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Synthesis of [1,2-^{3}H]ethylamine hydrochloride and [^{3}H]-labeled apadenoson for a human ADME study

Yang Hong,[a]* Samuel J. Bonacorsi Jr,[a] Yuan Tian,[a] Sharon Gong,[a] Donglu Zhang,[a] W. Griffith Humphreys,[a] Balu Balasubramanian,[a] Edward H. Cheesman,[b] Zhiqin Zhang,[b] James F. Castner,[b] and Paul D. Crane[b]

Tritium-labeled [1,2-^{3}H]ethylamine hydrochloride was prepared through a multiple-step sequence in high radioactive specificity. The labeled amine was isolated in high purity following cartridge filtration and used subsequently in the synthesis of [*N*-ethyl-1,2-^{3}H]apadenoson, an adenosine A_{2a} receptor agonist. The overall yield for this transformation was 56% and the radiochemical purity of the final product was greater than 99%.

Keywords: apadenoson; adenosine A_{2a} receptor agonist; tritium-NMR (T-NMR); [^{3}H]ethylamine hydrochloride

Introduction

Apadenoson (Figure 1) is a C2 and 5′-substituted adenosine analog with selective agonist activity for the adenosine A_{2a} receptor.[1,2] This compound induces coronary vasodilatation during myocardial perfusion imaging. It is currently in clinical development and undergoing testing as a pharmacological stress agent. Owing to its high potency and associated low dose, a tritium-labeled analog of apadenoson with high specific radioactivity was required for determination of the absorption, distribution, metabolism, and excretion (ADME) properties of the molecule. The primary goal of the ADME study was to obtain a pharmacokinetic profile of the compound relative to the profile of total drug-related materials and to determine major clearance routes for the compound. The compound for human clinical analysis was labeled in a metabolically stable pharmacophore of the molecule. The suitability of the ethylamine moiety was established through a separate BDC rat study using the same tritium-labeled substrate planned for the human study. The metabolic stability of [^{3}H]apadenoson was examined following administration of a single 3-μg/kg (250 μCi/kg) intravenous dose of [^{3}H]apadenoson. Radioactivity profiling of plasma, urine, and fecal extract by high-performance liquid chromatography (HPLC) separation, fraction collection, and scintillation counting showed that no tritiated water was formed. Total radioactivity was also measured in plasma and urine samples before and after drying. The results of this study showed no loss of label due to either metabolism or chemical exchange.[3]

The radiochemical synthesis of [^{3}H]apadenoson required efficient methodology to prepare [1,2-^{3}H]ethylamine hydrochloride. A product with high purity and specific radioactivity was obtained in excellent yield by tritium reduction of a commercially available precursor, *N*-vinylphthalimide, followed by hydrolysis. The product was conveniently isolated by rotary evaporation and solid-phase extraction. The synthesis of tritium-labeled apadenoson was designed around the use of this precursor, [1,2-^{3}H]ethylamine hydrochloride, which was incorporated into apadenoson. This route proved to be a concise and efficient method for the preparation of tritiated apadenoson.

Results and discussion

The synthesis of [1,2-^{3}H]ethylamine hydrochloride began with tritium reduction of commercially available *N*-vinylphthalimide followed by the classical Gabriel synthesis (Scheme 1).[4] *N*-Vinylphthalimide was reduced in ethanol using carrier-free tritium gas in the presence of a Pd/C catalyst. Pure [*N*-ethyl-1,2-^{3}H]phthalimide was obtained after catalyst removal by filtration. The [*N*-ethyl-1,2-^{3}H]phthalimide was then refluxed overnight in 6 N hydrochloric acid. The resulting [1,2-^{3}H]ethylamine hydrochloride was isolated after solvent removal and solid-phase extraction through C-18 SepPak cartridges. The product, in ethanol, was determined to be 95% radiochemically pure by HPLC, and stored as a solution until utilized in the synthesis. The overall yield for these two steps was 82%, based on the *N*-vinylphthalimide precursor utilized in the tritium reduction.

[a]*Bristol-Myers Squibb Research and Development, Route 206 and Province Line Road, Princeton, NJ 08540, USA*

[b]*Bristol-Myers Squibb Medical Imaging, 331 Treble Cove Road, Billerica, MA 01862, USA*

**Correspondence to: Yang Hong, Department of Chemical Synthesis, The Bristol-Myers Squibb Research and Development, Route 206 and Province Line Road, Princeton, NJ 08540, USA.*
E-mail: yang.hong@BMS.com

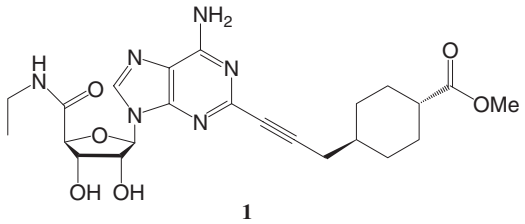

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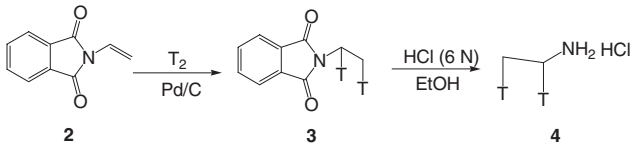
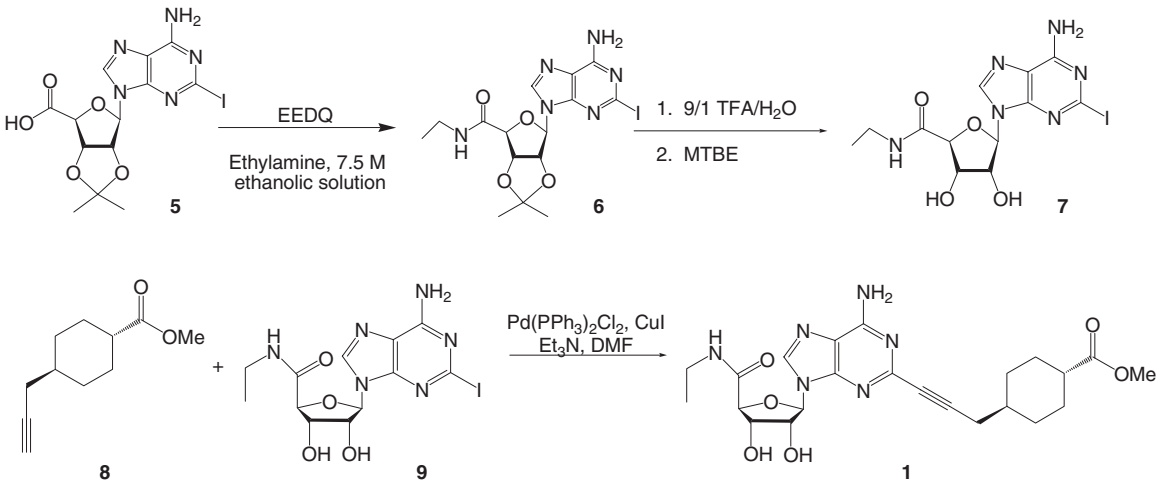
Figure 1. Structure of apadenoson.

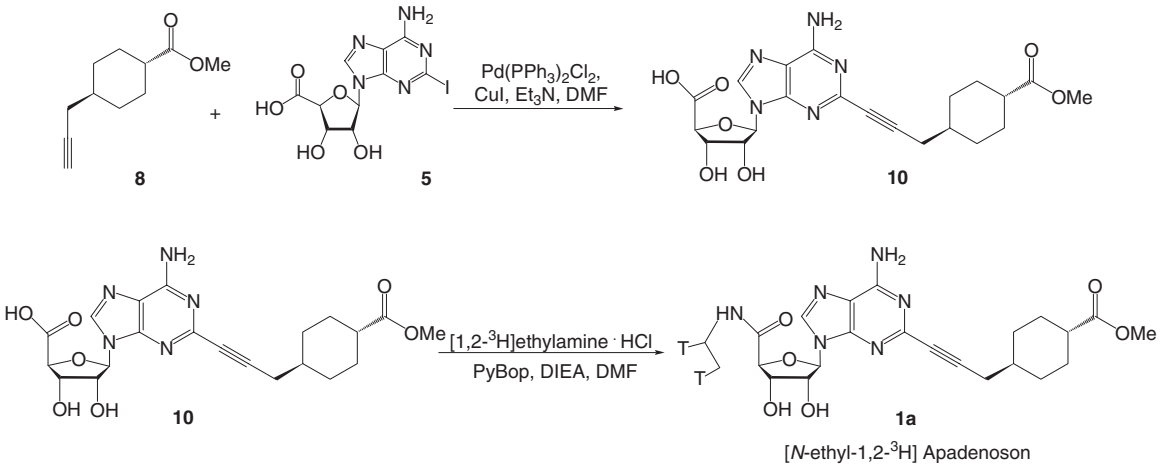


2 3 4

Scheme 1



Scheme 2



Scheme 3

Scheme 2 shows the synthetic route for the preparation of unlabeled apadenoson, in which the *N*-ethyl amide linkage is formed prior to deprotection and Sonogashira coupling. In this sequence an excess of ethylamine was used to drive the reaction to completion.

The modified synthesis to produce [^{3}H]apadenoson is illustrated in Scheme 3. There were several advantages of this route, including introduction of labeled ethylamine in the final synthetic step. In addition, complications of running a Sonogashira reaction on a micro-molar scale were avoided. A more

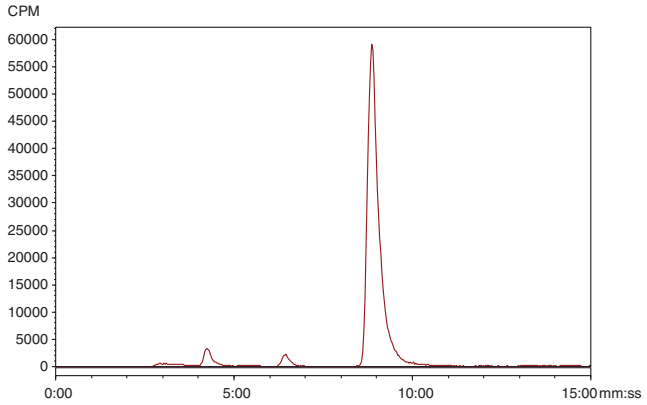


Figure 2. Radio-HPLC analysis of the amidation reaction [*N*-ethyl-1,2-^{3}H]apadenoson (9.1 min component).

Table 1. Tritium mass distribution and average specific radioactivity of [*N*-ethyl-1,2-^{3}H]apadenoson

Unlabeled	17%
Mono-tritiated analog	23%
Di-tritiated	26%
Tri-tritiated	24%
Tetra-tritiated	9%
Average specific radioactivity	48 Ci/mmol

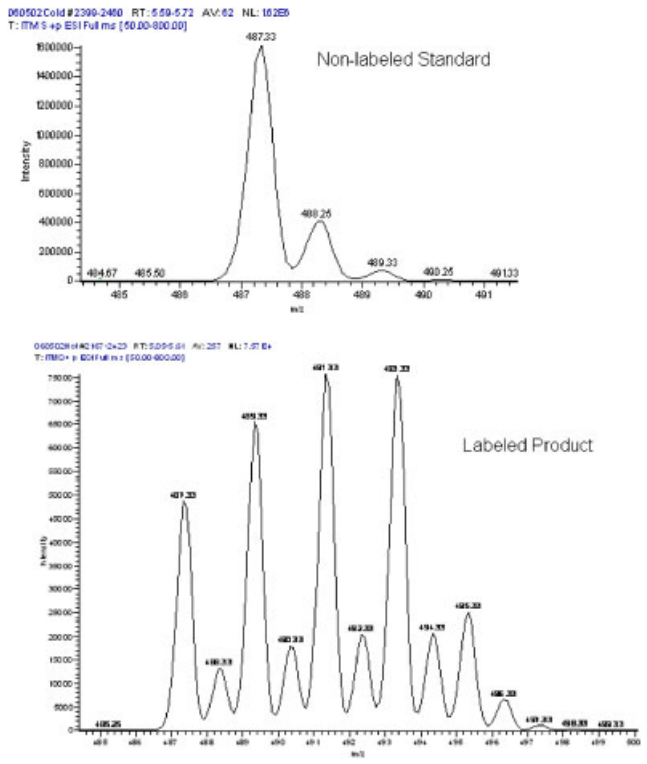


Figure 3. Mass analysis of [*N*-ethyl-1,2-^{3}H]apadenoson.

efficient coupling reagent benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop) was also adopted to improve the reactivity to form the amide. This modification

Table 2. Tritium NMR: multiple species of [*N*-ethyl-1,2-^{3}H]apadenoson

Chemical shift δ	Assignment
0.99, 1.01, 1.04, 1.07	$-CT_3$, $-CHT_2$, $-CH_2T$
3.20, 3.22, 3.24, 3.26, 3.28, 3.30	$-CT_2-$, $-CHT-$

was critical for the success of the synthesis and avoided the use of a volatile-free amine. The improved synthetic efficiency of PyBop led to quantitative consumption of radiolabeled ethylamine hydrochloride as the limiting reagent.

In the final step of the synthesis, ethanol was removed from the solution of [1,2-^{3}H]ethylamine hydrochloride by rotary evaporation to dryness and the labeled ethylamine hydrochloride was dissolved in dry dimethyl formamide (DMF). An excess of the intermediate acid **10** was added to the solution along with PyBop and *N*,*N*-diisopropylethylamine (DIEA). Radiometric HPLC analysis showed concomitant consumption of [1,2-^{3}H]ethylamine hydrochloride and the formation of [*N*-ethyl-1,2-^{3}H]apadenoson. The reaction was complete in less than 30 min as indicated by HPLC (Figure 2). The crude product was purified by preparative reverse-phase HPLC giving 170 mCi (56%) of pure [*N*-ethyl-1,2-^{3}H] apadenoson with high specific radioactivity.

The LC-MS analysis of the tritium-labeled product (Table 1 and Figure 3) shows a mixture of isotopic isomers containing 0–4 tritons in each molecule. The average specific activity of this mixture was 48 Ci/mmol. Tritium NMR analysis of the [*N*-ethyl-1,2-^{3}H] apadenoson (Figure 4 and Table 2) showed multiple peaks for the isotopomers with a 5.6:1 ratio of methyl/methylene tritium labeling. The observed distribution of tritium was likely the result of T/H exchange that occurred during heterogeneous catalytic reduction, which involves a complicated mechanism.[5] This ratio was consistent throughout the multi-step reactions.

The synthesis of clinical supplies of [*N*-ethyl-1,2-^{3}H]apadenoson was carried under GLP conditions and was fully characterized for use in human clinical studies. Long-term stability and formulation suitability studies were carried out to determine the stability of the molecule. Overall, the compound was found to be stable for several months if stored as a dilute solution in absolute ethanol at low temperature.

Experimental

Materials: Compounds **6** and **7** were synthesized at Bristol–Myers Squibb, Medical Imaging, Discovery Chemistry Department. Compound **5** was prepared by a modification of the procedure reported by Cristalli *et al.*[6] Compound **8** was prepared by a modification of the procedure reported by Rieger *et al.*[7] *N*-Vinylphthalimide and Pd/C, 10% were purchased from Sigma-Aldrich. Carrier-free tritium gas was purchased from American Radiolabeled Chemicals Inc. All other reagents were obtained from commercial sources as ACS reagent grade or higher. C18 Sep-Pak® Cartridges were purchased from Waters Corporation. HPLC was performed using an Agilent ChemStation HPLC system (Agilent Technologies) equipped with a IN/US β-RAM detector (IN/US Systems). Tritium NMR spectra were recorded on a Bruker DMX-300MHz spectrometer at 320 MHz. LC-MS was determined on an LXQ Mass Spectrometer (Thermo Electron Corp.). The tritiation manifold used in all

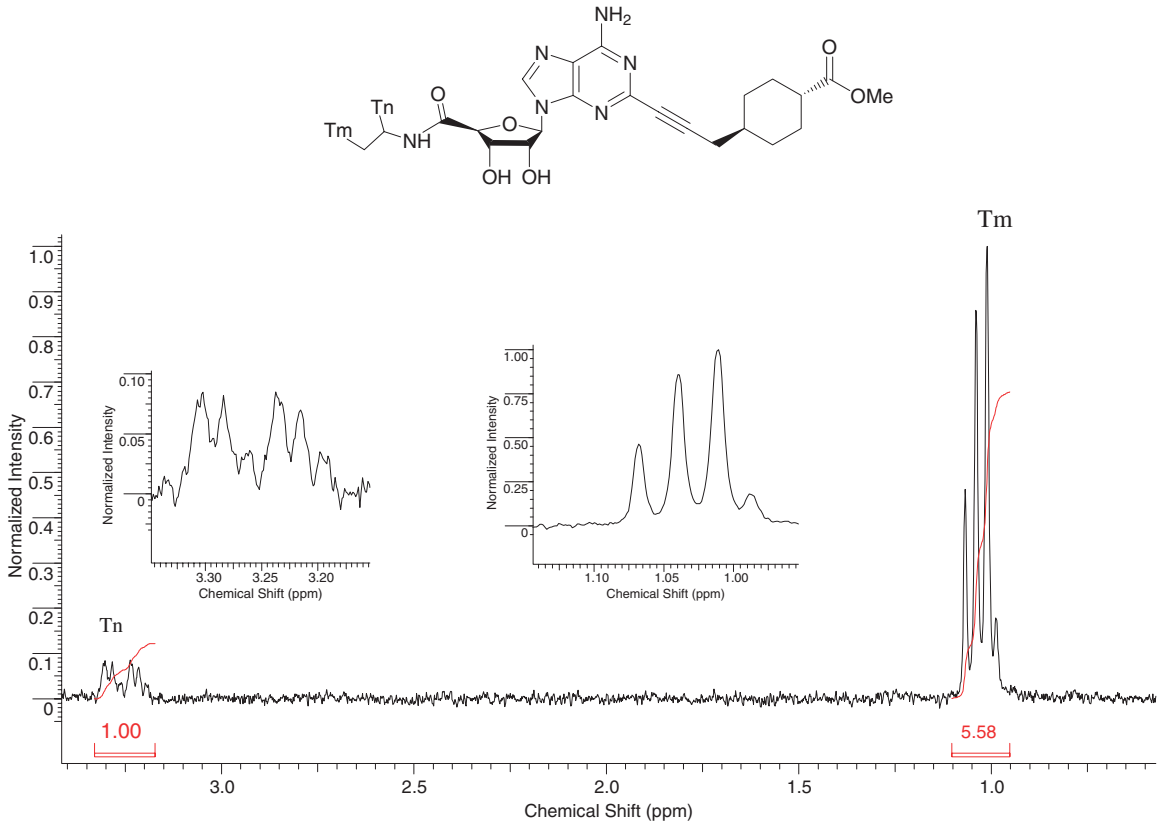


Figure 4. Tritium NMR (T–H decoupled) of [*N*-ethyl-1,2-^{3}H]apadenoson.

syntheses was purchased from IN/US Systems, Inc. (model: TRISORBER®).

[*N*-Ethyl-1,2-^{3}H]phthalimide (3): Compound **2** (3.4 mg, 20 μmol) was dissolved in EtOH (0.7 mL) and 4 mg of 10% Pd/C added. The mixture was then reduced under an atmosphere of 2000 mCi of carrier-free tritium gas using a commercial tritiation manifold at room temperature for 2 h. Labile tritium was removed by distillation and rotary evaporation of the ethanol solution. The [*N*-ethyl-1,2-^{3}H]phthalimide product was dissolved in EtOH (5 mL) and assayed by liquid scintillation counting to give 550 mCi of the tritium-labeled product (99% by analytical HPLC). Tritium distribution of the compound was determined by ^{3}H NMR (320 MHz, chloroform-d, T-H decoupled) δ ppm 1.21–1.29 (methyl, ^{3}H), 3.69–3.75 (methylene, ^{3}H), methyl/methylene ^{3}H ratio, 5:1.

Analytic HPLC method: Column: BDS Hypersil C18, 250 × 4.6 mm, 5 μm; solvent: A, water with 0.1% TFA; B, acetonitrile with 0.1% TFA; flow: 1 ml/min; wavelength 255 nm; T_R = 8.7 min. The column was eluted with 40% acetonitrile in water with 0.1% TFA for 10 min and eluted with acetonitrile with 0.1% TFA afterwards.

[1,2-^{3}H]Ethylamine hydrochloride (4): Compound **3** (400 mCi in 4 ml EtOH) was mixed with 4 ml HCl (6 N). The solution was heated to 105°C and refluxed overnight to achieve complete hydrolysis. The [1,2-^{3}H]ethylamine hydrochloride solution was concentrated by rotary evaporation to 2 mL by rotary evaporation. The solution was then filtered successively through two Waters C18 SepPak cartridges connected in series. The cartridges were rinsed with water (2 × 2 mL) to achieve complete recovery of the radioactivity. The combined aqueous solutions were evaporated to dryness and dissolved in ethanol (5 mL) to give 322.5 mCi of **4** (95% radiochemically pure by HPLC). Tritium distribution of the compound was determined by ^{3}H NMR (320 MHz, d_4-methanol, T–H decoupled) δ ppm 1.17–1.25 (methyl, ^{3}H), 2.93–3.01 (methylene, ^{3}H), methyl/methylene ^{3}H ratio, 5.5:1.

Analytical HPLC conditions: Column: BDS Hypersil C18, 250 × 4.6 mm, 5 μm; solvent: A, water with 0.1% TFA; B, acetonitrile with 0.1% TFA; flow: 1 ml/min; T_R = 4 min; gradient table: 0–2 min 0–5% B; 2–8 min 5% B; 8–12 min 5–100% B, 15 min 100% B.

(2S,3R,4S,5R)-5-(6-amino-2-(3-((1r,4r)-4-(methoxycarbonyl)cyclohexyl)prop-1-ynyl)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-carboxylic acid (10): Compounds **5** (710 mg, 1.75 mmol) and **8** (471 mg, 2.6 mmol) were dissolved in dry DMF (1 mL) and triethylamine (2.5 mL) under nitrogen. The solution was vacuum purged twice with nitrogen flushing followed by the addition of $Pd(Ph_3)_2Cl_2$ (92.3 mg, 0.13 mmol) and CuI (50 mg, 0.262 mmol). The solution was stirred for 1.5 h until **5** was consumed. At this point, the solvents were removed under vacuum and the residual oil run through a short silica plug (HOAc/MeOH/THF 1:10:90) to afford 532 mg of crude amber solid after solvent removal. This was purified by preparative HPLC: Phenomenex Luna C-18 (10 μ, 100 A, 41.2 × 250 mm) A: 0.1% TFA; B: 90% ACN/0.1% TFA; flow rate = 20 mL/min.

Time (min)	0	4	5	20
%B	15	15	30	50

The product-containing fractions were combined and lyophilized to afford product **10** as a white powder (255 mg, 32%). HRMS: calculated $m/z = 482.16462$ $(M+Na)^+$, observed $m/z = 482.16467$ $(M+Na)^+$; ^1H NMR (DMSO-d_6, 300.13 MHz): 1.10 (dddd, 2H, $J = 3.3, 12.8, 12.8, 12.8$ Hz), 1.36 (dddd, 2H, $J = 3.3, 12.8, 12.8, 12.8$ Hz), 1.52 (m, 1H), 1.91 (m, 4H), 2.25 (dddd, 1H, $J = 3.4, 3.4, 12.2, 12.2$ Hz), 2.36 (d, 2H), 3.58 (s, 3H), 4.28 (dd, 1H, $J = 2.5, 4.5$ Hz), 4.41 (d, 1H), 4.46 (dd, 1H, $J = 4.5, 6.5$ Hz), 6.02 (d, 1H), 5.5–6.7 (b, 3H) 7.67 (bs, 2H), 8.50 (s, 1H) ^{13}C NMR: 25.54, 28.22, 30.98, 35.77, 42.05, 51.24, 73.30, 74.06, 81.12, 82.62, 85.65, 86.77, 118.07, 139.87, 144.74, 149.51, 155.10, 171.91, 175.35.

[N-Ethyl-1,2-^{3}H]apadenoson, [(1R,4R)-methyl 4-(3-(6-amino-9-((2R,3S,4R,5S)-5-(1,2-^{3}H ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9H-purin-2-yl)prop-2-ynyl)cyclohexanecarboxylate] (1a): Compound **4** (300 mCi, 6.25 μmol) was dried under vacuum in a 50-mL round-bottomed flask. Compound **10** (6 mg, 13 μmol), (benzotriazol-1-yloxy)tripyrrolidino phosphonium hexafluorophosphate (10 mg, 19.2 μmol), and DMF (5 mL) were added to the reaction flask at room temperature giving a clear solution. Excess DIEA (0.2 mL) was then added and the solution was stirred at room temperature. After 1.5 h HPLC indicated complete consumption of the [^{3}H]ethylamine and formation of compound **1a**. At this stage, the reaction was terminated by addition of water (20 mL). The resulting solution was passed through four Sep-Pak C18 cartridges (1 mL size) connected in series. The cartridges were washed with 10 mL of water first and the compound was eluted afterward with ethanol (4×2 mL). The ethanol fractions were combined and concentrated to a small volume for HPLC purification.

Preparative HPLC conditions: LUNA 5μ C18(2) (10×250); solvent: A, water 1% TEAA; B, acetonitrile; flow: 4.7 mL/min; UV = 254 nm; gradient: 0–35 min 25% B; 35–45 min 25–100% B.

Analytic HPLC condition: Column: BDS Hypersil C18, 250×4.6 mm, 5 μm; solvent: A, water with 0.1% TFA; B, acetonitrile with 0.1% TFA; flow: 1 mL/min; UV = 254 nm; $T_R = 8.7$ min; 30% B isocratic.

Fractions containing pure product were pooled and concentrated by rotary evaporation. The final product was reconstituted in ethanol to afford 170 mCi (56% yield) of **1a** with a radiochemical purity >99%. The specific radioactivity was measured to be 48.07 Ci/mmol by LC-MS and tritium distribution of the compound was determined by ^{3}H NMR (320 MHz, d6-DMSO, T–H decoupled) δ ppm 0.99–1.07 (methyl, ^{3}H), 3.20–3.30 (methylene, ^{3}H), methyl/methylene ^{3}H ratio, 5.58:1.

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