Electronic Supplementary Information

A metal-free fluorescence turn-on molecular probe for detection of nucleoside triphosphates

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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. IR spectra were measured on a Jasco FT/IR-430 spectrometer. ^{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. Melting points were obtained in open glass capillary tubes using an apparatus from Büchi and are quoted uncorrected. 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±5 °C, 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.

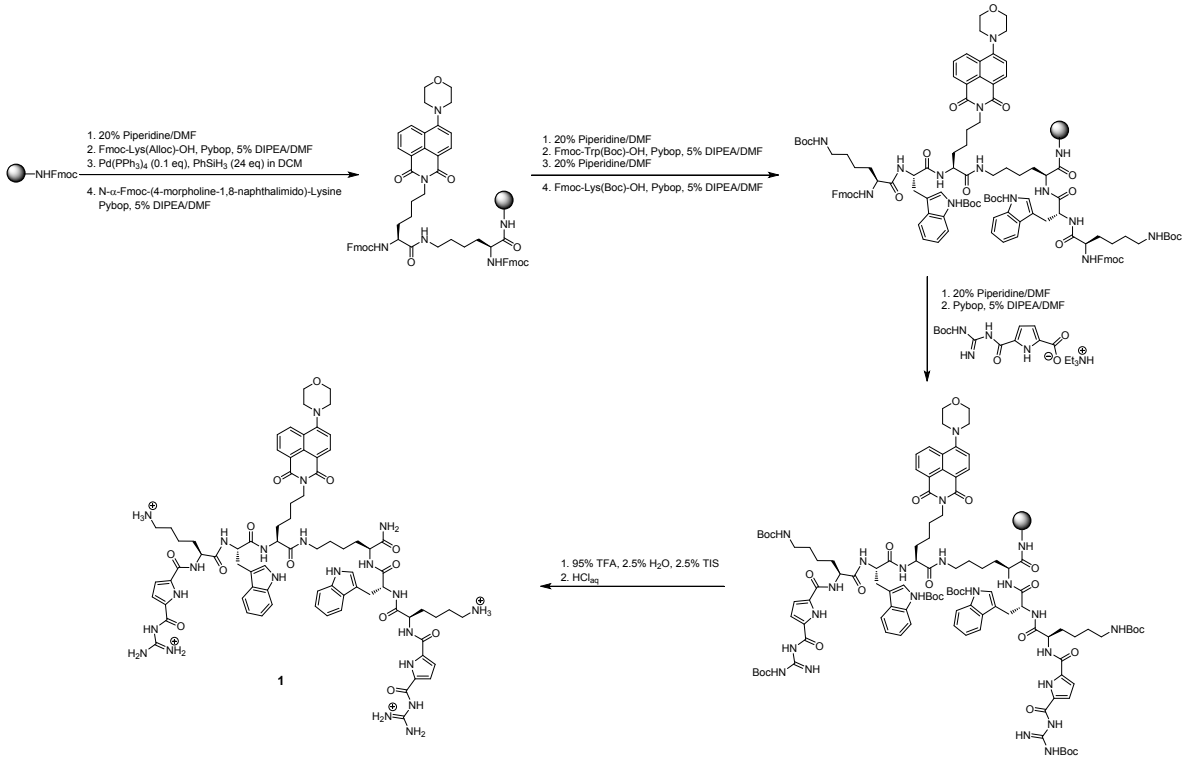
Standard Fmoc solid phase peptide synthesis techniques (SPPS): Each amino acid was attached using 0.6 mmol/g loading Fmoc Rink amide resin under microwave radio (20 W, 60±5 °C, 20 min) Then, the resin was washed 3×8 mL with DMF, 3×8 mL with DCM, 3×8 mL with DMF (ca. 5 min each) to remove the last traces 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 probe 1 and characterization:

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, tryptophan and lysine, as well as the tert-butoxycarbonyl (Boc)-protected guanidiniocarbonyl pyrrole (GCP)[2] motif were coupled



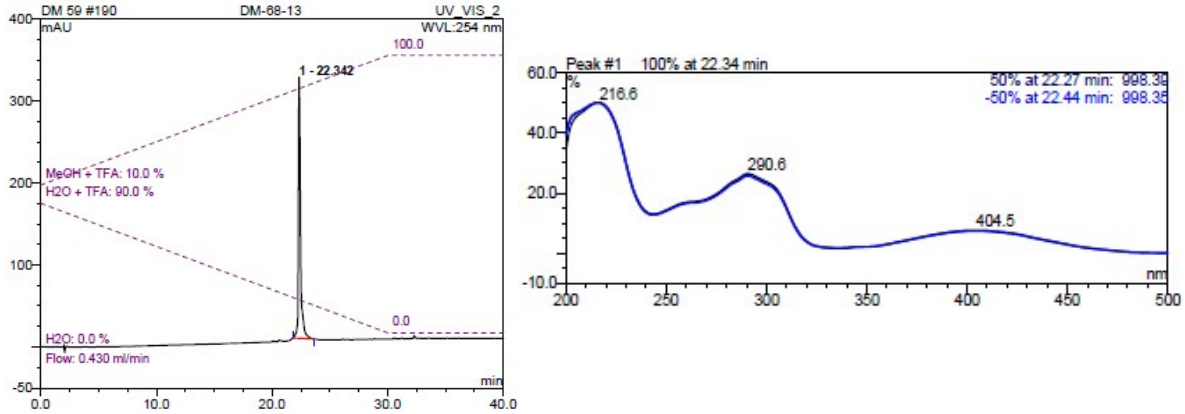
Scheme S1. Microwave assisted SPPS of the peptidic probe **1**. Coupling steps were carried out by irradiating the resin for 20 min at 20 W with a maximum temperature of 60 °C. Fmoc-removal was achieved by irradiating the solid support for 1 min and 5 min (20 W, max. 60 °C).

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.

Probe 1 (73.0 mg, 47.8 µmol, Yield 30%, purity HPLC 99%). ^{1}H NMR (500 MHz, DMSO-d_6): δ[ppm] 1.26-1.33 (m, 10H), 1.54-1.71 (m, 14H), 2.72 (m, 4H), 2.93-3.01 (m, 4H), 3.09-3.15 (m, 6H), 3.87 (m, 4H), 3.95 (q, *J* = 6.1 Hz, 4H), 4.14-4.18 (m, 1H), 4.24-4.26 (m, 1H), 4.39 (m, 1H), 4.52 (m, 1H), 6.87 (m, 2H), 6.90 (t, *J* = 6.2 Hz, 2H), 7.00 (q, *J* = 6.1 Hz, 2H), 7.13 (m, 2H), 7.25 (d, *J* = 6.8 Hz, 2H), 7.28 (d, *J* = 6.7 Hz, 2H), 7.50 (d, *J* = 6.5 Hz, 1H), 7.53 (d, *J* = 6.5 Hz, 1H), 7.56 (m, 2H), 7.73-7.76 (m, 2H), 7.85 (t, *J* = 5.2 Hz, 1H), 7.91 (br, 6H), 7.98 (d, *J* = 6.0 Hz, 1H), 8.23 (d, *J* = 6.1 Hz, 1H), 8.27 (d, *J* = 6.2 Hz, 1H), 8.33-8.37 (dd, *J* = 3.7 Hz, 2.9 Hz, 2H), 8.39-8.43 (m, 3H), 8.52 (br, 4H), 8.60-8.62 (t, *J* = 6.4 Hz, 1H), 8.69 (br, 4H), 10.81 (s, 1H), 10.84 (s, 1H), 12.09 (s, 2H), 12.47 (s, 2H). ^{13}C NMR (125.8 MHz, DMSO-d_6) δ: 22.46, 22.74, 22.96, 26.61, 27.26, 27.44, 28.79, 31.29, 31.63, 31.73, 32.06, 38.50, 38.59, 40.05, 52.49, 52.70, 52.93, 53.02, 53.65, 66.21, 66.77, 109.88, 110.02, 111.26, 113.68, 114.45, 115.03, 115.81, 118.22, 118.37, 120.81, 122.53, 122.56, 123.56, 123.61, 125.22, 125.58, 125.59, 126.09, 127.28, 128.38, 129.06, 130.49, 130.69, 132.21, 132.35, 136.00, 155.40, 155.58, 156.64, 159.02, 159.77, 163.01, 163.52, 170.04, 171.11, 171.33, 171.56, 171.61, 171.65, 173.63.

RP-HPLC chromatogram of Probe 1:



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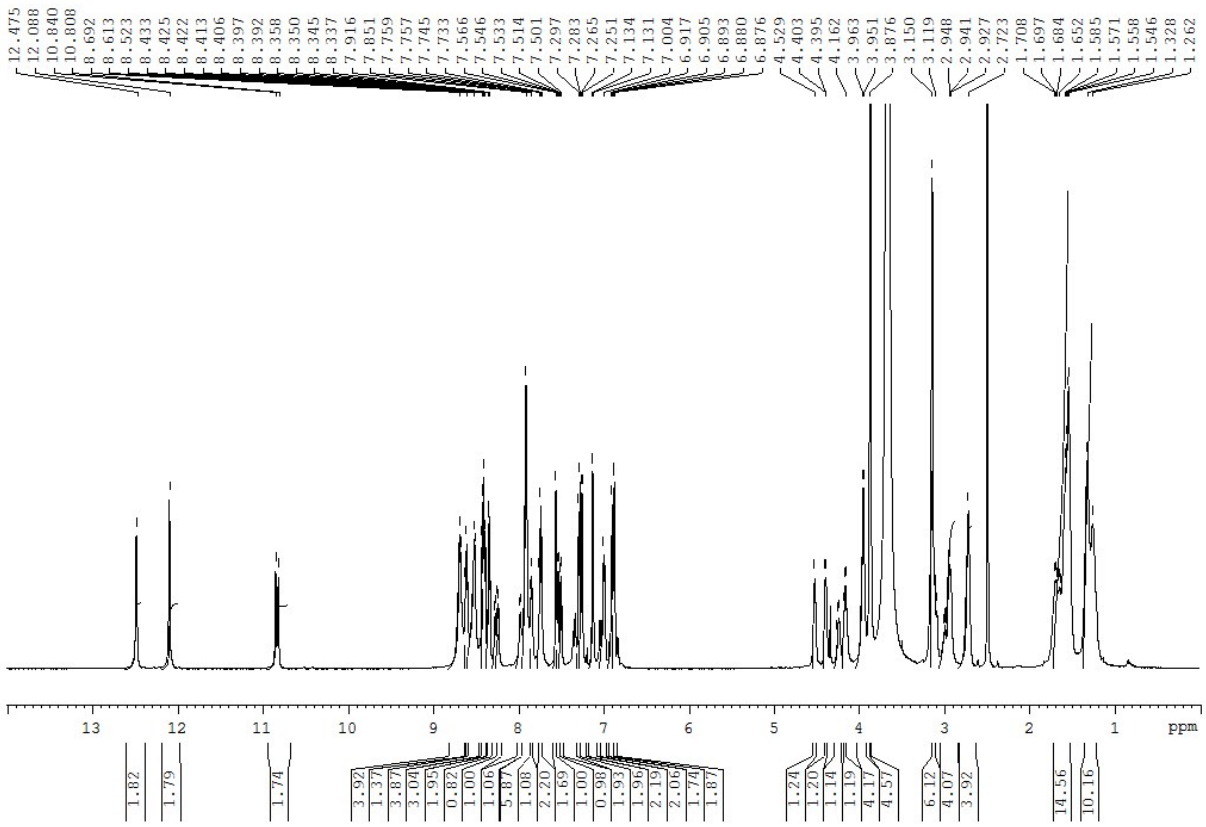
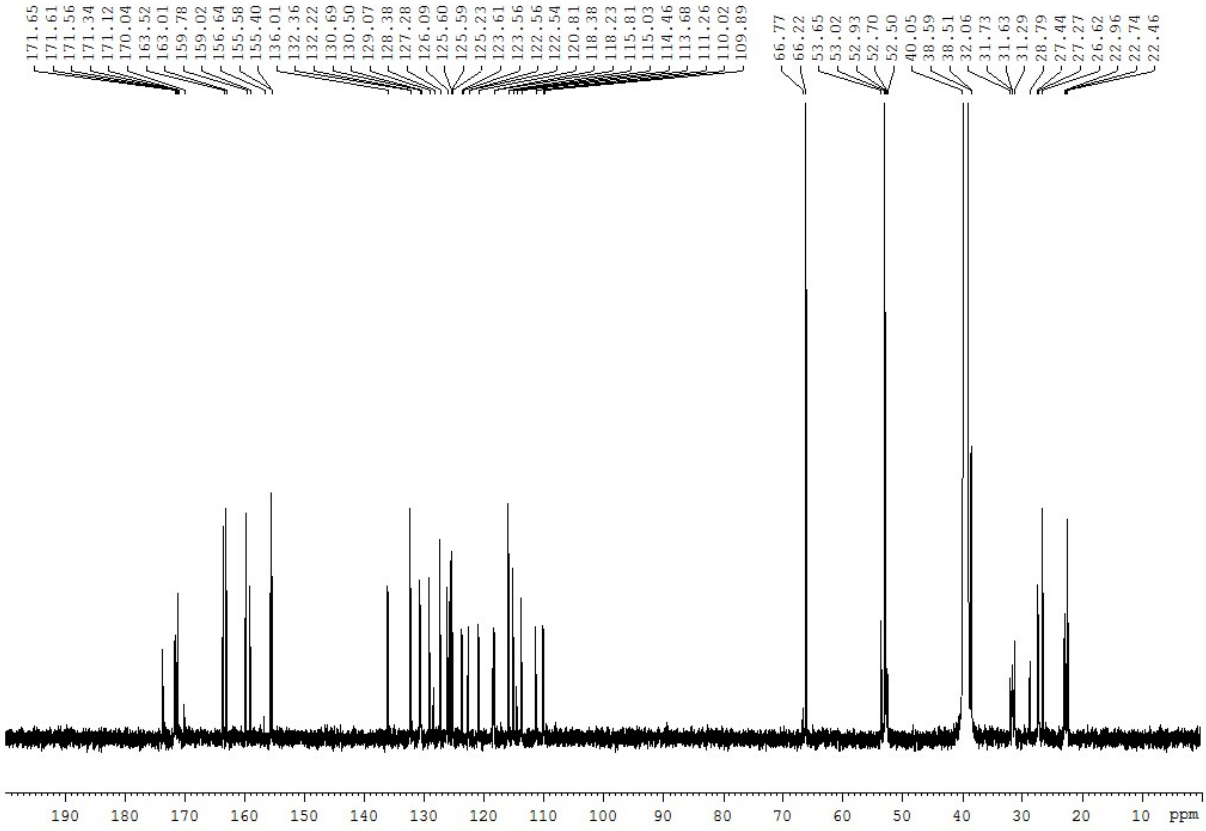
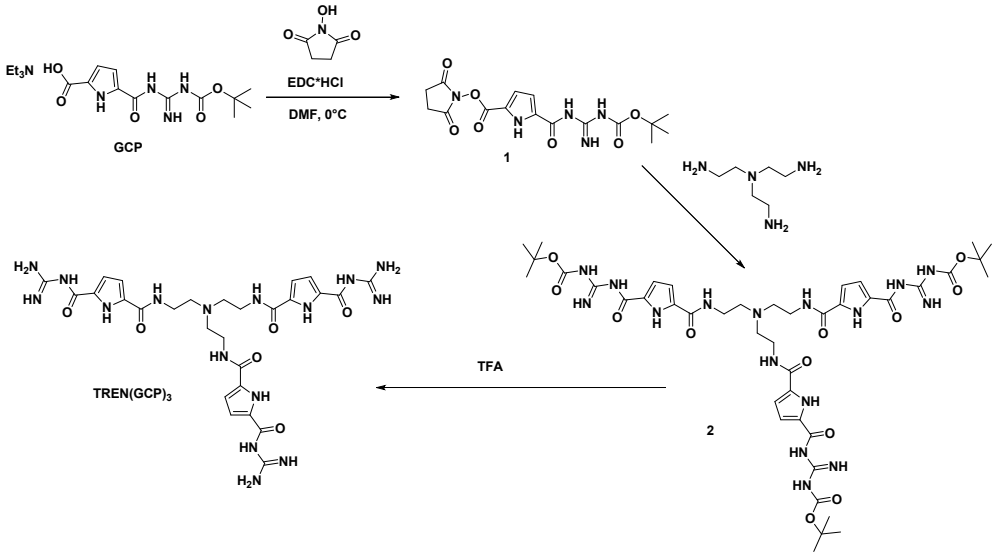


125.8 MHz ^{13}C NMR Bruker AVANCE 500 (DRX)



Synthesis of TREN(GCP)$_3$:



Compound 1: 2.00 g (5.03 mmol) of GCP and 0.93 g (8.12 mmol) of N-Hydroxysuccinimide were suspended in 50 ml of DMF at 0 °C. After 30 min, 1.56 g (8.11 mmol) of EDC.HCl was added and the mixture was stirred for 2h at 0 ºC. Then, the solution was stirred at room temperature overnight. The yellowish solution was diluted in water and extracted with ethyl acetate tow times. The organic layer was washed with brine and then, it was dried with $MgSO_4$ to get 1.87 g of **1** as a brownish solid (95%). LRMS (ESI): m/z calculated for $C_{16}H_{18}N_5O_7^-$ [M-H]$^-$: 392.12; found: 392.16. ^{1}H-NMR (300 MHz, DMSO-*d*^{6}) δ [ppm]: 1.47 (s, 9H, 3 x CH_3), 2.86 (s, 4H, 2 x CH_2), 6.87 (br.s, 1H, GCP-CH_{ar}), 7.10-7.09 (d, 1H, GCP-CH_{ar}), 8.59 (br.s, 1H, NH), 9.36 (br.s, 1H, NH), 10.67 (br.s, 1H, NH), 12.34 (br.s, 1H, NH).

Compound 2: To a solution of **1** (203.67 mg, 0.52 mmol) in 3 mL of DMF tris(2-aminoethyl)amine (25.00 µL, 0.17 mmol) was added, and the mixture was stirred overnight. Then, the solution was diluted in water and extracted with ethyl acetate tow times. The organic layer was washed with brine and dried with $MgSO_4$.The crude was purified to get 100 mg (61%) of **2** as a brownish solid. LRMS (ESI): m/z calculated for $C_{42}H_{61}N_{16}O_{12}^+$ [M+H]$^+$: 981.5; found: 981.6. ^{1}H-NMR (500 MHz, DMSO-*d*^{6}) δ [ppm]: 1.45 (s, 27H, 9 x CH_3), 2.66 (s, 6H, 3 x CH_2), 3.32 (s, 6H, 3 x CH_2), 6.75-6.80 (m, 6H, 6 x GCP-CH_{ar}), 8.33 (br.s, 3H, 3 x NH), 8.54 (br.s, 3H, 3 x NH), 9.30 (br.s, 3H, 3 x NH), 10.84 (br.s, 3H, 3 x NH), 11.47 (br.s, 3H, 3 x NH).

Compound TREN(GCP)3: To a solution of **2** (70 mg, 0.071 mmol) in 2.00 mL of DCM trifluoroacetic acid (0.49 mL, 6.42 mmol) was added. The mixture was stirred overnight and then, it was concentrated to dryness. After purification step it was got 35 mg (72%) as a desired product as a white solid. LRMS (ESI): m/z calculated for C27H37N16O6$^+$ [M+H]$^+$: 681.3; found: 681.3. ^{1}H-NMR (500 MHz, DMSO-*d*^{6}) δ [ppm]: 2.71 (s, 6H, 3 x CH_2), 3.67 (s, 6H, 3 x CH_2), 6.86 (m, 3H, 3 x GCP-CH_{ar}), 7.12 (m, 3H, 3 x GCP-CH_{ar}), 8.40-8.49 (m, 12H, 3 x NH, 3 x NH_3^+), 8.76 (br.s, 3H, 3 x NH), 11.32 (br.s, 3H, 3 x NH), 12.33 (br.s, 3H, 3 x NH).

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 **probe 1** 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.

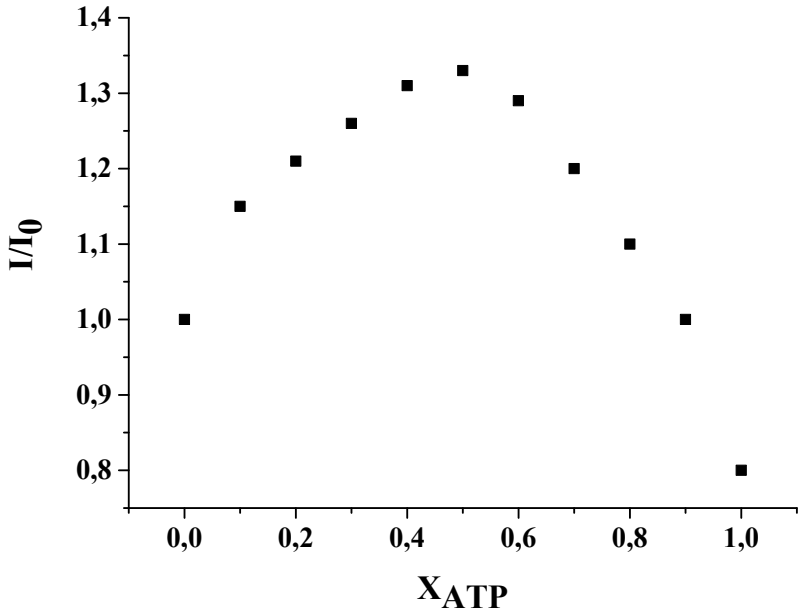


Fig. S1 Job plot for the determination of the stoichiometry of probe **1** and ATP in the complex. A series of solutions containing probe **1** and ATP were prepared such that the sum of the total concentration of probe **1** and ATP remained constant (10 μM). The mole fraction (X) of ATP was varied from 0.1 to 1.0.

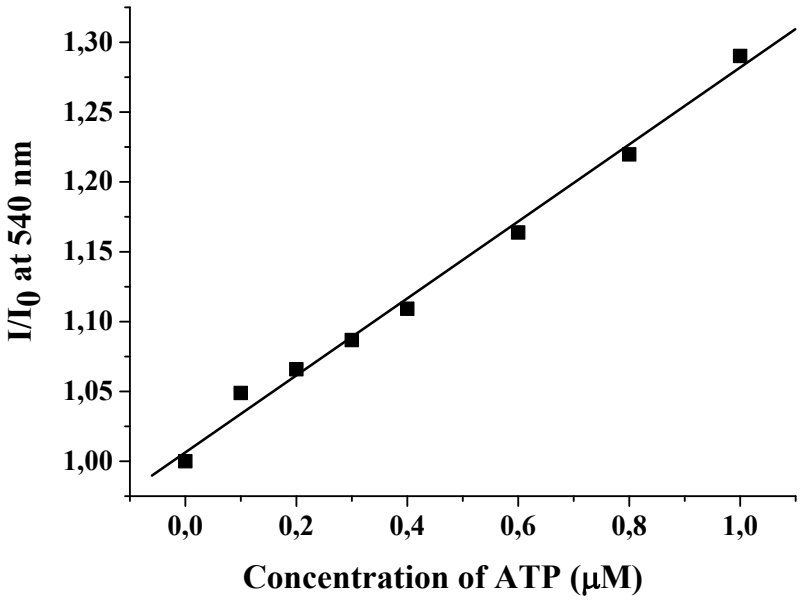


Fig. S2 Determination of detection limit of ATP using probe **1** (20.0 μM) (λ_{ex} = 410 nm) in 10 mM HEPES buffer, pH = 7.4.

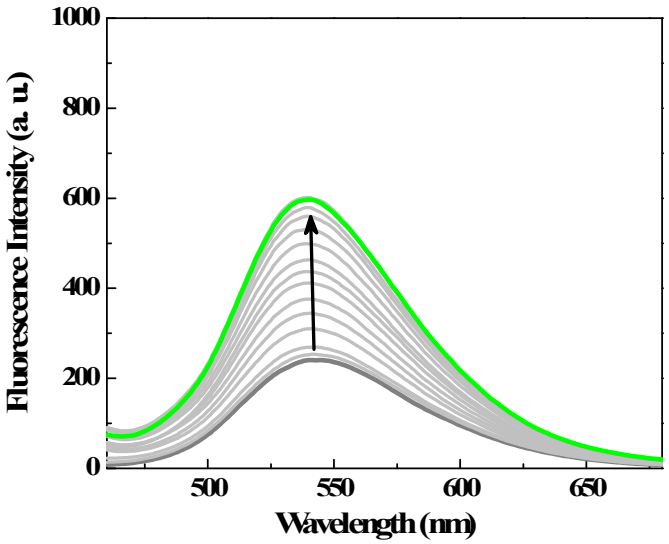


Fig. S3 Fluorescence emission spectra of probe **1** (20.0 μM) (λ_{ex} = 410 nm) with increasing concentration (0.0, 2.0, 4.0, 6.0, 8.0, 10.0, 13.0, 16.0, 19.0, 22.0, 25.0, 28.0, 31.0, 34.0, 37.0, 40.0 μM) of ADP in 10 mM HEPES buffer, pH = 7.4.

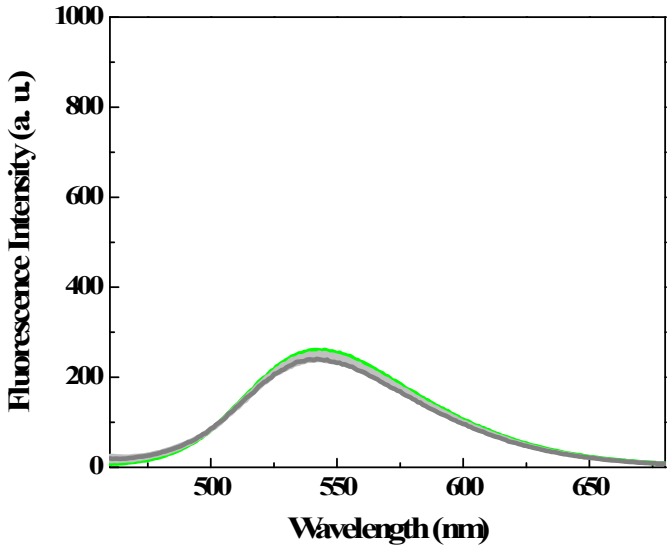


Fig. S4 Fluorescence emission spectra of probe **1** (20.0 μM) (λ_{ex} = 410 nm) with increasing concentration (0.0, 2.0, 4.0, 6.0, 8.0, 10.0, 13.0, 16.0, 19.0, 22.0, 25.0, 28.0, 31.0, 34.0, 37.0, 40.0 μM) of AMP in 10 mM HEPES buffer, pH = 7.4.

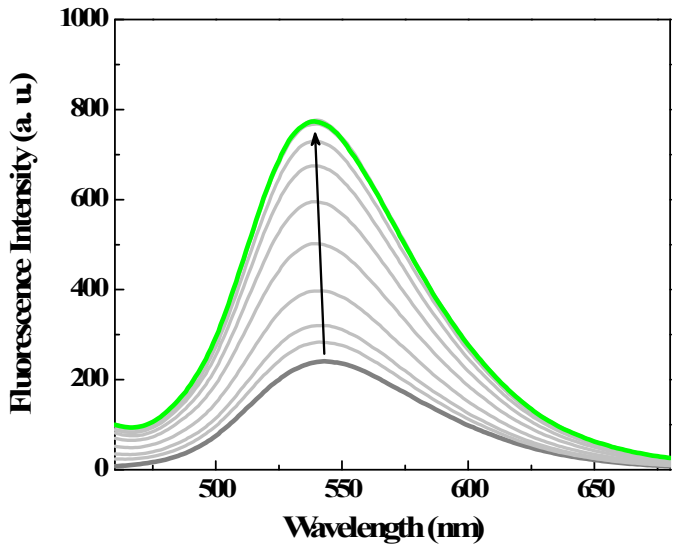


Fig. S5 Fluorescence emission spectra of probe **1** (20.0 μM) (λ_{ex} = 410 nm) with increasing concentration (0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 μM) of GTP in 10 mM HEPES buffer, pH = 7.4.

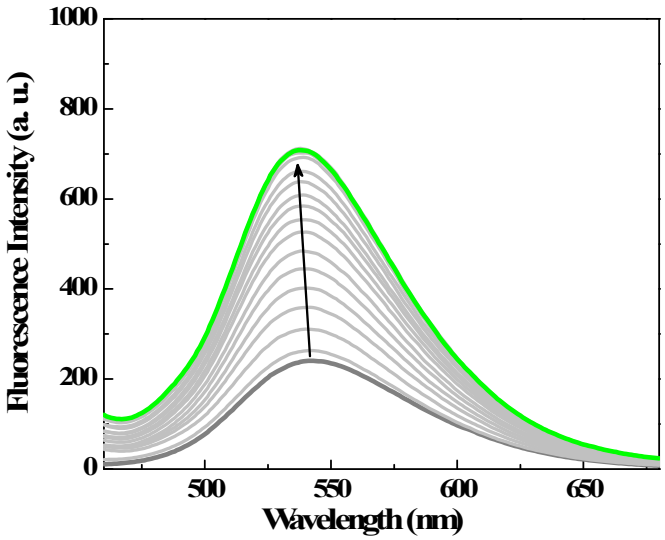


Fig. S6 Fluorescence emission spectra of probe **1** (20.0 μM) (λ_{ex} = 410 nm) with increasing concentration (0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0 μM) of CTP in 10 mM HEPES buffer, pH = 7.4.

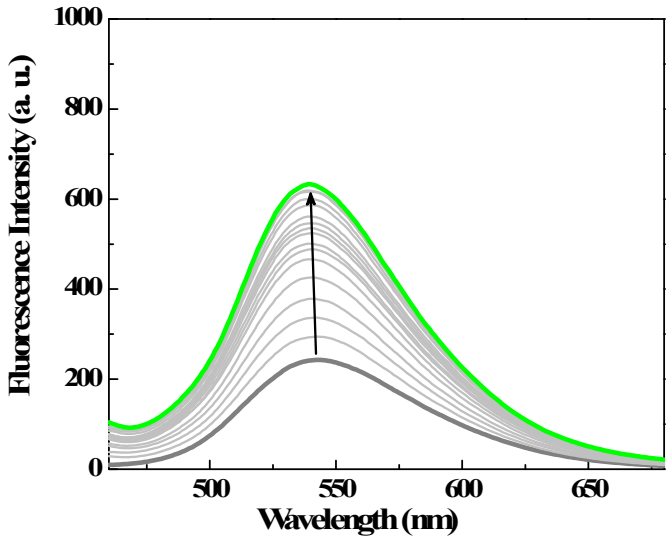


Fig. S7 Fluorescence emission spectra of probe **1** (20.0 μM) (λ_{ex} = 410 nm) with increasing concentration (0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0 μM) of TTP in 10 mM HEPES buffer, pH = 7.4.

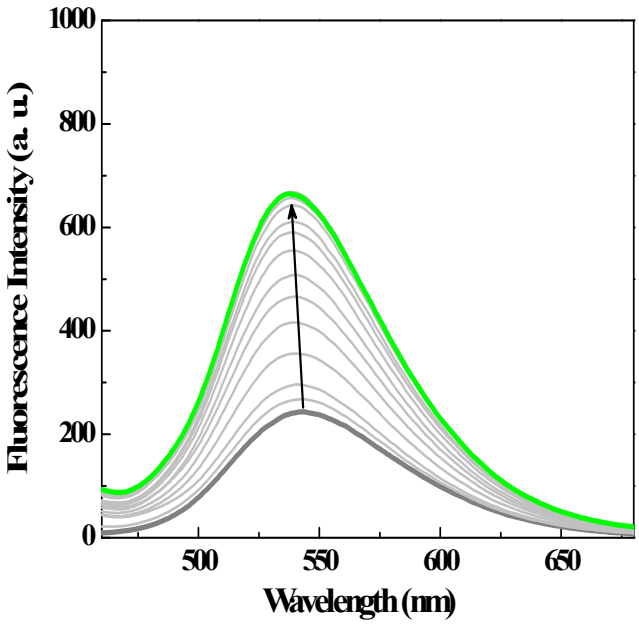


Fig. S8 Fluorescence emission spectra of probe **1** (20.0 μM) (λ_{ex} = 410 nm) with increasing concentration (0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 12.0 μM) of UTP in 10 mM HEPES buffer, pH = 7.4.

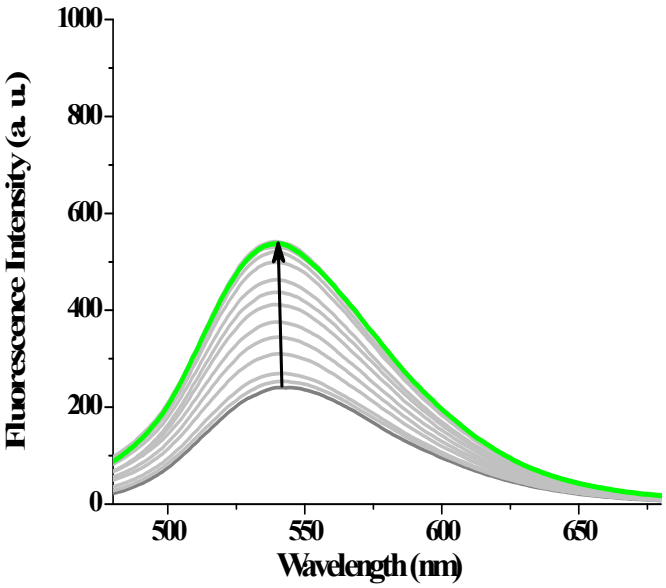


Fig. S9 Fluorescence emission spectra of probe **1** (20.0 μM) (λ_{ex} = 410 nm) with increasing concentration (0.0, 2.0, 4.0, 6.0, 8.0, 10.0, 13.0, 16.0, 19.0, 22.0, 25.0, 28.0, 31.0, 34.0, 37.0, 40.0 μM) of UDP in 10 mM HEPES buffer, pH = 7.4.

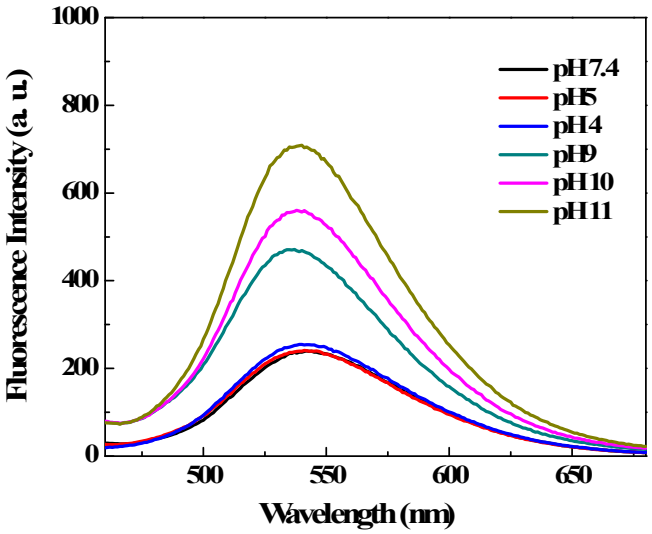


Fig. S10 Fluorescence emission spectra of probe **1** (20.0 μM) (λex = 410 nm) with at different pH in 10 mM HEPES buffer.

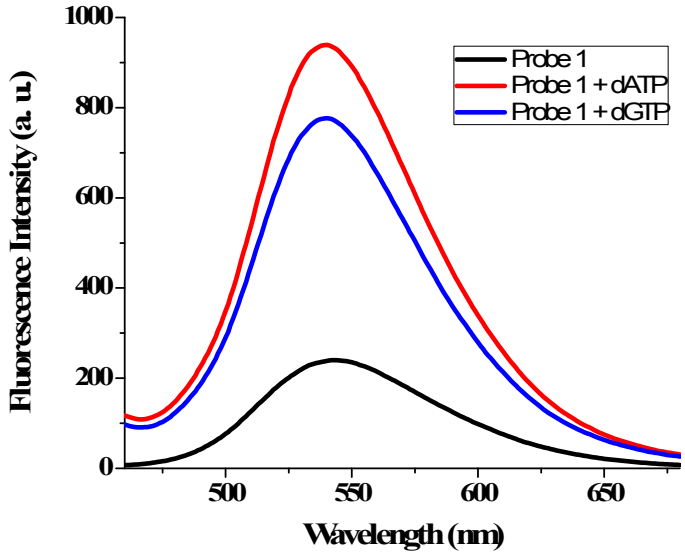
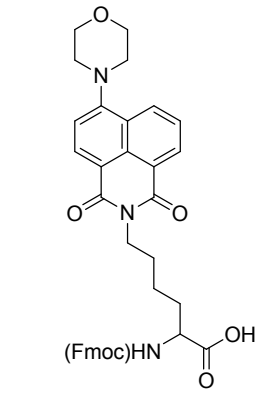
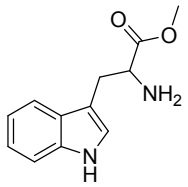


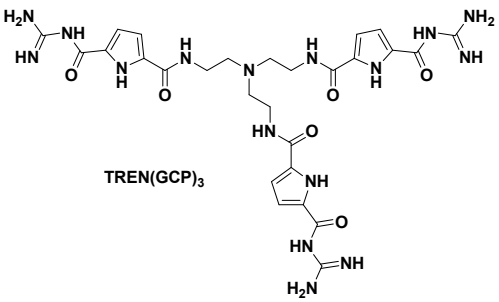
Fig. S11 Fluorescence emission spectra of probe **1** (20.0 μM) (λ_{ex} = 410 nm) upon addition of 10.0 μM of dATP and dGTP respectively in 10 mM HEPES buffer.



Naphthalimide precursor (nap)



Trp-OMe



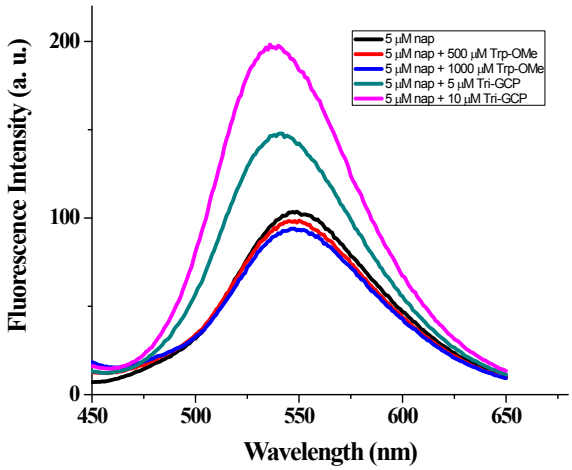


Fig. S12 Fluorescence control study of 5.0 μM naphthalimide precursor (nap) (λ_{ex} = 410 nm) in presence of Trp-OMe and TREN(GCP)$_3$ respectively in 10 mM HEPES buffer.

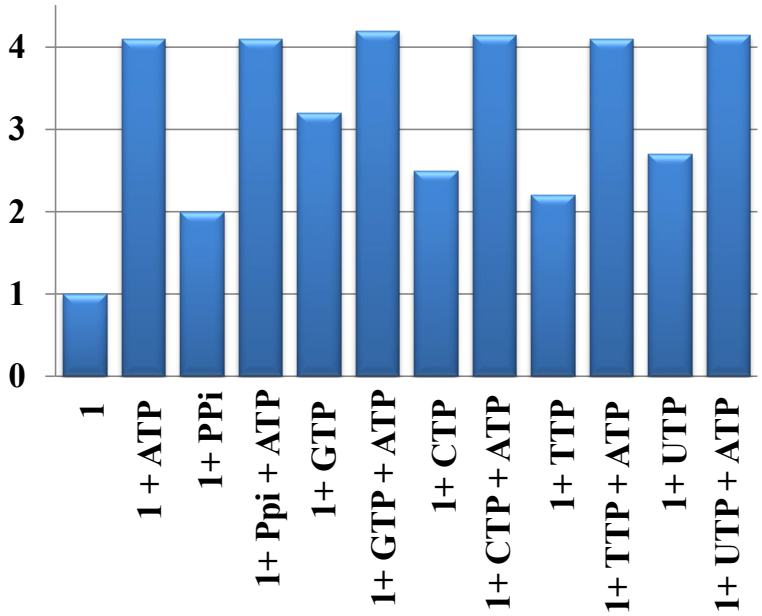


Fig. S13 Competitive fluorescence study for ATP detection in presence of other polyphosphates. Probe **1** (20.0 μM) (λ_{ex} = 410 nm) was treated with 10.0 μM of ATP in presence of other polyphosphates (10.0 μM) respectively in 10 mM HEPES buffer.

5. Molecular modeling studies

Virtual molecular studies were carried out by a conformational search using Macro Model[3] with the OPLS2005 forcefield, GB/SA solvent model for water, 250000 iterations and a convergence threshold of 0.05. The conformational search has been processed with mixed torsional / low-mode sampling with 1000 maximum steps and 100 steps per rotatable bond. The collected conformations have been in a energy window of 5 kJ/mol and have been of similar shape, so that the shown structure (**Fig. 4**) represents all found conformations in that specific energy window. PyMOL[4] was used for illustration.

6. Cell image experiments

The Human cervix carcinoma cell lines HeLa (ATCC-No.CCL-2) were obtained from the American Type Culture Collection and maintained as recommended in complete Dulbecco`s Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum, 1 % Glutamine and 1 % Antibiotic-Antimycotic (Invitrogen) at 37° C in a humidified atmosphere of 5 % CO_2. 1×10^4 cells per well were seeded in 96 well cell culture plates (Greiner bio-one) in a total medium volume of 100 µl 24 h before imaging. Afterwards, probe 1 were added to the culture medium in desired concentrations and incubated for 30 min. The cells were extensively washed with PBS buffer for three times and examined with inverted fluorescence microscope (Axiovert 200M, Carl Zeiss) with a 10X air objective. Images were processed and analyzed using MetaMorph 6.3r6 (Molecular Devices) and Adobe Photoshop CS4 (Adobe Systems).

7. Cytotoxicity assay:

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

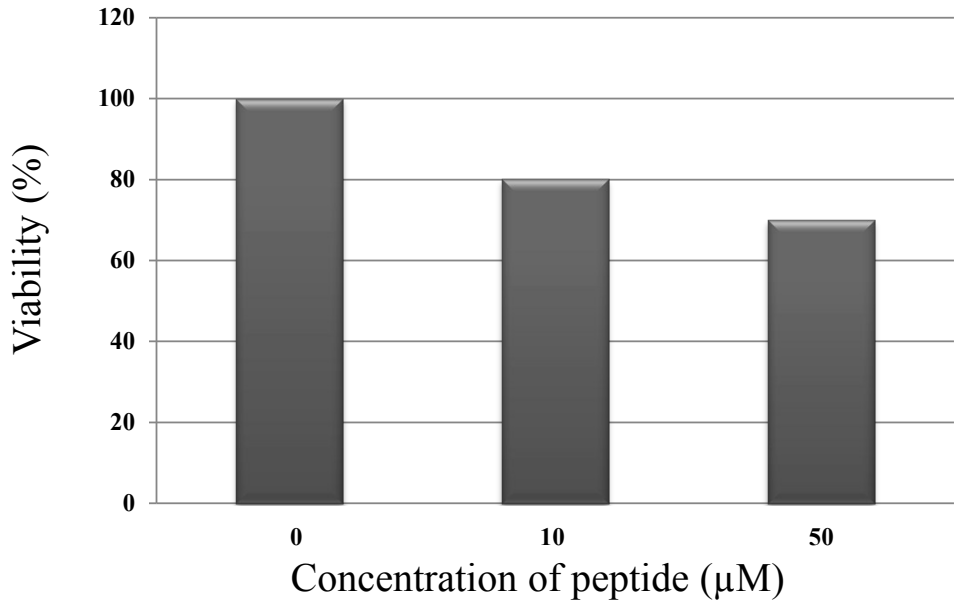


Fig. S14 Cell viability values (%) estimated by MTT proliferation test at different concentrations of probe **1**. HeLa cells were cultured in the presence of probe **1** at 37 °C for 24 h.

10. References

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