

# TOTAPOL: A Biradical Polarizing Agent for Dynamic Nuclear Polarization Experiments in Aqueous Media

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**Abstract:** In a previous publication, we described the use of biradicals, in that case two TEMPO molecules tethered by an ethylene glycol chain of variable length, as polarizing agents for microwave driven dynamic nuclear polarization (DNP) experiments. The use of biradicals in place of monomeric paramagnetic centers such as TEMPO yields enhancements that are a factor of approximately 4 larger ( $\epsilon \sim 175$  at 5 T and 90 K) and concurrently the concentration of the polarizing agent is a factor of 4 smaller (10 mM electron spins), reducing the residual electron nuclear dipole broadening. In this paper we describe the synthesis and characterization by EPR and DNP/NMR of an improved polarizing agent 1-(TEMPO-4-oxy)-3-(TEMPO-4-amino)propan-2-ol (TOTAPOL). Under the same experimental conditions and using 2.5 mm magic angle rotors, this new biradical yields larger enhancements ( $\epsilon \sim 290$ ) at lower concentrations (6 mM electron spins) and has the additional important property that it is compatible with experiments in aqueous media, including salt solutions commonly used in the study of proteins and nucleic acids.

## 1. Introduction

The sensitivity in solid-state NMR (ssNMR) experiments can be enhanced by 2–3 orders of magnitude by dynamically polarizing the nuclear spin system prior to recording the NMR spectrum.<sup>1–12</sup> This procedure involves microwave irradiation of the electron paramagnetic resonance (EPR) spectrum of either an endogenous or exogenous paramagnetic species present in the sample and results in the transfer of the greater spin polarization of the electrons to the nuclei. However, to perform dynamic nuclear polarization (DNP) experiments at magnetic fields used in contemporary NMR experiments (5–20 T), the following three requirements must be satisfied. First, a high-

frequency (140–600 GHz), high-power ( $\sim 10$  W) microwave source is required to drive the continuous-wave (CW) DNP transitions associated with the second-order electron–nuclear dipolar interactions. To date this requirement has been satisfied by utilizing gyrotrons<sup>13–16</sup> since they operate in the requisite frequency range and produce the required microwave powers. Second, the relaxation times of the spin systems in the experiment dictate that it be optimally performed at low temperatures (usually  $\leq 90$  K), and to obtain high-resolution spectra of solids, magic-angle spinning (MAS) is incorporated into the experiment.<sup>4–6,9,11</sup> Thus, multiple resonance—i.e., <sup>1</sup>H, <sup>13</sup>C, <sup>15</sup>N, and e<sup>−</sup>—low-temperature MAS probes are required for proper execution of DNP experiments.

The third requirement is the presence of a suitable paramagnetic center that acts as source of polarization. This paramagnetic center should (a) be compatible with the polarization mechanism that yields the optimal signal enhancement, namely the three-spin thermal mixing (TM)<sup>4,17</sup> or cross effect (CE),<sup>18–23,24</sup> (b)

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be useful in polarizing a large array of samples ranging from small molecules to proteins, (c) produce large signal enhancements at a reduced concentration of paramagnetic species, and (d) be soluble in aqueous media. In a previous publication,<sup>12</sup> we described the use of biradicals that satisfy the first three of these criteria and yield improved DNP enhancements. In particular, we reported that bis-TEMPO-*n*-ethylene glycol (BTnE), where TEMPO is 2,2,6,6-tetramethylpiperidin-1-oxyl and *n* = 2 indicates a tether of two ethylene glycol units, produced DNP enhancements of ~175 at 90 K and 5 T. Further, this was accomplished at a reduced radical concentration (~5 mM biradicals or 10 mM electron spins, as opposed to ~40 mM monomeric TEMPO), thus reducing the electron nuclear dipolar broadening. The design, synthesis, and characterization of an improved polarizing agent, 1-(TEMPO-4-oxy)-3-(TEMPO-4-amino)propan-2-ol (TOTAPOL), satisfying the first three as well as the fourth requirement, is the topic of this paper. Specifically, we have prepared a biradical consisting of two TEMPO molecules tethered with a three carbon chain that increases the average electron–electron dipole coupling constant from ~0.5 MHz, in the typical 40 mM solution of TEMPO that we use for DNP experiments, to ~30 MHz in the biradical. At 140 GHz this yields a maximum enhancement of ~290, and again the electron concentration is reduced by a factor of 6 from the typical level of 40 to 6 mM. Very importantly, TOTAPOL has hydroxyl and secondary amine moieties on the tether and these functional groups increase the solubility of the biradical in aqueous media so that it is compatible with a variety of biological systems where DNP experiments are currently performed.

In this article we describe the synthesis of TOTAPOL and several EPR and DNP-enhanced NMR experiments that characterize the molecule and illustrate its utility as an effective polarizing agent. These include a comparison of the enhancements obtained with TEMPO and several other biradicals, as well as its compatibility with low-temperature MAS experiments in glycerol/water mixtures.

## 2. Experimental Section

**2.1. Synthesis of TOTAPOL. 2.1.a. General Experimental Conditions.** 4-Hydroxy-TEMPO (**1**) and 4-amino-TEMPO (**2**), containing ≥97.0% free radical, were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Anhydrous CH<sub>3</sub>CN was purchased from the same company as a Sure-Seal bottle. All other chemicals were of reagent grade and used as received. For NMR analysis, TEMPO radicals were reduced to *N*-hydroxy compounds by ascorbic acid in methanol. NMR spectra were recorded on a Bruker Advance-400 or Varian Mercury-300 spectrometer, and chemical shifts were referenced to residual solvent peaks. IR spectra were obtained on a Nicolet 8700 FT-IR spectrometer, in which the sample was drop-casted on a KBr disk. High-resolution mass spectra were obtained on a Bruker Daltonics APEX II 3T FT-ICR-MS.

**2.1.b. 4-(2,3-Epoxypropoxy)-2,2,6,6-tetramethyl-1-piperidin-1-oxyl [4-(2,3-Epoxypropoxy)-TEMPO] (**3**).** In a 100 mL round-bottom flask equipped with a stirring bar were combined tetrabutylammonium hydrogen sulfate (0.136 g, 4 mol %), 50% w/w aqueous NaOH (10 mL), and epichlorohydrin (3.91 mL, 50 mmol). To the mixture was added 4-hydroxy-TEMPO (1.73 g, 10 mmol) in portions, and the

mixture was stirred overnight at room temperature. It was then poured into water and extracted with ethyl acetate. The organic layer was washed with brine, dried over MgSO<sub>4</sub>, and evaporated under reduced pressure. The resulting crude product was purified by column chromatography (dichloromethane, methanol), providing a quantitative yield of red, viscous oil. IR (KBr disk, cm<sup>-1</sup>): 2975, 2937, 1636, 1464, 1384, 1363, 1244, 1179, 1095. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ 7.12 (bs, 1H), 3.69 (dd, 1H, *J* = 11, 2.7 Hz), 3.60 (m, 1H), 3.24 (dd, 1H, *J* = 11, 6.6 Hz), 3.04 (m, 1H), 2.70 (*pseudo-t*, 1H, *J* = 5.1 Hz), 2.52 (dd, 1H, *J* = 5.1, 2.7 Hz), 1.86 (dd, 2H, *J* = 12, 3.6 Hz), 1.24 (*pseudo-t*, 2H, *J* = 11 Hz), 1.05 (d, 12H, *J* = 11 Hz).

**2.1.c. 1-(2,2,6,6-Tetramethyl-1-oxy-4-piperidinyl)oxy-3-(2,2,6,6-tetramethyl-1-oxy-4-piperidinyl)amino-propan-2-ol [1-(TEMPO-4-oxy)-3-(TEMPO-4-amino)propan-2-ol] (TOTAPOL).** In a 100 mL round-bottom flask equipped with a stirring bar were combined 4-(2,3-epoxy-propoxy)-TEMPO (**3**) (1.62 g, 7 mmol), LiClO<sub>4</sub> (0.745 mg, 7 mmol), and 10 mL of anhydrous CH<sub>3</sub>CN under Ar. To the mixture was added a CH<sub>3</sub>CN (3 mL) solution of 4-amino-TEMPO (1.20 g, 7 mmol), and the mixture was stirred overnight at room temperature. Most of the solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography (dichloromethane, methanol). Yield: 1.98 g of orange-red solid. IR (KBr disk, cm<sup>-1</sup>): 3446, 2977, 2938, 1635, 1465, 1364, 1244, 1178, 1098. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 7.31 (bs, 1H), 7.15 (bs, 1H), 4.57 (bs, 1H), 3.93 (m, 1H), 3.69 (m, 1H), 3.57 (m, 1H), 3.41 (m, 2H), 3.34 (m, 1H), 3.01 (*pseudo-d*, 1H, *J* = 11 Hz), 2.80 (m, 1H), 1.96 (m, 2H), 1.86 (m, 2H), 1.52 (m, 2H), 1.23 (*pseudo-t*, 2H, *J* = 11 Hz), 1.04 (dd, 24H, *J* = 17, 3.9 Hz). HR-MS (ESI): calcd for C<sub>21</sub>H<sub>41</sub>N<sub>3</sub>O<sub>4</sub><sup>2+</sup>; [M + H]<sup>+</sup>, 400.3170; found, 400.3161.

**2.2. EPR Experiments.** For 9 GHz solution EPR experiments, the samples consisted of a capillary containing ~10 μL of 0.5 mM TOTAPOL in absolute ethanol since it has a relatively low dielectric constant that facilitates observation of solution EPR spectra. To increase the resolution of powder EPR spectra from frozen solutions, TOTAPOL was synthesized with <sup>15</sup>N, <sup>2</sup>H-labeled TEMPO's (CDN Isotope, Quebec, Canada). Samples for the X-band experiments were typically 60 μL of 0.5 mM <sup>15</sup>N, <sup>2</sup>H-TOTAPOL in the glass forming solvent <sup>2</sup>H<sub>6</sub>-DMSO/<sup>2</sup>H<sub>2</sub>O (6:4 w/w) in a 4 mm o.d. quartz tube. The 9 GHz CW EPR spectra were recorded with a Bruker EMX spectrometer with the sample immersed in liquid nitrogen (77 K) in a finger dewar (Wilmad WG-819-B-Q). Samples for 140 GHz experiments consisted of 0.4 μL of TOTAPOL in the same deuterated glass forming solution contained in a quartz capillary immersed in a helium cryostat (Oxford Instruments). The 140 GHz spectra were recorded with echo-detected experiments using a custom-designed spectrometer.<sup>13</sup> The rigid-limit for EPR spectra of TOTAPOL is reached below 100 K in the glass forming mixture; thus, the 20 and 77 K temperatures chosen for the above EPR experiments are sufficient for observing rigid limit EPR spectra.

**2.3. DNP-Enhanced NMR Experiments with TOTAPOL.** Solutions for the DNP/NMR experiments—2 M <sup>13</sup>C-urea or 0.2 M U-<sup>13</sup>C-<sup>15</sup>N-proline—were prepared in <sup>2</sup>H<sub>6</sub>-DMSO/<sup>2</sup>H<sub>2</sub>O/H<sub>2</sub>O (60:34:6 w/w/w) or <sup>2</sup>H<sub>8</sub>-glycerol/<sup>2</sup>H<sub>2</sub>O/H<sub>2</sub>O (60:25:15 w/w/w) and doped with 3–5 mM TOTAPOL (6–10 mM electron spins). The reduced <sup>1</sup>H concentration was required to optimize the signal enhancements and chosen to maintain effective proton homonuclear spin diffusion. DNP experiments using TOTAPOL as a polarizing agent were performed on a custom-designed DNP/NMR spectrometer and triple-resonance (e<sup>-</sup>, <sup>1</sup>H, and <sup>13</sup>C/<sup>15</sup>N) cryogenic (90 K) MAS probe (with a 4 or 2.5 mm rotor stator)<sup>9,25</sup> operating at 5 T (140 GHz EPR and 211 MHz <sup>1</sup>H NMR). The enhanced <sup>1</sup>H polarization developed by the microwave irradiation was detected indirectly via observation of the cross-polarized (CP) <sup>13</sup>C signals. The 140 GHz microwaves were generated by a gyrotron, a vacuum electron device capable of producing high-power (>10 W)

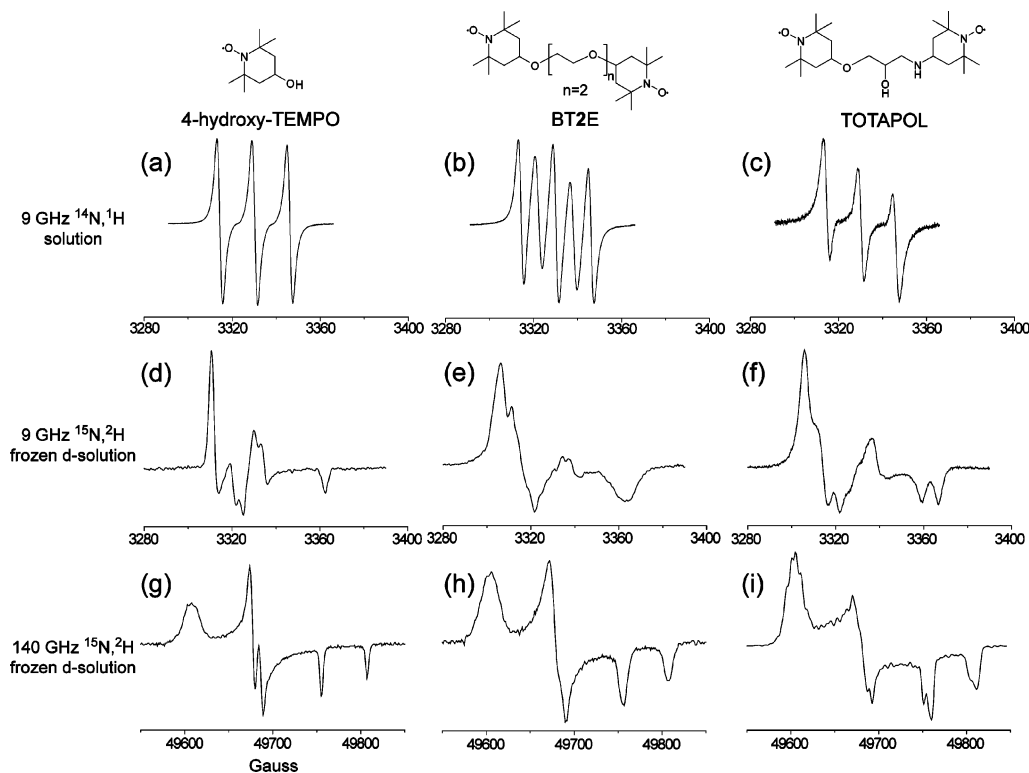
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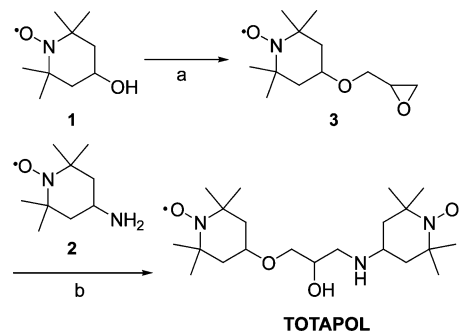
**Figure 1.** 9 and 140 GHz EPR spectra of TEMPO, BT2E, and TOTAPOL with the molecular structures shown in the top line. (a)–(c) are 9 GHz solution spectra illustrating the extra two lines in the spectrum from the transient proximity of two TEMPO moieties with a strong electron–electron  $J$ -coupling [compare (a) with (b)]. These lines in TOTAPOL are severely broadened in (c) by shorter lifetime of the transient proximity due to rigidity of the tether, which cannot bend the biradical easily. (d)–(f) illustrate the 9 GHz spectra obtained from frozen solutions at 77 K, and (g)–(i) illustrate the 140 GHz spectra from the same solutions frozen at 20 K.

millimeter waves.<sup>15</sup> Sapphire, rather than zirconia, rotors are preferred for the DNP experiments since they transmit microwaves with  $\sim 30\%$  less attenuation, and we have used rotors of two diameters, 2.5 and 4 mm, in the experiments reported here. As will be seen below, we find larger enhancements in the smaller diameter system probably because the microwave penetration is more complete. Finally, the 5 T magnet has a superconducting sweep coil used to vary the field by  $\pm 750$  G for EPR and DNP experiments, and that facilitates locating the maximum and minimum in the DNP enhancement curve from its field dependence.

### 3. Results and Discussion

The design of TOTAPOL was dictated by two considerations. First, following our successful experiments on the BTnE series of biradicals whose structures are illustrated in Figure 1 (top row), we wanted to prepare a radical with as short a linker as possible. Second, the new species was supposed to be soluble in aqueous media so that it could be used to polarize biological solids. These considerations motivated us to attempt to prepare a polarizing agent with a two carbon linkage, such as TEMPO–O–CH<sub>2</sub>–CH<sub>2</sub>–O–TEMPO which would be BT1E (vide infra), but despite exploration of several approaches, we were not successful. Subsequently, we did synthesize a biradical with a three carbon linker with asymmetric bridging atoms—N and O rather than two O's. In addition we added an –OH moiety to the central carbon to increase the solubility of the molecule –TEMPO–NH–CH<sub>2</sub>–CH(OH)–CH<sub>2</sub>–O–TEMPO. The fact that we obtained an enhancement factor of  $\sim 165$  (in 4 mm rotors) is probably due to the short electron–electron distance and the fortuitously correct orientation of the two TEMPO  $g$ -tensors. In a separate paper we describe radicals such as

#### Scheme 1. Synthesis of TOTAPOL<sup>a</sup>



<sup>a</sup> Conditions: (a) epichlorohydrin, tetrabutylammonium hydrogen sulfate, 50% w/w NaOH(aq), room temperature; (b) LiClO<sub>4</sub>, CH<sub>3</sub>CN, room temperature.

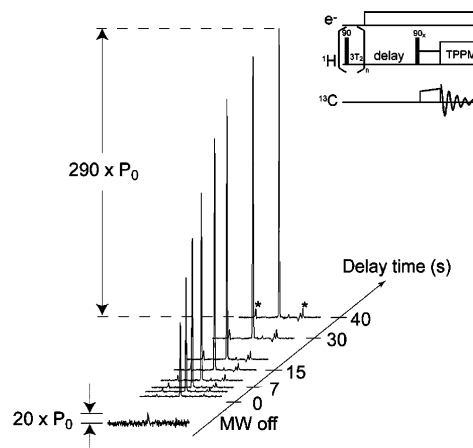
BTurea (two TEMPOs tethered by –NH–CO–NH–) that have a shorter linker but yield enhancements of only about 100 (again in 4 mm rotors). We believe the lower enhancement is due to a suboptimal orientation of the  $g$ -tensors of the two TEMPO moieties. In 2.5 mm rotors we observe larger enhancements from all of the biradicals (up to  $\sim 290$  from TOTAPOL), and we believe that this effect is due to more efficient microwave penetration of the sample as discussed further below.

1-(TEMPO-4-oxy)-3-(TEMPO-4-amino)propan-2-ol (TOTAPOL) was prepared according to the two-step reaction illustrated in Scheme 1. Note that the molecule is an asymmetric biradical with an ether and a secondary amino linkage in contrast to the symmetrical bis-TEMPO-ethylene oxide biradicals that we described previously.<sup>12</sup>

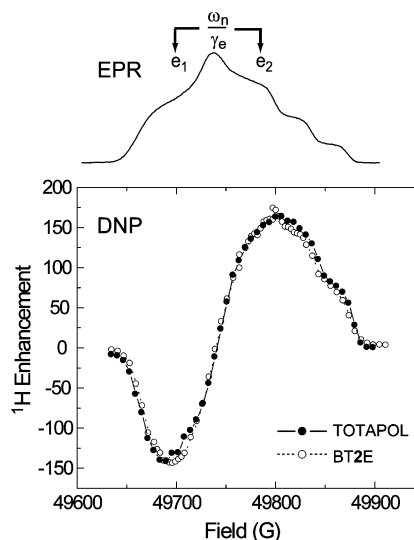
The EPR spectra of flexible biradicals such as BTnE, two TEMPO's tethered by  $n$  ethylene glycol units,<sup>12</sup> usually have two additional peaks yielding a quintet spectrum with the lines spaced at about half the  $^{14}\text{N}$  hyperfine splitting normally observed in TEMPO, 16.7 G. This five-line solution EPR spectrum is a result of the proximity of the two TEMPO radicals and arises when the average  $J$ -coupling (exchange integral) is  $\geq 10$  times stronger than the hyperfine coupling.<sup>26</sup> This phenomenon is illustrated in Figure 1a,b which shows the solution EPR spectra of 4-hydroxy-TEMPO and the biradical BT2E. There are three lines present in the TEMPO spectrum, but in the BT2E spectrum there are two additional lines arising from the strong  $J$ -coupling between the electrons. In contrast, the spectrum of TOTAPOL (Figure 1c) shows that the two additional biradical lines are broadened into the baseline due to the short tether ( $-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{NH}-$ ) that restricts the proximity of two TEMPO moieties.

Moreover, the EPR spectrum of 0.5 mM TOTAPOL in  $^2\text{H}_6$ -DMSO/ $^2\text{H}_2\text{O}$  (6:4 w/w) glass-forming solution at 77 K (Figure 1f) reveals a significantly broadened line shape with a resolved dipolar splitting at 3360 G. Note for comparison that neither the broadening nor the splitting is present in the spectrum of frozen monomeric TEMPO at 1 mM (Figure 1d). For TOTAPOL, the  $\sim 8$  G (Figure 1f,i) splitting at  $g_{zz}$ <sup>27</sup> results from an intramolecular electron–electron interaction, which is mainly attributed to a dipolar coupling. As a comparison, the powder EPR spectra of BT2E reveal broadened features (Figure 1e,h). Analyses of the powder EPR line shapes yielded similar electron–electron distances ( $\sim 12.8$  Å) for both BT2E and TOTAPOL, even though the two molecular tethers are composed of different numbers of atoms. Since an electron–electron dipolar splitting can be observed in the powder EPR spectra (Figure 1f,i), we surmise that the conformational flexibility of the tether in TOTAPOL may be decreased by its short length. In contrast, the longer and more flexible tether in BT2E probably permits a distribution of orientations of the  $g$ -tensors of the two TEMPO moieties and thereby transforms the dipolar splitting visible at 3360 G to a broad peak (Figure 1e). Similar dipolar splitting and broadening are present in the 140 GHz biradical spectra (Figure 1h,i), where inhomogeneous broadening due to the larger  $g$ -anisotropy dominates the line shape. Both the solution and solid-state EPR spectra of TOTAPOL provide information on the distance between the two TEMPO moieties, and a detailed analysis of these line shapes will be the subject of a future publication.

The pulse sequence for DNP-enhanced  $^{13}\text{C}$ -CPMAS NMR experiments is shown in the inset of Figure 2. The  $^1\text{H}$  polarization is initially saturated by a series of  $90^\circ$  pulses followed by a delay of  $3T_2$ . Next, the microwave irradiation is applied to dynamically polarize the  $^1\text{H}$ 's or, in the absence of microwaves, the thermal equilibrium polarization develops. Finally, the  $^1\text{H}$  polarization is transferred to  $^{13}\text{C}$  via cross-polarization and observed in the presence of TPPM decoupling.<sup>28</sup> The Fourier transforms of the FIDs are shown (Figure 2) as a series of spectra with various microwave irradiation periods,



**Figure 2.** Illustration of the growth of the nuclear polarization as a result of microwave irradiation. Integration of the spectral intensities with and without irradiation yields a  $^1\text{H}$  enhancement of  $\sim 290$  measured indirectly through  $^{13}\text{C}$  CP signal using the pulse sequence shown in the inset. The measurements were performed on a sample of 3 mM TOTAPOL and 2 M  $^{13}\text{C}$ -urea in  $^2\text{H}_6$ -DMSO/ $^2\text{H}_2\text{O}$ /H $_2\text{O}$  (60:34:6 w/w/w) at 90 K, 5 T, and  $\omega_r/2\pi = 7$  kHz MAS (spinning sidebands marked by asterisks). The time constant associated with the growth is  $\sim 9$  s, approximately the nuclear  $T_1$  of the sample.



**Figure 3.** Top: EPR absorption of TOTAPOL measured on the 140 GHz spectrometer. The line width is much greater than the  $^1\text{H}$  Larmor frequency ( $\omega_n/2\pi$ ) and therefore can encompass two coupled electron spins with the correct EPR frequency separation,  $\omega_n$ , required for the cross effect. Bottom: Solid circles show the field dependence of the  $^1\text{H}$  enhancement (in a 4 mm rotor) in a DNP experiment using the biradical polarizing agent TOTAPOL. The field dependence from BT2E, shown as open circles, is essentially identical with that of TOTAPOL. The lines connecting the data points are for guidance of the eye.

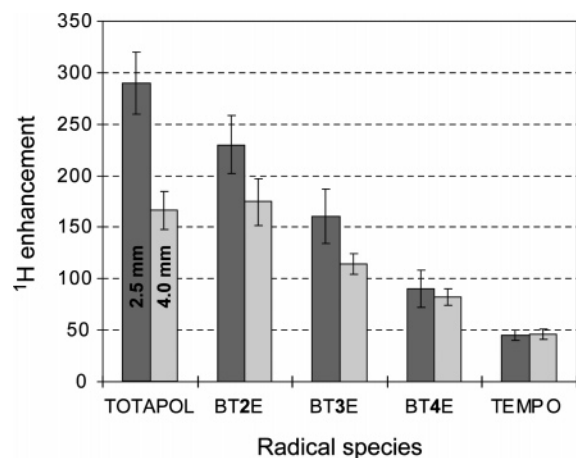
leading to an enhancement factor for  $^1\text{H}$  polarization  $\epsilon = 290 \pm 30$  determined by comparing the saturated NMR signals after 40 s delay with and without microwaves. Note that the error for the enhancement factor was determined primarily by the uncertainty in measuring the intensity of the unenhanced NMR signal.

In the top half of Figure 3 we show the EPR absorption of TOTAPOL recorded at 140 GHz/5 T. This inhomogeneous line shape supports the cross-effect mechanism in which two dipole-coupled electrons, separated by  $\omega_n/2\pi$  in the EPR spectrum, execute a three-spin electron–electron–nucleus process involving the mutual flip of an electron and a second electron separated

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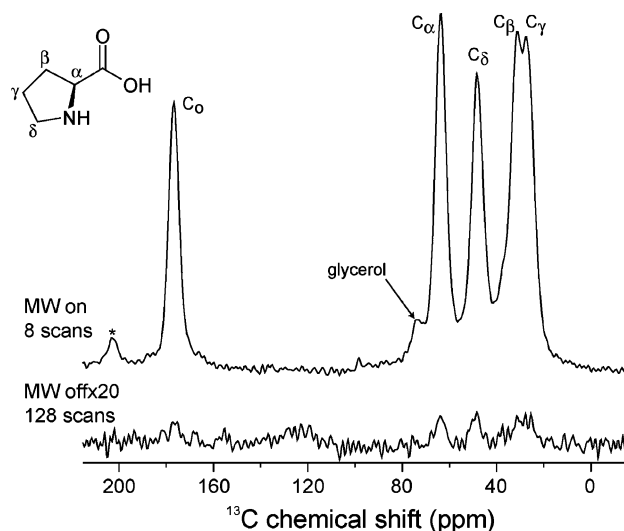


**Figure 4.** Histogram of DNP enhancements (with error bars) in 4 mm (light gray) and 2.5 mm (dark gray) rotors with the TOTAPOL, a series of BT $n$ E biradicals, and monomeric TEMPO. The data illustrate that TOTAPOL yields the largest enhancement, especially when the microwave penetration is optimized in 2.5 mm rotors.

by  $\omega_n/2\pi$  and a nuclear spin. The energy difference matches the nuclear Larmor frequency and results in nuclear spin flips that generate the enhanced nuclear polarization. The correct frequency separation of the two electrons is provided by the fact that the two TEMPO moieties have relative  $g$ -tensor orientations that satisfy the correct matching condition  $\omega_{2e} - \omega_{1e} = \omega_n$ . The field dependence of the DNP enhancement with TOTAPOL is shown in the bottom half of Figure 3, together with the enhancement curve of BT2E. To facilitate observation of NMR signals in the absence of microwave irradiation, the field-dependent DNP experiments were performed using a 4 mm sapphire rotor. The DNP enhancement ( $\epsilon \sim 165$ ) in 4 mm rotors (25  $\mu$ L) yielded by TOTAPOL was smaller than that observed ( $\epsilon \sim 290$ ) in 2.5 mm rotors (9  $\mu$ L), presumably because of limited microwave penetration by the larger sample volume. However, the shape of the curve in Figure 3 and the optimal external magnetic field for the maximum DNP enhancement showed no dependence within experimental error on the average microwave power at the sample.<sup>7</sup> In particular, TEMPO-based biradicals exhibit a universal DNP enhancement profile since the electron–electron dipolar splittings are relatively small compared to the inhomogeneous broadening of EPR line shapes.

As is illustrated in Figure 3, the enhancement profile curves generally resemble the first derivative of the EPR absorptive line shapes, having the maxima and minima of about 165 and  $-140$ , respectively, separated by 107 G ( $\sim 300$  MHz). The enhancement curves can be compared with the EPR line shape in the top half of Figure 3. Although the TOTAPOL chain is shorter than in BT2E and probably less flexible, we nevertheless observe essentially the same shape for the enhancement curve for each case. The shape and amplitude of the two curves depend on the average electron–electron distance and relative orientations of the two  $g$ -tensors, which must be very nearly the same in the two molecules.

Figure 4 compares the enhancement obtained with TOTAPOL with those from the BT $n$ E ( $n = 2, 3, 4$ ) series and monomeric TEMPO. The comparison was made using both 4 and 2.5 mm rotors to demonstrate the effect of microwave penetration into samples. Reducing the number of atoms separating the two TEMPO moieties increases the electron–electron dipolar interaction and the observed enhancement. Since the DNP



**Figure 5.** DNP enhanced spectrum of a 9  $\mu$ L sample of 0.2 M proline solution doped with 5 mM TOTAPOL. An enhancement of  $\sim 240$  was observed in the experiment with 20 s of microwave irradiation. The sample was prepared in  $^2\text{H}_8$ -glycerol/ $^2\text{H}_2\text{O}/\text{H}_2\text{O}$  (60:25:15 w/w/w), and the spectra were recorded with  $\omega_r/2\pi = 7.4$  kHz MAS (2.5 mm rotor) at 90 K and 5 T. The asterisk indicates a rotational sideband.

enhancement strongly depends on the electron–electron dipolar interaction, it appears to be optimized for BT2E and TOTAPOL at  $\epsilon \sim 165$  in a 4 mm rotor. In a 2.5 mm rotor, where the microwave penetration is more complete, the maximum enhancement from TOTAPOL grows to  $\epsilon \sim 290$ . Note that the increase in  $\epsilon$  in going from 4 to 2.5 mm rotors is  $\sim 1.75$  for TOTAPOL whereas for BT2E it is  $\sim 1.36$ . The underlying reason for this difference is not clear at present. It could be related to differences in the electronic relaxation times of the two biradicals. For systems which are sample-volume limited the smaller rotor offers advantages in sensitivity. However, when this is not the case then the 4 mm system could yield improved signal-to-noise.

A very important feature of TOTAPOL is its solubility in water due to the  $-\text{OH}$  group on the central carbon of the tethering chain and the  $-\text{NH}-$  linkage of the TEMPO moiety. Further, TOTAPOL is soluble in 200 mM salt, solvent conditions that are common in preparation of protein samples, and it is stable in glycerol/water (6:4 w/w) at  $-10$   $^\circ\text{C}$  for months. In contrast, BT2E is insoluble in all of these media. To illustrate the utility of TOTAPOL in these circumstances, we show in Figure 5 a DNP-enhanced high-resolution  $^{13}\text{C}$  NMR spectrum of 0.2 M proline in  $^2\text{H}_8$ -glycerol/ $^2\text{H}_2\text{O}/\text{H}_2\text{O}$  (60:25:15 w/w/w) which exhibits an enhancement of  $240 \pm 40$  using 5 mM TOTAPOL at 90 K and 5 T. Note the enhancement with proline is slightly smaller than the 290 observed with  $^{13}\text{C}$ -urea. In addition, we have used TOTAPOL to polarize samples of membrane proteins such as bacteriorhodopsin<sup>29</sup> and amyloid peptide (e.g., GNNQQNY) nanocrystals<sup>30,31</sup> in glycerol/water solvents, so we believe that TOTAPOL should be a widely applicable polarizing agent.

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#### 4. Conclusions

To summarize, we have synthesized a new TEMPO-based biradical, TOTAPOL, which produces larger enhancements than those observed with BT2E and, very importantly, is soluble in aqueous media containing salt and glycerol at concentrations typically found in protein solutions. These properties should make this molecule widely applicable to DNP investigations involving biological systems. The sizes of the enhancements presently observed reach 290 in a 2.5 mm rotor or 165 in a 4 mm rotor at 90 K and 5 T. As mentioned above, we have already used TOTAPOL in a number of other systems—in particular it

has yielded interesting results in solid-state NMR studies of bR and GNNQQNY—and observed larger enhancements than obtained with monomeric TEMPO. Thus, it would appear TOTAPOL will find wide applicability in DNP-enhanced NMR spectroscopy of biological systems.

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