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

Anil P. Jagtap,[1] Michel-Andreas Geiger,[2] Daniel Stöppler,[2] Marcella Orwick-Rydmark,[2] Hartmut Oschkinat,[2]* Snorri Th. Sigurdsson[1]*

[1]University of Iceland, Department of Chemistry, Science Institute, Dunhaga 3, 107 Reykjavik, Iceland. [2]Leibniz-Institut für Molekulare Pharmakologie im Forschungsverbund Berlin e.V. (FMP), Campus Berlin-Buch, Robert-Rössle-Str. 10, 13125 Berlin, Germany.

**Corresponding authors: email: snorrisi@hi.is, oschkinat@fmp-berlin.de*

Table of contents

Abbreviations

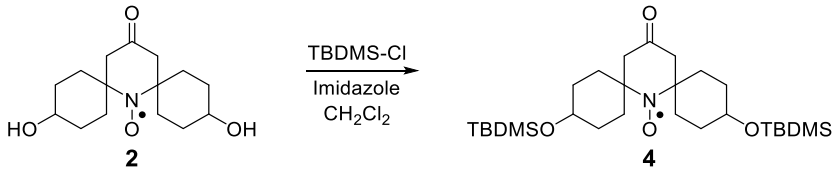
HRMS	high-resolution mass spectrometry
TLC	thin layer chromatography
TBDMS-Cl	*tert*-butyldimethylsilyl chloride
m-CPBA	*meta*-chloroperbenzoic acid
PMA	phosphomolybdic acid
THF	tetrahydrofuran
CDI	carbonyldiimidazole
Et_3N	triethylamine
TBAF	tetra-n-butylammonium fluoride
DNP	dynamic nuclear polarization
MAS	magic angle spinning
GDH	glycerol-d_8, D_2O, H_2O (60/30/10 v/v/v)
TPPM	two pulse phase modulation
EPR	electron paramagnetic resonance
SNR	Signal-to-noise ratio per unit time
ssNMR	solid-state nuclear magnetic resonance

Synthetic procedures

General materials and methods

All reagents were purchased from Sigma-Aldrich and used without further purification. TLC was carried out using glass plates pre-coated with silica gel (0.25 mm, F-254) from Silicycle, Canada. All compounds were visualized by UV light and by staining with PMA. Flash column chromatography was performed using ultra-pure flash silica gel (Silicycle, 230-400 mesh, size 60 Å). All moisture and air-sensitive reactions were carried out in oven-dried glassware under an inert argon atmosphere. Nitroxide radicals show broadening and loss of NMR signals due to their paramagnetic nature,[1] and therefore, integration of the NMR peaks was not done. HRMS analyses of all organic compounds were performed on Bruker, MicrOTOF-Q, equipped with an electron spray ionization module, in a positive ion mode.

Synthetic protocols



Compound 4

A solution of **2** (0.020 g, 0.07 mmol) in CH_2Cl_2 (3 mL) was treated with imidazole (0.019 g, 0.28 mmol) and TBDMS-Cl (0.026 g, 0.177 mmol) and the resulting solution was stirred at 24 °C for 12 h. After addition of CH_2Cl_2 (15 mL), the organic layer was washed successively with water (10 mL) and brine (10 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to obtain the crude product, which was purified by flash column chromatography (silica) using a gradient elution (EtOAc:pet. ether; 0:100 to 2:98) to give **4** (0.034 g, 94% yield) as a yellow solid.

TLC (Silica gel, 10% MeOH in CH_2Cl_2), R_f (**2**) = 0.2, R_f (**4**) = 1, PMA active.

(Silica gel, 20% EtOAc in pet. ether), R_f (**4**) = 0.6, PMA active.

HRMS: calculated for $C_{27}H_{52}NO_4Si_2$: 510.3435, found 533.3374 $(M+Na)^+$.

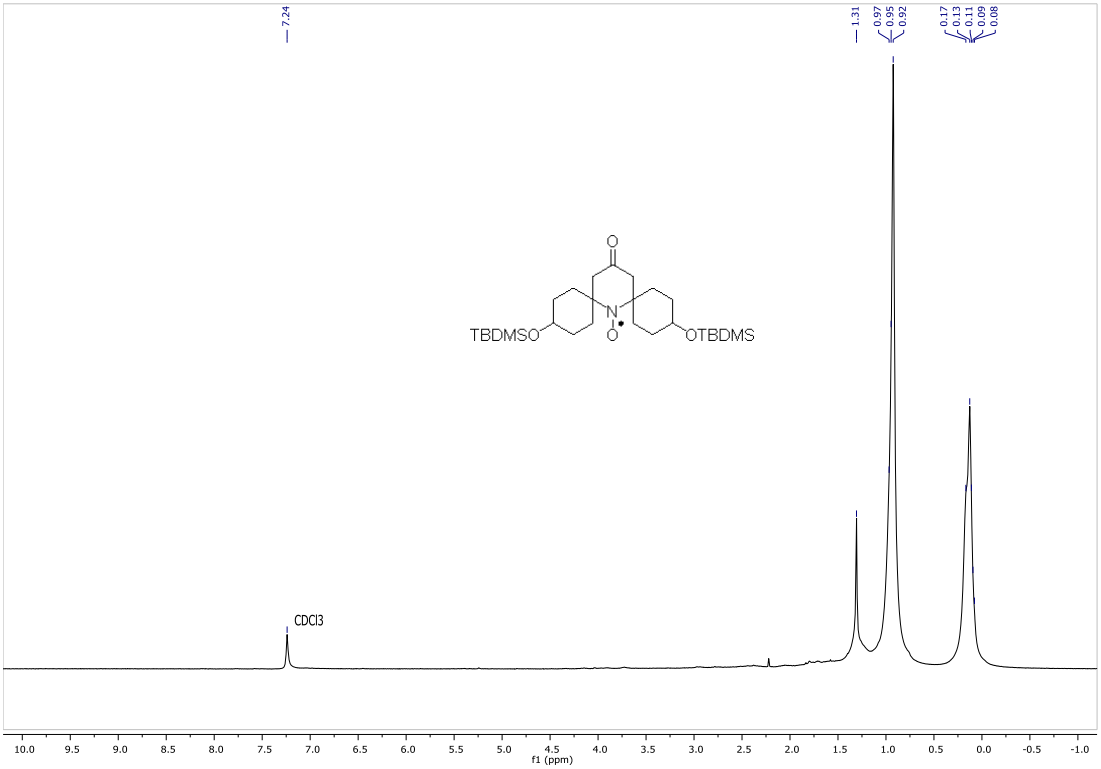
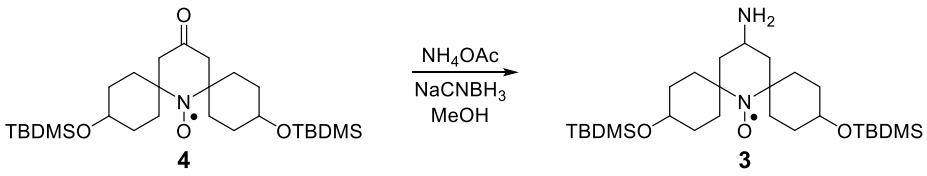


Figure S1. ^{1}H NMR spectrum of compound **4** in $CDCl_3$.



Compound 3

Ammonium acetate (0.150 g, 1.95 mmol) was added to a solution of **4** (0.1 g, 0.195 mmol) in MeOH (4 mL) and stirred for 2 h, followed by portion-wise addition of $NaCNBH_3$ (0.016 g, 0.254 mmol). The reaction mixture was stirred at 24 °C for 12 h, the solvent was removed *in vacuo* and a saturated solution of $NaHCO_3$ (5 mL) added. The aqueous layer was extracted with CH_2Cl_2 (3 x 15 mL), the combined organic layer dried over anhydrous sodium sulfate, filtered and concentrated to obtain the crude product. Purification was performed by flash column chromatography (silica) using a gradient elution ($MeOH:CH_2Cl_2$; 0:100 to 4:96) to give **3** (0.057 g, 57% yield) as a yellow solid.

TLC (Silica gel, 20% EtOAc in pet. ether), R_f (**4**) = 0.6, R_f (**3**) = 0.0, PMA active.

(Silica gel, 10% MeOH in CH_2Cl_2), R_f (**3**) = 0.2, PMA active.

HRMS: calculated for $C_{27}H_{55}N_2O_3Si_2$: 511.3751, found 512.3831 $(M+H)^+$.

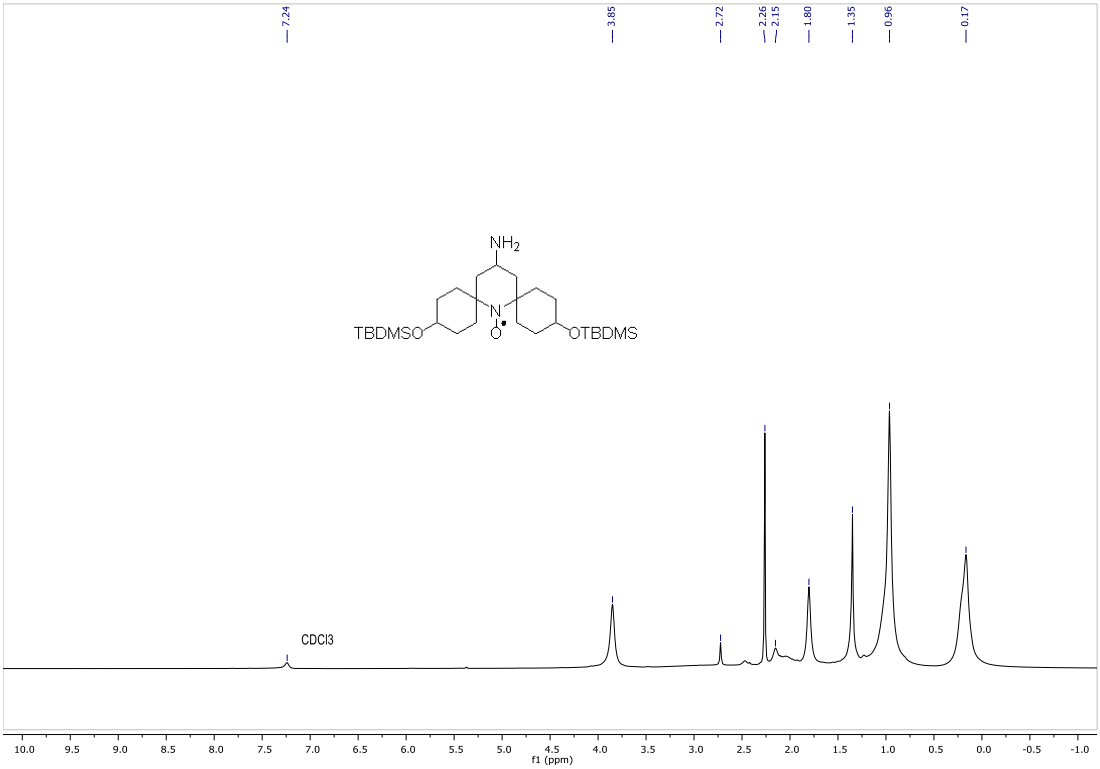
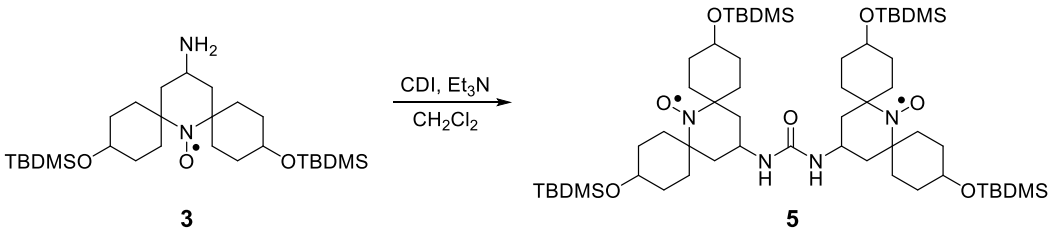


Figure S2. ^1H NMR spectrum of compound **3** in $CDCl_3$.



3 **5**

Compound 5

Carbonyldiimidazole (0.021 g, 0.13 mmol) and triethylamine (0.071 mL, 0.52 mmol) were added to a solution of **3** (0.132 g, 0.26 mmol) in CH_2Cl_2 (6 mL) and stirred at 24 °C for 12 h. The reaction mixture was diluted with CH_2Cl_2 (10 mL) and washed consecutively with H_2O (10 mL) and brine (15 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated to obtain the crude product, which was purified by flash column chromatography (silica) using a gradient elution (EtOAc:Pet. ether; 0:100 to 15:85) to afford **5** as an orange solid (0.043 g, 70% yield based on recovery of starting material).

TLC (Silica gel, 10% MeOH in CH_2Cl_2), R_f (**3**) = 0.2, R_f (**5**) = 0.8, PMA active.

HRMS: calculated for $C_{55}H_{108}N_4O_7Si_4$: 1048.7295, found 1071.7159 $(M+Na)^+$.

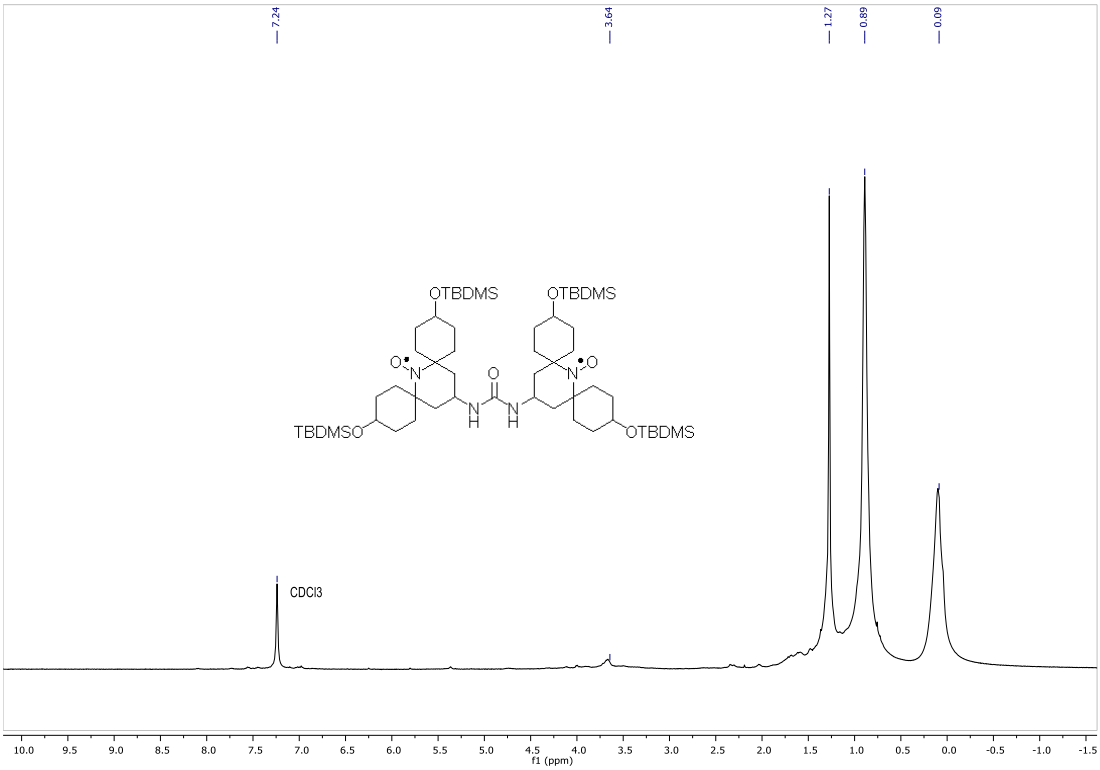
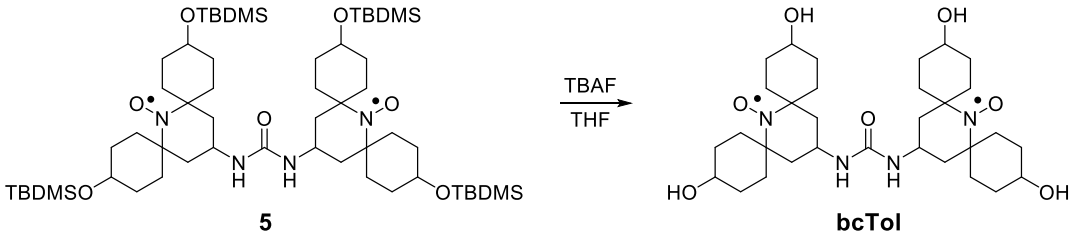


Figure S3. ^{1}H NMR spectrum of compound **5** in $CDCl_3$.



bcTol

A solution of **5** (0.105 g, 0.1 mmol) in THF (7 mL) was treated dropwise with TBAF (2.6 mL, 2.6 mmol, 1M in THF) and stirred for 48 h at 24 °C. The solvent was removed *in vacuo* and the crude material was purified by flash column chromatography (silica) using a gradient elution (MeOH:CH_2Cl_2; 0:100 to 20:80) to give **bcTol** (0.051 g, 89% yield) as a pale yellow solid.

TLC (Silica gel, 20% MeOH in CH_2Cl_2), R_f (**5**) = 1, R_f (**bcTol**) = 0.2, PMA active.

HRMS: calculated for $C_{31}H_{52}N_4O_7$: 592.3836, found 615.3730 $(M+Na)^+$.

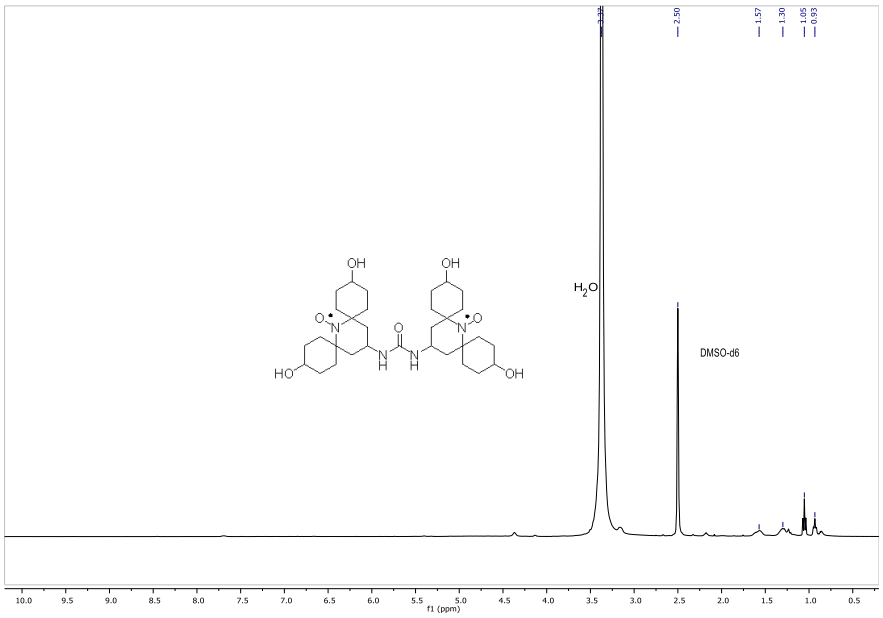


Figure S4. ^{1}H NMR spectrum of **bcTol** in DMSO-d6.

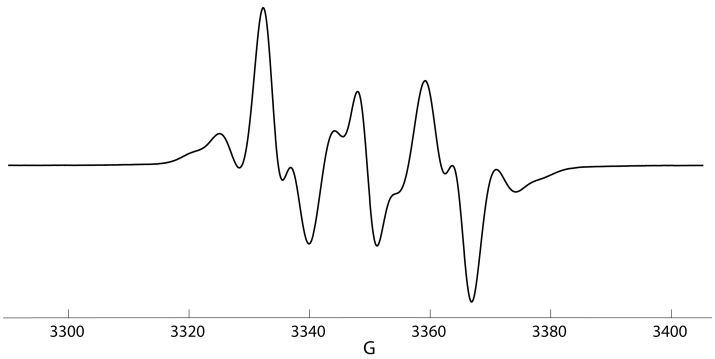


Figure S5. EPR spectrum of **bcTol**, in MeOH (1 mM) at 25 °C.

DNP measurements

General information

All samples were measured on a 400 MHz (9.4 T) wide-bore spectrometer (Bruker Avance III console) combined with a Bruker 263 GHz gyrotron (~5 W at the end of the waveguide). The variable temperature was adjusted with a Bruker Cryo-NMR cooling cabinet. All samples were measured in the triple resonance mode of the Cryo-MAS probe. Standard 3.2 mm zirconium rotors from Bruker were used for all measurements, at magic angle spinning frequencies of 8000 Hz for proline (25 µl Volume), 8889 Hz for SH3 and channelrhodopsin respectively. Between on/off comparisons with and without microwaves, the temperature was equilibrated for 10 minutes before data acquisition. All on/off comparisons were recorded with 16 scans and 4 dummy scans (for the channelrhodopsin sample 128 scans and 16 dummy scans) and processed with Topspin version 2.1. ^{1}H-DNP signals were recorded via a cross polarization (^{1}H-^{13}C) MAS experiment with TPPM decoupling.[2] Spectra for enhancement determination were recorded under the same conditions with and without microwave irradiation. At each temperature, the proton 90° pulse and proton T_1 were evaluated and the recycle delay set to 1.3 x T_1. The T_1 times were measured with an inversion recovery experiment with 2 scans and 1 dummy scans (for channelrhodopsin 8 scans and 2 dummy scans) and fit in the Topspin relaxation module. The Sample temperature was calibrated using KBr[3], T_1 and chemical shift value measurements. The reported sample temperatures correspond to the calibrated temperature with microwave irradiation.

Sample preparation

The SH3 samples were crystallized and prepared for DNP experiments according to previously published procedures.[4] The channelrhodopsin samples were prepared as described in Bruun & Stoeppler et al.[5] Instead of using TOTAPOL, 20 mM **bcTol** in GDH was added to the pellet containing C1C2 reconstituted with C(12)-C(15)-C(20)-^{13}C-labeled retinal in lipid vesicles.

Determination of signal-to-noise per unit time (SNR)

In order to evaluate the performance of **bcTol** in more detail, we conducted signal-to-noise per 10 min measurement time (^{10m}SNR) determinations. We used 7.0 mg microcrystalline SH3 in GDH with 20 mM **bcTol** and, as a comparison, a sample of 7.2 mg microcrystalline SH3 in GDH with 20 mM **AMUPol**. At 110 K, 181 K and 200 K, the ^{10m}SNR of both samples for both with and without microwave irradiation was determined. The reported

values are averages of five measurements. One-dimensional ^{1}H-^{13}C-CP spectra were recorded at 8889 Hz MAS with an acqusition time of 30 ms. The recycle delay was set to 1.3 x T_1 (^{1}H) and the number of scans adjusted to complete each measurement within 10 min., whereas all other conditions were kept identical to the measurements described under General Information.

For data evaluation, we used Bruker TopSpin 3.2 software. All spectra for SNR determinations were processed without window function and recorded with a spectral width of 1380 ppm with the transmitter frequency offset at 102 ppm. Two baseline corrections with a polynomial degree of five were applied, the first between 750 ppm and -550 ppm and the second from -300 ppm to -550 ppm. The SNR was determined for the carbonyl resonances (194 ppm to 164 ppm) using the region ranging from -350 ppm to -450 ppm as noise. To normalize the ^{10m}SNR according to the amount of protein, the ^{10m}SNR value was divided by 7 for **bcTol** (7.0 mg protein in the sample containing 20 mM **bcTol**) and 7.2 for **AMUPol** (7.2 mg protein in the sample containing 20 mM **AMUPol**).

Dependence of MAS frequency of the enhancement for bcTol

The MAS frequency dependence of the DNP ^{1}H-^{13}C-CP signal was recorded at 110 K. No significant changes of the enhancement values were observed between 2 and 12 kHz. This differs from the results obtained for TOTAPOL where a significant loss was observed.[6] The shown results are consistent with the observation reported for **AMUPol** where the authors suggest that the spinning independency might be due to a lower temperature dependence and to stronger electron-electron dipolar coupling.[7]

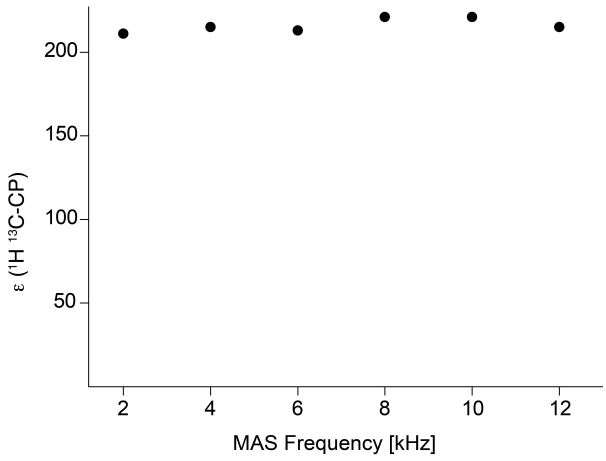


Figure S7. DNP signal enhancement ^{1}H^{13}C-CP as a function of MAS frequency (kHz) at 263 GHz (400 MHz). The sample contains 0.25 M U^{13}C-^{15}N proline (25 µL in GDH). For each data point 16 scans and 4 dummy scans where recorded with and without microwave irradiation.

Signal to noise per 10 minutes

Signal to noise values per 10 minutes of ^{1}H-^{13}C-CP experiments were recorded as a function of the recycle delay (d1) under constant microwave irradiation at 180 K in order to confirm the maximum sensitivity for d_1=1.3 x T_1. The apparent ^{1}H-T_1 (4.1 s) time was determined in a separate measurement via an inversion recovery experiment. The curve (Figure S8) shows an increase of the enhancement values until a maximum at approximately 5 s, followed by smaller values towards longer recycle delays. This is in accordance with the predicted value (5.3 s) from NMR theory.

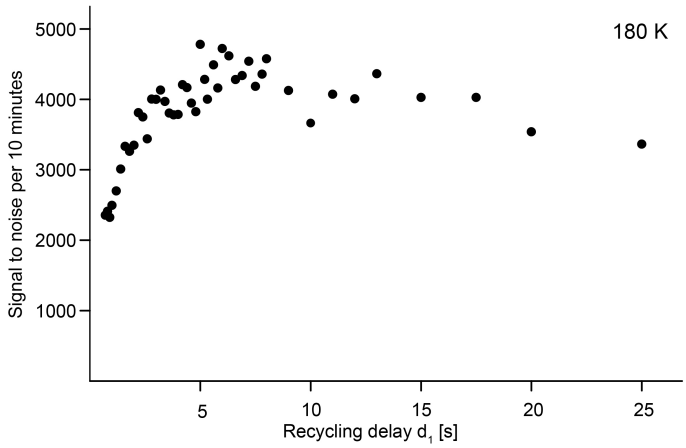


Figure S8. Signal to noise values per 10 minutes as a function of recycling delay d_1 recorded at 8.9 MAS spinning rate in a 3.2 mm zirconia rotor for a sample containing SH3 (7.2 mg), bcTol (20 mM) (^{1}H-T_1 of 4.1 s was determined via inversion recovery experiment) measured at 9.4 T and ~5 W microwave power at the end of the probe waveguide**.**

References

1. (a) T. D. Lee and F. W. Keana, *J. Org. Chem.*, 1975, **40**, 3145-3147; (b) Y. J. Li, X. G. Lei, X. Li, R. G. Lawler, Y. Murata, K. Komatsu and N. J. Turro, *Chem. Commun.*, 2011, **47**, 12527-12529.
2. A. E. Bennett, C. M. Rienstra, M. Auger, K. V. Lakshmi and R. G. Griffin, *J. Chem. Phys.*, 1995, **103**, 6951-6958.
3. K. R. Thurber and R. Tycko, *J. Magn. Reson.*, 2009, **196**, 84-87.
4. (a) Ü. Akbey, H. Oschkinat and B. J. van Rossum, *J. Am. Chem. Soc.*, 2009, **131**, 17054-17055; (b) Ü. Akbey, S. Lange, W. T. Franks, R. Linser, K. Rehbein, A. Diehl, B. J. van Rossum, B. Reif and H. Oschkinat, *J. Biomol. NMR*, 2010, **46**, 67-73.
5. S. Bruun, D. Stoeppler, A. Keidel, U. Kuhlmann, M. Luck, A. Diehl, M.-A. Geiger, D. Woodmansee, D. Trauner, P. Hegemann, H. Oschkinat, P. Hildebrandt and K. Stehfest, *Biochemistry*, 2015, **54**, 5389-5400.
6. (a) M. Rosay, L. Tometich, S. Pawsey, R. Bader, R. Schauwecker, M. Blank, P. M. Borchard, S. R. Cauffman, K. L. Felch, R. T. Weber, R. J. Temkin, R. G. Griffin and W. E. Maas, *Phys. Chem. Chem. Phys.*, 2010, **12**, 5850-5860; (b) F. Mentink-Vigier, Ü. Akbey, Y. Hovav, S. Vega, H. Oschkinat and A. Feintuch, *J. Magn. Reson.*, 2012, **224**, 13-21.
7. C. Sauvée, M. Rosay, G. Casano, F. Aussenac, R. T. Weber, O. Ouari and P. Tordo, *Angew. Chem. Int. Ed.*, 2013, **52**, 10858-10861.