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ONE-STEP PREPARATION OF [2,3-^{3}H]1-AMINOCYCLOPROPANECARBOXYLIC ACID: A USEFUL LIGAND FOR STRYCHNINE-INSENSITIVE GLYCINE RECEPTORS

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SUMMARY

Catalytic hydrogenation of 1-aminocyclopropenecarboxylic acid under tritium gas afforded [2,3-^{3}H]1-aminocyclopropanecarboxylic acid with specific activity 26 Ci/mmol, determined by a combination of ^{1}H and ^{3}H NMR. Pilot radioligand binding assays indicate this compound will be a useful probe for the NMDA receptor-associated strychnine-insensitive glycine receptor.

Key Words: 1-aminocyclopropanecarboxylic acid, glycine, strychnine-insensitive, radioligand, tritiated, ^{3}H NMR

INTRODUCTION

The crucial role of glycine as a component of the NMDA-gated cation channel supramolecular complex (1) has prompted considerable research in this area. Radioreceptor binding studies, which have provided significant insights in many other related areas, have been hampered both by the relatively low affinity of glycine for strychnine-insensitive receptors (2,3) and its high concentration in the mammalian brain. Our discovery that 1-aminocyclopropanecarboxylic acid (ACPC) (4) is two to three times as potent as glycine in competitively inhibiting [^{3}H]glycine binding to these strychnine-insensitive receptors suggested that it might be a useful ligand for receptor binding. Indeed, the preparation of [2,3-^{3}H]ACPC has been reported while our work was in progress (5). We now wish to report a somewhat simpler, higher yield synthesis of high specific activity [2,3-^{3}H]ACPC, requiring less handling of radioactive materials and the determination of the specific activity utilizing ^{3}H. We also report some results demonstrating the usefulness of [2,3-^{3}H]ACPC in radioligand binding assays.

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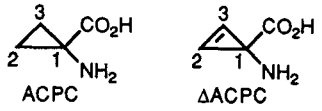


RESULTS

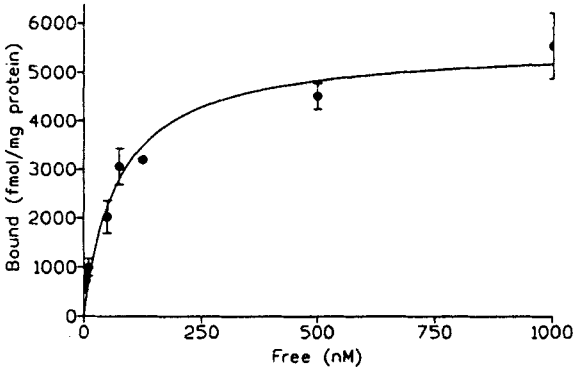
Chemistry

Exposure of 1-aminocyclopropenecarboxylic acid (ΔACPC) (6) to tritium gas over a palladium catalyst afforded a single radiolabeled product, which coeluted with authentic unlabeled ACPC. After purification of this product by preparative thin layer chromatography (TLC), it was found to be pure by TLC and ^{1}H NMR. The radiochemical purity was established by TLC-radioscan and ^{3}H NMR. High performance liquid chromatography (HPLC) analysis following the literature (5) was found to be unsatisfactory for purity and specific activity determinations since, in our hands, using a radially compressed Novapak column and eluting with 0.05% trifluoroacetic acid (TFA), were observed for ACPC as well as for a number of other α-amino acids. Doubling the concentration of TFA reduced the area of the early eluting component. The retention volume of glycine methyl ester was almost the same as that of the late eluting components in the spectra of ACPC and of serine. The specific activity was determined by recording the ^{3}H NMR spectrum in the presence of [^{3}H]phenylalanine of known specific activity. [2,3-^{3}H]ACPC was obtained in 20% yield; the specific activity was determined to be 26 Ci/mmol.

Pharmacology

The binding of [2,3-^{3}H]ACPC to membranes from rat forebrain was saturable (Figure 1), with B_{max} and K_D values of 5541 ± 414 fmol/mg protein and 252 ± 66 nM (n = 4). The dissociation rate upon addition of 1 mM glycine was 0.010 sec^{-1}.

Figure 1
Saturation analysis of the binding of [2,3-^{3}H]ACPC to rat cortical membranes, carried out as described in the Experimental Section. The K_D and B_{max} values of [2,3-^{3}H]ACPC in this representative experiment were 73 nM and 5541 fmol/mg protein, respectively (r^2 = 0.97).



DISCUSSION

The literature preparation of [2,3-^{3}H]ACPC (5) had involved the use of t-Boc protected ΔACPC to provide solubility in aprotic solvent, and a 41-fold molar excess of tritium. Recovery of purified [2,3-^{3}H]ACPC corresponded to 0.08% radiochemical yield and to 4% chemical yield. Our preparation required less handling of radioactive material (one step), used less tritium, and afforded the final product in higher chemical and radiochemical yield. Thus, we used unprotected ΔACPC in tetrahydrofuran spiked with sufficient methanol to achieve solubility, and 1.5-fold molar excess of tritium. The reaction was rapid (45 min) and the purified product was recovered in 4% radiochemical yield and 20% chemical yield.

Attempts to determine the specific activity following the published HPLC procedure (5) were unsatisfactory due to the observation of two components. Based on our limited HPLC study we concluded that, in the presence of 0.05-0.1% TFA, α-amino acids existed as a mixture of protonated and unprotonated forms which eluted separately. The early eluting component would be the protonated material, while the late eluting component would be the neutral form. This situation precluded quantitation since the late eluting signal was very broad and sometimes hard to distinguish from the baseline. Attempts to use OPA derivatives were also unsuccessful due to the short lifetime of these derivatives.

These difficulties led us to examine the use of NMR for the determination of the specific activity. Both ^{1}H and ^{3}H NMR spectra of [2,3-^{3}H]ACPC had signals between 1.10 and 1.40 ppm; no other signals were observed. For [2',6'-^{3}H]phenylalanine the ^{3}H NMR spectrum exhibited a sharp signal at 7.4 ppm; in the aromatic region of the ^{1}H NMR spectrum it had two signals between 7.3 and 7.4 ppm. There was no overlap between any of the [2',6'-^{3}H]phenylalanine and [2,3-^{3}H]ACPC signals. Since the specific activity of [2',6'-^{3}H]phenylalanine was known to be 40 Ci/mmol, it followed that each mole contained 1.38 atoms ^{3}H and 3.62 atoms ^{1}H in the aromatic region. From this information the integrated areas of a single ^{3}H and a single ^{1}H were computed. These areas were then used to determine the number of moles of ^{3}H and of ^{1}H associated with ACPC, and this information was used to calculated the ^{3}H content per mole of [2,3-^{3}H]ACPC. The specific activity was obtained by multiplying by 29 Ci/mmol. Because the accuracy for integration of NMR signals is only 95%, this approach to the determination of the specific activity is certainly not as accurate as quantitation by HPLC, GC or UV. However, it provides a useful method in cases as difficult as ACPC. To our knowledge this approach has not been previously utilized.

The data obtained for [2,3-^{3}H]ACPC binding at the strychnine-insensitive glycine site resemble those of [^{3}H]glycine and appear to be in general accord with the data reported in abstract form on [^{3}H]ACPC binding to brain (7). The findings indicate that [2,3-^{3}H]ACPC may become a useful biochemical and pharmacological probe since it will allow ligand binding studies to be performed using a filtration assay. In contrast, studies with [^{3}H]glycine are routinely performed with a centrifugation assay due to its high dissociation rate. Furthermore, the use of a radioligand with partial agonist (4,8) properties may provide unique insights into the role and regulation of strychnine-insensitive glycine receptors.

EXPERIMENTAL SECTION

[2,3-$^3H_2$]1-Aminocyclopropanecarboxylic Acid ([2,3-^{3}H]ACPC)

To a solution of ΔACPC hydrochloride hydrate (6) (5 mg; 0.033 mmol) in MeOH (0.1 mL) and dry THF (0.7 mL) was added 10% Pd/C (5 mg) and the mixture was stirred under T_2(g) (5 Ci, 0.085 mmol) for 1 h. The reaction mixture was filtered through celite into a 25 mL round bottomed flask, the catalyst was washed with MeOH and the combined solvents were removed under vacuum. The residue was subjected to tritium exchange in MeOH (3 x 2 mL) and was diluted to 250 mL with $CHCl_3$ for overnight storage.

The solvent was removed and the residue was streaked onto two 20 cm x 20 cm x 0.25 mm SiO_2 TLC plates and eluted with n-BuOH:H_2O:HOAc (4:1:1). A portion of the plate was visualized by treatment with ninhydrin and the band corresponding to ACPC (R_f 0.29; ΔACPC has R_f 0.17) was removed and eluted with MeOH. The material was filtered into a 100 mL volumetric flask. The solvent was blown off and the residue was diluted to volume with water. The amount of ACPC recovered was 318.5 mCi. The absence of ΔACPC was confirmed by HPLC. The radiochemical purity was checked by TLC-radioscan (as above) and by HPLC-radioscan and was found to be greater than 99%. The HPLC conditions were: C_{18} Nova-Pak 60Å, 6 μm, 8 x 100 mm cartridge (Waters); H_2O (0.05% TFA) at a flow rate of 1 mL/min; UV 205 nm. The retention time of ACPC was 2.58 min and that of ΔACPC was 1.85 min. The injected quantity was 0.1 mCi (4 nmol).

Tissue Preparation

Male Sprague-Dawley rats (200-250 g) were sacrificed by decapitation and their brains were rapidly removed. Forebrains were dissected on an ice-cold plate and rapidly frozen on aluminum foil over solid CO_2. The tissue was then homogenized in approximately 50 volumes of sterile, ice-cold

Hepes-Tris Buffer (5 mM Hepes, 4.5 mM Tris, pH 7.8) which had been filtered through 0.2 μ nitrocellulose filters to avoid bacterial contamination which has been found to alter binding of glycinergic ligands (unpublished observations). The homogenate was centrifuged for 20 min at 20,000 x g (0°C) and the supernatant was discarded. The membranes were then washed twice in Hepes-Tris buffer containing 1 mM EDTA, resuspended in Hepes-Tris buffer and centrifuged. This step was repeated 3-4 times. The final pellets were resuspended in Hepes-Tris buffer and stored at -70°C in approximately 5 volumes of buffer. For assays the frozen homogenates were thawed, centrifuged at 20,000 x g for 20 min and the supernatant discarded. The pellet was resuspended in the required volume of fresh Hepes-Tris buffer, to which had been added sufficient $MgCl_2$ to result in 10 mM final concentration.

Binding Assays

Assays were performed in triplicate using 5-10 mg wet weight of tissue and 10-400 nM [2,3-^{3}H]ACPC or 20 nM [2,3-^{3}H]ACPC and 20-1000 nM ACPC in a total volume of 0.5-1.0 mL. Nonspecific binding was defined by 1 mM glycine. The suspensions were incubated at room temperature for 1 h. Incubations were terminated with 3 washes of buffer containing 10 mM $MgCl_2$ through Whatman GF/C filters, using a Brandel 48R filtering manifold (Brandel Instruments, Gaithersburg, MD). Radioactivity retained on the filters was counted in a Beckman LS-5801 liquid scintillation counter. Data were analyzed using iterative curve fitting routines (GraphPad-Inplot 4.01, San Diego, CA).

CONCLUSIONS

High specific activity tritium labeled ACPC can be used in a filtration assay to directly measure the strychnine-insensitive glycine receptors associated with the NMDA receptor, thus providing a powerful biochemical and pharmacological probe. It can be prepared in one step in 20% yield.

The use of ^{3}H and ^{1}H NMR provides new methodology for the determination of specific activity of high specific activity compounds lacking a chromophore.

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