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Succinyl-DOTOPA: An effective triradical dopant for low-temperature dynamic nuclear polarization with high solubility in aqueous solvent mixtures at neutral pH

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Abstract

We report the synthesis of the nitroxide-based triradical compound succinyl-DOTOPA and the characterization of its performance as a dopant for dynamic nuclear polarization (DNP) experiments in frozen solutions at low temperatures. Compared with previously described DOTOPA derivatives, succinyl-DOTOPA has substantially greater solubility in glycerol/water mixtures with pH > 4 and therefore has wider applicability. Solid state nuclear magnetic resonance (ssNMR) measurements at 9.39 T and 25 K, with magic-angle spinning at 7.00 kHz, show that build-up times of DNP-enhanced, cross-polarized ^{13}C ssNMR signals are shorter and that signal amplitudes are larger for glycerol/water solutions of L-proline containing succinyl-DOTOPA than for solutions containing the biradical AMUPol, with electron spin concentrations of 15 mM or 30 mM, resulting in greater net sensitivity gains from DNP. In similar measurements at 90 K, AMUPol yields greater net sensitivity, apparently due to its longer electron spin-lattice and spin-spin relaxation times. One- and two-dimensional ^{13}C ssNMR measurements at 25 K on the complex of the 27-residue peptide M13 with the calcium-sensing protein calmodulin, in glycerol/water with 10 mM succinyl-DOTOPA, demonstrate the utility of this compound in DNP-enhanced ssNMR studies of biomolecular systems.

Graphical Abstract

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Keywords

solid state NMR; dynamic nuclear polarization; low-temperature magic-angle spinning

Introduction

The introduction of nitroxide-based biradicals as dopants for dynamic nuclear polarization (DNP) experiments on frozen solutions in 2004 [1, 2] was a major contributing factor for the growth of DNP-related activity in the solid state nuclear magnetic resonance (ssNMR) community. A variety of biradical dopants have been developed subsequently with improved performance under various experimental conditions [3–17]. In 2010, we reported that the nitroxide-based triradical compound DOTOPA has favorable properties for low-temperature DNP, under certain conditions producing larger DNP-enhanced signals and more rapid build-up of nuclear spin polarizations under microwave irradiation [18]. We subsequently described a series of DOTOPA derivatives that have greater solubility at neutral or basic pH in water/glycerol mixtures than does the original DOTOPA compound [19]. Favorable properties of DOTOPA have also been reported by others [20].

Although we have used DOTOPA and DOTOPA derivatives in several published studies [21–26], their solubility is still a limitation, leading to sample-to-sample variations in DNP-enhanced signals and build-up times. Our previously described DOTOPA derivatives are also not useful in experiments where low glycerol-to-water ratios are required, such as studies of transient intermediates that are prepared by rapid mixing and freeze-trapping [27].

In this paper, we report the synthesis and DNP properties of a new derivative, succinyl-DOTOPA, which is highly soluble at neutral and basic pH. In experiments on frozen glycerol/water solutions of ^{13}C -enriched L-proline at 9.4 T, 25 K, and magic-angle spinning (MAS) frequencies up to 7 kHz, we show that succinyl-DOTOPA produces greater DNP-enhanced, cross-polarized ^{13}C NMR sensitivity than does the commercially available biradical AMUPol [6] at equivalent electron spin concentrations. At 90 K, greater sensitivity is obtained with AMUPol, apparently because the 1.5 W microwave power available to us in these experiments is not sufficient to saturate electron spin transitions in succinyl-DOTOPA. Measurements of temperature-dependent electron spin relaxation times T_{1e} and T_{2e} provide insight into the DNP properties of these compounds.

Materials and methods

Synthesis of 4-{N,N-di-[2-(succinate, sodium-trimethylamine salt)-3-(TEMPO-4'-oxy)-propyl]}-amino TEMPO (succinyl-DOTOPA)

Succinyl-DOTOPA was prepared by reacting DOTOPA with succinic anhydride (Fig. 1). Succinic anhydride and sodium bicarbonate were purchased from Sigma–Aldrich, and Mallinckrodt, respectively. DOTOPA was synthesized as described previously [18]. All solvents were reagent grade and used without further purification. Silica column chromatography was performed on silica gel (130–270 mesh, 60 Å pore size, Sigma-Aldrich). Liquid chromatography-mass spectrometry (LC-MS) data were recorded on a Thermo Scientific UltiMate 3000 LC-MS system, equipped with a MSQ Plus single quadrupole mass spectrometer with electrospray ionization.

Succinic anhydride (1.0 g, 10 mmol) was mixed with sodium bicarbonate (1.0 g, 12 mmol) and DOTOPA (314 mg, 0.5 mmol) in 7.5 ml ethyl acetate as described by Lugemwa *et al.* [28]. The mixture was stirred at room temperature overnight. After filtration, the residue was washed with ethyl acetate (5.0 ml) three times and dried under nitrogen gas. After loading the filtrate on the silica gel column using CH_2Cl_2 with 0.5% triethylamine, 1–3% methanol with 0.5% triethylamine was applied to elute the final product. After removing the organic solvents and drying under vacuum overnight, we obtained a 90% yield of red oily product. LC-MS data (Figs. S1a and S1b) indicated a mass of 828.10 Da ($\text{C}_{41}\text{H}_{71}\text{N}_4\text{O}_{13}^{3*}$, theoretical average mass = 828.02 Da).

The solubility of succinyl-DOTOPA in glycerol/water (40:60 volume ratio) as a function of pH at 24° C was determined by preparing a solution with [succinyl-DOTOPA] = 48 mM at pH 9.5, then reducing the pH in steps of 0.5 units by addition of HCl. After each step, insoluble material was removed by centrifugation and the concentration was determined by optical absorbance at 430 nm, using an extinction coefficient for nitroxide groups equal to $11\text{ cm}^{-1}\text{ M}^{-1}$. Results in Fig. S1c indicate a solubility above 15 mM at pH < 4.0.

Sample preparations

$^{13}\text{C}_5$, ^{15}N -L-proline was dissolved at a concentration of 100 mM in $^2\text{H}_2\text{O}$: $^{12}\text{C}_3$, $^2\text{H}_8$ -glycerol: $^1\text{H}_2\text{O}$ (50:40:10 ratio of initial volumes) with 50 mM phosphate buffer, pH 7.5. $^{12}\text{C}_3$, $^2\text{H}_8$ -glycerol was purchased from Cambridge Isotope Laboratories, Inc. Stock solutions of 75

mM succinyl-DOTOPA and 754 mM AMUPol (based on absorbance at 430 nm, with an extinction coefficient for nitroxide groups of $11 \text{ cm}^{-1} \text{ M}^{-1}$) were prepared in $^2\text{H}_2\text{O}$ with 100 mM phosphate buffer, pH 8, and were included in the glycerol/water mixtures to achieve the desired dopant concentrations. An additional sample of $^{13}\text{C}_5$, ^{15}N -L-proline in the same solvent mixture was prepared with 5 mM DyCl_3 and 5 mM disodium ethylenediaminetetraacetate (5 mM Dy-EDTA).

For measurements on the calmodulin/M13 complex, 20 μl of a 11 mM solution of unlabeled calmodulin in $^2\text{H}_2\text{O}$ and 9.2 μl of a 12 mM solution of selectively ^{15}N , ^{13}C -labeled M13 in $^2\text{H}_2\text{O}$ were combined with 3.5 μl of 326 mM CaCl_2 in $^2\text{H}_2\text{O}$, 7.5 μl of 200 mM Tris buffer in $^1\text{H}_2\text{O}$ (pH 8.0), 5.0 μl of 75 mM succinyl-DOTOPA in $^2\text{H}_2\text{O}$, and 30 μl of $^{12}\text{C}_3$, $^2\text{H}_8$ -glycerol. M13 was synthesized on the 0.1 mmol scale by automated solid-phase methods (Protein Technologies Tribute synthesizer) using standard fluorenylmethyloxycarbonyl (Fmoc) chemistry and a Fmoc-Met-Wang resin (0.53 mEq/g, Peptides International, Inc.), and was purified by high-performance liquid chromatography, using a water/acetonitrile gradient with 0.1% trifluoroacetic acid and a preparative C18 reverse-phase column. Bovine calmodulin was obtained as a lyophilized powder from Ocean Biologics.

ssNMR measurements

DNP-enhanced ssNMR measurements were performed at 9.4 T (400.9 MHz and 100.8 MHz for ^1H and ^{13}C NMR frequencies, respectively), with a Bruker Avance III spectrometer console and the home-built DNP-MAS ssNMR probe described previously [25, 29], which uses zirconia rotors (O'Keefe Ceramics, Inc.) with 4.0 mm outer diameters and maximum sample volumes of 80 μl . Rotor caps and drive tips were obtained from Revolution NMR, LLC. An extended interaction oscillator (Communications and Power Industries, LLC) and quasi-optical interferometer (Thomas Keating Ltd.) provided circularly polarized microwaves at 263.9 GHz with 1.5 W power [25].

Sample solutions in MAS rotors were frozen by immersion in liquid nitrogen, then loaded into the pre-cooled DNP-MAS ssNMR probe below the NMR magnet. The probe was then raised into the magnet, with the transfer line (carrying helium or nitrogen) attached and with the sample spinning (and remaining frozen) throughout this process.

One-dimensional (1D) ^{13}C ssNMR spectra were obtained with ^1H - ^{13}C cross-polarization (CP), using a ^{13}C radio-frequency (RF) field strength of 29 kHz and ^1H RF field strengths of 43 kHz, 37 kHz, and 33 kHz at ν_{MAS} values of 7.00, 4.00 and 2.00 kHz, respectively. Two-pulse phase modulated (TPPM) ^1H decoupling [30] was applied during ^{13}C signal acquisition, with an 80 kHz decoupling field. ^1H polarization build-up times T_{DNP} (or spin-lattice relaxation times T_1 for the Dy^{3+} -doped sample described below) were measured with the saturation-recovery method, using a train of 16 $\pi/2$ pulses with 10 ms delays to saturate the initial nuclear spin polarizations of both ^1H and ^{13}C . In all other measurements, recycle delays were then set to $1.26T_{\text{DNP}}$ (or $1.26T_1$). DNP signal enhancement factors ϵ for ^{13}C are ratios of signal areas with and without microwave irradiation, from spectra obtained with ^1H - ^{13}C CP. Signal areas were obtained by integrating the full ^{13}C spectra, including MAS sidebands.

Sample cooling and temperature control

For measurements at a nominal sample temperature of 25 K, samples were cooled with helium as previously described [25, 29, 31]. The liquid helium tank gauge pressure (2.5 psi) and helium transfer line needle valve opening were adjusted to produce a temperature of 25 ± 2 K for a sample of KBr powder at MAS frequency $\nu_{\text{MAS}} = 7.00$ kHz, as determined from the temperature-dependent ^{79}Br spin-lattice relaxation rate [32]. Sample temperatures were not significantly different at $\nu_{\text{MAS}} = 2.0$ kHz or 4.0 kHz. The same conditions, which correspond to a liquid helium consumption rate of approximately 2.0 liters per hour, were then used for DNP measurements on frozen proline solutions.

For measurements at a nominal sample temperature of 90 K, a liquid helium tank (Cryofab model CMSH-10) was filled with liquid nitrogen instead of liquid helium. The same transfer line was used (Janis Research model FHT-ST) and no other modifications were required for liquid nitrogen cooling. At 90 K and $\nu_{\text{MAS}} = 7.0$ kHz, liquid nitrogen consumption was 0.5 liters per hour.

During measurements at 90 K, sample temperatures were determined from the temperature-dependent peak wavelength of photoluminescence (PL) from a thin film of CdSeS/ZnS core/shell quantum dots (QDs), which was painted on the outer surface of the MAS rotor as previously described [33]. QDs were purchased from Ocean NanoTech (product number QSP-665) and co-dissolved with varnish (Lake Shore Cryotronics, VGE-7031) in toluene, with approximately 5 μl of varnish and 0.15 mg of QDs in 100 μl toluene. The QD solution was applied to the MAS rotor surface and dried in air to create the desired film. PL spectra were excited by a battery-powered laser operating at 405 nm with 200 mW power and detected with a fiber optic spectrometer (StellarNet, Inc., model CXR-100). To carry the excitation and PL emission light, two multimode optical fibers (400 μm diameter, 0.39 numerical aperture, Thorlabs part number FT400UMT) were inserted into the MAS module of our DNP-MAS ssNMR probe through holes in the Teflon fitting that connects the transfer line to the MAS model (see Fig. 1 of ref. [25]). In this way, the ends of the two optical fibers were positioned between turns of the solenoidal radio-frequency (RF) sample coil, approximately 2 mm from the MAS rotor surface.

Fig. S2 shows the temperature dependence of the PL peak wavelength, calibrated against sample temperatures that were determined simultaneously from ^{79}Br spin-lattice relaxation rates of a spinning KBr powder sample, cooled with liquid helium. Although the QDs used in these measurements have longer PL wavelengths than the QDs used in the original demonstration of this method for determining sample temperatures under MAS [33], the sensitivity to temperature is approximately the same (i.e., 13 nm change in PL peak from 50 K to 250 K). Based on the PL spectrum, which was monitored continuously during DNP measurements, sample temperatures were maintained at 90 ± 2 K.

EPR measurements

For temperature-dependent electron paramagnetic resonance (EPR) measurements, dopants were dissolved in $^2\text{H}_8$ -glycerol/ $^2\text{H}_2\text{O}$ (40:60 ratio of initial volumes) at 0.1 mM concentrations. Electron spin-lattice (T_{1e}) and spin-spin (T_{2e}) relaxation times were

measured from 20 K to 90 K in 10 K increments with the magnetic field at 1.1956 T, using a Bruker Elexsys EPR spectrometer. T_{1e} measurements used an echo-detected inversion-recovery pulse sequence ($\pi - \text{trec} - \pi/2 - \delta t - \pi - \delta t$, with $\delta t = 0.6 \mu\text{s}$ and incremented t_{rec}). T_{2e} measurements used a standard spin echo sequence ($\pi/2 - t_{\text{echo}}/2 - \pi - t_{\text{echo}}/2$ with incremented t_{echo}).

Results

Fig. 2 shows 1D ^{13}C and ^1H ssNMR spectra of a frozen $^{13}\text{C}_5, ^{15}\text{N}$ -L-proline solution containing 10 mM succinyl-DOTOPA at 25 K with $\nu_{\text{MAS}} = 2.00, 4.00, \text{ and } 7.00 \text{ kHz}$. Spectra are shown with and without microwave irradiation. For comparison, spectra of a solution containing 15 mM AMUPol, so that the total electron spin concentration $c_{e,\text{tot}}$ is also 30 mM, are shown in Fig. S3. Spectra of these samples at 90 K are shown in Figs. S4 and S5. Spectra of frozen $^{13}\text{C}_5, ^{15}\text{N}$ -L-proline solutions with $c_{e,\text{tot}} = 15 \text{ mM}$ and $c_{e,\text{tot}} = 45 \text{ mM}$ (5 mM and 15 mM succinyl-DOTOPA, 7.5 mM and 22.5 mM AMUPol) were also obtained (data not shown).

Fig. 3 shows the DNP build-up times T_{DNP} for all six samples at the two temperatures and three values of ν_{MAS} , obtained from saturation-recovery measurements with ^1H - ^{13}C CP and ^{13}C signal detection. The saturation-recovery data and single-exponential fits are shown in Figs. S6 and S7. Values of T_{DNP} are roughly inversely proportional to dopant concentration, consistent with the simplistic idea that (on the average) each dopant molecule polarizes a volume around itself that is inversely proportional to the dopant concentration. For the same value of $c_{e,\text{tot}}$, T_{DNP} is smaller when succinyl-DOTOPA is used, consistent with the idea that the fraction of dopant molecules that participate in the cross-effect DNP mechanism is larger for the triradical succinyl-DOTOPA than for the biradical AMUPol. Values of T_{DNP} at 25 K and 90 K are nearly equal and do not depend strongly on ν_{MAS} , especially for samples that contain succinyl-DOTOPA.

The DNP-enhanced ^{13}C ssNMR sensitivity is proportional to the absolute ^{13}C signal area with microwave irradiation and inversely proportional to $T_{\text{DNP}}^{0.5}$. Signal areas divided by $T_{\text{DNP}}^{0.5}$ are plotted as functions of $c_{e,\text{tot}}$ in Fig. 4, for the three values of ν_{MAS} , two temperatures, and two dopants. Under our experimental conditions, succinyl-DOTOPA produces significantly greater cross-polarized ^{13}C ssNMR sensitivity at 25 K, with $c_{e,\text{tot}} = 15 \text{ mM}$ or $c_{e,\text{tot}} = 30 \text{ mM}$. At 90 K, AMUPol produces greater or equal sensitivity. In addition, for samples containing succinyl-DOTOPA, sensitivities at 25 K are greater than at 90 K by factors of 9–12 at $\nu_{\text{MAS}} = 7.00 \text{ kHz}$, factors of 9–18 at $\nu_{\text{MAS}} = 4.00 \text{ kHz}$, and factors of 14–26 at $\nu_{\text{MAS}} = 2.00 \text{ kHz}$. For samples containing AMUPol, sensitivities at 25 K are greater than at 90 K by factors of 3–6 at $\nu_{\text{MAS}} = 7.00 \text{ kHz}$, factors of 3–11 at $\nu_{\text{MAS}} = 4.00 \text{ kHz}$, and factors of 9–10 at $\nu_{\text{MAS}} = 2.00 \text{ kHz}$. Leavesley *et al.* have also reported that dopants with multiple unpaired electrons yield smaller T_{DNP} values and greater overall sensitivity in DNP experiments at 4 K without MAS [20].

Data in Fig. 4 show that the differences in ^{13}C ssNMR sensitivity at 25 K between samples containing succinyl-DOTOPA or AMUPol become smaller at higher electron spin concentrations. At an electron spin concentration of 45 mM, the average volume per electron

spin is $36.8 \text{ nm}^3 = (3.33 \text{ nm})^3$. Given that the diameter of a succinyl-DOTOPA molecule is roughly 1.5 nm, one expects the distinction between triradical and biradical dopants to become less pronounced at such high concentrations.

These measurements of sensitivity do not include the temperature dependence of noise in the spectra. In practice, noise in our measurements does not change significantly between 90 K and 25 K because only the sample coil is cooled by the cryogenics, whereas other components of the probe circuit remain near 180 K.

Tables 1 and 2 contain the complete results for ^1H and ^{13}C signals, DNP enhancement factors ϵ (ratio of signal areas with and without microwave irradiation), T_{DNP} values, and ^{13}C sensitivities for samples containing succinyl-DOTOPA or AMUPol. For comparison, results are also given for a frozen glycerol/water solution of $^{13}\text{C}_5, ^{15}\text{N}$ -L-proline containing 5 mM Dy-EDTA, which reduces the ^1H T_1 value but does not produce DNP [31]. Compared with this Dy^{3+} -doped sample, samples with 5 mM or 10 mM succinyl-DOTOPA exhibit roughly 30-fold greater cross-polarized ^{13}C sensitivity with microwave irradiation at 25 K and $\nu_{\text{MAS}} = 7.00 \text{ kHz}$.

Fig. 5 compares cross-polarized ^{13}C signal areas without microwave irradiation for samples containing succinyl-DOTOPA, AMUPol, and Dy-EDTA. At 25 K, samples containing either succinyl-DOTOPA or AMUPol show strong depolarization effects [26, 34], *i.e.*, reduced signal areas relative to the sample containing Dy-EDTA. The extent of depolarization increases with increasing $c_{\text{e,tot}}$ and with increasing ν_{MAS} . With $c_{\text{e,tot}} = 15 \text{ mM}$ and $c_{\text{e,tot}} = 30 \text{ mM}$ and with $\nu_{\text{MAS}} = 7.00 \text{ kHz}$, samples containing AMUPol show larger depolarization effects than do samples containing succinyl-DOTOPA. The opposite is true under certain other conditions.

Differences between samples containing succinyl-DOTOPA or AMUPol may be attributed in part to differences in electron spin relaxation properties. Fig. 6 shows the temperature dependences of $T_{1\text{e}}$ and $T_{2\text{e}}$ for the two dopants in frozen glycerol/water solutions, measured at an EPR frequency of 33.6 GHz. $T_{1\text{e}}$ values for AMUPol are significantly larger than those for succinyl-DOTOPA. Assuming that this is also true at 263.9 GHz, the larger $T_{1\text{e}}$ value at 25 K may contribute to the stronger dependence of depolarization on ν_{MAS} for AMUPol in Fig. 5a.

At 25 K and $c_{\text{e,tot}} = 15 \text{ mM}$, depolarization effects at $\nu_{\text{MAS}} = 2.00 \text{ kHz}$ are small, suggesting that $T_{1\text{e}}$ values for both AMUPol and succinyl-DOTOPA are less than approximately 0.5 ms at 263.9 GHz, since depolarization is expected to be negligible when $\nu_{\text{MAS}}T_{1\text{e}} < 1$ [26]. At 25 K, $c_{\text{e,tot}} = 15 \text{ mM}$, and $\nu_{\text{MAS}} = 7.00 \text{ kHz}$, greater depolarization is observed for the sample containing AMUPol, suggesting that $T_{1\text{e}}$ for AMUPol is approximately 100 μs , while $T_{1\text{e}}$ for succinyl-DOTOPA is somewhat shorter. With $c_{\text{e,tot}} = 30 \text{ mM}$ or 45 mM, samples containing either AMUPol or succinyl-DOTOPA show significant depolarization at $\nu_{\text{MAS}} = 2.00 \text{ kHz}$, consistent with simulations showing that intermolecular electron-electron couplings can enhance depolarization [26]. The observation that samples containing succinyl-DOTOPA exhibit greater depolarization (*i.e.*, smaller cross-polarized ^{13}C ssNMR signals) with $c_{\text{e,tot}} = 30 \text{ mM}$ or 45 mM and with $\nu_{\text{MAS}} = 2.00 \text{ kHz}$ or 4.00 kHz is not readily

explained. Apparently, the quantitative aspects of depolarization can be sensitive to multiple parameters, including T_{1e} , ν_{MAS} , dopant concentration, and dopant structure (*e.g.*, intramolecular electron-electron distances and nitroxide orientations). Reductions in ssNMR signals due to other paramagnetic effects [35] may also contribute to the results in Fig. 5.

At 90 K, T_{2e} for AMUPol is also significantly larger than T_{2e} for succinyl-DOTOPA. The relatively rapid electron spin-lattice and spin-spin relaxation in succinyl-DOTOPA at 90 K may reduce the achievable level of saturation of electron spin transitions, leading to the lower DNP-enhanced ^{13}C sensitivity for samples containing succinyl-DOTOPA at 90 K under our experimental conditions. We estimate that the Rabi frequency for electron spins in our experiments is approximately 200 kHz (corresponding to 0.5 W of circularly polarized microwave power on a 1 cm² area). Higher microwave powers are likely to produce greater DNP-enhanced ^{13}C sensitivity for samples containing succinyl-DOTOPA at 90 K. In earlier experiments on a frozen solution containing a different DOTOPA derivative (DOTOPA-ethanol), we found that DNP-enhanced ^{13}C signals became nearly independent of microwave power above 0.8 W at 25 K, but continued to increase beyond 1.2 W at 100 K [25].

Discussion

Data presented above show that succinyl-DOTOPA is a useful DNP dopant for ssNMR measurements on biological or organic molecules in frozen glycerol/water solutions. Of particular interest for experiments in our laboratory [23, 27], DNP build-up times at 25 K are consistently shorter in samples that contain succinyl-DOTOPA than in samples that contain AMUPol (Fig. 3), and cross-polarized ^{13}C sensitivity at $\nu_{\text{MAS}} = 7.00$ kHz is greater by factors of 2.3 and 1.4 at electron spin concentrations of 15 and 30 mM, respectively (Fig. 4). The higher sensitivity under these experimental conditions is due to both the smaller T_{DNP} values and the larger DNP-enhanced ^{13}C signals, which reflect a weaker depolarization effect for succinyl-DOTOPA (Fig. 5). The weaker depolarization effect apparently results at least in part from a shorter T_{1e} (Fig. 6).

At 90 K, samples containing AMUPol show greater cross-polarized ^{13}C sensitivity at $\nu_{\text{MAS}} = 7.00$ kHz (Fig. 4). This observation is probably a consequence of lower saturation of electron spin transitions, due to the short T_{1e} and T_{2e} values of succinyl-DOTOPA at 90 K (Fig. 6).

At $\nu_{\text{MAS}} = 7.00$ kHz, the highest ^{13}C sensitivity in our DNP-enhanced measurements at 25 K (Table 1, 5 mM succinyl-DOTOPA) is greater than the highest ^{13}C sensitivity at 90 K (Table 2, 15 mM AMUPol) by a factor of 6.1. This corresponds to a reduction in the time required to achieve a given signal-to-noise ratio by a factor of 37. Because experiments at 25 K require cooling with liquid helium, in practice our experiments at 25 K are typically limited to total measurement times of 12 h or less [23, 27]. Comparable experiments at 90 K would require measurement times of about 18 days. At 25 K and $\nu_{\text{MAS}} = 7.00$ kHz, the ^{13}C sensitivity for a sample containing 5 mM succinyl-DOTOPA is greater than the ^{13}C sensitivity for a Dy³⁺-doped sample by a factor of 31, meaning that use of DNP can reduce total measurement times by factors greater than 900.

In a previous publication, we described several DOTOPA derivatives (DOTOPA-Ethanol, DOTOPA-NH, DOTOPA-4OH, DOTOPA-3OH-Methoxy) with solubilities slightly above 10 mM in glycerol/water mixtures at pH 7.4 [19]. At 25 K, $\nu_{\text{MAS}} \approx 6.7$ kHz, and 10 mM dopant concentration, these compounds produced T_{DNP} values in the 2.6–3.8 s range and ϵ values in the 94–128 range (for cross-polarized ^{13}C ssNMR signals of melittin). These T_{DNP} and ϵ values are very similar to those of succinyl-DOTOPA under similar experimental conditions. Thus, the DNP performance of DOTOPA derivatives is largely independent of variations in their chemical structures. The primary advantage of succinyl-DOTOPA is its greater solubility above pH 5 (Fig. S1c). This is an important practical advantage. Experiments with the previously-described DOTOPA derivatives required that they be dissolved in dimethyl sulfoxide (DMSO) before addition to the glycerol/water solution, resulting in final DMSO contents of roughly 2–5%. The previously-described DOTOPA derivatives were also not useful in experiments with relatively low glycerol concentrations [27], due to insufficient solubility.

Finally, to illustrate the applicability to systems of genuine biochemical interest, Fig. 7 shows 1D and 2D ^{13}C - ^{13}C spectra of a frozen solution of the complex of calmodulin with the 27-residue peptide M13 [36], with uniform ^{15}N and ^{13}C labeling of four amino acids in M13. This sample contained 1.5 mM M13, 3.0 mM calmodulin, and 15 mM CaCl_2 and was doped with 10 mM succinyl-DOTOPA. The sample volume was 75 μl , corresponding to 113 nmol of M13. Spectra were recorded at 25 K with $\nu_{\text{MAS}} = 7.00$ kHz. The value of T_{DNP} for cross-polarized ^{13}C signals was determined to be 4.6 s at 25 K. Comparison of signal areas in 1D spectra with and without microwave irradiation (Fig. 7a) indicates an apparent DNP enhancement factor of $\epsilon \approx 140$. A 2D spectrum with good signal-to-noise was obtained in 40 min (Fig. 7b).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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- The nitroxide-based triradical succinyl-DOTOPA is highly soluble in glycerol/water solutions and performs well in low-temperature dynamic nuclear polarization experiments.
- At 25 K, 9.39 T, and 7.00 kHz magic-angle spinning, succinyl-DOTOPA gives higher overall cross-polarized ^{13}C solid state NMR sensitivity than does the biradical AMUPol.
- At 90 K, AMUPol gives higher overall sensitivity, attributable to its longer electron spin relaxation times.
- Experimental results for a peptide/calmodulin complex in glycerol/water at 25 K verify the utility of succinyl-DOTOPA.

Fig. 1:
Synthesis of succinyl-DOTOPA (iii) by reaction of DOTOPA (i) with succinic anhydride (ii).

Fig. 2:

^{13}C (left) and ^1H (right) ssNMR spectra of a frozen glycerol/water solution of ^{15}N , ^{13}C -L-proline containing 10 mM succinyl-DOTOPA at 25 K, with MAS frequencies of 7.00 kHz (a), 4.00 kHz (b), and 2.00 kHz (c). The magnetic field strength was 9.39 T. Blue spectra were obtained with microwave irradiation and with two scans, using 1.5 W of circularly polarized microwaves at 263.9 GHz. Red spectra were obtained without microwave irradiation and with 32 scans.

Fig. 3:

Dependence of the build-up time for cross-polarized ^{13}C ssNMR signals under microwave irradiation (T_{DNP}) on MAS frequency and dopant concentration at 25 K and 90 K, for frozen glycerol/water solutions of ^{15}N , ^{13}C -L-proline containing either succinyl-DOTOPA (left) or AMUPol (right). Values of T_{DNP} were determined from single-exponential fits to build-up curves in Figs. S6 and S7.

Fig. 4:

Dependence of the net DNP-enhanced, cross-polarized ^{13}C sensitivity (^{13}C signal area in arbitrary units with microwave irradiation divided by $T_{\text{DNP}}^{0.5}$) on electron spin concentration and temperature for frozen glycerol/water solutions of ^{15}N , ^{13}C -L-proline containing either succinyl-DOTOPA (blue or green symbols at 25 K or 90 K, respectively) or AMUPol (red or yellow symbols at 25 K or 90 K, respectively). Data are shown for MAS frequencies of 7.00 kHz (left), 4.00 kHz (middle), and 2.00 kHz (right).

Fig. 5: Dependences of cross-polarized ^{13}C signal areas, without microwave irradiation, on MAS frequency for frozen glycerol/water solutions of ^{15}N , ^{13}C -L-proline containing succinyl-DOTOPA (blue symbols), AMUPol (red symbols), or Dy-EDTA (black symbols) with the indicated concentrations. Data are shown for measurements at 25 K (a) and 90 K (b).

Fig. 6: Temperature-dependences of the electron spin-lattice (T_{1e} , left) and spin-spin (T_{2e} , right) relaxation times in frozen glycerol/water solutions of succinyl-DOTOPA (blue symbols) and AMUPol (red symbols) at 1.1956 T.

Fig. 7:

(a) 1D ^{13}C NMR spectra at 25 K of the M13/ Ca^{2+} /calmodulin complex in frozen glycerol/water containing 10 mM succinyl-DOTOPA. Spectra were recorded with microwave irradiation and 4 scans (blue) or without microwave irradiation and 128 scans (red). The sample contains 1.5 mM of the 27-residue M13 peptide, uniformly ^{15}N , ^{13}C -labeled at Phe8, Ile9, Ala10, and Val11. The MAS frequency was 7.00 kHz. (b) 2D ^{13}C - ^{13}C NMR spectrum of the same sample at 25 K with microwave irradiation, using a 25 ms spin diffusion mixing period between t_1 and t_2 periods. A 1D slice at 65 ppm is shown above the 2D spectrum. The 2D spectrum was acquired in 40 min, using a 5.9 s recycle delay, 100 complex t_1 points, and a 40.0 μs t_1 increment. Contour levels increase by successive factors of 1.3.

Table 1:

Summary of measurements of ^{13}C and ^1H signal amplitudes, DNP enhancement factors s , and build-up times T_{DNP} at 25 K. Although signal amplitudes are in arbitrary units, all ^{13}C signal amplitudes in Tables 1 and 2 are on the same scale and can be directly compared with one another. All ^1H signal amplitudes are also on the same scale and can be directly compared with one another. The ^{13}C sensitivity is defined as the ^{13}C signal amplitude with microwaves on divided by the square root of T_{DNP} . For the sample doped with Dy-EDTA, build-up times are spin-lattice relaxation times T_1 , independent of microwave irradiation. ^{13}C T_{DNP} values are build-up times with ^1H - ^{13}C cross-polarization.

sample	MAS frequency (kHz)	^{13}C signal, μwaves on, 2 scans	^{13}C signal, μwaves off, 32 scans	^{13}C e	^{13}C T_{DNP} (or T_1)	^1H signal, μwaves on, 2 scans	^1H signal, μwaves off, 32 scans	^1H e	^1H T_{DNP} (or T_1)	relative ^{13}C sensitivity
5 mM succinyl-DOTOPA	2.0	611	158	61.9	5.40 ± 0.49	138	83	26.7	4.56 ± 0.01	263
	4.0	526	106	79.0	5.37 ± 0.11	118	52	36.6	4.92 ± 0.01	227
	7.0	401	78	82.8	5.46 ± 0.07	139	41	54.9	5.54 ± 0.01	172
10 mM succinyl-DOTOPA	2.0	555	86	102.9	2.90 ± 0.15	129	50	41.7	2.26 ± 0.02	326
	4.0	381	72	84.1	2.62 ± 0.06	111	28	64.3	2.78 ± 0.01	235
	7.0	260	45	92.1	2.58 ± 0.14	100	23	68.3	3.19 ± 0.01	161
15 mM succinyl-DOTOPA	2.0	393	62	100.8	1.34 ± 0.08	117	24	78.7	0.72 ± 0.01	339
	4.0	307	53	92.3	2.08 ± 0.02	98	21	73.0	1.16 ± 0.02	213
	7.0	173	32	85.3	1.99 ± 0.03	89	17	86.5	1.51 ± 0.01	123
7.5 mM AMUPol	2.0	479	154	49.7	7.35 ± 0.19	114	59	31.1	5.38 ± 0.01	177
	4.0	419	103	65.2	9.17 ± 0.17	103	52	31.4	6.34 ± 0.01	138
	7.0	244	30	128.5	10.32 ± 0.26	89	25	57.7	8.83 ± 0.01	76
15 mM AMUPol	2.0	418	132	50.7	3.32 ± 0.08	116	57	32.9	3.08 ± 0.01	229
	4.0	447	99	71.9	3.78 ± 0.09	104	49	33.7	3.36 ± 0.01	230
	7.0	244	30	128.5	4.63 ± 0.03	96	26	59.3	3.83 ± 0.01	114
22.5 mM AMUPol	2.0	570	80	114.1	3.00 ± 0.23	257	54	76.5	2.93 ± 0.01	329
	4.0	364	67	87.2	3.12 ± 0.07	105	33	50.9	3.25 ± 0.02	206
	7.0	222	38	98.8	3.00 ± 0.14	97	20	76.4	3.32 ± 0.01	128

sample	MAS frequency (kHz)	¹³ C signal, μ waves on, 2 scans	¹³ C signal, μ waves off, 32 scans	¹³ C ϵ	¹³ C T _{DNP} (or T ₁)	¹ H signal, μ waves on, 2 scans	¹ H signal, μ waves off, 32 scans	¹ H ϵ	¹ H T _{DNP} (or T ₁)	relative ¹³ C sensitivity
5 mM Dy-EDTA	2.0		167	–	4.17 $\pm$ 0.02		96	–	5.08 $\pm$ 0.01	5.1
	4.0		153	–	3.00 $\pm$ 0.03		95	–	4.85 $\pm$ 0.01	5.5
	7.0		164	–	3.31 $\pm$ 0.01		74	–	4.11 $\pm$ 0.01	5.6

Table 2:

Same as Table 1, but for measurements at 90 K. ^{13}C T_{DNP} values are build-up times with ^1H - ^{13}C cross-polarization.

sample	MAS frequency (kHz)	^{13}C signal, μwaves on, 2 scans	^{13}C signal, μwaves off, 32 scans	^{13}C e	^{13}C T_{DNP} (or T_1)	^1H signal, μwaves on, 2 scans	^1H signal, μwaves off, 32 scans	^1H e	^1H T_{DNP} (or T_1)	Relative ^{13}C sensitivity
5 mM succinyl-DOTOPA	2.0	44	26	26.9	5.49 ± 0.60	16	20	13.5	4.85 ± 0.01	19
	4.0	56	31	29.1	5.34 ± 0.08	20	18	17.8	5.21 ± 0.01	24
	7.0	44	25	28.5	5.38 ± 0.03	20	18	18.2	5.26 ± 0.01	19
10 mM succinyl-DOTOPA	2.0	36	24	23.7	2.78 ± 0.11	14	18	13.1	2.80 ± 0.02	21
	4.0	27	22	19.5	2.42 ± 0.43	14	17	13.9	2.74 ± 0.01	16
	7.0	21	18	19.3	2.54 ± 0.12	17	16	17.3	2.34 ± 0.02	13
15 mM succinyl-DOTOPA	2.0	15	18	12.9	1.56 ± 0.05	10	17	10.1	1.37 ± 0.01	13
	4.0	18	18	15.6	1.61 ± 0.12	12	16	12.2	1.42 ± 0.01	12
	7.0	15	15	15.8	1.76 ± 0.03	13	15	14.1	1.51 ± 0.01	10
7.5 mM AMUPol	2.0	55	18	48.0	8.59 ± 0.05	25	14	29.3	8.98 ± 0.01	20
	4.0	55	12	75.7	8.09 ± 0.08	27	14	31.4	8.03 ± 0.02	18
	7.0	84	15	89.2	8.50 ± 0.03	44	13	51.9	8.21 ± 0.01	26
15 mM AMUPol	2.0	42	16	43.0	4.24 ± 0.11	22	14	24.6	5.35 ± 0.02	23
	4.0	135	17	129.2	4.29 ± 0.02	61	14	67.8	4.62 ± 0.01	69
	7.0	60	11	83.0	4.04 ± 0.03	49	13	58.9	4.81 ± 0.02	28
22.5 mM AMUPol	2.0	66	15	72.4	2.48 ± 0.24	26	7	60.8	2.85 ± 0.01	38
	4.0	32	9	56.7	1.72 ± 0.15	15	11	21.9	2.52 ± 0.01	18
	7.0	36	7	84.6	2.47 ± 0.15	21	10	35.0	2.69 ± 0.01	21
5 mM Dy-EDTA	2.0	–	46	–	5.37 ± 0.02	–	25	–	4.53 ± 0.01	1.2
	4.0	–	35	–	5.92 ± 0.01	–	19	–	5.23 ± 0.02	0.9
	7.0	–	30	–	6.17 ± 0.02	–	20	–	5.29 ± 0.02	0.8