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²H,¹⁵N-Substituted Nitroxides as Sensitive Probes for Electron Paramagnetic Resonance Imaging

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Electron paramagnetic resonance imaging (EPRI) using nitroxides is an emergent imaging method for studying in vivo physiology, including O_2 distribution in various tissues. Such imaging capabilities would allow O_2 mapping in tumors and in different brain regions following hypoxia or drug abuse. We have recently demonstrated that the anion of 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (2) can be entrapped in brain tissue to quantitate O_2 concentration in vivo. To increase the sensitivity of O_2 measurement by EPR imaging, we synthesized 3-carboxy-2,2,5,5-tetra(²H₃)methyl-1-(3,4,4-²H₃,1-¹⁵N)-pyrrolidinyloxyl (7). EPR spectroscopic measurements demonstrate that this fully isotopically substituted nitroxide markedly improves signal-to-noise ratio and, therefore, the sensitivity of EPR imaging. The new isotopically substituted nitroxide shows increased sensitivity to changes in O_2 concentration, which will enable more accurate O_2 measurement in tissues using EPRI.

Introduction

Electron paramagnetic resonance imaging (EPRI) is an emergent magnetic resonance imaging modality that can be used to study a wide range of physiology.¹ For example, molecular oxygen, being paramagnetic, broadens the electron paramagnetic resonance (EPR) spectral lines of other paramagnetic species, including trityl radicals² and

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nitroxides.³ Using these spin probes, one can make reliable, minimally invasive measurements of O_2 concentration in vivo. Besides the significance of O_2 generally in brain metabolism, the importance of O_2 measurement in brain stems from the suggestion that oxygen-centered free radicals are responsible for methamphetamine-induced neurotoxicity,⁴ leading to altered O_2 levels in the affected regions of the brain. Therefore, it is clinically important to map the regions of decreased O_2 . A major obstacle to using EPR imaging to quantify O_2 in brain tissue is the difficulty of transporting O_2 -sensitive imaging probes across the blood—brain barrier.

As EPRI probes, nitroxides have many desirable qualities, including ease of preparation, chemical flexibility, and high stability at physiologic pH and temperature. We have

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Reagents and conditions: (a) ${}^{15}ND_4Cl$, MgO; (b) D_2O_2 , D_2O ; (c) (i) D_2 , Pd/C, CH₃OD, (ii) DCl, D_2O ; (d) (i) Br_2 , CHCl₃, (ii) NaNO₂, D_2O ; (e) (i) KOD, (ii) DCl; (f) CH₃CO₂CH₂Br, K₂CO₃, CH₃CN.

recently synthesized nitroxide labile esters⁵ (e.g., 1) that can cross the blood-brain barrier to enter brain tissue, where they are converted by enzymatic hydrolysis to 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (2). This compound, being anionic at physiologic pH, becomes entrapped in the brain and can report O₂ concentration through EPRI. Based on this success,⁵ we describe the synthesis of 3-carboxy-2,2,5,5-tetra(${}^{2}H_{3}$)methyl-1-(3,4,4- ${}^{2}H_{3}$,1- ${}^{15}N$)pyrrolidinyloxyl (7), wherein isotopic substitution with ${}^{15}N$ and ${}^{2}H$ are expected to improve detection limits and signal-to-noise ratio (SNR) for EPRI. The rationale for isotopic substitution is straightforward. In an isotopically unmodified nitroxide, hyperfine interaction with the spin-1 ¹⁴N nucleus splits the electron resonance into three spectral lines, each with roughly one-third the total signal intensity. Because EPRI measures the amplitude of only one spectral line, the signal contained in the other two lines is wasted. The spin-1/2 ¹⁵N nucleus splits the nitroxide resonance into only two lines, each of which contains 50% more signal than any line in the ¹⁴N EPR spectrum. Additionally, in isotopically unmodified nitroxides, hyperfine interactions with ¹H nuclei broaden the EPR spectral lines, with corresponding reduction of peak amplitudes. Complete isotopic substitution with 2 H, which has a weaker nuclear magnetic moment, should significantly reduce line broadening and thereby increase spectral peak amplitude and SNR. Lastly, O₂ concentrations are estimated from the width of the EPR spectral lines. We demonstrate that in comparison with the unmodified nitroxide 2, the isotopically substituted nitroxide 7 has a relative line width dependence on O_2 concentration that is 2.6-fold steeper, which makes the latter a more sensitive probe for O_2 measurement in vivo.



Results and Discussion

In preparing isotopic-substituted nitroxide 7, 15 N must be introduced in the first step, forming 4-oxo-2,2,6,6-tetra(2 H₃)-

methyl(3,3,5,5⁻²H₄,1⁻¹⁵N)piperidine (3), prior to subsequent reactions, including ring contraction. We originally considered using the procedure of Rozantsev,⁶ which called for the condensation of acetone with ammonia. The reported low yield (19%) for this reaction, however, compelled us to seek an alternative synthetic pathway. A review of the literature uncovered the procedure of Lin et al.,^{7a} in which heating perdeuteroacetone and ¹⁴ND₄Cl with MgO in a sealed vessel gave the desired piperidine with 56% yield. ¹⁵ND₄Cl is not commercially available. Therefore, it was prepared by isotope exchange: ¹⁵NH₄Cl was dissolved in D₂O and lyophilized; the process was repeated four times.

In our hands, heating perdeuteroacetone and $^{15}ND_4Cl$ with MgO in a sealed vessel yielded 3 in ~40% yield (Scheme 1). Whether 3 was purified by distillation made little difference in the yield of the next reaction; therefore, crude 3 was used directly in the next step to ensure efficient use of isotopically substituted material. The oxidation of 3 with D_2O_2 in D_2O afforded nitroxide 4 in reasonable yield.

Although it has been reported⁸ that addition of HCl to isotopically unmodified nitroxide **4** followed by bromination gave the corresponding **6** in good yield via the intermediate **5**, Marc and Pecar⁹ found that reducing **4** by catalytic hydrogenation prior to bromination gave superior results. For the isotopically unmodified **4**, this procedure should give a nearly quantitative yield of **5**. In the case of ²H-substituted nitroxide **4**, however, the procedure could lead to hydrogendeuterium exchange, owing to keto-enol tautomerization (Scheme 2). To eliminate this possibility, we initially considered using perdeuterated ammonium formate as the electron source for reduction¹⁰ but ultimately decided to reduce piperidinyloxyl **4** to the hydroxylamine **5** with D₂ over Pd/C in CH₃OD.

Subsequent bromination of 5 at a single position α to the carbonyl, achieved by dropwise addition of Br₂ in

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SCHEME 2



chloroform over 45 min, followed by NaNO₂ oxidation, afforded the bromopiperidinyloxyl 6,⁸ with no evidence of dibromination.¹¹ Importantly, piperidinyloxyl 4, which was recovered by chromatography, was recycled through the reaction to bring the total yield of 6 to 64%. Favorskii rearrangement of 6 with KOD⁸ gave the desired carboxylic acid 7.

Figure 1 shows EPR spectra for the K⁺ salts of nitroxides 2 and 7, each at 100 μ M in air-equilibrated H₂O ([O₂] = 0.25 mM). Although the samples are at the same concentration, nitroxide 7 exhibits significantly larger and narrower EPR spectral peaks. Using relatively low magnetic fields permits EPR imaging with low-frequency electromagnetic radiation, which penetrates tissue well but reduces SNR. Nitroxide 7 remedies this deficit with much narrower, and thus larger, spectral peaks. Using nitroxide 7 improves the limit of detection and enables imaging with higher contrast.

Figure 2 shows the dependence of the EPR line width on O₂ concentration for nitroxides 7 and 2. As O₂ concentration varies, the relative change in line width for nitroxide 7 is much larger than for nitroxide 2. Linear least-squares fits of the data show that the line-broadening effect of O_2 is 2.74fold greater for nitroxide 7 than for nitroxide 2. Thus, nitroxide 7 is more O₂-sensitive and capable of resolving smaller changes in O_2 than nitroxide 2. In EPR imaging, overmodulation is often used to improve SNR, at the cost of spectral line broadening. The threshold modulation amplitude at which significant line broadening occurs is lower for 7 than for 2. Therefore, overmodulation could reduce the difference in the line-broadening effect of O_2 on the two nitroxides. The spectra in Figure 2 were acquired at a modulation amplitude of 0.125 G, at which line broadening was negligible for both nitroxides (Figure S1, Supporting Information). We measured the dependence of the linewidths of 7 and 2 on O_2 at an increased modulation amplitude of 0.5 G (Figure S2, Supporting Information). At this larger modulation amplitude, the difference in the linebroadening effect of O₂ is reduced from 2.74-fold to 2.60fold. Thus, even under typical conditions of overmodulation, nitroxide 7 is still far superior to nitroxide 2 as an O_2 sensor.

Isotopic substitution of 15 N and 2 H in the pyrrolidinyloxyl ring is a significant advancement in using nitroxides as oxygen-sensitive probes in EPR imaging. We have shown previously that nitroxide 1, the isotopically unmodified analogue of labile ester 8, is a pro-imaging agent that can cross the blood-brain barrier and be converted to nitroxide 2 in brain tissue.¹² Therefore, the isotopically substituted



FIGURE 1. EPR spectra of nitroxides 2 and 7 (K⁺ salt, each at 100 μ M in H₂O equilibrated with air). Both spectra were acquired with identical spectrometer settings (see the Experimental Section) and are represented on the same intensity scale. The hyperfine splittings and linewidths are indicated.



FIGURE 2. Relative linewidths of nitroxides 2 and 7 at different O_2 concentrations in water. For each nitroxide, the linewidths were normalized to the value measured at 0.003 mM O_2 (in N₂-sparged water). Solid lines are least-squares fits of the data; the slope of each line is indicated on the graph.

labile ester 8, like its isotopically unmodified counterpart, is expected to cross the blood-brain barrier. When introduced into live animals, ester 8 will enable us to determine the actual extent to which isotopic substitution improves O_2 imaging in vivo. These studies are underway.

Experimental Section

General Materials and Methods. Reagents and solvents from commercial vendors were used without further purification. Silica gel (230–400 mesh) was used for column chromatography.

4-Oxo-2,2,6,6-tetra(2 H₃)**methyl-(3,3,5,5-** 2 H₄,1- 15 N)**piperidine (3).** This compound was prepared following the general procedure of Lin et al.^{7a} with minor modifications. To generate 15 ND₄Cl, 15 NH₄Cl (10 g) was dissolved in D₂O (99.9%; 15 mL); the solution was evaporated to dryness under vacuum. This procedure was repeated a total of four times.

In a glovebox under positive internal N_2 pressure, ${}^{15}ND_4Cl$ (3.5 g, 60 mmol) was added to a 250-mL round-bottom flask

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containing oven-dried anhydrous Na₂CO₃ (3.18 g, 30 mmol) and MgO (3.0 g, 75 mmol). Thereafter, perdeuteroacetone (12.5 mL, 150 mmol; 99.9%) was introduced into the flask by canula under N2 pressure. While still under N2 atmosphere, the flask was sealed with a rubber septum. The reaction mixture was kept for 3 days at 50 °C in an oil bath and then allowed to cool. Perdeuterocetone (20 mL) was added to the flask, and the resulting mixture was filtered. The filter cake was crushed into a fine powder, washed with dry ether and perdeuteroacetone (1:1 mixture, 20 mL), and again filtered; this procedure was repeated three more times. The combined filtrates were concentrated on a rotary evaporator to give a red liquid (5.5 g), a portion of which was distilled to yield a yellow liquid (bp 60-64 °C at 12 mmHg),⁷ which solidified upon cooling. In trial runs, we found that distillation of 3 did not significantly affect the yield of 4; therefore, crude 3 was used for the next reaction without further purification.

4-Oxo-2,2,6,6-tetra(²H₃)methyl-1-(3,3,5,5-²H₄,1-¹⁵N)piperidinyloxyl (4). To a solution of crude 4-oxo-2,2,6,6-tetra(²H₃)-methyl-(1,3,3,5,5-²H₅,1-¹⁵N)piperidine (3) (5.5 g, 34 mmol) dissolved in D₂O (60 mL) were added oven-dried Na₄EDTA (0.55 g, 1.5 mmol) and oven-dried Na₂WO₄ (0.55 g, 1.7 mmol). Upon dissolution of the salts, D₂O₂ (30% in D₂O, 6 mL) was added, and the reaction was allowed to proceed in the dark for 10 days. The reaction mixture was filtered and extracted with ether (3 × 50 mL). The ether extract was first washed with cold dilute DCl (10% in D₂O, 2 × 20 mL) and then with saturated Na₂CO₃ in D₂O (10 mL). Thereafter, the solution was dried over anhydrous MgSO₄, filtered, and reduced to dryness on a rotary evaporator. This residue was chromatographed (hexane/Et₂O, 2:1) to yield 4-oxo-2,2,6,6-tetra-(²H₃)methyl-1-(3,3,5,5-²H₄,1-¹⁵N)piperidinyloxyl **4** as a red oil, which solidified in the cold (2.8 g, 51%): IR (CHCl₃) 1720 cm⁻¹ (C=O). Anal. Calcd for C₉²H₁₆¹⁵NO₂: C, 57.69; ²H, 8.61; ¹⁵N, 7.40. **4-Oxo-2,2,6,6-tetra(²H₃)methyl-1-(²H)hydroxy-(3,3,5,5-²H₄, ¹⁵N)**

1-¹⁵N)piperidine (²H)Hydrochloride (5). The general procedure of Marc and Pecar⁹ was used with minor modifications. 4-Oxo-2,2,6,6-tetra(²H₃)methyl-1-(3,3,5,5-²H₄,1-¹⁵N)piperidinyloxyl (4) (2.8 g, 15 mmol) was dissolved in CH₃OD (30 mL), and 5% Pd/C (50 mg) was added. Deuterium gas (99%) was gently bubbled into the reaction mixture for several min and the flask was sealed. The flask was periodically recharged with D₂ over the next several hours. After being stirred overnight, the reaction mixture was filtered through Celite. The colorless filtrate was acidified with 4 M DCl (in D₂O, 2.5 mL) and reduced to dryness on a rotary evaporator. The residue was washed with dry ether $(2 \times 20 \text{ mL})$ to remove any remaining nitroxide and dried, in vacuo, to yield 4-oxo-2,2,6,6-tetra(²H₃)methyl-1-(²H)hydroxyl- $(3,3,5,5-{}^{2}H_{4},1-{}^{15}N)$ piperidine (²H) hydrochloride (5) as a white solid (2.7 g, 80%). Compound 5 is very hygroscopic and was therefore used immediately in the monobromination reaction to yield 6, as described below.

3-Bromo-4-oxo-2,2,6,6-tetra(2 H₃)**methyl-1-**(**3,5,5-** 2 H₃,**1-** 15 N)**piperidinyloxyl (6).** The procedure of Sosnovsky and Cai⁸ was used with minor modifications. Br₂ (2.14 g, 11.9 mmol) in CHCl₃ (10 mL) was added dropwise over 45 min at room temperature to a stirred solution of 4-oxo-2,2,6,6-tetra(2 H₃)methyl-1-(2 H)hydroxyl-(3,3,5,5- 2 H₄,1- 15 N)piperidine (2 H)hydrochloride (**5**) (2.7 g, 11.9 mmol) in CHCl₃ (25 mL); thereafter, the reaction mixture was stirred for 2.5 h. Thereafter, a solution of NaNO₂ (1.85 g, 27 mmol) in D₂O (10 mL) was added dropwise over 10 min to the vigorously stirred reaction mixture; stirring was continued for another 15 min. The organic phase was washed with D₂O, dried over anhydrous MgSO₄, filtered, and evaporated to dryness under reduced pressure. Chromatography (hexane/Et₂O, 2:1) yielded two fractions: (1) 3-bromo-4-oxo-2,2,6,6-tetra(2 H₃)methyl-1-(3,5,5- 2 H₃,1- 15 N)piperidinyloxyl (**6**) and (2) nitroxide **4**. Rereducing the recovered piperidinyloxyl **4** with D₂ over 5% Pd/C (25 mg) in CH₃OD (30 mL) followed by bromination and then oxidation with NaNO₂ led to a 64% overall yield of piperidinyloxyl **6** (2.0 g). Recrystallization from hexane yielded light orange crystals, whose purity was confirmed by HPLC (Figure S3, Supporting Information): mp = 81-82 °C; IR (CHCl₃) 1730 cm⁻¹ (C=O); HRMS (ESI) calcd for C₉¹H²H₁₅¹⁵NO₂⁷⁹Br [M+H]⁺ 265.12773, found 265.11910; calcd for C₉¹H²H₁₅¹⁵NO₂⁸¹Br [M + H]⁺ 267.12558, found 267.12953.

3-(²H)Carboxy-2,2,5,5-tetra(²H₃)methyl-1-(3,4,4-²H₃,1-¹⁵N)pyrrolidinyloxyl (7). The general procedure of Sosnovsky and Cai⁸ was used, with minor modifications. KOD (1 M in D₂O, 5 mL) was added to 3-bromo-4-oxo-2,2,6,6-tetra(²H₃)methyl-1-(3,5,5-²H₃,1-¹⁵N)piperidinyloxyl (**6**) (0.52 g, 2 mmol). With stirring over the next 2 h, nitroxide **6** dissolved completely. The alkaline solution was extracted with Et₂O (3 × 20 mL), cooled in an ice bath, and adjusted to pH 3 with dilute DCl (10% in D₂O). This acidic solution was extracted with Et₂O (3 × 20 mL); the combined extract was dried over anhydrous MgSO₄, filtered, and evaporated under reduced pressure to yield 3-carboxy-2,2,5,5-tetra(²H₃)methyl-1-(3,4,4-²H₃,1-¹⁵N)pyrrolidinyloxyl (7) as a yellow solid (0.25 g, 61%). Recrystallization from CHCl₃/ hexane gave a yellow powder: mp = 190 – 194 °C dec;¹³ IR (CHCl₃) 3500 cm⁻¹ (broad peak, OH), 1711 cm⁻¹ (C=O).

3-Acetoxymethoxycarbonyl-2,2,5,5-tetra(²H₃)**methyl-1-(3,4,4-**²H₃,1-¹⁵N)**pyrrolidinyloxyl (8).** The synthesis followed our reported procedure.¹⁴ Bromomethyl acetate (0.044 g, 0.03 mL, 0.33 mmol) was added to a solution of 3-carboxy-2,2,5,5-tetra(²H₃)methyl-1-(3,4,4-²H₃,1-¹⁵N)pyrrolidinyloxyl (7) (0.050 g, 0.25 mmol) and K₂CO₃ (0.070 g, 0.50 mmol) in acetonitrile (dried over CaH₂, 10 mL); the mixture was stirred overnight at room temperature. Thereafter, this mixture was filtered through Celite, and the filtrate was evaporated to dryness. The oily residue was chromatographed (hexane/ethyl acetate, 5:1) to yield a thick oil, which was crystallized from hexane to yield 3-acetoxymethoxycarbonyl-2,2,5,5-tetra(²H₃)methyl-1-(3,4,4-²H₃,1-¹⁵N)pyrroldinyloxyl (8) as a yellow solid (0.050 g; 74%): mp = 77-78 °C; IR (CHCl₃) 1763 cm⁻¹ (C=O); HRMS (ESI) calcd for C₁₂¹H₆²H₁₅¹⁵NO₅ [M + H]⁺ 275.23316, found 275.23085. Anal. Calcd for C₁₂²H₁₅¹⁴F¹⁵NO₅: C, 52.52; ²H + ¹H, 7.35; ¹⁵N, 5.10. Found: C, 52.76; ²H + ¹H, 7.42; ¹⁵N, 5.12.

EPR Spectroscopy. EPR spectra were recorded on an X-band spectrometer at the following settings: microwave power, 20 mW; microwave frequency, 9.55 GHz; field set, 3335 G for ¹⁴N or 3324 G for ¹⁵N; modulation frequency, 1 kHz; modulation amplitude, 0.125 G; field sweep, 4 G at 13.3 G min⁻¹. These settings encompassed the central spectral peak of the ¹⁴N spectrum, or the first spectral peak of the ¹⁵N spectrum. For recording the complete spectrum of either nitroxide, the field set was 3335 G, and field sweep was 50 G at 13.3 G min⁻¹. Digital acquisition of EPR spectra was through EWWIN software.

EPR Spectral Linewidth Measurements. To assess the effect of O₂ on the EPR linewidths of the nitroxides, deionized H₂O (18.3 M Ω ·cm resistivity) was sparged with N₂, equilibrated with air, or sparged with O₂ at 24 °C for 30 min, to yield solutions containing O₂ at 0.003, 0.25, and 1.25 mM (O₂-saturated¹⁵), respectively. Submillimolar O₂ concentrations were determined using a dissolved O₂ meter. Stock solutions (10 mM) of the K⁺ salt of nitroxide **2** or **7** were diluted 500-fold into the gas-equilibrated H₂O samples to a final nitroxide concentration of 20 μ M. Each solution was transferred into a flat quartz EPR cell previously purged with the appropriate gas. The quartz cell was sealed, and immediately positioned in the EPR spectrometer. Duplicate spectroscopic measurements on the samples were

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performed in random order. Reported linewidths are the peakto-peak width of the central spectral line of **2** and the first spectral line of **7**.

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Supporting Information Available: Supplemental methods and figures. This material is available free of charge via the Internet at http://pubs.acs.org.