Electronic Supplementary Information

Arginine Mimetic Appended Peptide-Based Probes for Fluorescence Turn-On Detection of 14-3-3 Proteins

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1. Materials and devices:

Solvents were dried and distilled before use. Millipore water was obtained with a Micropure apparatus from TKA. Lyophilization was carried out with an Alpha 1-4 2D plus freeze drying apparatus from Christ. Analytical TLC was carried out on SiO_2 aluminium foils ALUGRAM SIL G/UV_{254} from Macherey-Nagel. Reversed phase column chromatography was done with an Armen Instrument Spot Flash Liquid Chromatography MPLC apparatus with RediSep C-18 Reversed-Phase columns. The purity of the compounds was determined with the help of an HPLC apparatus from Dionex containing the following components: P680 HPLC pump, ASI-100 Automated Sample Injector and UVD 340U detector. A SupelcosilTM LC-18 column (25 cm × 4.6 mm, 5 µm) from Supelco or a YMC ODS-A column (15 cm × 3.0 mm, 5 µm) was utilized. Ultrapure water and HPLC-grade solvents were used as eluents. Detection was achieved with the help of a UV detector. ^{1}H- and ^{13}C-NMR spectra were recorded on DRX 500 MHz spectrometer from Bruker at ambient temperature. The chemical shifts are reported in parts per million (ppm) relative to the deuterated solvent DMSO-d_6. All mass spectra were received by using a Bruker BioTOF III. Determination of pH values was carried out with a pH-Meter 766 Calimatic from Knick. Fluorescence spectra were obtained with a Varian Cary Eclipse spectrometer.

2. General procedure for the synthesis of the peptide:

Fmoc Removal: The Fmoc protecting group was cleavage by treatment with 20% piperidine in DMF (2 × 6 mL, 5 min each) under microwave radio condition (20 W, 50± ºC, 5 min). Then, the resin was washed 3 × 8 mL with DMF, 3 × 8 mL with DCM, 3 × 8 mL with DMF (ca. 5 min each) to remove the last traces of piperidine. A positive Kaiser test confirmed the cleavage of the Fmoc group and the presence of free amino function.

Alloc deprotection: The Alloc protecting group was removed with $Pd(PPh_3)_4$ (0.1 eq) in the presence of $PhSiH_3$ (24 eq) in DCM for 20 min followed by washing with DCM (3 × 5 mL) and DMF (3 × 5 mL). A positive Kaiser test confirmed the cleavage of the Alloc group and the presence of free amino function.

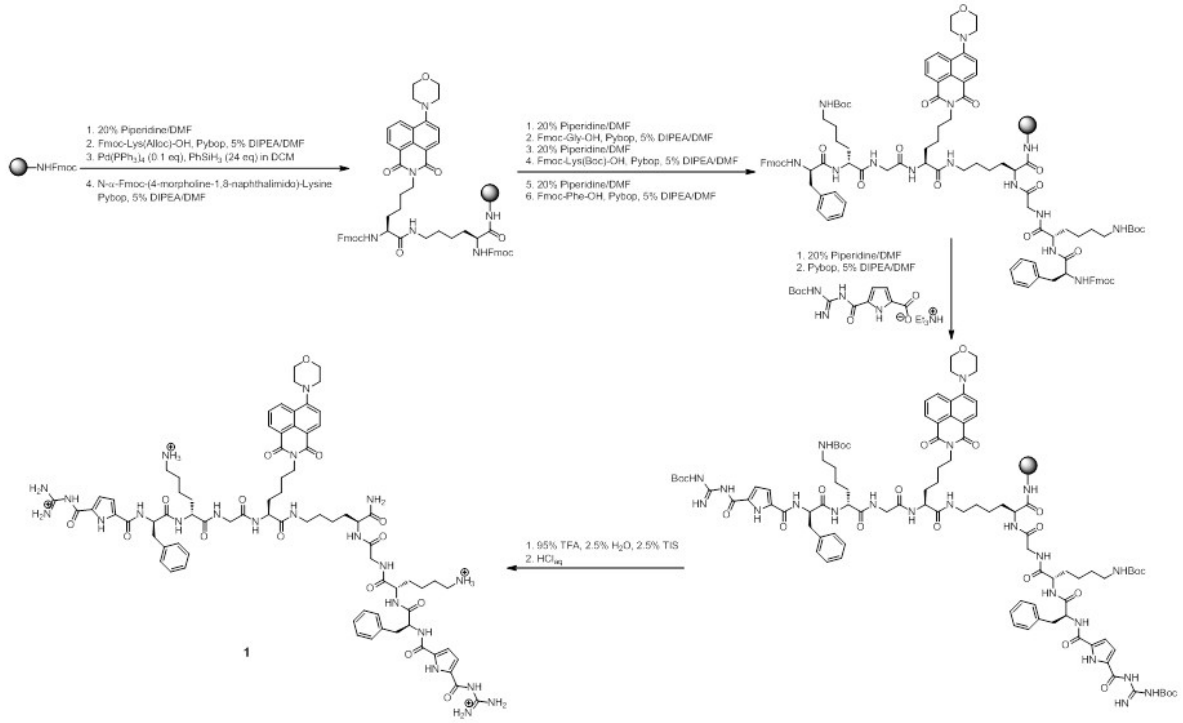
Standard Fmoc solid phase peptide synthesis techniques (SPPS): Each amino acid was attached using 0.6 mmol/g loading Fmoc Rink amide resin under microwave radio (20 W, 60±5 °C, 20 min) Then, the resin was washed 3 × 8 mL with DMF, 3 × 8 mL with DCM, 3 × 8 mL with DMF (ca. 5 min each) to remove the last traces of the amino acid. A negative Kaiser test confirmed the attachment of the corresponding amino acid.

Cleavage from the Resin: Cleavage of the product from the resin was achieved by treatment with a mixture of TFA/H_2O/triisopropylsilane (95:2.5:2.5) for 3 h. The yellow cleavage mixture was collected by filtration and the resin was washed twice with pure TFA (6 mL). The filtrates were combined and concentrated under vacuum to obtain an oily residue. The peptide was precipitated by adding dry diethyl ether to the oil, following by centrifugation of the mixture. The precipitate was dissolved in water (25 mL), and the mixture was freezedried in vacuum. The resulting solid was purified by MPLC on C18 reversed phase silica gel (MeOH/water, 0.1% TFA). Then the product was dissolved three times in HCl_{aq} (0.1 N) plus one time in water and consequently freezedried to obtain white solid.

Purity of the peptides was checked by HPLC on a RP18-column using water/MeOH (with 0.05% TFA) as solvent.

3. Synthesis of peptides and their characterization:

Peptide 1: Rink amide resin (200 mg, 0.8 mmol/g, 1 equiv.) was weighed out into plastic peptide synthesis vessel and allowed to swell in DCM/DMF (5.0/5.0 mL) for 2 h. The Fmoc protection group was removed by treatment with piperidine. After an intensive washing cycle with DMF, Fmoc-Lys(Alloc)-OH was attached as a spacer to the solid support. The coupling step was repeated to assure complete conversion of all accessible amino groups on the resin. After the removal of Alloc protecting group, the N-α-Fmoc-(4-morpholine-1,8-naphthalimido)-Lysine[1] was coupled. After deprotection of the Fmoc group with piperidine/DMF, the next two amino acids, lysine and phenylalanine, as well as the tert-butoxycarbonyl (Boc)-protected guanidiniocarbonyl pyrrole (GCP)[1–3] motif were coupled

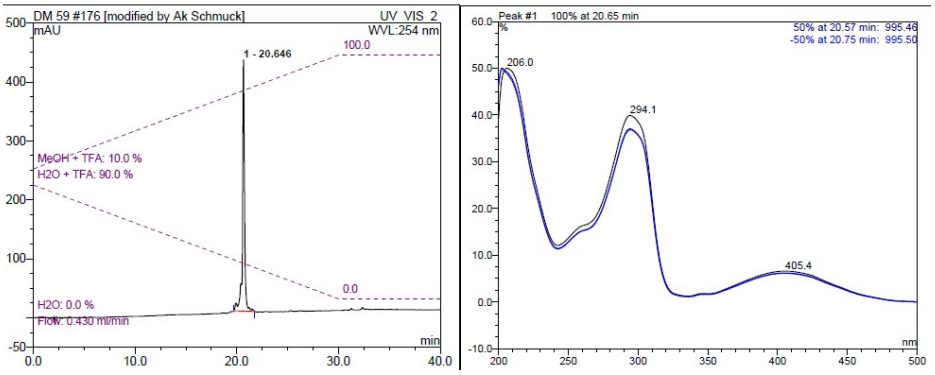


Scheme S1. Microwave assisted SPPS of the peptidic probe **1**. Coupling steps were carried out by irradiating the resin for 20 min at 20 W with a maximum temperature of 60°C. Fmoc-removal was achieved by irradiating the solid support for 1 min and 5 min (20 W, max. 60°C).

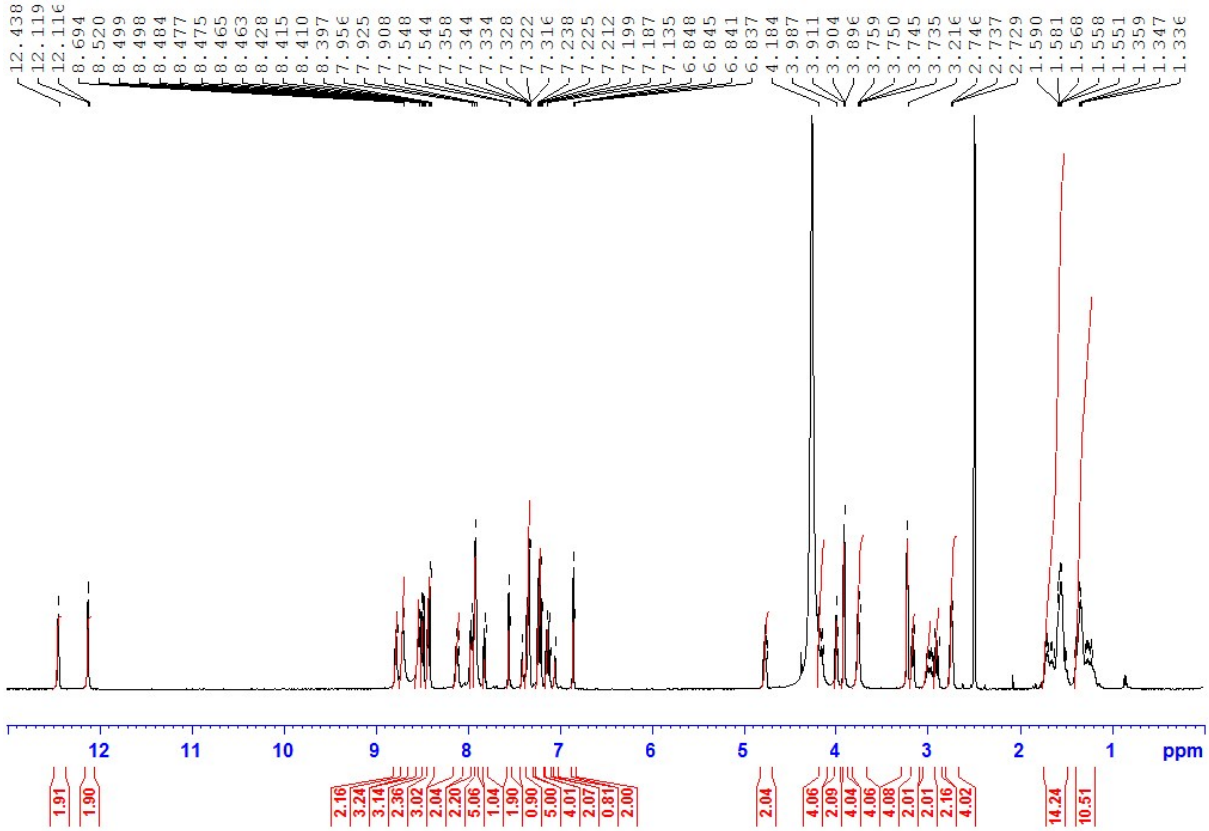
similarly using six equivalents of each reactant. After the resin was thoroughly washed and dried, the receptor was cleaved from the solid support; the Boc-protected side chains were deprotected at the same time without microwave irradiation by utilizing a cleavage mixture composed of trifluoroacetic acid (TFA)/water/triisopropylsilane (TIS) (95:2.5:2.5). After purification by means of precipitation and reversed-phase medium-pressure liquid chromatography (MPLC) with 0.1% TFA in H_2O/MeOH and consequent anion exchange, probe **1** was obtained as hydrochloric salt in 30% yield and more than 95% purity.

Peptide 1 (72.0 mg, 46 µmol, Yield 28%, purity HPLC 99%). ^{1}H NMR (500 MHz, DMSO-d_6): δ[PPM] 1.21-1.40 (m, 10H), 1.49-1.71 (m, 14H), 2.73 (dd, *J* = 4.4 Hz, 4.0 Hz, 4H), 2.87-2.91 (m, 2H), 2.93-3.02 (m, 2H), 3.14 (t, *J* = 3.3 Hz, 1H), 3.16 (t, *J* = 3.5 Hz, 1H), 3.21 (m, 4H), 3.73-3.76 (dd, *J* = 4.6 Hz, 2.7 Hz, 4H), 3.90 (t, *J* = 3.8 Hz, 4H), 3.98 (t, J = 6 Hz, 2H), 4.12-4.18 (m, 4H), 4.74-4.78 (m, 2H), 6.83-6.85 (m, 2H), 7.04 (br, 1H), 7.09-7.14 (m, 2H), 7.18-7.23 (m, 4H), 7.31-7.35 (m, 5H), 7.39 (br, 1H), 7.53-7.55 (dd, J = 3.1 Hz, 1.9 Hz, 2H), 7.79-7.82 (t, J = 6.2 Hz, 1H), 7.90-7.96 (m, 7H), 8.08-8.12 (m, 2H), 8.39-8.42 (m, 3H), 8.46-8.51 (m, 5H), 8.69 (br, 3H), 8.75-8.78 (t, J = 6.3 Hz, 2H), 12.11 (br, 2H), 12.43 (br, 2H). ^{13}C NMR (125.8 MHz, DMSO-d_6): δ[PPM] 22.17, 22.74, 22.98, 26.49, 27.42, 28.72, 31.22, 31.63, 32.08, 37.26, 38.08, 38.43, 38.51, 40.05, 41.99, 52.17, 52.37, 52.62, 53.05, 54.26, 66.20, 113.39, 114.43, 115.11, 118.82, 115.98, 122.61, 125.39, 125.63, 126.16, 126.23, 128.04, 128.07, 128.35, 129.13, 129.15, 130.58, 130.72, 132.20, 132.23, 138.03, 155.47, 155.55, 158.83, 159.70, 163.01, 163.53, 168.46, 168.50, 170.01, 171.13, 171.36, 171.72, 173.63. MALDI-TOF MS: cald 1559.7655 for $C_{76}H_{99}N_{22}O_{15}^{+}$, found 1559.4594.

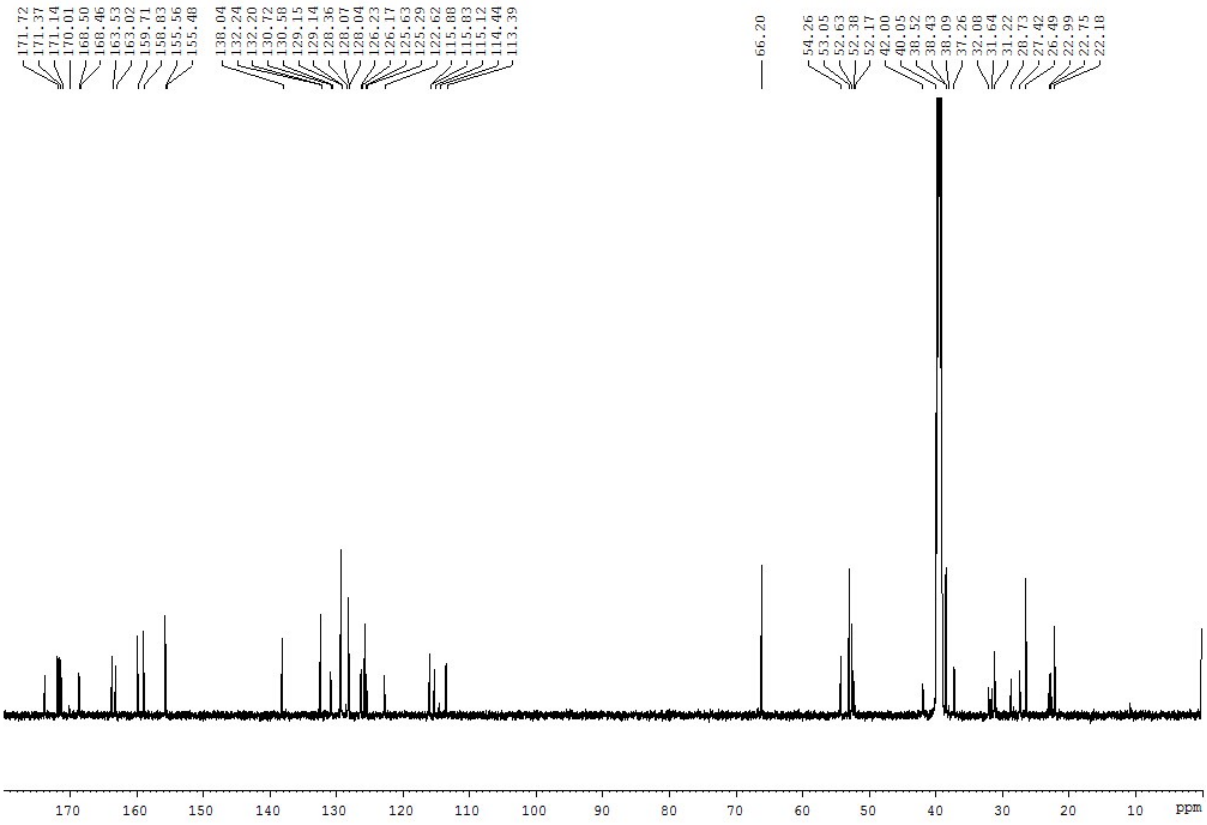
RP-HPLC chromatogram of Peptide 1:



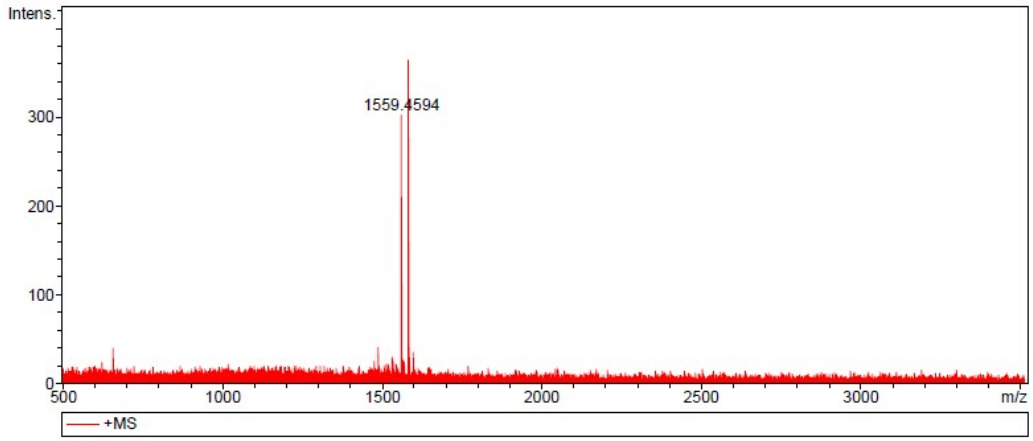
500 MHz ^{1}H NMR Bruker AVANCE 500 (DRX)



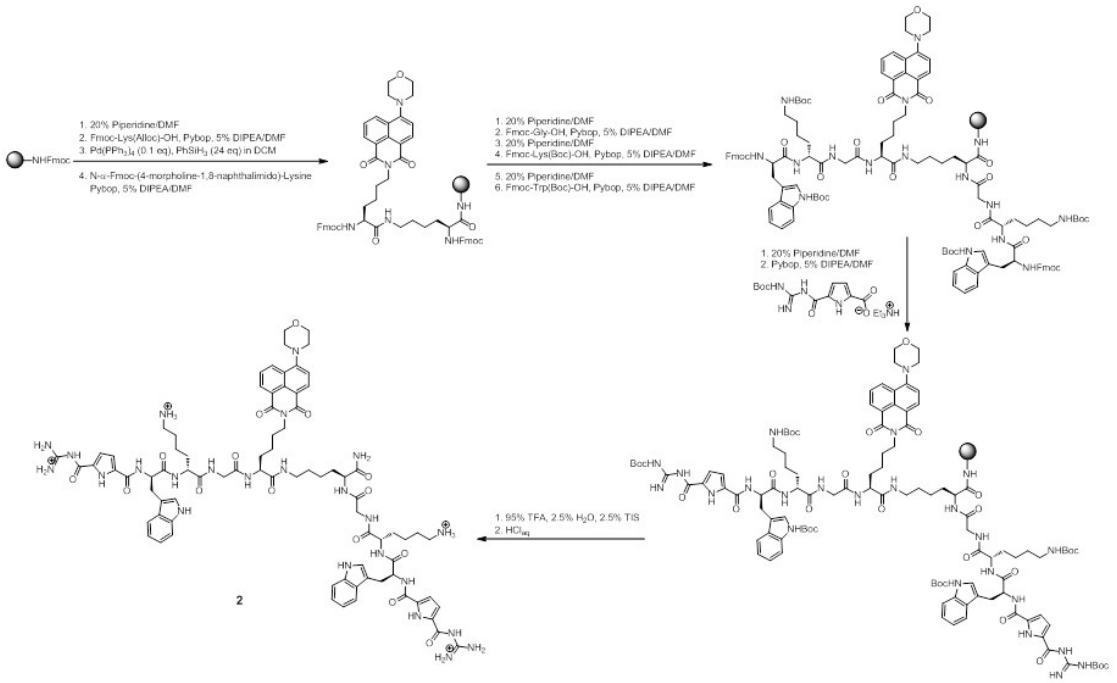
125.8 MHz ^{13}C NMR Bruker AVANCE 500 (DRX)



MALDI-TOF MS spectra of Peptide 1:



Peptide 2: Rink amide resin (200 mg, 0.8 mmol/g, 1 equiv.) was weighed out into plastic peptide synthesis vessel and allowed to swell in DCM/DMF (5.0/5.0 mL) for 2 h. The Fmoc protection group was removed by treatment with piperidine. After an intensive washing cycle with DMF, Fmoc-Lys(Alloc)-OH was attached as a spacer to the solid support. The coupling step was repeated to assure complete conversion of all accessible amino groups on the resin.

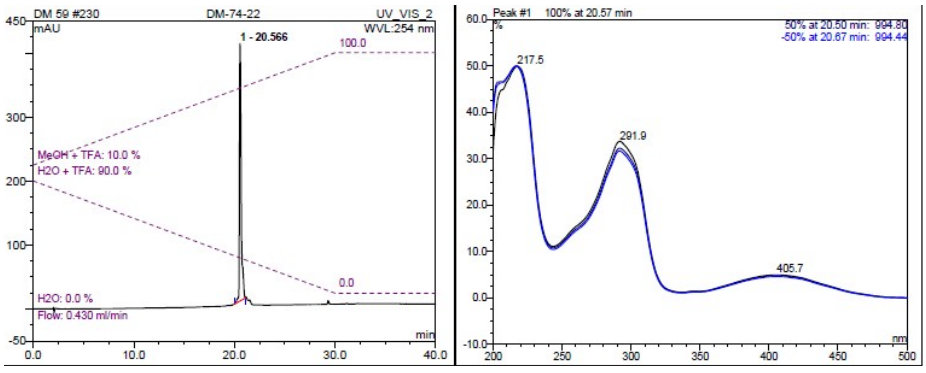


Scheme S2. Microwave assisted SPPS of the peptidic probe **2**. Coupling steps were carried out by irradiating the resin for 20 min at 20 W with a maximum temperature of 60°C. Fmoc-removal was achieved by irradiating the solid support for 1 min and 5 min (20 W, max. 60°C).

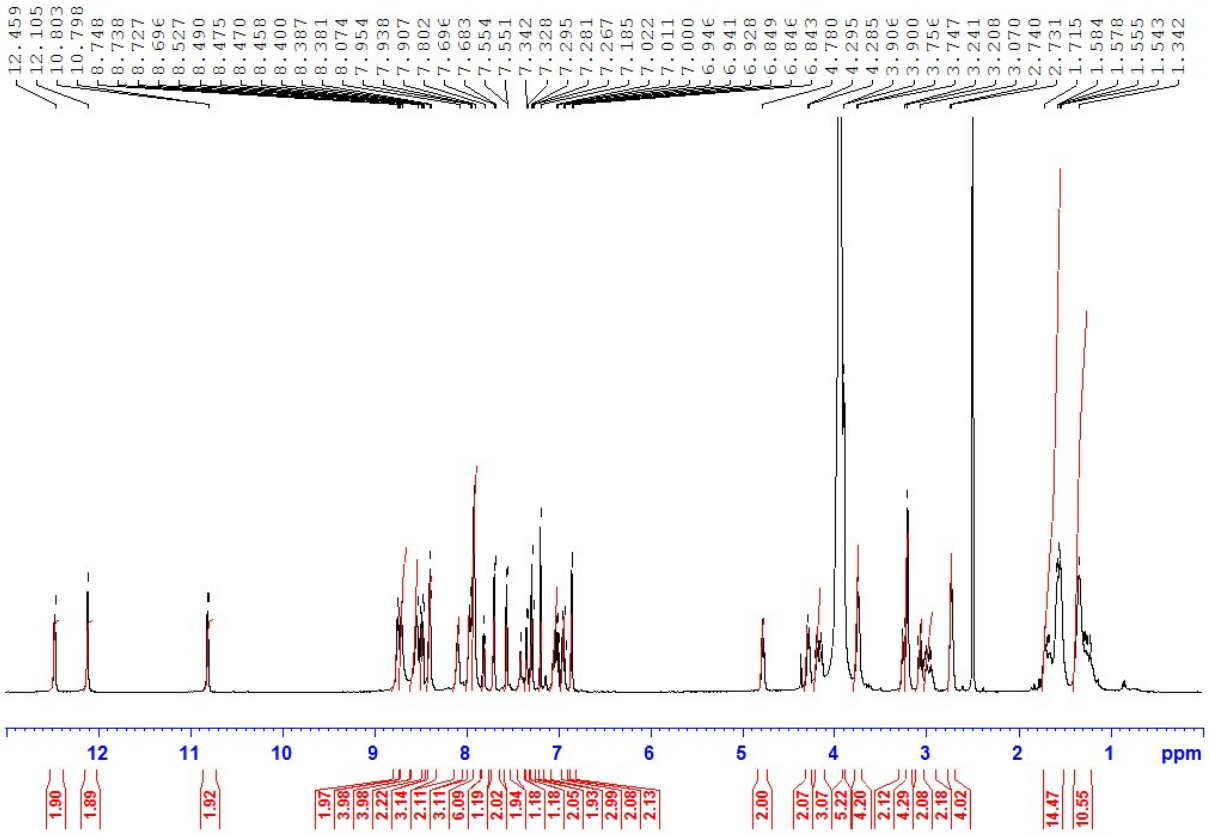
After the removal of Alloc protecting group, the N-α-Fmoc-(4-morpholine-1,8-naphthalimido)-Lysine[4] was coupled. After deprotection of the Fmoc group with piperidine/DMF, the next two amino acids, lysine and tert-butoxycarbonyl (Boc)-protected tryptophan, as well as the Boc-protected guanidiniocarbonyl pyrrole (GCP)[2] motif were coupled similarly using six equivalents of each reactant. After the resin was thoroughly washed and dried, the receptor was cleaved from the solid support; the Boc-protected side chains were deprotected at the same time without microwave irradiation by utilizing a cleavage mixture composed of trifluoroacetic acid (TFA)/water/triisopropylsilane (TIS) (95:2.5:2.5). After purification by means of precipitation and reversed-phase medium-pressure liquid chromatography (MPLC) with 0.1% TFA in H_2O/MeOH and consequent anion exchange, probe **2** was obtained as hydrochloric salt in 30% yield and more than 95% purity.

Peptide 2 (75.0 mg, 45.7 µmol, Yield 28%, purity HPLC 99%).^{1}H NMR (500 MHz, DMSO-d_6): δ[PPM] 1.21-1.34 (m, 10H), 1.54-1.71 (m, 14H), 2.73 (m, 4H), 2.94-3.02 (m, 2H), 3.04-3.08 (t, *J* = 9.1 Hz, 2H), 3.20-3.26 (m, 6H), 3.74 (m, 4H), 3.90 (m, 5H), 4.12-4.20 (m, 3H), 4.26-4.29 (m, 2H), 4.75-4.79 (m, 2H), 6.84 (m, 2H), 6.91-6.95 (m, 2H), 6.98-7.04 (m, 3H), 7.18 (br, 2H), 7.28 (t, *J* = 6 Hz, 2H), 7.32 (d, *J* = 6.8 Hz, 1H), 7.40 (m, 1H), 7.55 (m, 2H), 7.68 (d, J = 6.5 Hz, 2H), 7.80 (t, *J* = 6.3 Hz, 1H), 7.90-7.95 (m, 9H), 8.07-8.09 (m, 2H), 8.38-8.40 (m, 3H), 8.45-8.48 (dd, *J* = 6.1 Hz, 2.5 Hz, 2H), 8.52 (br, 4H), 8.69 (br, 4H), 8.73 (t, J = 4.7 Hz, 2H), 10.79 (d, *J* = 2.6 Hz, 2H), 12.10 (br, 2H), 12.45 (br, 2H). ^{13}C NMR (125.8 MHz, DMSO-d_6): δ[PPM] 22.20, 22.77, 23.00, 26.51, 27.42, 27.57, 28.72, 31.15, 31.62, 32.07, 38.45, 38.55, 40.05, 41.96, 42.03, 52.42, 52.66, 53.05, 53.70, 66.21, 66.76, 110.15, 111.30, 113.42, 114.44, 115.11, 115.83, 115.89, 118.20, 118.61, 120.86, 122.62, 123.74, 125.29, 125.61, 126.16, 127.21, 128.37, 129.13, 130.58, 130.73, 132.24, 132.34, 136.02, 155.47, 155.57, 158.90, 159.71, 163.03, 163.54, 168.51, 168.56, 170.04, 171.19, 171.76, 171.79, 173.70. MALDI-TOF MS: cald 1637.7873 for $C_{80}H_{101}N_{24}O_{15}^+$, found 1638.4191.

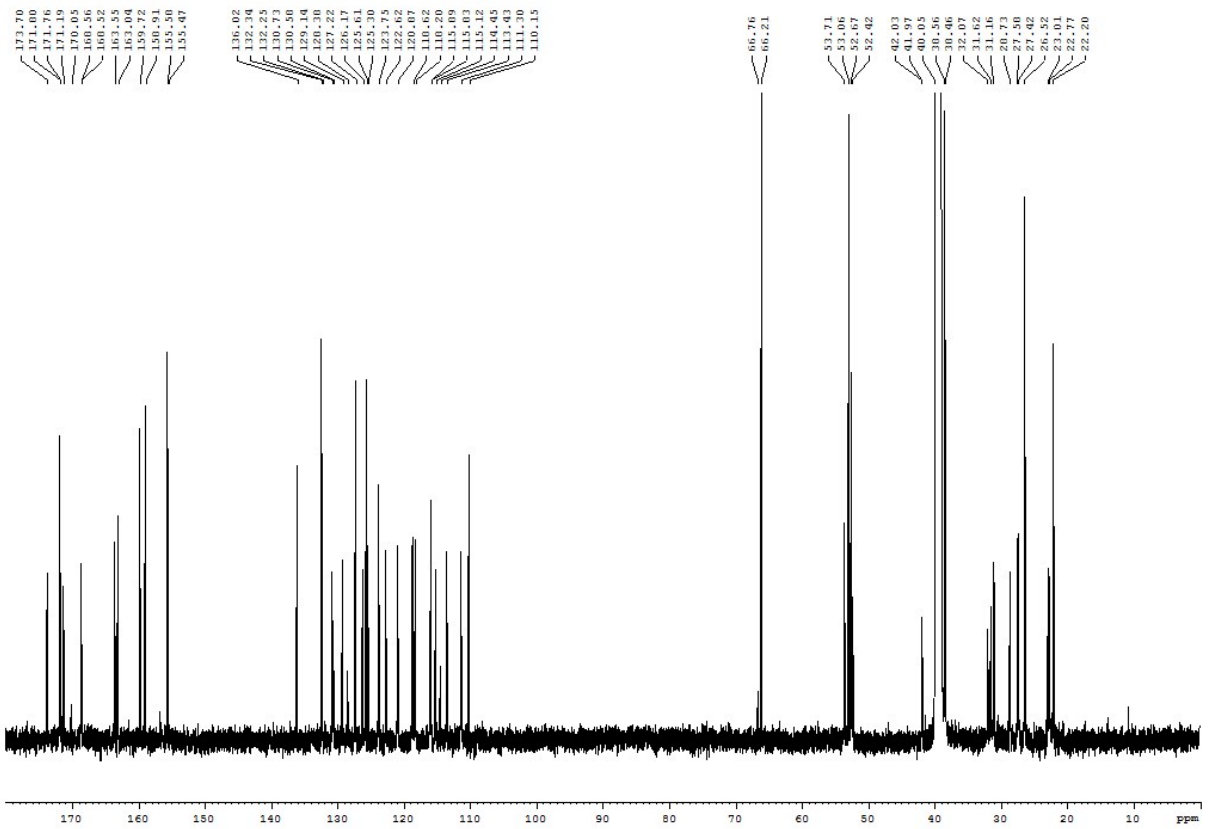
RP-HPLC chromatogram of Peptide 2:



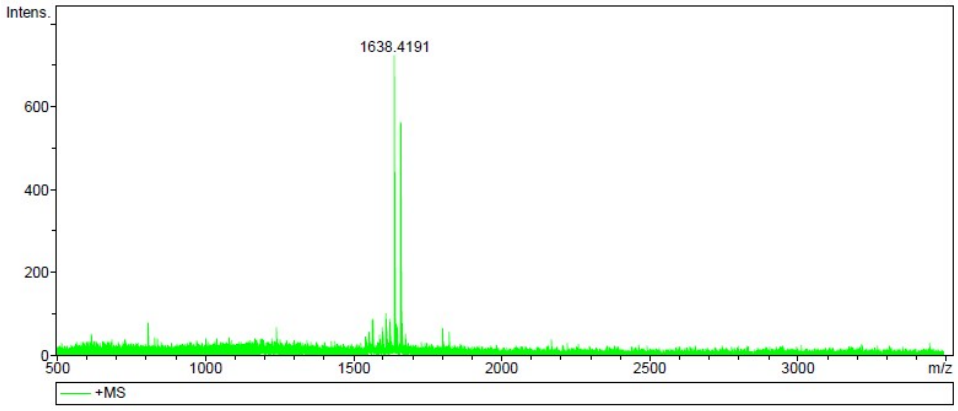
500 MHz ^{1}H NMR Bruker AVANCE 500 (DRX)



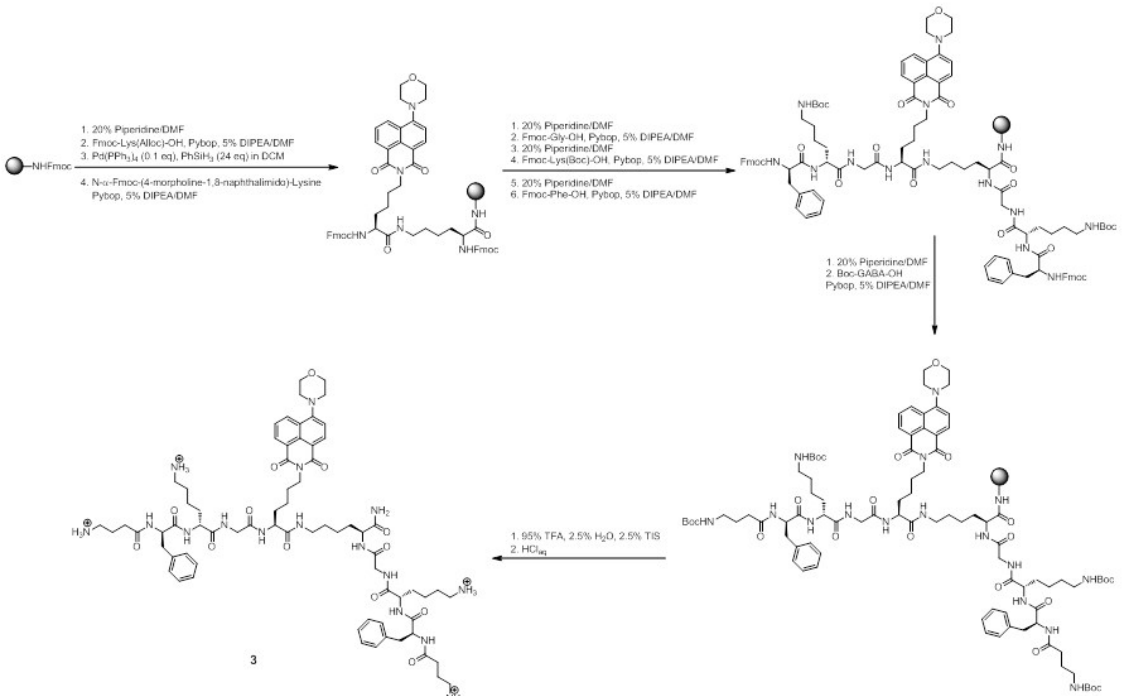
125.8 MHz ^{13}C NMR Bruker AVANCE 500 (DRX)



MALDI-TOF MS spectra of Peptide 2:



Peptide 3: Rink amide resin (200 mg, 0.8 mmol/g, 1 equiv.) was weighed out into plastic peptide synthesis vessel and allowed to swell in DCM/DMF (5.0/5.0 mL) for 2h. The Fmoc protection group was removed by treatment with piperidine. After an intensive washing cycle with DMF, Fmoc-Lys(Alloc)-OH was attached as a spacer to the solid support. The coupling step was repeated to assure complete conversion of all accessible amino groups on the resin. After the removal of Alloc protecting group, the N-α-Fmoc-(4-morpholine-1,8-naphthalimido)-Lysine[1] was coupled. After deprotection of the Fmoc group with

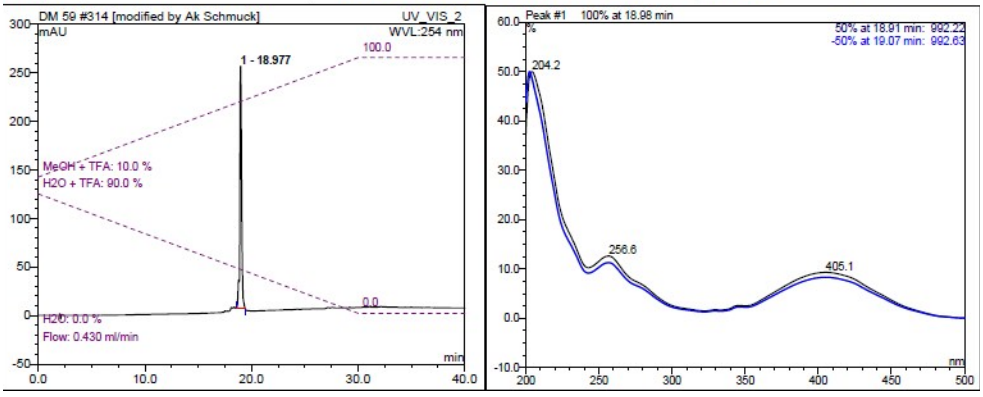


Scheme S3. Microwave assisted SPPS of the peptidic probe **3**. Coupling steps were carried out by irradiating the resin for 20 min at 20 W with a maximum temperature of 60°C. Fmoc-removal was achieved by irradiating the solid support for 1 min and 5 min (20 W, max. 60°C).

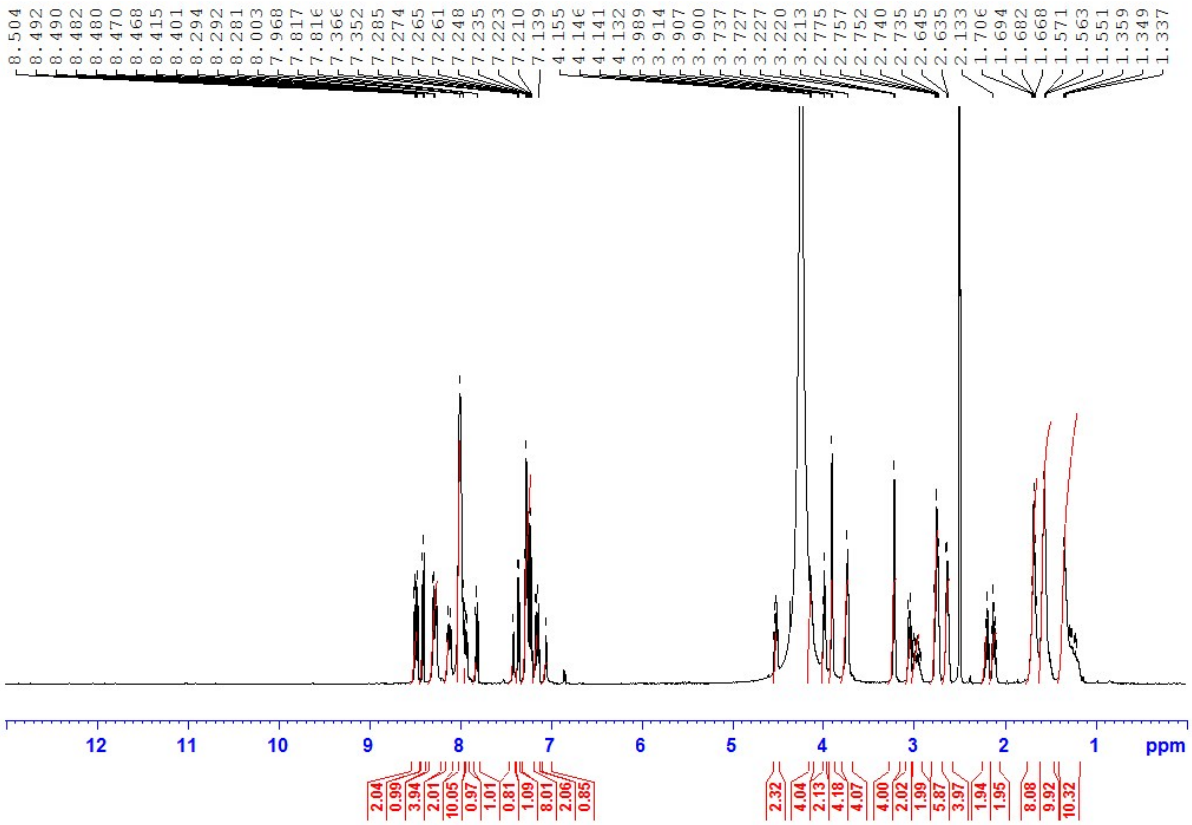
piperidine/DMF, the next two amino acids, lysine and phenylalanine, as well as the γ-(Boc-amino)butyric acid motif were coupled similarly using six equivalents of each reactant. After the resin was thoroughly washed and dried, the receptor was cleaved from the solid support; the Boc-protected side chains were deprotected at the same time without microwave irradiation by utilizing a cleavage mixture composed of trifluoroacetic acid (TFA)/water/triisopropylsilane (TIS) (95:2.5:2.5). After purification by means of precipitation and reversed-phase medium-pressure liquid chromatography (MPLC) with 0.1% TFA in H_2O/MeOH and consequent anion exchange, probe **3** was obtained as hydrochloric salt in 30% yield and more than 95% purity.

Peptide 3 (57.0 mg, 41 µmol, Yield 26%, purity HPLC 99%).^{1}H NMR (500 MHz, DMSO-d_6): δ[PPM] 1.21-1.35 (m, 10H), 1.56 (m, 10H), 1.65-1.70 (m, 8H), 2.09-2.14 (m, 2H), 2.17-2.22 (m, 2H), 2.62-2.65 (m, 4H), 2.73-2.77 (m, 6H), 2.92-3.02 (m, 2H), 3.04-3.06 (m, 2H), 3.22 (m, 4H), 3.72 (m, 4H), 3.90 (t, *J* = 3.8 Hz, 4H), 3.98 (t, *J* = 6.2 Hz, 2H), 4.11-4.15 (m, 4H), 4.49-4.54 (m, 2H), 7.05 (br, 1H), 7.12-7.17 (m, 2H), 7.21-7.28 (m, 8H), 7.35 (d, J = 6.8 Hz, 1H), 7.41 (br, 1H), 7.80-7.83 (t, *J* = 6.2 Hz, 1H), 7.92-7.93 (d, *J* = 6.7 Hz, 1H), 8.00 (m, 10H), 8.09-8.14 (m, 2H), 8.25-8.30 (m, 4H), 8.40 (d, J = 6.7 Hz, 1H), 8.46-8.50 (m, 2H). ^{13}C NMR (125.8 MHz, DMSO-d_6): δ[PPM] 22.13, 22.76, 23.01, 23.11, 26.46, 27.42, 28.70, 31.18, 31.59, 31.87, 32.04, 37.26, 38.21, 38.44, 38.52, 40.05, 41.94, 42.02, 52.42, 52.55, 52.65, 53.05, 54.16, 54.19, 66.20, 114.18, 114.44, 115.13, 115.89, 116.09, 125.30, 126.20, 126.23, 128.00, 128.03, 128.36, 129.14, 129.19, 129.20, 130.60, 130.73, 132.25, 138.05, 155.49, 163.02, 163.54, 168.49, 168.53, 171.19, 171.38, 171.39, 171.50, 171.75, 171.77, 173.69. MALDI-TOF MS: cald 1373.5711 for $C_{70}H_{101}N_{16}O_{13}^{+}$, found 1373.7729.

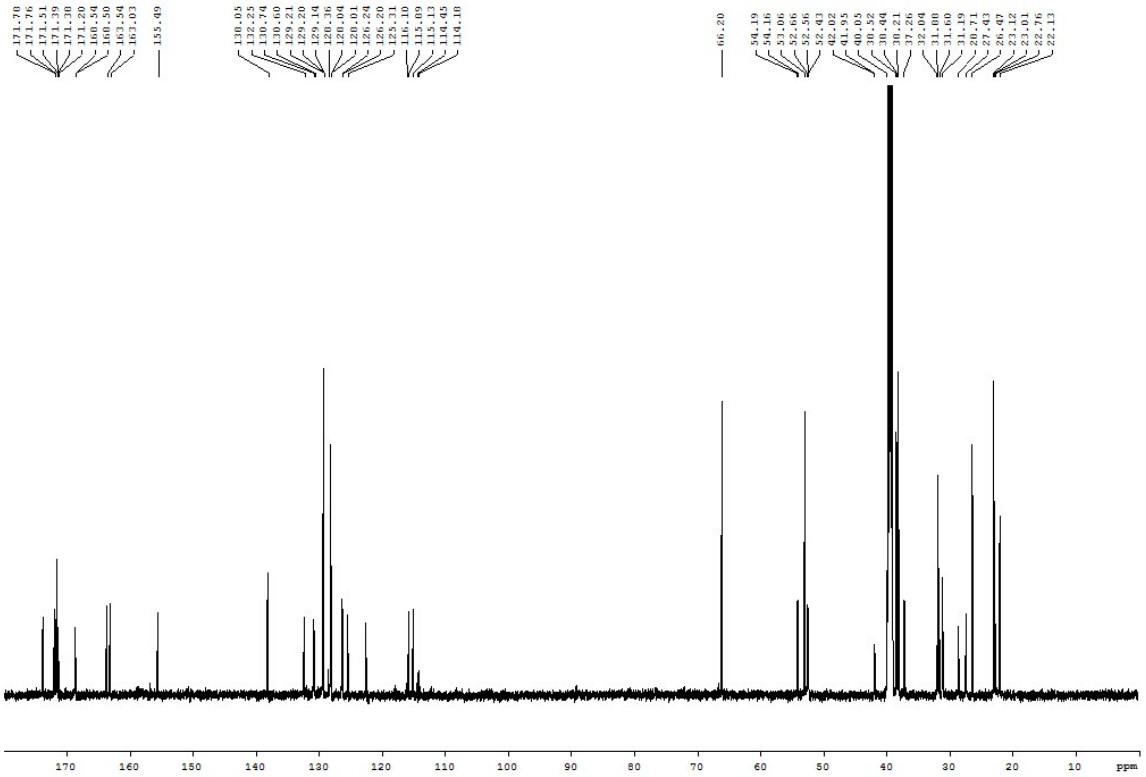
RP-HPLC chromatogram of Peptide 3:



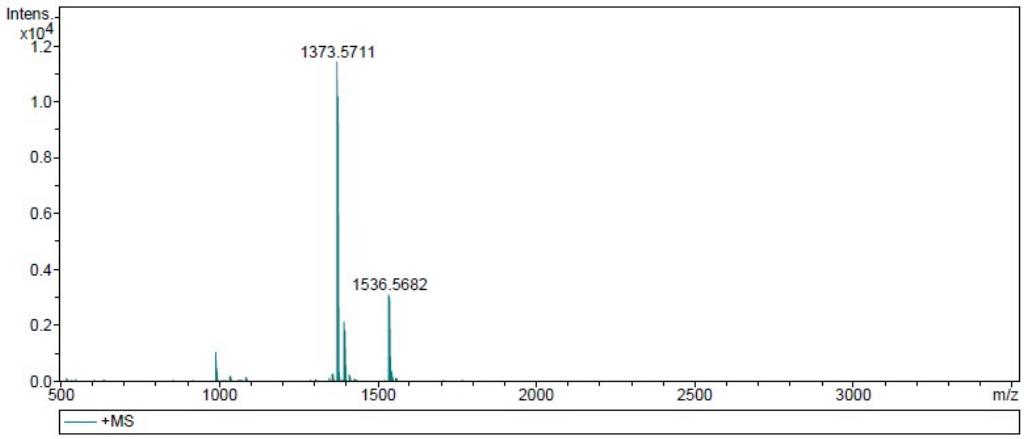
500 MHz ^{1}H NMR Bruker AVANCE 500 (DRX)



125.8 MHz ^{13}C NMR Bruker AVANCE 500 (DRX)



MALDI-TOF MS spectra of Peptide 3:



4. Fluorescence experiments:

General procedures: All fluorescence spectra were recorded using a VARIAN Fluorescence Spectrophotometer at 25°C. 1 cm cells were used for emission titration. For fluorescence titrations stock solution of peptides was prepared (c = 1000 μM) in 10 mM HEPES buffer (pH = 7.4). The slit widths were set to 5 nm for excitation and emission. The data points were collected at 1 nm increments with a 0.1 s integration period. All spectra were corrected for intensity using the manufacturer-supplied correction factors and corrected for background fluorescence and absorption by subtracting a blank scan of the buffer system.

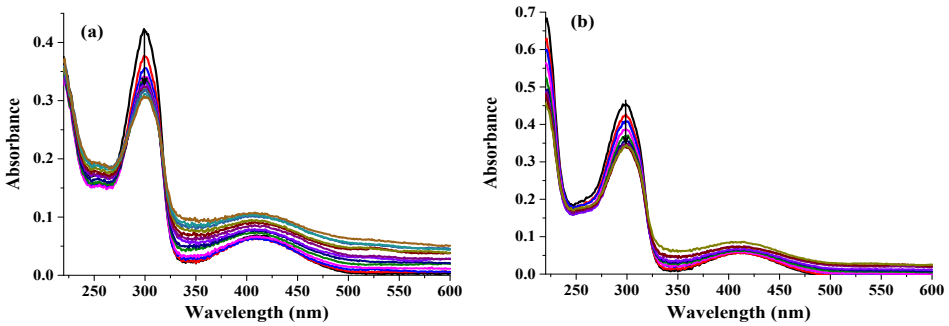


Fig. S1 UV/Vis spectra of: a) probe **1** (10.0 μM) with increasing concentration (0-3.0 μM) of 14-3-3ζ protein and b) probe **2** (10.0 μM) with increasing concentration (0-2.0 μM) of 14-3-3ζ protein in 10 mM HEPES buffer, pH = 7.4.

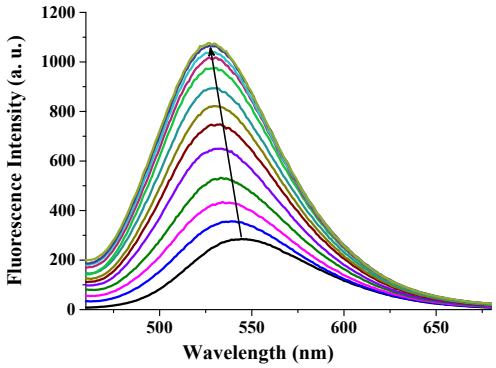


Fig. S2 Fluorescence emission spectra of probe **1** (5.0 μM) with increasing concentration (0-3.6 μM) of 14-3-3β protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

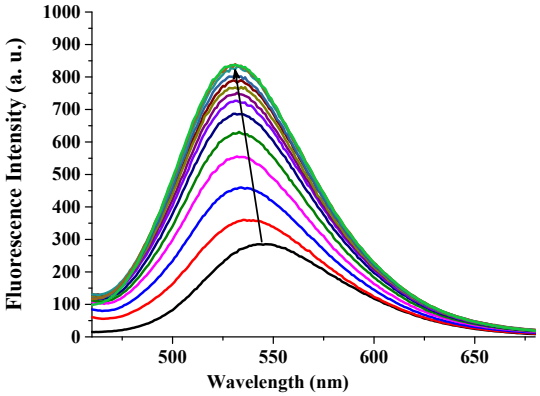


Fig. S3 Fluorescence emission spectra of probe **1** (5.0 μM) with increasing concentration (0-3.6 μM) of 14-3-3ε protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

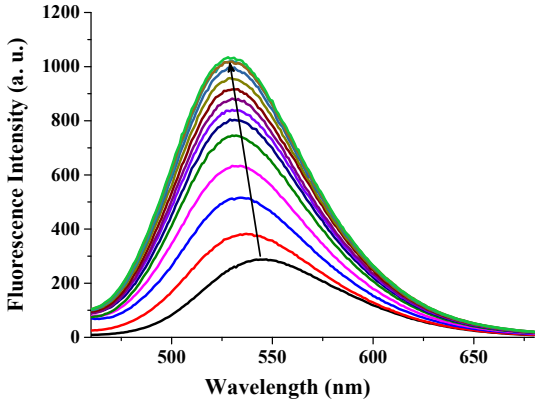


Fig. S4 Fluorescence emission spectra of probe **1** (5.0 μM) with increasing concentration (0-3.6 μM) of 14-3-3γ protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

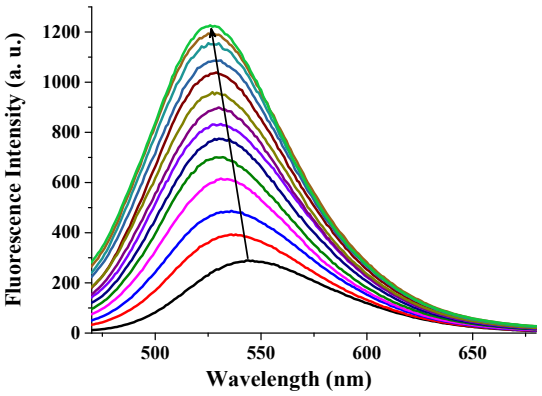


Fig. S5 Fluorescence emission spectra of probe **1** (5.0 μM) with increasing concentration (0-3.6 μM) of 14-3-3η protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

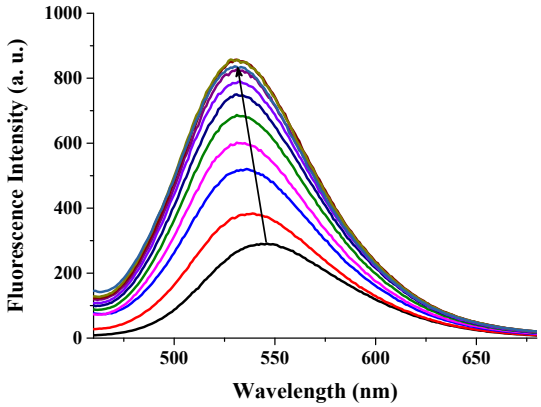


Fig. S6 Fluorescence emission spectra of probe **1** (5.0 μM) with increasing concentration (0-3.6 μM) of 14-3-3σ protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

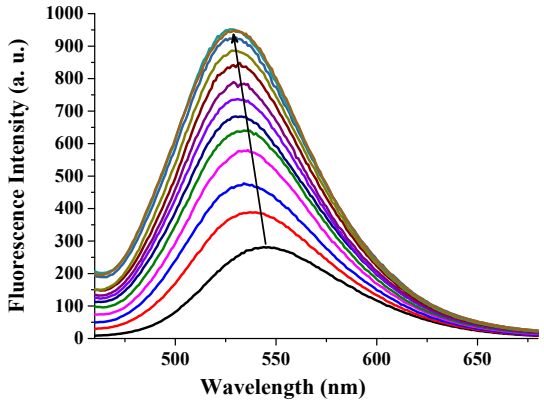


Fig. S7 Fluorescence emission spectra of probe **1** (5.0 μM) with increasing concentration (0-3.6 μM) of 14-3-3τ protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

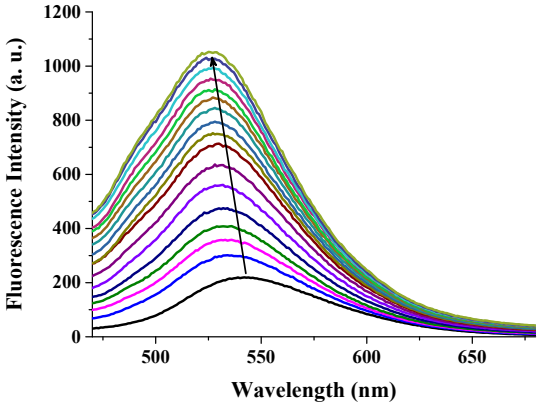


Fig. S8 Fluorescence emission spectra of probe **2** (5.0 μM) with increasing concentration (0-5.0 μM) of 14-3-3β protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

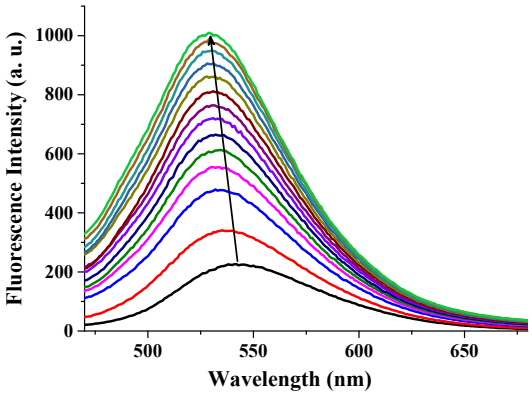


Fig. S9 Fluorescence emission spectra of probe **2** (5.0 μM) with increasing concentration (0-5.0 μM) of 14-3-3ε protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

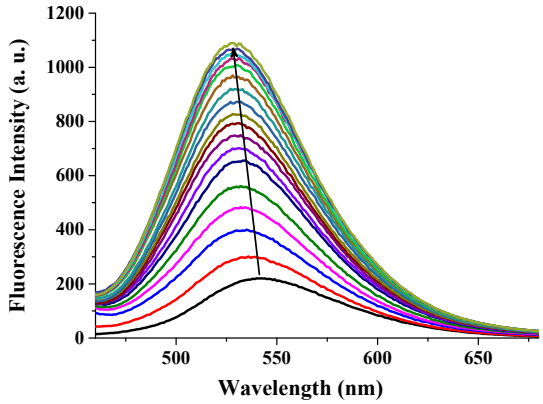


Fig. S10 Fluorescence emission spectra of probe **2** (5.0 μM) with increasing concentration (0-5.0 μM) of 14-3-3γ protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

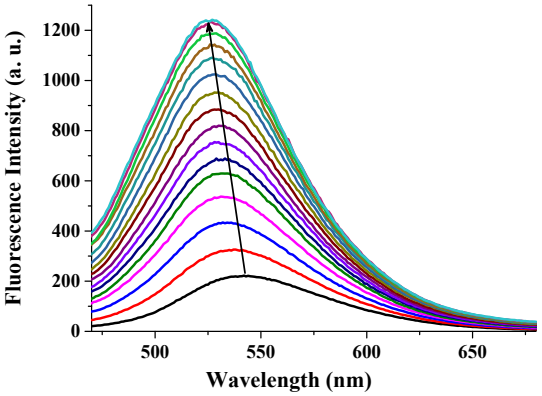


Fig. S11 Fluorescence emission spectra of probe **2** (5.0 μM) with increasing concentration (0-5.0 μM) of 14-3-3η protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

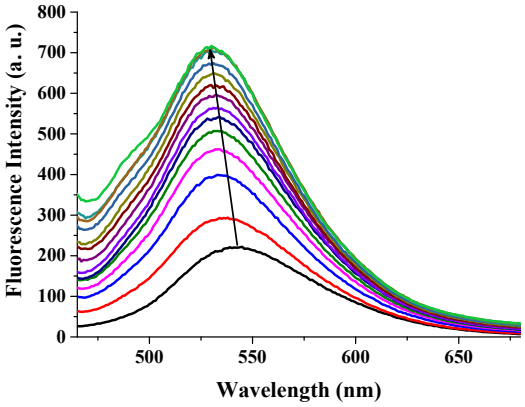


Fig. S12 Fluorescence emission spectra of probe **2** (5.0 μM) with increasing concentration (0-5.0 μM) of 14-3-3σ protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

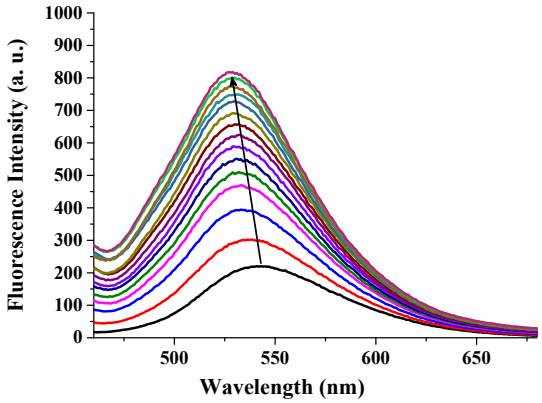


Fig. S13 Fluorescence emission spectra of probe **2** (5.0 μM) with increasing concentration (0-5.0 μM) of 14-3-3τ protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

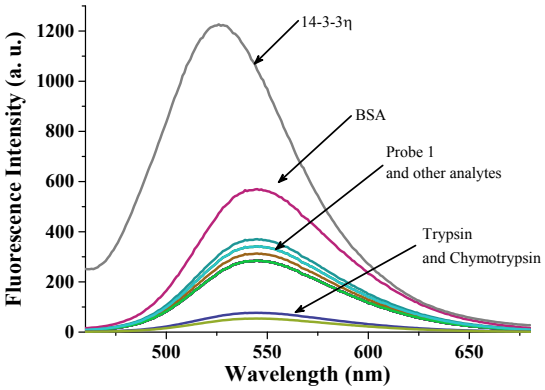


Fig. S14 Fluorescence spectra of probe **1** (5.0 μM) (λ_{ex} = 410 nm) in presence of one equivalents of different biomolecules in 10 mM HEPES buffer, pH = 7.4.

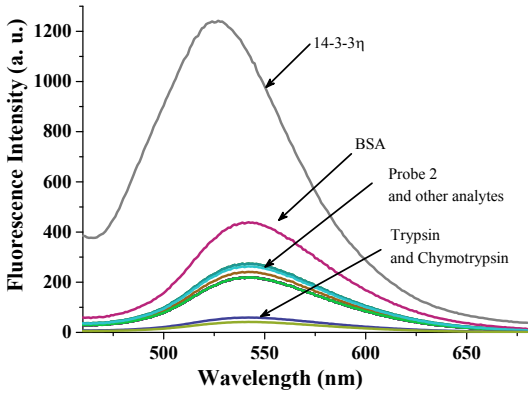


Fig. S15 Fluorescence spectra of probe **2** (5.0 μM) (λ_{ex} = 410 nm) in presence of one equivalents of different biomolecules in 10 mM HEPES buffer, pH = 7.4.

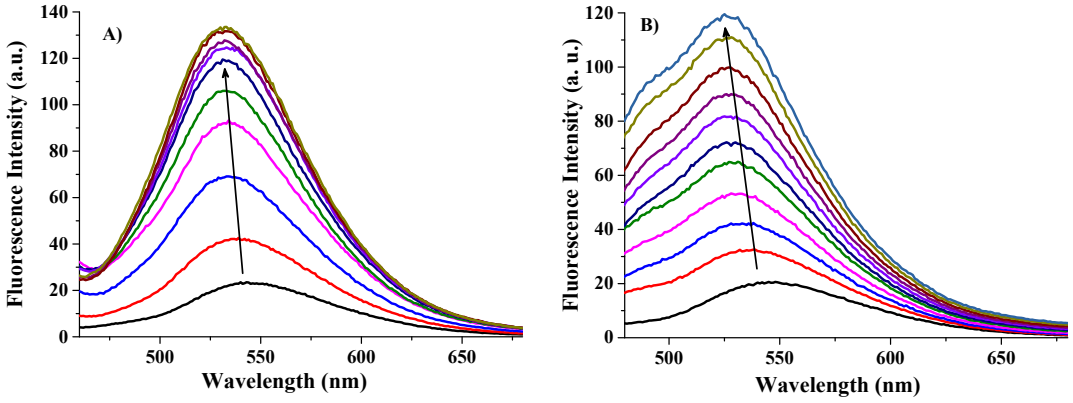


Fig. S16 Fluorescence emission spectra of probe **3** (5.0 μM) with increasing concentration (0-3.0 μM) of A) 14-3-3ζ protein and B) BSA protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

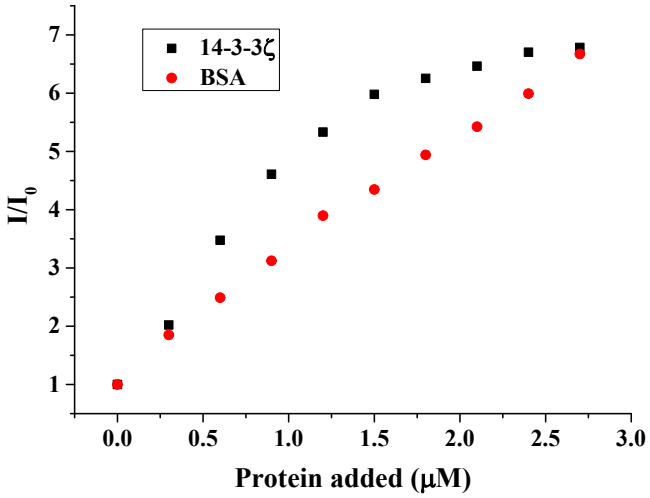


Fig. S17 Fluorescence response of probe **3** (5.0 μM) with increasing concentration 14-3-3ζ protein and BSA protein in 10 mM HEPES buffer, pH = 7.4 (λ_{ex} = 410 nm).

5. **Cytotoxicity assay:**

Alamar Blue Cell Viability Assay: Hela cells were grown 24h and incubated with peptide **1** and **2**. Afterwards cells were incubated with Alamar Blue dye (*Invitrogen*, 10% v/v) for 3h at 37°C at 5% CO_2. Fluorescence was measured at 590 nm using a multimode reader (GloMax-Multi+DetectionSystem, *Promega*).

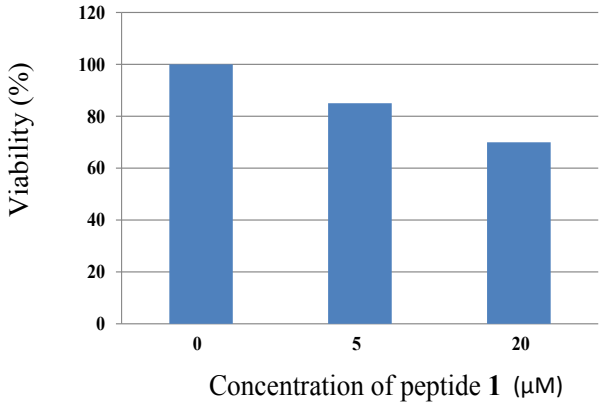


Fig. S18 Cell viability values (%) estimated by MTT proliferation test at different concentrations of peptide **1**. HeLa cells were cultured in the presence of peptide **1** at 37°C for 24h.

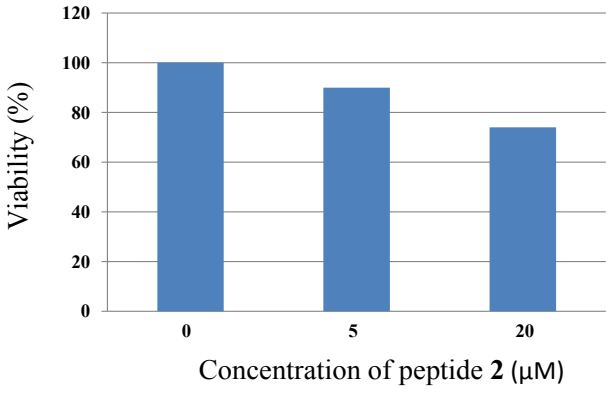


Fig. S19 Cell viability values (%) estimated by MTT proliferation test at different concentrations of peptide **2**. HeLa cells were cultured in the presence of peptide **2** at 37°C for 24h.

6. Computational studies:

The Cartesian coordinates of the human 14-3-3η isoform were taken from the Protein Data Bank (PDB id. 2C63).[5] The fluorophore in probes **1** and **2** (naphthalimide moeity) were docked into two binding sites using AutoDock4:[6] the first one centered on Phe122, where several organic compounds and natural products have been co-crystallized with this adaptor protein (e.g. fusicoccin A);[7] the second one in the outer face of the helices α6 and α8, where most of the sequence variability are found between isoforms of 14-3-3. In both cases, a grid of 60x60x60 Å was used. After visual inspection, a best pose in each case was selected as initial guess for the following force-field MD simulation.

The parameters of the naphthalimide fluorophore were computed following the standard procedure in AMBER. The ESP point charges were computed after fitting the surface electrostatic potential (at the level HF/6-31G*) using the program antechamber and the force-field parameters were computed using the program parmchk2, both of them implemented in AmberTools17. The complex of the protein with the embedded in a truncated octahedral box of ca. 29000 TIP3P water molecules that extended 15 Å away from any solute atom and 24 Na^+ ions were added to ensure charge neutrality.[8] The system was relaxed by energy minimization in three consecutive steps (3x5000 cycles), in which after the first 1000 cycles the minimization method was switched from steepest descendent to conjugate graduate. The resulting system was heated from 100 to 300 K during 20 ps with a time step of 2 fs and with the position of the solute atoms restrained (20 kcal mol^{-1} $Å^{-2}$). The weak-coupled algorithm was employed for the temperature regulation and the simulation was run with fixed volume (NVT ensemble).[9] The harmonic restraints were gradually reduced in five steps until they were completely removed where the harmonic restraint was reduced from 40 to 0 kcal mol^{-1} $Å^2$. Then, the density of the system was equilibrated for 20 ps using a time step of 2 fs by fixing the pressure, using the Berendsen barostat with isotropic pressure scaling (NPT ensemble), and allowing the volume of the box to change. The cutoff distance for the nonbonded interactions was 10 Å and periodic boundary conditions were used. Electrostatic interactions were treated by using the smooth particle mesh Ewald (PME) method with a grid spacing of 1 Å.[10] The SHAKE algorithm[11] was applied to all bonds involving hydrogen atoms. The molecular dynamics (MD) simulation protocol made use of the pmemd_cuda.SPFP module for Graphics Processor Units (GPUs) implemented in the AMBER16 suite of programs.[12]

In order to carried out a vibrational sampling of the fluorophore for computing the emission spectrum, a Born-Oppenheimer (BO) QM/MM MD simulation of 0.2 ps was run from the final snapshot of the force-field MD simulation. The QM region (the heterocycles of the fluorophore) were simulated in the first excited singlet state (S1) using the interface of Amber with Gaussian09.[13] The fluorophore was described with PBE-D3 and the def2-SVP basis set for all the atoms.

Ensembles of 54 snapshots were used for computing the vertical deexcitations by means of time-dependent density functional theory in the Tamm-Dancoff approximation from the S1 state using the Gaussian 09 package, version D.01. The DFT functional PBE was used in combination with the triple-zeta def2-TZVP basis set. The environment was represented in form of point atomic charges. The emission spectra were obtained by convolution of the 54 deexcitation between the electronic states $S_1 \rightarrow S_0$ by Lorentzian functions with a full width at half maximum of 0.50 eV. The wave packet analysis was carried out with the software TheoDORE[14] that analyzes the one-electron transition density matrix (1TDM).[15] The computed emission spectra in solution and in the binding site 1 are shifted less than 0.5 eV compared to the experimental spectrum.

7. References

[1] C. Schmuck, L. Geiger, *Chem. Commun.* **2005**, 772–774.
[2] C. Schmuck, M. Heil, J. Scheiber, K. Baumann, *Angew. Chemie Int. Ed.* **2005**, *44*, 7208–7212.
[3] C. Schmuck, *Chem. – A Eur. J.* **2000**, *6*, 709–718.
[4] J. Wu, A. Zawistowski, M. Ehrmann, T. Yi, C. Schmuck, *J. Am. Chem. Soc.* **2011**, *133*, 9720–9723.
[5] X. Yang, W. H. Lee, F. Sobott, E. Papagrigoriou, C. V Robinson, J. G. Grossmann, M. Sundström, D. A. Doyle, J. M. Elkins, *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 17237–17242.
[6] URL: http://autodock.scripps.edu/wiki/AutoDock4/
[7] M. Bartel, A. Schäfer, L. M. Stevers, C. Ottmann, *Future Med. Chem.* **2014**, *6*, 903–921.
[8] W. L. Jorgensen, J. Chandrasekhar, J. D. Madura, R. W. Impey, M. L. Klein, *J. Chem. Phys.* **1983**, *79*, 926–935.
[9] T. Morishita, *J. Chem. Phys.* **2003**, *119*, 7075–7082.
[10] T. Darden, D. York, L. Pedersen, *J. Chem. Phys.* **1993**, *98*, 10089–10092.
[11] S. Miyamoto, P. A. Kollman, *J. Comput. Chem.* **2004**, *13*, 952–962.
[12] D. A. Case, V. Babin, J. T. Berryman, R. M. Betz, Q. Cai, D. S. Cerutti, T. E. Cheatham, T. A. Darden, R. E. Duke, H. Gohlke, A. W. Goetz, S. Gusarov, N. Homeyer, P. Janowski, J. Kaus, I. Kolossváry, A. Kovalenko, T. S. Lee, S. LeGrand, T. Luchko, R. Luo, B. Madej, K. M. Merz, F. Paesani, D. R. Roe, A. Roitberg, C. Sagui, R. Salomon-Ferrer, G. Seabra, C. L. Simmerling, W. Smith, J. Swails, Walker, J. Wang, R. M. Wolf, X. Wu, P. A. Kollman, *{Amber 14}*, **2014**.
[13] Gaussian09, revision D.01. 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, Ö. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, and D. J. Fox, Gaussian 09 (Gaussian, Inc., Wallingford CT, 2009)

[14] URL: http://theodore-qc.sourceforge.net/doc.html

[15] F. Plasser, H. Lischka, *J. Chem. Theory Comput.* **2012**, *8*, 2777–2789.