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

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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\(_4\)Cl (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\(_2\)Cl\(_2\) (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\(_3\)): \(\delta = 1.72\) (s, 1 H, NH), 1.62 (s, 12 H, CH\(_3\)); \(^1^3\)C-NMR (126 MHz, CDCl\(_3\)): \(\delta = 123.2\) (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\(_2\)SO\(_4\) (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\(_2\)SO\(_4\) 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): \(\delta = 10.60\) (s, 1 H, CONH), 2.72 (s, 1H, NH), 1.27 (s, 12H, CH\(_3\)); \(^1^3\)C-NMR (126 MHz, [D\(_6\)]DMSO): \(\delta = 177.8\) (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-\(\text{L}-\text{Hpg-OH}\) 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\(_2\)CO\(_3\) (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-\(\text{L}-\text{Hpg-OH}\) (4.90 g, 91 %, 16.3 mmol). \(^1\)H-NMR (300 MHz, [D\(_6\)]DMSO): \(\delta = 12.67\) (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, CH₂), 5.05 (d, J = 7.9 Hz, 1 H, α-CH); ¹³C-NMR (126 MHz, [D₆]DMSO): δ = 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 (CH₂), 57.5 (α-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 C₁₆H₁₅NO₅Na [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 NaHCO₃ (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 MgSO₄, 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). ¹H-NMR (300 MHz, [D₆]DMSO): δ = 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, α-CH), 5.12 (s, 2 H, CH₂), 5.06 (s, 2 H, CH₂); ¹³C-NMR (126 MHz, [D₆]DMSO): δ = 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 CH₂), 65.5 (Cbz CH₂), 57.6 (α-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 C₂₃H₂₁NO₅Na [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 CH₂Cl₂ (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 NaHCO₃ (50 mL) and the resulting solution extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were washed with brine (3 × 20 mL), dried over MgSO₄ and concentrated in vacuo. Pyridine was removed by codistillation 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). ¹H-NMR (300 MHz, [D₆]DMSO): δ = 7.44-7.06 (m, 14 H, phenyl CH), 5.95 (d, J = 7.0 Hz, 1 H, NH),
Bn$_2$-L-Hpg(Tf)-OBn (8) A solution of Cbz-L-Hpg(Tf)-OBn (2.00 g, 3.82 mmol) and (CH$_3$)$_2$S (8.40 mL, 113 mmol) in TFA (34 mL) was stirred at 25 °C for 17 h. Trifluoroacetic acid was subsequently removed by codistillation 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$_3$): $\delta$ = 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, $\alpha$-CH), 3.76 (d, $J$ = 13.9 Hz, 2 H$_a$, N(CH$_2$H$_b$-phenyl)$_2$), 3.67 (d, $J$ = 13.9 Hz, 2 H$_b$, N(CH$_2$H$_b$-phenyl)$_2$); $^{13}$C-NMR (126 MHz, CDCl$_3$): $\delta$ = 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 ($\alpha$-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$_{24}$H$_{19}$F$_3$NO$_7$S [M-H]: 522.0840, found 522.0840.

Bn$_2$-4-pinacolboronyl-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$_2$(dpff) (247 mg, 338 µmol), and dpff (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 % EtOAc in pentane to yield 9 (1.74 g, 90 %, 3.18 mmol). $^1$H-NMR (300 MHz, CDCl$_3$): $\delta$ = 7.76 (m, 3.18 mmol).
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, \( \alpha\)-CH), 3.73 (s, 4 H, CH\(_2\)), 1.34 (s, 12 H, CH\(_3\)); \(^{13}\)C-NMR (126 MHz, CDCl\(_3\)): \( \delta = 171.7 \) (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 (\( \alpha\)-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\(_4\)OAc (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\(_2\)O (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\(_3\)): \( \delta = 8.19 \) (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, \( \alpha\)-CH), 3.79 (s, 4 H, CH\(_2\)); \(^{13}\)C-NMR (126 MHz, CDCl\(_3\)): \( \delta = 171.5 \) (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 (\( \alpha\)-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\(_3\)N (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\(_3\)): \( \delta = 7.51-7.19 \) (m, 17 H, phenyl CH), 7.06 (m, 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, \( \alpha\)-CH), 3.82 (d, \( J = 14.0 \) Hz, 2 H, N(CH\(_3\)H\(_2\)-phenyl)\(_2\)), 3.72 (d, \( J = 14.0 \) Hz, 2 H, N(CH\(_3\)H\(_2\)-phenyl)\(_2\)), 1.69
(s, 1 H, NH), 1.53 (s, 12 H, CH₃); \(^{13}\)C-NMR (126 MHz, CDCl₃): \(\delta = 176.4 \) (CONR₂), 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 (CH₂), 65.4 (α-C), 56.0 (C(CH₃)₂), 54.2 (CH₂), 28.5 (CH₃); 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 C₃₇H₄₀N₃O₄ [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 Bn₂-4-(3,3,5,5-tetramethyl-2,6-dioxopiperazin-1-yl)-L-Phg-OBn (500 mg, 849 µmol) and 20 % Pd(OH)₂/C (50 % H₂, 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 NaHCO₃ (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 MgSO₄ and concentrated in vacuo. The residue was purified by flash chromatography using CH₂Cl₂/MeOH/AcOH (98:2:0.1 ? 97:3:0.1) to provide 11 (423 mg, 92 %, 781 µmol). \(^1\)H-NMR (600 MHz, [D₆]DMSO): \(\delta = 8.25 \) (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, α-CH), 4.29 (d, \(J = 7.3 \) Hz, 2 H, Fmoc CH₂), 4.23 (t, \(J = 7.3 \) Hz, 1 H, Fmoc CH), 1.56 (s, 1 H, NH), 1.40 (s, 12 H, CH₃); \(^{13}\)C-NMR (126 MHz, [D₆]DMSO): \(\delta = 176.5 \) (CONR₂), 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 CH₂), 57.7 (α-C), 55.3 (C(CH₃)₂), 46.6 (Fmoc CH), 28.0 (CH₃); ESI-MS: \(m/z \) (%) = 564.2 (100) [M+Na]⁺, 540.2 (100) [M-H]; HR-ESI-MS: \(m/z \) calculated for C₃₁H₃₀N₃O₆ [M-H]⁻: 540.2140, found 540.2140.

Fmoc-4-(3,3,5,5-tetramethyl-2,6-dioxo-4-oxylpiperazin-1-yl)-L-Phg-OH (I) 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 CH₂Cl₂ (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$_2$Cl$_2$/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$_3$O$_7$ [M-H]$^-$: 555.2011; found 555.2013.
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 NaHCO₃ 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 Ac₂O/DIEA/DMF (1:1:18). Cleavage from the resin was carried out using TFA/H₂O/TIS (90:5:5, v/v/v) (for **P1** and **P2**) or TFA/H₂O/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-AKAAAAKAAKA-TOPP-KAAAA-NH₂ (C₁₁₅H₁₈₆N₃₄O₃₁, 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, tᵣ = 14.9 min; HR-ESI-MS: m/z calculated for C₁₁₅H₁₈₉N₃₄O₃₁ [M+3H]³⁺: 847.4747, found 847.4749.
(P2) Ac-AAAAKYAKAAAAAAKAAKAYKAAAA-NH₂ (C₁₀₁H₁₆₈N₃₀O₂₇, 2234.6)
RP-HPLC-column: Phenomenex Jupiter (C18, 300 Å, 5 µm, 250 x 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, tᵣ = 25.3 min; HR-ESI-MS: m/z calculated for C₁₀₁H₁₇₁N₃₀O₂₇ [M+3H]³⁺:
745.4304, found 745.4308.

(P3) Ac-AAAAK MTSSL AKAAAAAKAAA MTSSL KAAAA-NH₂ (C₁₀₇H₁₈₈N₃₂O₂₇S₄,
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, tᵣ= 19.62 min; HR-ESI-MS: m/z calculated for C₁₀₇H₁₉₁N₃₂O₂₇S₄

(P4) Ac-AAAAKCAKAAAAAAKAAKACKAAAA-NH₂ (C₈₉H₁₆₅N₃₀O₂₅S₂, 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, tᵣ= 16.69 min; HR-ESI-MS: m/z calculated for C₈₉H₁₆₃N₃₀O₂₅S₂ [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.\cite{2}

![Figure S1](image)

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

Figure S2 shows the conformers B\( \alpha \) and B\( \beta \) of the compound 12, which were calculated using the B3LYP method.

![Figure S2](image)
Figure S2: Geometry optimizations and frequency calculations of the conformations $B\alpha$ (a, b, c) and $B\beta$ (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 $B\alpha$ exists in the ground state and the conformation $B\beta$ with $C_{2v}$ symmetry is energetically 1.4 kJ/mol above the ground state (Table S1). The angle $\lambda$, 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).

<table>
<thead>
<tr>
<th>Conformation of 12</th>
<th>$\Delta E$ / kJ/mol</th>
<th>Angle $\lambda$ / °</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B\alpha$</td>
<td>0.0°</td>
<td>6.5</td>
</tr>
<tr>
<td>$B\beta$</td>
<td>1.4</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table S1: The energy difference and the angle $\lambda$ of the conformers $B\alpha$ and $B\beta$ of the compound 12. *Zero-point energy = -878.795016 a. u.

To determine the geometry of TOPP in an $\alpha$-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 ($\varphi = -52^\circ$, $\psi = -53^\circ$). 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 $\lambda$ are shown in the Table S2.

<table>
<thead>
<tr>
<th>Conformation of 13</th>
<th>$\Delta E$ / kJ/mol</th>
<th>Angle $\lambda$ / °</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B\alpha$</td>
<td>0.0°</td>
<td>8.1</td>
</tr>
<tr>
<td>$B\beta$</td>
<td>4.8</td>
<td>3.2</td>
</tr>
<tr>
<td>$B\gamma$</td>
<td>4.9</td>
<td>3.2</td>
</tr>
</tbody>
</table>
Table S2: The energy difference and angle $\lambda$ of the conformers $B\alpha$, $B\beta$ und $B\gamma$ 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:

<table>
<thead>
<tr>
<th>Compound &amp; Conformation</th>
<th>$\Delta$E / kJ/mol</th>
<th>Angle $\lambda$/ °</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 $P\alpha$</td>
<td>0.0$^a$</td>
<td>7.7</td>
</tr>
<tr>
<td>12 $P\beta$</td>
<td>1.5</td>
<td>0.0</td>
</tr>
<tr>
<td>13 $P\alpha$</td>
<td>0.00$^b$</td>
<td>3.4</td>
</tr>
<tr>
<td>13 $P\beta$</td>
<td>0.30</td>
<td>10.6</td>
</tr>
<tr>
<td>13 $P\gamma$</td>
<td>0.34</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table S3: The energy difference and angle $\lambda$ of the conformers 12 $P\alpha$, 12 $P\beta$ und 13 $P\alpha$, 13 $P\beta$, 13 $P\gamma$. 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.
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.
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.
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)

\[ p = \text{pump} \]
\[ o = \text{observe} \]

EtOH/MeOH/H₂O

EtOH/MeOH/TFE

EtOH/MeOH/H₂O
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; π/2 – π = 16 – 32 ns; π$_{\text{ELDOR}}$ = 36 ns; SPP = 50; SRT = 5 ms; acquisition time: 12 h. The Pake pattern distortion is produced by the two different DEER set-up.
Figure S7. X-Band time-domain DEER signal of double-labeled P1: sum of 11 field values ($B_{\text{obs}} = 3453.5 - 3466$ G, with 2.5 G step). $T = 50$ K; $\pi/2 - \pi = 16 - 32$ ns; $\pi_{\text{ELDOR}} = 36$ 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; $\tau_{\text{ELDOR}} = 16$ 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