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Supporting Information

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TOPP—A Novel Nitroxide-Labeled Amino Acid for EPR Distance Measurements**

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Supporting Information

S1. Synthesis of Fmoc-TOPP-OH (1)

2,2'-Iminobis(2-methylpropionitrile) (4)[1] Acetone (71.2 mL, 970 mmol) was added to an ice-cold solution of KCN (78.1 g, 1.20 mol) and NH_4Cl (77.0 g, 1.44 mol) in aq ammonia (38 %, 500 mL) over 1 h and the solution was stirred at 25 °C for 5 d. The solution was extracted with CH_2Cl_2 (3 × 250 mL), the combined organic layers dried over $MgSO_4$, and the solvent was removed *in vacuo*. The residue was distilled under reduced pressure (52 °C, 20 mbar) to give 2-amino-2-methylpropionitrile (60.8 g, 720 mmol), which was then refluxed at 100 °C under reduced pressure (50 ? 20 mbar) for 3 d, and concentrated *in vacuo* to obtain pure **4** (35.5 g, 48 %, 235 mmol). ^{1}H-NMR (300 MHz, $CDCl_3$): *d* = 1.72 (s, 1 H, NH), 1.62 (s, 12 H, CH_3); ^{13}C-NMR (126 MHz, $CDCl_3$): *d* = 123.2 (CN), 49.0 ($C(CH_3)_2$), 29.0 (CH_3).

3,3,5,5-Tetramethylpiperazine-2,6-dione (2)[1] 2,2'-Iminobis(2-methylpropionitrile) (17.1 g, 112 mmol) was added to H_2SO_4 (124 mL) at 5 °C over 2 h and the solution was stirred at 25 °C for 3 d. The solution was heated to 100 °C for 1 h and left overnight at room temperature. The solution was, then, poured onto ice (1.5 kg), neutralized to pH 7 by addition of 10N NaOH and concentrated. The residue was suspended in MeOH (1 L), Na_2SO_4 was removed by filtration and the filtrate concentrated *in vacuo*. The crude product was washed with water (200 mL) and pentane (200 mL) to provide **2** (11.8 g, 62 %, 69 mmol) as a colorless powder. ^{1}H-NMR (300 MHz, [D_6]DMSO): *d* = 10.60 (s, 1H, CONH), 2.72 (s, 1H, NH), 1.27 (s, 12H, CH_3); ^{13}C-NMR (126 MHz, [D_6]DMSO): *d* = 177.8 (CONH), 54.6 ($C(CH_3)_2$), 27.7 (CH_3); ESI-MS: *m/z* (%) = 363.2 (100) $[2M+Na]^+$, 169.1 (100) $[M-H]^-$.

Cbz-L-Hpg-OH A solution of Cbz-Cl (50 % in toluene, 6.10 mL, 18.2 mmol) in dioxane (30 mL) was added to an ice-cold suspension of L-4-hydroxyphenylglycine (3.00 g, 17.9 mmol) in aq Na_2CO_3 (10 %, 40 mL). The suspension was stirred at 0 °C for 30 min and at 25 °C for 1 h. The organic solvent was removed under reduced pressure and the aqueous residue was poured into ice water (100 mL) and extracted with EtOAc (3 × 50 mL). The aqueous layer was acidified with 2N HCl to pH 2 and extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with water (30 mL), dried over $MgSO_4$ and concentrated *in vacuo* to yield pure Cbz-L-Hpg-OH (4.90 g, 91 %, 16.3 mmol). ^{1}H-NMR (300 MHz, [D_6]DMSO): *d* = 12.67 (s, 1 H, COOH), 9.43 (s, 1 H, OH), 7.89 (d, *J* = 7.9 Hz,

1 H, NH), 7.46-7.26 (m, 5 H, phenyl CH), 7.24-7.18 (m, 2 H, phenyl CH), 6.77-6.70 (m, 2 H, phenyl CH), 5.06 (s, 2 H, CH_2), 5.05 (d, *J* = 7.9 Hz, 1 H, *a*-CH); ^{13}C-NMR (126 MHz, [D_6]DMSO): *d* = 172.2 (COOH), 156.9 (C-OH), 155.6 (CONH), 136.8 (phenyl C), 128.8 (phenyl CH), 128.1 (phenyl CH), 127.6 (phenyl CH), 127.5 (phenyl CH), 127.1 (phenyl C), 115.0 (phenyl CH), 65.4 (CH_2), 57.5 (*a*-C); ESI-MS: *m/z* (%) = 324.2 (50) $[M+Na]^+$, 340.2 (100) $[M+K]^+$, 641.2 (34) $[2M+K]^+$, 300.1 (43) $[M-H]^-$, 623.1 (100) $[2M+Na-2H]^-$, 940.2 (100) $[3M+K-2H]^-$; HR-ESI-MS: *m/z* calculated for $C_{16}H_{15}NO_5Na$ $[M+Na]^+$: 324.0842, found 324.0846.

Cbz-L-Hpg-OBn (6) BnBr (2.30 mL, 19.3 mmol) was added dropwise to an ice-cold suspension of Cbz-L-Hpg-OH (4.80 g, 15.9 mmol) and $NaHCO_3$ (1.40 g, 16.7 mmol) in dry DMF (80 mL). The suspension was stirred at 25 °C for 15 h, diluted with water (120 mL) and extracted with EtOAc (3 × 50 mL). The extracts were washed with water (20 mL), brine (3 × 20 mL), dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by flash chromatography using EtOAc/pentane (1:1) to provide **6** (3.90 g, 63 %, 9.96 mmol). ^{1}H-NMR (300 MHz, [D_6]DMSO): *d* = 9.48 (s, 1 H, OH), 8.11 (d, *J* = 7.6 Hz, 1 H, NH), 7.50-7.12 (m, 10 H, phenyl CH), 7.24-7.17 (m, 2 H, phenyl CH), 6.76-6.69 (m, 2 H, phenyl CH), 5.21 (d, *J* = 7.6 Hz, 1 H, *a*-CH), 5.12 (s, 2 H, CH_2), 5.06 (s, 2 H, CH_2); ^{13}C-NMR (126 MHz, [D_6]DMSO): *d* = 170.7 (COOBn), 157.1 (C-OH), 155.7 (CONH), 136.7 (phenyl C), 135.6 (phenyl C), 128.9 (phenyl CH), 128.1 (phenyl CH), 127.7 (phenyl CH), 127.6 (phenyl CH), 127.5 (phenyl CH), 127.3 (phenyl CH), 126.0 (phenyl C), 115.0 (phenyl CH), 65.9 (Bn CH_2), 65.5 (Cbz CH_2), 57.6 (*a*-C); ESI-MS: *m/z* (%) = 414.2 (100) $[M+Na]^+$, 390.1 (100) $[M-H]^-$, 781.3 (58) $[2M-H]^-$; HR-ESI-MS: *m/z* calculated for $C_{23}H_{21}NO_5Na$ $[M+Na]^+$: 414.1312, found 414.1314.

Cbz-L-Hpg(Tf)-OBn (7) Triflic anhydride (2.50 mL, 14.9 mmol) was added to an ice-cold solution of Cbz-L-Hpg-OBn (3.80 g, 9.70 mmol) and pyridine (2.40 mL, 29.8 mmol) in dry CH_2Cl_2 (30 mL). The solution was stirred at 0 °C for 15 min and at 25 °C for 20 min. The reaction was quenched with saturated aq $NaHCO_3$ (50 mL) and the resulting solution extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were washed with brine (3 × 20 mL), dried over $MgSO_4$ and concentrated *in vacuo*. Pyridine was removed by codestillation with toluene under reduced pressure and the residue was purified by flash chromatography using EtOAc/pentane (1:4) to provide **7** (4.80 g, 94 %, 9.17 mmol). ^{1}H-NMR (300 MHz, [D_6]DMSO): *d* = 7.44-7.06 (m, 14 H, phenyl CH), 5.95 (d, *J* = 7.0 Hz, 1 H, NH),

5.42 (d, *J* = 7.0 Hz, 1 H, *a*-CH), 5.14-5.03 (m, 4 H, CH_2); ^{13}C-NMR (126 MHz, [D_6]DMSO): *d* = 169.6 (COOBn), 155.1 (CONH), 149.3 (phenyl C), 137.2 (phenyl C), 135.8 (phenyl C), 134.5 (phenyl C), 129.0 (2 phenyl CH), 128.5 (phenyl CH), 128.3 (phenyl CH), 128.1 (phenyl CH), 127.9 (phenyl CH), 121.7 (phenyl CH), 118.6 (q, *J* = 320.6 Hz, CF_3), 67.9 (Bn CH_2), 67.4 (Cbz CH_2), 57.2 (*a*-C); ESI-MS: *m/z* (%) = 546.1 (100) $[M+Na]^+$, 1069.2 (38) $[2M+Na]^+$, 522.1 (100) $[M-H]^-$; HR-ESI-MS: *m/z* calculated for $C_{24}H_{19}F_3NO_7S$ $[M-H]^-$: 522.0840, found 522.0840.

Bn$_2$-L-Hpg(Tf)-OBn (8) A solution of Cbz-L-Hpg(Tf)-OBn (2.00 g, 3.82 mmol) and $(CH_3)_2S$ (8.40 mL, 113 mmol) in TFA (34 mL) was stirred at 25 °C for 17 h. Trifluoroacetic acid was subsequently removed by codestillation with toluene at room temperature. The crude intermediate and $NaHCO_3$ (1.90 g, 22.6 mmol) were suspended in dry DMSO (20 mL) and BnBr (8.10 mL, 68.2 mmol) was added dropwise. The suspension was stirred at 25 °C for 25 h, diluted with water (120 mL) and extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with water (20 mL) and brine (3 × 20 mL) and excess benzyl bromide was removed under high vacuum. The residue was purified by flash chromatography using 3 % EtOAc in pentane to provide **8** (1.50 g, 69 %, 2.63 mmol). ^{1}H-NMR (300 MHz, $CDCl_3$): *d* = 7.44-7.16 (m, 19 H, phenyl CH), 5.31 (d, *J* = 12.1 Hz, 1 H, CH_2), 5.20 (d, *J* = 12.1 Hz, 1 H, CH_2), 4.62 (s, 1 H, *a*-CH), 3.76 (d, *J* = 13.9 Hz, 2 H_a, $N(CH_aH_b$-phenyl$)_2$), 3.67 (d, *J* = 13.9 Hz, 2 H_b, $N(CH_aH_b$-phenyl$)_2$); ^{13}C-NMR (126 MHz, $CDCl_3$): *d* = 170.9 (COOBn), 148.8 (phenyl C), 138.8 (phenyl C), 137.3 (phenyl C), 135.4 (phenyl C), 130.5 (phenyl CH), 128.6 (phenyl CH), 128.5 (phenyl CH), 128.3 (phenyl CH), 127.2 (phenyl CH), 121.1 (phenyl CH), 118.6 (q, *J* = 320.6 Hz, CF_3), 66.5 (CH_2), 64.9 (*a*-C), 54.3 (CH_2); ESI-MS: *m/z* (%) = 570.2 (100) $[M+H]^+$, 592.1 (46) $[M+Na]^+$, 568.1 (100) $[M-H]^-$; HR-ESI-MS: *m/z* calculated for $C_{30}H_{27}F_3NO_5S$ $[M+H]^+$ 570.1557, found 570.1556.

Bn$_2$-4-pinacolboryl-L-Phg-OBn (9) A suspension of Bn$_2$-L-Hpg(Tf)-OBn (2.00 g, 3.51 mmol), bis(pinacolato)diboron (1.08 g, 4.25 mmol), KOAc (1.04 g, 10.6 mmol), $PdCl_2$(dppf) (247 mg, 338 µmol), and dppf (196 mg, 354 µmol) in degassed dioxane (35 mL) was stirred at 80 °C under argon atmosphere for 10 h. The suspension was diluted with EtOAc (150 mL), filtered through Celite, washed with brine (3 × 20 mL), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by flash chromatography using a gradient of 3? 10 % EtOAc in pentane to yield **9** (1.74 g, 90 %, 3.18 mmol). ^{1}H-NMR (300 MHz, $CDCl_3$): *d* = 7.76 (m_c,

2 H, phenyl CH), 7.39-7.17 (m, 17 H, phenyl CH), 5.30 (d, *J* = 12.2 Hz, 1 H, CH_2), 5.18 (d, *J* = 12.2 Hz, 1 H, CH_2), 4.66 (s, 1 H, *a*-CH), 3.73 (s, 4 H, CH_2), 1.34 (s, 12 H, CH_3); ^{13}C-NMR (126 MHz, $CDCl_3$): *d* = 171.7 (COOBn), 139.6 (phenyl C), 139.3 (phenyl C), 135.7 (phenyl C), 134.6 (phenyl CH), 128.7 (phenyl CH), 128.4 (phenyl CH), 128.2 (phenyl CH), 128.1 (phenyl CH), 126.9 (phenyl CH), 83.8 ($C(CH_3)_2$), 66.2 (CH_2), 65.9 (*a*-C), 54.2 (CH_2), 25.0 (CH_3), 24.9 (CH_3); ESI-MS: *m/z* (%) = 548.3 (100) $[M+H]^+$, 570.3 (100) $[M+Na]^+$; HR-ESI-MS: *m/z* calculated for $C_{35}H_{39}BNO_4$ $[M+H]^+$ 548.2973, found 548.2972.

Bn_2-4-dihydroxyborane-L-Phg-OBn (3) Water (380 mL), NH_4OAc (2.20 g, 28.5 mmol) and $NaIO_4$ (6.20 g, 29.0 mmol) were added to a solution of Bn_2-4-pinacolboryl-L-Phg-OBn (5.30 g, 9.69 mmol) in acetone (430 mL). The suspension was stirred at 25 °C for 2 d. Acetone was removed under reduced pressure and the aqueous residue was extracted with Et_2O (3 × 100 mL). The combined organic layers were washed with brine (3 × 20 mL), dried over $MgSO_4$ and concentrated *in vacuo* to yield **3** (4.00 g, 89 %, 8.60 mmol). ^{1}H-NMR (300 MHz, $CDCl_3$): *d* = 8.19 (s, 1 H, B-OH), 8.17 (s, 1 H, B-OH), 7.51-7.20 (m, 19 H, phenyl CH), 5.36 (d, *J* = 12.2 Hz, 1 H, CH_2), 5.23 (d, *J* = 12.2 Hz, 1 H, CH_2), 4.74 (s, 1 H, *a*-CH), 3.79 (s, 4 H, CH_2); ^{13}C-NMR (126 MHz, $CDCl_3$): *d* = 171.5 (COOBn), 141.3 (phenyl C), 139.2 (phenyl C), 135.7 (phenyl C), 135.5 (phenyl CH), 128.7 (phenyl CH), 128.5 (phenyl CH), 128.4 (phenyl CH), 128.3 (phenyl CH), 128.2 (phenyl CH), 127.0 (phenyl CH), 66.3 (CH_2), 65.9 (*a*-C), 54.3 (CH_2); ESI-MS: *m/z* (%) = 466.2 (100) $[M+H]^+$; HR-ESI-MS: *m/z* calculated for $C_{29}H_{29}BNO_4$ $[M+H]^+$: 466.21896, found 466.21807.

Bn_2-4-(3,3,5,5-tetramethyl-2,6-dioxopiperazin-1-yl)-L-Phg-OBn (10) A suspension of Bn_2-4-dihydroxyborane-L-Phg-OBn (4.00 g, 8.60 mmol), 3,3,5,5-tetramethylpiperazine-2,6-dione (1.46 g, 8.59 mmol), $Cu(OAc)_2$ (1.56 g, 8.59 mmol), molecular sieve (4 g, 4 Å, powder) and Et_3N (1.66 mL, 12.0 mmol) in dry DMSO (180 mL) was stirred at 25 °C under oxygen atmosphere for 14 d. The suspension was filtered through Celite, diluted with water (200 mL) and extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with brine (3 × 30 mL), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by flash chromatography using EtOAc/pentane (1:2) to yield **10** (3.94 g, 78 %, 6.68 mmol). ^{1}H-NMR (300 MHz, $CDCl_3$): *d* = 7.51-7.19 (m, 17 H, phenyl CH), 7.06 (m_c, 2 H, phenyl CH), 5.36 (d, *J* = 12.2 Hz, 1 H, CH_2), 5.19 (d, *J* = 12.2 Hz, 1 H, CH_2), 4.69 (s, 1 H, *a*-CH), 3.82 (d, *J* = 14.0 Hz, 2 H_a, $N(CH_aH_b$-phenyl$)_2$), 3.72 (d, *J* = 14.0 Hz, 2 H_b, $N(CH_aH_b$-phenyl$)_2$), 1.69

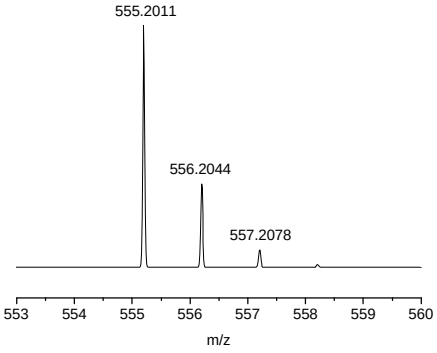
(s, 1 H, NH), 1.53 (s, 12 H, CH_3); ^{13}C-NMR (126 MHz, $CDCl_3$): *d* = 176.4 ($CONR_2$), 171.3 (COOBn), 139.0 (phenyl C), 136.7 (phenyl C), 135.6 (phenyl C), 134.8 (phenyl C), 129.3 (phenyl CH), 128.7 (phenyl CH), 128.5 (phenyl CH), 128.4 (phenyl CH), 128.3 (phenyl CH), 128.2 (phenyl CH), 128.1 (phenyl CH), 126.9 (phenyl CH), 66.3 (CH_2), 65.4 (*a*-C), 56.0 (*C*(CH_3)$_2$), 54.2 (CH_2), 28.5 (CH_3); ESI-MS: *m/z* (%) = 590.3 (100) $[M+H]^+$, 612.3 (67) $[M+Na]^+$, 1201.6 (26) $[2M+Na]^+$, 588.3 (100) $[M-H]^-$, 634.3 (99) $[M+HCOO^-]^-$, 634.3 (23) $[2M+HCOO^-]^-$; HR-ESI-MS: *m/z* calculated for $C_{37}H_{40}N_3O_4$ $[M+H]^+$: 590.3013, found 590.3013.

Fmoc-4-(3,3,5,5-tetramethyl-2,6-dioxopiperazin-1-yl)-L-Phg-OH (11) A suspension of Bn_2-4-(3,3,5,5-tetramethyl-2,6-dioxopiperazin-1-yl)-L-Phg-OBn (500 mg, 849 µmol) and 20 % $Pd(OH)_2$/C (50 % H_2O, 100 mg, 71.2 µmol) in MeOH (20 mL) was shaken in Parr apparatus under hydrogen atmosphere (70 psi) at 25 °C for 20 h. The resulting suspension was diluted with MeOH (230 mL), filtered and concentrated *in vacuo*. A suspension of crude intermediate, Fmoc-OSu (286 mg, 848 µmol) and $NaHCO_3$ (143 mg, 1.70 mmol) in dry DMF (5 mL) was stirred at 25 °C for 21 h. The suspension was diluted with water (20 mL), acidified with 2N HCl to pH 2 and extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (3 × 15 mL), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by flash chromatography using CH_2Cl_2/MeOH/AcOH (98:2:0.1 ? 97:3:0.1) to provide **11** (423 mg, 92 %, 781 µmol). ^{1}H-NMR (600 MHz, [D_6]DMSO): *d* = 8.25 (d, *J* = 8.0 Hz, 1 H, CONH), 7.92-7.08 (m, 12 H, 8 Fmoc CH, 4 phenyl CH), 5.24 (d, *J* = 8.0 Hz, 1 H, *a*-CH), 4.29 (d, *J* = 7.3 Hz, 2 H, Fmoc CH_2), 4.23 (t, *J* = 7.3 Hz, 1 H, Fmoc CH), 1.56 (s, 1 H, NH), 1.40 (s, 12 H, CH_3); ^{13}C-NMR (126 MHz, [D_6]DMSO): *d* = 176.5 ($CONR_2$), 171.6 (COOH), 155.8 (CONH), 143.7 (Fmoc C), 140.6 (Fmoc C), 136.9 (phenyl C), 135.7 (phenyl C), 128.5 (phenyl CH), 128.2 (phenyl CH), 127.6 (phenyl CH), 127.0 (phenyl CH), 125.3 (phenyl CH), 120.0 (phenyl CH), 65.9 (Fmoc CH_2), 57.7 (*a*-C), 55.3 (*C*(CH_3)$_2$), 46.6 (Fmoc CH), 28.0 (CH_3); ESI-MS: *m/z* (%) = 564.2 (100) $[M+Na]^+$, 540.2 (100) $[M-H]^-$; HR-ESI-MS: *m/z* calculated for $C_{31}H_{30}N_3O_6$ $[M-H]^-$ 540.2140, found 540.2140.

Fmoc-4-(3,3,5,5-tetramethyl-2,6-dioxo-4-oxylpiperazin-1-yl)-L-Phg-OH (1) mCPBA (296 mg, 70-75 %, 1.24 mmol) was added to an ice-cold solution of Fmoc-4-(3,3,5,5-tetramethyl-2,6-dioxopiperazin-1-yl)-L-Phg-OH (361 mg, 667 µmol) in dry CH_2Cl_2 (90 mL). The solution was stirred at 0 °C for 15 min and at 25 °C for 5 h. The solvent was removed under reduced

pressure and the residue was purified by flash chromatography using CH_2Cl_2/MeOH/AcOH (99.4:0.5:0.1 ? 98.4:1.5:0.1) to provide **1** (300 mg, 81 %, 539 µmol) as a yellow powder. ESI-MS: *m/z* (%) = 579.2 (100) $[M+Na]^+$, 555.2 (100) $[M-H]^-$; HR-ESI-MS: *m/z* calculated for $C_{31}H_{29}N_3O_7$ $[M-H]^-$: 555.2011; found 555.2013.

calculated



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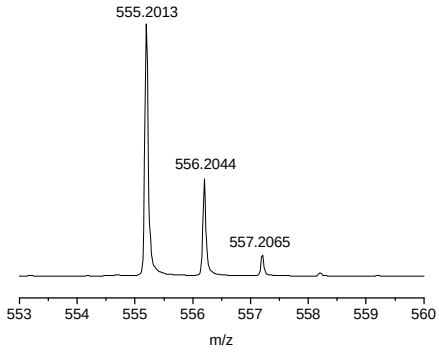
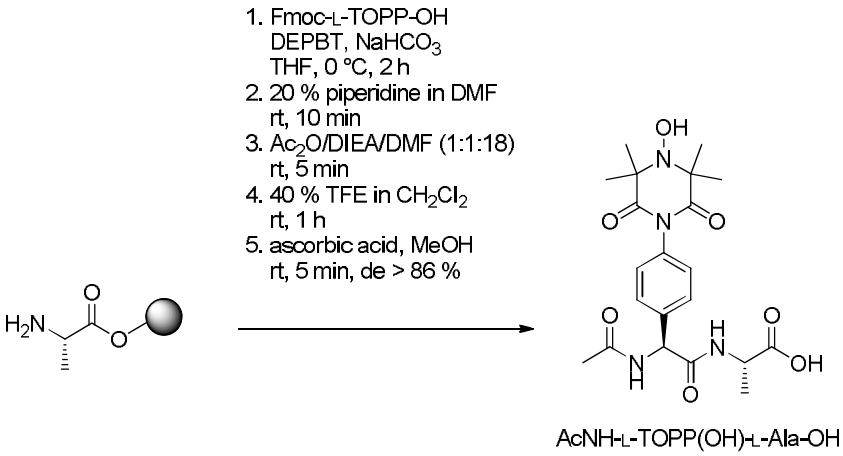
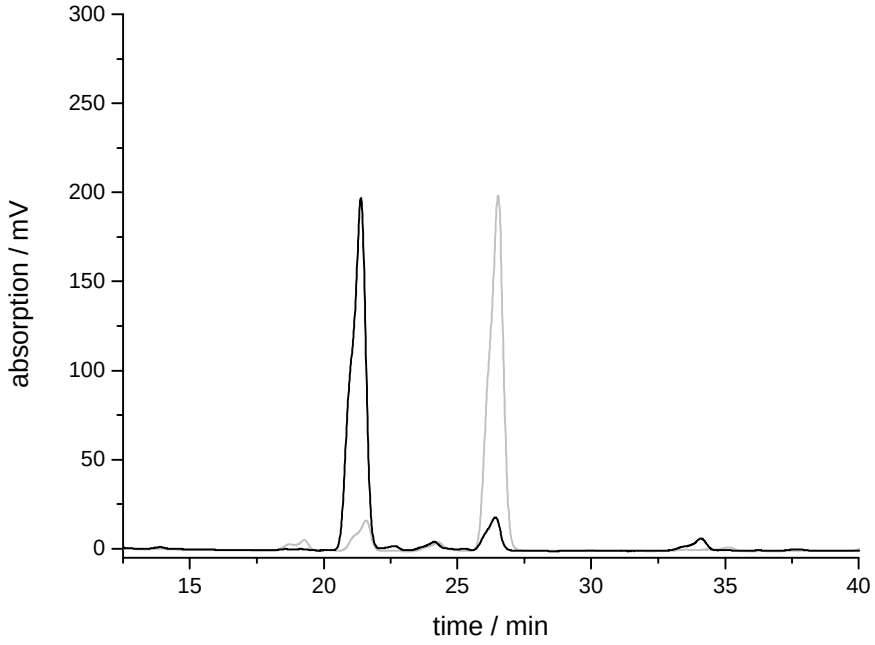


S2. Racemization study of TOPP





AcNH-L-TOPP(OH)-**L**-Ala-OH (black), AcNH-L-TOPP(OH)-**D**-Ala-OH (gray)

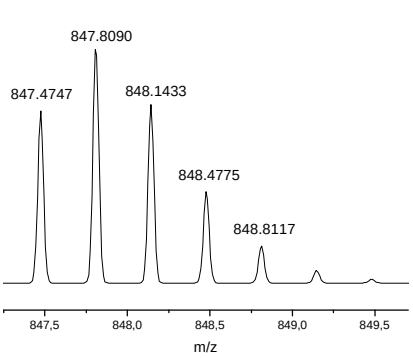
RP-HPLC-column: *Phenomenex* Jupiter (C18, 300 Å, 5 µm, 250 × 4.6 mm); gradient: water/TFA (100:0.1) ? acetonitrile/TFA (100:0.1) in 60 min; flow rate 1.0 mL/min; wavelength: 235 nm.

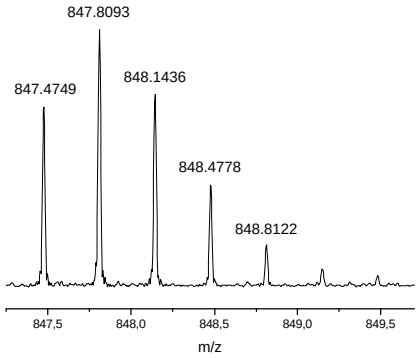
S3. Synthesis of Peptides P1, P2 and P3

For the synthesis of peptides **P1**, **P2** and **P3** low loaded (0.29 mmol/g) Rink amide MBHA resin was used. The first amino acid was coupled three times using diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) as coupling reagents. The proteinogenic amino acids were activated with HBTU/HOBt and DIEA and coupled using microwave irradiation. Fmoc-TOPP-OH (**1**) was activated with DEPBT and $NaHCO_3$ in dry THF and coupled at 0 °C for 2 h and then at 25 °C for 30 min. Fmoc protecting group was cleaved by piperidine/DMF (1:4). The terminal amino group of the peptides was acetylated with Ac_2O/DIEA/DMF (1:1:18). Cleavage from the resin was carried out using TFA/H_2O/TIS (90:5:5, v/v/v) (for **P1** and **P2**) or TFA/H_2O/EDT/TIS (94/2.5/2.5/1, v/v/v/v) (for **P3** precursor peptide (**P4**)). The hydroxylamine group of the crude peptide **P1** was oxidized to nitroxide with $Cu(OAc)_2$ before RP-HPLC purification. The regeneration of the nitroxide was proven by mass spectrometry. MTSSL labeling of cysteine residues was performed with peptide **P4** (1.0 mM) in a phosphate buffer/acetonitrile (4/1, v/v) solution and MTSSL (5 eq. per cysteine residue). The solution was stirred under argon atmosphere at 25 °C for 12 h. The target peptide **P3** was obtained by RP-HPLC.

(**P1**) Ac-AAAAK-TOPP-AKAAAAAKAAKA-TOPP-KAAAA-NH_2 ($C_{115}H_{186}N_{34}O_{31}$, 2540.9) RP-HPLC-column: *Phenomenex* Jupiter (C18, 300 Å, 5 µm, 250 × 10 mm); gradient: 25 ? 50 % B in 30 min, A = water/TFA (100:0.1), B = acetonitrile/water/TFA (80:20:0.1); flow rate 3.0 mL/min, t_R = 14.9 min; HR-ESI-MS: *m/z* calculated for $C_{115}H_{189}N_{34}O_{31}$ $[M+3H]^{3+}$: 847.4747, found 847.4749.

calculated found





(**P2**) Ac-AAAAKYAKAAAAAKAAKAYKAAAA-NH_2 ($C_{101}H_{168}N_{30}O_{27}$, 2234.6)
RP-HPLC-column: *Phenomenex* Jupiter (C18, 300 Å, 5 µm, 250 × 10 mm); gradient: 5 ? 40 % B in 30 min, A = water/TFA (100:0.1), B = acetonitrile/water/TFA (80:20:0.1); flow rate 3.0 mL/min, t_R = 25.3 min; HR-ESI-MS: *m/z* calculated for $C_{101}H_{171}N_{30}O_{27}$ $[M+3H]^{3+}$: 745.4304, found 745.4308.

(**P3**) Ac-AAAAK MTSSL AKAAAAAKAAKA MTSSL KAAAA-NH_2 ($C_{107}H_{188}N_{32}O_{27}S_4$, 2483.10)
RP-HPLC-column: *MN* Nucleodur 100 (C18, 300 Å, 5 µm, 250 x 21 mm); gradient: 20 ? 60 % B in 30 min, A = water/TFA (100:0.1), B = acetonitrile/water/TFA (80:20:0.1), flow rate = 10 mL/min, t_R=19.62 min; HR-ESI-MS: *m/z* calculated for $C_{107}H_{191}N_{32}O_{27}S_4$ $[M+3H]^{3+}$: 828.1143, found 828.1141.

(**P4**) Ac-AAAAKCAKAAAAAKAAKACKAAAA-NH_2 ($C_{89}H_{160}N_{30}O_{25}S_2$, 2114.54)
RP-HPLC-column: *MN* Nucleodur 100 (C18, 300 Å, 5 µm, 250 x 21 mm); gradient: 15 ? 60 % B in 30 min, A = water/TFA (100:0.1), B = acetonitrile/water/TFA (80:20:0.1), flow rate 10 mL/min, t_R=16.69 min; HR-ESI-MS: *m/z* calculated for $C_{89}H_{163}N_{30}O_{25}S_2$ $[M+3H]^{3+}$: 705.3939, found 705.3944.

S4. Characterization of the molecular structure of the spin label TOPP by DFT calculations

The hybrid functionals B3LYP and PBE0 were used for calculations of the molecular geometry of the model compounds **12** and **13** (Figure S1). The geometry optimizations and frequency calculations were performed using the basis set 6-311G (d, p) in the gas phase or in the dipole field of water. Therefore, the program package GAUSSIAN 09 was used.[2]

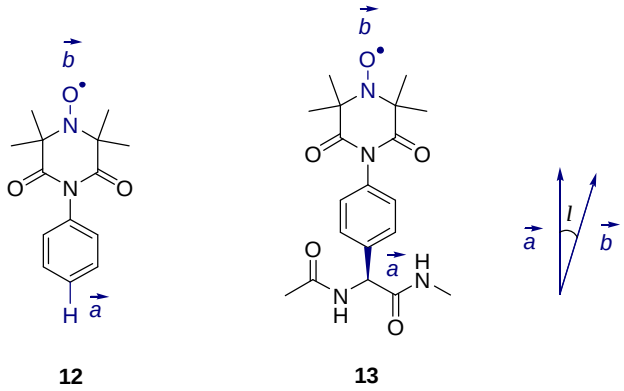


Figure S1: Structures of the calculated compounds **12** and **13** and the angle l formed by the vectors $\vec{a}$ and $\vec{b}$.

Figure S2 shows the conformers *Ba* and *Bb* of the compound **12**, which were calculated using the B3LYP method.

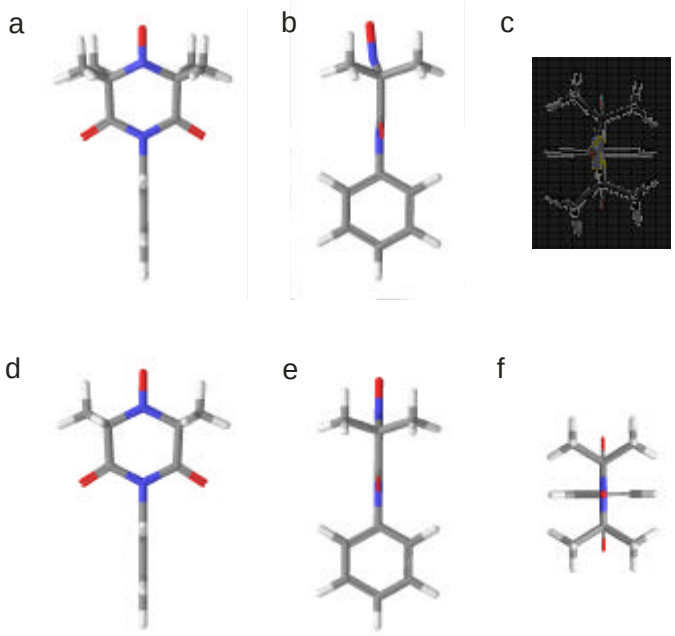


Figure S2: Geometry optimizations and frequency calculations of the conformations *Ba* (a, b, c) and *Bb* (d, e, f) of the compound **12** were obtained with the B3LYP method and the basis set 6-311G (d, p) in the gas phase at 298 K.

The conformation *Ba* exists in the ground state and the conformation *Bb* with C_{2v} symmetry is energetically 1.4 kJ/mol above the ground state (Table S1). The angle l, enclosed by the CH bond and nitroxide bond was calculated by the following formula:

$$\cos\lambda = \frac{\vec{a} \cdot \vec{b}}{|\vec{a}| \cdot |\vec{b}|}$$

Here, vector $\vec{a}$ is described by the CH bond and vector $\vec{b}$ by the NO bond (Figure S1).

Conformation of **12**	*D* E / kJ/mol	Angle l / °
Ba	0.0[a]	6.5
Bb	1.4	0.0

Table S1: The energy difference and the angle l of the conformers *Ba* and *Bb* of the compound **12**. [a] Zero-point energy = -878.795016 a. u.

To determine the geometry of TOPP in an *a*-helical secondary structure in water (25 °C, 1 atm), the amino acid **13** was used as a model compound (Figure 5). The calculations were carried out with fixed dihedral angles (φ = -52°, ψ = -53°). The simulation of the solvent was carried out by the continuum model (IEFPCM). Three conformational isomers of the compound **13** with the corresponding energy differences and the angles l are shown in the Table S2.

Conformation of **13**	*D* E / kJ/mol	Angle l / °
Ba	0.0[a]	8.1
Bb	4.8	3.2
Bg	4.9	3.2

Table S2: The energy difference and angle l of the conformers *Ba*, *Bb* und *Bg* of compound **13**. Geometry optimization and frequency calculation were obtained with the B3LYP method and the basis set 6-311G (d, p) in water (298 K, 1 atm). [a] Zero-point energy = -1334.144545 a. u.

Calculations with PBE0 method show comparable results:

Compound & Conformation	D E / kJ/mol	Angle l / °
12 *Pa*	0.0[a]	7.7
12 *Pb*	1.5	0.0
13 *Pa*	0.00[b]	3.4
13 *Pb*	0.30	10.6
13 *Pg*	0.34	3.3

Table S3: The energy difference and angle l of the conformers **12** *Pa*, **12** *Pb* und **13** *Pa*, **13** *Pb*, **13** *Pg*. Geometry optimization and frequency calculation were obtained with the PBE0 method and the basis set 6-311G (d, p) in the gas phase (**12**) and in water (**13**) at 298 K. [a] Zero-point energy = -877.782029 a. u. [b] Zero-point energy = -1332.618222 a. u.

S5. CW EPR

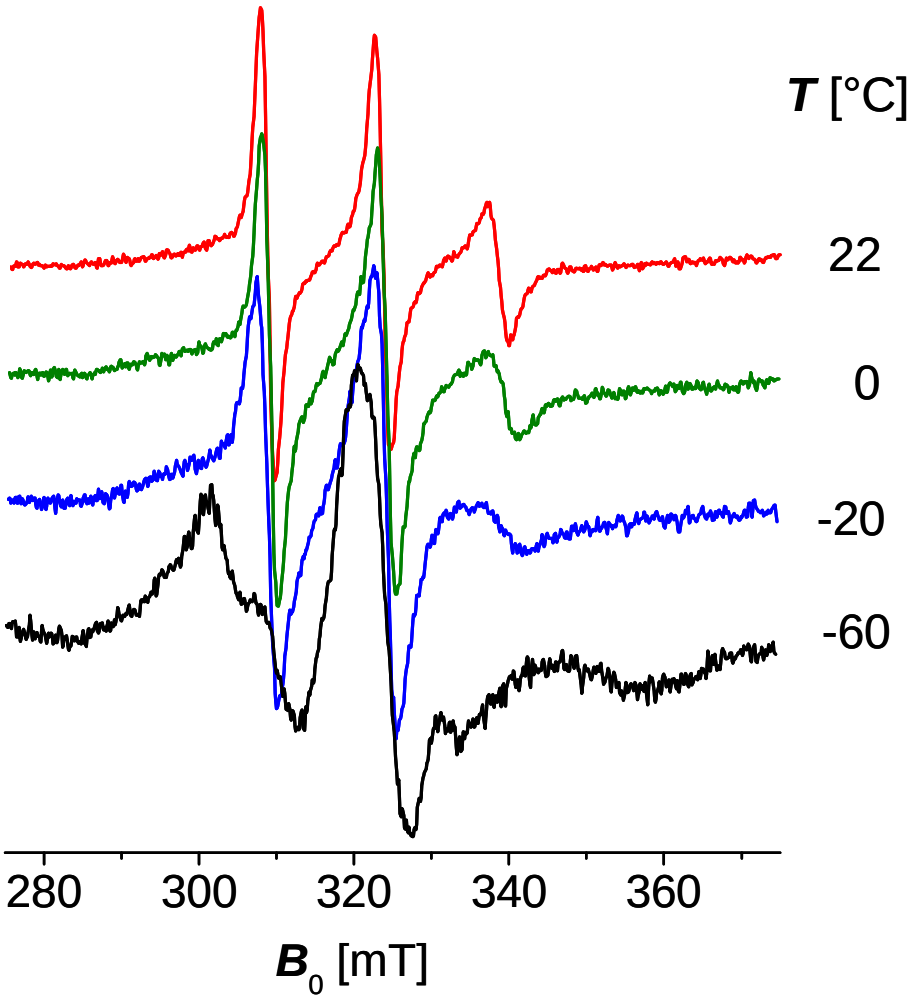


Figure S3. Continuous-wave (CW) EPR spectra for the peptide **P1** collected at temperatures 20 to –60 °C. 50 μM of the labeled system, dissolved in EtOH/MeOH/TFE (40:40:20). Spectra have been recorded with modulation amplitude of 1.5 G, modulation frequency 100 kHz, sweep-width 100 G.

S6. CW EPR

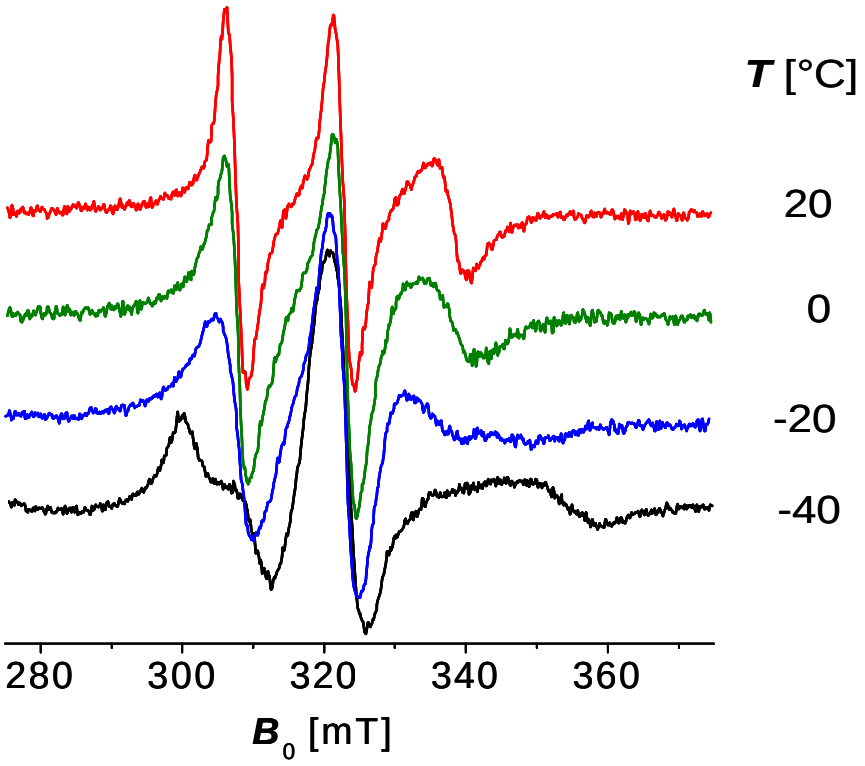


Figure S4. Continuous-wave (CW) EPR spectra for the peptide **P1** collected at temperatures 20 to –60 °C. 50 μM of the labeled system, dissolved in TFE/glycerol (90:10). Spectra have been recorded with modulation amplitude of 1.5 G, modulation frequency 100 kHz, sweep-width 100 G.

S7. CW EPR

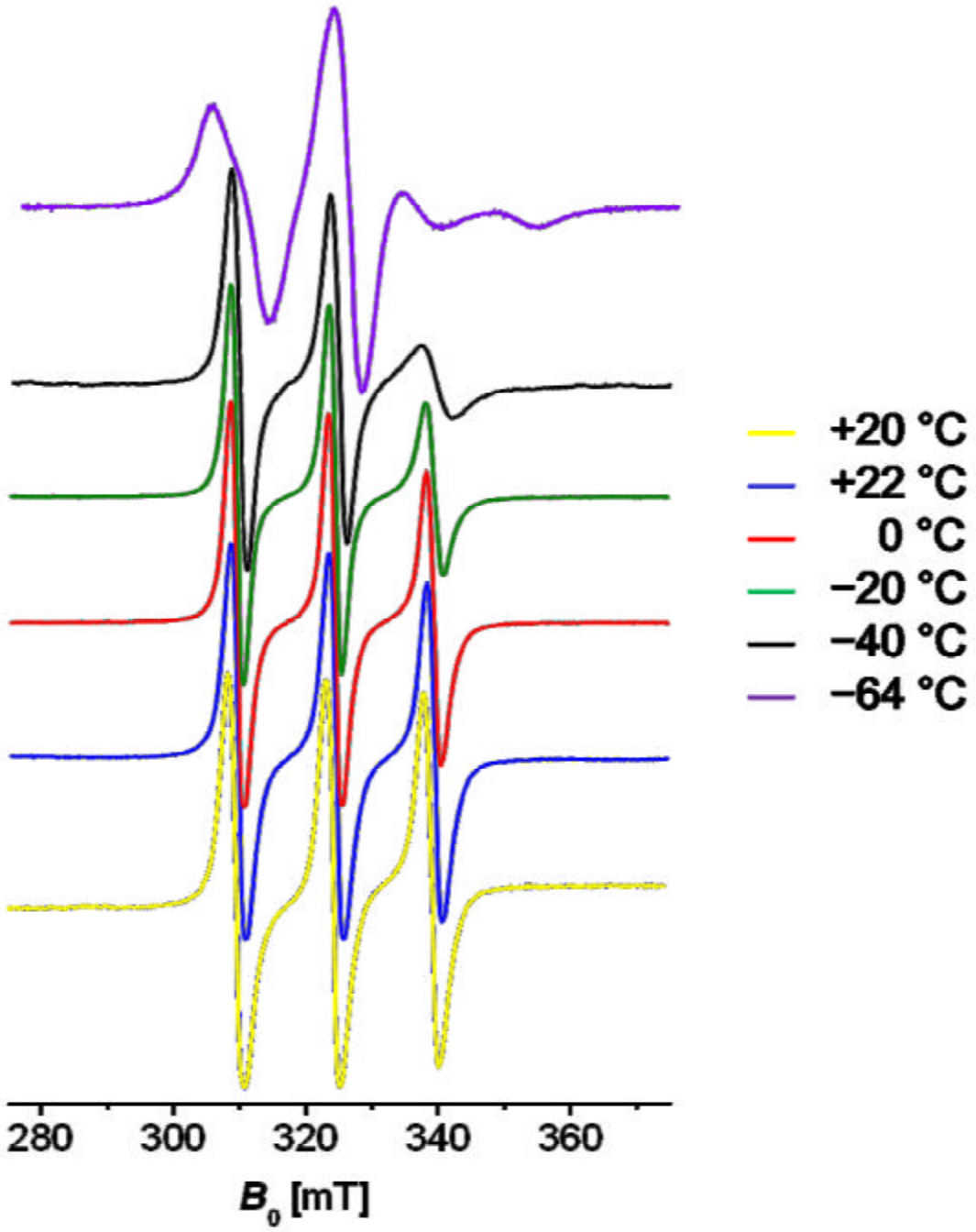
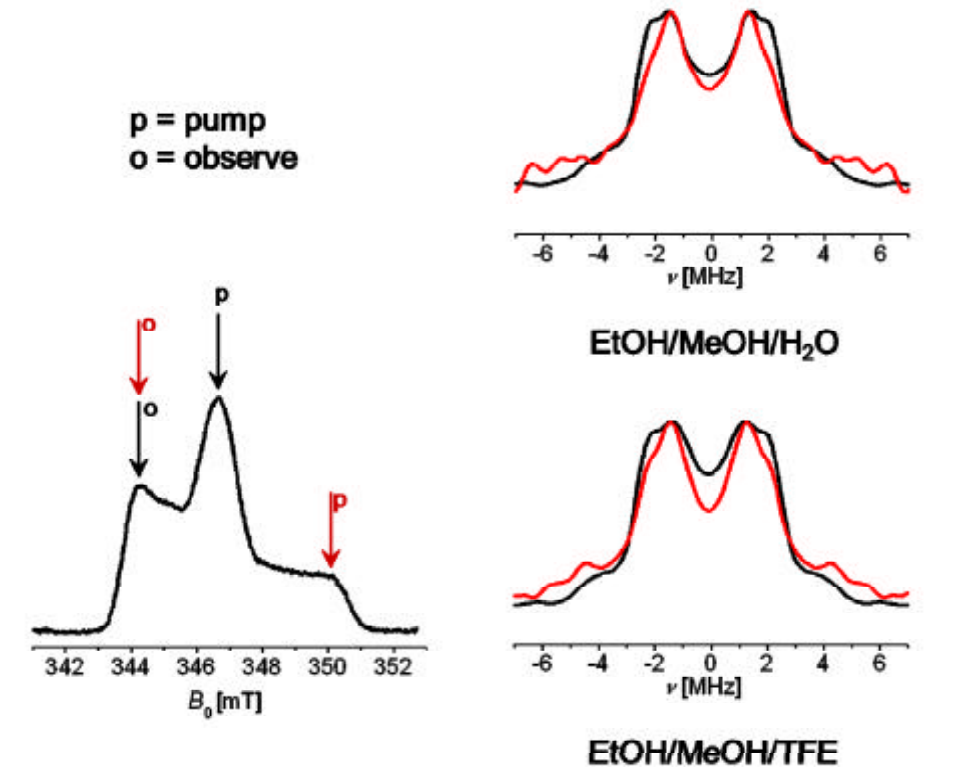
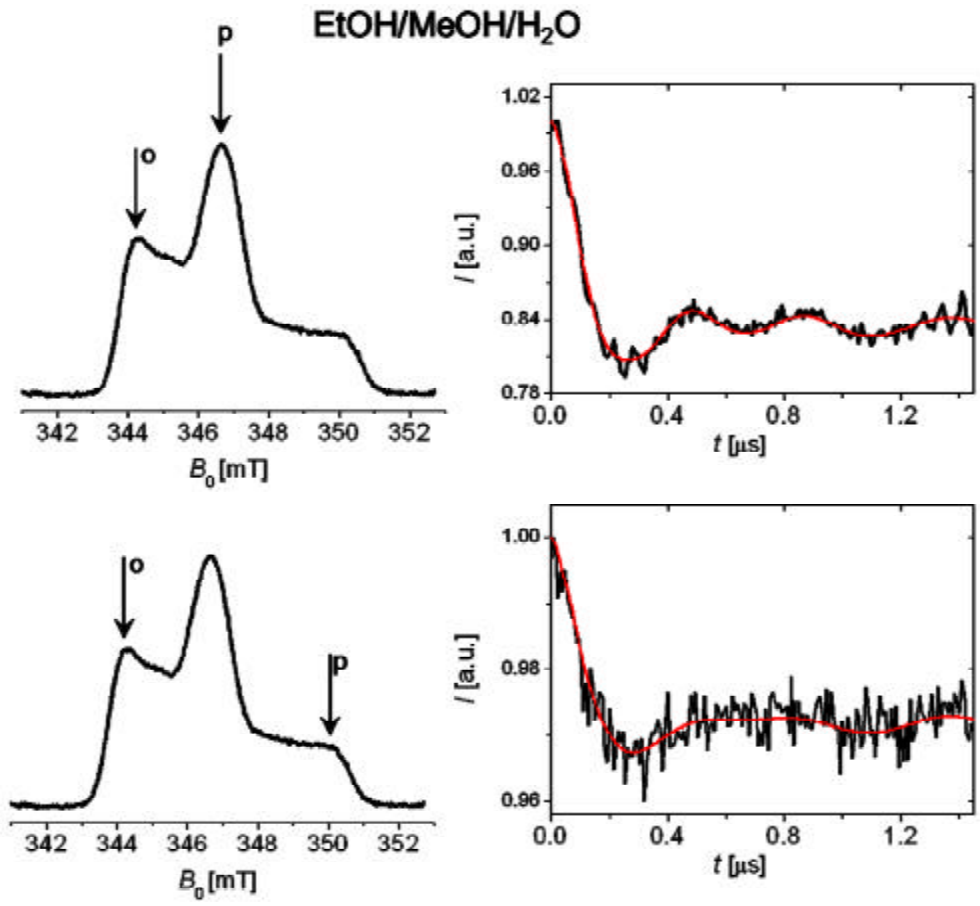


Figure S5. Continuous-wave (CW) EPR spectra for the monomer **Fmoc-TOPP-OH** collected at temperatures 20 to –64 °C. 100 μM of the labeled system, dissolved in TFE/glycerol (90:10). Spectra have been recorded with modulation amplitude of 1.5 G, modulation frequency 100 kHz, sweep-width 100 G.

S8. Orientation Selection Experiments at low field (X-band)





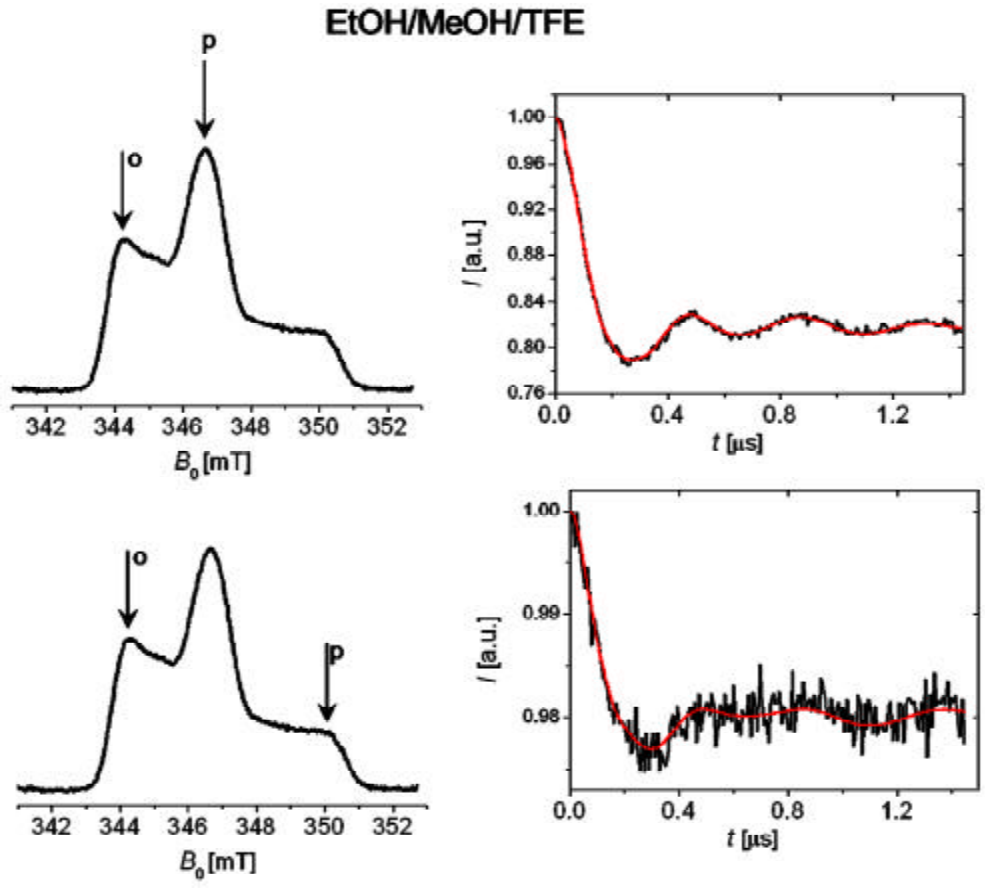


Figure S6. Double-labeled **P2** in TFE/EtOH/MeOH (40:40:20). *Left*: Field swept echo-detected spectrum and DEER set-up; two different Δν values, corresponding to 70 (black) and 150 (red) MHz, respectively. *Right*: Dipolar spectra obtained from the Fourier transform of the DEER signals and dipolar evolutions after background subtraction. The DEER experiment was carried out on a Bruker ELEXYSIS 580 pulsed EPR spectrometer at 50 K; $\pi/2 - \pi = 16 - 32$ ns; $\pi_{ELDOR} = 36$ ns; SPP = 50; SRT = 5 ms; acquisition time: 12 h. The Pake pattern distortion is produced by the two different DEER set-up.

S9. Orientation Averaging Experiments

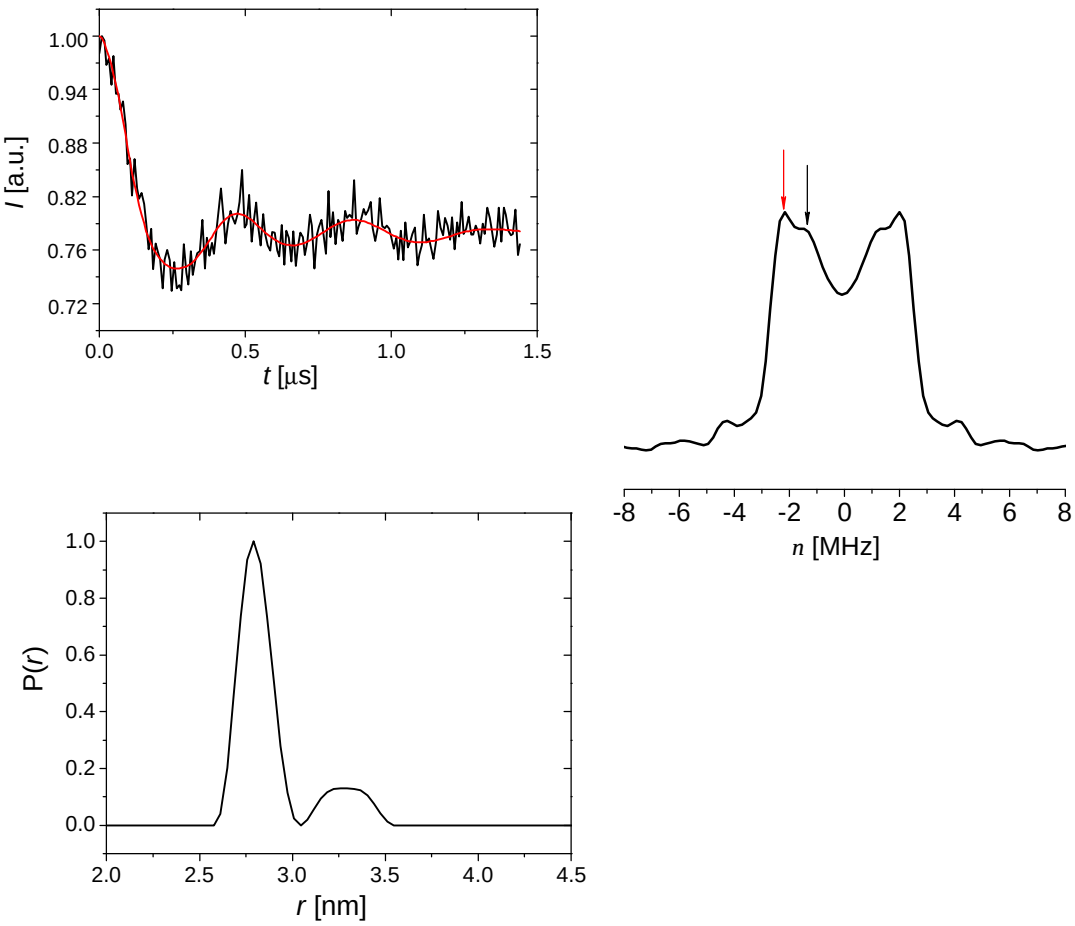


Figure S7. X-Band time-domain DEER signal of double-labeled **P1**: sum of 11 field values (B_{obs} = 3453.5 – 3466 G, with 2.5 G step). T = 50 K; $\pi/2 - \pi$ = 16 – 32 ns; π_{ELDOR} = 36 ns; SPP = 50; SRT = 5 ms; 10 scans for each field value.

S10. DEER experiment with hard pulses

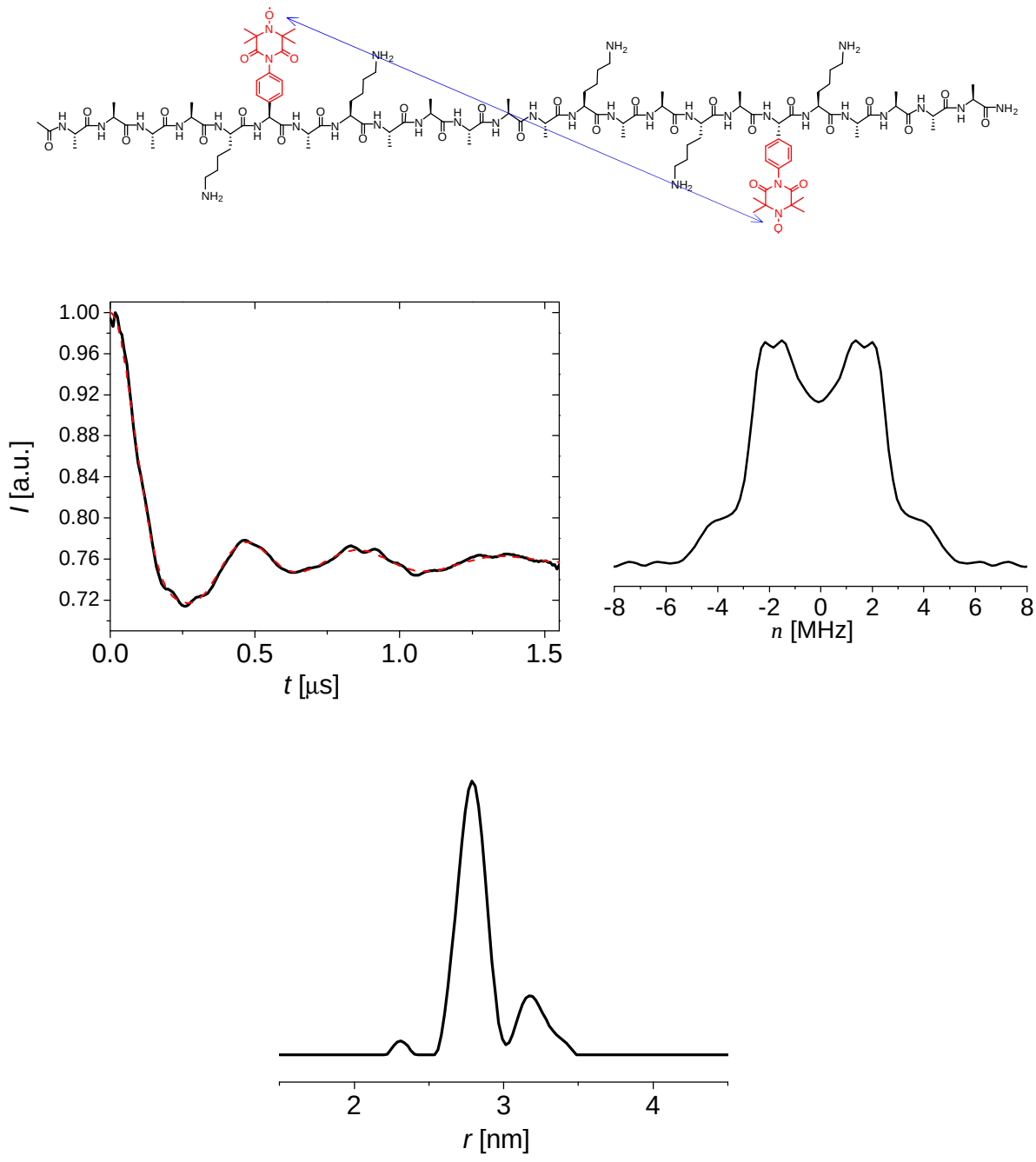
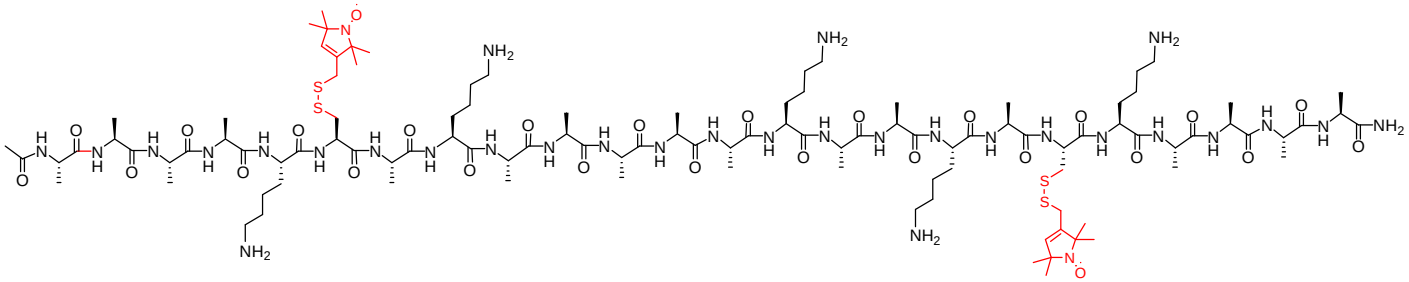


Figure S8. Time-domain DEER signal of double-labeled **P1** in TFE/EtOH/MeOH (40:40:20). The black line is the background-subtracted experimental data and the red line is the time-domain simulation of the data performed by DeerAnalysis2009. Dipolar spectrum obtained from the Fourier transform of the DEER signal; best fit of the distance distribution obtained from Tikhonov regularization. The DEER experiment was carried out on a Bruker ELEXYSIS 580 pulsed EPR spectrometer at 40 K; $\pi/2 - \pi = 8 - 16$ ns; $\pi_{ELDOR} = 16$ ns; SPP = 50; SRT = 5 ms; scans = 264; acquisition time: 14 h.

S11. Structure for the peptide **P3**



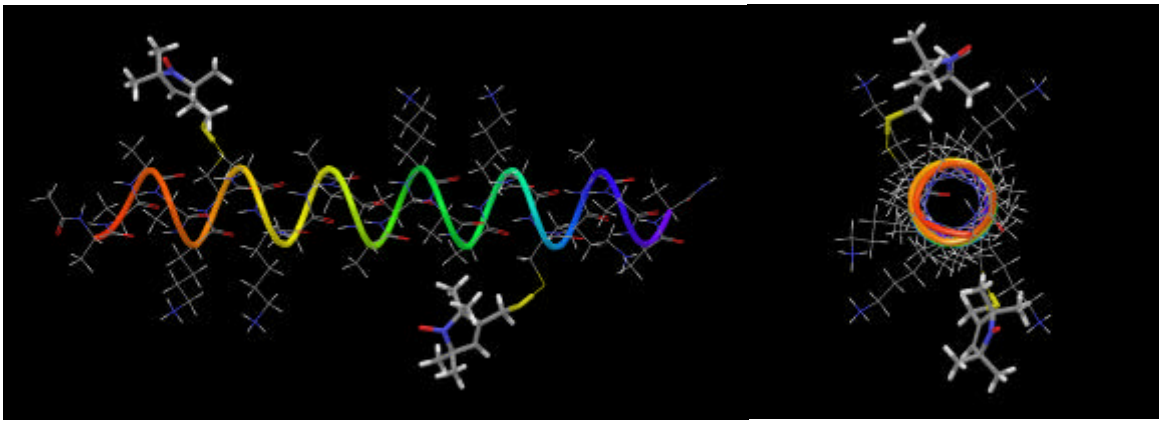


Figure S9. Distance between the spin labels in the MTSSL-modified peptide is highly dependent on the conformation of the spin labels. Here, the distance is 2.21 nm, which fits well with the measured 2.26 nm.

S12. Literature

[1] C. E. Ramey, J. J. Luzzi (Ciba-Geigy AG), US-3920659, **1975**.

[2] Gaussian 09, Revision A.02, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, and D. J. Fox, Gaussian, Inc., Wallingford CT, **2009**.