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bcTol: a highly water-soluble biradical for efficient dynamic nuclear polarization of biomolecules†

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Dynamic nuclear polarization (DNP) is an efficient method to overcome the inherent low sensitivity of magic-angle spinning (MAS) solid-state NMR. We report a new polarizing agent (bcTol), designed for biological applications, that yielded an enhancement value of 244 in a microcrystalline SH3 domain sample at 110 K.

Magic-angle spinning (MAS) NMR is now routinely applied to study structure and dynamics of biological systems, with a focus on membrane proteins,[1] protofibrils,[2] and microcrystalline protein preparations.[3] A limiting factor in exploiting the full power of MAS NMR in structural biology is its low sensitivity. This shortcoming has been addressed by the application of dynamic nuclear polarization (DNP), which involves the transfer of electron spin polarization to the spin states of nuclei in the investigated biological macromolecule.[4] The theoretical maximum NMR signal enhancement (ε) of DNP is γ_e/γ_n, where γ_e and γ_n are the gyromagnetic ratios of the electron and nucleus (γ_e/γ_H = 658, γ_e/γ_{13C} = 2618 and γ_e/γ_{15N} = 6494). Among the mechanisms that contribute to DNP, the cross-effect (CE) has yielded so far the highest nuclear polarization at magnetic fields in the range of 4.7–14.1 T.[5] The CE arises from the interaction of three spins, namely two electrons and one nucleus, and is most efficient when the Larmor frequencies of the two electrons are separated by the nuclear Larmor frequency.[6]

Nitroxide biradicals, in which two 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO) units are connected by a linker, have been shown to be particularly useful polarizing agents for CE DNP,[7] such as **bTurea**[8] (Fig. 1). The DNP enhancement not only depends on the electron–electron distance, but also on the relative orientations of the TEMPO units.[7c] The electron relaxation properties of the biradicals also effect the DNP process. For this reason, low temperatures (<200 K) and glass-forming solvents such as mixtures of 60% glycerol-d_8, 30% D_2O and 10% H_2O (GDH) are applied; the glassy matrix has the advantage of distributing radicals and analytes appropriately to avoid electron relaxation enhancement through aggregation that may take place upon freezing.[5,9] Chemical fine-tuning of the structures of biradicals by replacement of the methyl groups on the TEMPO moieties with spirocyclohexyl[10] or CD_3 groups,[7d,11] has also yielded significantly higher DNP efficiency by increasing electron and nuclear relaxation times.

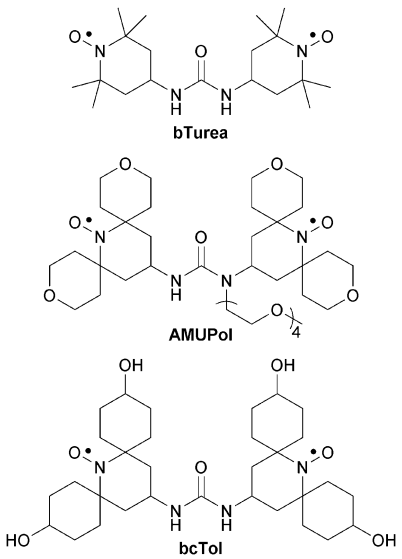

Fig. 1 Structures of **bTurea**, **AMUPol** and **bcTol**.

A drawback of many biradicals is their hydrophobic nature, making them less suited for studies of biological systems, primarily due to low solubility in glycerol/water mixtures. Furthermore, it is more likely that such biradicals show high affinity to hydrophobic surface areas of proteins or to membranes[12] and the concomitant paramagnetic relaxation enhancement (PRE) effects can reduce signal intensities and broaden the

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signals of nuclei in their vicinity.[13] Polarizing agents that are most suitable for biological applications should thus show minimal binding to the proteins or associated membranes. For these reasons, considerable efforts have been devoted to making biradicals more soluble in aqueous media and glycerol/water mixtures, for example by noncovalent complexation of hydrophobic radicals with cyclodextrin[14] and micelles.[15] Another approach involves covalent addition of solubility-supporting tags. One example is **AMUPol**,[16] a pentaethylene glycol derivative of **bTurea** (Fig. 1), that is soluble in water or GDH in concentrations of up to 30 mM. Despite these successes, biradicals that have high solubility in aqueous solutions and GDH and minimized protein binding properties, while maintaining large DNP enhancements, are still in high demand.

Here we report the synthesis of a new water-soluble biradical using a novel synthetic strategy for its preparation and its application to NMR studies of biological samples. The synthesis approach replaces the methyl groups of TEMPO with spirocyclohexanolyl groups, forming the **bTurea**-derivative **bcTol** [bis-(spirocyclohexyl-TEMPO-alcohol)-urea] (Fig. 1), leading to substantially enhanced solubility in aqueous-based solvents, while minimizing the binding to hydrophobic surfaces of proteins.

Synthesis of **bcTol** started with the condensation of acetonine (**1**) with 4-hydroxycyclohexanone, followed by oxidation to yield the dihydroxy biradical **2**[17] (Scheme 1). The hydroxyl groups of **2** were protected as silyl ethers and the ketone was subjected to reductive amination to yield amine **3**. Compound **3** was reacted with carbonyldiimidazole, followed by deprotection of the hydroxyl groups to give **bcTol**, which showed excellent solubility in GDH (150 mM), water (100 mM) and glycerol (240 mM). Furthermore, **bcTol** dissolves immediately in these solvents without the need for sonication.[16] The crystallinity and high solubility of **bcTol** in GDH, and even in glycerol alone, simplifies handling of the radical and preparation of its stock solutions.

The DNP performance of **bcTol** was investigated using samples of proline, of a water-soluble protein, and of a membrane protein

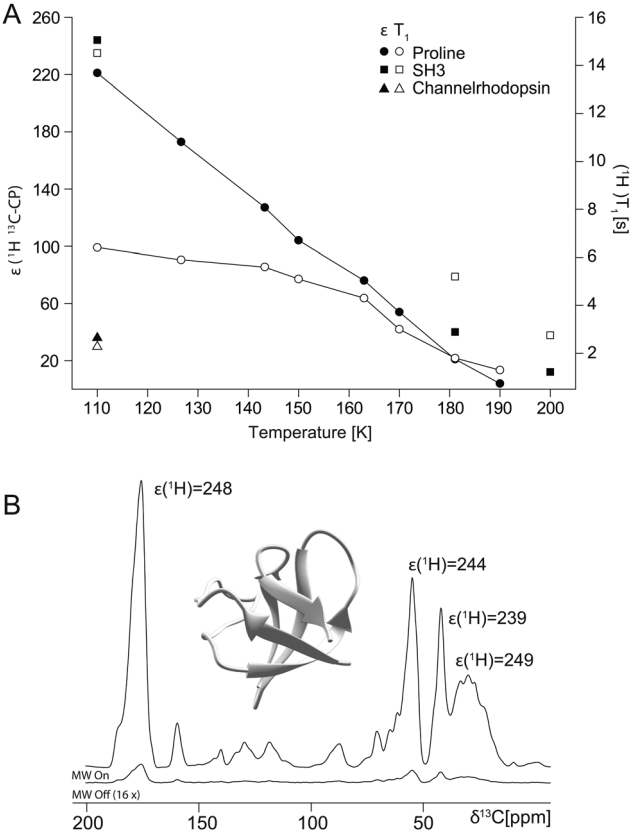
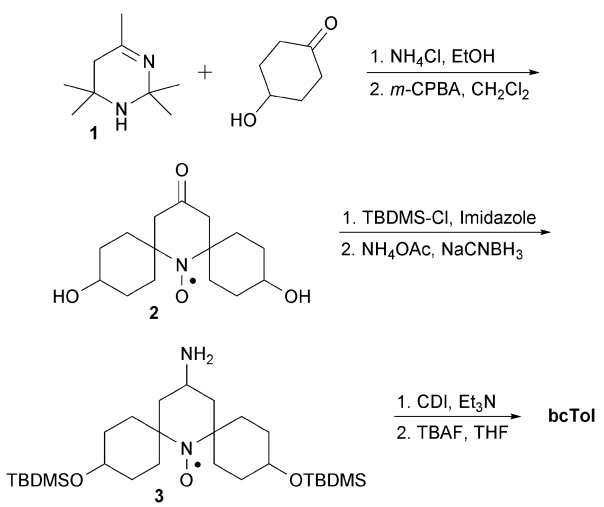


Fig. 2 (A) ^{1}H-DNP-signal enhancement (ε filled symbols) and T_1 (open symbols) for proline, microcrystalline SH3 and channelrhodopsin as a function of temperature using **bcTol** as a polarizing agent. The proline (0.25 M) was uniformly ^{13}C-, ^{15}N-labeled. Spectra were recorded in glycerol-d_8/D_2O/H_2O (60/30/10 v/v/v) containing **bcTol** (10 mM), measured at 9.4 T in a 3.2 mm zirconia rotor at 8 kHz MAS. T_1 was measured *via* an inversion recovery experiment with ^{1}H–^{13}C-CP. (B) A sample of SH3 (7.0 mg) containing **bcTol** (20 mM) (18.78 s recycle delay) measured with and without microwave irradiation at 9.4 T (110 K, 16 scans, 4 dummy scans, 5 W microwave power at end of probe waveguide). Inset shows a ribbon representation of the three-dimensional structure of the SH3 protein (PDB entry 1U06).



Scheme 1 Synthesis of **bcTol**. TBDMS-Cl *tert*-butyldimethylsilyl chloride; CDI, carbonyldiimidazole.

contained in zirconium rotors. The signal enhancements and apparent proton T_1 values of proline were determined as a function of temperature (Fig. 2A). All enhancements for the proline sample were determined using 1.3 × T_1 (^{1}H) as the recycle delay and 8000 Hz MAS-frequency, which represents the best compromise between undesired sample heating and spectral resolution. At 110 K, an enhancement of 221 ± 8 was obtained for proline. The enhancement decreased nearly linearly with temperature to around 21 ± 5 at 181 K. The apparent proton T_1 values also decreased strongly with temperature, by more than a factor of five.

To investigate the potential of **bcTol** as a polarizing agent in a biological context, we used samples containing a microcrystalline preparation of ^{2}H, ^{13}C, and ^{15}N-labeled (80% ^{1}H-backexchanged) α-spectrin Src homology 3 (SH3) domain in GDH.[3a] A maximum enhancement of 244 ± 5 was observed at 110 K and 8889 Hz MAS at a radical concentration of 20 mM (Fig. 2A). The enhancement factor decreased to 40 ± 4 at 181 K, while at

Table 1 Values of signal-to-noise-ratio per unit time (10 min, ^{10m}SNR) measured by ^{13}C-CP-MAS experiments with and without microwave irradiation (ON and OFF, respectively) for a microcrystalline SH3 sample with 20 mM **bcTol** and 20 mM **AMUPol**. NH protons were initially back exchanged to 80% and 1.5 times v/v d_8-glycerol was added, relative to all water, including crystal water. Measurements were taken in 3.2 mm zirconia rotors containing 7.2 mg SH3 for the **AMUPol** sample and 7 mg for the **bcTol** sample at 8.8 kHz MAS

T [K]	^{10m}SNR$_{ON}$		^{10m}SNR$_{OFF}$		$\varepsilon_{on/off}(^1H)$	
	bcTol	**AMUPol**	**bcTol**	**AMUPol**	**bcTol**	**AMUPol**
110	9473 ± 474	9497 ± 188	45 ± 3	49 ± 2	211 ± 26	187 ± 12
181	1667 ± 74	1056 ± 51	40 ± 2	36 ± 2	42 ± 4	26 ± 4
200	180 ± 16	656 ± 21	13 ± 1	35 ± 2	14 ± 2	17 ± 2

200 K the enhancement was still 12 ± 2. All enhancements for the SH3 domain samples were determined by scaling the signal intensities of the carbonyl resonances between spectra with and without microwave irradiation. The apparent proton T_1 (Fig. 2A) dropped from 14.5 s at 110 K to 5.1 s at 181 K and further to 2.7 s at 200 K.

Since radical- or temperature-dependent changes in apparent proton T_1-values, thermal noise, heterogeneous line width and depolarisation effects[18] – together with the different Boltzmann distributions – are as relevant for the overall sensitivity as the enhancement, we determined the signal-to-noise-ratio per unit measurement-time of 10 min (^{10m}SNR) at 110 K, 181 K and 200 K (Table 1), with the relaxation delay adjusted to 1.3 × T_1 for maximizing the sensitivity. Since the samples were prepared in a reproducible manner, the radical performance can be compared to that of other radicals by normalizing the ^{10m}SNR values to the amount of protein. At 110 K, the sample containing 20 mM **bcTol** yielded a ^{10m}SNR of 9473 ± 474 with 7.0 mg of protein and thus a ^{10m}SNR per mg of 1353 ± 68, whereby a sample prepared with 20 mM **AMUPol** containing 7.2 mg of SH3 yielded a comparable ^{10m}SNR per mg of 1319 ± 26. At 181 K, the situation was more in favour of **bcTol**, with ^{10m}SNR per mg values of 238 ± 11 and 147 ± 7 for **bcTol** and **AMUPol**, respectively. Surprisingly, the drop in ^{10m}SNR per mg between the two temperatures is only a factor of 6 for **bcTol** but 9 for **AMUPol**. A comparison of the values obtained for the radicals at 200 K is less reliable since this temperature is too close to the phase transition temperature, causing unexpectedly large variations in the values between samples. Comparison of ^{10m}SNRoff per mg values from the **AMUPol** (6.8) and **bcTol** (6.4) samples with that of a sample without radical (12.5) at 110 K highlights the depolarisation effects[18] of the radicals, pointing to the significantly higher SNR when no radical is present. However, DNP always yields SNR that is orders of magnitude larger.

The performance of **bcTol** was tested further with a sample of the membrane protein channelrhodopsin[19] in liposomes at 110 K (Fig. 2A). Protein and lipid signals showed enhancements of 36 ± 6, which is an improvement by a factor of more than three compared to polarisation by the biradical TOTAPOL[7b] ($\varepsilon \approx 10$). An apparent proton T_1 of 2.3 s was observed.

Measurements at higher temperatures result in a reduction in heterogeneous broadening that may become too severe in biological studies at temperatures around 110 K.[20] Therefore, we exploited the increase in enhancement by **bcTol** for improving resolution in two-dimensional ^{13}C–^{13}C dipolar-assisted-rotational-resonance (DARR) correlations of the SH3 sample by measuring at 181 K (Fig. 3A).[21] 768 t1-increments were recorded in 5.8 h at an enhancement of 40 ± 4 using a DARR mixing time of 25 ms. The cross sections shown in Fig. 3B indicate ample signal-to-noise, even without the application of a window function in F_2 that is enabled by the choice of a sufficiently long acquisition time. Overall, the spectrum strongly resembles the corresponding room temperature spectra,[3a] with a somewhat increased line width as indicated by the analysis of the cross sections taken through the cross peaks Ala55 Cβ–Cα, Leu10 Cβ–Cα and Ala55 Cα–Cβ yielding values of 135, 137, and 177 Hz in F_2, respectively (Fig. 3B). We estimate that the spectral resolution observed at 181 K is sufficient for obtaining sequence-specific resonance assignments on the basis of a set of three-dimensional spectra in case of our SH3 domain sample.

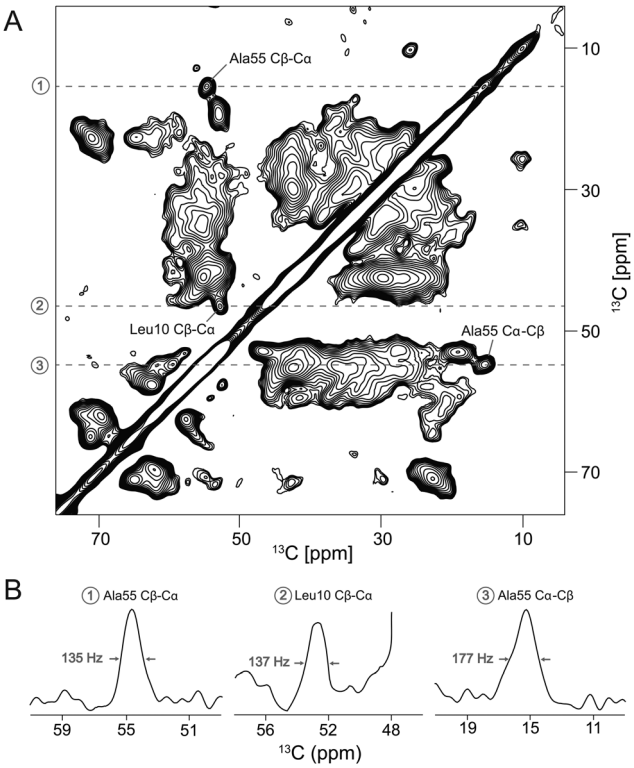


Fig. 3 DNP enhanced ^{13}C–^{13}C correlation spectrum of microcrystalline SH3 at 100 MHz carbon frequency (at 9.4 T), recorded at 181 K. (A) 2D ^{13}C–^{13}C DARR spectrum recorded with 25 ms mixing time. The dashed lines indicate positions of cross sections for evaluation of line widths. (B) Cross sections for selected cross peaks as indicated in (A), along with their line widths. To enable the evaluation of line width, the spectrum was recorded with a sufficiently long acquisition time and processed without application of a window function in F_2.

Compared with other known biradicals, **bcTol** has structural features that could potentially reduce non-specific or even specific binding to proteins. First, the high solubility of **bcTol** in aqueous-based solvents indicates increased polarity, which will likely decrease its affinity to hydrophobic surfaces. Second, the potential

hydrogen bond-donating hydroxyl groups in **bcTol** remain more or less in the same orientation relative to each other, and require matching geometries for multivalent binding to a protein surface.

In conclusion, we have described the preparation of the new biradical **bcTol** that facilitates high DNP enhancements and displays unparalleled solubility in water, GDH and glycerol. Measurements of signal-to-noise per unit time suggest a comparable DNP performance of **bcTol** at 110 K to that of **AMUPol**, but remarkably a less severe drop in DNP enhancement when measuring at 181 K (factors of 6 and 9 for **bcTol** and **AMUPol**, respectively). 2D spectra of the SH3 domain sample recorded at 181 K and with an enhancement of 40 $\pm$ 4 show acceptable resolution for structural studies. Therefore, this biradical is particularly well-suited for investigation of biomolecules by MAS DNP NMR spectroscopy. The incorporation of spirocyclohexanolyl groups represents a new strategy for preparation of efficient and water-soluble radicals for DNP.

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