TfNN\(^{15}\)N: A \(\gamma^{15}\)N-Labeled Diazotransfer Reagent for the Synthesis of \(\beta^{15}\)N-Labeled Azides

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**ABSTRACT:** Azides are infrared (IR) probes that are important for structure and dynamics studies of proteins. However, they often display complex IR spectra owing to Fermi resonances and multiple conformers. Isotopic substitution of azides weakens the Fermi resonance, allowing more accurate IR spectral analysis. Site-specifically \(^{15}\)N-labeled aromatic azides, but not aliphatic azides, are synthesized through nitrosation. Both \(^{15}\)N-labeled aromatic and aliphatic azides are synthesized through nuclophilic substitution or diazo-transfer reaction but as an isotopomeric mixture. We present the synthesis of TfNN\(^{15}\)N, a \(\gamma^{15}\)N-labeled diazo-transfer reagent, and its use to prepare \(\beta^{15}\)N-labeled aliphatic as well as aromatic azides.

**INTRODUCTION**

Various spectroscopic techniques have been used to study the structures and dynamics of proteins. Fluorophores are widely used probes for studying changes in the protein structure. However, the introduction of relatively large fluorophores significantly disturbs the native structure.

Infrared (IR) probes, such as CO, \(\text{CN}^*\), and SCN, which directly convey intramolecular bonding vibrations, are relatively small, thus minimizing native structure disturbance. IR probes have been used as site-specific probes of biomolecules because of their sensitivity to the local environment. However, IR spectral analysis of biomolecules is difficult because their IR signals often overlap with those of peptides. Therefore, IR probes with isotopic labels or a signal in the transparent window region between 1800 and 2500 cm\(^{-1}\) are used.

Azides have considerable potential as vibration probes of biomolecules due to their IR absorption in the transparent window region of the spectrum. In addition, the molar extinction coefficient of the azide probe is approximately 5–19 times larger than that of the CN probe. Therefore, azide probes may be used for low-concentration peptides or proteins. Azides are also used in site-specific “click chemistry”.

However, short vibrational lifetimes and Fermi resonance are disadvantages in the IR spectral analysis of azide. In the presence of Fermi resonance, the IR absorption spectrum is complex, which hampers the spectral analysis for probing structural changes in proteins or surrounding solvents. Furthermore, whether the IR spectrum is complicated by Fermi resonance or multiple conformation is unclear.

Accidental Fermi resonance can be detected by FTIR absorption and 2DIR spectroscopies. However, the complex spectra, due to Fermi resonance, are challenging to analyze. Generally, isotopic substitution overcomes spectral interference by Fermi resonance because its effect is reduced by increasing the energy difference between the fundamental and overtone (or combination) modes.

Three synthetic routes are known for preparing \(^{15}\)N-labeled azides (Scheme 1). First, nucleophilic substitution reaction, wherein halides or good leaving groups are substituted with \(^{15}\)N\(^{-}\), is the most commonly used method for the synthesis of \(^{15}\)N-labeled aliphatic azides. This method was used by Brewer and co-workers to prepare azido isotomers of 2’-azido-2’-deoxyuridine (dU-NNN) as a mixture of dU-\(^{15}\)NNN and dU-NN\(^{15}\)N. Although there was a slight frequency red-shift of dU-\(^{15}\)NNN (2111 cm\(^{-1}\)) and dU-NN\(^{15}\)N (2089 cm\(^{-1}\)) relative to dU-NNN (2111 cm\(^{-1}\)), IR spectral analysis of the two-isotopomer mixture was still difficult because their IR spectra overlapped.

Second, nitrosation of aryl hydrazine with Na\(^{15}\)NO\(_2\) is useful for the synthesis of site-specifically \(^{15}\)N-labeled aromatic azides. This method was used by Brewer and co-workers to prepare azido isotomers of 3-azidopyridine (PyrNNN) in a site-specific manner. In the IR spectrum of PyrNNN, a complex band containing the Fermi resonance was observed at 2075–2150 cm\(^{-1}\). The IR bands of Pyr-\(^{15}\)NNN, PyrNN\(^{15}\)N, and PyrN\(^{15}\)NN were observed at 2121, 2080, and 2067 cm\(^{-1}\), respectively. The IR spectrum of PyrNN\(^{15}\)N was still complex, due to Fermi resonance, but those of Pyr-\(^{15}\)NNN and PyrN\(^{15}\)NN revealed one band. However, unlike aryl or carbonyl hydrazine, alkyl hydrazine has the limitation that it cannot be rearranged through nitrosation to produce azides.
That mixture was then used to prepare a mixture reagents such as imidazole-1-sulfonyl azide (ImSO₂N₃) and 2-labeled azides, but its IR spectrum still overlapped with that of modulated the accidental Fermi resonance occurring in the IR spectroscopies. Nitrosation usually occurs at the terminal γ-N atom as suggested by Wong’s mechanism.14 Accordingly, the α-, β-, and γ-15N-labeled diazo-transfer reagents furnish the unlabeled, γ-, and β-15N-labeled azides, respectively. Brewer and co-workers synthesized azido isotopomers of tri fuoromethanesulfonazides (TfNNN), a diazo-transfer reagent, as a mixture of Tf15NNN and TfNN15N isotopomers of tri fuoromethanesulfonic anhydride (Tf2O) with Na15NO2,15 Such a mixture was also obtained by the nitrosation of 1,3-dimethylimidazolidinone hydrazone with Na15NO2. Such a mixture was also obtained by the nitrosation of 1,3-dimethylimidazolidinone hydrazone with Na15NO2. Therefore, the NNN. Such frequency di ff erences between dU-15NNN and dU-NN15N. Therefore, the 15N-Labeled Azides

\[ R - X_1 \quad \xrightarrow{\text{Nucleophilic substitution}} \quad \text{Na}^{15}\text{NNN} \quad \xrightarrow{\text{X}_2} \quad R - X_2 \]

where \( R = \text{aromatic or aliphatic} \), \( X_1 = \text{leaving group} \), and \( X_2 = 15\text{NNN} \) or\( 15\text{NN} \) or\( 15\text{NN} \) or\( 15\text{NN} \).

(b) Nitrosation

\[ R - \text{NHNN}_2 \quad \xrightarrow{\text{Na}^{15}\text{NO}_2 \text{H}^{+}} \quad R - \text{NN}^{15}\text{N} \]

where \( R = \text{aromatic} \).

(c) Diazo-transfer

\[ \text{Tf} - \text{Cl} \quad \xrightarrow{\text{Na}^{15}\text{NNN}} \quad \text{Tf} - X_1 \quad \xrightarrow{\text{R - NH}_2} \quad R - X_2 \]

where \( R = \text{aromatic or aliphatic} \), \( X_1 = 15\text{NNN} \) or\( 15\text{NN} \) or\( 15\text{NN} \) or\( 15\text{NN} \), and \( X_2 = \text{NN} \) or\( 15\text{NN} \).

(d) This work

\[ \text{Tf} - \text{NHNNBoc} \quad \xrightarrow{\text{Na}^{15}\text{NO}_2 \text{H}^{+}} \quad \text{Tf} - \text{NN}^{15}\text{N} \quad \xrightarrow{\text{R - NH}_2} \quad R - \text{NN}^{15}\text{N} \]

where \( R = \text{aromatic or aliphatic} \).

Finally, the diazo-transfer reaction of primary amines is an efficient method for the syntheses of both 15N-labeled aromatic and aliphatic azides.11,13 Diazo-transfer occurs via nucleophilic attack of amine on the azido group of the reagent at its terminal γ-N atom as suggested by Wong’s mechanism.14 Accordingly, the α-, β-, and γ-15N-labeled diazo-transfer reagents furnish the unlabeled, γ-, and β-15N-labeled azides, respectively. Brewer and co-workers synthesized azido isotopomers of trifluoromethanesulfonyl azides (TfNNN), a diazo-transfer reagent, as a mixture of Tf15NNN and TfNN15N (α- and γ-15N-labeled ones) by the nucleophilic substitution reaction of trifluoromethanesulfonic anhydride (Tf2O) with Na15NO2.15 That mixture was then used to prepare a mixture of dU-NNN and dU-15NNN. The IR spectrum of dU-N15NN (2069 cm⁻¹) exhibited a red-shift of 42 cm⁻¹ from that of dU-NNN. Such frequency difference is greater than those for dU-15NNN and dU-NN15N. Therefore, the β-15N-labeled azide modulated the accidental Fermi resonance occurring in the unlabeled azide by the largest frequency shift among the single-labeled azides, but its IR spectrum still overlapped with that of the unlabeled azide. Azido isotopomers of other diazo-transfer reagents such as imidazole-1-sulfonyl azide (ImSO₂N₃) and 2-azido-1,3-dimethylimidazolium hexafluorophosphate (ADMP) were also synthesized as an isotopomeric mixture. A mixture of α- and γ-15N-labeled azido isotopomers of ImSO₂N₃ was synthesized by nucleophilic substitution using Na15NO₂. Recently, we found that a 1:1 mixture of α- and γ-15N-labeled azido isotopomers of ADMP was synthesized by nitrosation of 1,3-dimethylimidazolidine hydrazone with Na15NO₂. Such a mixture was also obtained by the nucleophilic substitution reaction of 2-chloro-1,3-dimethylimidazolinium chloride with Na15NO₂.

Taken together, site-specifically 15N-labeled aromatic azides, but not aliphatic azides, can be synthesized through nitrosation. Both 15N-labeled aromatic and aliphatic azides can be synthesized by nucleophilic substitution or diazo-transfer reaction but as an isotopomeric mixture (15NN, N-15NN, or -15NN, -NN). That is, a synthetic method for preparing site-specifically 15N-labeled aliphatic azides has not been established yet. In particular, β-15N-labeled azides are demanded to facilitate the IR spectral analysis of the azide probe by decreasing the Fermi resonance effect. Herein, we report the synthesis of Tf15NNN, a γ-15N-labeled diazo-transfer reagent, via nitrosation of TfNNNH₂ with Na15NO₂, and its use to prepare β-15N-labeled aliphatic as well as aromatic azides.

RESULTS AND DISCUSSION

A γ-15N-labeled diazo-transfer reagent was designed based on TfN₄ an early model diazo-transfer reagent.16 Like aryl or carbonyl hydrazine, sulfonyl hydrazine may rearrange to produce γ-15N-labeled azides,17 but not aliphatic azides, can be synthesized through nitrosation with Na15NO₂.18

Scheme 1. (a–d) Syntheses of Site-Specifically 15N-Labeled Azides

Scheme 2. Synthesis of Tf15NN 1

Tf15NN 1 (or TfNNN 1) was synthesized by nitrosation of in situ generated TfNNNH₂, 1° with Na15NO₂ (or NaNO₂) (Scheme 2). TfNNNH₂ could not be obtained upon treatment of Tf₂O with NH₂NH₂18,19 Instead, it was generated in situ from TfNNNH₂Boc 1°, which was synthesized using Tf₂O and NH₂NH₂Boc. Hydrazine precursor 1° is a more stable and easier-to-use solid than its derived hydrazine 1°. After the removal of Boc in 1° with trifluoroacetic acid (TFA), it was subjected without purification to direct reaction with Na15NO₂ to afford the desired product 1. This indicates that nitrosation occurs under acidic conditions without being severely affected by the cleavage product of Boc. TfNNN 1 present in the organic layer (CH₂Cl₂) obtained through the work-up process was used without further purification for subsequent spectral analyses and diazo-transfer reactions because of its low boiling point.22 Currently, the yields in the preparation of TfNNN 1 are inconsistent. However, hydrazine is easily obtained via its precursor for TfN₂ but not for ImSO₂N₂.

The synthesis of TfNN15N was confirmed by 15N NMR and IR spectroscopies. Nitrosation usually occurs at the β-N of hydrazine, forming the γ-15N-labeled azide. Occasionally, however, nitrosation occurs at the α-N of hydrazine, forming not only γ-15N-labeled but also β-15N-labeled azide.23 The 15N NMR spectrum of Tf15NNN shows only one peak at −139.08 ppm, confirming the synthesis of the γ-15N-labeled azide via nitrosation at the β-N of hydrazine. The IR spectrum of Tf15NNN shows a strong, broad band at 2126 cm⁻¹, confirming the synthesis of the γ-15N-labeled azide (Figure 1). The IR band of TfNNN appears at 2155 cm⁻¹, which is blue-shifted by approximately 28 cm⁻¹ from that of TfNN15N. Note that TfNN15N and TfNNN show the shoulder peaks at 2155 and 2128 cm⁻¹, respectively, which arise from Fermi resonance. Although site-specific isotopic substitutions are confirmed through the observed frequency shift, accurate
analysis of the IR spectrum is difficult because of Fermi resonance.

Scheme 3. Syntheses of Azides 3 by Diazo-Transfer Reactions of Amines 2 with TfNN15N 1

With TfNN15N in hand, we then explored the diazo-transfer reaction of various amines (Scheme 3). Three representative amines used were Ac-DAP-NHMe-TFA 2a,6a H-Phe-ObBu-HCl 2b, and Ac-Phe(p-NH2)-OMe 2c, which are aliphatic amines bonded to primary and secondary carbons, and aromatic amines, respectively. Upon the reaction with TfNN15N, they were converted to the β-15N-labeled aliphatic and aromatic azides 3a–3c in moderate yields.24

The syntheses of the β-15N-labeled aliphatic and aromatic azides were confirmed by 1H NMR, 13C NMR, and IR spectroscopies. First, the 1H NMR spectra of AlaN15NN (Ac-Ala(N15NN)-NHMe, 3a) and AlaNNN (Ac-Ala(NNN)-NHMe, 6a) 3a revealed that the splitting pattern of the signal for two Hβ's, Hβ1 and Hβ2, at 3.45–3.80 ppm was different between 3a and 3a' (Figure 2). The signal for the two Hβ's in 3a and 3a' is split into 16 and 8 peaks, respectively, which can be explained as follows. Each of the two Hβ's exhibits a different signal, which appear at 3.73 and 3.53 ppm for 3a and 3.72 and 3.54 ppm for 3a'. Each of these two signals is further split into eight and four peaks for 3a and 3a', which is due to the coupling of Hβ1 with Hβ1′ and Hγ, and β-15Nα for 3a but with Hβ2 and Hγ for 3a'. Thus, the 1H NMR spectrum confirms the presence of β-15Nα in 3a. The 13C NMR spectrum of 3a shows the J1 and J2 couplings of β-15Nα with adjacent Cβ (J1(β-15Nα,Cβ) = 1.9 Hz) and Cγ (J2(β-15Nα,Cγ) = 1.9 Hz), which also confirms the presence of β-15Nα in 3a. The IR spectra of 3a and 3a' exhibit one band at 2061 and 2104 cm−1, respectively (Figure 3). A red-shift of 43 cm−1 is due to replacing β-Nα with β-15Nα. Thus, the IR spectrum also confirms the presence of β-15Nα in 3a. The same patterns were also observed in the 1H NMR, 13C NMR, and IR spectra of other azides 3b, 3b', 3c, and 3c' (Figures S1–S6 of the Supporting Information): 1H NMR spectra (500 MHz, CDCl3) of 3b, δ 3.91 (dd, J = 8.5, 6.0 Hz, 1H); 3b', δ 3.91 (dd, J = 8.0, 6.0 Hz, 1H); 13C NMR spectra (125 MHz, CDCl3) of 3b, J(β-15Nα,Cδ) = 1.9 Hz, J(β-15Nα,Cγ) = 1.9 Hz; 3c, J(β-15Nα,Cδ) = 2.8 Hz; IR spectra of 3b, 2062 cm−1; 3b', 2112 cm−1; 3c, 2090 cm−1; 3c', 2143 cm−1.

**CONCLUSIONS**

In conclusion, we synthesized TfNN15N, a γ-15N-labeled diazotransfer reagent, via nitrosation of TfNH2 with Na15NO2. We then demonstrated that it could be used to prepare both β-15N-labeled aliphatic and aromatic azides. TfNN15N is the first example of a site-specifically 15N-labeled diazo-transfer reagent, which can provide site-specifically 15N-labeled aliphatic azides for the first time. β-15N-Labeled azides display a larger frequency red-shift than α- and γ-15N-labeled azides compared to the unlabeled one. Thus, β-15N-labeled azides render the IR spectral analysis of azide probes much easier because a more significant decrease in the Fermi resonance effect is attained.25,26
EXPERIMENTAL SECTION

General. 1H and 13C NMR spectra were recorded on a Bruker Ascend 500 NMR spectrometer. 15N NMR spectra were recorded on an Agilent DD2 700 NMR spectrometer. Chemical shifts (δ) and coupling constants (J) are reported in parts per million (ppm) and hertz (Hz), respectively. 1H NMR spectra are referenced to TMS (0.03% v/v tetramethylsilane in CDCl3) as an internal standard. 13C NMR spectra are referenced to the solvent (13C: CDCl3, δ 77.00 ppm) as an internal standard. High-resolution mass spectra (HRMS) were recorded on a JEOL JMS-700 mass spectrometer using the fast atom bombardment (FAB) technique. IR spectra were measured on a Bruker VERTEX 70 FTIR spectrometer equipped with a HgCdTe detector. The samples 1 and 3 were dissolved in DMF at 0.3 M. IR spectra were measured with a frequency resolution of 1 cm⁻¹ in 12 scans using a CaF2 cell (2 mm thickness) confined with a Teflon spacer (25 μm thickness). Thin-layer chromatography (TLC) was performed on silica gel 60 F254 precoated plates (0.25 mm thickness, Merck, Darmstadt). Flash chromatography was carried out on silica gel 60 (230–400 mesh, Merck). Reagent-grade chemicals were purchased from Sigma-Aldrich, Alfa Aesar, and TCI and used as received unless otherwise specified. Amino acids (H-DAP(Boc)-OMe, H-Phe-Obu-HCl 2b, and Ac-p-aminophenyl azide (TfNN15N, 1)) were purchased from BACHEM. Sodium nitrite (15N, 98%+) was purchased from Cambridge Isotope Laboratories. TIN NhHBOc 1‴ was prepared as reported previously.

Preparation of 15N-Labeled Trifluoromethanesulfonfyl Azide (TfNHN15N, 1). To a cooled (0 °C) and stirred solution of TIN NhHBOc 1‴ (2.38 g, 9.0 mmol) in CH2Cl2 (40 mL) was added trifluoroacetic acid (15 mL). After stirring at 0 °C for 1 h, a solution of NaN3O2 (931 mg, 13.3 mmol) in H2O (10 mL) was added. After stirring at 0 °C for a further 1 h, the reaction mixture was treated with saturated aqueous Na2CO3 solution (200 mL). The organic layer was collected and washed with DMSO (10 mL). Amino acids (H-Phe-OH, H-Boc-Phe-OH, H-Phe-Obu-HCl 2b, and Ac-Phe-Obu-HCl 2c) were purchased from BACHEM. Sodium nitrite (15N, 98%+) was purchased from Cambridge Isotope Laboratories. TIN NhHBOc 1‴ was prepared as reported previously.

General Procedure for the Preparation of β-15N-Labeled Azides 3. To a stirred solution of amine 2 (1.0 mmol) in H2O/MeOH (1:2, 15 mL) were added CuSO4, K2CO3 (691 mg of 5 mg, 50 + mmol),14 and then a solution of TIN NhN15N 1 (~3 mmol) in CH2Cl2 (15 mL). After stirring vigorously at room temperature for 12 h, the reaction mixture was concentrated in vacuo. The residue was purified by flash chromatography to give azide 3.

Ac-Ala(N15NN)-NHMe (3a). Ac-Dap-NHMe-TFA 2a66 (273 mg, 1.0 mmol) was treated according to the general procedure (flash chromatography, MeOH/CH2Cl2 = 1:50) to give 3a (155 mg, 83%) as a white solid. TLC (MeOH/CH2Cl2 = 1:15) Rf = 0.38; 1H NMR (500 MHz, CDCl3) δ 6.69 (brs, 1H), 6.63 (d, J = 7.5 Hz, 1H), 5.62 (d, J = 7.6, 6.4, 5.1 Hz, 1H), 3.73 (dd, J = 12.4, 5.1, 3.6 Hz, 1H), 3.53 (dd, J = 12.3, 6.3, 3.8 Hz, 1H), 2.84 (d, J = 4.5 Hz, 3H), 2.06 (s, 3H), 18C NMR (125 MHz, CDCl3) δ 170.55, 169.69, 52.24 (d, J = 1.9 Hz), 51.88 (d, J = 1.9 Hz, 26.45, 23.13; 15N NMR (70 MHz, CDCl3) δ −133.45; HRMS (FAB+) for C12H16N2O2 (MH+), calcd 247.0981, found 247.0986. 15N-Phe-Obu (3b). H-Phe-Obu-HCl 2b (258 mg, 1.0 mmol) was treated according to the general procedure (flash chromatography, CH2Cl2/n-hexene = 1:1) to give 3b (189 mg, 76%) as a colorless oil. TLC (CH2Cl2/n-hexene = 1:3) Rf = 0.32; 1H NMR (500 MHz, CDCl3) δ 7.34–7.23 (m, 5H), 3.91 (dd, J = 8.5, 6.0, 5.0 Hz, 1H), 3.13 (dd, J = 14.0, 6.0 Hz, 1H), 2.99 (dd, J = 14.0, 8.5 Hz, 1H), 1.45 (s, 9H); 13C NMR (125 MHz, CDCl3) δ 168.99, 136.18, 129.26, 128.56, 127.11, 83.00, 63.60 (d, J = 1.9 Hz), 37.55 (d, J = 1.8 Hz), 27.94; 15N NMR (70 MHz, CDCl3) δ −135.09; HRMS (FAB+) for C12H16N2O2 (MH+), calcd 249.1369, found 249.1371.

Ac-Phe(p-N15NN)-OMe (3c). Ac-Phe(p-NH2)-OMe 2c (236 mg, 1.0 mmol) was treated according to the general procedure (flash chromatography, EtOAc/n-hexene = 1:1) to give 3c (91.1 mg, 35%) as a yellow solid. TLC (EtOAc/n-hexene = 3:1) Rf = 0.41; 1H NMR (500 MHz, CDCl3) δ 7.08 (d, J = 8.0 Hz, 2H), 6.96 (d, J = 8.0 Hz, 2H), 5.93 (d, J = 7.0 Hz, 1H), 4.87 (q, J = 6.5 Hz, 1H), 3.74 (s, 3H), 3.14 (dd, J = 13.5, 6.0 Hz, 1H), 3.06 (dd, J = 14.0, 5.5 Hz, 1H), 1.99 (s, 3H); 13C NMR (125 MHz, CDCl3) δ 171.93, 169.50, 138.97, 132.57, 130.59, 119.15 (d, J = 2.8 Hz), 53.11, 52.39, 37.29, 23.14; 15N NMR (70 MHz, CDCl3) δ −137.72; HRMS (FAB+) for C12H13N3O3 (MH+), calcd 264.1115, found 264.1110.

ASSOCIATED CONTENT

# Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c04679.

NMR and IR spectra of compounds (PDF)

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Notes

The authors declare no competing financial interest.

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Additional quantities ($x$, $n$) should be added when the reaction does not reach completion. For example, the reaction of $\text{TNN}^\text{N}_1$ and Ac-Phe-(p-NH$_2$)-OMe 2c required $x = 691$ mg, $n = 5.0$ mmol.

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