Radiosynthesis of Carbon-11 Labeled Puromycin as a Potential PET Candidate for Imaging Protein Synthesis *in Vivo*

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Supporting Information

ABSTRACT: In order to address the limitations associated with the present range of PET radiotracers used for imaging protein synthesis *in vivo* we have synthesized a candidate PET radiotracer based on Puromycin (3, PURO), a protein synthesis inhibitor. The desmethylPURO 9 precursor for radiolabeling with carbon-11 radioisotope was synthesized in two steps employing EDC/HOBt amide coupling in overall 76% yield. Optimal conditions for radiolabeling were then established via methylation/deprotection sequence. Under these conditions as determined by NMR analysis 9 showed partial stability (ca. 80%) under acidic conditions. Limited evidence of stereochemical stability of 3 was also found. The radiolabeling of intermediate [^{11}C]12 was accomplished with up to 57% conversion from [^{11}C]iodomethane. An automated method was then developed for high radioactivity radiosynthesis to produce [^{11}C]3 ([^{11}C]PURO) in $16 \pm 6\%$ ($n = 3$) decay corrected radiochemical yields.

KEYWORDS: Positron emission tomography, puromycin, rate of protein synthesis

The rate of protein synthesis (RPS), which represents one of the hallmarks of cancer, is a target for imaging with positron emission tomography (PET). To image RPS *in vivo*, protein building blocks, amino acids, for example, radiolabeled with PET radionuclides (e.g., fluorine-18; $t_{1/2} = 109.8$ min or carbon-11; $t_{1/2} = 20.4$ min) have been employed extensively.¹

To date, the most commonly used amino acid for PET imaging is [^{11}C]methionine ([^{11}C]1, [^{11}C]MET, Chart 1).^{1,2} The radiolabeling of [^{11}C]1 is straightforward, and [^{11}C]1 is successful in crossing the blood–brain barrier (BBB). The highest accumulation of [^{11}C]1 was found in the organs with the highest rate of protein synthesis *in vivo*: brain, liver, and pancreas.³ Although PET imaging with radiolabeled amino acids is routinely used, quantification of the PET data is complicated due to amino acid involvement in other metabolic processes (e.g., amino acid active transport).¹ In 1993, a study compared three radiolabeled amino acids: [^3H]1, [^{18}F]tyrosine ([^{18}F]TYR, structure not shown), and [^{14}C]leucine ([^{14}C]2, [^{14}C]LEU, Chart 1)⁴ for their ability to image protein synthesis *in vivo*. The authors found that the rate of protein synthesis was reduced in brain and pancreas only with [^{14}C]2 under blocking conditions using the protein synthesis inhibitor cyclohexamide in excess amounts. Subsequently, [^{11}C]2 emerged as a superior PET radiotracer for imaging protein synthesis *in vivo*;^{5–7} however, it is not without limitations. Amino acid [^{11}C]2 is radiolabeled with the short-lived carbon-11, and the radiosynthesis is lengthy and challenging. This is primarily because it requires carbon-11 HCN, which is not readily available from the cyclotron.^{8,9} In addition, the radiosynthesis requires chiral

Chart 1. (A) Structures of Most Commonly Employed Amino Acids for PET Imaging of Protein Synthesis and (B) Structures of Puromycin Derivatives and Aminoacyl-tRNA

prep-HPLC separation to obtain the L-form from the crude mixture. Furthermore, quantitative PET analysis requires arterial blood sampling coupled with metabolite analysis,

Received: March 2, 2016

Accepted: April 12, 2016

Published: April 12, 2016

which involves trapping and measuring of gaseous [^{11}C]CO $_2$ radiometabolite.¹⁰

In order to overcome difficulties associated with the PET analysis using amino acid based radiotracers, our group sought to explore a potential PET radiotracer based on the Puromycin (3, PURO, Chart 1) scaffold.¹¹ Amide 3 is an aminonucleoside antibiotic from *Streptomyces alboniger* and a protein synthesis inhibitor. As such, 3 exhibits inhibitory activity via the covalent attachment to the nascent protein chains.^{12–14} Eukaryotic ribosome can then insert 3 (PURO) in place of aminoacyl-tRNA (5, Chart 1) causing premature termination of translation, resulting in truncated proteins. The concept of imaging protein synthesis *in vivo* using protein synthesis inhibitor has been employed previously.¹⁵ In 2012, the Salic group¹⁶ employed 4, (O-PropagylPURO, Chart 1) and fluorescence imaging was accomplished via the [2 + 3] cycloaddition between 4 and fluorophore tagged azide. The authors were successful in establishing that protein synthesis was indeed inhibited as quantified by fluorescence measurement in cultured cells as well as *ex vivo*.

To assess this concept for imaging RPS *in vivo* by PET we selected [^{11}C]3 (O-carbon-11 labeled 3 (PURO), Chart 1) as a potential radiotracer candidate. In analogy to fluorescence imaging with 4,¹⁶ the concept of a PET PURO radiotracer would be based on accumulation of radioactivity from [^{11}C]3 within the ribosome, which can then be visualized and quantified by PET. However, several challenges were considered in the design and synthesis of [^{11}C]3 arising from the chemical scaffold of 3 having the following features: five stereocenters, three reactive exchangeable functional groups (two hydroxyl and one amine), and one labile amide bond. Herein, we report the synthesis of a respective precursor for the radiosynthesis of [^{11}C]3, NMR experimental evidence for its chemical and stereochemical stability as well as carbon-11 modular radiosynthesis of [^{11}C]3.

The required desmethylPURO radiolabeling precursor phenol 9 (Scheme 1) also has an advantage of being a

Scheme 1. Concise Synthesis of Common Intermediate/Precursor 9 for Derivatization of 3 and Radiosynthesis of [^{11}C]3

common intermediate for further derivatization of 3.^{17,18} In their synthesis of 4, Salic and co-workers used commercially available puromycin aminonucleoside (6, Scheme 1) and utilized a disuccinimidyl (DSC) ester coupling (not shown) to form the critical amide bond in the framework of 3.¹⁶ In the synthesis of 9, tyrosine 8 was initially used to form the

corresponding DSC ester 11. However, with the more reactive free phenol functionality in the molecule, only a mixture of inseparable products was obtained. Faced with the complications with the DSC ester coupling method, we revisited the synthesis of the 3 (PURO) manifold^{17,18} and developed a two-step synthesis of 9 as depicted in Scheme 1. The two hydroxyl groups in 6 were protected with TBS to facilitate the subsequent amide coupling by avoiding side reactions observed when unprotected 6 was employed. Only a modest 30% yield of 9 was reached when unprotected 6 was employed for the coupling directly. Similarly, protected precursor 9 is less likely to undergo side reactions during radiolabeling. In the second step, TBS-protected aminonucleoside 7 was coupled to commercially available Boc-protected tyrosine 8 using HOBt/EDC coupling conditions¹⁹ to afford 9 in 86% yield.

With 9 in hand, we next explored conditions for methylation of the phenolic functionality in 9 (Table 1), which could then

Table 1. Conditions for the Nonradioactive methylation of DesmethylPURO 9

Entry	Base	Temp. (°C)	Time (h)	NMR conversion (%)
1	aq. NaOH	70	0.3	99 ^a
2	aq. NaOH	90	0.1	84
3	K ₂ CO ₃	RT	20	33
4	K ₂ CO ₃	70	1	73
5	aq. K ₂ CO ₃	70	0.3	82
6	aq. K ₂ CO ₃ ^b	70	1	18
7	NaH	RT	20	mix
8	aq. TBAOH	70	0.3	mix

^aVery low mass recovery (ca. 50%). ^bReaction done in MeCN as solvent.

be translated to the radiosynthesis. Several bases were investigated (entries 1–8), with NaOH (entries 1 and 2) and K₂CO₃ (entries 3–6) showing good conversions to product. However, with NaOH at 70 and 90 °C, low mass recovery (50%) was found or the conversion to product was lower, respectively (entries 1 vs 2). To avoid the possibility of racemization with NaOH, further optimization of conditions was conducted with K₂CO₃ and showed higher yield with higher reaction temperature but shorter reaction time (entry 3 vs 4 and 5). The yield was significantly reduced with MeCN as solvent (entry 6) and interestingly an aqueous solution of K₂CO₃ yielded 82% of 12 (entry 5). A homogeneous reaction mixture was particularly well suited for automation of radiolabeling procedure.

In order to establish optimal reaction conditions for removal of TBS and Boc protecting groups in one step, acidic hydrolysis of 12 was investigated. Complete decomposition, particularly seen through cleavage of the amide bond, was observed when 12 was treated with 2 M aq. HCl for 1 h at either 100 or 80 °C. Similarly, 12 decomposed upon treatment with 1.25 M methanolic HCl at 60 °C over 1 h as well as at ambient

temperature over 30 min. Initial failed attempts to achieve complete deprotection of **12** prompted us to investigate acidic hydrolysis in an NMR experiment using a DCl/D₂O/MeOD-*d*₄ mixture with **12**. Successful monitoring of the reaction progress was dependent on the solubility of **12**, which dissolved only partly in DCl/D₂O for which reason MeOD-*d*₄ was added to ensure solubility was complete. The mixture was heated to 70 °C and NMR analysis performed at intervals indicated in Table 2. An unidentified byproduct was observed and accounted for

Table 2. Time-Dependent Experiments for the One-Step Deprotection to Obtain 3 (NMR Monitored)

Entry	Time (min)	SM:product:byproduct
1	10	62:42:10
2	20	10:19:16
3	30	0:10:14
4	60	0:10:22

in molar ratios with starting material (SM), product, and byproduct. SM was consumed within 30 min (entries 1–3) and the amount of byproduct increased with time (entries 1–4). After 10 min (entry 1) there was 37% of product and 9% of byproduct, and this ratio changed to 42% product and 35% byproduct after 20 min (entry 2). With time more SM was consumed, but the relative amount of desired product did not improve. For this reason 10 min was set as the optimal reaction time. Although the NMR data showed no change in chemical shifts for the desired product once formed (see Supporting Information), actual ratios of SM/product/byproduct suggested partial instability of **3** (entry 3 vs 4).

Both TBS and Boc protecting groups were removed in a single step with aqueous hydrochloric acid; however, further NMR experiment was required to confirm the stereochemical identity and determine stability of formed product **3**.

Both commercially available **3** and fully protected intermediate **12** were treated with 2 M aq. DCl in DMF-*d*₇ at 80 °C for 10 min and then neutralized with aq. NaOD and subjected to analysis by NMR (Table 3). These reaction conditions were selected considering two requirements for the choice of solvent. Methylation step was performed in DMF (**9** to **12**, Table 1), and the deprotection under radiochemical conditions was foreseen without previous removal of DMF from the reaction mixture. Also, suggested instability of **3** (Table 2) could likely be due to the presence of protic solvent (MeOD-*d*₄). Analysis of NMR spectral data revealed 82% intact sample after the treatment of **3** with acid. Gratifyingly, under the same hydrolysis conditions **12** afforded 77% desired product identical to commercially available **3** when comparing corresponding chemical shifts. The NMR data also indicated presence of byproduct in 18% and 23% from **3** and **12**, respectively (Table 3). This strongly suggests that although the removal of TBS and Boc protecting groups yielded **3**, under acidic conditions **3** was partly chemically unstable. Due to minimally different chemical shifts among epimers of **3**¹⁸ stereochemical identity of

Table 3. Comparison of Commercially Available 3 and Synthesized Radiolabelling Intermediate 12 in One-Step Hydrolysis (NMR Monitored)

SM	R ₁	R ₂	PURO (3) (%)	byproduct (%)
3	H	H	82	18
12	TBS	Boc	77	23

formed **3** could not be unambiguously determined by NMR despite good chemical shift agreement between synthesized **3** and that commercially sourced. However, a doubling of peaks would be expected should epimerization occur, and this was not observed.

Next, we explored methylation/deprotection sequence under radiochemical (i.e., *hot*) conditions. For this purpose, aq. K₂CO₃ was used, [¹¹C]iodomethane produced from cyclotron-generated [¹¹C]CO₂ was trapped, in DMF and this solution was added to radiolabeling precursor **9**. To reduce the reaction time, reaction temperature was increased to 70, 75, or 80 °C (Table 4). Aliquots were analyzed by HPLC after 5 and 10 min

Table 4. Radiochemistry of 9 (DesmethylPURO) To Obtain [¹¹C]3

Entry	Temp. (°C)	Time (min)	Conversion (%)
1	70	5	40
2	75	5	40
3	80	5	47
4	70	10	57
5	75	10	44
6	80	10	20

and the results revealed no significant difference among three temperatures at 5 min (entries 1–3). Longer reaction time was proven beneficial (entries 1 vs 4, 2 vs 5), unless temperature was 80 °C in which case decomposition of both radiolabeled precursor and formed product was predominant (entries 3 vs 6). The deprotection step was performed under acidic conditions previously established with nonradioactive **12** (i.e., *cold*) to afford [¹¹C]**3** as confirmed by HPLC coinjection with commercially available **3**.

For initial proof of principle, development method based on [¹¹C]CH₃I trapping was sufficient to demonstrate that [¹¹C]**3** can be prepared; however, automated radiosynthesis was

required to enable production of $[^{11}\text{C}]\mathbf{3}$ on larger scale. We selected to use iPHASE module²⁰ with an attached semiprep HPLC. The iPHASE module contains a small volume (1 mL) reactor for which reason the radiosynthesis was modified as follows; i) the volumes of acid and base were reduced while increasing their concentration (4 M vs previously used 2 M HCl and NaOH); ii) the reaction temperature was kept at 75 °C for both methylation and deprotection; and iii) the reaction time was reduced to 5 min for each step. Before injection onto semiprep HPLC the crude reaction mixture was neutralized with aq. NaOH to ensure pH was >7. Within 70 min from the end-of-bombardment (EOB) ca. 350 MBq of $[^{11}\text{C}]\mathbf{3}$ was isolated with 66 GBq/ μmol specific activity in $16 \pm 6\%$ ($n = 3$) decay corrected radiochemical yield and >99% radiochemical purity at end-of-synthesis (EOS).

In conclusion, we have prepared **9** (desmethylPURO precursor) in two steps from commercially available materials in 76% overall yield improving the published synthesis of similar PURO derivatives. NMR analysis of **3** and fully protected radiolabeling intermediate **12** showed partial (ca. 80%) chemical stability of **3** and limited evidence provided support for the lack of epimerization. Optimal conditions were found for nonradioactive $[^{12}\text{C}]$ - and radioactive $[^{11}\text{C}]$ -methylation/deprotection sequence to produce $[^{11}\text{C}]\mathbf{3}$ using $[^{11}\text{C}]$ iodomethane. An automated method was then developed to enable preparation of $[^{11}\text{C}]\mathbf{3}$ in $16 \pm 6\%$ ($n = 3$) decay corrected radiochemical yield with high radiochemical purity (>99%) and specific activity of 66 GBq/ μmol at EOS. The radiosynthesis of $[^{11}\text{C}]\mathbf{3}$ included a two-step radiolabeling followed by semipreparative HPLC purification and C18 Light Sep-Pak trapping, and $[^{11}\text{C}]\mathbf{3}$ was produced within 70 min from EOB. *In vitro* and *in vivo* studies are ongoing in our group to evaluate $[^{11}\text{C}]\mathbf{3}$ as a potential PET radiotracer for imaging protein synthesis and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.6b00093.

Full experimental procedures and characterization of all compounds, results of NMR experiments, and radio and UV traces for $[^{11}\text{C}]\mathbf{3}$ (PDF)

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge Dr. Lindsay McMurray (WBIC) and Mr. Robert Bielik (CRUK) for technical assistance with the production of $[^{11}\text{C}]\text{CH}_3\text{I}$ and working with iPHASE as well as many useful discussions. Dr. Mark A. Sephton (Discovery from Charles River, UK) is acknowledged for proofreading the manuscript. NMSF (EPSRC) at the University of Swansea is acknowledged for running HRMS data.

■ ABBREVIATIONS

$[^{11}\text{C}]\text{MET}$, carbon-11 methionine; PET, positron emission tomography; $[^3\text{H}]\text{MET}$, tritiated methionine; $[^{18}\text{F}]\text{TYR}$, fluorine-18 tyrosine; $[^{14}\text{C}]\text{LEU}$, carbon-14 leucine; $[^{11}\text{C}]\text{LEU}$, carbon-11 leucine; PURO, puromycin; tRNA, transcription ribonucleic acid; NMR, nuclear magnetic resonance; DSC, disuccinimydil; TBS, *tert*-butyldimethylsilyl; DMF, *N,N'*-dimethylformamide; Boc, *tert*-butoxycarbonyl; HOBt, hydroxybenzotriazole; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide; HPLC, high pressure liquid chromatography; HCN, hydrogen cyanide; EOB, end-of-bombardment; EOS, end-of-synthesis; RPS, rate of protein synthesis

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