropic EAA receptors can, based on ligand interactions and molecular cloning, be classified into three main groups: i) *N*-methyl-D-aspartic acid (NMDA) receptors, ii) (*RS*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) receptors, and iii) kainic acid (KAIN) receptors (Figure 1) [2-4]. [^{3}H]AMPA and [^{3}H]KAIN are used as ligands for binding studies of the respective receptors. No selective agonist radioligand for studies of NMDA receptors is available. NMDA receptor sites are, at the present time, being studied using [^{3}H]-(*S*)-glutamic acid under conditions, where AMPA and KAIN receptors are blocked, or by using radiolabelled competitive or non-competitive NMDA receptor antagonists [5-7]. The selective agonist, NMDA, has previously been labelled, but studies with [^{3}H]NMDA have revealed a low affinity and a ratio of specific to non-specific binding too low for practical use in binding assays [5].



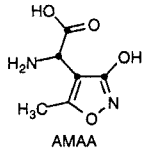
(*S*)-Aspartic Acid



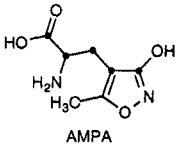
(*S*)-Glutamic Acid



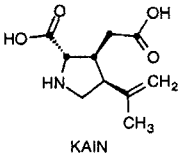
NMDA



AMAA



AMPA



KAIN

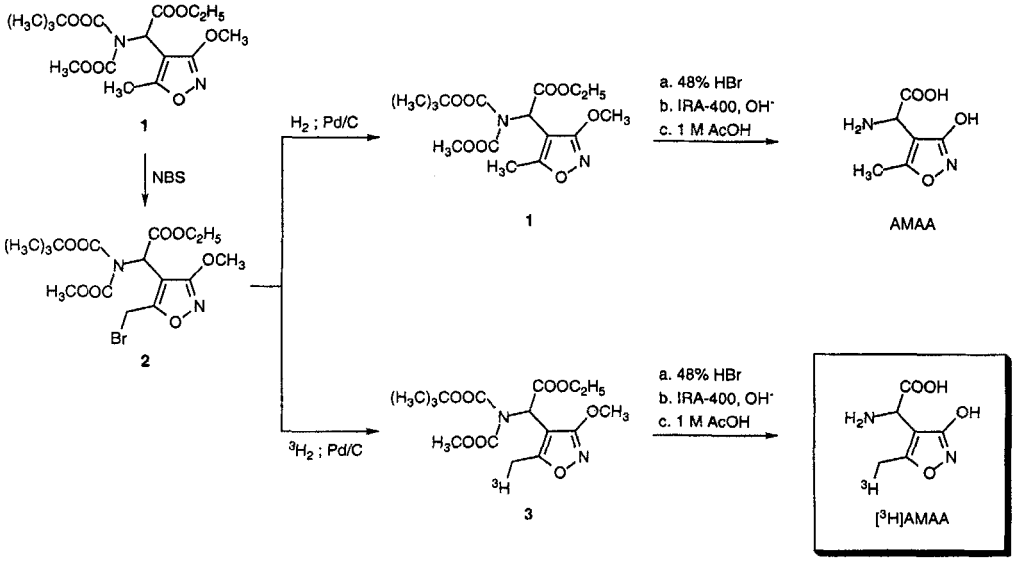
Figure 1. Chemical structures of some key excitatory amino acid receptor agonists.

The lower homologue of AMPA, (*RS*)-2-amino-2-(3-hydroxy-5-methylisoxazol-4-yl)acetic acid (AMAA) [8], is an analogue of aspartic acid and NMDA and is a potent and selective NMDA receptor agonist with no affinity for AMPA or KAIN sites [9,10]. As observed for NMDA itself, the NMDA receptor agonist activity of AMAA resides predominantly in the (*R*)-isomer, which is at least an order of magnitude more potent than (*S*)-AMAA [11]. In order to examine the potential of AMAA as a radioligand for NMDA receptor sites, we have developed a convenient synthesis of [^{3}H]AMAA.

RESULTS AND DISCUSSION

Chemistry

The synthesis of the [^{3}H]AMAA is outlined in Scheme 1. The starting material, **1** [12], is a protected derivative of AMAA containing an *N*,*N*-diprotected amino group. Bromination of compound **1** using *N*-bromosuccinimide (NBS) to give the bromomethyl analogue, **2**, has previously been performed in the synthesis of an analogue of AMAA [12].



Scheme 1. Synthesis of [^{3}H]AMAA.

N,N-Diprotection of the amino group proved to be necessary in order to accomplish bromination of the 5-methyl group. Attempts to brominate the corresponding *N*-mono-*tert*-butoxycarbonyl and *N*-mono-methoxycarbonyl protected analogues of compound **1** primarily led to bromination of the 4-glycinyl moiety leading to the formation of a number of side products. In both cases, significant amounts of non-, 5-mono-, and 5-dibrominated derivatives of ethyl 2-(3-methoxy-5-methylisoxazol-4-yl)-2-oxoacetate could be isolated. However, when the diprotected derivative, **1**, was used, none of these α-oxo-esters were formed. Low pressure hydrogenation of **2** was carried out in ethanol in the presence of Pd/C at room temperature. After 1 hr, analysis of the reaction mixture by TLC showed complete conversion to compound **1**. After workup and purification, **1** was isolated in 73% yield. Deprotection of compound **1** in 48% hydrobromic acid afforded AMAA which upon ion-exchange chromatography was isolated as a zwitterion.

Catalytic tritiation of compound **2** was carried out using tritium gas (1 atm) at room temperature in the presence of Pd/C in methanol containing two equivalents of triethylamine to neutralize the acid formed. After 1 hr, the solvent was evaporated and the residue was treated with methanol and again evaporated to semidryness under reduced pressure. This process was repeated twice to remove any labile tritiated compounds in the product. Finally, the methanol solution was filtered to remove the catalyst and lyophilized overnight. The crude product was analyzed by HPLC and tritium NMR.

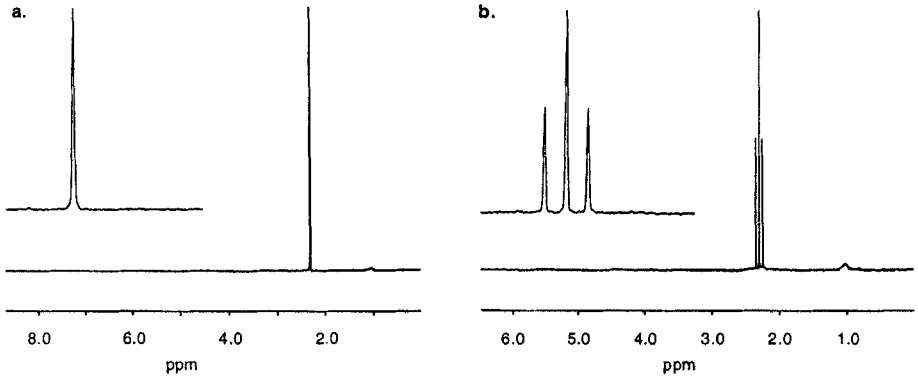


Figure 2. ^{1}H-Decoupled ^{3}H-NMR spectrum (a) and ^{1}H-coupled ^{3}H-NMR spectrum (b) of compound **3**.

HPLC analysis was carried out using a reverse phase column and showed complete conversion of compound **2** into the desired tritiated compound, **3**. Purified **3** had a specific activity of 25 Ci/mmol and a radiochemical purity of >98%.

^{3}H-NMR of the product, **3**, was recorded in CD_3OD solution. Figure 2 shows the proton decoupled ^{3}H-NMR spectrum of compound **3** as well as the corresponding proton coupled spectrum. The tritium spectrum of the crude reaction mixture contains a sharp singlet at 2.29 ppm for the tritium in the 5-methyl group of compound **3**. A trace of impurity considered not to be related to the product is visible around 1 ppm. The insert shows the expansion of the signal at 2.29 ppm. The proton coupled spectrum shows a triplet due to coupling of the two protons on the methyl group with the tritium atom. Again, the insert shows the expansion of this signal, and it is clear from the symmetry of the triplet that the tritium coupling to the two protons is identical and that it is located on the same carbon atom. The proton spectrum of this compound (not shown) confirms these findings.

Deprotection of **3** with 48% hydrobromic acid followed by ion-exchange chromatography and HPLC purification gave [^{3}H]AMAA with a radiochemical purity of >97%.

Receptor Binding

Standard methods were employed in an effort to detect specific binding of [^{3}H]AMAA to rat brain homogenate, including the use of a variety of buffers (0.05 M, pH 7.2: Tris acetate, HEPES KOH, or TES), temperatures (2°C, 23°C, or 37°C), and rat brain preparations (crude whole forebrain, synaptosomal forebrain, or synaptosomal cerebellum) prepared either immediately, before assaying, or following 2-5 days at -80°C. AMAA (10^{-4} M) or (*S*)-glutamic acid (10^{-4} M) displaceable binding of [^{3}H]AMAA could not be observed using either centrifugation or filtration methods. The failure to detect specific binding of the ligand may be due to a low density of AMAA sensitive receptors and/or to the relatively low affinity of AMAA for the NMDA excitatory amino acid receptor subtype [10].

In summary, we have developed a convenient synthesis of [^{3}H]AMAA, which was obtained with a specific activity of 25 Ci/mmol. ^{3}H-NMR analysis was carried out on the intermediate, compound **3**. Our efforts to develop a binding assay using [^{3}H]AMAA as ligand for AMAA sites of the NMDA receptor have not been successful.

EXPERIMENTAL SECTION

General procedures

Melting points were determined in capillary tubes and are uncorrected. Infrared spectra were obtained on a Perkin-Elmer grating infrared spectrophotometer. 200 MHz ^{1}H-NMR spectra were recorded on a Bruker AC-200 F spectrometer. TMS was used as internal standard except for compounds dissolved in D_2O, where 1,4-dioxane (δ 3.70 ppm) was used. ^{3}H-NMR analyses were performed using a 300 MHz IBM instrument. 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. HPLC analyses were performed with Waters and Shimadszu HPLC units equipped with UV and radiochemical detectors and a Spherisorb C8-column (5 μm, 4.2 × 250 mm). Methanol-water (7:3) containing 0.1% trifluoroacetic acid was used as mobile phase at a flow rate of 1.0 ml·min^{-1}.

Ethyl (*RS*)-2-(*N*-*tert*-butoxycarbonyl-*N*-methoxycarbonyl)amino-2-(3-methoxy-5-methylisoxazol-4-yl)acetate (1)

To a solution of ethyl (*RS*)-2-(*N*-*tert*-butoxycarbonyl-*N*-methoxycarbonyl)amino-2-(5-bromomethyl-3-methoxyisoxazol-4-yl)acetate (**2**) [12] (20 mg; 0.044 mmol) in ethanol (5 ml) was added Pd/C (40 mg; 5%). A stream of hydrogen was passed through the mixture for 1hr. After removal of the catalyst by filtration through Celite, the solvent was removed under reduced pressure. The residue gave upon CC [eluents: toluene containing ethyl acetate (10%)] **1** (12 mg; 73%) as a yellowish oil. TLC, IR and ^{1}H-NMR data of the isolated compound were identical with those of authentic **1** [12].

(*RS*)-2-Amino-2-(3-hydroxy-5-methylisoxazol-4-yl)acetic Acid Hydrate (AMAA Hydrate)

A solution of **1** (40 mg; 0.11 mmol) in aqueous hydrobromic acid (2 ml; 48%) was refluxed for 1 hr and then evaporated to dryness under reduced pressure. The residue was placed on a strongly basic ion-exchange column (Amberlite IRA-400) (5 ml) for 1 hr and then eluted with 1 M acetic acid. Evaporation of appropriate fractions followed by recrystallization (water - ethanol) gave AMAA hydrate (10 mg; 47%) m.p. 194-195°C. TLC, IR, and ^{1}H-NMR data of this compound were identical with those of authentic AMAA hydrate [11].

Tritium Labelling

Tritium labelling studies were carried out at the National Tritium Labelling Facility (NTLF), Berkeley, CA. Compound **2** [12] (19 mg) was dissolved in methanol (1.2 ml). Triethylamine (2 µl) was added and the reaction flask attached to the manifold. The catalyst, 10% Pd/C (20 mg) was placed in a glass spoon over the reaction flask. After degassing under nitrogen several times, tritium gas was vented into the flask at 1 atmosphere. The solution was cooled and stirred. The catalyst was added and the reaction was allowed to proceed at room temperature. After 1 hr, excess tritium gas was reabsorbed on a charcoal trap cooled with liquid nitrogen and then reabsorbed into an uranium bed. The reaction mixture was evaporated partially under high vacuum, and methanol was added twice and evaporated to remove any labile tritium compounds in the product. The catalyst was then filtered off using a glass fiber filter and the product frozen in liquid nitrogen and lyophilized overnight. The final product, **3**, was analyzed by HPLC and by ^{3}H-NMR. Water was then added to the crude product and the mixture was extracted with ethyl acetate. After drying and evaporation of the organic phase, the residue was treated with 48% hydrobromic acid and passed through an ion-exchange resin as described above for AMAA hydrate. The fractions containing radiolabelled AMAA were finally purified by HPLC.

Table 1. Physicochemical data on tritiated compounds

	Compound **3**	[^{3}H]AMAA
HPLC retention time	6.0 min	4.0 min
Chemical purity	>98%	>97%
Radiochemical purity	>98%	>97%
Specific activity	25 Ci/mmol	25 Ci/mmol

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