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Radiosynthesis of [^{13}N]dantrolene, a positron emission tomography probe for breast cancer resistant protein, using no-carrier-added [^{13}N]ammonia

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ABSTRACT

Dantrolene (**1**) is a substrate for breast cancer resistant protein, which is widely distributed in the blood–brain-barrier, intestine, gall bladder, and liver. PET study with **1** labeled with a positron emitter can be used to visualize BCRP and to elucidate the effect of BCRP on the pharmacokinetics of drugs. The objective of this study was to label **1** using nitrogen-13 (^{13}N, a positron emitter; half-life: 9.9 min). Using no-carrier-added [^{13}N]NH_3 as the labeling agent, we synthesized [^{13}N]dantrolene ([^{13}N]**1**) for the first time. The reaction of carbomyl chloride **2b** with [^{13}N]NH_3 gave an unsymmetrical urea [^{13}N]**3**, followed by cyclization of [^{13}N]**3** to afford [^{13}N]**1**. Due to its instability, **2b** was prepared in situ by treating amine **5** with triphosgene in a ratio of 4 to 1 and used for subsequent [^{13}N]ammonolysis without purification.

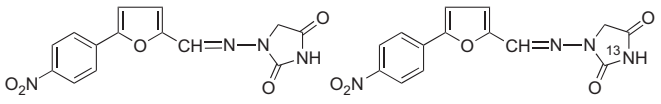
1. Introduction

Dantrolene (**1**, 1-{[5-(4-nitrophenyl)-2-furyl]methylideneamino} imidazolidine-2,4-dione; Scheme 1) is a skeletal muscle relaxant that is currently used for specific and effective treatment of malignant hyperthermia.[1,2] It is also used for the management of neuroleptic malignant syndrome, muscle spasticity (e.g., after strokes, in paraplegia, cerebral palsy, or patients with multiple sclerosis), ecstasy intoxication, serotonin syndrome, and 2,4-dinitrophenol poisoning.[3–6] Recently, it has been shown that **1** is a substrate for breast cancer resistance protein (BCRP).[7] BCRP, one of the ATP-binding cassette (ABC) transporters, was initially isolated from atypical multidrug-resistant MCF-7 human breast cancer cells, and is an efflux transporter with wide substrate specificity recognizing molecules of either negative or positive charge, organic anions, and sulfate conjugates.[8,9] It has been reported that BCRP plays an important role in the absorption (small intestine), distribution (placenta and blood–brain-barrier), and elimination (liver and small intestine) of drugs.[10] BCRP, like other ABC transporters, is assumed to limit the uptake of drugs and other xenobiotic compounds from the blood into the brain.[7,10,11]

Positron emission tomography (PET) is a useful molecular imaging tool using radioactive probes labeled with positron-emitting isotopes, such as ^{11}C, ^{18}F, ^{13}N, and ^{15}O. PET can be used to investigate the pharmacokinetic and pharmacodynamic profiles of drugs in the living body and to elucidate the therapeutic efficacy, side and toxic effects, and potential action mechanisms of drugs.[12–15] The significant effectiveness of PET motivated us to label **1** with a positron emitter and to use this PET probe for imaging BCRP in the blood–brain-barrier for the first time and examining the relationship between BCRP and brain uptake of **1** in rodents and primates.

The aim of this study was to label **1** with ^{13}N (positron emitter, half-life: 9.965 min; 100% β^+, decay) and to synthesize 1-{[5-(4-nitrophenyl)-2-furyl]methylideneamino}-3-[^{13}N]imidazolidine-2,4-dione ([^{13}N]dantrolene, [^{13}N]**1**, Scheme 1). Although **1** has been previously labeled with [^{11}C]$COCl_2$ in our laboratory,[16] a useful ^{13}N-labeled PET ligand has several favorable features. For example, PET imaging using an ^{13}N-ligand can be repeated on the same individual within a shorter period of time and often results in much lower radiation burden on the subject than using an ^{11}C-ligand.

So far, no ^{13}N-labeled PET ligands except [^{13}N]NH_3[17–19] have been developed for clinical use. A main reason is that the labeling technique using ^{13}N and [^{13}N]NH_3 has not been well established.



dantrolene (**1**) [^{13}N]dantrolene ([^{13}N]**1**)

Scheme 1. Chemical structures of dantrolene (**1**) and [^{13}N]dantrolene ([^{13}N]**1**).

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Radiosynthesis with ^{13}N must be accomplished within a short time compatible with its short half-life, using only a limited step sequence. Another characteristic of radiosynthesis involves the efficient production and application of anhydrous [^{13}N]NH_3 with high specific activity as a labeling agent.[20–22] Because of its short half-life and the ease of contamination by nitrogen carrier in air, it was difficult to produce ^{13}N-labeled ligands with high specific activity (>37 GBq/mmol) and enough radioactivity to carry out conventional PET studies.

Ten years ago, Suzuki et al. developed an automated synthesis system for producing anhydrous [^{13}N]NH_3 (>37 GBq/mmol).[23,24] Using this synthesis system, we synthesized [^{13}N]thalidomide[25] and [^{13}N]carbamazepine,[26] two therapeutic drugs. We used small-animal PET to determine the pharmacokinetics in vivo of two PET probes. To widen the usefulness of the labeling technique with high specific activity [^{13}N]NH_3, we selected **1** as a target compound in the present study, with the expectation that [^{13}N]**1** could become a promising PET probe for imaging BCRP. Here, we synthesized [^{13}N]**1** using no-carrier-added [^{13}N]NH_3 equipped with an entirely-automated synthesis system for the first time.

2. Results and discussion

2.1. Synthetic route

Regarding the chemical structure of **1** with a hydantoin ring, we planned to label this compound using [^{13}N]NH_3 as the labeling agent. This approach involves (1) the production of anhydrous [^{13}N]NH_3,[22,23] (2) [^{13}N]ammonolysis of 4-nitrophenylcarbamate **2a** or carbamoyl chloride **2b** with [^{13}N]NH_3, and (3) cyclization of unsymmetrical urea [^{13}N]**3** to construct the [^{13}N]hydantoin ring, as shown in the retro-synthetic route (Scheme 2).

2.2. Production of anhydrous [^{13}N]NH_3

Anhydrous [^{13}N]NH_3 gas used for radiosynthesis was produced by the nuclear ^{16}O (p, α) ^{13}N reaction[22,23] by irradiating a target containing H_2O and EtOH (10 mM). The irradiated solution was quickly passed through a pre-conditioned cation exchange column to concentrate [^{13}N]NH_3 onto this column. The [^{13}N]NH_3 was released with aqueous KOH from the column, dried by flowing through another column filled with CaO, and then trapped into a cooled vial containing various solvents and solutions. The specific activity of anhydrous [^{13}N]NH_3 in the present study was estimated to 37–100 GBq/μmol (n > 20) at the end of [^{13}N]NH_3 production.

2.3. [^{13}N]Ammonolysis

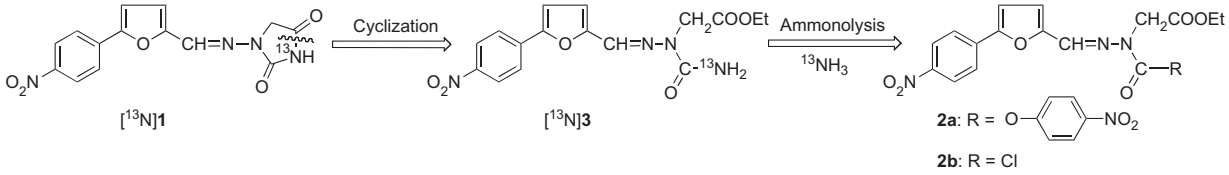
2.3.1. [^{13}N]Ammonolysis of 4-nitrophenylcarbamate 2a with [^{13}N]NH_3

As a substrate for [^{13}N]ammonolysis, 4-nitrophenylcarbamate **2a** was synthesized according to Scheme 3. Condensation of 5-(4-nitrophenyl)-2-furaldehyde (**4**) with ethyl hydrazinoacetate hydrochloride gave a Schiff's base **5** in 69% yield. Reaction of **5** with 4-nitrophenylcarbamoyl chloride at room temperature for 12 h afforded **2a** in a chemical yield of 64%.

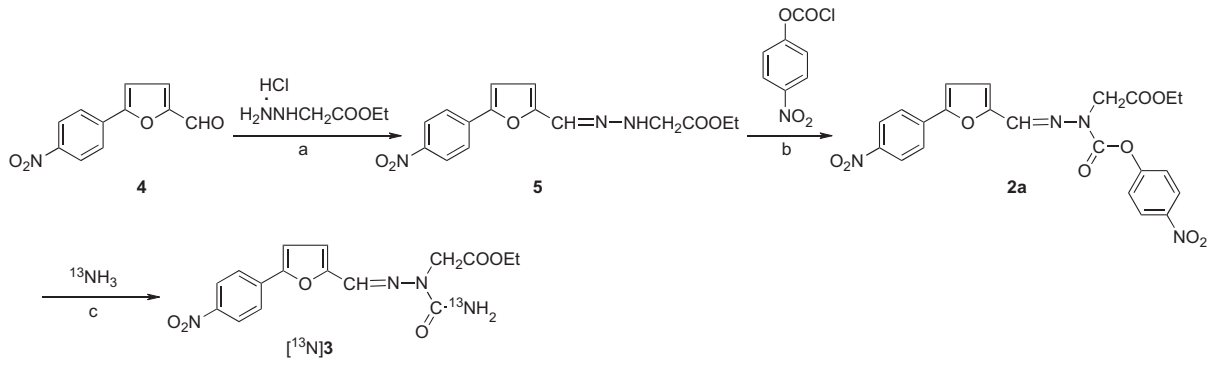
Conditions for the [^{13}N]ammonolysis of **2a** with no-carrier-added [^{13}N]NH_3 were examined. Reactions of **2a** with [^{13}N]NH_3 were performed in anhydrous THF, dichloroethane (DCE), CH_3CN and DMF in the presence of various bases (K_2CO_3, pyridine, Et_3N, DMAP, i-Pr_2NEt and lutidine), respectively. HPLC was used to analyze the incorporation efficiency of [^{13}N]**3** in the reaction mixture. Among all conditions investigated, when i-Pr_2NEt and DCE were used, **5a** reacted with [^{13}N]NH_3 at 75 °C for 3 min to afford [^{13}N]**3** with the highest radioactivity-incorporating yield (decay-corrected) of 60% in the reaction mixture. Thus, DCE and i-Pr_2NEt were used as a solvent and base for the following [^{13}N]ammonolysis in the present study.

2.3.2. One-pot synthesis of [^{13}N]3 via carbamoyl chloride 2b

To achieve higher efficiency of [^{13}N]ammonolysis in a short period of time, the more reactive carbomyl chloride **2b** was used as another substrate in place of **2a** (Table 1). Amine **5** was firstly treated with triphosgene to give **2b** in situ. After excess $COCl_2$ was removed, **2b** was not separated from the reaction mixture due to its

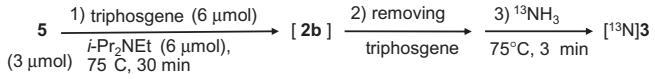


Scheme 2. Retrosynthesis of [^{13}N]**1**.



Scheme 3. Chemical synthesis and [^{13}N]ammonolysis of **2a**. Reagents and conditions: (a) DMF, H_2O, room temperature, 24 h, 69%; (b) Et_3N, CH_2Cl_2, room temperature, 12 h, 64%; (c) base (K_2CO_3, pyridine, Et_3N, DMAP, i-Pr_2NEt or lutidine), solvent (THF, DCE, CH_3CN or DMF), 75 °C, 3 min, 2–60%.

Table 1
One-pot synthesis of [^{13}N]**3** by reacting **5** with triphosgene, followed by [^{13}N]ammonolysis-**1**



[^{13}N]NH_3 (nmol)	Addition of NH_3 (nmol)	Yield* (%)
~1	None	No product
~1	100	34
~1	10,000	75

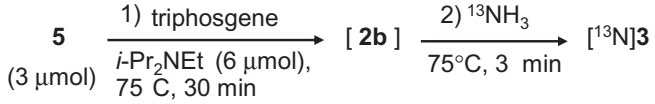
* Radioactivity-incorporating yield (decay-corrected) for [^{13}N]**3** was measured by analytical HPLC. All results are the mean (n = 3) with a maximum range of ±5%.

instability. The reaction mixture containing **2b** was directly used to react with [^{13}N]NH_3 by the one-pot synthetic procedure.

Table 1 shows [^{13}N]ammonolysis efficiency according to the conditions listed. When no-carrier added [^{13}N]NH_3 was used, no [^{13}N]**3** was produced in the reaction mixture. By the addition of 100 nmol NH_3 to the mixture, [^{13}N]**3** was produced in 34% yield. Addition of 10^4 nmol NH_3 increased the [^{13}N]ammonolysis yield to 75%. The difference in results between the addition of NH_3 and non-addition of NH_3 was explained by the reaction scale of PET chemistry. Since the NH_3 carrier contained in the no-carrier-added (cyclotron-produced) [^{13}N]NH_3 for the present radiosynthesis was only about 1 nmol, [^{13}N]NH_3 was easily exhausted by trace $COCl_2$ and HCl, resulting from the acylating process. Although as much unreacted $COCl_2$ as possible was removed before [^{13}N]ammonolysis, trace $COCl_2$ left in the mixture was enough to react with [^{13}N]NH_3, which stopped the subsequent [^{13}N]ammonolysis. Utilization of the carrier improved the reaction efficiency, but the yielded [^{13}N]**3** with too low specific activity was not useful for PET study, especially for brain imaging.

Considering the difference between radiosynthesis in PET chemistry and conventional chemical synthesis, more efforts were made using no-carrier-added [^{13}N]NH_3 to achieve [^{13}N]ammonolysis by the one-pot synthesis of [^{13}N]**3** from **5** (Table 2). Our approach was to control the amount of triphosgene relative to amine **5** to guarantee the complete consumption of $COCl_2$. Table 2 shows the one-pot synthesis results of [^{13}N]**3** according to the reaction conditions listed. When 1 μmol triphosgene (equal to 3 μmol $COCl_2$) was treated with 3 μmol **5**, [^{13}N]**3** was formed but its yield was not reproducible. When 0.75 μmol triphosgene (2.5 μmol $COCl_2$) was used, high and reproducible efficiency of [^{13}N]ammonolysis was achieved. This result suggested that no $COCl_2$ was left in the reaction mixture after acylation of **5**. Further decreasing the amount of triphosgene decreased the yield of [^{13}N]**3**. Thus, the 1–4 ratio of triphosgene to **5** was suitable for achieving high incorporation efficiency of [^{13}N]**3** via **2b** in situ.

Table 2
One-pot synthesis of [^{13}N]**3** by reacting **5** with triphosgene, followed by [^{13}N]ammonolysis-**2**



Triphosgene (mmol)	Yield* (%)
1	0–34
0.75	86
0.3	53

* Radioactivity-incorporating yield (decay-corrected) for [^{13}N]**3** was measured by analytical HPLC. All results are the mean (n = 3) with a maximum range of ±5%.

2.5. Cyclization of [^{13}N]3

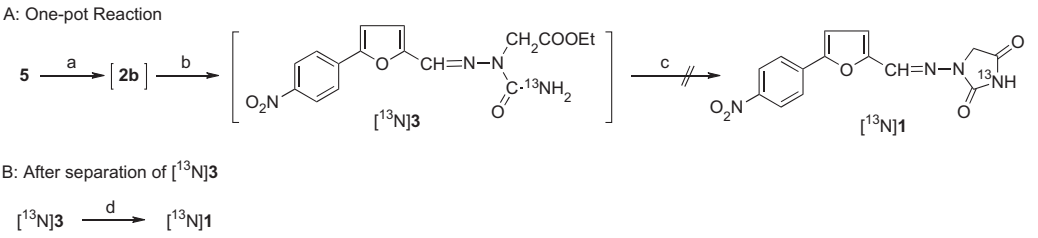
The cyclization of [^{13}N]**3** was attempted without any further purification by the addition of conventional coupling agents, such as 1,1′-carbonyldiimidazole (CDI),[26,27] 1,1′-carbonyl-di-(1,2,4-trizole) diimidazole (CDT),[27] trifluoromethanesulfonic acid anhydride ($(CF_3SO_2)_2O$),[28] or bis(trimethylsilyl)trifluoroacetamide (BSTFA),[29] in a one-pot reaction, followed by heating the reaction mixtures for at least 3 min at 100–120 °C. However, these treatments did not give [^{13}N]**1** presumably due to perturbation of the reaction mixture by excess **5** and i-Pr_2NEt.

Next, [^{13}N]**3** was separated from the reaction mixture and then cyclized (Scheme 4B). Semi-preparative HPLC purification (eluent: CH_3CN/H_2O, 4/6) for the reaction mixture gave a radiochemically pure fraction corresponding to [^{13}N]**3** (Fig. 1A). When the fraction was heated at 95 °C to remove all solvents, we unexpectedly found that [^{13}N]**3** was gradually converted to [^{13}N]**1** during the heating process. As shown in Figure 1, heating [^{13}N]**3** in CH_3CN and H_2O for 1 min and 2 min gave [^{13}N]**1** in 17% (B) and 74% (C) accounting for the total radioactivity in the HPLC fraction, respectively. After heating the radioactive fraction for 3 min, [^{13}N]**3** was efficiently converted to [^{13}N]**1** (Fig. 1D). The efficient cyclization of [^{13}N]**3** in a short period of time pave the way for the rapid synthesis of [^{13}N]**1** using an automated system.

2.6. Automated synthesis of [^{13}N]1

According to the optimized reaction conditions examined above, we synthesized [^{13}N]**1** using an entirely-automated synthesis system[30] (Scheme 5).

Amine **5** (3 μmol) was reacted with triphosgene (0.75 μmol) in DCE in the presence of i-Pr_2NEt to give a mixture containing **2b**. This mixture was immediately set to a reaction vial for the subsequent [^{13}N]ammonolysis. After irradiation, [^{13}N]NH_3 was recovered from the cyclotron target, dried and trapped in the above mixture. The reaction vial was heated at 75 °C for 3 min. Semi-preparative HPLC purification for the [^{13}N]ammonolysis mixture gave a radioactive fraction of [^{13}N]**3**. This fraction was collected and



Scheme 4. Cyclization of [^{13}N]**3**. Reagents and conditions: (a) i-Pr_2NEt, DCE, 75 °C, 30 min (b) [^{13}N]NH_3, DCE, 75 °C, 3 min; (c) condensation agent (CDI, CDT, $(CF_3SO_2)_2O$ or BSTFA), DCE, 100–120 °C, 1–3 min; (d) CH_3CN, H_2O, 95 °C, 1–3 min, 17–100%.

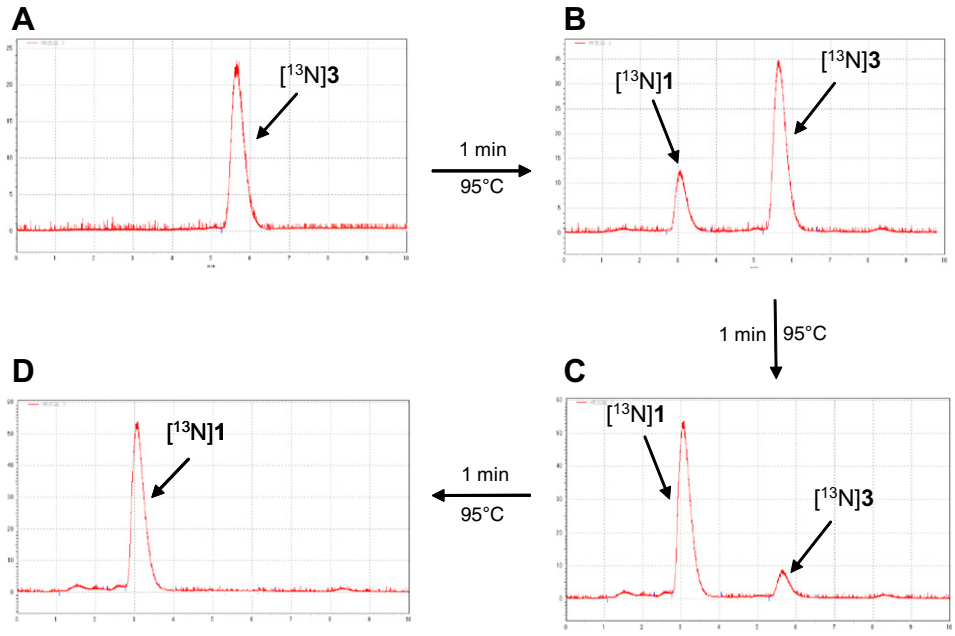
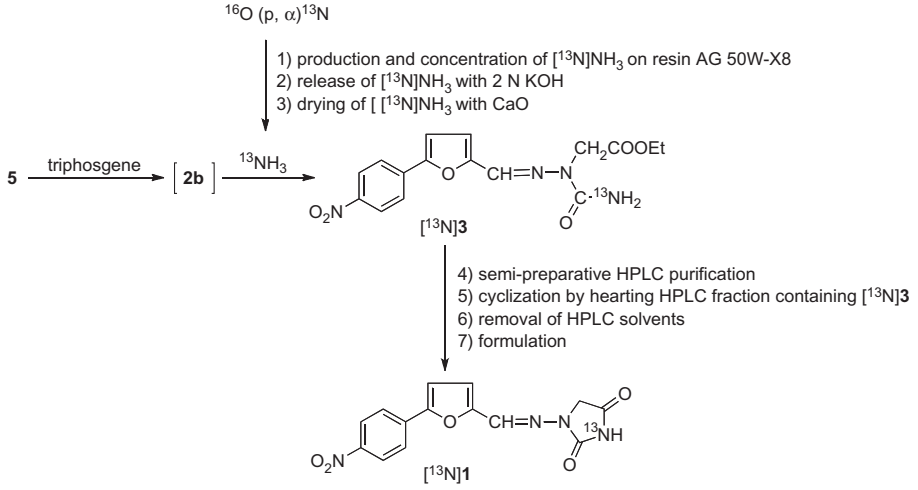


Figure 1. HPLC analytical chromatograms reflecting the cyclization process of [^{13}N]**3** (retention time: 5.5 min) to form [^{13}N]**1** (retention time: 3.1 min) in CH_3CN and H_2O at 95 °C: (a) before heating the HPLC fraction containing [^{13}N]**3**; (b) heating [^{13}N]**3** for 1 min; (c) heating [^{13}N]**3** for 2 min; (d) heating [^{13}N]**3** for 3 min. The analytical conditions were as follows: Shiseido Capcell Pak C_{18} (4.6 mm $\phi \times$ 250 mm), H_2O/CH_3CN (6/4), 1.0 mL/min, 254 nm. The identity of [^{13}N]**3** or [^{13}N]**1** was confirmed by co-injection with the non-radioactive **3** or **1**.



Scheme 5. Automated synthesis of [^{13}N]**1** using no-carrier-added [^{13}N]NH_3.

heated for 3 min at 95 °C in a sealed flask. After all solvents were removed, [^{13}N]**1** was obtained as a formulated product.

Starting from cyclotron-produced [^{13}N]NH_3 with total radioactivity of 6.6–7.5 GBq at a beam current of 15 μA after 25 min proton bombardment, [^{13}N]**1** was obtained with 270–480 MBq (n = 7) as an injectable solution at the end of synthesis. This amount of radioactivity is enough for in vitro and in vivo evaluation by animal experiments. The automated reaction sequence took 17 ± 2 min from the end of bombardment.

Identity, radiochemical purity and specific activity of [^{13}N]**1** were measured using analytical HPLC. Identity was confirmed by co-injecting with non-radioactive **1**. The radiochemical purity of [^{13}N]**1** was higher than 98% and specific activity was about 30 GBq/μmol at the end of synthesis, as determined from the mass measured from the HPLC UV analysis. In the final product solution, no significant peak relative to **5** and its decomposition products was detected by HPLC. Moreover, the radiochemical purity of [^{13}N]**1** remained >95% after maintaining the product at room temperature for at least 40 min, and this product was stable for performing evaluation. These analytical results were in compliance with our in-house quality control/assurance specifications.

3. Conclusions

[^{13}N]**1**, a PET probe for BCRP, was synthesized using no-carrier-added [^{13}N]NH_3 equipped with an automated system for the first time. The entire automated synthesis was reproducible and reliable, giving [^{13}N]**1** in sufficient radiochemical yield and purity for animal experiments. PET studies with [^{13}N]**1** will be used to visualize BCRP in the blood–brain-carrier for the first time and to elucidate the influence of BRCP on rodent brain uptakes of drugs.

4. Experimental section

Melting points were measured using a micro melting point apparatus (MP-500P, Yanaco, Tokyo) and are uncorrected. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a JNM-GX-270 spectrometer (JEOL, Tokyo) with tetramethylsilane as an internal standard. All chemical shifts (d) were reported in parts per million (ppm) downfield from the standard. High resolution (HR) MS (FAB) was obtained on a JEOL NMS-SX102 spectrometer (JEOL). Column chromatography was performed on Merck Kieselgel gel 60 F_{254} (70–230 mesh). Nitrogen-13 (^{13}N) was produced by the ^{15}O (p, α) ^{13}N nuclear reaction using a CYPRIS HM-18 cyclotron (Sumitomo Heavy Industry, Tokyo). Radioactivity was measured with a dose calibrator (IGC-3R Curiemeter, Aloka, Tokyo). HPLC was performed using a JASCO HPLC system (JASCO, Tokyo): effluent radioactivity was monitored using a NaI (Tl) scintillation detector system. Dantrolen (**1**) was purchased from Aldrich–Sigma (Milwaukee, WI). If not otherwise stated, chemicals were purchased from Aldrich–Sigma and Wako Pure Industries (Osaka) with the highest grade commercially available.

4.1. Chemical synthesis

4.1.1. Ethyl 2-{2-[5-(4-nitrophenyl)furfurylidene]hydrazino} acetate (5)

Ethyl hydrazinoacetate hydrochloride (8.8 g, 57 mmol) in water (20 mL) was added to a solution of 5-(4-nitrophenyl)-2-furaldehyde (**4**; 12.4 g, 57 mmol) in DMF (100 mL). This mixture was stirred at room temperature for 24 h. After DMF and H_2O were removed under reduced pressure, the obtained residue was washed with water and extracted with CH_2Cl_2. The organic layer was washed with saturated NaCl, dried over Na_2SO_4 and removed. The crude product was purified by column chromatography on silica gel under *n*-hexane/AcOEt (3:1) as eluent to give **5** (12.5 g, 69%) as a yellow powder; mp: 104–105 °C. ^{1}H NMR (300 MHz, $CDCl_3$) d: 8.23 (2H, d, J = 8.8 Hz), 7.82 (2H, d, J = 8.8 Hz), 7.57 (1H, s), 6.90 (1H, d, J = 3.7 Hz), 6.62 (1H, d, J = 3.7 Hz), 4.24 (2H, q, J = 7.2 Hz), 4.07 (2H, s), 1.31 (3H, t, J = 7.0 Hz). HRMS (FAB) calcd for $C_{15}H_{16}N_3O_5$, 318.1090; found, 318.1053.

4.1.2. 4-Nitrophenyl 1-(2-ethoxy-2-oxoethyl)-2-[(5-(4-nitrophenyl)furan-2-yl)methylene]hydrazinecarboxylate (2a)

4-Nitrophenyl chloroformate (240 mg, 1.2 mmol) was added slowly to a mixture of **5** (317 mg, 1.0 mmol) and Et_3N (0.21 mL, 1.5 mmol) in dry CH_2Cl_2 (3 mL) at 0 °C. The reaction mixture was stirred at room temperature for 12 h. After removal of CH_2Cl_2 and Et_3N under reduced temperature, the crude product was purified by column chromatography on silica gel under hexane/AcOEt (from 4/1 to 3/1) to give **2a** (309 mg, 64%) as an orange powder; mp: 142–144 °C. ^{1}H NMR (300 MHz, $CDCl_3$) d: 8.26–8.33 (4H, m), 7.88 (2H, d, J = 8.8 Hz), 7.60 (1H, s), 7.45 (1H, s), 6.89–6.97 (2H, m), 4.80 (2H, s), 4.32 (2H, q, J = 7.1 Hz), 1.35 (3H, t, J = 7.1 Hz). HRMS (FAB) calcd for $C_{22}H_{19}N_4O_9$; 483.1152; found, 483.1163.

4.1.3. Ethyl 2-{1-carbamoyl-2-[(5-(4-nitrophenyl)furan-2-yl)methylene]hydrazinyl}ethanoate (3)

To a solution of anhydrous ammonia (about 5%) in DMF (10 mL) **2a** (100 mg, 0.2 mmol) was added. The reaction mixture was stirred at room temperature for 12 h. After removal of DMF under reduced pressure, the crude product was purified by column chromatography on silica gel under CH_2Cl_2/CH_3OH (from 0% to 3%) to give **3** (47 mg, 65.2%) as a yellow powder. ^{1}H NMR (DMSO-d_6) d: 8.28 (2H, d, J = 8.8 Hz), 7.84 (2H, d, J = 8.8 Hz), 7.30 (1H, s), 6.95 (1H, d, J = 3.7 Hz), 6.79 (1H, d, J = 3.7 Hz), 4.78 (2H, s), 4.26 (2H, q, J = 7.2 Hz), 1.30 (3H, t, J = 7.1 Hz); HRMS (FAB) calcd for $C_{16}H_{17}N_4O_6$, 361.1148; found, 361.1100.

4.2. Production system of automated synthesis

Radiosynthesis was performed using an automated unit for the quick production of ^{13}N-labeled compounds using anhydrous [^{13}N]NH_3.[22,23,30] This system was designed to minimize the inner volume of the production line and required amount of reagent, which decreased the radioactivity loss in the line to a low level. The system enabled one to carry out all the procedures from production of [^{13}N]NH_3 to formulation of [^{13}N]**1**.

4.3. Production of anhydrous [^{13}N]NH_3 gas

Before irradiation, an aqueous ethanol solution (10 mM) was saturated with pure oxygen gas by bubbling for about 30 min. This solution was loaded into the target chamber. The target was irradiated at 15 μA for 15–25 min with 18 MeV protons (15.7 MeV on target) from the cyclotron. The irradiated solution was quickly passed through the preconditioned column (1 mm $\phi \times 40$ mm) filled with cation exchange resin AG 50W-X8 and [^{13}N]NH_3 in irradiated water was concentrated on this column. Then, [^{13}N]NH_3 was eluted with aqueous KOH (30 μL, 2 N) under He gas flow, desiccated through the small column (3 mm $\phi \times 30$ mm, kept at 150 °C) filled with CaO (250 mg), and flowed into a vial (Pyrex, 5 mL) containing various anhydrous organic solvents (1 mL) cooled at −15°C. The production time of [^{13}N]NH_3 was about 4 min from the end of bombardment.

4.4. [^{13}N]Ammonolysis of 2a using no-carrier-added [^{13}N]NH_3

In a hot cell, a solution of [^{13}N]NH_3 (5–18 MBq) in DCE (100 μL) was added to a reaction vial (Pyrex, 5 mL) containing **2a** (1.45 mg, 3 μmol), anhydrous THF, CH_3CN, DMF or DCE (200 μL), and various bases (K_2CO_3, pyridine, Et_3N, DMAP, *i*-Pr_2NEt or lutidine: 6 μmol). Each reaction mixture was sealed and heated at 75 °C for 3 min in a hot oil bath. The reaction vial was rapidly cooled and the efficiency of [^{13}N]ammonolysis was evaluated for each reaction.

The incorporation yield (decay-corrected) of radioactivity for [^{13}N]**3** was measured by analytical HPLC using the following condition: Shiseido Capcell Pak C_{18} column: 4.6 mm $\phi \times 250$ mm; CH_3CN/H_2O: 4/6; flow rate: 1.0 mL/min; 254 nm; retention time: 5.6 min. The identity of [^{13}N]**3** was confirmed by co-injection with the non-radioactive **3**.

4.5. [^{13}N]Ammonolysis of 2b using no-carrier-added [^{13}N]NH_3

4.5.1. One-pot synthesis by removing excess $COCl_2$

A solution of triphosgene (1.78 mg, 6 μmol) in DCE (100 μL) was added to a reaction vial containing a mixture of **5** (1.08 mg, 3 μmol) and *i*-Pr_2NEt (1 mL, 6 μmol) in DCE (100 μL). The mixture was heated at 75 °C under N_2 for 30 min. After excess $COCl_2$ was removed from the reaction mixture with N_2 flow, an anhydrous [^{13}N]NH_3 solution (30–150 MBq) in DCE (200 μL) was added to the mixture containing **2b**. The incorporation yield (decay-corrected) of radioactivity for [^{13}N]**3** was measured by analytical HPLC. For comparison, NH_3 solution with a designated concentration (1 mM or 100 mM) in DCE (100 μL) was mixed with the no-carrier-added (cyclotron-produced) [^{13}N]NH_3 in advance for performing [^{13}N]ammonolysis.

4.5.2. One-pot synthesis by controlling the amount of triphosgene

A solution of triphosgene (90–270 mg, 0.3–1 μmol) in DCE (100 μL) was added to a reaction vial containing **5** (1.08 mg, 3 μmol) and *i*-Pr_2NEt (1 mL, 6 μmol) in DCE (100 μL). The mixture was heated at 75 °C under N_2 for 30 min. Then, an anhydrous solution of [^{13}N]NH_3 (30–180 MBq) in DCE (200 μL) was added into the

reaction vial. The reaction mixture was continuously heated for 3 min at 75 °C. The radioactivity-incorporating yield (decay-corrected) for [^{13}N]**3** was measured by analytical HPLC.

4.6. Radiosynthesis of [^{13}N]1

A mixture of **5** (1.08 mg, 3 μmol), triphosgene (216 mg, 0.75 μmol) and *i*-Pr_2NEt (1 mL, 6 μmol) in DCE (300 μL) was heated under N_2 at 75 °C for 30 min to give **2b**. This mixture was not separated and was used directly for subsequent [^{13}N]ammonolysis. After irradiation at a beam current of 15 μA for 25 min, anhydrous [^{13}N]NH_3 gas was produced, dried, and trapped in the reaction mixture containing **2b**. This mixture was heated at 75 °C for 3 min. After the reaction, the mixture was diluted with HPLC mobile phase (500 μL), and transferred onto a column (Shiseido Capcell Pak C_{18}: 10 mm ϕ × 250 mm). Elution with CH_3CN/H_2O (4/6) at a flow rate of 4 mL/min gave a radioactive fraction corresponding to pure [^{13}N]**1** (retention time: 4.2 min). This fraction was collected in a flask which was heated at 75 °C for 3 min without evaporation of the HPLC solvents. After the 3-min heating, the solvents were removed at 95 °C under reduced pressure. The residue was dissolved in ethanol (10 μL) and sterile isotonic saline (3 mL), and passed through a 0.22-μm Millipore filter to give the formulated product of [^{13}N]**1**. The total synthesis time was about 17 min from the EOB. At the end of the syntheses, 420 MBq of [^{13}N]**1** was obtained as an iv injectable solution. The radiochemical purity of [^{13}N]**1** was measured by analytical HPLC (Shiseido Capcell Pak C_{18} column: 4.6 mm ϕ × 250 mm; CH_3CN/H_2O: 4/6; flow rate: 1.0 mL/min; retention time: 3.1 min. The identity of [^{13}N]**1** was confirmed by co-injection with the authentic **1**. The specific activity of [^{13}N]**1** was calculated by comparing the assayed radioactivity to the carrier at the UV peak of 254 nm. The amount of carrier was quantified using a calibration curve obtained with 0.5–10.0 mg/mL solutions of the authentic **1**.

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