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Synthetic approach for unsaturated precursors for parahydrogen induced polarization of choline and its analogs†

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Reported here are (i) a new synthetic approach for preparation of (ii) a new compound class, of –OH, for example, an –OH group is replaced with acetyl protecting group, protected 1,2-dehydrocholine analogs and (iii) a new synthetic route for betaine aldehyde. The C=C bond of 1,2-dehydrocholine moiety can be used for molecular addition of parahydrogen producing –OH protected hyperpolarized choline by parahydrogen-induced polarization (PHIP). The reported synthetic approach allows for incorporation of ^{15}N and deuterium labels, which are necessary for preparation of highly polarized PHIP contrast agents. Isotope labeling with ^{15}N and/or deuterium was conducted. Hyperpolarized ^{15}N-choline enabled by the reported synthetic approach can be potentially used as an imaging biomarker of cancer similar to choline positron emission tomography tracers.

Keywords: parahydrogen; NMR; PASADENA; PHIP; hyperpolarization; contrast agent; choline; ^{15}N; MRI

Introduction

Nuclear magnetic hyperpolarization significantly increases nuclear spin alignment by several orders of magnitude.[1] This allows for a major NMR sensitivity enhancement enabling detection of dilute metabolites in humans.[2] As a result, molecular MR imaging of diseases, such as cancer with impaired metabolism, can report on disease grading,[3] progression, response to treatment,[4] and so on. Hyperpolarized imaging is similar to positron emission tomography (PET) imaging, because both imaging modalities use exogenous contrast agents that enable molecular imaging. In contrast to PET, hyperpolarized imaging can provide more metabolic information as it can distinguish between injected contrast agent and its *in vivo* metabolites by using molecular signatures discerned by MR. The main shortcoming of hyperpolarized contrast agents is that their lifetime is limited by spin lattice relaxation time T_1. As a result, ^{13}C-enriched carboxyl sites of key biomolecules are exploited, because of their relatively long T_1 with respect to that of protons or ^{13}C sites with directly attached hydrogens. Hyperpolarized 1-^{13}C-pyruvate is the leading ^{13}C molecular contrast agent tested in phase I clinical trials for imaging of prostate cancer in men.[2,5] It reports on elevated rate of glycolysis[6] and has *in vivo* T_1 on the order of tens of seconds, which is sufficient for tracer injection and *in vivo* conversion to 1-^{13}C-lactate.

Hyperpolarized ^{15}N-choline[7] is an attractive contrast agent for molecular imaging of cancer proliferation primarily for the following reasons: (i) known rapid uptake by tumors,[8] (ii) long >2 min *in vivo* ^{15}N T_1,[9] and most importantly, (iii) its known efficacy for imaging and staging of cancer, as used in the form of PET imaging agent C11-choline or F18-choline.[10,11] Furthermore, over-expression of choline kinase and increase of choline kinase activity leading to elevated choline uptake and elevated levels of phosphocholine in tumors have been linked to tumor progression, invasiveness, drug resistance and hypoxia.[12,13]

Although 1-^{13}C-pyruvate can be efficiently polarized by dynamic nuclear polarization (DNP) to ^{13}C nuclear spin polarization $P \sim 30\%$ in approximately 1 h, DNP polarization of ^{15}N-choline is significantly less successful with $P \sim 5\%$ and polarization time of ~3 h. A faster and more efficient approach would clearly be useful for advancement of hyperpolarized ^{15}N-choline as an imaging molecular probe in preclinical models of human cancer and in potential clinical trials.

Parahydrogen-induced polarization (PHIP)[14] is a chemical hyperpolarized method that takes advantage of a singlet state of parahydrogen. The spin order of the parahydrogen molecule can be preserved during its molecular addition to an unsaturated molecular precursor. Once the symmetry of the singlet state is broken, the spin order can be converted to a large net magnetization[15] of ^{13}C site[16,17] via the mechanism of spin–spin couplings with polarization P approaching ~100%. In practice, several ^{13}C hyperpolarized compounds with $P > 10\%$ were reported using PHIP, and parahydrogen and synthesis allow dramatically enhanced nuclear alignment phenomenon[18] including 1-^{13}C-succinate[19] shown to be useful for interrogating

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† *Supporting information may be found in the online version of this article.*

cancer metabolism.[20,21] PHIP is an ultrafast polarization method capable of polarizing contrast agents within seconds to polarization of 20% and more.[22]

The PHIP requires an unsaturated carbon–carbon bond for molecular addition of parahydrogen. Therefore, a typical molecular frame is C=C–S, Figure 1A, where S can be ^{13}C, ^{15}N or any other spin ½ nucleus preferably without any directly attached protons[23,24] allowing for long *in vivo* T_1 of more than 40 s. Unfortunately, PHIP polarization of ^{15}N compounds was limited only to ^{15}N trimethylethyl ammonium. Although ultra-long *in vitro* ^{15}N $T_1 = 400$ s was reported,[25] this agent has no metabolic relevance, and it is in fact highly neurotoxic.

The requirement for an unsaturated carbon–carbon bond severely limits the number of biomolecules that can be directly polarized by PHIP. For, example, 1-^{13}C-pyruvate and ^{15}N-choline are not amenable to PHIP, because the molecular framework is either not feasible or unstable as in the case of unsaturated molecular precursor for ^{15}N-choline. The corresponding enol would simply collapse to betaine aldehyde, Figure 1B. A potential solution to circumvent this challenge is the use of –OH protected, for example, an –OH group is replaced with acetyl protecting group, unsaturated precursors. This approach was recently demonstrated for PHIP of ethanol[26] and 1-^{13}C-phospholactate[27] by using acetate and phosphate moieties as protecting groups, respectively. The latter showed nearly complete molecular hydrogenation using water soluble Rh-based[28] catalyst and sufficiently long ^{13}C T_1 suitable for *in vivo* imaging. Unlike 1-^{13}C-phosphoenolpyruvate, which is a PHIP precursor for hyperpolarized 1-^{13}C-phospholactate[27] and is commercially available, unsaturated PHIP precursors for protected ^{15}N-choline have not been reported to the best of our knowledge.

Here, a new synthetic approach for a new class of compounds, unsaturated protected cholines, is described and exemplified by acetyl 1,2-dehydrocholine. This synthetic scheme allows for incorporation of ^{15}N and deuterium labels. Although the requirement for spin ^{15}N labeling is straightforward as it allows for sensitivity increase by >200 fold compared with the natural abundance level of ^{15}N, complete or partial deuteration of the precursor is necessary to simplify the PHIP spin system to three spins—^{15}N and two nascent protons from parahydrogen—to maximize the quantum yield of the polarization transfer sequence.[16] The latter should lead to high PHIP polarization in future studies, which are outside the scope of this work.

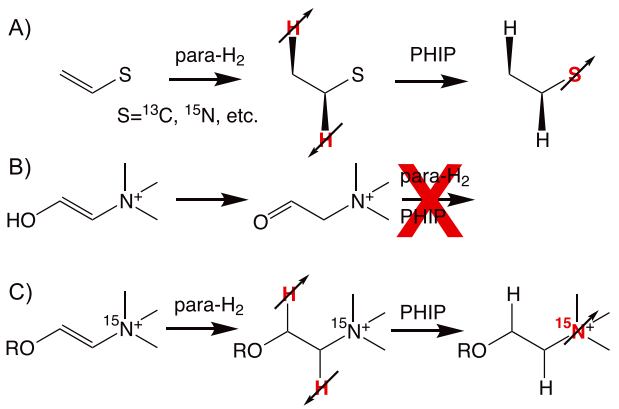

Figure 1. The molecular diagrams of parahydrogen induced polarization (PHIP). (A) General diagram of spin ½ S nucleus (S = ^{13}C, ^{15}N, etc.) polarization using spin order of parahydrogen (para-H_2) added to an unsaturated carbon–carbon bond via molecular mechanism. (B) Diagram showing collapse of C=C bond in unstable hypothetical precursor 1,2-dehydrocholine leading to formation of betaine aldehyde that is not suitable for PHIP hyperpolarization. (C) The diagram of molecular hydrogenation of OH protected 1,2-dehydrocholine that can be potentially used for PHIP hyperpolarized leading to hyperpolarized protected ^{15}N-choline.

Experimental section

General

All solvents were purchased from common vendors and were used as received. All new compounds were characterized by ^1H and ^{13}C NMR and HR-MS. High-resolution mass spectra were recorded using a Synapt hybrid quadrupole/oa-TOF Mass Spetrometer (Waters Corp., Milford, MA) equipped with a dual chemical ionization/electrospray (ESCI) source. A post-acquisition gain correction was applied using a solution of 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, FW 614.88, Sigma-Aldrich 3050 Spruce St. St. Louis, MO 63103, USA) as the lock mass spray.

Synthesis of new compounds

2,2-Dihydroxy-N,N,N-trimethylethanaminium bromide or betaine aldehyde bromide (2a)

Allyltrimethylammonium bromide[29] **1a** (3 g, 16.7 mmol) was dissolved in methanol (300 mL) and water (2 mL). A small amount of Rose bengal was added, and the solution was cooled to −78 °C. Ozone was passed through the solution until the pink color of Rose bengal almost completely disappeared, and solution was flushed with air for 20 min. Dimethyl sulfide (30 mL) was added, and the reaction mixture was left at room temperature overnight. The solvent was removed under reduced pressure, and the resulting residue was washed with anhydrous acetone (3 × 50 mL). The solid residue was dried *in vacuo* for an additional 20 min. Then it was precipitated from anhydrous MeOH by acetone with 5:95 volume ratio. Crystals were washed with anhydrous ether, yielding betaine aldehyde bromide **2a** with 62% yield (2.07 g, 10.3 mmol).

2-Acetoxy-N,N,N-trimethylethenaminium bromide (3a)

Betaine aldehyde bromide **2a** (0.20 g, 1.0 mmol) dried overnight in lyophilizer was suspended in 20 mL of acetic anhydride. After 12 h, reaction mixture was filtered, and the filtrate was evaporated to dryness. Mixture of *E*- and *Z*-acetyl 1,2-dehydrocholine bromide was obtained as a major product with 20% yield (0.045 g), *E*:*Z* ratio of 1:0.46. The structure of the major isomer was elucidated using ^1H, ^{13}C, COSY, HMBC, select NOE high-resolution NMR spectra. *E*-acetyl 1,2-dehydrocholine bromide: ^1H (DMSO-d_6, 400 MHz) δ: 7.93 (d, 1H, J(HH) = 11.6 Hz), 6.87 (d, 1H, J(HH) = 11.6 Hz), 3.34 (s, 9H), 2.23 (s, 3H); $^{13}C\{^1H\}$ (DMSO-d_6, 100 MHz) δ: 167.2, 136.3, 127.3, 55.2, 20.2. *Z*-acetyl 1,2-dehydrocholine bromide: ^1H (DMSO-d_6, 400 MHz) δ: 7.30 (d, 1H, J(HH) = 5.6 Hz), 5.92 (d, 1H, J(HH) = 5.6 Hz), 3.40 (s, 9H), 2.30 (s, 3H); ^1H (D_2O, 400 MHz) δ: 7.26 (d, 1H, J(HH) = 5.2 Hz), 5.76 (d, 1H, J(HH) = 5.6 Hz), 3.37 (s, 9H), 2.23 (s, 3H).

Allyltrimethylammonium chloride (1b)

Trimethylamine hydrochloride (5 g, 52 mmol) was dissolved in dry ethanol (200 mL). The solution was cooled to 0 °C, and ice cold 1 M NaOH ethanol solution (52 mL, 52 mmol) was added slowly. After 10 min, allyl bromide (4.5 mL, 52 mmol) was added drop-wise. The reaction mixture was left overnight at room temperature and then concentrated at reduced pressure by using a rotary evaporator. The resulting solid was re-suspended in 300 mL of dry ethanol. The solution was filtered to remove sodium chloride/bromide salt. The resulting filtrate was passed through an ion exchange column (~400 mL of IRA-400 Cl beads, total volume capacity 1.6 eq/L). The column was washed with excess ethanol, and the resulting solution was concentrated *in vacuo*. The solid residue was precipitated from ethanol (100 mL) by addition of diethyl ether (700 mL) resulting in allyltrimethylammonium chloride **1b** (5 g) with 71% yield.[30]

Betaine aldehyde chloride (2b)

Allyltrimethylammonium chloride **1b** (2 g, 15 mmol) was dissolved in methanol (300 mL) and water (2 mL) solution. A small amount of Rose bengal was added, and the solution was cooled to −78 °C. Ozone was passed through the solution until the pink color of Rose bengal completely disappeared. Dimethyl sulfide (30 mL) was added, and the reaction mixture was left at room temperature overnight. The solvent was removed at reduced pressure, and the resulting residue was washed with anhydrous acetone (3 × 50 ml). The solid residue was dried *in vacuo* for an additional 20 min. Then it was precipitated from 60 mL of anhydrous methanol followed by the addition of anhydrous diethyl ether (750 mL). Betaine aldehyde chloride[31] **2b** (1.77 g) was produced with 76% yield as a white, extremely hygroscopic solid. ^{1}H (DMSO-d_6, 400 MHz) δ: 6.73 (d, 2H, J(HH) = 6.1 Hz), 5.26 (p, 1H, J(HH) = 5.8 Hz), 3.26 (d, 2H, J(HH) = 5.4 Hz) 3.13 (s, 9H); ^{13}C (DMSO-d_6, 100 MHz) δ: 84.9, 68.5, 53.4. Note: upon dehydration ^{1}H NMR peak at 6.73 ppm disappears and ^{1}H NMR peak at 5.25 ppm collapses into a triplet. ^{1}H (D_2O, 400 MHz) δ: 5.46 (t, 1H, J(HH) = 5.5 Hz), 3.33 (d, 2H, J(HH) = 5.5 Hz), 3.13 (s, 9H); ^{13}C{^{1}H} (D_2O, 100 MHz) δ: 84.9 (d, J = 3.9 Hz), 68.4 (t, J = 3.0 Hz), 53.4 (dd, J_1 = 3.0 Hz, J_2 = 3.9 Hz). Note: upon prolonged storage in D_2O for more than 4 h, ^{1}H NMR peak at 3.33 ppm and ^{13}C NMR peak at 68.4 ppm disappear because of H/D exchange.

2-Acetoxy-N,N,N-trimethylethenaminium chloride (3b)

Betaine aldehyde chloride **2b** (1.57 g, 10.1 mmol) was suspended in 400-mL acetic anhydride. After 24 h, reacted solution was evaporated to dryness. The residue was submerged in additional 200-mL acetic anhydride and evaporated to dryness. After 4 h *in vacuo*, the residue was partially re-dissolved in 250-mL acetic anhydride and filtrated. Filtrate was precipitated by addition of dry diethyl ether (1500 mL) producing a mixture of *E*- and *Z*-acetyl 1,2-dehydrocholine chloride **3b** and diacetylbetaine aldehyde **4b** (1.2 g) molar ratio of *Z*-:*E*-:diac of 0.25:1.00:0.74. Note: *E*-/*Z*- ratio varies from batch to batch and depends on storage time. *E*-acetyl 1,2-dehydrocholine chloride ^{1}H (DMSO-d_6, 600 MHz) δ: 7.91 (d, 1H, J(HH) = 11.6 Hz), 6.97 (d, 1H, J(HH) = 11.6 Hz), 3.37 (s, 9H), 2.22 (s, 3H); ^{13}C{^{1}H} (DMSO-d_6, 125 MHz) δ: 167.2, 136.2, 127.4, 55.1, 20.3; ^{1}H (D_2O, 500 MHz) δ: 7.90 (d, 1H, J(HH) = 11.5 Hz), 6.58 (d, 1H, J(HH) = 11.5 Hz), 3.31 (s, 9H), 2.15 (s, 3H); ^{13}C{^{1}H} (D_2O, 125 MHz) δ: 169.5, 136.8, 127.1, 55.8, 19.9. *Z*-acetyl 1,2-dehydrocholine chloride ^{1}H (DMSO-d_6, 600 MHz) δ: 7.28 (d, 1H, J(HH) = 5.6 Hz), 5.95 (d, 1H, J(HH) = 5.6 Hz), 3.42 (s, 9H), 2.30 (s, 3H); ^{1}H (D_2O, 500 MHz) δ: 7.27 (d, 1H, J(HH) = 5.5 Hz), 5.77 (d, 1H, J(HH) = 5.5 Hz), 3.38 (s, 9H), 2.24 (s, 3H); ^{13}C{^{1}H} (D_2O, 125 MHz) δ: 167.3, 132.2, 119.0, 56.3, 19.8. Diacetylbetaine aldehyde ^{1}H (DMSO-d_6, 600 MHz) δ: 6.99 (t, 1H, J(HH) = 4.8 Hz), 3.88 (d, 2H, J(HH) = 4.9 Hz), 3.22 (s, 9H), 2.10 (s, 6H); ^{13}C{^{1}H} (DMSO-d_6, 125 MHz) δ: 168.1, 84.5, 64.2, 53.5, 20.5; ^{1}H (D_2O, 500 MHz) δ: 7.08 (t, 1H, J(HH) = 4.4 Hz), 3.78 (d, 2H, J(HH) = 4.4 Hz), 3.21 (s, 9H), 2.09 (s, 6H); ^{13}C{^{1}H} (D_2O, 125 MHz) δ: 170.6, 84.5, 65.1, 54.6, 20.0.

^{15}N-Allyltrimethylammonium bromide (1c)

It was prepared from $Me_3^{15}N$•HCl, 98% ^{15}N, Sigma-Aldrich-Isotec, Miamisburg, OH, #608831, (0.96 g, 10 mmol) in manner similar to that of **1b**, but without the anion exchange. Yield = 1.34 g (74%). ^{1}H (CD_3OD, 400 MHz) δ: 6.10 (m, 1H), 5.74 (m, 1H), 5.71 (d of m, 1H), 4.00 (d, 2H, J(HH) = 7.5 Hz), 3.13 (d, 9H, J(H-^{15}N) = 0.7 Hz); ^{13}C{^{1}H} (CD_3OD, 100 MHz) δ: 129.5 (d, J(^{13}C-^{15}N) = 1.6 Hz), 126.5, 69.5 (d, J(^{13}C-^{15}N) = 4.3 Hz), 53.2 (d, J(^{13}C-^{15}N) = 5.8 Hz); ^{15}N{^{1}H} (CD_3OD, 40 MHz, auto calibrated) δ: 48.87. HR-MS calculated for $C_6H_{14}^{15}N$: 101.1091; found: 101.1092 (0.99 ppm).

^{15}N-2-Acetoxy-N,N,N-trimethylethenaminium bromide (3c)

^{15}N-betaine aldehyde bromide was prepared in a manner similar to that of **2a** from compound **1c** (1.30 g). No purification was performed, and it was used directly in the next step. Acetyl ^{15}N-1,2-dehydrocholine bromide **3c** was prepared and purified in a manner similar to that of **3b**. The reaction produced acetyl ^{15}N-1,2,-dehydrocholine bromide **3c** and diacetyl-^{15}N-betaine aldehyde bromide **4c** (1.09 g) with molar ratio of *Z*-:*E*-:diac of 0.3:1.0:0.9. *E*-acetyl ^{15}N-1,2-dehydrocholine bromide ^{1}H (DMSO-d_6, 400 MHz) δ: 7.92 (dd 1H, J(H-^{15}N) = 1.5 Hz and J(HH) = 11.6 Hz), 6.89 (dd, 1H, J(H-^{15}N) = 3.2 Hz and J(HH) = 11.6 Hz), 3.35 (broad s, 9H), 2.23 (s, 3H); ^{13}C{^{1}H} (DMSO-d_6, 100 MHz) δ: 167.2, 136.3 (d, J(^{13}C-^{15}N) = 2.4 Hz), 127.3 (d, J(^{13}C-^{15}N) = 9.6 Hz), 55.1 (d, J(^{13}C-^{15}N) = 5.1 Hz), 20.2; ^{15}N (DMSO-d_6, 40 MHz, auto calibrated) δ: 51.3; ^{1}H (D_2O, 400 MHz) δ: 7.87 (dd, 1H, J(H-^{15}N) = 1.5 Hz and J(HH) = 11.6 Hz), 6.54 (dd, 1H, J(H-^{15}N) = 3.4 and J(HH) = 11.6 Hz), 3.28 (d, 9H, J(H-^{15}N) = 0.6 Hz), 2.11 (s, 3H); ^{15}N{^{1}H} (D_2O, 40 MHz, auto calibrated) δ: 50.1. *Z*-acetyl ^{15}N-1,2-dehydrocholine bromide ^{1}H (DMSO-d_6, 400 MHz) δ: 7.29 (t, 1H, J(HH) = 5.5 Hz), 5.92 (dd, 1H, J(H-^{15}N) = 3.6 Hz and J(HH) = 5.5 Hz), 3.41 (d, 9H, J(H-^{15}N) = 0.6 Hz), 2.30 (s, 3H); ^{13}C{^{1}H} (DMSO-d_6, 100 MHz) δ: 165.8, 131.6, 119.9 (d, J(^{13}C-^{15}N) = 7.9 Hz), 55.7 (d, J(^{13}C-^{15}N) = 5.0 Hz), 20.5; ^{15}N (DMSO-d_6, 40 MHz, auto calibrated) δ: 52.0; ^{1}H (D_2O, 400 MHz) δ: 7.24 (t, 1H, J(HH) = 5.6 Hz), 5.73 (dd, 1H, J(H-^{15}N) = 3.7 Hz and J(HH) = 5.6 Hz), 3.34 (d, 9H, J(H-^{15}N) = 0.6 Hz), 2.21 (s, 3H); ^{15}N{^{1}H} (D_2O, 40 MHz, auto calibrated) δ: 50.4. ^{15}N-diacetylbetaine aldehyde bromide ^{1}H (DMSO-d_6, 400 MHz) δ: 6.99 (dt, 1H, J(H-^{15}N) = 1.2 Hz and J(HH) = 4.8 Hz), 3.84 (d, 2H, J(HH) = 4.8 Hz), 3.21 (s, 9H), 2.10 (s, 6H); ^{13}C{^{1}H} (DMSO-d_6, 100 MHz) δ: 168.1, 84.5, 64.3 (d, J(^{13}C-^{15}N) = 5.2 Hz), 53.6 (d, J(^{13}C-^{15}N) = 5.1 Hz), 20.5; ^{15}N {^{1}H} (DMSO-d_6, 40 MHz, auto calibrated) δ: 48.1; ^{1}H (D_2O, 400 MHz) δ: 7.05 (dt, 1H, J(H-^{15}N) = 1.7 Hz and J(HH) = 4.5 Hz), 3.75 (d, 2H, J(HH) = 4.5 Hz), 3.17 (s, 9H), 2.06 (s, 6H); ^{15}N{^{1}H} (D_2O, 40 MHz, auto calibrated) δ: 47.1.

^{15}N-Allyltrimethylammonium-d_{12} bromide (1d)

It was prepared from $(CD_3)_3^{15}$N•HCl, 98% ^{15}N, 99% D, Sigma-Aldrich-Isotec, #591815, (2.00 g, 19 mmol) and allylbromide-d_5 (2.51 g, 20 mmol) in a manner similar to that of **1b** except eliminating the anion-exchange step. Yield = 84% (3.12 g). ^{13}C{^{1}H} (CD_3OD, 100 MHz) δ: 128.9 (m), 125.9 (t), 68.3 (m), 52.2 (m); ^{15}N{^{1}H} (CD_3OD, 40 MHz, auto calibrated) δ: 47.4. HR-MS calculated for $C_6^{15}ND_{14}$: 115.1970; found: 115.1971 (0.87 ppm).

^{15}N-2-Acetoxy-N,N,N-trimethylethenaminium-d_{11} bromide (3d)

^{15}N-betaine aldehyde-d_{14} bromide was prepared in a manner similar to that of **2c** starting from compound **1d** (0.95 g, 4.7 mmol). Methanol-d_1 and acetone-d_6 were used instead of regular methanol and acetone. No purification was performed and ^{15}N-betaine aldehyde-d_{14} bromide was used directly in the next step. Acetyl ^{15}N-1,2-dehydrocholine-d_{11} bromide **3d** was prepared from crude ^{15}N-betaine aldehyde-d_{14} bromide in a manner similar to that of **3c**. No recrystallization was attempted. The reaction produced acetyl ^{15}N-1,2-dehydrocholine-d_{11} bromide **3d** and diacetyl-^{15}N-betaine aldehyde-d_{11} bromide **4d**. Yield over two steps = 0.99 g with molar ratio of *Z*-:*E*-:diac was 0.9:1.0:1.2. *E*-acetyl ^{15}N-1,2-dehydrocholine-d_{11} bromide ^{1}H (D_2O, 400 MHz) δ: 6.55 (m, 0.62H), 2.11 (s, 3H). *Z*-acetyl ^{15}N-1,2-dehydrocholine-d_{11} bromide ^{1}H (D_2O, 400 MHz) δ: 5.75 (dd, 0.79H), 2.21 (s, 3H). Diacetyl-^{15}N-betaine aldehyde-d_{11} bromide ^{1}H (D_2O, 400 MHz) δ: 2.09 (s, 6H).

Results and discussion

Despite scarce reports[32,33] of ketone-derived ylides, neither unsaturated choline analog nor any closely related compounds have been reported. Therefore, the synthetic approach to protected ^{15}N-choline PHIP precursor was divided into two parts: (i) development of synthetic methodology without enrichment by stable isotopes and (ii) synthesis of ^{15}N and deuterium isotopically enriched molecular precursors. A number of retrosynthetic approaches can be envisioned, Figure 2. Advantages and disadvantages of these retrosynthetic strategies are briefly discussed in the succeeding paragraphs.

The direct coupling approach # 1 shown in Figure 2 is potentially viable because of the relative ease of preparation of acetoxy-,[34] silyloxy-,[35] benzoxy-haloalkenes.[36] Unfortunately, direct replacement of vinylic halogen is extremely difficult, because alkylic halides, which are more active, react rather slowly with trimethylamine.[37]

Both ethoxy-[38] and benzoxy-acetylenes[36] shown in approach #2, Figure 2, have been prepared previously by relatively

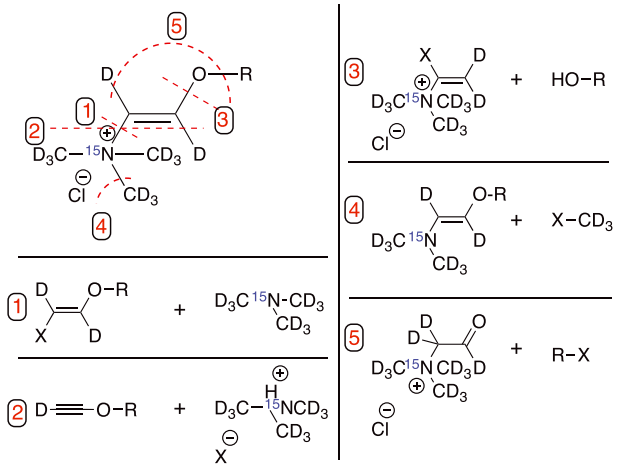

Figure 2. Five potential retrosynthetic approaches for preparation of protected ^{15}N-choline PHIP precursor. The desired precursor shown in upper left corner has ^{15}N label and 11 deuterium labels. The outlined five strategies are depicted with dashed red lines highlighting potential molecular assembling routes. X refers to a halogen atom. R refers to a protecting group such as alkyl, silyl or carbonyl.

straightforward methodologies. Ethoxyacetylene has been shown to react with trimethylamine, albeit with reverse regioselectivity, yielding (α-ethoxyvinyl)trialkylammonium hydroxides[39] unsuitable for ^{15}N-choline PHIP.

Herkes and Simons[40] have shown a possibility of alkoxide addition to 1-bromovinyltrimethylammonium bromide, approach #3, Figure 2. The reaction with methanol (R = $-CH_3$) produced a mixture of 1-methoxyvinyltrimethylammonium bromide as well as an unknown product that was assigned as 2-methoxyvinyltrimethylammonium bromide. Despite all isolation attempts of 2-methoxyvinyltrimethylammonium bromide by fractional precipitation employing ethanol-ether or by recrystallization (acetonitrile) resulted in 1-methoxyvinyltrimethylammonium bromide as the only product.[40]

Brookhart and co-workers[41] have recently developed a transformation capable of producing silyl enol ethers similar to that shown in approach #4, Figure 2. Although this is by far the most promising methodology among all other approaches discussed earlier, it has two major disadvantages. First, isotopic labeling would require rather complex multistep synthesis. Second, this methodology is limited to the ethyl dimethyl silyl protecting group.

Enolization following by subsequent protection has been implemented in preparation of unsaturated t-butyl substituted phosphocholine analogs.[33] This methodology relies on stability of ylides derived from 2-trialkylammonium ketones with bulky substituents on the other side of the carbonyl group, approach #5, Figure 2.[32] Unfortunately, this methodology is not applicable to the 2-trimethylammonium ethanal, betaine aldehyde, because of the competing aldol condensation. Therefore, a methodology for a betaine aldehyde enolization-protection under the mild conditions was developed.

A previously reported procedure[31] required 2-day-long reaction at 100 °C in a sealed tube and produced only 2.3% betaine aldehyde yield after two steps. Insertion of rather expensive deuterium and ^{15}N spin labels via betaine aldehyde required significantly more efficient synthetic route, which was not previously available to the best of our knowledge. Moreover, although betaine aldehyde with natural abundance level of ^{15}N and deuterium isotopes is commercially available, it is very expensive for synthetic studies and large-scale production of contrast agents. For example, the Sigma-Aldrich price was $594 per 100 mg. Significantly more efficient and arguably more cost-effective methodology for preparation of betaine aldehyde is presented here. Moreover, betaine aldehyde produced in this fashion is amenable to isotopic ^{15}N enrichment using relatively inexpensive, commercially available trimethylamine hydrochloride with ^{15}N and/or deuterium isotopically enriched nuclei.

Allyltrimethylammonium bromide (**1a**) synthesized using previously described procedure[29] was ozonized in MeOH/H_2O and reduced with dimethyl sulfide in a manner similar to that described by Pappas and Keaveney, Figure 3, yielding betaine aldehyde (**2a**).[42]

This procedure, Figure 3A, allows producing large quantities of betaine aldehyde in its bromide form. Betaine aldehyde is a stable 1,1-diol (stable hydrated form of betaine aldehyde) even in its crystalline form. Several procedures, i–vi, Figure 3B, were evaluated for converting compound **2a** to a stable protected PHIP choline precursor with a double carbon–carbon bond. Either starting material or polymerized products were obtained in all cases. However, betaine aldehyde was found to react with excess acetic anhydride, Figure 3C, resulting in acetyl 1,2-dehydrocholine with ~20% yield and with variable ratios of *E* and *Z* isomer.

Although the primary goal of this study is the development of an efficient synthetic route to a water-stable, isotopically enriched unsaturated choline PHIP precursor, the chemistry described here is new as well as the reaction mechanism, which is of interest from a fundamental perspective. The compound **3a** was exhaustively characterized by multinuclear high-resolution NMR spectroscopy: 1D proton spectroscopy, 1D ^{13}C spectroscopy, COSY, ^{1}H-^{13}C HMBC and selective NOE, Figs. S1–S7, supplementary material. These studies provided peak assignments and structural characterization, Fig. S8.

Acetyl 1,2-dehydrocholine bromide (**3a**) is soluble in water, which is one of the major prerequisites for biomedical

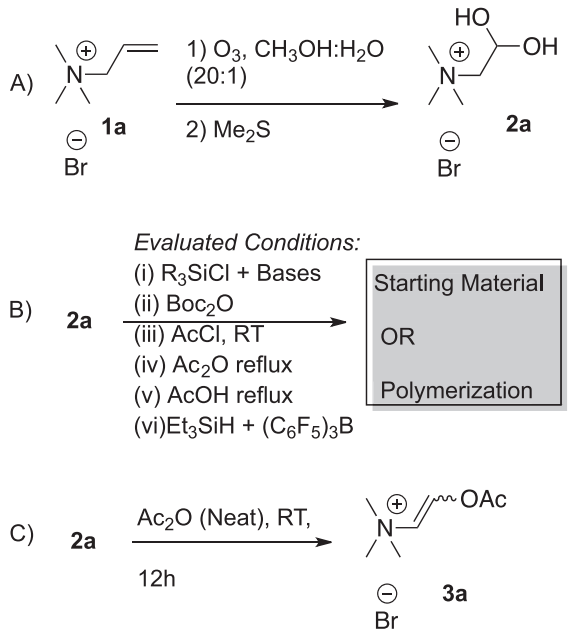

Figure 3. Synthetic scheme for preparation of betaine aldehyde bromide (**2a**) and acetyl protected 1,2-dehydrocholine bromide (**3a**). (A) New betaine aldehyde synthesis. (B) Unsuccessful attempts to convert betaine aldehyde into protected 1,2-dehydrocholine. (C) Successful preparation scheme for acetyl 1,2-dehydrocholine.

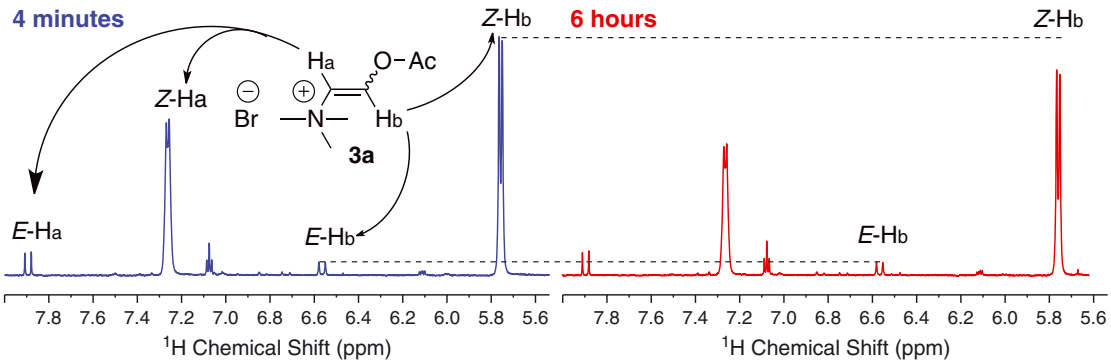

Figure 4. Proton high-resolution NMR spectra of compound **3a**: spectrum in blue color represents 'alkene region' of **3a** after being dissolved for 4 min in D_2O, spectrum in red corresponds to the same sample after 6 h.

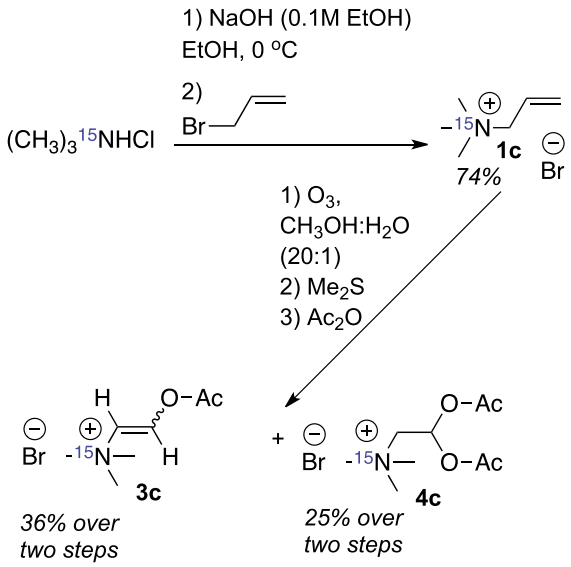

Figure 5. Synthetic scheme for preparation of acetyl ^{15}N-1,2-dehydrocholine bromide (**3c**) using ^{15}N-trimethylammonium hydrochloride as a starting material. Achieved percentage yields are provided.

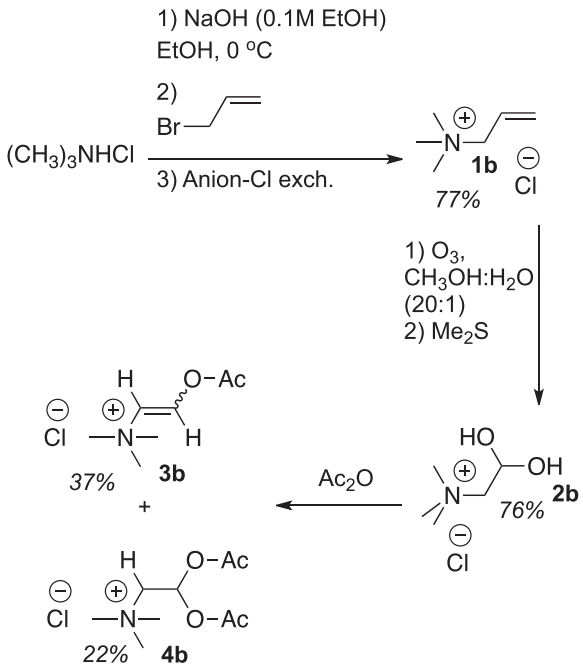

Figure 6. Synthetic scheme for preparation of betaine aldehyde chloride (**2b**) and acetyl protected 1,2-dehydrocholine chloride (**3b**) using trimethylammonium hydrochloride as a starting material enabling potential introduction of ^{15}N spin label from commercially available $(CH_3)_3$ ^{15}N•HCl and $(CD_3)_3$ ^{15}N•HCl. Achieved percentage yields are provided.

application of PHIP polarized contrast agents. A crude reaction mixture containing product **3a** and starting material **2a** was dried at reduced pressure and re-dissolved in 99.8% deuterium oxide. Proton high-resolution NMR spectra recorded after 4 min and 6 h, Figure 4, demonstrate the stability of this new compound in aqueous medium. Although a modest ~15% decomposition of **3a** was observed after 6 h as measured by NMR signal intensities, the slow decay rate is very encouraging, because the PHIP polarization process takes less than 2 min. Therefore, overall **3a** stability in aqueous medium is satisfactory.

The starting material to introduce the ^{15}N PHIP spin label is $(CH_3)_3^{15}$N•HCl (^{15}N-trimethylamine hydrochloride, Figure 5) rather than allyltrimethylammonium bromide shown in Figures 3A. As a result of required chloride as a starting material, the previously described procedure[29] was modified to accommodate bromide to chloride ion substitution. The synthetic scheme shown in Figure 6 allows potentially introducing the ^{15}N spin label from commercially available $(CH_3)_3^{15}$N•HCl and $(CD_3)_3^{15}$N•HCl. The viability of this modified synthetic route was initially tested with non-isotopically enriched compounds, because the latter are significantly less expensive than isotopically enriched ones. Moreover, stable ^{15}N and D isotopes exponentially complicate structure characterization and validation of final products and intermediates by both high-resolution NMR and mass spectrometry because of the presence of hetero-nuclear spin-spin couplings and the complex distribution of molecular masses in the case of partial enrichment.

In the first step, $(CH_3)_3$ N•HCl, trimethylamine hydrochloride, was converted to its free base by reaction with ethanolic solution of sodium hydroxide and reacted with allyl bromide. Anion exchange on an IRA-400 Cl column afforded known allyltrimethylammonium chloride (**1b**),[30] which in turn was converted to betaine aldehyde (**2b**) by an ozonolysis-reduction procedure described for (**2a**). In the absence of extensive drying of betaine aldehyde chloride improved overall yield of the desired product (**3b**) but produced substantial quantities of a side product 1,1-diacetyl choline chloride (**4b**). The final product was evaluated by means of 1D

and 2D high-resolution NMR spectroscopy in both DMSO-d_6 as well as aqueous medium, 99.8% D_2O: 1D proton spectroscopy, 1D ^{13}C spectroscopy, ^{1}H-^{13}C HSQC and NOESY, Figs. S9–S21, supplementary material. The proven stability and solubility in water, and improved yield of the final product are essential for biomedical application of this class of molecules as hyperpolarized contrast agents.

Although the ultimate goal is the development of a fully ^{15}N and deuterium labeled acetyl ^{15}N-1,2-dehydrocholine-d_{11} chloride analog, the preparation of ^{15}N-1,2-dehydrocholine chloride was developed first, because the former requires more expensive starting materials and significantly complicates structure characterization and validation by both high-resolution NMR and mass spectrometry because of the presence of 11 deuterons. No anion exchange was performed to minimize potential loss of expensive ^{15}N isotope label. Previous experience has shown that ozonolysis-reduction produces relatively pure betaine aldehyde chloride. Moreover, betaine aldehyde is extremely hygroscopic, and it is prone to undergo partial polymerization during extensive exposure to room temperature. Therefore, its recrystallization was replaced by repeated acetone washes in this procedure. Crude ^{15}N-labeled betaine aldehyde bromide was applied to the final step producing a mixture of desired acetyl ^{15}N-1,2-dehydrocholine bromide (**3c**) as well as diacetyl ^{15}N-choline bromide (**4c**) after recrystallization with relatively good yields. The presence of **3c** and **4c** was confirmed by means of 1D high-resolution NMR spectroscopy, Figs. S25–S29 (supplementary material): 1D proton, $^{13}C\{^{1}H\}$, $^{15}N\{^{1}H\}$ NMR spectroscopy and high-resolution mass spectrometry. We also report 1D proton, $^{13}C\{^{1}H\}$, $^{15}N\{^{1}H\}$ high-resolution NMR spectra of compound **1c**, Figs. S22–S24, supplementary material.

Although compound **3c** carries a ^{15}N spin label for preparation of potential PHIP, long-lived hyperpolarized states, it has too many protons with the anticipated outcome of dramatically decreased PHIP polarizing efficiency. This problem is usually mitigated by simplifying the spin system of the PHIP contrast agent to three spins: a long-lived heteronucleus and two nascent protons after parahydrogen addition. The key prerequisite for the molecular structure of unsaturated ^{15}N PHIP precursor is the absence of any *J*-coupled protons to the ^{15}N heteronucleus. This has been demonstrated with many ^{13}C hyperpolarized contrast agents, when protons adjacent to the double bond were replaced with deuterons.[19,21,43,44] To introduce deuterium in the molecular structure of PHIP precursor, ^{15}N-trimethylamine-d_9 hydrochloride and allyl bromide-d_5 were used as starting materials, Figure 7. Note that deuteration of the protecting acetyl group is not necessary, because its methyl protons are not *J*-coupled to ^{15}N. Preparation of **1d** was similar to that of **1b**, whereas synthesis of final product **3d** required deuterated solvents D_2O and methanol-d_1 during the ozonolysis step. Because of relatively high cost of ^{15}N-trimethylamine-d_9 hydrochloride, no recrystallization of final product **3d** was attempted. The presence of **3d** and **4d** was confirmed by means of 1D high-resolution NMR spectroscopy, Figs. S25–S29 (supplementary material): 1D proton and $^{13}C\{^{1}H\}$ NMR spectroscopy. We also report $^{13}C\{^{1}H\}$, $^{15}N\{^{1}H\}$ high-resolution NMR spectra of compound **1d**, Figs. S30–S31, supplementary material.

^{15}N enrichment is nearly 100% in starting materials as confirmed by expected proton splitting patterns. Deuterium enrichment is expected to be similar to ^{15}N for non-exchangeable sites, but it is likely to be more challenging for exchangeable sites in the final product or intermediates. For example, deuterons of methyl quaternary amine and deuteron in position Hb in **3d** are non-exchangeable during synthesis progress. As a result, the residual protons are almost non-detectable by proton NMR spectroscopy, Figure 8. However, deuterons in position Ha can potentially exchange during synthesis. A likely step is conversion of ^{15}N-betaine aldehyde-d_{11} into **3d**. This step

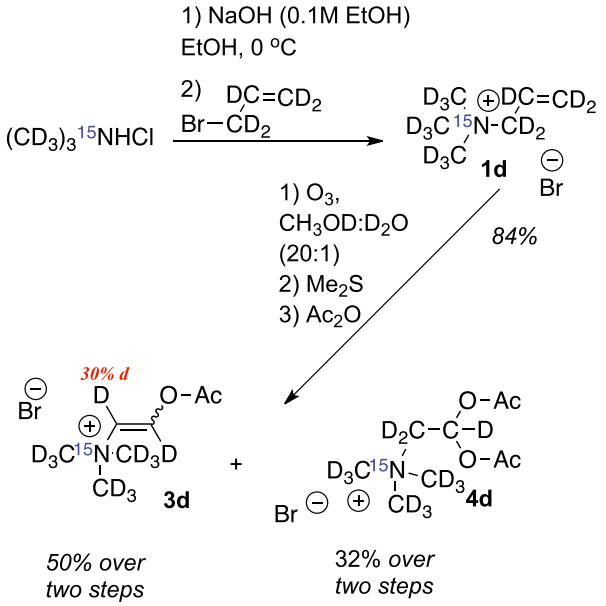

Figure 7. Synthetic scheme for preparation of acetyl ^{15}N-1,2-dehydrocholine-d_{11} bromide (**3d**) using ^{15}N-trimethylamine-d_9 hydrochloride and allyl bromide-d_5 as starting materials. Achieved percentage yields are provided. Note the deuterium position susceptible to isotopic exchange and achieved % deuteration.

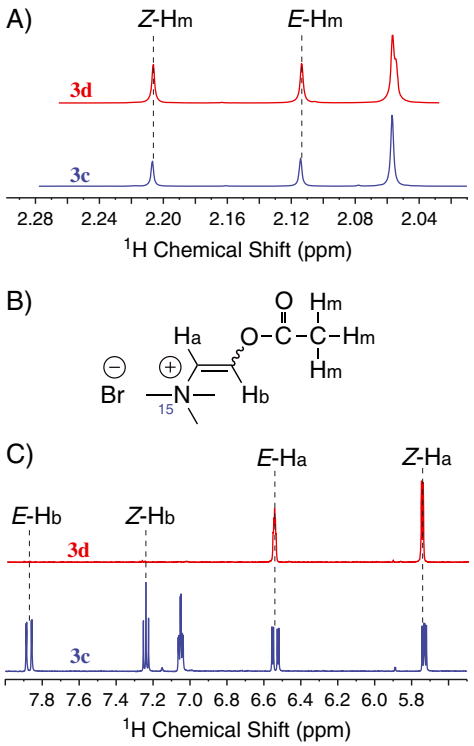

Figure 8. Proton high-resolution NMR spectra of compounds **3d** and **3c** and molecular diagram of acetyl ^{15}N-1,2-dehydrocholine showing proton positions. (A) Acetyl methyl spectral region used for 'fingerprinting' of compound **3d** lacking most of its other protons. (B) Molecular diagram of acetyl ^{15}N-1,2-dehydrocholine. (C) Vinyl spectral region used for % D.

requires large quantities of acetic anhydride, and a small acetic acid impurity is a likely explanation for acid catalyzed deuterium-hydrogen exchange via keto-enol tautomerization. As a result, deuteration of position Ha is only 30%, Figure 7. This potentially can be improved by additional purification of the acetic anhydride, which will be addressed in the future work.

The PHIP hydrogenation is not stereospecific[27] in the sense that both *E*- and *Z*-acetyl ^{15}N-1,2-dehydrocholine-d_{11} can serve as a potentially new precursor for PHIP hydrogenation with parahydrogen to produce ^{15}N hyperpolarized protected choline in a manner similar to recently demonstrated phosphate-protected ^{13}C hyperpolarized phospholactate-1-^{13}C.[27] Although the mechanism of this newly reported synthetic route for preparation of –OH protected unsaturated choline analogs is outside of the scope of this work, we can speculate that *Z*-isomer is produced as the major and perhaps the only product of this transformation, Figures 6 and 7. This speculation is supported by observation that a significantly greater proportion of *Z*-isomer is detected by NMR in the first 5–8 h of the final reaction step with acetic anhydride. Subsequent NMR measurements consistently detected more *E*-isomer and diacetylbetaine aldehyde. The latter is expected because of formation of the side product acetic acid during reaction of acetic anhydride and betaine aldehyde. Produced in this manner, acetic acid is likely responsible for yielding diacetylbetaine aldehyde and catalyzing *Z*- to *E*-isomer conversion. Although the presence of both *E*- and *Z*- isomers is not likely to impact PHIP hyperpolarization efficiency, diacetylbetaine aldehyde is clearly an undesirable product that cannot contribute to production of hyperpolarized ^{15}N choline analogs because it lacks a double C=C bond.

Use of a phosphate group for protection of C=C–O– has been shown as a promising strategy significantly expanding the range of potential biomolecules amenable to PHIP hyperpolarization.[27] The transformation of betaine aldehyde to 1,2-dehydrocholine analogs as demonstrated here can be potentially tailored to preparation of ^{15}N-1,2-dehydrophosphocholine-d_{11}. This is highly desirable, because phosphate protecting groups are resilient to hydrogenation. Therefore, PHIP hydrogenation of ^{15}N-1,2-dehydrophosphocholine-d_{11} would yield hyperpolarized ^{15}N-phosphocholine-d_{11} suitable for biomedical applications as a metabolic contrast agent without any additional chemical modifications. PHIP hydrogenation of the acetyl ^{15}N-1,2-dehydrocholine-d_{11} synthesized here could yield acetyl ^{15}N-choline-d_{11}. However, the latter is highly neurotoxic and therefore requires chemical modifications before it can be used in biomedicine. These hydrogenation or/and other chemical modifications are much needed for biomedical use of this class of contrast agents, but well beyond the scope of this study.

Conclusion

Presented here is a robust synthetic approach for preparation of protected 1,2-dehydrocholine compounds. The synthetic approach allows for convenient incorporation of ^{15}N and deuterium spin labels in a manner suitable for PHIP hyperpolarization of protected ^{15}N-choline based contrast agents. The method is exemplified with natural abundance and ^{15}N and deuterium spin labeled acetyl 1,2-dehydrocholine derivatives as described in US Patent Application US2012/0225020. Ac–O–C=C– motifs were successfully PHIP hyperpolarized earlier[26] suggesting that acetyl protected cholines can be polarized as well and hyperpolarized ^{15}N-choline can be produced using a recently demonstrated strategy.[26]

Moreover, this synthetic approach could also be extended to 1,2-dehydrophosphocholine PHIP precursor. The latter may be more suitable for molecular hydrogenation with water soluble Rh^+ based catalysts, because the negative charge of the phosphate protecting group could significantly accelerate the hydrogenation rate of otherwise positively charged choline moiety. Although PHIP of ^{15}N-phosphocholine using 1,2-dehydrophosphocholine as a precursor remains to be demonstrated in future studies, complete hydrogenation in PHIP of ^{13}C-phospolactate was successfully demonstrated[27] suggesting that phosphocholine should also be amenable by PHIP.

Hyperpolarized ^{15}N-choline is a potential molecular contrast agent of cancer, reporting on elevated choline uptake and elevated rate of phosphocholine synthesis due to hypoxia, HIF-1α over-expression and so on. Hypoxia is a universal hallmark of cancer.[45,46] Therefore, hyperpolarized ^{15}N-choline enabled by the reported synthetic approach can be potentially used as a very specific imaging biomarker of cancer similar to 1-^{13}C-pyruvate reporting on a different hallmark of cancer, elevated aerobic glycolysis.[46,47]

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Conflict of Interest

The authors did not report any conflict of interest.

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