Radiosynthesis of a carbon-11 labeled tetrahydrobenzisoxazole derivative as a new PET probe for γ-secretase imaging in Alzheimer's disease

Zhidong Xu[a,c], Caihong Miao[c], Fugui Dong[c], Limeng Jia[c], Wei Li[c], Min Wang[b], Wenqing Liu[a,*], Qi-Huang Zheng[b,*]

[a]*College of Chemical & Pharmaceutical Engineering, Key Laboratory of Molecular Chemistry for Medicine of Hebei Province, Hebei University of Science & Technology, Shijiazhuang, Hebei 050018, China*

[b]*Department of Radiology and Imaging Sciences, Indiana University School of Medicine, 1345 West 16th Street, Room 202, Indianapolis, IN 46202, USA*

[c]*Key Laboratory of Medicinal Chemistry and Molecular Diagnosis of Ministry of Education, College of Chemistry and Environmental Science, Hebei University, Baoding, Hebei 071002, China*

***Corresponding authors:**

Wenqing Liu, Ph.D.

College of Chemical & Pharmaceutical Engineering

Key Laboratory of Molecular Chemistry for Medicine of Hebei Province

Hebei University of Science & Technology

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Shijiazhuang, Hebei 050018, China

E-mail: wenq.liu@hebust.edu.cn

Qi-Huang Zheng, Ph.D.

Department of Radiology and Imaging Sciences

Indiana University School of Medicine

1345 West 16th Street, L3-202

Indianapolis, IN 46202, USA

E-mail: qzheng@iupui.edu

Abstract

To develop PET radiotracers for imaging of Alzheimer's disease, a new carbon-11 labeled potent and selective γ-secretase modulator (GSM) has been synthesized. The reference standard tetrahydrobenzisoxazole derivative **8** and its desmethylated precursor **9** were synthesized from cyclohex-2-en-1-one and 3-hydroxy-4-nitrobenzaldehyde in eight and nine steps with 11% and 5% overall chemical yield, respectively. The radiotracer [^{11}C]**8** was prepared from its corresponding precursor **9** with [^{11}C]CH_3OTf through *O*-^{11}C-methylation and isolated by RP-HPLC combined with SPE in 45-50% radiochemical yield, based on [^{11}C]CO_2 and decay corrected to EOB. The radiochemical purity was >99%, and the molar activity (A_m) at EOB was 555-740 GBq/μmol.

Keywords: γ-Secretase; Carbon-11 labeled γ-secretase modulator (GSM); Radiosynthesis; Positron emission tomography (PET); Alzheimer's disease (AD).

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is recognized by the World Health Organization (WHO) as a global public health priority (Maia and Sousa, 2019). Although the precise pathological mechanism of AD remains unclear, it is commonly accepted that AD is characterized by the formation of two different insoluble protein aggregates, β-amyloid (Aβ) plaques and phosphorylated tau-induced neurofibrillary tangles, which are named amyloid hypothesis and tau hypothesis (Crump et al., 2013; Mach, 2014). The first AD

pathological protein Aβ is closely linked to the second AD pathological protein tau. Furthermore, Aβ has been shown to drive tau pathology *in vivo* (Xia, 2019). Aβ peptides, the major constituents of amyloid plaques, are considered to be involved in the degeneration and loss of neurons as well as the onset of AD (Zhao et al., 2017). Aβ peptide is produced by the cleavage of a larger transmembrane-spanning protein called amyloid precursor protein (APP) *via* cleaving enzymes. There are two pathways for cleaving APP into smaller fragments: amyloidogenic and nonamyloidogenic pathways (Mach, 2014). The nonamyloidogenic pathway is the cleavage by the enzyme α-secretase, resulting in the formation of a soluble Aβ peptide. The amyloidogenic pathway is a two-step sequential cleavage, first by the enzyme β-secretase (also known as BACE-1, beta-site APP-cleaving enzyme) then by γ-secretase, producing the most amyloidogenic and neurotoxic $A\beta_{42}$, which is the most prone to aggregation, forming aggregates of insoluble fibrils in the brain (Goldstein et al., 2007). Based on amyloid hypothesis, the significant research efforts have been devoted to discover effective and safety anti-amyloid agents as disease modifying agents for AD (MacLeod et al., 2015), and the APP cleaving enzymes BACE-1 and γ-secretase have become attractive therapeutic targets for AD (Maia and Sousa, 2019). Many anti-amyloid agents including BACE-1 inhibitors, γ-secretase inhibitors (GSIs) and modulators (GSMs) have been identified in the AD pipeline (Kumar et al., 2018; Prati et al., 2017; Tam et al., 2019; Wolfe, 2012).

Consequently BACE-1 and γ-secretase have become interesting imaging targets in AD, as the development of imaging agents parallels the drug development process (Agdeppa and Spilker, 2009; Schmidt et al., 2005). Advanced biomedical imaging technique positron emission tomography (PET) is a promising modality for AD, and significant advances have occurred in

this field of molecular imaging (Frisoni et al., 2017; Johnson et al., 2012). There is a growing interest in design and evaluation of new PET radiotracers for *in vivo* imaging of BACE-1, and several BACE-1 radiotracers have been developed (Kawai et al., 2013; Lang et al., 2012; Nordeman et al., 2014; Takano et al., 2019; Zhang et al., 2018), and representative carbon-11 and fluorine-18 labeled BACE-1 inhibitors including [^{11}C]Me-NCFB, [^{11}C]BSI-IV and [^{18}F]PF06684511 are shown in Figure 1. In this project of new radiotracer development for AD, we focus on γ-secretase as an imaging target. However, so far no development of carbon-11 or fluorine-18 labeled GSIs and/or GSMs has been reported. Unfortunately, the development of GSIs for the treatment of AD has shown several side effects, especially Notch-related adverse effects as a major obstacle, and thus, the drug discovery efforts have shifted to the development of GSMs (Zhang et al., 2014; Zhao et al., 2017). Recently a new series of tetrahydrobenzisoxazoles as GSMs with nanomolar potency for potential treatment of AD has been developed, and the lead compound, an enantiomer of 3-(3-methoxy-4-(4-methyl-1*H*-imidazol-1-yl)phenyl)-5,6-dihydrobenzo[*d*]isoxazol-7(4*H*)-one (**8**), exhibited excellent potency (IC_{50} 39 nM for $A\beta_{42}$) and selectivity ($A\beta_{Total}/A\beta_{42}$ = 499) (Zhao et al., 2017). This GSM has the combination of favorable *in vitro* activity to $A\beta_{42}$, and *O*-methyl position amenable to labeling with carbon-11, therefore, its carbon-11 labeled radioligand is expected to have high specific binding. Here, we report the design, synthesis and labeling of a carbon-11 labeled GSM 3-(3-[^{11}C]methoxy-4-(4-methyl-1*H*-imidazol-1-yl)phenyl)-5,6-dihydrobenzo[*d*]isoxazol-7(4*H*)-one ([^{11}C]**8**) (Figure 1) as a new potential PET probe for imaging of γ-secretase in AD, for the first time.

Insert Figure 1 about here

2. Results and discussion

2.1. Chemistry

Synthesis of the reference standard **8** and its corresponding desmethylated precursor 5-(7-((4-fluorophenyl)amino)-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-yl)-2-(4-methyl-1*H*-imidazol-1-yl)phenol (**9**) is depicted in Scheme 1, according to the reported procedures (Zhao et al., 2017) with modifications. The bromination of commercially available cyclohex-2-en-1-one in the presence of potassium peroxymonosulfate (OXONE) and HBr (48%) gave an important intermediate **1** in 73% yield (Kim and Park, 2004; Kobayahsi et al., 2014). Compound **2** was prepared through an *O*-methylation from the commercially available 3-hydroxy-4-nitrobenzaldehyde by reacting with CH_3I in the presence of K_2CO_3 in 90% yield. Compound **3** was converted from **2** *via* an oximation reaction in 80% yield. To prepare the isoxazol **4**, a one-pot [3+2] cycloaddition was used for the reaction of **3** with *N*-chlorosuccinimide (NCS), followed by **1** in the presence of $NaHCO_3$ in 64% yield. The nitro group of **4** was reduced by $SnCl_2$/HCl to generate amine **5** in 74% yield. Then we modified the reported synthetic approach for **8** (Zhao et al., 2017). Compound **6** was prepared by an azidation of **5**, followed by a nucleophilic substitution with KI in 61% yield (Coleman et al., 2000; Muppidi et al., 2014). Imidization of **6** by reacting with 4-fluoroaniline in the presence of $Ti(OPr\text{-}i)_4$, followed by a reduction in the presence of $NaBH_4$ generated compound **7** with 84% yield. The standard **8** was prepared from **7** through a CuI with (1*R*,2*R*)-N^1,N^2-dimethylcyclohexane-1,2-diamine catalyzed

N-arylation in 83% yield (Antilla et al., 2004). The overall chemical yield of **8** was improved through the modification of the published synthetic method, which shortened the reaction steps. Compound **8** is a chiral compound. The method for resolution of the enantiomers by a chiral OD column with a mixed solvent of 2-propanol and hexanes was mentioned in the literature (Zhao et al., 2017), but the experimental details were not provided, the absolute configuration (*R*/*S* system) was not assigned, and the enantiomeric purities were not reported. Due to a preparative chiral OD column not available in this laboratory, compound **8** was not further resolved to two enantiomers. In addition, the resolution of a chiral compound does not affect its radiosynthesis, because a *R*- or *S*-enantiomer or a racemic mixture undergoes the same radiosynthetic pathway, ^{11}C-methylation. The desmethylated precursor **9** was obtained by the reaction of **8** with BBr_3 in CH_2Cl_2 in 44% yield.

Insert Scheme 1 about here

2.2. Radiochemistry

Synthesis of the radiotracer [^{11}C]**8** is shown in Scheme 2. The precursor **9** underwent *O*-^{11}C-methylation (Gao et al., 2018; Wang et al., 2018) using the reactive ^{11}C-methylating agent [^{11}C]methyl triflate ([^{11}C]CH_3OTf) (Jewett, 1992; Mock et al., 1999) in acetonitrile at 80 °C under basic conditions (2 N NaOH). The product was isolated by semi-preparative reversed-phase (RP) high performance liquid chromatography (HPLC) with a C-18 column, and then concentrated by solid-phase extraction (SPE) (Wang et al., 2011, 2012a) with a disposable C-18

Light Sep-Pak cartridge to produce the corresponding pure radiolabeled compound [^{11}C]**8** in 45-50% radiochemical yield, decay corrected to end of bombardment (EOB), based on [^{11}C]CO_2.

Insert Scheme 2 about here

The radiosynthesis was performed in a home-built automated multi-purpose ^{11}C-radiosynthesis module (Mock et al., 2005a, b; Wang et al., 2012b). Our radiosynthesis module facilitated the overall design of the reaction, purification and reformulation capabilities in a fashion suitable for adaptation to preparation of human doses. The radiosynthesis includes three stages: 1) labeling reaction; 2) purification; and 3) formulation. The overall synthesis time was 35-40 min from EOB. Our module is also designed to allow in-process measurement of ^{11}C-tracer molar activity (A_m, GBq/μmol at EOB) using semi-preparative RP-HPLC (Mock et al., 2005a). At the end of synthesis (EOS), the A_m of ^{11}C-tracer was determined again by analytical RP-HPLC, calculated, decay corrected to EOB, and based on [^{11}C]CO_2. Both semi-preparative and analytical RP-HPLC methods gave similar A_m values. The A_m of [^{11}C]**8** at EOB was in a range of 555-740 GBq/μmol. The general method to increase the A_m of ^{11}C-tracer produced in our radiochemistry facility has been detailed in our previous work (Gao et al., 2018).

Chemical purity and radiochemical purity were determined by analytical HPLC (Zheng and Mock, 2005). The chemical purity of the precursor and reference standard was >95%. A representative analytical RP-HPLC chromatographic profile for the radiotracer [^{11}C]**8** and co-injection of [^{11}C]**8** with the reference standard **8**, Radio-HPLC (**A**) and UV-HPLC (**B**) traces for [^{11}C]**8**; Spike-Radio-HPLC (**C**) and Spike-UV-HPLC (**D**) traces for [^{11}C]**8** with **8**, is shown in

Figure 2. The radiochemical purity of [^{11}C]**8** was >99% determined by radio-HPLC through γ-ray (PIN diode) flow detector as indicated in Figure 2, **A** or **C**. The chemical purity of [^{11}C]**8** was simultaneously determined by UV-HPLC through UV flow detector as indicated in Figure 2, **B** or **D**. The minor impurities included its corresponding labeling precursor **9** and a few unknown UV peaks from the EtOH-saline used in tracer formulation after HPLC-SPE purification. However, there is no chemical purity of the radiotracer release limit in PET tracer production, because the radiosynthesis is a micro-scale synthesis, and the radiotracer prepared is very trace amount.

Insert Figure 2 about here

3. Experimental

3.1. General

All commercial reagents and solvents were purchased from Sigma-Aldrich and Fisher Scientific, and used without further purification. [^{11}C]CH_3OTf was prepared according to a literature procedure (Mock et al., 1999). Melting points were determined on WRR apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II 600 MHz or BUXI-I NMR Fourier transform spectrometer at 400, 600 or 100 MHz, respectively. Chemical shifts (δ) are reported in parts per million (ppm) relative to an internal standard tetramethylsilane (TMS, δ 0.0) (^{1}H NMR) and to the solvent signal (^{13}C NMR), and coupling constants (J) are reported in

hertz (Hz). Liquid chromatography-mass spectra (LC-MS) analysis was performed on AB Sciex 4000Q Trap instrument, consisting of an 1100 series HPLC connected to a diode array detector and a 1946D mass spectrometer configured for positive-ion/negative-ion electrospray ionization (ESI). The high resolution mass spectra (HRMS) were obtained using a Waters/Micromass LCT Classic spectrometer. Chromatographic solvent proportions are indicated as volume : volume ratio. Thin-layer chromatography (TLC) was run using HS silica gel GF254 uniplates (5 × 10 cm^2). Plates were visualized under UV light. Normal phase flash column chromatography was carried out on Combiflash Rf 150 silica gel 60 (300-400 mesh) with a forced flow of the indicated solvent system in the proportions described below. The purity of the synthesized compounds was confirmed by RP-HPLC using an Agilent Extend 5 TC-C18 column (4.6 × 250 mm, 5 μm) on a Thermo UltiMate 3000 HPLC system. All moisture- and air-sensitive reactions were performed under a positive pressure of nitrogen maintained by a direct line from a nitrogen source. Analytical RP HPLC was performed using a Prodigy (Phenomenex) 5 μm C-18 column, 4.6 × 250 mm, mobile phase 65% CH_3CN/35% 4.0 mM CH_3COONa, flow rate 1.0 mL/min; UV (254 nm) and γ-ray (PIN diode) flow detectors. Semi-preparative RP HPLC was performed using a Prodigy (Phenomenex) 5 μm C-18 column, 10 × 250 mm; 70% CH_3CN:30% H_2O mobile phase; 6 mL/min flow rate; UV (254 nm) and γ-ray (PIN diode) flow detectors. C18 Light Sep-Pak cartridges were obtained from Waters Corporation (Milford, MA). Sterile Millex-FG 0.2 μm filter units were obtained from Millipore Corporation (Bedford, MA).

*3.2. 2-Bromocyclohex-2-en-1-one (**1**)*

To a solution of cyclohex-2-en-1-one (0.48 mL, 5.0 mmol) and potassium peroxymonosulfate (OXONE, 3.7 g, 6.0 mmol) in CH_2Cl_2 (20 mL), aqueous solution of HBr (48%, 5.5 mL) was added slowly at room temperature (RT) under stirring. After the reaction was continued at RT for 2 h, 4 mL of triethylamine was added, and then the reaction mixture was stirred for another 2 h. The reaction was quenched with 2 N HCl (20 mL), and the resulted reaction mixture was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layer was washed with 1 N NaOH, water, brine, dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under vacuum. The crude product was purified by silica gel column chromatography with petroleum ether (PE)/EtOAc (100:1 to 10:1) as eluent to afford **1** as a white solid (0.64 g, 73%), mp 72.3-73.0 ºC. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.43 (t, J = 4.8 Hz, 1H), 2.64 (t, J = 6.6 Hz, 2H), 2.48-2.45 (m, 2H), 2.10-2.06 (m, 2H). HRMS (ESI, *m/z*): calcd for C_6H_7BrONa ($[M+Na]^+$) 196.9572, found 196.9570.

*3.3. 3-Methoxy-4-nitrobenzaldehyde (**2**)*

To a mixture of 3-hydroxy-4-nitrobenzaldehyde (5 g, 29.9 mmol) and K_2CO_3 (3.7 g, 6.0 mmol) in DMF (25 mL), CH_3I (2.3 mL, 38.9 mmol) was added slowly at RT under N_2. After the reaction was stirred at RT for 10 h, the reaction mixture was poured into ice water (30 mL), and the resulted mixture was extracted with EtOAc (3 × 100 mL). The combined organic layer was washed with water, brine, dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under vacuum to afford **2** as a pale yellow solid (4.9 g, 90%), which was used without further purification, mp 97.7-98.2 ºC. ^{1}H NMR (600 MHz, $CDCl_3$): δ 10.06 (s, 1H), 7.93 (d, J = 8.4 Hz, 1H), 7.61 (s, 1H), 7.55 (d, J = 7.8 Hz, 1H), 4.04 (s, 3H).

*3.4. (E)-3-Methoxy-4-nitrobenzaldehyde oxime (**3**)*

To a mixture of **2** (5.0 g, 27.6 mmol) in anhydrous ethanol (30 mL), $NH_2OH{\cdot}HCl$ (2.9 g, 41.4 mmol) was added slowly at RT under N_2. After the reaction was stirred at RT for 8 h, the reaction mixture was poured into ice water (50 mL), and the resulted mixture was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layer was washed with water, brine, dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under vacuum. The resulted crude product was purified by silica gel column chromatography with PE/EtOAc (10:1 to 1:1) as eluent to afford **3** as a pale yellow solid (4.3 g, 80%), mp 146.8-147.3 ºC. ^{1}H NMR (600 MHz, DMSO-d6): δ 11.76 (s, 1H), 8.24 (s, 1H), 7.92 (d, J = 8.4 Hz, 1H), 7.53 (s, 1H), 7.34 (d, J = 8.4 Hz, 1H), 3.95 (s, 3H). HRMS (ESI, *m/z*): calcd for $C_8H_8N_2O_4Na$ ($[M+Na]^+$) 219.0376, found 219.0374.

*3.5. 3-(3-Methoxy-4-nitrophenyl)-5,6-dihydrobenzo[d]isoxazol-7(4H)-one (**4**)*

To a solution of **3** (1.5 g, 7.5 mmol) in DMF (30 mL), NCS (1.2 g, 9.0 mmol) was added slowly at 0 ºC under N_2. After the reaction mixture was stirred at 0 ºC for 15 min, and then at RT for 2 h, **1** (2.0 g, 11.2 mmol) and $NaHCO_3$ (1.0 g, 15.0 mmol) were added. The reaction was continued at RT for 12 h. 30 mL of cold water was added to the reaction mixture, and the resulted solution was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layer was washed with water, brine, dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under vacuum. The resulted crude product was purified by silica gel column chromatography with PE/EtOAc (10:1 to 1:1) as eluent to afford **4** as a pale yellow solid (1.38 g, 64%), mp 161.4-162.0 ºC. ^{1}H NMR

(600 MHz, $CDCl_3$): δ 7.96 (d, J = 8.4 Hz, 1H), 7.59 (s, 1H), 7.33 (d, J = 8.4 Hz, 1H), 4.05 (s, 3H), 3.00 (t, J = 6.0 Hz, 2H), 2.74 (t, J = 6.6 Hz, 2H), 2.34-2.30 (m, 2H). LC-MS (ESI, *m/z*): calcd for $C_{14}H_{13}N_2O_5$ ($[M+H]^+$) 289.08, found 289.10.

*3.6. 3-(4-amino-3-methoxyphenyl)-5,6-dihydrobenzo[d]isoxazol-7(4H)-one (**5**)*

To a solution of **4** (1.2 g, 4.1 mmol) in anhydrous ethanol (20 mL), $SnCl_2$ (3.9 g, 20.0 mmol) and 1 N HCl (5 mL) were added at RT, and then the reaction mixture was heated to 55 ºC and stirred for 2 h. After the reaction mixture was cooled to RT, 100 mL of CH_2Cl_2 was added, and the pH was adjusted to 10 by adding 1 N NaOH. After the precipitate was removed by filtration, the filtrate was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layer was washed with water, brine, dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under vacuum. The resulted crude product was purified by silica gel column chromatography with PE/EtOAc (10:1 to 1:4) as eluent to afford **5** as a yellow solid (0.78 g, 74%), mp 172.5-173.1 ºC. ^{1}H NMR (600 MHz, DMSO-d6): δ 7.18 (s, 1H), 7.13 (d, J = 7.8 Hz, 1H), 6.75 (d, J = 7.8 Hz, 1H), 5.30 (s, 2H), 3.84 (s, 3H), 2.94 (t, J = 6.0 Hz, 2H), 2.64 (t, J = 6.6 Hz, 2H), 2.18-2.14 (m, 2H). LC-MS (ESI, *m/z*): calcd for $C_{14}H_{15}N_2O_3$ ($[M+H]^+$) 259.11, found 259.10.

*3.7. 3-(4-Iodo-3-methoxyphenyl)-5,6-dihydrobenzo[d]isoxazol-7(4H)-one (**6**)*

Solution A: To a solution of **5** (0.5 g, 1.9 mmol) in 10 N HCl (5 mL), $NaNO_2$ (0.15 g, 2.2 mmol) was added at 0 ºC, and then stirred for 1 h. To a solution of KI (0.45 g, 2.7 mmol) in water (5 mL), Solution A was added slowly at 0 ºC in 30 min, and then the mixture was warmed up to RT

and continued stirring for 12 h. After 20 mL of water was added, the reaction mixture was extracted with EtOAc (3 × 50 mL). The combined organic layer was washed with water, brine, dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under vacuum. The resulted crude product was purified by silica gel column chromatography with PE/EtOAc (10:1 to 4:1) as eluent to afford **6** as a white solid (0.43 g, 61%), mp 163.3-164.5 ºC. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (d, J = 8.0 Hz, 1H), 7.28 (s, 1H), 6.98 (d, J = 8.0 Hz, 1H), 3.95 (s, 3H), 2.95 (t, J = 6.0 Hz, 2H), 2.71 (t, J = 6.4 Hz, 2H), 2.31-2.24 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.21, 161.09, 160.29, 158.73, 140.02, 129.66, 127.75, 121.20, 109.55, 88.91, 56.57, 38.56, 24.31, 21.63. HRMS (ESI, *m/z*): calcd for $C_{14}H_{14}INO_3$ ($[M+H]^+$) 369.9935, found 369.9932.

3.8. N-(4-Fluorophenyl)-3-(4-iodo-3-methoxyphenyl)-4,5,6,7-tetrahydrobenzo[d]isoxazol-7-amine (7)

To a solution of **6** (0.5 g, 1.4 mmol) and 4-fluoroaniline (0.46 g, 4.1 mmol) in DMF (5 mL), $Ti(OPr\text{-}i)_4$ (0.43g, 1.5 mmol) was added at RT, and then the mixture was warmed up to 60 ºC, and continued stirring for 6 h. After the reaction mixture was cooled to 0 ºC, ethanol (5 mL) and $NaBH_4$ (0.08 g, 2.1 mmol) were added, and the reaction was continued at 0 ºC for 1 h. After the solid was remove by filtration, the filtrate was extracted with EtOAc (5 × 30 mL). The combined organic layer was washed with water, brine, dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under vacuum. The resulted residue was purified by silica gel column chromatography with PE/EtOAc (20:1 to 5:1) as eluent to afford **7** as a white solid (0.53 g, 84%), mp 59.1-61.5 ºC. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.86 (d, J = 5.2 Hz, 1H), 7.27 (d, J = 1.2 Hz, 1H), 7.00 (dd, J = 5.2, 1.2 Hz, 1H), 6.95-6.92 (m, 2H), 6.71-6.69 (m, 2H), 6.67 (d, J = 3.6 Hz,

1H), 3.93 (s, 3H), 3.81-3.80 (m, 1H), 2.70-2.62 (m, 2H), 2.17-2.12 (m, 1H), 2.00-1.87 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.67, 159.53, 158.51, 155.18, 142.85, 139.79, 131.00, 121.23, 115.95, 115.73, 114.70, 114.63, 113.13, 109.55, 87.96, 56.45, 47.67, 29.49, 21.26, 20.21. LC-MS (ESI, *m/z*): calcd for $C_{20}H_{19}FIN_2O_2$ ($[M+H]^+$) 465.05, found 465.06.

*3.9. N-(4-Fluorophenyl)-3-(3-methoxy-4-(4-methyl-1H-imidazol-1-yl)phenyl)-4,5,6,7-tetrahydrobenzo[d]isoxazol-7-amine (**8**)*

To a mixture of CuI (0.18 g, 0.9 mmol), Cs_2CO_3 (0.63 g, 1.9 mmol) and 4-methyl-1*H*-imidazole (0.08 g, 0.9 mmol) in anhydrous DMF (2 mL), **7** (0.5 g, 1.1 mmol) and (1*R*,2*R*)-N^1,N^2-dimethylcyclohexane-1,2-diamine (0.13 g, 0.9 mmol) were added at RT. The reaction mixture was purged with Ar_2, and then heated to 90 ºC and continued stirring for 24 h under Ar_2. After the reaction mixture was cooled to RT, H_2O (10 mL) was added, and the resulted solution was extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with water, brine, dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under vacuum. The resulted residue was purified by silica gel column chromatography with PE/EtOAc (100:1 to 20:1) as eluent to afford **8** as a white solid (0.38 g, 83%), mp 210.3-211.3 ºC. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.08 (s, 1H), 7.56 (s, 1H), 7.40-7.35 (m, 2H), 7.01-6.97 (m, 3H), 6.78-6.75 (m, 2H), 4.74 (d, J = 4.8 Hz, 1H), 3.96 (s, 3H), 3.90-3.89 (m, 1H), 2.79-2.70 (m, 2H), 2,36 (s, 3H), 2.20-2.18 (m, 1H), 2.07-1.94 (m, 3H). LC-MS (ESI, *m/z*): calcd for $C_{24}H_{24}FN_4O_2$ ($[M+H]^+$) 419.19, found 419.10.

*3.10. 5-(7-((4-Fluorophenyl)amino)-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-yl)-2-(4-methyl-1H-imidazol-1-yl)phenol (**9**)*

To a solution of **8** (0.35 g, 0.84 mmol) in CH_2Cl_2 (15 mL), BBr_3 (0.65 mL) was added slowly at 0 ºC, and the reaction was continued at 0 ºC for 20 h. The reaction mixture was poured into ice water (20 mL), and then the CH_2Cl_2 was removed under reduced pressure. The resulted aqueous solution was extracted with EtOAc (3 × 40 mL), and the combined organic layer was washed with water, brine, dried over anhydrous Na_2SO_4 and filtered. The organic solvent was evaporated under vacuum, and the crude product was purified by silica gel column chromatography with CH_2Cl_2/MeOH (100:1 to 15:1) as eluent to afford **9** as a white solid (0.15 g, 44%), mp 251.1-253.2 ºC. ^{1}H NMR (400 MHz, DMSO-d6): δ 10.53 (s, 1H), 7.93 (s, 1H), 7.51-7.49 (m, 2H), 7.30 (dd, J = 8.0, 1.6 Hz, 1H), 7.27 (s, 1H), 7.03-6.98 (m, 2H), 6.82-6.78 (m, 2H), 6.03 (d, J = 8.8 Hz, 1H), 4.83-4.79 (m, 1H), 2.73-2.63 (m, 2H), 2.20 (s, 3H), 2.12-1.85 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d6): δ 170.11, 159.09, 156.18, 153.88, 150.74, 144.81, 137.09, 129.09, 126.50, 125.75, 118.85, 116.67, 115.89, 115.81, 115.67, 114.09, 114.02, 112.96, 46.37, 29.21, 21.18, 20.34, 14.03. HRMS (ESI, *m/z*): calcd for $C_{23}H_{23}FN_4O_2$ ($[M+H]^+$) 405.1721, found 405.1716.

*3.11. 3-(3-[^{11}C]Methoxy-4-(4-methyl-1H-imidazol-1-yl)phenyl)-5,6-dihydrobenzo[d]isoxazol-7(4H)-one ([^{11}C]**8**)*

[^{11}C]CO_2 was produced by the ^{14}N(p,α)^{11}C nuclear reaction in the small volume (9.5 cm^3) aluminum gas target provided with the Siemens RDS-111 Eclipse cyclotron. The target gas consisted of 1% oxygen in nitrogen purchased as a specialty gas from Praxair, Indianapolis, IN.

Typical irradiations used for the development were 58 μA beam current and 15 min on target. The production run produced approximately 25.9 GBq of [^{11}C]CO_2 at EOB. Desmethylated precursor **9** (0.1-0.3 mg) was dissolved in CH_3CN (300 μL). To this solution was added aqueous NaOH (2 N, 2 μL). The mixture was transferred to a small reaction vial. No-carrier-added (high molar activity) [^{11}C]CH_3OTf that was produced by the gas-phase production method (Mock et al., 1999) within 12 min from [^{11}C]CO_2 through [^{11}C]CH_4 and [^{11}C]CH_3Br with silver triflate (AgOTf) column was passed into the reaction vial at RT until radioactivity reached a maximum (2 min), and then the reaction vial was isolated and heated at 80 °C for 3 min. The contents of the reaction vial were diluted with aqueous $NaHCO_3$ (0.1 M, 1 mL). The reaction vial was connected to a 3-mL HPLC injection loop. The labeled product mixture solution was injected onto the semi-preparative HPLC column for purification. The product fraction was collected in a recovery vial containing 30 mL water. The diluted tracer solution was then passed through a C-18 Sep-Pak Light cartridge, and washed with water (3 × 10 mL). The cartridge was eluted with EtOH (3 × 0.4 mL) to release the labeled product, followed by saline (10-11 mL). The eluted product was then sterile-filtered through a Millex-FG 0.2 μm membrane into a sterile vial. Total radioactivity (2.99-3.95 GBq) was assayed and total volume (10-11 mL) was noted for tracer dose dispensing. The overall synthesis time including HPLC-SPE purification and reformulation was 35-40 min from EOB. The decay corrected radiochemical yield was 45-50%. Retention times (t_R) in the analytical RP-HPLC system were: t_R **9** = 3.75 min, t_R **8** = 7.74 min, and t_R [^{11}C]**8** = 7.85 min. Retention times in the semi-preparative RP-HPLC system were: t_R **9** = 4.02 min, t_R **8** = 7.40 min, and t_R [^{11}C]**8** = 7.56 min.

4. Conclusion

In summary, multiple step synthetic route with moderate to high yields has been developed to produce the precursor **9**, the reference standard **8**, and the target PET radiotracer [^{11}C]**8.** The radiosynthesis employed [^{11}C]CH_3OTf for *O*-[^{11}C]methylation at the phenyl hydroxyl position of the precursor, followed by product purification and isolation by a semi-preparative RP-HPLC combined with SPE. [^{11}C]**8** was obtained in high radiochemical yield, and high radiochemical purity, with a reasonably short overall synthesis time, and high molar activity. As the first attempt to develop molecular imaging probe for γ-secretase, a new carbon-11 labeled potent and selective GSM has been successfully radiosynthesized. This will facilitate studies to evaluate [^{11}C]**8** as a new potential PET agent for imaging of γ-secretase in AD.

Conflict of interest statement

The authors declare that they have no conflict of interest relevant to this article.

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Figure and Scheme Legends

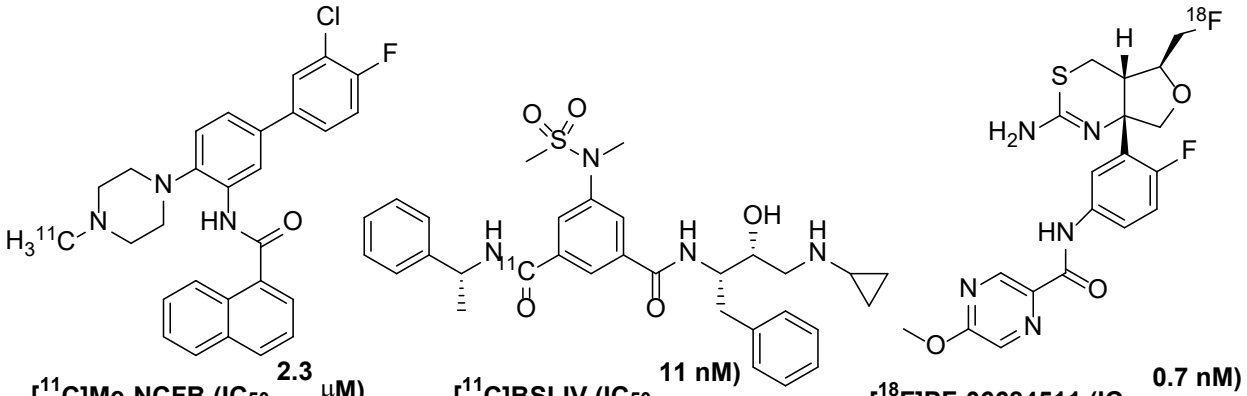
Figure 1. PET radiotracers for BACE-1 and γ-secretase.

Figure 2. A representative analytical RP-HPLC chromatographic profile for the radiotracer [^{11}C]**8** and co-injection of [^{11}C]**8** with the reference standard **8**, Radio-HPLC (**A**) and UV-HPLC (**B**) traces for [^{11}C]**8**; Spike-Radio-HPLC (**C**) and Spike-UV-HPLC (**D**) traces for [^{11}C]**8** with **8**. Analytical RP-HPLC conditions were a Prodigy (Phenomenex) 5 μm C-18 column, 4.6 × 250 mm; mobile phase 65% CH_3CN/35% 4.0 mM CH_3COONa; flow rate 1.0 mL/min; UV (254 nm) and γ-ray (PIN diode) flow detectors.

Scheme 1. Synthesis of the reference standard **8** and precursor **9**. Conditions: (i) potassium peroxymonosulfate (OXONE), HBr (48%), CH_2Cl_2, room temperature (RT); (ii) CH_3I, K_2CO_3, DMF, RT; (iii) $NH_2OH{\cdot}HCl$, ethanol, RT; (iv) *N*-chlorosuccinimide (NCS), **1**, $NaHCO_3$, DMF, RT; (v) $SnCl_2$, 1 N HCl, ethanol, 55 ºC; (vi) (a) $NaNO_2$, 10 N HCl, H_2O, 0 ºC, (b) KI, H_2O, RT; (vii) (a) 4-fluoroaniline, Ti(OPr-i)$_4$, DMF, 60 ºC, (b) $NaBH_4$, ethanol, 0 ºC; (viii) 4-methyl-1*H*-imidazole, CuI, Cs_2CO_3, (1*R*,2*R*)-N^1,N^2-dimethylcyclohexane-1,2-diamine, DMF, 90 ºC; (ix) BBr_3, CH_2Cl_2, 0 ºC.

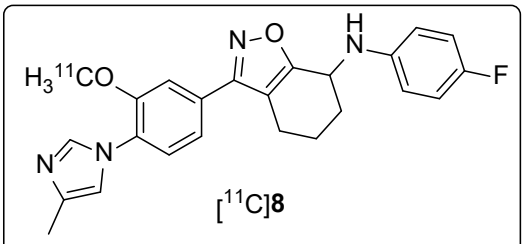
Scheme 2. Synthesis of the radiotracer [^{11}C]**8**. Conditions: (i) (a) [^{11}C]CH_3OTf, CH_3CN, 2 N NaOH, 80 ºC, 3 min, (b) HPLC-SPE.

Figure 1.



[^{11}C]Me-NCFB (IC$_{50}$ 2.3 μM) **[^{11}C]BSI-IV (IC$_{50}$ 11 nM)** **[^{18}F]PF-06684511 (IC$_{50}$ 0.7 nM)**

Radiolabled BACE-1 inhibitors

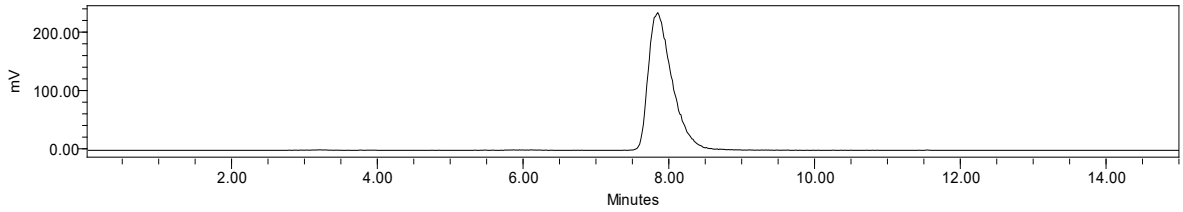


[^{11}C]**8**

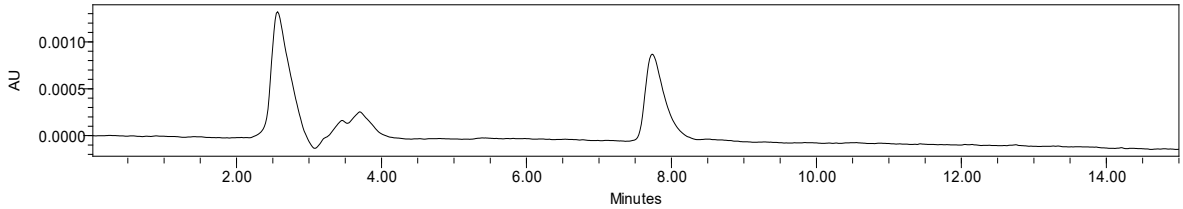
Carbon-11 labeled GSM

Figure 2.

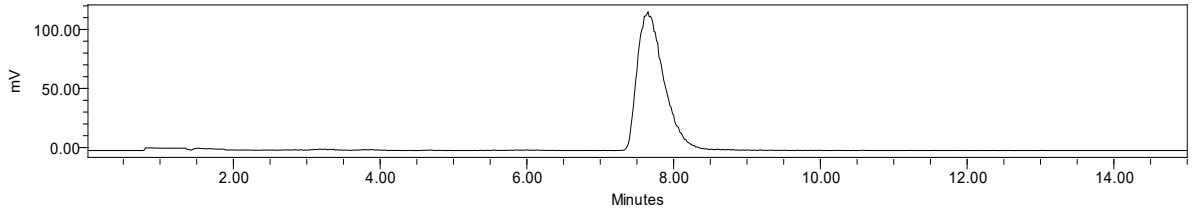
A. Analytical radioactive HPLC trace for [^{11}C]**8**, Retention time (t_R) = 7.85 min



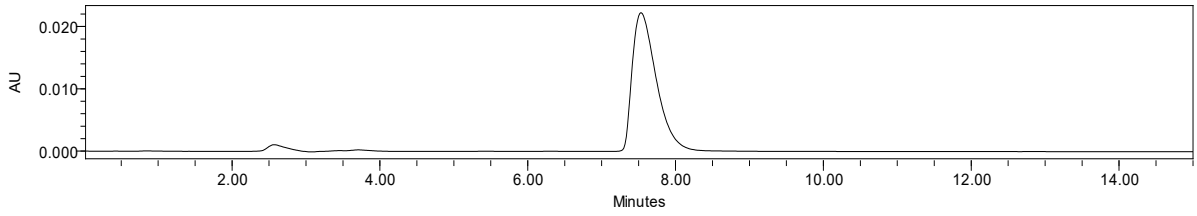
B. Analytical UV HPLC trace for [^{11}C]**8**, t_R = 7.74 min



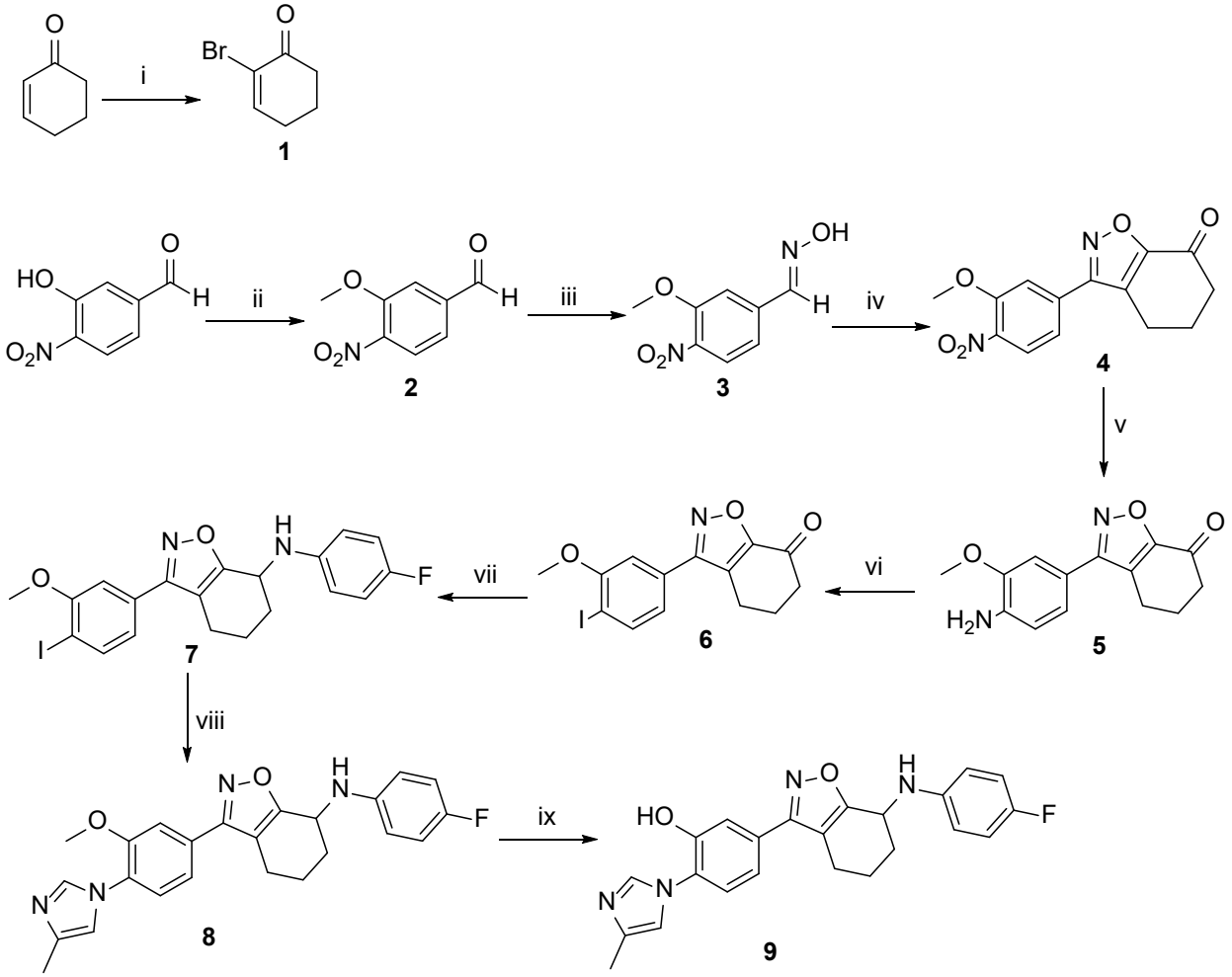
C. Analytical radioactive HPLC trace for [^{11}C]**8** with **8**, t_R = 7.65 min



D. Analytical UV HPLC trace for [^{11}C]**8** with **8**, t_R = 7.53 min



Scheme 1.



Scheme 2.