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Synthesis and hyperpolarized ^{15}N NMR studies of ^{15}N-choline-d_{13}

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ABSTRACT

^{15}N-Choline-d_{13} was synthesized as a new biomarker for dynamic nuclear polarization, and the lifetime of the polarized signal governed by the spin–lattice relaxation time T_1 was measured. ^{15}N-Choline-d_{13} exhibited a T_1 that was over twice as long as that of ^{15}N-choline, and the hyperpolarized ^{15}N signal could be observed for 1 h.

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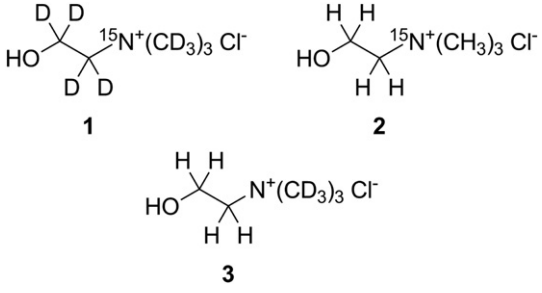
1. Introduction

Techniques to retain highly polarized spins in solution via dynamic nuclear polarization (DNP) have enabled highly sensitive NMR spectroscopic measurements and magnetic resonance spectroscopy (MRS) imaging for nuclei with spin ½ quantum numbers.[1] Particularly, greater than 10,000-fold signal enhancements of hyperpolarized ^{13}C nuclei in short acquisition times were accomplished when the nuclei had relatively long spin–lattice relaxation times T_1.[2–4] This technique has been applied in real-time metabolic studies of tumor tissue and cancers with several ^{13}C-labeled substrates.[5–9]

Choline is a precursor of cellular phospholipid metabolism as well as the neurotransmitter acetylcholine, and it has been applied as a biomarker for cancer detection and metabolic response using ^{1}H and ^{31}P MRI,[10] PET,[11,12] and SPECT.[13] Recently, choline with a relatively long T_1 for the ^{15}N nucleus has been of interest as a useful substrate for DNP ^{15}N NMR and MRS studies.[14–18] Erykyn and co-workers described the high polarization level, long T_1, and long lifetime for the ^{15}N signal[14] in hyperpolarized ^{15}N-choline (**2**) by DNP. Independently, Denisov and co-workers reported that natural-abundance ^{15}N-choline with deuterated methyl groups [choline-trimethyl-d_9 (**3**)] prolonged the T_1 of the ^{15}N signal more than that of **2**.[15] Since tumor uptake of choline is reached a maximum at 3 min after injection,[19] T_1s and lifetimes of ^{15}N signals for hyperpolarized **2** and **3** may be insufficient for in vivo metabolic imaging. During our investigation of useful substrates for DNP, we obtained DNP ^{13}C NMR measurements of deuterium-enriched glucoses, and discovered that *C*-perdeuterated glucose (glucose-*C*-d_7) exhibited a longer T_1 and higher polarization level for the ^{13}C signals than those of glucose samples without deuterium or with partial C-deuteration.[20] Therefore, we synthesized the *C*-perdeuterated ^{15}N-choline [^{15}N-choline-d_{13} (**1**)], and measured ^{15}N NMR spectra of these substrates after hyperpolarization by a solution DNP procedure. Here, we describe the synthesis of ^{15}N-choline-d_{13} (**1**) and ^{15}N NMR profiles of hyperpolarized **1** by DNP.



2. Results and discussion

^{15}N-Choline-d_{13} (**1**) was synthesized from ethylene glycol-d_4 (**4**, 98% ^{2}H-enrichment) in five steps, as shown in Scheme 1. Although installation of a trimethyl ammonium group is possible via a one-step substitution reaction with trimethylamine, ^{15}N-trimethylamine-d_9 is

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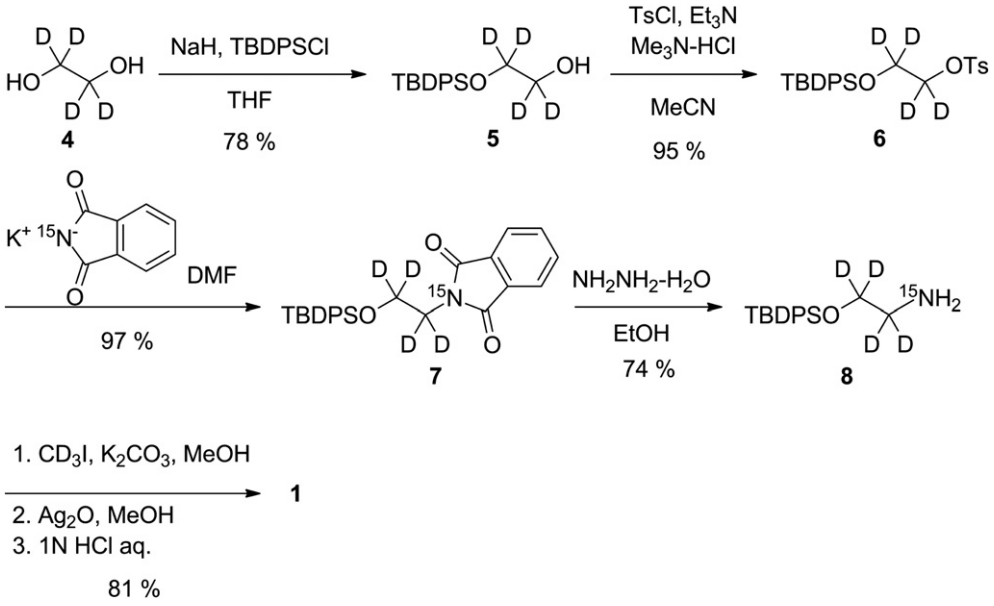

Scheme 1. Synthesis of ^{15}N-choline-d_{13} (**1**).

relatively expensive. So, we opted for a three-step conversion through treatment of **6** with ^{15}N-labeled potassium phthalimide, hydrazinolysis, and methylation with iodomethane-d_3. The monosilyl alcohol (**5**) prepared from **4** was converted into the tosylate (**6**), which was treated with ^{15}N-labeled potassium phthalimide (98% ^{15}N-enrichment) to afford compound **7**. Reaction of **7** with hydrazine gave the ^{15}N-labeled primary amine (**8**). Treatment of **8** with iodomethane-d_3 (99.5% ^{2}H-enrichment) and then silver oxide generated deuterated choline iodide through permethylation of the amino group and deprotection of the silyl group. ^{15}N-Choline-d_{13} chloride (**1**), with calculated enrichments of 96% ^{2}H and 98% ^{15}N, was obtained through an ion-exchange step in 43% overall yield.

The ^{15}N NMR experiments of the cholines were carried out using a 9.4 T wide-bore NMR spectrometer equipped with a 5 mm CH dual probe. The conventional ^{15}N NMR spectrum of **1** (13 mM in D_2O) at thermal equilibrium (Boltzmann) polarization was measured using 3072 scans with a 10° flip angle and 10 s repetition time, as shown in Fig. 1a. Hyperpolarization of the cholines was performed using a HyperSense DNP polarizer (Oxford Instruments) employing OX63 as a radical source under microwave irradiation at 94 GHz in the DMSO-d_6/D_2O glassing solvent system at 1.4 K for 3 h. After dissolution with D_2O, the ^{15}N NMR spectrum (Fig. 1b) of hyperpolarized **1** (2.2 mM) was immediately recorded with a single acquisition using a 10° flip angle at 298 K. The signal-to-noise ratio (SNR) of the ammonium nitrogen signal (41.72 ppm) for thermal equilibrium **1** was 5.65, while hyperpolarized **1** had an SNR of 285. From comparison of these ^{15}N signal intensities in the hyperpolarized and thermal equilibrium NMR spectra, the enhancement factor for hyperpolarized **1** was found to be 1.68×10^4. As 3.3×10^{-6}% was accounted for by the thermal polarization $P(^{15}N)$ at 298 K and 9.4 T, the enhanced polarization P(DNP) for **1** was found to be 5.5%, which was nearly equal to that of **2** (5.4%), as calculated in the same manner.

Fig. 2 shows the time-dependent decays of the ^{15}N signal intensities for the hyperpolarized cholines (**1**–**3**) recorded every 10 s using a 10° flip angle. The T_1 values were estimated from similarities between these observed intensities and curves simulated on the basis of the equation reported by Day et al.[21] The T_1s of the hyperpolarized ^{15}N-choline (**2**) and natural-abundance choline-trimethyl-d_9 (**3**) were found to be 200±5 and 380±70 s, respectively, which were agreed well with previously reported data.[14,15]

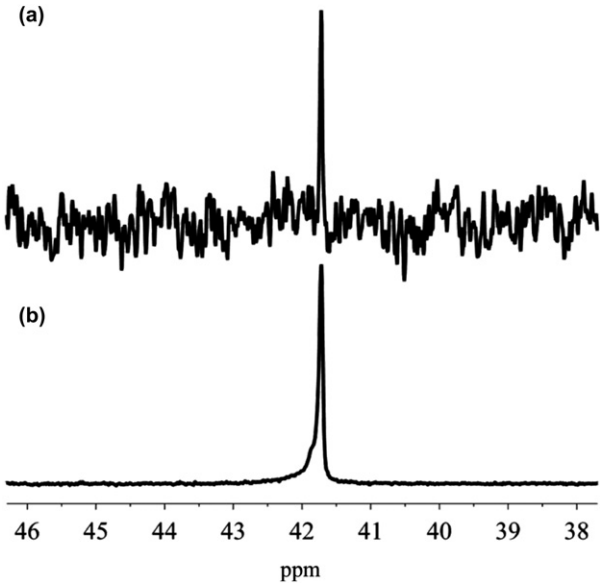

Fig. 1. (a) ^{15}N NMR spectrum of thermal equilibrium ^{15}N-choline-d_{13} (**1**, 13 mM) measured with 3072 scans using a 10° flip angle and 10 s repetition period. SNR was 5.65. (b) ^{15}N NMR spectrum of hyperpolarized ^{15}N-choline-d_{13} (**1**, 2.2 mM) recorded with a single acquisition using a 10° flip angle. SNR was 285. A small shoulder of the signal may be derived from the residual ^{15}N-choline-d_{12}.

Similarly, the T_1 of hyperpolarized ^{15}N-choline-d_{13} (**1**) was found to be 580±10 s. The perdeuterated choline **1** exhibited 2.9- and 1.5-fold increases in T_1 versus those of **2** and **3**, respectively, indicating that perdeuteration of the carbon nuclei around the ammonium nitrogen in choline was effective in prolonging T_1. Comparing of the T_1 for **1** with that of **3**, the T_1 of choline seems to be proportional to deuterium numbers.

Fig. 3a and b show the time-dependent ^{15}N NMR spectra of hyperpolarized **1** and **2** (final concentration: 1.8 mM), respectively, which were recorded using a 10 mm CH probe with a maximum of 180 repetitions of 30 s acquisition duration employing a 10° radio frequency flip angle. Although the signal intensity of the ammonium nitrogen of **1** at 20 min (40 repetitions, Fig. 3c) retained one-

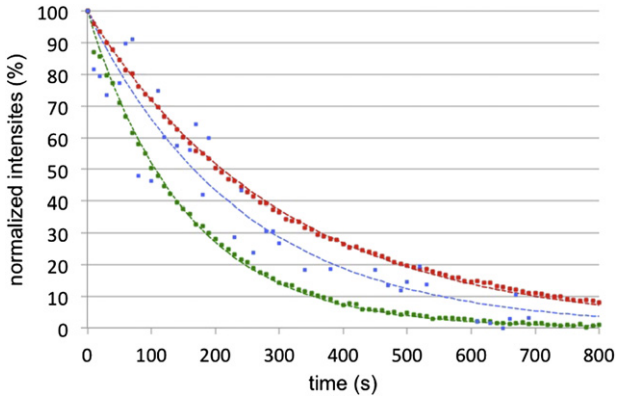

Fig. 2. Time-dependent decays of ^{15}N signal intensities for hyperpolarized cholines (**1**–**3**). Filled red, green, and blue squares represent the observed signal intensities of hyperpolarized ^{15}N-choline-d_{13} (**1**), ^{15}N-choline (**2**), and choline-d_9 (**3**), respectively. Dotted green, blue, and red lines represent the fitting curves calculated for T_1 at 200 s, 380 s, and 580 s, respectively. The signal intensities of **3** showed uneven because of natural abundance for ^{15}N and high noise level.

fifth of that at the first acquisition, the ammonium nitrogen signal in **2** had completely disappeared (Fig. 3d). The ammonium nitrogen signal for **1** was confirmed even at 60 min (3600 s, 120 repetitions, Fig. 3e).

3. Conclusions

In the present study, perdeuterated ^{15}N-labeled choline (**1**) was synthesized and the properties of hyperpolarized **1** were measured. This study showed that perdeuteration of the carbon nuclei attached to the ammonium nitrogen in choline prolonged the ^{15}N spin–lattice relaxation time T_1. The 1 h survival of the hyperpolarized signal for **1** was remarkable. This may have been derived from an increase in the dipolar interaction. To the best our knowledge, the lifetime of hyperpolarized signal for **1** is the longest in the molecular probes for solution DNP reported up to now. These results indicate that perdeuterated ^{15}N-labeled choline may be useful for extended observation of choline metabolism in cells, ex vivo or in vivo.

4. Experimental

4.1. General information and materials

All NMR spectra were recorded on a Varian 400MRWB spectrometer. ^{1}H NMR and ^{13}C NMR chemical shifts were reported in parts per million (ppm) from tetramethylsilane (TMS) as an internal standard. ^{2}H NMR chemical shifts were reported in parts per million (ppm) from $CDCl_3$ (δ 7.26) or D_2O (δ 4.80) as an external standard. Chemical shifts in the ^{15}N NMR spectra were based on

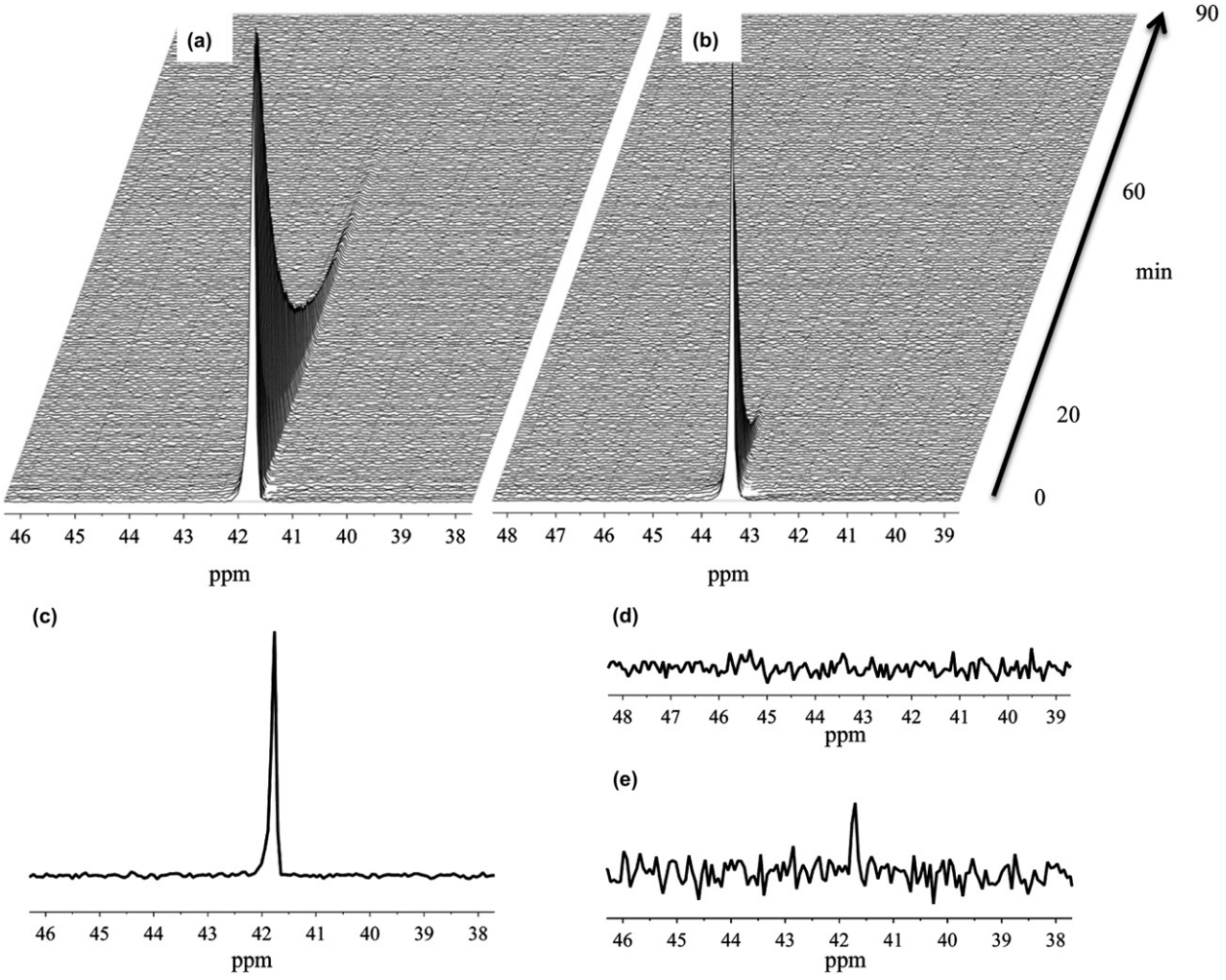

Fig. 3. ^{15}N NMR spectra of hyperpolarized ^{15}N-labeled cholines (**1** and **2**) measured using 10 mm CH probe. Individual spectra were processed with 5.0 Hz Gaussian line broadening. (a) and (b) Time-dependent ^{15}N NMR spectra of hyperpolarized ^{15}N-choline-d_{13} (**1**) and ^{15}N-choline (**2**), respectively. A shoulder of the signal in Fig. 3a may be derived from the residual ^{15}N-choline-d_{12}. (c) and (d) ^{15}N NMR spectra of hyperpolarized ^{15}N-choline-d_{13} (**1**) and ^{15}N-choline (**2**), respectively, 20 min after the start of measurement. (e) ^{15}N NMR spectrum of hyperpolarized ^{15}N-choline-d_{13} (**1**) 60 min after the start of measurement.

parts per million from ^{15}N-choline (**3**) (δ 43.37), as described in the previous paper.[14] Measurements of DNP ^{15}N NMR spectra are described below. Electrospray ionization-mass spectrometry (ESI-MS) results were obtained on JEOL JMS-T100LC and Thermo Scientific EXACTIVE spectrometers, while high-resolution field desorption mass spectrometry (HRFDMS) measurements were obtained on a JEOL JMS-T100GCV spectrometer. Elemental analyses were obtained using a J-Science Lab MICRO CORDER JM10 instrument. Ethylene-d_4 glycol (99.5% D), methyl-d_3 iodide (98% D), and potassium ^{15}N-phthalimide (98% ^{15}N) were obtained from Sigma–Aldrich. ^{15}N-Choline chloride and choline-trimethyl-d_9 chloride were purchased from CIL. Commercially available materials were used as received without further purification. All reaction experiments were carried out under argon in flame-dried glassware using standard inert atmosphere techniques for introducing reagents and solvents, unless otherwise noted.

4.2. Synthesis

*4.2.1. 2-tert-Butyldiphenylsilyloxy[$^2H_4$]ethanol **5**.* To a stirred suspension of sodium hydride (60% oil dispersion, 70.0 mg, 1.74 mmol) in THF (1 mL) was added ethylene-d_4 glycol (**4**, 88.4 μL, 1.58 mmol) in THF (34 mL), and the mixture was refluxed with stirring for 1 h. The reaction mixture was cooled to 0 °C and treated with *tert*-butyldiphenylsilyl chloride (452 μL, 1.74 mmol) at room temperature for 16 h. After evaporation of the solvent, the residue was partitioned between EtOAc and water. The organic phase was washed with saturated aqueous $NaHCO_3$ and then brine, dried over $MgSO_4$, and evaporated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc, 10:1) to afford the crude monosilyl alcohol (**5**, 375 mg, 1.23 mmol, 78%). Compound **5**: colorless oil; ^1H NMR ($CDCl_3$) δ 1.07 (9H, s), 7.40 (4H, m), 7.44 (2H, m), 7.68 (4H, m); ^{13}C NMR ($CDCl_3$) δ 19.2, 26.8 (3C), 62.9 (1C, m), 64.1 (1C, m), 127.8 (4C), 129.8 (2C), 133.2 (2C), 135.5 (4C); ^2H NMR ($CHCl_3$) δ 3.66, 3.75; GCFDMS *m*/*z* 247.1 (M−*t*-Bu)$^+$; HRGCFDMS *m*/*z* 247.11264 (M−*t*-Bu)$^+$, calcd for $C^1_{14}H^2_{11}H_4O_2Si$, 247.10924. Anal. Calcd for $C^1_{18}H^2_{20}H_4O_2Si$ requires: C, 71.00%. Found: C, 70.95%.

*4.2.2. 2-tert-Butyldiphenylsilyloxy[$^2H_4$]ethyl 4-methylbenzenesulfonate **6**.* To a solution of compound **5** (328 mg, 1.08 mmol), triethylamine (375 μL, 2.69 mmol), and trimethylammonium chloride (103 mg, 1.08 mmol) in acetonitrile (2 mL) was added a solution of *p*-chlorotoluenesulfonyl chloride (308 mg, 1.62 mmol) in acetonitrile (1 mL) at 0 °C, and stirring was continued for 1 h. After the solvent was evaporated, the reaction mixture was partitioned between EtOAc and saturated aqueous citric acid. The organic phase was washed with brine, dried over $MgSO_4$, and evaporated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc, 20:1). Tosylate **6** (470 mg, 1.02 mmol) was obtained in 95% yield. Compound **6**: colorless oil; ^1H NMR ($CDCl_3$) δ 1.00 (9H, s), 2.44 (3H, s), 7.31 (2H, d, *J*=6.5 Hz), 7.36 (4H, m), 7.43 (2H, m), 7.60 (4H, m), 7.87 (2H, d, *J*=6.5 Hz); ^{13}C NMR ($CDCl_3$) δ 19.1, 21.7, 26.7 (3C), 60.7 (1C, m), 70.2 (1C, m), 127.7 (4C), 128.0 (2C), 129.8 (4C), 132.9 (2C), 133.0, 135.6 (4C), 144.7; ^2H NMR ($CHCl_3$) δ 3.77, 4.07; ESI-MS *m*/*z* 481.2 (M+Na)$^+$; HRESI-MS *m*/*z* 481.17811 (M+Na)$^+$, calcd for $C^1_{25}H^2_{26}H_4O_4SSiNa$, 481.17773.

*4.2.3. 2-tert-Butyldiphenylsilyloxy[$^2H_4$]ethyl 1H-[^{15}N]isoindole-1,3(2H)-dione **7**.* To a solution of compound **6** (419 mg, 913 μmol) in dimethylformamide (4 mL) was added potassium ^{15}N-phthalimide (204 mg, 1.10 mmol), and the mixture was refluxed for 2 h. After the solvent was evaporated, the reaction mixture was partitioned between EtOAc and saturated aqueous $NaHCO_3$. The organic phase was washed with brine, dried over $MgSO_4$, and evaporated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc, 10:1), and compound **7** (384 mg, 884 μmol) was obtained in 97% yield. Compound **7**: colorless oil; ^1H NMR ($CDCl_3$) δ 0.98 (9H, s), 7.30 (4H, m), 7.37 (2H, m), 7.59 (4H, m), 7.71 (2H, m), 7.82 (2H, m); ^{13}C NMR ($CDCl_3$) δ 19.1, 26.7 (3C), 39.3 (m), 60.0 (m), 123.2 (2C), 27.6 (4C), 129.7 (2C), 132.1, 132.2, 133.2 (2C), 133.8 (2C), 135.5 (4C), 168.2, 168.3; ^2H NMR ($CHCl_3$): δ 3.88; ^{15}N NMR ($CDCl_3$) δ 152.8; ESI-MS *m*/*z* 457.2 (M+Na)$^+$; HRESI-MS *m*/*z* 457.18778 (M+H)$^+$, calcd for $C^1_{26}H^2_{23}H^{15}_4NO_3SiNa$, 457.18738.

*4.2.4. 2-tert-Butyldiphenylsilyloxy[$^2H_4$]ethan[^{15}N]amine **8**.* To a solution of compound **7** (384 mg, 884 μmol) in ethanol (8 mL) was added hydrazine monohydrate (130 μL, 2.68 mmol), and the mixture was refluxed for 1 h. After the precipitate was removed, the solvent was evaporated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH, 30:1) to afford amine **8** (200 mg, 656 μmol) in 74% yield. Compound **8**: colorless oil; ^1H NMR ($CDCl_3$) δ 1.06 (9H, s), 7.37 (4H, m), 7.43 (2H, m), 7.67 (4H, m); ^{13}C NMR ($CDCl_3$) δ 19.1, 26.9 (3C) 127.7 (4C), 129.7 (2C), 135.5 (4C); ^2H NMR ($CHCl_3$) δ 2.79, 3.66; ^{15}N NMR ($CDCl_3$) δ 31.1; GCFDMS *m*/*z* 305.2 (M+H)$^+$; HRGCFDMS *m*/*z* 305.20385 (M+H)$^+$, calcd for $C^1_{18}H^2_{21}H^{15}_4NO_3Si$, 305.20051.

*4.2.5. ^{15}N-Choline-d_{13} chloride **1**.* A solution of compound **8** (88.6 mg, 291 μmol) in methanol (1 mL) was treated with methyl-d_3 iodide (184 μL, 2.96 mmol) and potassium carbonate (450 mg, 3.25 mmol) at room temperature for 16 h. After the precipitate was removed, the solvent was evaporated in vacuo. To the residue was added EtOAc, and the insoluble materials were collected by filtration. These crude materials were dissolved in methanol, and then, silver oxide (137 mg, 592 μmol) was added. After stirring for 15 h at room temperature and removal of the precipitate, to the reaction mixture was added, dropwise, 1 M aqueous hydrochloric acid until pH 4, followed by evaporation of solvent. After the addition of EtOH and removal of the insoluble material, the solvent was evaporated to obtain ^{15}N-choline-d_{13} (**1**, 36.2 mg, 236 μmol) in 81% yield. Compound **1**: colorless oil; ^{13}C NMR (CD_3OD) δ 54.4 (3C, m), 57.1 (m), 68.6 (m); ^2H NMR (H_2O) δ 2.96 (9×^2H), 3.27 (2×^2H), 3.84 (2×^2H); ^{15}N NMR (D_2O) δ 41.72; ESI-MS *m*/*z* 118.2 (M−Cl)$^+$; HRESI-MS *m*/*z* 118.18569 (M−Cl)$^+$, calcd for $C^1_5H^2H^{15}_{13}NO$, 118.18562.

4.3. DNP ^{15}N NMR

4.3.1. Polarization of cholines by DNP. Samples were polarized in a HyperSense DNP polarizer (Oxford Instruments Molecular Biotools, U.K.). Each of **1** and **2** together with 15 mM of trityl free radical (OX63, GE Healthcare) was dissolved in DMSO-d_6/D_2O. The mixture was placed in the 3.35 T superconducting magnet of the DNP polarizer, frozen at 1.4 K, and irradiated with 94 GHz microwaves for 3 h. Instantaneous dissolution of the polarized and frozen sample was performed by the addition of heated and pressurized D_2O (3 mL) containing 0.025% EDTA (189 °C, 10 bar). The sample was then transferred immediately via a thin Teflon® tube into an NMR tube positioned in a broadband probe in a 9.4 T wide-bore magnet (Varian). Final concentrations of **1** and **2** were fixed at 2.2 and 2.4 mM, respectively. Natural-abundance choline-trimethyl-d_9 (**3**, 100 mg) was hyperpolarized as described above, with a final concentration of 134 mM.

4.3.2. Time-dependent ^{15}N NMR spectra and T_1 calculation of hyperpolarized cholines. The time-dependent ^{15}N NMR spectra of the hyperpolarized samples were recorded with 80 repetitions of a 10 s acquisition employing a 10° radio frequency flip angle, when the 5 mm dual probe was used. Spectral width and data points were 5 kHz and 5000, respectively. The FID data were processed with 0.5 Hz Gaussian line broadening. SNRs of the ammonium nitrogens of hyperpolarized **1** (2.4 mM) and **2** (2.1 mM) in the first ^{15}NMR spectra were 285 and 303, respectively. The thermal ^{15}N NMR

spectra of **1** (13.0 mM) and **2** (35.6 mM) were measured with the same flip angle, 10 s repetition time, 3072 scans, and an experiment duration of ca. 8.5 h. SNRs of **1** and **2** were 5.65 and 15.4, respectively. Enhancement factors for **1** and **2** were estimated as 1.68×10^4 and 1.64×10^4, respectively, by comparison of SNRs between the hyperpolarized and thermal spectra. For the 10 mm dual probe, measurements of time-dependent ^{15}N NMR spectra of hyperpolarized **1** and **2** (both 1.8 mM) were performed with 180 repetitions of a 30 s acquisition employing a 10° radio frequency flip angle. Spectral width and data points were 50 kHz and 50,000, respectively. The FID data were processed with 0.5 Hz Gaussian line broadening.

The T_1 values were obtained on the basis of comparison of the time-dependent signal decays of the polarized cholines measured by 5 mm probe, with fitting curves simulated by the equation reported by Day et al.[21]

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