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$^{2}H,^{15}N$–Substituted Nitroxides as Sensitive Probes for Electron Paramagnetic Resonance Imaging

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Abstract

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 ($^{2}H_3$)methyl-1-(3,4,4-$^{2}H_3$,1-^{15}N)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 nitroxides3. 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.

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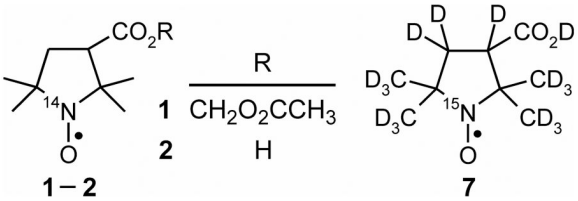
As EPRI probes, nitroxides have many desirable qualities, including ease of preparation, chemical flexibility, and high stability at physiologic pH and temperature. We have recently synthesized nitroxide labile esters[5] (e.g., **1**) that can cross the blood-brain barrier to enter brain tissue, where 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 O_2 concentration through EPRI. Based on this success,[5] we describe the synthesis of 3-carboxy-2,2,5,5-tetra($^2H_3$)methyl-1-(3,4,4-$^2H_3$,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 ^{14}N 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-½ ^{15}N nucleus splits the nitroxide resonance into only two lines, each of which contains 50% more signal than any line in the ^{14}N EPR spectrum. Additionally, in isotopically unmodified nitroxides, hyperfine interactions with ^{1}H 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, O_2 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 linewidth 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($^2H_3$)methyl(3,3,5,5-$^2H_4$,1-^{15}N)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 $^{14}ND_4Cl$ with MgO in a sealed vessel gave the desired piperidine with 56% yield. $^{15}ND_4Cl$ is not commercially available. Therefore, it was prepared by isotope exchange: $^{15}NH_4Cl$ was dissolved in D_2O and lyophilized; the process was repeated 4 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 reported[8] 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 Pecar[9] 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 ^{2}H-substituted nitroxide **4**, however, the procedure could lead to hydrogen-deuterium exchange, owing to keto-enol tautomerization (Scheme 2). To eliminate this possibility, we initially considered using perdeuterated ammonium formate as the electron source for reduction,[10] but ultimately decided to reduce piperidinyloxyl **4** to the hydroxylamine **5** with D_2 over Pd/C in CH_3OD.

Subsequent bromination of **5** at a single position α to the carbonyl, achieved by dropwise addition of Br_2 in chloroform over 45 min, followed by $NaNO_2$ 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 KOD[8] gave the desired carboxylic acid **7**.

Figure 1 shows EPR spectra for the K^+ salts of nitroxides **2** and **7**, each at 100 μM in air-equilibrated H_2O ($[O_2]$ = 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 linewidth on O_2 concentration for nitroxides **7** and **2**. As O_2 concentration varies, the relative change in linewidth 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 2.74-fold greater for nitroxide **7** than for nitroxide **2**. Thus, nitroxide **7** is more O_2-sensitive and capable of resolving smaller changes in O_2 than nitroxide **2**. In EPR imaging, over-modulation 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, over-modulation 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 (Supplemental Figure S1). We measured the dependence of the linewidths of **7** and **2** on O_2 at an increased modulation amplitude of 0.5 G (Supplemental Figure S2). At this larger modulation amplitude, the difference in the line-broadening effect of O_2 is reduced from 2.74-fold to 2.60-fold. Thus even under typical conditions of over-modulation, 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 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($^2H_3$)methyl-(3,3,5,5-$^2H_4$,1-^{15}N)piperidine (3)

This compound was prepared following the general procedure of Lin, *et al.*[7a] with minor modifications. To generate $^{15}ND_4Cl$, $^{15}NH_4Cl$ (10 g) was dissolved in D_2O (99.9%; 15 mL); the solution was evaporated to dryness under vacuum. This procedure was repeated a total of 4 times.

In a glove box under positive internal N_2 pressure, $^{15}ND_4Cl$ (3.5 g, 60 mmol) was added to a 250-mL round bottom flask containing oven-dried anhydrous Na_2CO_3 (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 N_2 pressure. While still under N_2 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 mm Hg),[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($^2H_3$)methyl-1-(3,3,5,5-$^2H_4$,1-^{15}N)piperidinyloxyl (4)

To a solution of crude 4-oxo-2,2,6,6-tetra($^2H_3$)methyl-(1,3,3,5,5-$^2H_5$,1-^{15}N)piperidine (**3**) (5.5 g, 34 mmol) dissolved in D_2O (60 mL), oven-dried Na_4EDTA (0.55 g, 1.5 mmol) and oven-dried Na_2WO_4 (0.55 g, 1.7 mmol) were added. Upon dissolution of the salts, D_2O_2 (30% in D_2O, 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 D_2O, 2 × 20 mL) and then with saturated Na_2CO_3 in D_2O (10 mL). Thereafter, the solution was dried over anhydrous $MgSO_4$, filtered, and reduced to dryness on a rotary evaporator. This residue was chromatographed (hexane:Et_2O, 2:1) to yield 4-oxo-2,2,6,6-tetra($^2H_3$)methyl-1-(3,3,5,5-$^2H_4$,1-^{15}N)piperidinyloxyl **4** as a red oil, which solidified in the cold (2.8 g, 51%). IR ($CHCl_3$): 1720 cm^{-1} (C=O). Anal. calculated for $C_9{}^2H_{16}{}^{15}NO_2$: C, 57.69; ^2H, 8.61; ^{15}N, 7.48. Found: C, 57.57; ^2H, 8.58; ^{15}N, 7.40.

4-Oxo-2,2,6,6-tetra($^2H_3$)methyl-1-(^2H)hydroxy-(3,3,5,5-$^2H_4$,1-^{15}N)piperidine (^2H) hydrochloride (5)

The general procedure of Marc and Pecar[9] was used with minor modifications. 4-Oxo-2,2,6,6-tetra($^2H_3$)methyl-1-(3,3,5,5-$^2H_4$,1-^{15}N)piperidinyloxyl (**4**) (2.8 g, 15 mmol) was dissolved in CH_3OD (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 D_2 over the next several hours. After stirring overnight, the reaction mixture was filtered through Celite. The colorless filtrate was acidified with 4 M DCl (in D_2O, 2.5 mL) and reduced to dryness on a rotary evaporator. The residue was washed with dry ether (2 × 20 mL) to remove any remaining nitroxide and dried, *in vacuo*, to yield 4-oxo-2,2,6,6-tetra($^2H_3$)methyl-1-(^2H)hydroxyl-(3,3,5,5-$^2H_4$,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($^2H_3$)methyl-1-(3,5,5-$^2H_3$,1-^{15}N)piperidinyloxyl (6)

The procedure of Sosnovsky and Cai[8] was used with minor modifications. Br_2 (2.14 g, 11.9 mmol) in $CHCl_3$ (10 mL) was added dropwise over 45 min at room temperature to a stirred solution of 4-oxo-2,2,6,6-tetra($^2H_3$)methyl-1-(^2H)hydroxyl-(3,3,5,5-$^2H_4$,1-^{15}N)piperidine

(^{2}H)hydrochloride (**5**) (2.7 g, 11.9 mmol) in $CHCl_3$ (25 mL); thereafter the reaction mixture was stirred for 2.5 h. Thereafter, a solution of $NaNO_2$ (1.85 g, 27 mmol) in D_2O (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 D_2O, dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. Chromatography (hexane:Et_2O, 2:1) yielded two fractions: 1) 3-bromo-4-oxo-2,2,6,6-tetra($^2H_3$)methyl-1-(3,5,5-$^2H_3$,1-^{15}N) piperidinyloxyl (**6**), and 2) nitroxide **4**. Re-reducing the recovered piperidinyloxyl **4** with D_2 over 5% Pd/C (25 mg) in CH_3OD (30 mL) followed by bromination and then oxidation with $NaNO_2$ 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 (Supplemental Figures S3): mp = 81–82°C; IR ($CHCl_3$): 1730 cm^{-1} (C=O). HRMS (ESI) calcd for $C_9{}^1H^2H_{15}{}^{15}NO_2{}^{79}Br$ $[M + H]^+$ 265.12773, found 265.11910; calcd for $C_9{}^1H^2H_{15}{}^{15}NO_2{}^{81}Br$ $[M + H]^+$ 267.12558, found 267.12953.

3-(^{2}H)Carboxy-2,2,5,5-tetra($^2H_3$)methyl-1-(3,4,4-$^2H_3$,1-^{15}N)pyrrolidinyloxyl (7)

The general procedure of Sosnovsky and Cai[8] was used, with minor modifications. KOD (1 M in D_2O, 5 mL) was added to 3-bromo-4-oxo-2,2,6,6-tetra($^2H_3$)methyl-1-(3,5,5-$^2H_3$,1-^{15}N) piperidinyloxyl (**6**) (0.52 g, 2 mmol). With stirring over the next 2 h, nitroxide **6** dissolved completely. The alkaline solution was extracted with Et_2O (3 × 20 mL), cooled in an ice bath, and adjusted to pH 3 with dilute DCl (10% in D_2O). This acidic solution was extracted with Et_2O (3 × 20 mL); the combined extract was dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure to yield 3-carboxy-2,2,5,5-tetra($^2H_3$)methyl-1-(3,4,4-$^2H_3$,1-^{15}N)pyrrolidinyloxyl (**7**) as a yellow solid (0.25 g, 61%). Recrystallization from $CHCl_3$/hexane gave a yellow powder, mp = 190 – 194 °C (with decomposition);[13] IR ($CHCl_3$): 3500 cm^{-1} (broad peak, OH), 1711 cm^{-1} (C=O).

3-Acetoxymethoxycarbonyl-2,2,5,5-tetra($^2H_3$)methyl-1-(3,4,4-$^2H_3$,1-^{15}N)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($^2H_3$)methyl-1-(3,4,4-$^2H_3$,1-^{15}N)pyrrolidinyloxyl (**7**) (0.050 g, 0.25 mmol) and K_2CO_3 (0.070 g, 0.50 mmol) in acetonitrile (dried over CaH_2, 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($^2H_3$)methyl-1-(3,4,4-$^2H_3$,1-^{15}N)pyrroldinyloxyl (**8**) as a yellow solid (0.050 g; 74%), mp = 77–78°C; IR ($CHCl_3$): 1763 cm^{-1} (C=O). HRMS (ESI) calcd for $C_{12}{}^1H_6{}^2H_{15}{}^{15}NO_5$ $[M + H]^+$ 275.23316, found 275.23085. Anal. calculated for $C_{12}{}^2H_{15}{}^1H_5{}^{15}NO_5$: C, 52.52; ^{2}H + ^{1}H, 7.35; ^{15}N, 5.10. Found: C, 52.76; ^{2}H + ^{1}H, 7.42; ^{15}N, 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 ^{14}N or 3324 G for ^{15}N; 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 ^{14}N spectrum, or the first spectral peak of the ^{15}N 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 O_2 on the EPR linewidths of the nitroxides, deionized H_2O (18.3 MΩ·cm resistivity) was sparged with N_2, equilibrated with air, or sparged with O_2 at 24°C for

30 min, to yield solutions containing O_2 at 0.003 mM, 0.25 mM and 1.25 mM (O_2-saturated15), respectively. Submillimolar O_2 concentrations were determined using a dissolved O_2 meter. Stock solutions (10 mM) of the K^+ salt of nitroxide **2** or **7** were diluted 500-fold into the gas-equilibrated H_2O samples to a final nitroxide concentration of 20 μM. 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 performed in random order. Reported linewidths are the peak-to-peak width of the central spectral line of **2**, and the first spectral line of **7**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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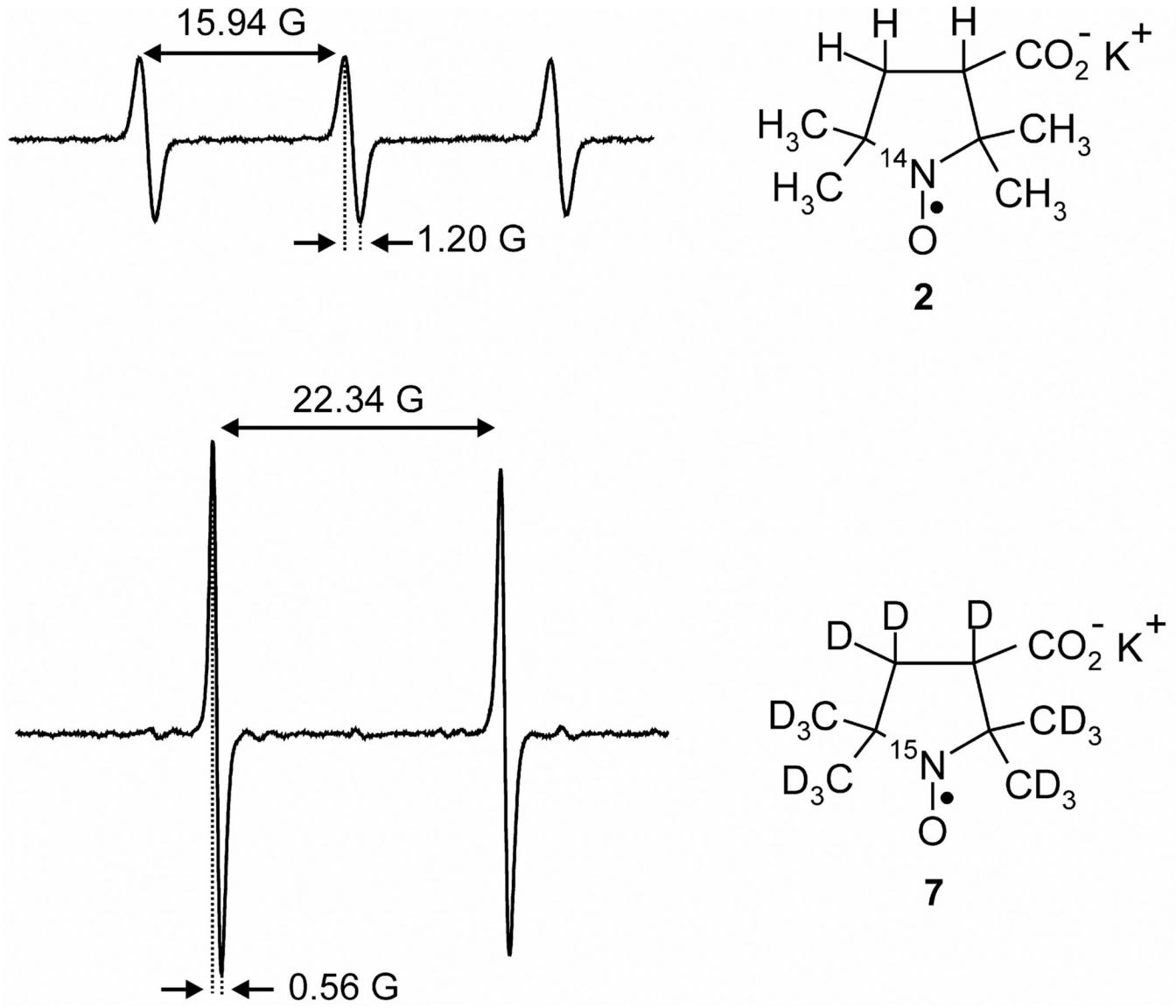


Figure 1.

EPR spectra of nitroxides **2** and **7** (K^+ salt, each at 100 μM in H_2O equilibrated with air). Both spectra were acquired with identical spectrometer settings (see Experimental section) and are represented on the same intensity scale. The hyperfine splittings and linewidths are indicated.

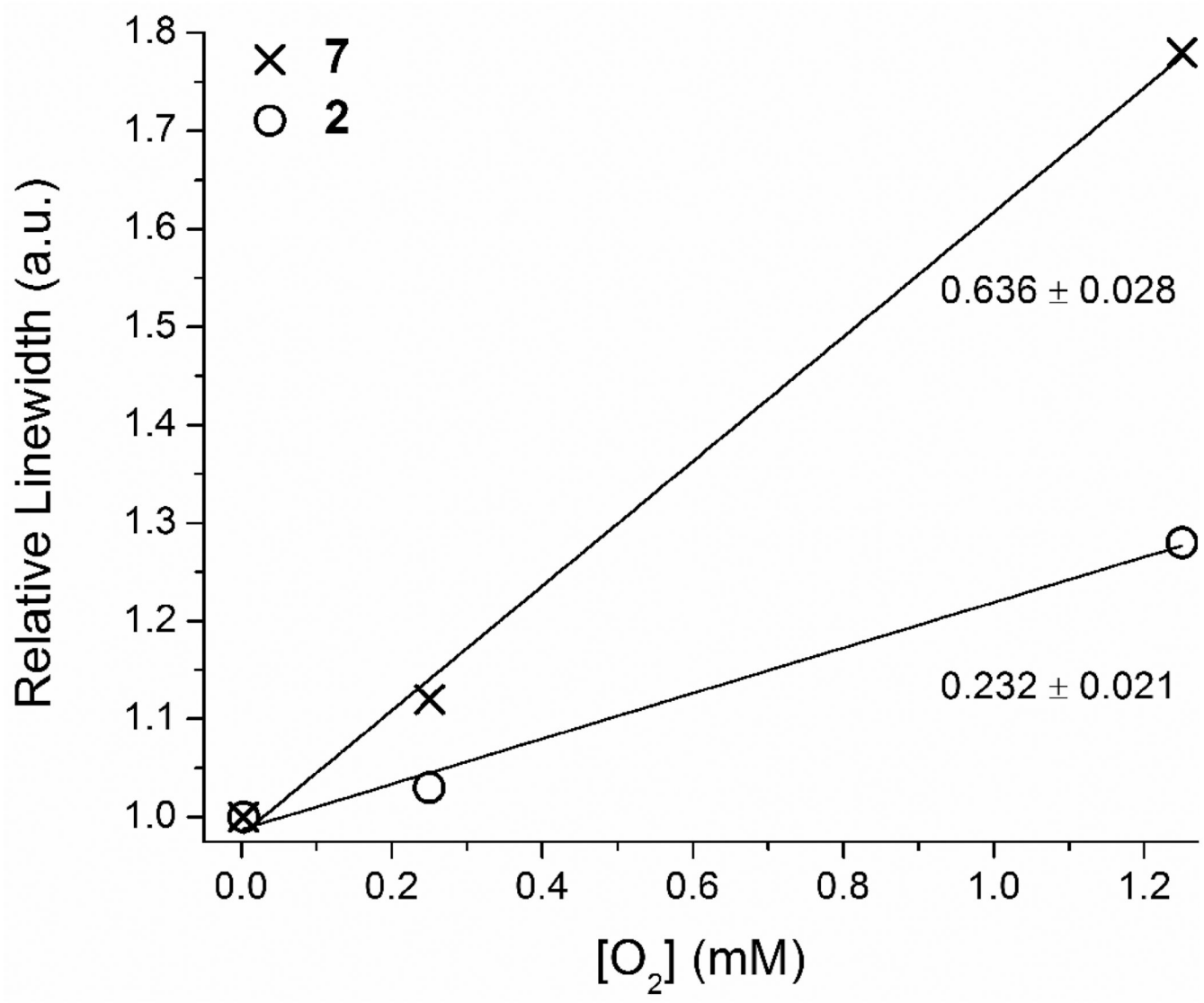
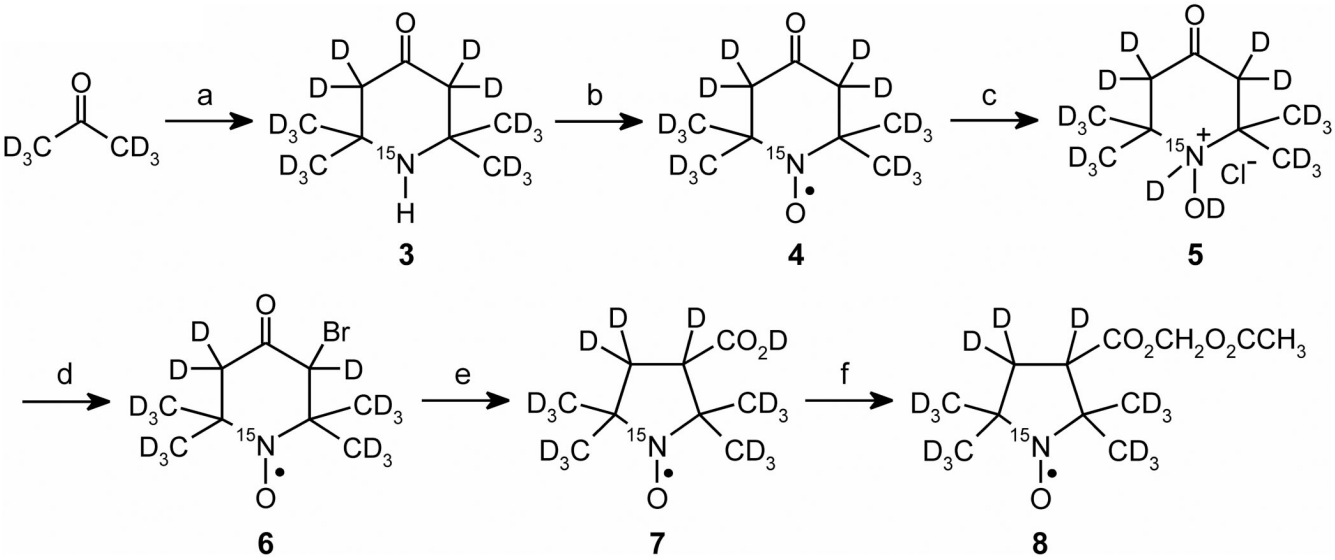
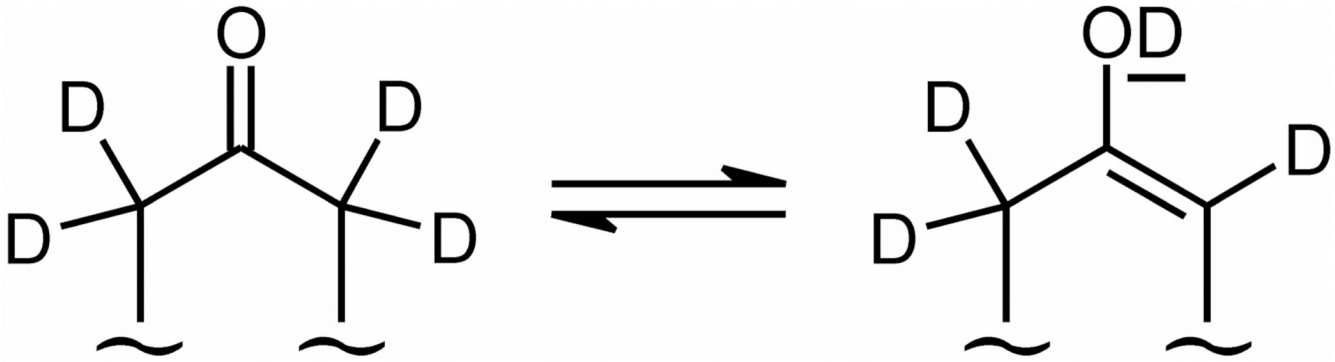


Figure 2.
Relative linewidths of nitroxides **2** and **7** at different O_2 concentrations in water. For each nitroxide, the linewidths were normalized to the value measured at 0.003 mM O_2 (in N_2-sparged water). Solid lines are least-squares fits of the data; the slope of each line is indicated on the graph.



Scheme 1.

Reagents and conditions: (a) $^{15}ND_4Cl$, MgO; (b) D_2O_2, D_2O; (c) *i.* D_2, Pd/C, CH_3OD, *ii.* DCl, D_2O; (d) *i.* Br_2, $CHCl_3$, *ii.* $NaNO_2$, D_2O; (e) *i.* KOD, *ii.* DCl; (f) $CH_3CO_2CH_2Br$, K_2CO_3, CH_3CN.



Scheme 2.