Convergent synthesis of $^{13}$N-labelled Peptidic structures using aqueous $[^{13}\text{N}]\text{NH}_3$

Julia E. Blower, Samuel F. Cousin and Antony D. Gee*

Abstract

Background: Nitrogen-13 has a 10-min half-life which places time constraints on the complexity of viable synthetic methods for its incorporation into PET imaging agents. In exploring ways to overcome this limitation, we have used the Ugi reaction to develop a rapid one-pot method for radiolabelling peptidic molecules using $[^{13}\text{N}]\text{NH}_3$ as a synthetic precursor.

Methods: Carrier-added $[^{13}\text{N}]\text{NH}_3$ (50 $\mu$L) was added to a solution of carboxylic acid, aldehyde, and isocyanide in 2,2,2-TFE (200 $\mu$L). The mixture was heated in a microwave synthesizer at 120 °C for 10 min. Reactions were analysed by radio-HPLC and radio-LCMS. Isolation of the target $^{13}$N–labelled peptidic Ugi compound was achieved via semi-preparative radio-HPLC as demonstrated for Ugi1.

Results: Radio-HPLC analysis of each reaction confirmed the formation of radioactive products co-eluting with their respective reference standards with radiochemical yields of the crude products ranging from 11% to 23%. Two cyclic $\gamma$-lactam structures were also achieved via intra-molecular reactions. Additional radioactive by-products observed in the radio-chromatogram were identified as $^{13}$N–labelled di-imines formed from the reaction of $[^{13}\text{N}]\text{NH}_3$ with two isocyanide molecules. The desired $^{13}$N–labelled Ugi product was isolated using semi-preparative HPLC.

Conclusion: We have developed a one-pot method that opens up new routes to radiolabel complex, peptidic molecules with $^{13}$N using aqueous $[^{13}\text{N}]\text{NH}_3$ as a synthetic precursor.

Keywords: PET chemistry, Nitrogen-13, $[^{13}\text{N}]$ammonia, Multicomponent reaction, Peptide, Ugi

Background

Nitrogen-13 ($t_{1/2} = 9.97$ min; 100% $\beta^+$ decay) is used clinically in the field of positron emission tomography (PET). Compared to other cyclotron-produced positron emitters such as $^{18}$F and $^{11}$C, $^{13}$N has been largely overlooked as a viable option for radiolabelling as its short half-life poses challenges to the development of synthetic methods. Despite its limited use, $^{14}$N offers many favourable attributes that may be complementary to conventional $^{11}$C and $^{18}$F labelling: the ubiquity of nitrogen in endogenous molecules and pharmaceuticals allows direct labelling of molecules of interest without interfering with their biological activity, enabling study of authentic biogenic molecules, and the short half-life offers the ability to repeat PET scans on the same individual within a short time and without excessive radiation dose to the patient. Cyclotron-produced $^{15}$N is most...
commonly generated by the proton bombardment of $^{16}$O$\text{H}_2\text{O}$ via the nuclear reaction $^{16}$O(p, $\alpha$)$^{13}$N to produce $^{13}$N$\text{NH}_3$ (Wieland et al., 1991), which is the only routinely-used $^{13}$N radiopharmaceutical (for myocardial perfusion imaging) but can also be a potentially useful radiolabelling precursor (Kumata et al., 2010; Kumata et al., 2012; Gomez-Vallejo et al., 2014).

Multi-component reactions are powerful tools in the efficient and rapid generation of diverse libraries of compounds, and are particularly useful for high-throughput screening. The four-component Ugi reaction combines a carboxylic acid, an amine (sometimes ammonia), an aldehyde or ketone, and an isocyanide to create $\alpha$-aminoacyl amide derivatives - peptidomimetic structures with proven pharmaceutical application (Domling & Ugi, 2000). Notably, the one-pot synthesis of the local anaesthetic Xylocaine and the subsequent development and marketing of at least 12 other anaesthetics is based on this scaffold (Hulme & Gore, 2003). The Ugi reaction is therefore an attractive method for the rapid radiolabelling of peptides with short-lived PET radionuclides, useful in diagnosis, drug discovery and as a research tool in understanding molecular mechanisms in vivo. Here we report the radiolabelling of $\alpha$-aminoacyl amide derivatives with $^{13}$N, via the four-component coupling (4-CC) Ugi synthesis, using aqueous $^{13}$N$\text{NH}_3$ as a precursor.

**Methods**

**General**

Benzaldehyde (≥99%), levulinic acid (98%), $p$-toluic acid (98%), tert-butyl isocyanide (98%) and trifluoroacetic acid (99%) were purchased from Sigma-Aldrich. Benzyl isocyanide (98%) and 2,2,2-trifluoroethanol (99.8%) were purchased from Acros Organics. 1,1,3,3-tetramethylbutyl isocyanide and ammonia solution (28–30%) were purchased from Merck KGaA. Acetic acid (~100%) was purchased from AnalaR. Methanol (HPLC grade) was purchased from Fisher.

Reactions were carried out using a CEM Discover microwave synthesis unit.

$^1$H–NMR and $^{13}$C–NMR spectra were obtained using a Bruker DRX 400 MHz spectrometer. Mass spectrometry was performed on an Agilent Technologies 6520 Accurate-Mass Q-TOF LC/MS connected to an Agilent Technologies 1200 HPLC system with UV detector and autosampler.

Radio-HPLC analysis was performed on an Agilent Technologies 1200 Series with UV detector (254 nm) and Lablogic $\beta$ + radio-detector using an Agilent Eclipse XDB-C18 column (5 $\mu$m, 4.6 $\times$ 150 mm). The following mobile phase conditions were used: solvent A: H$_2$O + 0.1% TFA; solvent B: MeOH +0.1% TFA; time:%B 0:5, 1:5, 10:95, 18:5, 23:5. Flow rate was 1 mL/min.

**Reference standard synthesis**

For reference standard compounds 1–5, benzaldehyde (2 mmol), the respective carboxylic acid ($p$-toluic acid (2, 4) or acetic acid (1, 3, 5), 2 mmol), the respective isocyanide (tert-butyl isocyanide (4, 5), benzyl isocyanide (3) or 1,1,3,3-tetramethylbutyl isocyanide (1, 2), 2 mmol) and ammonia (excess, ~ 3 mmol) were combined in 2,2,2-trifluoroethanol (TFE, 2 mL) in a microwave tube. For reference standard compounds 6–7, levulinic acid (2 mmol), the respective isocyanide (benzyl isocyanide (6) or tert-butyl isocyanide
(7), 2 mmol) and ammonia (excess, ~ 3 mmol) were combined in TFE (2 mL) in a microwave tube. The mixture was heated at 100 °C for 30 min in a microwave synthesis unit. The reaction mixture was cooled to room temperature and the solvent was removed under vacuum. The crude product was filtered and washed with ice-cold methanol. The final product was isolated using semi-preparative HPLC and lyophilised.

**Radiochemistry**

**[^13N]NH₃ production**

Aqueous [^13N]NH₃ was produced on a CTI RDS 112 biomedical cyclotron via the ¹⁶O(p,α[^13N]) nuclear reaction. The target contained 8 mL H₂O with 5 mM ethanol. Concentration of [^13N]NH₃ into a 1 mL volume was carried out using a weak cation exchange Sep-Pak (Accell Plus CM Light, Waters.) and eluted with saline (0.9%, 1 mL).

**Radiolabelling of 1–7**

For radiolabelling of 1–5, benzaldehyde (48.5 μmol), the respective carboxylic acid (p-toluic acid (2, 4) or acetic acid (1, 3, 5), 48.5 μmol), the respective isocyanide (tert-butyl isocyanide (4, 5), benzyl isocyanide (3) or 1,1,3,3-tetramethylbutyl isocyanide (1, 2), 48.5 μmol), ammonium hydroxide solution (28–30%, 10 μL, 148 μmol) and [^13N]NH₃ (50 μL) were combined in 2,2,2-trifluoroethanol (200 μL) in a microwave tube. For radiolabelling of 6–7, levulinic acid (48.5 μmol), the respective isocyanide (benzyl isocyanide (6) or tert-butyl isocyanide (7), 48.5 μmol), ammonium hydroxide solution (28–30%, 10 μL, 148 μmol) and [^13N]NH₃ (50 μL) were combined in 2,2,2-trifluoroethanol (200 μL) in a microwave tube. The mixture was heated at 120 °C for 10 min in a microwave synthesis unit with stirring. The reaction mixture was cooled to room temperature and analysed via radio-HPLC. The desired ^13N–labelled Ugi product was isolated using semi-preparative HPLC.

**Results**

**Reference standard synthesis**

Based on methods developed by Thompson et al. (Thompson & Chen, 2009), reference standards 1–7 were successfully synthesised and isolated. LC/MS analysis of the crude product mixtures confirmed the presence of the desired 4-CC products. Full characterisation of the final isolated products can be found in the Additional file 1.
Fig. 1 Synthesis of $^{13}\text{N}$-labelled $\alpha$-aminoacyl amide derivatives (1–5) and $\gamma$-lactams (6–7).

Table 1 Reaction optimisation

<table>
<thead>
<tr>
<th>Entry</th>
<th>Solvent</th>
<th>$T$ (°C)</th>
<th>Time (min)</th>
<th>NH$_3$ (μL)</th>
<th>RCY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TFE</td>
<td>100</td>
<td>15</td>
<td>5</td>
<td>13 ± 1.4</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>100</td>
<td>15</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>MeCN</td>
<td>100</td>
<td>15</td>
<td>5</td>
<td>9 ± 1.5</td>
</tr>
<tr>
<td>4</td>
<td>TFE</td>
<td>80</td>
<td>15</td>
<td>5</td>
<td>10 ± 0.6</td>
</tr>
<tr>
<td>5</td>
<td>TFE</td>
<td>120</td>
<td>15</td>
<td>5</td>
<td>14 ± 1.2</td>
</tr>
<tr>
<td>6</td>
<td>TFE</td>
<td>150</td>
<td>10</td>
<td>5</td>
<td>12 ± 1.5</td>
</tr>
<tr>
<td>7</td>
<td>TFE</td>
<td>120</td>
<td>10</td>
<td>5</td>
<td>14 ± 1.1</td>
</tr>
<tr>
<td>8</td>
<td>TFE</td>
<td>120</td>
<td>5</td>
<td>5</td>
<td>12 ± 0.5</td>
</tr>
<tr>
<td>9</td>
<td>TFE</td>
<td>120</td>
<td>10</td>
<td>10</td>
<td>23 ± 0.5</td>
</tr>
<tr>
<td>10</td>
<td>TFE</td>
<td>120</td>
<td>10</td>
<td>20</td>
<td>16 ± 0.7</td>
</tr>
</tbody>
</table>

$^a$ Reaction conditions: benzaldehyde (4.95 μL), acetic acid (2.78 μL), 1,1,3,3-tetramethylbutyl isocyanide (8.52 μL), $^{13}$NH$_3$ (50 μL), solvent (200 μL). $^b$ 28% NH$_3$ in H$_2$O. $^c$ Radiochemical yield, based on radio-HPLC analysis of the crude product.
RCY (Table 1, entries 4–8). A reaction time of 10 min at a temperature of 120 °C was found to give optimal RCY (Table 1, entry 7). However, halving the reaction time only marginally reduced the RCY from 14% to 12%. Thus, taking into account radioactive decay, a shorter reaction time of 5 min would ultimately give higher activity yield (Table 1, entry 8), and would be more practically useful in a clinical scenario. The RCY was found to have the greatest dependency on the amount of carrier ammonia added. Increasing the amount of carrier ammonia from 5 μL (74 μmol) to 10 μL (148 μmol) led to an increase in RCY from 14% to 23%, respectively (Table 1, entries 7 and 9). A further increase to 20 μL (296 μmol) resulted in a decrease in RCY to 16% (Table 1, entry 10). Under no-carrier-added conditions, the desired labelled product was not observed. Increasing the volume of the aqueous [13N]NH3 in the reaction from 50 μL to 100 μL resulted in a significant decrease in RCY.

The established optimum conditions of 1 were applied to the radiosynthesis of a small library of compounds. 13N–labelling of target structures 1–7 was confirmed by the co-elution of the non-radioactive standards and radio-LC/MS analysis. The radiochemical yield of these compounds ranged from 11 to 23% (Table 2). The molar activity of [13N]NH3 used in these experiments was 2.64 ± 0.12 GBq/μmol. In addition to the target Ugi compound and un-reacted [13N]NH3, another unknown radiolabelled species was observed (Fig. 2). However, semi-preparative HPLC could be used to isolate the desired 13N–labelled Ugi product. To demonstrate this, isolation of 1 was carried out, achieving 96% radiochemical purity (Fig. 3) and an activity yield between 4 and 6% (based on 24 min preparation time).

To identify the radioactive by-products, analogous reactions using stable isotope labelling with 14N and 15N were carried out, followed by LC/MS analysis to establish a molecular weight associated with the by-products. In each case, the molecular weight corresponded to the combination of one [14/15N]NH3 molecule and two isocyanide molecules, suggesting the formation of labelled di-imine structures (Fig. 4).

**Discussion**

Ammonia is rarely used as the amine component in the Ugi reaction, as it reportedly leads to low yields and extensive by-product formation. A common by-product is the six-component coupling (6-CC) product formed through participation of the solvent (usually methanol) (Ackermann et al., 2012; Kazmaier & Hebach, 2003). Thompson et al. synthesised a library of structures via the Ugi reaction using ammonia, and replacing methanol with the less nucleophilic solvent TFE suppressed formation of the 6-CC product in favour of the desired 4-CC product (Thompson & Chen, 2009). Therefore, in the present study TFE was used for all reactions. Using this strategy, reference standards 1–7 were successfully synthesised. LC/MS analysis of the crude product mixtures confirmed the presence of the desired 4-CC product, with no evidence for a 6-CC product.

The radiolabelling of structures 1–7 via the Ugi reaction using aqueous carrier-added [13N]NH3 as a precursor was successful, with RCYs ranging from 11 to 23%. Under the tested conditions, the reaction did not proceed in the absence of carrier-added ammonia and the RCY varied significantly depending on the amount of carrier ammonia added: reducing the amount of ammonia from 10 μL (2.9 equivalents) to near stoichiometric amounts (5 μL, 1.4 equivalents) reduced the RCY; increasing to 20 μL (6 equivalents), also reduced the RCY. Therefore, 10 μL was selected for all subsequent
reactions. We note that under these specific high temperature, closed vessel microwave conditions, the amount of ammonia in the solution versus the gas phase (and thus unavailable for reaction) has not been quantified. The necessary addition of carrier ammonia to this reaction mixture will inherently result in a tracer with low molar activity, potentially preventing the targeting of low-abundance receptors. The detrimental effect on the RCY upon increasing the volume of aqueous [^{13}\text{N}]NH_3 suggests this reaction could be affected by the presence of water and may benefit from anhydrous conditions.

The use of microwave technology is often beneficial when rapid chemistry is required, particularly when using such short-lived isotopes as nitrogen-13. A limited number of experiments using conventional heating methods showed the target product could be obtained, but in lower RCY than that obtained using microwave heating. This

<table>
<thead>
<tr>
<th>Entry</th>
<th>Product</th>
<th>RCY(^a) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>23 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>23 ± 2.1</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>16 ± 0.6</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="Structure 4" /></td>
<td>15 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="Structure 5" /></td>
<td>12 ± 1.8</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6.png" alt="Structure 6" /></td>
<td>11 ± 0.6</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7.png" alt="Structure 7" /></td>
<td>15 ± 1.5</td>
</tr>
</tbody>
</table>

\(^a\) n = 3. Determined by radio-HPLC (not isolated). Reaction conditions: aldehyde (48.5 μmol), carboxylic acid (48.5 μmol), isocyanide (48.5 μmol), [^{13}\text{N}]NH_3 (50 μL), carrier ammonia (10 μL, 148 μmol) combined in TFE (200 μL) and heated with stirring to 120°C for 10 min in a microwave reactor.
indicates that the use of microwave technology is indeed effective in this case (see Additional file 1).

Despite the presence of un-reacted $[^{13}\text{N}]\text{NH}_3$ and radioactive by-products in the crude reaction mixture, the desired $^{13}\text{N}$–labelled Ugi product could be easily isolated using semi-preparative HPLC, as demonstrated for reaction 1. The UV chromatogram of the final isolated product showed two major products co-eluting with the radioactive product. These are likely to be the non-radioactive $\alpha$-aminoacyl amide Ugi product, and a structurally similar $\alpha$-acyloxy amide, the product of the three-component coupling Passerini reaction between the isocyanide, carboxylic acid and aldehyde (see Additional file 1). This is consistent with our observations of the presence of the 3-component coupling Passerini product in the LC/MS analysis of the crude reaction mixture during synthesis of the non-radioactive reference standards, and in the analogous $^{15}\text{N}$ reactions. The radioactive by-products observed in the radio-chromatograms of all reactions 1–7 were identified as $^{13}\text{N}$–labelled di-imines in which one $[^{13}\text{N}]\text{NH}_3$ molecule reacts with the electrophilic carbon of two isocyanide molecules to form the $^{13}\text{N}$–labelled di-imine (Fig. 4).

Identification of these by-products further contributes to expanding the breadth of known radiochemistry available for radiosynthesis with $[^{13}\text{N}]\text{NH}_3$. Derivatisation of $R_1$ and $R_2$ substituents via the isocyanide and carboxylic acid moieties bearing targeting groups such as peptides, sugars or other functional groups, could enable rapid access to libraries of functionalised $^{13}\text{N}$–labelled compounds. Furthermore, it may be possible to label molecules of specific biological interest using this method, such as 1,4-benzodiazepine-2,5-diones which have shown promise as anticonvulsant agents, opiate receptor antagonists

![Fig. 2 HPLC chromatogram of the crude radiolabelled product (Table 1, entry 9).](image)

- **a** Radioactivity (counts per second); target compound 1 at $R_t$ 11.58 min; unreacted $[^{13}\text{N}]\text{NH}_3$ at $R_t$ 1.82 min; unknown by-product at $R_t$ 8.55 min.
- **b** UV absorption (254 nm) of reaction 1.
and as inhibitors of histone deacetylases which are linked to the pathogenesis of several cancers (Hulme et al., 1998; Loudni et al., 2007).

**Conclusion**

The novel methodology presented here demonstrates an efficient strategy for quickly obtaining libraries of diverse $^{13}$N–labelled peptidomimetics. The Ugi reaction has been successfully adapted to radiolabel a library of α-aminoacyl amide derivatives and γ-lactams with $^{13}$N from cyclotron-produced aqueous $[^{13}\text{N}]\text{NH}_3$ precursor in a one-pot method. This is also the first report of radiosynthesis with $^{13}$N using microwave...
heating technology. This work demonstrates that despite its short half-life, complex molecules can be rapidly labelled with $^{13}$N and as such, $^{13}$N should be regarded as a viable option for labelling peptides in the future.

**Additional file**

**Additional file 1**: Electronic Supplementary Information. (DOCX 77 kb)

**Abbreviations**

4-CC: Four-component coupling; 6-CC: Six-component coupling; DMF: Dimethyl formamide; PET: Positron emission tomography; RCY: Radiochemical yield; TFA: Trifluoroacetic acid; TFE: 2,2,2-trifluoroethanol

**Acknowledgements**

Not applicable

**Availability of data and materials**

The data set supporting the conclusion of this article is included within the article and its additional file.

**Authors’ contributions**

ADG and JEB jointly conceived the study. JEB designed and coordinated the study. JEB and SFC conducted the chemical synthesis. JEB and SFC conducted the radiochemistry development and optimisation. The manuscript was drafted by JEB and ADG. All authors read and approved the final manuscript.

**Funding**

This research was supported by the PET Centre at St Thomas’ Hospital and the Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy’s & St Thomas’ NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust. This research was also supported by The Centre of Excellence in Medical Engineering funded by the Wellcome Trust and EPSRC under grant number WT 088641/Z/09/Z, and the KCL and UCL Comprehensive Cancer Imaging Centre funded by CRUK and EPSRC in association with the MRC and Department of Health (England). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the DoH.

**Authors’ information**

Not applicable

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Competing interests**

The authors declare that they have no competing interests.

**Publisher’s Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 21 September 2017 Accepted: 1 December 2017
Published online: 19 December 2017

**References**


