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Synthesis of ^{14}N- and ^{15}N-labeled Trityl-nitroxide Biradicals with Strong Spin−Spin Interaction and Improved Sensitivity to Redox Status and Oxygen

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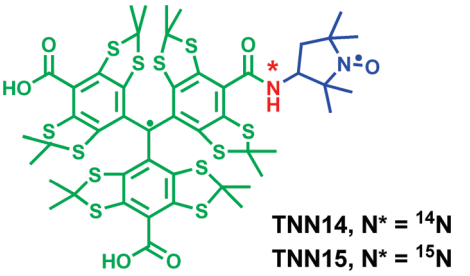

Simultaneous evaluation of redox status and oxygenation in biological systems is of great importance for the understanding of biological functions. Electron paramagnetic resonance (EPR) spectroscopy coupled with the use of the nitroxide radicals have been an indispensable technique for this application but are still limited by low oxygen sensitivity and low EPR resolution in part due to the moderately broad EPR triplet and spin quenching through bioreduction. In this study, we showed that these drawbacks can be overcome through the use of trityl-nitroxide biradicals allowing for the simultaneous measurement of redox status and oxygenation. A new trityl-nitroxide biradical **TNN14** composed of a pyrrolidinyl-nitroxide and a trityl and its isotopically labeled ^{15}N analogue **TNN15** were synthesized and characterized. Both biradicals exhibited much stronger spin−spin interaction with $J > 400$ G compared with that of the previous synthesized trityl-nitroxide biradicals **TN1** (∼160 G) and **TN2** (∼52 G) with longer linker chain length. The enhanced stability of **TNN14** was evaluated using ascorbate as reductant, and the effect of different types of cyclodextrins on its stability in the presence of ascorbate was also investigated. Both biradicals are sensitive to redox status, and their corresponding trityl-hydroxylamines resulting from the reduction of the biradicals by ascorbate share the same oxygen sensitivity. Of note is that the ^{15}N-labeled **TNN15-H** with an EPR doublet exhibits improved EPR signal amplitude as compared with that of **TNN14-H** with an EPR triplet. In addition, cyclic voltammetric studies verify the characteristic electrochemical behaviors of the trityl-nitroxide biradicals.

Introduction

Noninvasive measurement and monitoring of molecular oxygen (O_2) or "oximetry" and redox status are of importance for the understanding of biological functions, and the pathogenesis of various diseases. Nuclear magnetic resonance (NMR)- and low field electron paramagnetic resonance (EPR)-based techniques, coupled with the use of exogenous paramagnetic probes, are the most appropriate approaches for the noninvasive in vivo O_2 measurement and redox assessment because of the reasonable depth of magnetic field penetration in animal tissues.[1−4] In principle, EPR

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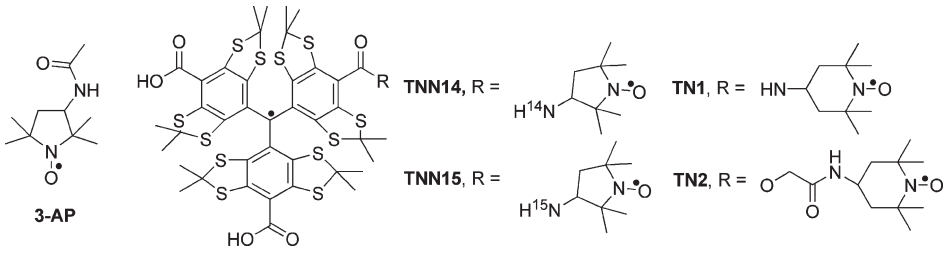
CHART 1. Molecular Strutures of Biradicals and Nitroxide 3-AP



methods (both spectroscopic and imaging) show better sensitivity to O_2 and redox measurement compared to NMR methods since the former have a much higher intrinsic sensitivity to exogenous probe concentration. EPR imaging has been widely used to measure oxygenation and redox status in living tissue and in vivo in mice.[5−8] Recently, a new MRI-based technique, commonly called Overhauser-enhanced MRI (OMRI), which combines the sensitivity of EPR with the advantage of MRI, has been employed for the measurement of tissue oxygenation and redox status.[9−11]

Among the commonly used paramagnetic probes, nitroxide radicals are the most popular, but their application in vivo was greatly limited by their rapid bioreduction and their relatively low sensitivity to O_2. Although the information on the redox status was extracted from the bioreduction of the nitroxides in the investigated systems by EPR imaging or OMRI, this spin quenching mechanism greatly limits the imaging resolution. Recent development of extraordinary stable trityl radicals has tremendous benefits in EPR imaging and OMRI.[12−21] To date, trityl radicals have been utilized to measure extracellular[22] and intracellular[17,23] oxygen level, superoxide radical anion,[24] and pH.[18,25] However, their application as redox probes has been a challenge due to their inertness to biological reductants such as ascorbate and glutathione.[16,24]

Recently, we described a novel strategy using trityl-nitroxide biradicals to simultaneously measure redox status and oxygenation through EPR spectrocopy.[26] As redox and O_2 probes, these biradicals possess several advantages compared to nitroxide radicals: (1) Their bioreduction results in the formation of the trityl-hydroxylamine monoradical whose signal intensity is enhanced, therefore effectively increasing the EPR signal and secondary sensitivity. In contrast, the EPR signals of simple nitroxides are completely quenched by bioreduction. (2) The resulting trityl radical from bioreduction has narrow singlet EPR line and high stability, which can further increase quality and resolution of EPR imaging in less time. (3) Introduction of an amide group into the trityl molecule affords a partial overlapping sharp triplet EPR signal that exhibits a higher O_2 sensitivity as compared with that of the usual trityl radical with a singlet EPR signal or the nitroxide radicals. As part of our continuing efforts in trityl-nitroxide biradical development, we herein report the synthesis of a new trityl-nitroxide biradical **TNN14** and its isotopically labeled analogue **TNN15** (Chart 1) with a short linker group. These two biradicals exhibit higher sensitivity for the simultaneous measurement of redox status and oxygenation as compared to the prior trityl-nitroxide biradicals with longer linker group. In addition, their electrochemical properties were also investigated.

Results and Discussion

The synthesis of the biradical **TNN14** and its analogue **TNN15** is shown in Scheme 1. While the nitroxide radical **^{14}NN** (also called 3-amino-PROXYL) is commercially available, its isotopical analogue **^{15}NN** was synthesized using 3-carboxy-proxyl (**3-CP**) as a starting material according to a previous procedure.[27] **3-CaP** was obtained by conjugation of **3-CP** with HOSu, followed by amidation using $^{15}NH_4Cl$. Subsequent treatment of **3-CaP** with sodium hypobromite at 0−70 °C for 3 h affords the ^{15}N-labeled nitroxide, **^{15}NN**, with a high yield of 52% from **3-CP**. The trityl radical **CT-03** was synthesized via the previously reported 4-step method[15] and linked with **^{14}NN** or **^{15}NN**, to afford the biradicals **TNN14** or

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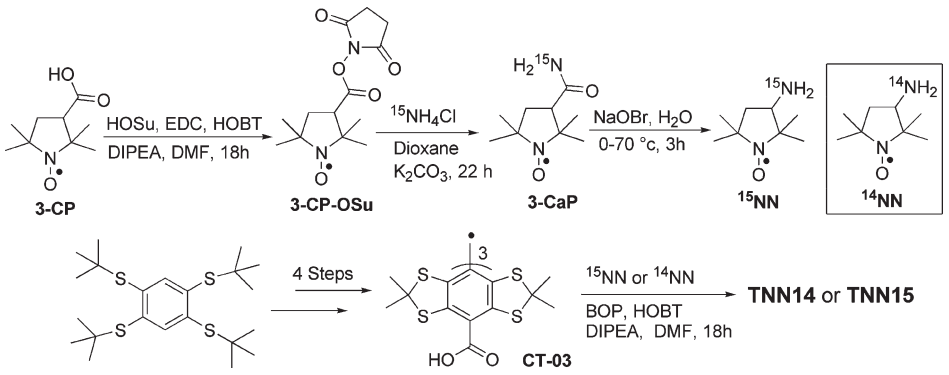
SCHEME 1. Synthesis of Biradicals TNN14 and TNN15



TNN15, which were characterized by HRMS and IR. The purity of both biradicals was determined by HPLC (>98%) and EPR method[17] (97 ± 1% for **TNN14** and 95 ± 1% for **TNN15**) (see Supporting Information). Interestingly, two close peaks were observed in their HPLC chromatograms indicating two components that were further determined to be two isomers by MS analysis (see Supporting Information). These two isomers are possibly diastereomers associated with the inherent chirality of the trityl moiety and C-3 of the nitroxide.

Figure 1 shows the room-temperature EPR spectra of **TNN14** and **TNN15**. Completely identical spectral profiles for both biradicals were observed, indicating that the isotopic labeling in the amide group did not affect the magnitude of spin−spin interaction between the two radical moieties. EPR spectra for both biradicals present a well-resolved triplet with the lines spaced by ~7.9 G, slightly less than half the ^{14}N hyperfine splitting of the five-membered ring nitroxide **3-AP** (16.1 G, Chart 1) in aqueous solution. Interestingly, the EPR triplet lines have almost the same line width and amplitude owing to the strong intramolecular spin−spin interaction. EPR spectral profile of **TNN14** is not affected at a concentration range of 10−500 μM (see Figure S3 in Supporting Information), further confirming the intramolecular nature of the spin interaction. Computer simulation[28] of these spectra gave a *J*-coupling value of >400 G for **TNN14** and **TNN15** at room temperature, which is much higher than the values of the previously reported biradicals, **TN1** (~160 G) and **TN2** (~52 G).[26] The high *J*-coupling values for **TNN14** and **TNN15** are most likely due to the short distance between the NO moiety and the central carbon of the trityl radical compared to those of **TN1** and **TN2**.

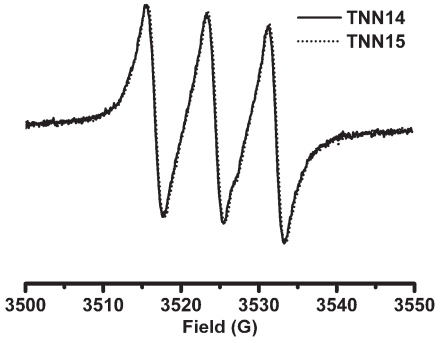


FIGURE 1. Room-temperature X-band EPR spectra of **TNN14** and **TNN15** in PBS (pH 7.4, 50 mM). Spectra were recorded with 10 mW microwave power and 1 G modulation amplitude.

The potential application of the new biradicals as redox probes was then evaluated using ascorbate, which is the main reductant of nitroxides in biological systems. The reduction of **TNN14** by ascorbate resulted in the formation of the monoradical trityl-hydroxylamine (**TNN14-H**) and afforded an intense partially overlapped EPR triplet signal (Figure 2A and inset). The formation of the signal was accompanied by a relatively slow decrease of the biradical signal due to the transformation of **TNN14** into **TNN14-H**. The unchanged UV absorbance around 469 nm proved that the trityl moiety is stable toward ascorbate (see Supporting Information). Addition of the oxidant $K_3Fe(CN)_6$ resulted in the recovery of the signal due to **TNN14** from **TNN14-H** (see Supporting Information), suggesting the reversibility of the reduction of the nitroxide moiety. Figure 2B shows the effect of the ascorbate concentration on the production of **TNN14-H**. Higher concentrations of ascorbate led to a faster increase in the signal intensity of the singlet peak and vice versa. According to the data shown in Figure 2B, the second order rate constant (k_2) for the **TNN14** reduction by ascorbate was determined to be 0.44 ± 0.07 M^{-1} s^{-1}. A similar k_2 value was observed for the nitroxide **3-AP** (Chart. 1) (0.36 ± 0.06 M^{-1} s^{-1}, see Supporting Information) which indicates that linkage of the nitroxide to the trityl does not significantly affect the reactivity of the nitroxide moiety. Moreover, **TNN14** has a much smaller k_2 compared with those of the piperidinyl nitroxide-trityl biradicals **TN1** (4.14 ± 0.14 M^{-1} s^{-1}) and **TN2** (3.48 ± 0.09 M^{-1} s^{-1}).[9] This result is consistent with the previous work showing the lower rates of reduction for the pyrrolidinyl nitroxide compared to the piperidinyl nitroxide due to the conformational flexibility of the latter compared to the former.[29]

Although the nitroxide bioreduction provides important information on the redox state in a given biological system, the bioreduction is often too fast to be monitored. In this study, the use of five-membered ring nitroxide for the construction of the trityl-nitroxide biradicals effectively slows their reduction by ascorbate as mentioned above. In order to further stabilize the biradicals and enhance their in vivo application potential, we investigated the effect of three different types of cyclodextrins (CDs) on the stability of the biradicals on the basis of the knowledge that CDs can effectively protect nitroxide radicals[30−32] and nitrone spin adducts[33−35] from bioreduction.

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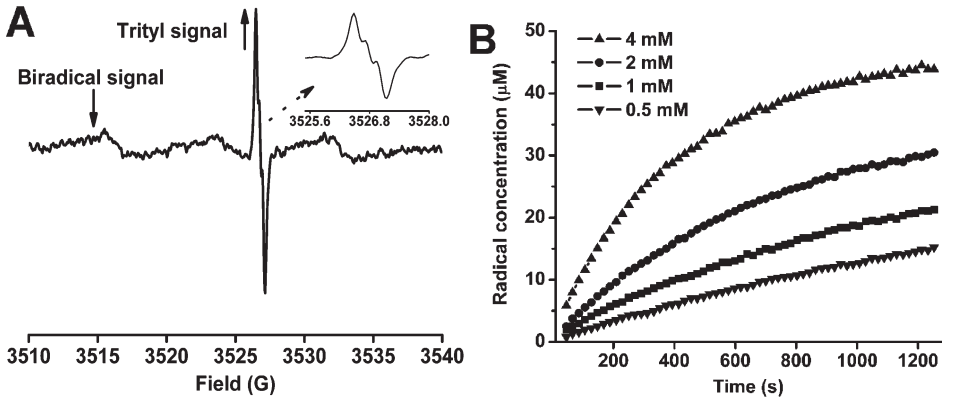

FIGURE 2. (A) EPR spectrum obtained from the reaction mixture containing **TNN14** (50 μM) and ascorbate (4 mM) in PBS (pH 7.4, 50 mM) after 45 s at room temperature; (↑) indicates the increasing signal of the trityl monoradical **TNN14-H** that resulted from the reduction of the biradical **TNN14**; (↓) shows the decreasing signal of the biradical **TNN14**; the inset shows an expanded portion of the spectrum of the trityl monoradical to better visualize its triplet hyperfine structure. To detect signals from both the biradical and the resulting trityl radical without any distortion, spectra were recorded with 1 mW microwave power and 0.08 G modulation amplitude. These low values of microwave power and modulation amplitude are required to prevent broadening or saturation of the trityl radical spectrum. (B) Plots of the **TNN14-H** concentration as a function of time at various ascorbate concentrations at room temperature.

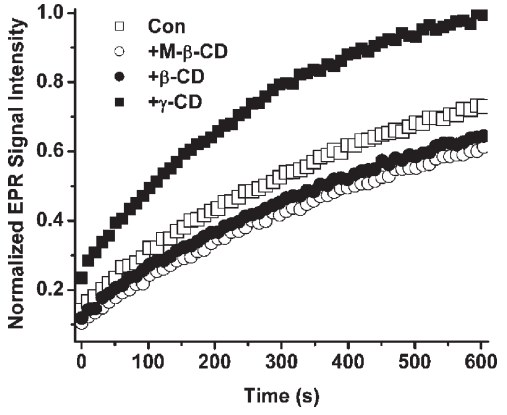

FIGURE 3. Effect of various cyclodextrins (2 mM) on the reduction of **TNN14** (50 μM) and ascorbate (4 mM) in PBS.

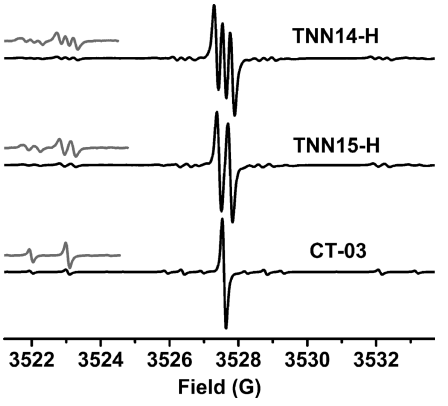

FIGURE 4. X-band EPR spectra of **TNN14-H**, **TNN15-H**, and **CT03** in PBS (pH 7.4, 50 mM) under anaerobic conditions. Gray lines show the enlarged (5×) portion of the spectrum. Spectra were recorded with 0.5 mW microwave power and 0.03 G modulation amplitude. These low values were required to prevent distortion of the very sharp trityl radical spectra detected under anaerobic conditions.

As shown in Figure 3, β-CD and its methylated analogue (M-β-CD) effectively decrease the reduction of **TNN14** by ascorbate as evidenced by the slower formation of the trityl signal intensity in the solution of **TNN14** containing ascorbate. Comparatively, γ-CD enhances the reaction between **TNN14** and ascorbate with 148% higher signal intensity in the presence of γ-CD than in the absence of γ-CD. The larger cavity size of γ-CD compared to β-CD could accommodate both the biradical, (or at least the nitroxide moiety), and ascorbate, therefore facilitating their reaction. Although high concentrations of β-CDs were used to provide protection to biradicals in this study, the covalent linkage of biradicals with methyl-β-CDs may offer better strategy for more stable biradicals with longer half-lives in biological milleiu.

Under anaerobic condition, the partially overlapped EPR triplet signal of **TNN14-H** (Figure 2A) becomes more pronounced (Figure 4, top) with a hyperfine splitting constant (hfc) of 0.23 G due to reduced Heisenberg exchange between **TNN14-H** and paramagnetic O_2. Comparatively, the trityl-hydroxylamine **TNN15-H** obtained through reduction of **TNN15** by ascorbate gave a well-resolved doublet with a hfc of 0.33 G under anaerobic condition (Figure 4). These additional hyperfine splittings observed from the trityl-hydroxylamines should be due to the amide-N ($I=1$ for ^{14}N and $I=1/2$ for ^{15}N) of the linker group, verifying our previous theoretical and experimental results showing long-range radical coupling.[26] Apart from the hyperfine interactions with the main nitrogen isotope (i.e., ^{14}N for **TNN14-H** and ^{15}N for **TNN15-H**), the interactions with the ^{13}C (natural abundance, 1.11%) mainly from the carbons of three aromatic groups and the other

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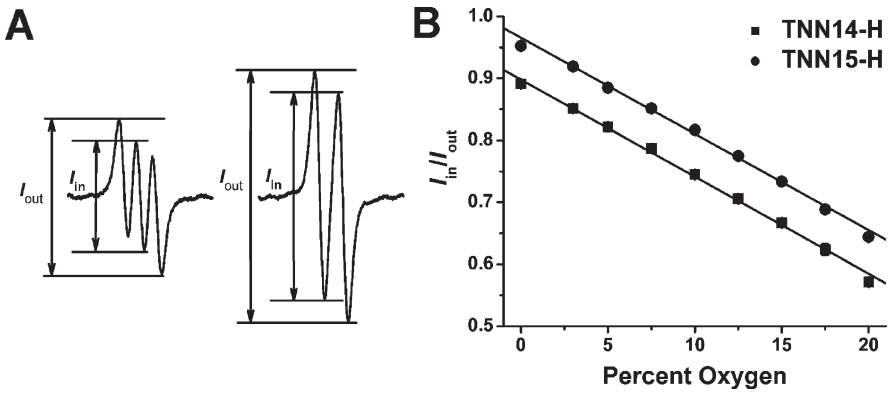

FIGURE 5. (A) EPR spectra of **TNN14-H** and **TNN15-H** denoting the spectral ratios I_{in} and I_{out} in the presence of 10% O_2−90% N_2. Spectra were recorded with 0.5 mW microwave power and 0.03 G modulation amplitude. (B) Plot of I_{in}/I_{out} as a function of percent oxygen. The trityl-hydroxylamines were generated by mixing ascorbate (4 mM) with the corresponding biradical (50 μM).

nitrogen isotope (natural abundance of ^{15}N (0.37%) for **TNN14-H** and ^{14}N (1%) for **TNN15-H** in our experiment) can be also observed, giving the weak flanking lines as shown in Figure 4.[36] Interestingly, the direct linkage of the amide group with the trityl moiety can further split these signals from **CT-03** into a doublet for **TNN15-H** or a triplet for **TNN14-H** (see the enlarged signals in Figure 4).

It has been demonstrated that the partially overlapped EPR peaks of the trityl radicals are sensitive to O_2 and the spectral ratio (I_{in}/I_{out}) exhibits enhanced sensitivity toward O_2, especially at low pO_2, relative to the line width.[21,26] It is therefore reasonable to use the ratio I_{in}/I_{out} to evaluate the oxygen sensitivity of **TNN14-H** and **TNN15-H**. Figure 5A shows the EPR spectra of **TNN14-H** and **TNN15-H**, which are denoted by the spectral intensities, I_{in} and I_{out}. As shown in Figure 5B, the spectral ratio I_{in}/I_{out} is highly sensitive to O_2 and both radicals have almost identical O_2 sensitivities with values of $1.55 \times 10^{-3}/\%O_2$ for **TNN14-H** and $1.53 \times 10^{-3}/\%O_2$ for **TNN15-H**. Although the isotopic ^{15}N labeling does not improve the oxygen sensitivity of **TNN15-H**, **TNN15-H** has 3/2 = 1.5 times larger EPR signal amplitude than **TNN14-H** at the same concentrations due to the reduced number of hyperfine lines (Figure 5A). Therefore, the isotopic ^{15}N labeling of the biradical can greatly improve its sensitivity and resolution for simultaneous measurement of redox status and oxygenation as compared to nitroxides and trityl radicals alone as well as the nonlabeled trityl-nitroxide biradicals, **TN1**, **TN2**, and **TNN14**.

It has been demonstrated that as neutral radicals, both trityl and nitroxide radicals can be either reduced or oxidized via a one-electron process. It is thus interesting to investigate the electrochemical behavior of the trityl-nitroxide biradicals. In doing so, cyclic voltammeric studies were carried out on the biradical **TNN14**. As shown in Figure 6, **TNN14** undergoes two one-electron reversible oxidations of **TNN14** to the corresponding trityl cation and oxammonium at $E_{1/2}$ (ox) = 0.515 V vs Ag/AgCl (ΔE_p = 64 mV) and 0.686 V vs Ag/AgCl (ΔE_p = 61 mV), respectively. A quasi-reversible one-electron reduction at $E_{1/2}$ (red) = −0.581 V vs Ag/AgCl (ΔE_p = 113 mV) was also observed, which was assigned to the reduction of the trityl moiety to the corresponding anion. Under our experimental conditions, the reduction of the nitroxide moiety was not observed, although

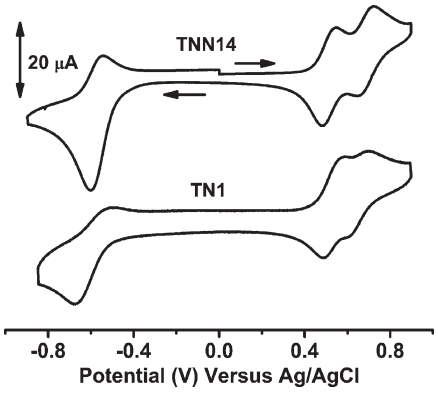

FIGURE 6. Cyclic voltammograms of **TNN14** (1 mM) and **TN1** (1 mM) in PBS (20 mM, pH 7.4) containing 0.15 M NaCl. Scan rate: 100 mV/s.

the above result has showed that the biradical **TNN14** can undergo one-electron reduction by ascorbate to the corresponding trityl-hydroxylamine. Compared to the redox potential of the parent trityl **CT-03** [$E_{1/2}$ (ox) = 0.45 V vs Ag/AgCl and $E_{1/2}$ (red) = −0.63 V vs Ag/AgCl],[16] the trityl moiety in **TNN14** is more difficultly oxidized [$E_{1/2}$ (ox) = 0.515 V vs Ag/AgCl] but easier to reduce [$E_{1/2}$ (red) = −0.581 V vs Ag/AgCl] because one of carboxylate groups in **CT-03** is replaced by a stronger electron-withdrawing amide group in the case of **TNN14**. Cyclic voltammetric studies were also carried out on the biradical **TN1**. The replacement of the pyrrolidinyl nitroxide in **TNN14** with a piperidinyl nitroxide makes the first oxidation ($E_{1/2}$ = 0.531 V) and reduction ($E_{1/2}$ = −0.587 V) slightly more difficult but easier for the second oxidation ($E_{1/2}$ = 0.655 V).

In summary, the newly synthesized trityl-nitroxide biradical **TNN14** and its ^{15}N-labeled analogue **TNN15** possess strong intramolecular spin−spin interaction (J > 400 G) due to the short distance between the two radical centers. Both biradicals are sensitive to the variation of the redox status. The use of five-membered ring nitroxide for the construction of the trityl-nitroxide biradicals as well as exogenous addition of β-CD and its methylated analogue M-β-CD effectively increases the stability of these two biradicals toward the reductant ascorbate, therefore enhancing their suitability for in vivo application. While both trityl-hydroxylamines **TNN14-H** and **TNN15-H** resulting from the reduction of the

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corresponding biradicals show high oxygen sensitivity, the ^{15}N-labeled **TNN15-H** with its doublet EPR signal exhibits improved EPR signal resolution compared with that of **TNN14-H** with its EPR triplet. Therefore the new probes also exhibit enhanced sensitivity for simultaneous measurement of redox status and oxygenation compared to either nitroxide or trityl radicals alone.

Experimental Section

EPR Measurements and Simulations. EPR measurements were carried out on a Bruker EMX-X band with an HS resonator at room temperature. General instrument settings were as follows: modulation frequency, 100 kHz; microwave frequency, 9.87 GHz; microwave power, 10 mW for biradicals and nitroxide radicals and 0.5−2 mW for the trityl-hydroxylamines; microwave frequency, 9.87 GHz; modulation amplitude, 1.0 G for biradicals and nitroxide radicals and 0.03−0.08 G for the trityl-hydroxylamines; receiver gain, $(1-10.00) \times 10^4$; time constant, 10.24−40.96 ms; sweep time, 10.49−41.94 s. Measurements were performed using 50 μL capillary tubes.

Simulations were carried out using a well-developed EPR simulation program (ROKI\EPR).[28] The fitting routine to determine the J values of the trityl-nitroxide biradicals was similar to the method described in our previous study.[26] Since we could not find any improvement about the quadratic error between experimental and calculated spectra when J was larger than 400 G, the J values for **TNN14** and **TNN15** were suggested to be >400 G.

Oxygen Sensitivity. Oxygen sensitivities of the trityl-hydroxylamine **TNN14-H** and **TNN15-H** were evaluated according to our previous method. In brief, ascorbic acid (4 mM) was added to a solution of the biradicals (50 μM) in PBS buffer. After 60 min, the solution was transferred into a gas-permeable Teflon tube (i.d. = 0.8 mm) and was sealed at both ends. The sealed sample was placed inside a quartz EPR tube with open ends. Nitrogen or N_2/O_2 gas mixture with varying concentrations of O_2 was allowed to bleed into the EPR tube and after about 4 min was changed into another gas mixture. EPR spectra were recorded using a model of incremental sweep. According to the resulting spectra, the spectral ratio (I_{in}/I_{out}) was calculated.

Cyclic Voltammetry. Cyclic voltammetry was performed on a potentiostat and computer-controlled electroanalytical system. Electrochemical measurements were carried out in a 10 mL cell equipped with a glassy carbon working electrode (7.07 mm^2), a platinum-wire auxiliary electrode, and a Ag/AgCl reference electrode. Solutions of biradicals (1 mM) were degassed by bubbling with the nitrogen gas before the detection. The redox potentials were calculated according to the relation $E = (E^a_p + E^c_p)/2$.

Reaction Kinetics of TNN14 with Ascorbate. Various concentrations of ascorbic acid (0.5, 1, 2, and 4 mM) were added to the solution of **TNN14** (50 μM) in PBS (50 mM, pH 7.4). Incremental EPR spectra were recorded 45 s after mixing. The concentration of the trityl-hydroxylamine **TNN14-H** at each time point was obtained by comparing their double integrated signal intensities relative to CT03 as standard. Since the ascorbic acid concentration (0.5, 1, 2, and 4 mM) used was in greater excess than the biradical concentration (50 μM), the reaction kinetics of the biradical with ascorbic acid is a pseudo-first-order reaction. The resulting curves in Figure 2B were fitted with the equation $\ln[(C_0 - C_t)/C_0] = -k_{obs}t$, where C_0 is the initial concentration of **TNN14**, C_t the concentration of the trityl-hydroxylamine **TNN14-H** at each time point, and k_{obs} the observed pseudo-first-order rate constant. Considering $k_{obs} = k_2[\mathrm{Asc}]$, the approximated second-order rate constant k_2 was finally calculated from the slope of the plot of k_{obs} versus [Asc].

Synthesis. 1-Oxyl-2,2,5,5-tetramethylpyrrolidinyl-3-carboxylic Acid *N*-Hydroxysuccinimide Ester (3-CP-OSu). To a solution of 3-carboxy-proxyl (0.53 g, 2.86 mmol), HOBT (1.61 g, 8.60 mmol), DIPEA (2.49 mL, 14.3 mmol), and *N*-hydroxysuccinimide (0.66 g, 5.73 mmol) in DMF (20 mL) was added a solution of EDCI (1.10 g, 5.73 mmol) in DMF (5 mL) at 0 °C. After addition, the reaction mixture was stirred for 18 h at room temperature. Solvents were removed under vacuum, and the residue was purified by flash column chromatography using the solvents ethyl acetate/petroleum ether (1:3, 1:2, and then 1:1 v/v) as eluents. A light yellowish solid (0.71 g) was obtained. Yield: 88%. IR (cm^{-1}, neat): 2978, 2937, 1810, 1783, 1735, 1464, 1427, 1366, 1304, 1245, 1200, 1092, 1065, 1047, 994, 958, 920, 899, 813, 735, 647. GC−MS: retention time, 5.90 min; $[M - C_4H_4NO_3 + H]^+$, 186.11(calculated), 186.00 (measured); $[M - C_9H_{15}NO_3 + H]^+$, 115.03 (calculated), 114.94 (measured).

3-Carbamoyl (^{15}N)-2,2,5,5-Tetramethylpyrrolidin-1-yloxy (3-CaP). To a solution of 3-CP-OSu (0.5 g, 1.77 mmol) and K_2CO_3 (0.3 g) in dioxane (5 mL) and water (1 mL) was added solid ^{15}NH4Cl (99% isotopic purity, 0.106 g, 1.94 mmol). The reaction mixture was stirred for 22 h at room temperature, filtered, and evaporated to dryness under vacuum. The residue was redissolved in 10 mL of ethyl acetate. After filtration, the filtrate was concentrated and separated by flash column chromatography using 2% methanol in dichlomethane as an eluent. The yellow solid was obtained and recrystallized from hexane and ethyl acetate to give the pure 3-Cap (0.31 g, 94%). IR (cm^{-1}, neat): 3346, 3195, 2977, 2934, 1668, 1462, 1426, 1365, 1321, 1283, 1244, 1171, 1154, 1102, 830, 739. GC−MS: retention time, 7.22 min; $[M]^+$, 186.13 (calculated), 185.99 (measured).

3-Amino (^{15}N)-2,2,5,5-Tetramethyl-1-pyrrolidin-1-yloxy (^{15}NN). To a solution of sodium hypobromite prepared from 0.36 g of NaOH (9 mmol), 4 mL of water, and 0.34 g of bromide (2.13 mmol) was added 0.28 g of 3-CaP (1.50 mmol) at 0 °C. The reaction mixture was stirred for 2 h at 0 °C, heated to 70 °C, and kept this temperature for 1 h. The reaction mixture was cooled with the ice bath, treated with 6 g KOH, and extracted with ether. The extract was dried on anhydrous Na_2SO_4, concentrated, and separated by flash column chromatography using $CH_2Cl_2/CH_3OH/NH_4OH$ = 10:1:0.05 as an eluent. A yellow solid (0.15 g) was obtained. Yield: 63%. IR (cm^{-1}, neat): 3483, 3363, 3291, 3198, 2972, 2933, 1610, 1463, 1363, 1315, 1254, 1228, 1195, 1164, 1102, 1059, 1024, 895, 834, 763, 673. GC−MS: retention time, 5.13 min; $[M]^+$, 158.13 (calculated), 157.99 (measured).

TNN14. To a solution of **CT-03** (100 mg, 0.1 mmol), HOBt (40.5 mg, 0.3 mmol), and BOP (46.4 mg, 0.105 mmol) in dry DMF (10 mL) was added DIPEA (90 μL) under N_2. The reaction mixture was stirred at room temperature for 20 min, and then ^{14}NN (16.5 mg, 0.105 mmol) in 5 mL of DMF was added dropwise. The resulting mixture was continuously stirred for 18 h at room temperature. Solvent was removed under vacuum, and the residue was dissolved in phosphate buffer (0.1 M, pH 7.4) and purified by column chromatography on reverse phase C-18 using water followed by 0−15% acetonitrile in water as eluants to give the biradical **TNN14** as a green solid (75 mg, 66%). Purity: >98% by HPLC (see Supporting Information) and 97 ± 1% versus TEMPOL determined as previously reported.[17] IR (cm^{-1}, neat): 3425.1, 2971, 2933, 1646, 1580, 1454, 1367, 1312, 1237, 1168, 1150, 1113, 886, 820, 725, 697. MS ($[M+H]^+$, m/z): 1139.038 (measured), 1139.071 (calculated); ($[M + Na]^+$, m/z): 1161.000 (measured), 1161.052 (calculated).

TNN15. To the solution of **CT-03** (63.3 mg, 63.3 μmol), HOBt (25.6 mg, 189.9 μmol) and BOP (29.4 mg, 66.5 μmol) in dry DMF (8 mL) was added DIPEA (60 μL) under N_2. The reaction mixture was stirred at room temperature for 20 min, and then ^{15}NN (10 mg, 63.3 μmol) in 5 mL of DMF was added dropwise. The resulting mixture was continuously stirred for 18 h at room temperature. Solvent was removed under vacuum, and the residue was dissolved in phosphate buffer (0.1 M, pH 7.4) and purified by column chromatography on reverse phase C-18 using water followed by 0−15% acetonitrile in water as eluants

to give the biradical **TNN15** as a green solid (45 mg, 62%). Purity: >98% by HPLC (See Supporting Information) and $95 \pm 1\%$ versus TEMPOL determined as previously reported.[17] IR (cm^{-1}, neat): 3423, 2973, 2918, 1646, 1579, 1455, 1367, 1238, 1150, 883, 727. MS ($[M+H]^+$, m/z):1140.044 (measured), 1140.068 (calculated); MS ($[M+K]^+$, m/z): 1177.985 (measured), 1178.024 (calculated).

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Supporting Information Available: GC−MS, IR, HRMS spectra and HPLC chromatograms and kinetic studies of the nitroxide radical 3-AP with ascorbate. This material is available free of charge via the Internet at http://pubs.acs.org.