Supplementary Information

Structure-Uptake Relationship Study of DABCYL-Derivatives Linked to Cyclic CPPs for Live-Cell Delivery of Synthetic Proteins

Abhishek Saha,[a] Shaswati Mandal,[a] Jan Vincent V. Arafiles,[b] Jacobo Gómez-González,[b] Christian P. R. Hackenberger,[*,b,c] and Ashraf Brik[*,a]

[a]Schulich Faculty of Chemistry, Technion-Israel Institute of Technology, Haifa, 3200008 (Israel).

[b]Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), Robert-Rössle-Strasse 10, Berlin 13125 (Germany)

[c]Department of Chemistry, Humboldt Universitätzu, Berlin Brook-Taylor-Str.2, Berlin 12489 (Germany)

*correspondence

Ashraf Brik, e-mail: abrik@technion.ac.il

Christian P. R. Hackenberger, e-mail: Hackenbe@fmp-berlin.de

Table of Contents:

Sl. No.	Contents	Page No.
1.	Materials and methods	4-5
2.	Fmoc-SPPS general procedure	5
3.	UPLC and HPLC for peptide analysis and purification	5-6
4.	Synthesis of DABCYL-cR10 and Cyanine5-labeled cyclic deca-arginine (cR10-Cy5) and its DABCYL conjugate (DABCYL-cR10-Cy5)	6-9
5.	Cell culture reagents	9
6.	General cell culture procedure	9-10
7.	General procedure of protein delivery experiments:	10
8.	General procedure of confocal microscopy and image analysis	10
9.	Time-lapse microscopy	11
10.	Fluorescence lifetime imaging microscopy (FLIM)	11-13
11.	Synthesis and characterization of DABCYL Derivatives	13-21
12.	Table of logP values of DABCYL-derivatives	22
13.	Synthesis of the activated form of cyclic deca-arginine(DABCYL derivatives), cR10DX(TNP)	23-31
14.	Synthesis of the activated form of cyclic deca-arginine, cR10-TNP	32-33
15.	Stability of trans-azobenzene conjugated peptides under experimental conditions and sunlight	34-35
16.	Synthesis of TAMRA-Cys-PEG-Ub(1-76)-$CONH_2$ (TAMRA-Ub)	36-37
17.	Synthesis of CPP-linked Ub conjugates (**Ub1**-**Ub14**)	38-45
18.	Representative images of live U2OS cell delivery of CPP-Ub conjugates (**Ub1**-**Ub13**)	46-51
19.	Statistical analysis of live-cell delivery of potent CPP-Ubs by Citation5 microscope	52
20.	Stability of **Ub11** in cell delivery assay conditions	53
21.	Cytosolic Ub delivery by the AzidoDABCYL CPP compared to the natural TAT CPP	54-56
22.	Live-cell Delivery comparison study between **Ub1** and **Ub11** in presence of serum	57

23.	Cell membrane integrity analysis using SYTOX™ Blue stain	58-59
24.	^{1}H and ^{13}C NMR spectra	60-77
25.	References	78

1. Materials and methods:

Reagents and solvents were purchased from Sigma, Alfa Aesar, and Fluka (used directly without any further purification unless otherwise stated). Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F254 (0.25 mm). The column chromatography was performed with Merck silica gel of 120-200 mesh. ^{1}H NMR and ^{13}C NMR were recorded at 300 or 400 MHz with a Bruker spectrometer. The chemical shifts were reported in parts per million (δ) using DMSO-d_6 and $CDCl_3$ as an internal solvent, coupling constants (*J*) were reported in hertz (Hz) and the abbreviations were reported as follows: s (singlet), brs (broad singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). High-resolution mass spectra (HRMS) were recorded at Xevo G2 Q-TOF mass spectrometer with a Z-spray source passing through UPLC (Acquity) using built-in software for analysis of the recorded data.

Resins were purchased from Creosalus, protected amino acids were purchased from GL Biochem, and activating reagents *O*-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU)], and 1-Hydroxybenzotriazolemonohydrate (HOBt) were purchased from Luxembourg Bio Technologies. Chemicals including all the protected amino acids used in peptides synthesis were purchased from Sigma Aldrich, Strem Chemicals, Alfa Aesar, and Chem-Implex. Analytical grade *N, N*-dimethylformamide (DMF) was purchased from Biotech. 7-Azabenzotriazol-1-acyloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) was procured from Novabiochem. 5-Carboxy-tetramethylrhodamine (TAMRA) and 4-Dimethylaminoazobenzene-4'-carboxylic acid (DABCYL, **D1**) were purchased from Tzamal D-Chem. 4-(dimethylamino)benzoic acid (**D2**) and 4-(phenylazo)benzoic acid (**D3**) were purchased from Sigma Aldrich. LCQ Fleet Ion Trap (Thermo Scientific) performed mass spectrometry analysis of synthesized protein and peptides.

List of the protected amino acids used in peptides and protein synthesis

Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Phe-OH, Fmoc-His(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Thr(^{t}Bu)-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Nle-OH, Fmoc-Leu-Ser($\psi^{Me,Me}$pro)-OH, Fmoc-Asp(O^{t}Bu)-(DMB)Gly-OH, Fmoc-Ile-Thr($\psi^{Me,Me}$Pro)-OH, Fmoc-Leu-Thr($\psi^{Me,Me}$Pro)-OH, Fmoc-Lys(Alloc)-OH, Fmoc-Glu(OAll)-OH, Boc-Cys(Trt)-OH.

2. Fmoc-SPPS general procedure:

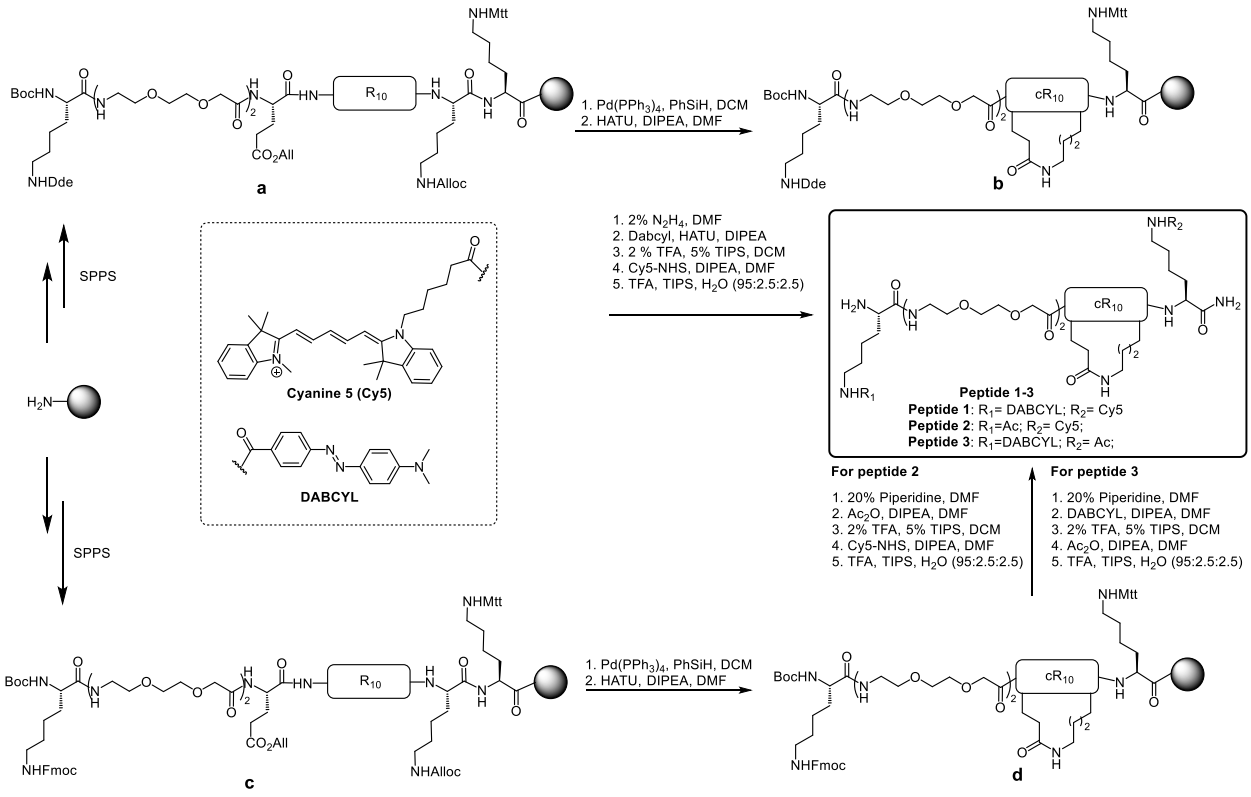
Fmoc-SPPS was carried on an automated peptide synthesizer (CS336X, CSBIO) in presence of 4 eq. of AA, 4 eq. of HCTU, and 8 eq. of DIEA to the initial loading of the resin for 1 h. The dipeptides were coupled manually using 2.5 eq. of AA, 2.5 eq. of HATU, and 5 eq. of DIEA to the initial loading of the resin for 2 h. To cleave the peptides from the solid support the resin was washed with DMF, MeOH, and DCM X5 and dried over a high vacuum. A cocktail of TFA/triisopropyl silane (TIS)/water (95:2.5:2.5) was added to the resin and the reaction mixture was shaken for 2 h at RT. The resin was filtered and the combined filtrate was added dropwise to a 10-fold volume of cold ether and centrifuged. The precipitate was dissolved in acetonitrile-water for freeze-drying in the lyophilizer to give the crude peptide.

3. UPLC and HPLC for peptide analysis and purification:

UPLC-UV traces for peptides were obtained on an ACQUITY H-class instrument (Waters Corporation, Milford, Massachusetts, USA) equipped with an ACQUITY UPLC®-BEH C18 1.7 µm, 2.1 x 50 mm column (Waters Corporation), applying a flow rate of 0.6 mL/min and using eluents A (99.9% H_2O, 0.1% TFA) and B (99.9% ACN, 0.1% TFA) in the corresponding linear gradient. UPLC-UV chromatograms were recorded at 220 nm. Gradient: 5% to 95% B in 15 min. Analytical HPLC was performed on a Thermo instrument (Dionex Ultimate 3000) using analytical

column XSelect (Waters, CSH C18, 3.5 µm, 4.6 × 150 mm) at a flow rate of 1.2 mL/min using linear gradient: 0% to 60% B in 30 min. Preparative HPLC was performed on a Thermo instrument (Dionex Ultimate 3000) using preparative column XSelect (Waters, C18, 10 µm, 250 × 19 mm) at a flow rate of 15 mL/min. semi-preparative HPLC was performed on a Thermo instrument (Dionex Ultimate 3000) using Phenomenex Jupiter C18 10 µm, 300 Å, 250 × 10 mm column, at a flow rate of 4 mL/min. All synthetic products were purified by HPLC and characterized by mass spectrometry using the LCQ Fleet Ion Trap (Thermo Scientific). All calculated masses have been reported as an average isotope composition. Buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in acetonitrile.

4. Synthesis of DABCYL-cR10 and Cyanine5-labeled cyclic deca-arginine (cR10-Cy5) and its DABCYL conjugate (DABCYL-cR10-Cy5):



Scheme 1: Synthesis of **Peptide 1-3**.

C-terminal amide R10 peptide derivatives were synthesized following standard Fmoc-peptide synthesis protocols on a 0.05 mmol scale using a 0.247 mmol/g loading *H-Rink* amide resin with a Liberty Blue automatic microwave-assisted peptide synthesizer from CEM Corporation. The amino acids were coupled in 5-fold excess using oxyme and DIC as an activating agent. Couplings were conducted for 2 min at 90 °C. Deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF for 1 min at 75 °C. Mtt deprotection was carried out by bubbling nitrogen through a resin suspension in DCM containing 1.5% TFA and 5% TIPS for 20 minutes (1.5 mL per 100 mg of resin) and this was repeated until achieving full deprotection. Dde deprotection was carried out by bubbling nitrogen through a resin suspension in DMF containing 2% hydrazine for 3 minutes (1 mL per 100 mg of resin). This was repeated three times and the Dde deprotection was monitored by UPLC-MS.

DABCYL was coupled using a 4-fold excess HATU as activating agent and activated for 1 min in DIEA/DMF 0.2 M before being added to the resin. These manual couplings were conducted for 120 min. The peptide **d** was acetylated with a solution of 0.8 ml Ac_2O, 2 ml of DIEA/DMF (0.2 M), and 3.2 ml of DMF for 40 min. Cy5 was coupled by using the corresponding NHS ester which was conducted in DIEA/DMF 0.2 M for 120 min.

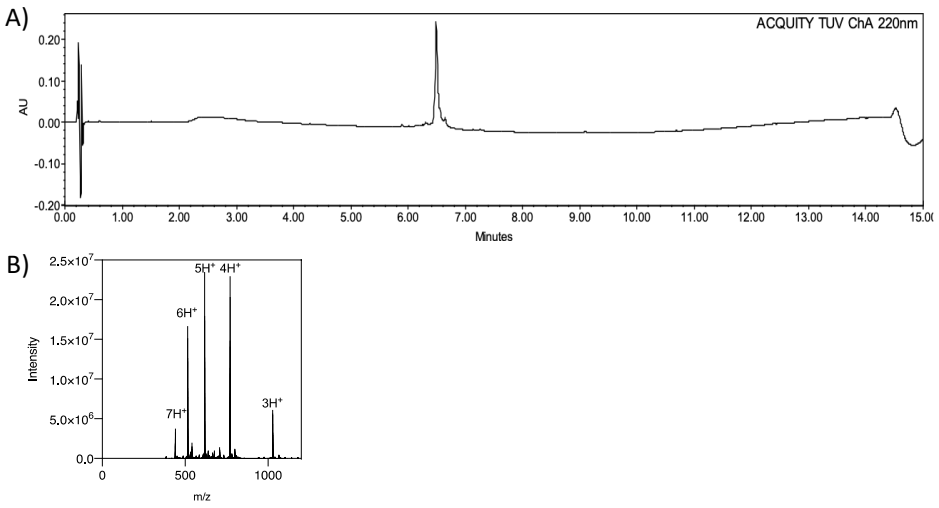


Figure S1. Synthesis of **Peptide 1** (H-K(Dabcyl)cR10K(Cy5)-$CONH_2$): (A) UPLC chromatogram of the purified **Peptide 1**. (B) Mass spectra corresponding to the peak wit retention time (t_R) of 2.3 min. MS (ESI): (5-95% B, t_R = 2.23 min) Calculated for $C_{142}H_{236}N_{55}O_{23}$ = 3079.90; calculated for $[M+4H]^{4+}$ = 770.97; found 770.96; calculated for $[M+5H]^{5+}$ = 616.98; found 616.97.

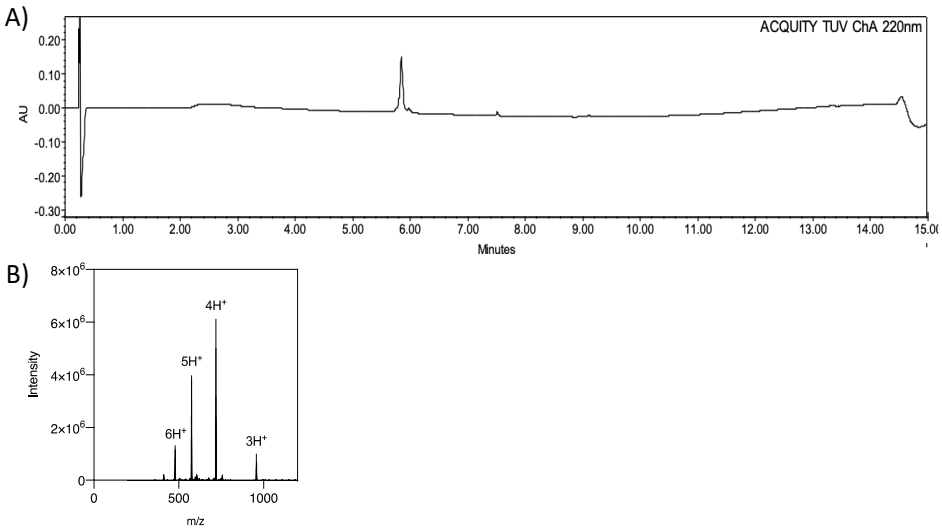


Figure S2. Synthesis of **Peptide 2** (H-K(Ac)cR10K(Cy5)-$CONH_2$): (A) UPLC chromatogram of the purified **Peptide 2**. (B) Mass spectra corresponding to the peak wit retention time (t_R) of 2.3 min. MS (ESI): (5-95% B, t_R = 2.10 min) Calculated for $C_{129}H_{225}N_{52}O_{23}$ = 2870.80; calculated for $[M+4H]^{4+}$ = 718.70; found 718.68; calculated for $[M+5H]^{5+}$ = 575.16; found 575.15.

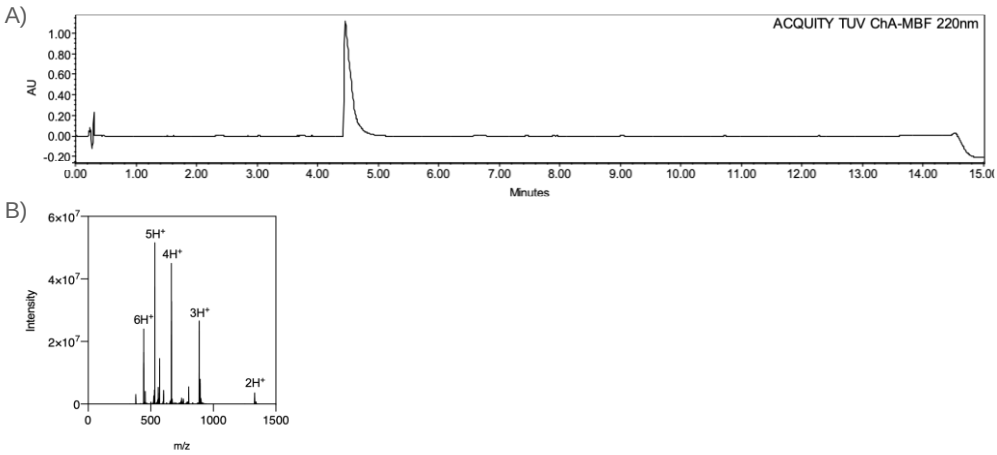


Figure S3. Synthesis of **Peptide 3**: (A) UPLC chromatogram of the purified **Peptide 3**. (B) Mass spectra corresponding to the peak wit retention time (t_R) of 4.5 min. MS (ESI): (5-95% B, t_R = 4.5 min) Calculated for $C_{112}H_{201}N_{53}O_{23}$ = 2658.19; calculated for $[M+4H]^{4+}$ = 665.50; found 665.54; calculated for $[M+5H]^{5+}$ = 532.63; found 532.60.

5. Cell culture reagents:

U2OS (HTB-96™) cells were purchased from ATCC®. Dulbecco's modified eagle's medium (DMEM), Fetal bovine serum (FBS), L-Gln, antibiotics (penicillin/streptomycin), trypsin/EDTA, and phosphate-buffered saline (PBS) were purchased from biological industries. Hoechst 33342 solution (20 mM) and SYTOX blue solution (5 mM) were purchased from Thermo-fisher. µ-Slide 8 well and µ-Slide 2 well for live-cell confocal microscopy were purchased from IBIDI and polylysine hydrobromide was purchased from Sigma.

6. General cell culture procedure:

U2OS (HTB-96™) cells were cultured in DMEM supplemented with 10% FBS, 0.2 mM L-Gln and antibiotics (penicillin/streptomycin) in a humidified 37 °C incubator at 5% CO_2. To detach cells from culture flasks, the media was aspirated and the flask was washed with sterile calcium and magnesium-free PBS before cells were treated with 0.25% Trypsin 0.02% EDTA solution and

returned to the incubation chamber for 5 min. Trypsin was quenched by adding the supplemented media. The cell suspension was collected and the cells were pelleted (2 min at 1,000xg). Media then aspired and the cell pellet was resuspended in fresh media. The cell density was determined using an automated cell counter (Countess II, Invitrogen) and seeded accordingly. For confocal microscopy and image stream analysis cells were seeded on IBIDI poly-L-lysine (PLL) 8 well µ-slides.

7. General procedure of protein delivery experiments[1]:

Cells in ~90% confluency were washed three times with warm PBS/serum-free DMEM followed by incubation for 1 h at 37 °C with PBS/serum-free medium containing CPP fused protein in dark. Cells were then washed three times with warm PBS followed by adding 0.1 mg/ml heparin sulfate in PBS and incubating for 5 min. After this step, cells were washed with a full culture medium. Cells were then stained using manufacturer standard Protocols with Hoechst (2 µg/ml) and imaged by CLSM or analyzed with Image Stream.

8. General procedure of confocal microscopy and image analysis:

The distribution of fluorescent proteins in live cells was analyzed using a confocal laser scanning microscope (Confocal Zeiss LSM 710) equipped with a 40× NA 1.2 water immersion objective lens having a 1 AU pinhole resolution limit. Different lasers were used for the different tags; UV laser (Hoechst, SYTOX blue) – 405 nm (10 mW), Green laser (TAMRA) – 543 nm (10 mW). During CLSM analysis the samples were kept at room temperature.

Image analysis was performed on all images collected using Fiji software. Relative fluorescence intensities were determined by first identifying the cytosolic compartment of individual cells by applying a cell masking algorithm based on Hoechst nuclear staining. The values presented are the averaged intensity of TAMRA.

9. Time-lapse microscopy:

U2OS cells (200000 cells/well) were seeded onto an 8-well glass-bottom slide (IBIDI). After 48 h of incubation, the cells were washed with PBS followed by the addition of 200 µL of FluorBrite DMEM (Gibco). The slide was mounted onto a Nikon-CSU spinning disc microscope with a CSU-X1 (Andor) and a live cell incubation chamber (OKOlab). Images were acquired using a PlanApo 60x NA 1.4 oil objective (Nikon) and an EMCCD (AU888, Andor). Images were captured every 15 sec. Diluted peptide solution was added (final concentration of 500 nM) after 180 sec of initial imaging, followed by additional imaging for 600 sec.

10. Fluorescence lifetime imaging microscopy (FLIM)[2]:

U2OS cells (200000 cells) were seeded onto an 8-well glass-bottom slide (IBIDI). After 48 h of incubation, cells were washed with PBS 3× and treated with 2 µM of Flipper-TR® probe (Spirochrome) in FluorBrite DMEM (Gibco) for 15 min according to manufacturer's protocol. Cells were washed once more with PBS and 200 µL FluorBrite DMEM was added onto the cells before mounting onto Leica TCS SP8 3X with fluorescence lifetime upgrade. Fluorescence lifetime images were taken every 15 sec for 1 min. In Figure 1C (i), 0s is indicated as before peptide addition. Immediately after image acquisition, indicated peptide (final concentration of 1 µM) was added, and further fluorescence lifetime images were taken at 15 sec intervals for 6 sec. For Figures 1B and C, 10 regions-of-interest (ROIs) from three independent biological replicates with high Cy5 photon counts the 30 sec after peptide addition (referred to as nucleation zones) were chosen. A double exponential fit was applied to the fluorescence decay on individual ROIs (represented as individual points on the graph), and the weighted average lifetimes were calculated and represented as red lines.

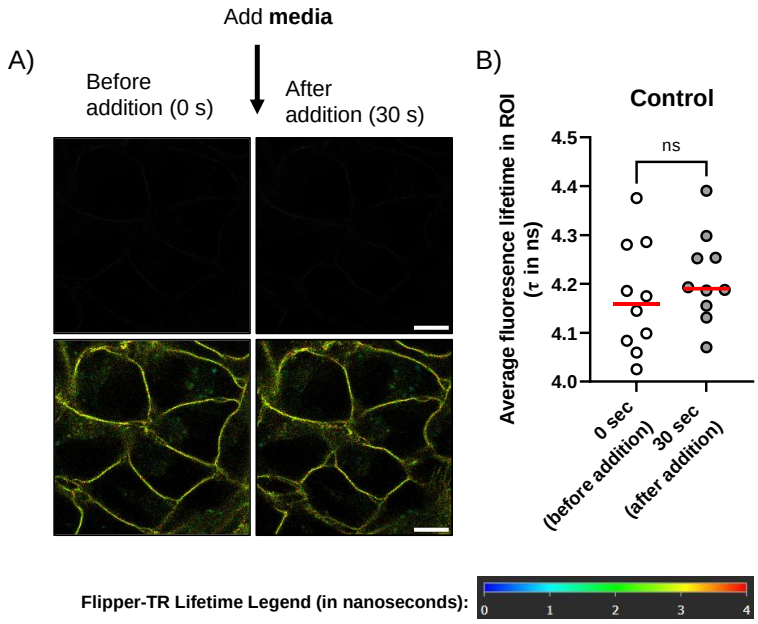


Figure S4. Fluorescence lifetime imaging microscopy (FLIM) images of U2OS cells before and after the addition of media (negative control). (A) The upper panel: represents Cy5 photon count and the lower panels show FastFLIM images. Scale bar = 10 µm. (B) Graph showing the fluorescence lifetime (τ in ns) of 10 nucleation zones across three biological replicates. Red lines indicate the mean τ. Statistical significance was determined by Student's *t*-test. (n.s., P>0.05).

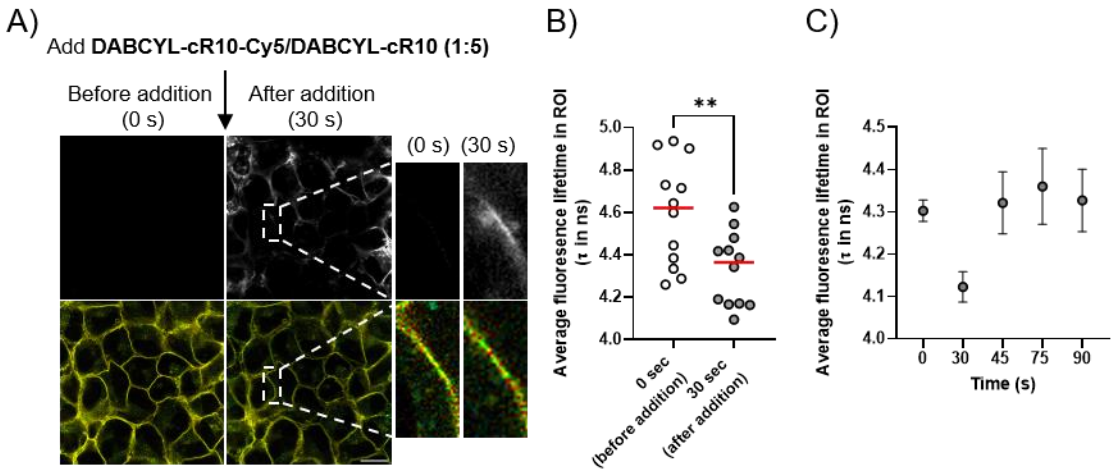
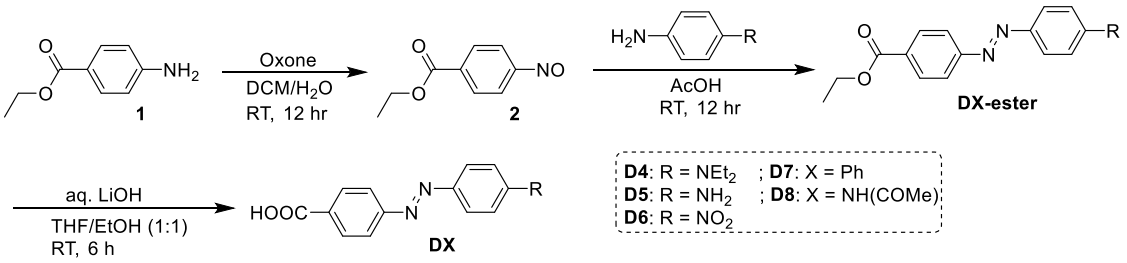


Figure S5. FLIM images of U2OS cells before and after the addition of 1:5 DABCYL-cR10-Cy5/DABCYL-cR10. (A) The upper panel: represents Cy5 photon count and the lower panels

show FastFLIM images. Scale bar = 20 µm. (B) Graph showing the fluorescence lifetime (τ in ns) of 12 nucleation zones across four biological replicates. Red lines indicate the mean τ. Statistical significance was determined by Student's *t*-test. (**, P<0.01). (C) Graph showing the change in fluorescence lifetime in selected nucleation zones over time (s). Data is presented as mean ± standard error (SE) of 8 selected nucleation zones across two biological replicates.

11. Synthesis and characterization of DABCYL-derivatives:

11.1. General synthetic procedure of D4-D8



Scheme S2: Synthesis of DABCYL derivatives; **D4**-**D8**.

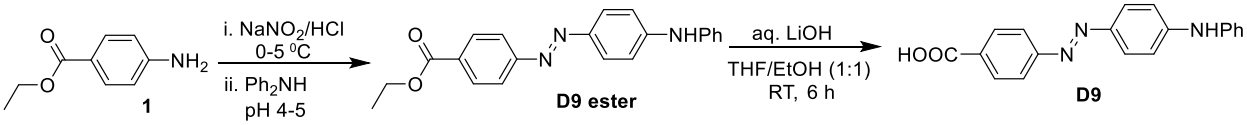
i) Synthesis of ethyl 4-nitrosobenzoate (2): Compound **2** was prepared using a modified literature procedure.[3] In brief, ethyl 4-aminobenzoate, **1** (165.2 mg, 1.0 mmol) was dissolved in distilled dichloromethane, to which an aqueous solution of oxone (1.0 mmol) was added. The reaction mixture was stirred overnight at room temperature and the progress of the reaction was monitored by TLC in the mixture of 3:1 dichloromethane/n-hexane. Subsequently, the two layers were separated and the aqueous layer was washed twice with dichloromethane. Then, the collective organic layer was sequentially washed with 1M HCl, saturated aq. $NaHCO_3$, and brine and the organic solution was dried over Na_2SO_4 and filtered. Finally, the filtered solvents were evaporated under reduced pressure and the resulting crude product was purified on a short pad of silica gel in dichloromethane/n-hexane (2:1). The isolated greenish solid product (95 % yield) was used in the next step without further purification.

ii) General procedure for the synthesis of D4-D8 ester[3]: In a Schlenk flask, ethyl 4-nitrosobenzoate, **2** (2.0 mmol) was dissolved in glacial acetic acid under an argon atmosphere. To this, substituted aromatic amine (1.0 mmol) was added to the green solution. The reaction mixture was allowed to stir at RT for 12 h under an argon atmosphere, an orange precipitate was formed. Then, the precipitate was filtered off, washed with AcOH, and air-dried. Finally, the crude product was purified by column chromatography on silica gel to give the desired product (orange or red solid) as a 70-75 % isolated yield.

iii) General procedure for the base-catalyzed hydrolysis of esters for the preparation of DABCYL derivatives, D4-D8:

The esters were hydrolyzed using a modified literature procedure with slight modifications.[4] In brief, a flask with a teflon-coated magnetic stirrer bar was charged with an ester compound (1.0 mmol) and LiOH•H_2O (3.0 mmol). To this, water/ethanol/tetrahydrofuran (1:1:1 in volume) were added and the mixture was stirred at ambient temperature for 6 h. The reaction was then quenched with 1 M aqueous HCl, and extracted with EtOAc (3 times). Finally, the combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was further purified by column chromatography on silica gel to give the desired product (orange/red solid) as a 98 % isolated yield.

11.2. General synthetic procedure of D9

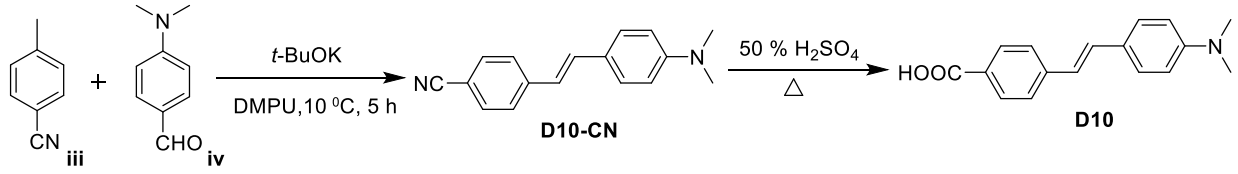


Scheme S3: Synthesis of DABCYL derivative; **D9**.

iv) General procedure of diazotization of (1) followed by coupling with diphenylamine for D9 synthesis[5]: Ethyl 4-aminobenzoate, **1** (2.0 mM) was dissolved in hydrochloric acid (1:1 v/v of conc. HCl and water) and cooled to 0-5 °C with stirring. To this, an aqueous solution of sodium

nitrite (2.0 mM) was added dropwise at 0-5 °C with constant stirring and left for 1 h while maintaining the temperature. Urea was added to the reaction mixture to quench the nitrous acid generated in the reaction. The Completion of the diazotization was monitored by TLC. Meanwhile, diphenylamine (2.0 mM) was dissolved in a mixture of 3:1 acetic acid/propionic acid and cooled to 0-5 °C in a salt-ice bath. The cold diazonium salt solution was added to this solution over 1 h with vigorous stirring in a dropwise manner. Finally, the pH of the reaction solution was adjusted to 4-5 with the addition of dilute potassium hydroxide solution. The mixture was further stirred for 1 h at 0-5 ºC and the resulting solid was filtered, washed with cold water, and dried in air to give the crude orange product, **D9 ester** in 62 % isolated yield. In the next step, base-catalyzed hydrolysis of this ester was performed according to the previous procedure in section (iii), to give the orange solid, **D9** with a 78 % isolated yield.

11.3. General synthetic procedure of D10

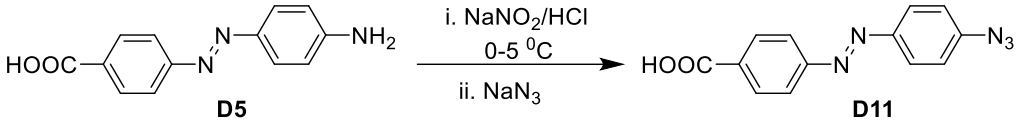


Scheme S4: Synthesis of DABCYL derivative; **D10**.

v) The base-catalyzed condensation of 4-(dimethylamino)benzaldehyde with methylbenzonitriles[6]**:** 4-(dimethylamino)benzaldehyde, **3** (1592 mg, 10 mmol) and 4-methylbenzonitrile, **4** (10 mmol) were dissolved in DMPU. This mixture was then added dropwise to a stirred solution of ^{t}BuOK (11 mmol) in DMPU at 20 °C under nitrogen. After stirring for 6 h, the reaction mixture was poured into ice water containing ammonium chloride (20 mmol) and extracted into dichloromethane and the resulting crude product was used as such in the next step without purification.

vi) General procedure for the acid-catalyzed hydrolysis of nitrile for the preparation of (E)-4-[2-(4-(Dimethylamino)phenyl)vinyl]benzoic Acid, D10[6]: The nitrile, **D10-CN** (1 mM) was heated to 90 °C with 50% sulfuric acid for 36 h. After cooling, the reaction mixture was diluted with water and the pH was adjusted to 3-4 by concentrated ammoniam hydroxide solution. The precipitate was collected, washed with water, and dried. Finally, the crude was purified by column chromatography on silica gel to yield an orange solid with a 65 % isolated yield.

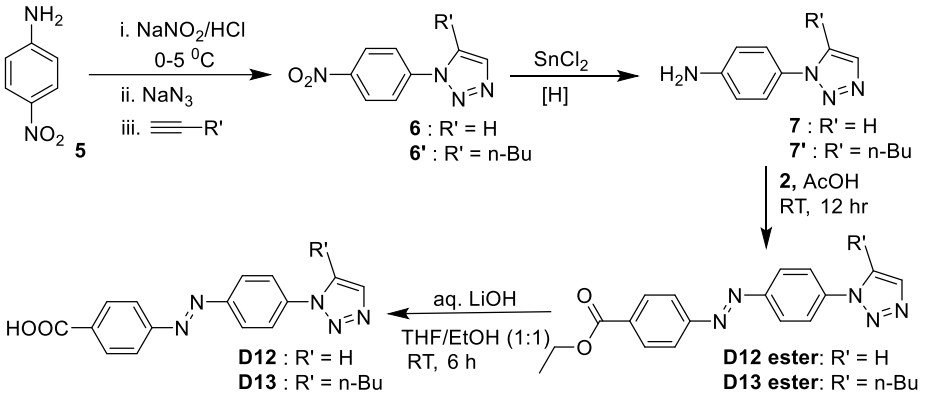
11.4. General synthetic procedure of D11



Scheme S5: Synthesis of DABCYL derivative; **D11**.

vii) General procedure for the synthesis of azides[7]: Solid **D5** (779.6 mg, 3.22 mmol) was dissolved in ethyl acetate and kept at 0 °C. Diazotisation of **D5** was performed similarly as discussed in previous section iv, using 3.87 mmol $NaNO_2$ to prepare its diazonium salt. NaN_3 (3.87 mmol) in water was added to a cold diazonium salt solution and allowed to stir at room temperature for 3 h. After completion of the reaction, the mixture was poured into water, extracted with ethyl acetate (3 times), dried over anhydrous sodium sulfate, and concentrated to give the crude product. Finally, the crude product was purified by column chromatography on silica gel to yield a red solid with a 60 % yield.

11.5. General synthetic procedure of D12-D13



Scheme S6: Synthesis of DABCYL derivatives; **D12**-**D13.**

viii) General procedure of azide-alkyne click reaction for the synthesis of triazoles[8]: Azidation of **5** was performed similarly as discussed in previous section vii. To the isolated solid azide (6.1 mmol) taken in a vial, acetylene (9.14 mmol), potassium carbonate (7.32 mmol), copper sulfate (1.22 mmol), and sodium ascorbate (2.44 mmol) were added and the mixture was dissolved in 1:1 methanol/water (v/v). The vial was sealed and the reaction mixture was stirred at RT for 48 h. Thereafter, NH_4OH solution (5% in water) was added dropwise to the reaction mixture. The two layers were separated and the aqueous layer was extracted thrice with ethyl acetate. The collective organic layer was washed sequentially with water (2 times), and brine. Subsequently, the organic solution was dried over Na_2SO_4 and filtered. The solvents were evaporated under reduced pressure and the resulting crude product was purified using column chromatography to give the solid product with a 55 % isolated yield.

ix) General procedure of $SnCl_2$ reduction for the synthesis of 7 and **7´**[9]**:** Triazole, **6** or **6´** and $SnCl_2$ were taken 1:4 (w/w) in a round flask and the mixture was suspended in ethanol. The solution was then refluxed for 6 h. Thereafter, ethanol was evaporated and dilute NaOH was added to quench excess $SnCl_2$. The aqueous mixture was extracted thrice with dichloromethane. The collective organic layer was washed sequentially with water and brine. Subsequently, the organic solution was dried over Na_2SO_4 and filtered. The filtered solvents were evaporated under

reduced pressure and the resulting crude product was used as such in the next step without purification.

In the next step, ethyl 4-nitrosobenzoate, **2** was dissolved in glacial acetic acid under an argon atmosphere and compound **7** or **7´** was added to the green solution, according to the previous section (ii) to get **D12 ester** and **D13 ester**. The crude product was used as such in the next step without purification. In the following step, base-catalyzed hydrolysis of the ester compounds was performed according to the previous section (iii) to get DABCYL derivatives **D12** and **D13.** Finally, the crude product was purified using column chromatography to give the solid product with a 65 % isolated yield.

11.6. Chemical characterization of the synthesized compounds:

Compound D4 ester: ^{1}H NMR (400 MHz, $CDCl_3$) δ_{ppm} 8.15 (d, *J* = 8.2 Hz, 2H), 7.90 – 7.85 (m, 4H), 6.72 (d, *J* = 12.0 Hz, 2H), 4.40 (q, *J* = 7.1 Hz, 2H), 3.45 (q, *J* = 7.0 Hz, 4H), 1.42 (t, *J* = 6.0 Hz, 3H), 1.22 (t, *J* = 6.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_{ppm} 166.5, 156.2, 150.8, 143.4, 130.6, 130.4, 126.0, 122.0, 111.1, 61.2, 44.9, 14.5, 12.8; HRMS (ESI): observed m/z for $C_{19}H_{23}N_3O_2$ $[M + H]^+$ 326.1871 (calcd. 326.1869).

Compound D4: ^{1}H NMR (400 MHz, DMSO-d_6) δ_{ppm} 13.08 (brs, 1H), 8.07 (d, *J* = 8.0 Hz, 2H), 7.84 – 7.81 (m, 4H), 6.83 (d, *J* = 12.0 Hz, 2H), 3.49 (q, *J* = 7.0 Hz, 4H), 1.17 (t, *J* = 8.0 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_{ppm} 167.4, 155.6, 151.2, 142.7, 131.2, 130.9, 126.2, 122.1, 112.0, 44.6, 13.0; HRMS (ESI): observed m/z for $C_{17}H_{19}N_3O_2$ $[M + H]^+$ 298.1558 (calcd.298.1556).

Compound D5 ester: ^{1}H NMR (400 MHz, $CDCl_3$) δ_{ppm} 8.16 (d, *J* = 8.5 Hz, 2H), 7.88 – 7.82 (m, 4H), 6.77 – 6.73 (m, 2H), 4.40 (q, *J* = 7.1 Hz, 2H), 4.14 (brs, 2H), 1.42 (t, *J* = 8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_{ppm} 165.3, 154.6, 149.3, 144.5, 129.9, 129.5, 124.6, 121.1, 113.6, 60.1, 13.3; HRMS (ESI): observed m/z for $C_{15}H_{15}N_3O_2$ $[M + H]^+$ 270.1238 (calcd. 270.1243).

Compound D5: ^{1}H NMR (400 MHz, DMSO-d_6) δ_{ppm} 8.06 (d, *J* = 8.0 Hz, 2H), 7.80 (d, *J* = 8.0 Hz, 2H), 7.70 (d, *J* = 8.0 Hz, 2H), 6.70 (d, *J* = 8.0 Hz, 2H), 6.28 (brs, 2H); ^{13}C NMR (100 MHz, DMSO-

d$_6$) δ$_{ppm}$ 167.3, 155.4, 153.8, 143.2, 130.9, 130.8, 126.1, 121.9, 113.7; HRMS (ESI): observed m/z for $C_{13}H_{11}N_3O_2$ $[M + H]^+$ 242.0929 (calcd. 242.0930).

Compound D6 ester: ^{1}H NMR (400 MHz, $CDCl_3$) δ$_{ppm}$ 8.41 – 8.37 (m, 2H), 8.23 – 8.20 (m, 2H), 8.08 – 8.03 (m, 2H), 8.01 – 7.98 (m, 2H), 4.41 (q, *J* = 7.1 Hz, 2H), 1.42 (t, *J* = 8.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ$_{ppm}$ 164.8, 154.4, 153.6, 148.1, 132.4, 129.7, 123.8, 122.7, 122.1, 60.4, 13.3; HRMS (APCI): observed m/z for $C_{15}H_{13}N_3O_2$ $[M + H]^+$ 300.1003 (calcd. 300.0979).

Compound D6: ^{1}H NMR (200 MHz, DMSO-*d*$_6$) δ$_{ppm}$ 13.36 (brs, 1H), 8.46 (d, *J* = 16 Hz, 2H), 8.20 – 8.02 (m, 6H); ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ$_{ppm}$ 167.1, 155.5, 154.3, 149.3, 131.2, 125.7, 124.2, 123.5; HRMS (ESI): observed m/z for $C_{13}H_9N_3O_4$ $[M - H]^-$ 270.0519 (calcd. 270.0515).

Compound D7 ester: ^{1}H NMR (400 MHz, $CDCl_3$) δ$_{ppm}$ 8.15 – 8.12 (m, 2H), 7.98 – 7.94 (m, 2H), 7.91 – 7.88 (m, 2H), 7.71 – 7.68 (m, 2H), 7.61 – 7.59 (m, 2H), 7.43 – 7.39 (m, 2H), 7.35 – 7.31 (m, 1H), 4.35 (q, *J* = 7.1 Hz, 2H), 1.36 (t, *J* = 8.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ$_{ppm}$ 165.1, 154.2, 150.7, 143.4, 139.0, 131.1, 129.6, 127.9, 127.0, 126.8, 126.2, 122.7, 121.6, 60.3, 13.3; HRMS (ESI): observed m/z for $C_{21}H_{18}N_2O_2$ $[M + H]^+$ 331.1448 (calcd. 331.1447).

Compound D7: ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ$_{ppm}$ 13.23 (brs, 1H), 8.13 – 8.07 (m, 2H), 8.0 – 7.87 (m, 6H), 7.76 – 7.73 (m, 2H), 7.50 – 7.37 (m, 3H); ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ$_{ppm}$ 167.2, 154.8, 151.5, 144.1, 139.3, 133.3, 131.2, 129.6, 128.9, 128.3, 127.4, 127.1, 124.0, 123.1; HRMS (ESI): observed m/z for $C_{19}H_{14}N_2O_2$ $[M + H]^+$ 303.1131 (calcd. 303.1134).

Compound D8 ester: ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ$_{ppm}$ 10.35 (brs, 1H), 8.09 (d, *J* = 8.4 Hz, 2H), 7.90 – 7.81 (m, 6H), 4.31 (t, *J* = 8.0 Hz, 2H), 2.11 (s, 3H), 1.33 (t, *J* = 8.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ$_{ppm}$ 169.4, 165.6, 155.0, 147.8, 143.6, 131.7, 130.8, 124.6, 122.8, 119.5, 61.5, 24.7, 14.6. HRMS (ESI): observed m/z for $C_{17}H_{17}N_3O_3$ $[M + H]^+$ 312.1348 (calcd. 312.1348).

Compound D8: ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ$_{ppm}$ 13.12 (brs, 1H), 10.34 (brs, 1H), 8.14 – 8.08 (m, 2H), 7.91 – 7.84 (m, 4H), 7.73 – 7.71 (m, 1H), 6.72 – 6.70 (m, 1H), 2.11 (s, 3H). ^{13}C NMR

(100 MHz, DMSO-d_6) δ_{ppm} 169.4, 167.3, 154.1, 154.9, 147.9; HRMS (ESI): observed m/z for $C_{15}H_{13}N_3O_3$ $[M + H]^+$ 284.1071 (calcd. 284.1035).

Compound D9 ester: ^{1}H NMR (400 MHz, DMSO-d_6) δ_{ppm} 9.05 (brs, 1H), 8.13 – 8.11 (m, 2H), 7.91 – 7.86 (m, 4H), 7.39 – 7.35 (m, 2H), 7.25 (d, *J* = 8.0 Hz, 2H), 7.19 (d, *J* = 8.0 Hz, 2H), 7.04 (t, *J* = 8.0 Hz, 1H), 4.36 (q, *J* = 7.1 Hz, 2H), 1.36 (t, *J* = 8.0 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_{ppm} 165.7, 155.5, 148.8, 145.3, 141.5, 130.9, 129.9, 126.0, 122.7, 122.5, 120.2, 115.2, 61.4, 14.7; HRMS (ESI): observed m/z for $C_{21}H_{19}N_3O_2$ $[M + H]^+$ 346.1552 (calcd. 346.1556).

Compound D9: ^{1}H NMR (400 MHz, DMSO-d_6) δ_{ppm} 13.13 (brs, 1H), 9.02 (brs, 1H), 8.10 (d, *J* = 8.1 Hz, 2H), 7.88 – 7.85 (m, 4H), 7.38 – 7.35 (m, 2H), 7.26 – 7.18 (m, 4H), 7.05 – 7.01 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_{ppm} 167.3, 155.3, 148.7, 145.3, 141.5, 131.0, 129.9, 125.9, 122.7, 122.4, 120.1, 115.2; HRMS (ESI): observed m/z for $C_{19}H_{15}N_3O_2$ $[M + H]^+$ 318.1241 (calcd. 318.1243).

Compound D10: ^{1}H NMR (300 MHz, DMSO-d_6) δ_{ppm} 12.76 (brs, 1H), 7.86 – 7.83 (m, 2H), 7.60 – 7.57 (m, 2H), 7.44 – 7.42 (m, 2H), 7.25 (d, *J* = 16.4 Hz, 1H), 7.00 (d, *J* = 16.6 Hz, 1H), 6.70 – 6.67 (m, 2H), 2.90 (s, 6H);^{13}C NMR (100 MHz, DMSO-d_6) δ_{ppm} 168.0, 150.1, 141.4, 130.1, 128.6, 128.4, 125.8, 124.2, 49.05; HRMS (ESI): observed m/z for $C_{17}H_{17}NO_2$ $[M + H]^+$ 268.1347 (calcd. 268.1338).

Compound D11: ^{1}H NMR (400 MHz, DMSO-d_6) δ_{ppm} 8.46 (d, *J* = 8.0 Hz, 2H), 8.18 – 8.12 (m, 4H), 8.04 (d, *J* = 8.1 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_{ppm} 167.3, 154.6, 149.5, 143.7, 131.1, 130.8, 125.2, 123.0, 120.7; HRMS (ESI): observed m/z for $C_{13}H_9N_5O_2$ $[M - H]^-$ 266.0682 (calcd. 266.0678).

Compound 6: ^{1}H NMR (400 MHz, DMSO-d_6) δ_{ppm} 9.05 (d, *J* = 1.1 Hz, 1H), 8.46 – 8.43 (m, 2H), 8.26 – 8.22 (m, 2H), 8.07 (d, *J* = 1.1 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_{ppm} 147.1, 141.4, 135.6, 126.0, 124.3, 121.1; HRMS (ESI): observed m/z for $C_8H_6N_4O_2$ $[M + H]^+$ 191.0560 (calcd. 191.0564).

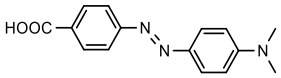
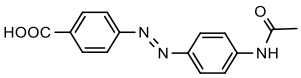
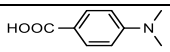
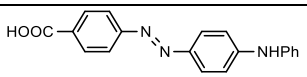
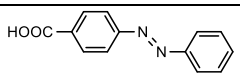
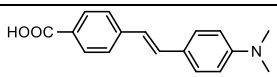
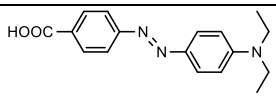
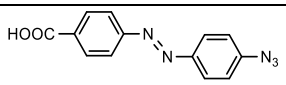
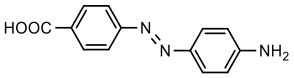
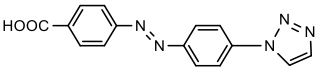
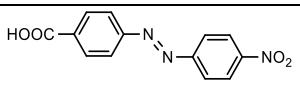
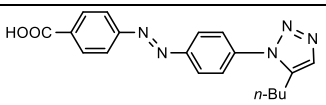
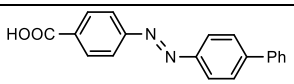
Compound 6´: ^{1}H NMR (400 MHz, $CDCl_3$) δ_{ppm} 8.48 – 8.44 (m, 1H), 7.96 – 7.93 (m, 1H), 7.82 (s, 1H), 2.79 – 2.75 (m, 1H), 1.58 – 1.51 (m, 1H), 1.33 – 1.23 (m, 1H), 0.84 (t, *J* = 8.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_{ppm} 152.7, 146.2, 144.0, 138.0, 131.2, 130.4, 117.6, 34.7, 27.9, 26.8, 18.7; HRMS (ESI): m/z calcd. for $C_{12}H_{14}N_4O_2$ $[M + H]^+$ 247.1199 (calcd. 247.1195).

Compound D12: ^{1}H NMR (400 MHz, DMSO-d_6) δ_{ppm} 13.26 (brs, 1H), 9.01 (d, *J* = 1.1 Hz, 1H), 8.21 – 8.16 (m, 6H), 8.15 – 8.01 (m, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_{ppm} 167.2, 154.6, 151.6, 139.3, 135.3, 131.2, 124.9, 123.9, 123.2, 121.4; HRMS (ESI): observed m/z for $C_{19}H_{23}N_3O_2$ $[M - H]^-$ 292.0836 (calcd. 292.0834).

Compound D13: ^{1}H NMR (400 MHz, DMSO-d_6) δ_{ppm} 13.25 (brs, 1H), 8.76 (s, 1H), 8.19 – 8.13 (m, 6H), 8.01 (d, *J* = 8.6 Hz, 2H), 2.73 (t, *J* = 8.0 Hz, 2H), 1.72 – 1.64 (m, 2H), 1.42 – 1.37 (m, 2H), 0.94 (t, *J* = 8.0 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_{ppm} 169.7, 161.3, 154.7, 149.0, 147.1, 145.1, 131.2, 125.0, 123.2, 120.9, 31.3, 25.1, 22.2, 14.2; HRMS (ESI): observed m/z for $C_{19}H_{23}N_3O_2$ $[M + H]^+$ 350.1617 (calcd. 350.1617).

M = formula weight

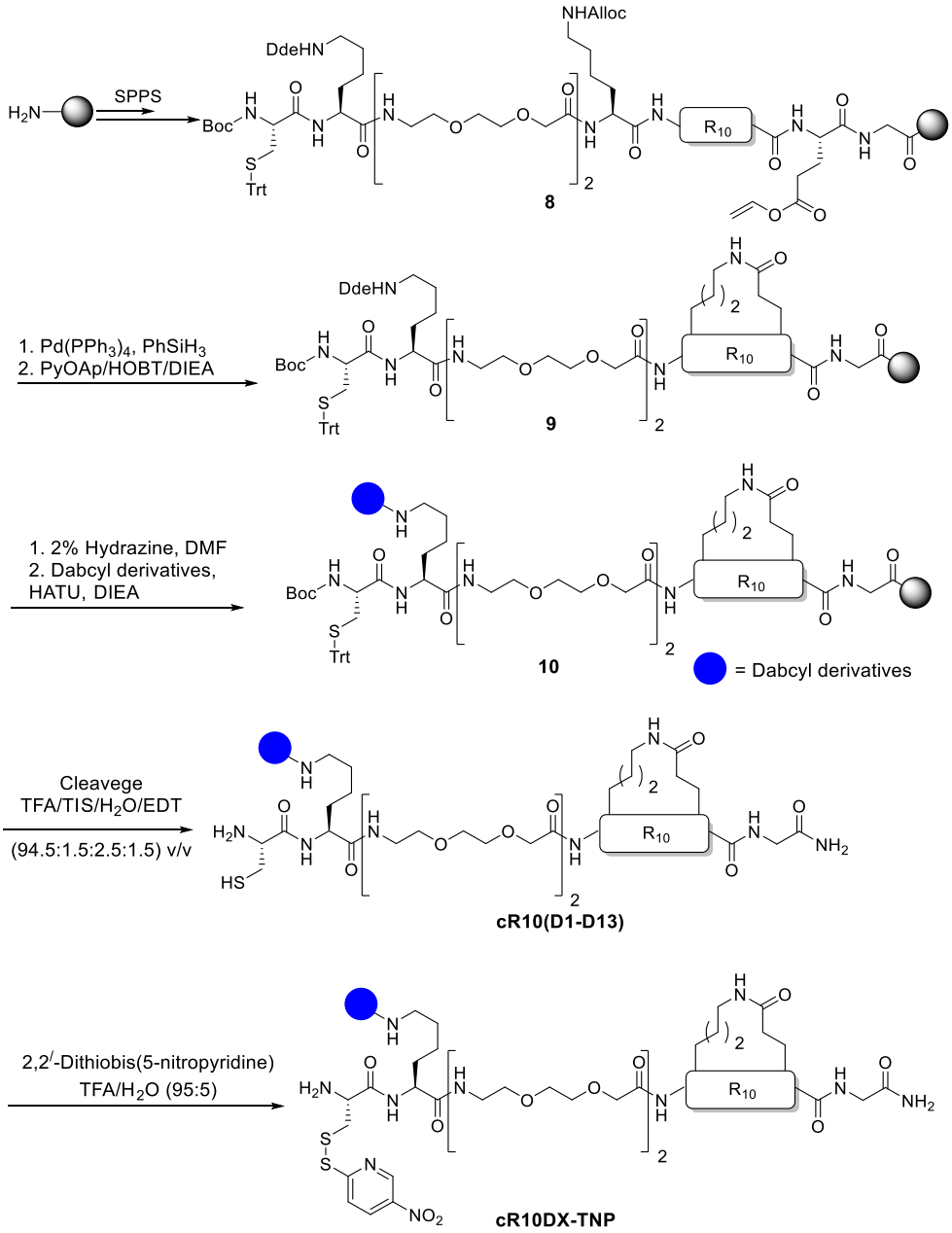
12. Table of logP values of DABCYL-derivatives

Entry of DABCYL-derivatives	log*P*	Entry of DABCYL-derivatives	log*P*
 **D1**	4.14	 **D8**	3.27
 **D2**	1.74	 **D9**	5.48
 **D3**	4.04	 **D10**	4.08
 **D4**	4.86	 **D11**	4.34
 **D5**	3.21	 **D12**	3.74
 **D6**	3.98	 **D13**	5.35
 **D7**	5.68		

*Theoretical log*P* values were calculated in Marvin Sketch (programme17.27).

13. Synthesis of the activated form of cyclic deca-arginine(DABCYL-derivatives), cR10DX(TNP):

cR10DX sequence: CK(DX)$(PEG)_2$KRrRrRrRrRrEG; DX = DABCYL-derivatives, capital letters 'R' and small letters 'r' correspond to L-arginine and D-arginine, respectively.



Scheme S7: Synthesis of activated **cR10D1**-**cR10D13**.

For synthesizing **cR10(D1-D13)** cell-penetrating peptides**,** all amino acids in the sequence were manually double coupled on pre-swollen Rink amide resin (0.1 mmol) using the standard procedure (section 2) to give **8**. Then, Alloc and OAllyl protecting groups were simultaneously removed with the mentioned conditions, and the cyclization was performed on the solid support using 5 eq. of PyAOP, 5 eq. of HOBT, and 10 eq. of DIEA in DMF (8 ml for 0.1 mmol) for 90 min to afford **9**. After cyclization, the sequence was completed and was followed by the Dde protecting group removal by three cycles of 2% hydrazine in DMF for 30 min each cycle to give **10**. Upon deprotection, DABCYL was coupled using 4 eq. DABCYL, 4 eq. HATU and 8 eq. DIEA for 2 h. The peptide was then cleaved with a cocktail of TFA/triisopropyl silane (TIS)/water/1,2 ethelenedithiol (94.5:1.5:2.5:1.5) was added to the resin and the reaction mixture was shaken for 1h at RT and precipitated in cold ether and centrifuged (in dark). Finally, the crude CPP was purified by preparative HPLC using a C18 column with a gradient of 0-60% buffer B over 30 min to give desired CPPs, **cR10(D1-D13)** in ~25-30 % yield. The Cys residue of **cR10DX** was then activated using 20 eq. DTNP in 95% TFA/H_2O for 1 h following DCM and cold ether washes to give TNP-linked CPPs, **cR10DX-TNP** (yield 80%) and used in the next step without further purification.

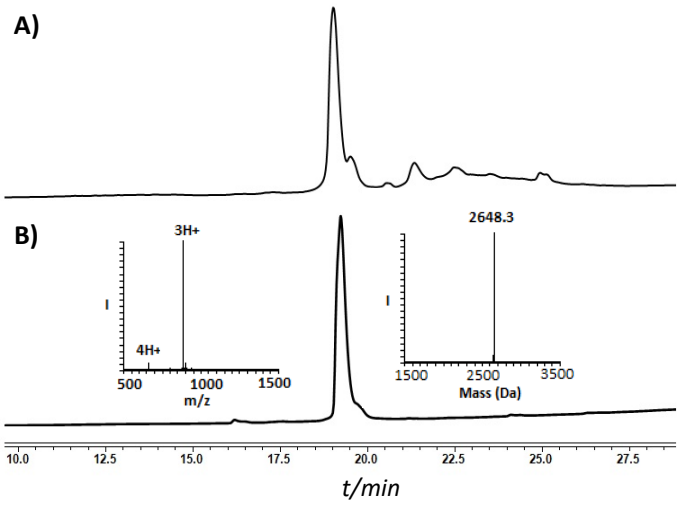


Figure S6. Synthesis of **cR10D1** peptides: (A) Analytical HPLC of crude **cR10D1**. (B) Analytical HPLC and mass analysis of purified **cR10D1** with the observed mass 2648.3 Da, calcd 2648 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

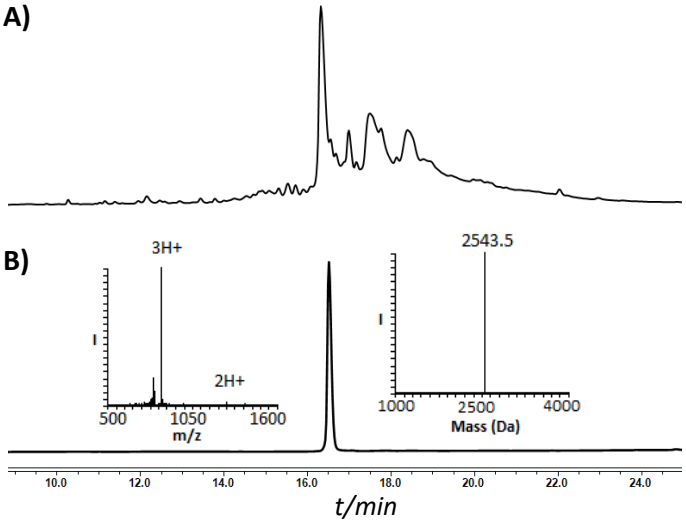


Figure S7. Synthesis of **cR10D2** peptides: (A) Analytical HPLC of crude **cR10D2**. (B) Analytical HPLC and mass analysis of purified **cR10D2** with the observed mass 2543.5 Da, calcd 2544 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

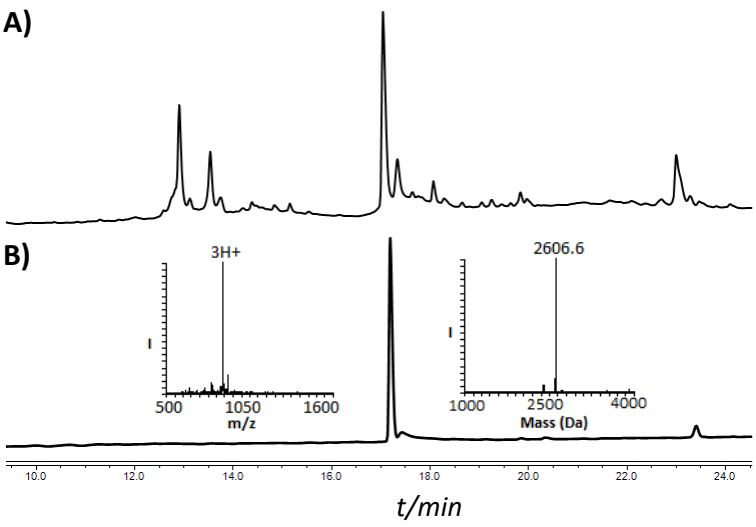


Figure S8. Synthesis of **cR10D3** peptides: (A) Analytical HPLC of crude **cR10D3**. (B) Analytical HPLC and mass analysis of purified **cR10D3** with the observed mass 2606.6 Da, calcd 2605 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

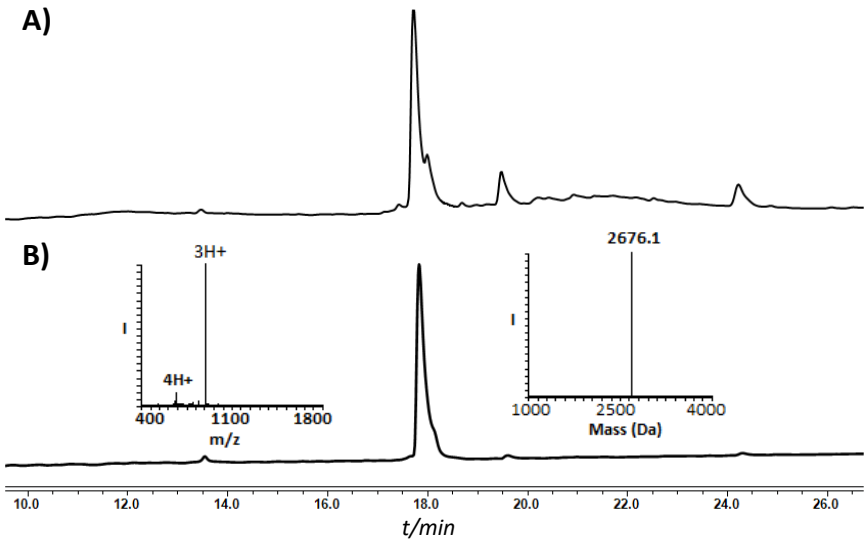


Figure S9. Synthesis of **cR10D4** peptides: (A) Analytical HPLC of crude **cR10D4**. (B) Analytical HPLC and mass analysis of purified **cR10D4** with the observed mass 2676.1 Da, calcd 2676 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

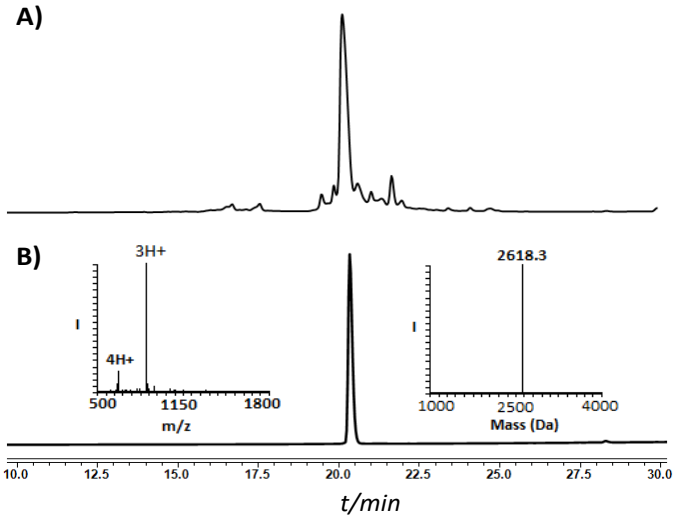


Figure S10. Synthesis of **cR10D5** peptides: (A) Analytical HPLC of crude **cR10D5**. (B) Analytical HPLC and mass analysis of purified **cR10D5** with the observed mass 2618.3 Da, calcd 2619 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

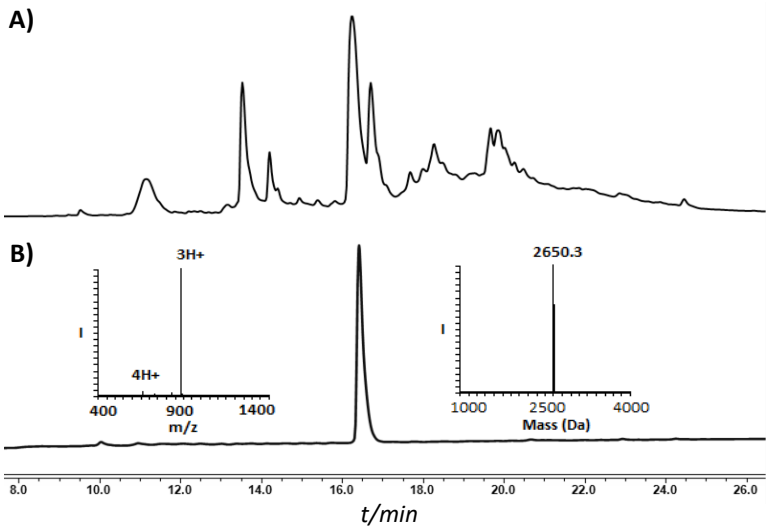


Figure S11. Synthesis of **cR10D6** peptides: (A) Analytical HPLC of crude **cR10D6**. (B) Analytical HPLC and mass analysis of purified **cR10D6** with the observed mass 2650.3 Da, calcd 2650 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

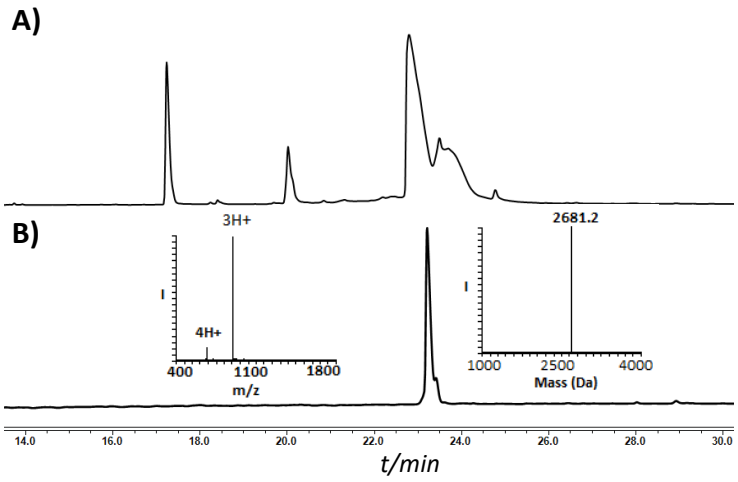


Figure S12. Synthesis of **cR10D7** peptides: (A) Analytical HPLC of crude **cR10D7**. (B) Analytical HPLC and mass analysis of purified **cR10D7** with the observed mass 2681.2 Da, calcd 2681 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

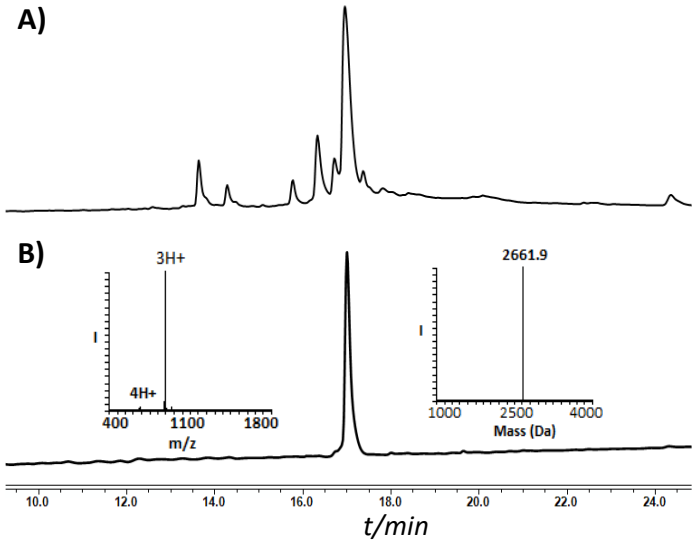


Figure S13. Synthesis of **cR10D8** peptides: (A) Analytical HPLC of crude **cR10D8**. (B) Analytical HPLC and mass analysis of purified **cR10D8** with the observed mass 2661.9 Da, calcd 2661.99 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

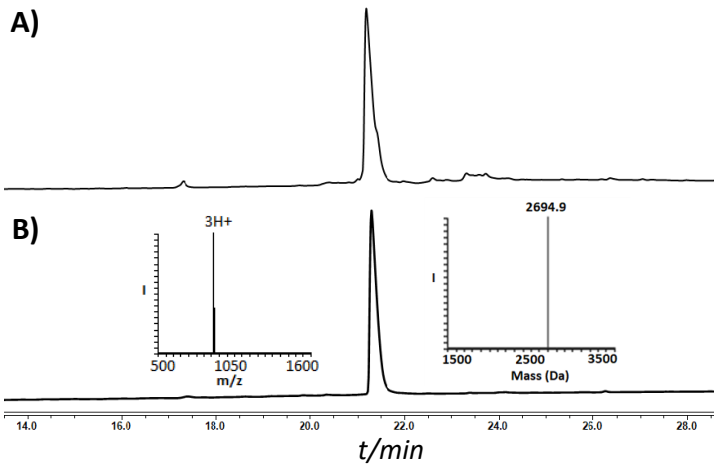


Figure S14. Synthesis of **cR10D9** peptides: (A) Analytical HPLC of crude **cR10D9**. (B) Analytical HPLC and mass analysis of purified **cR10D9** with the observed mass 2694.9 Da, calcd 2695.6 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

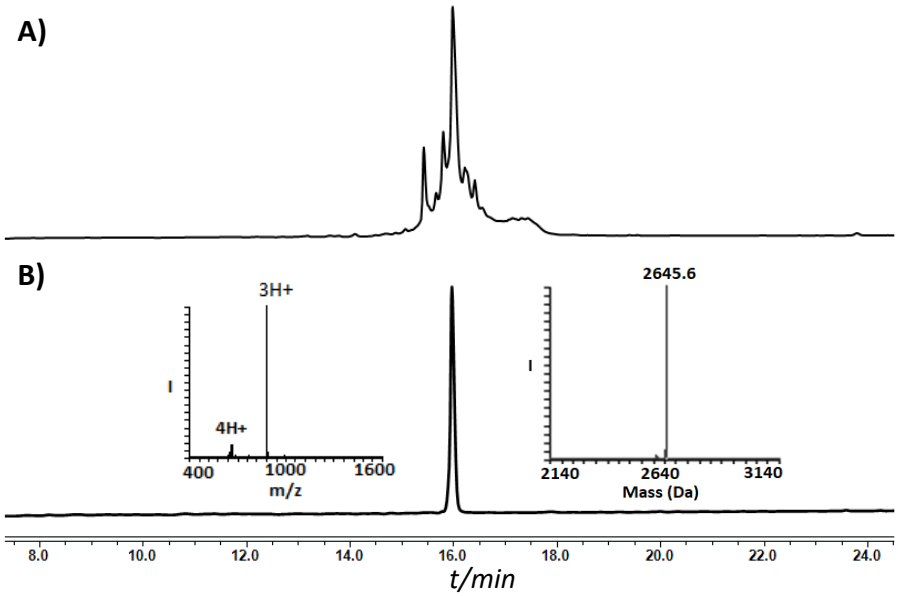


Figure S15. Synthesis of **cR10D10** peptides: (A) Analytical HPLC of crude **cR10D10**. (B) Analytical HPLC and mass analysis of purified **cR10D10** with the observed mass 2645.6 Da, calcd 2646 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

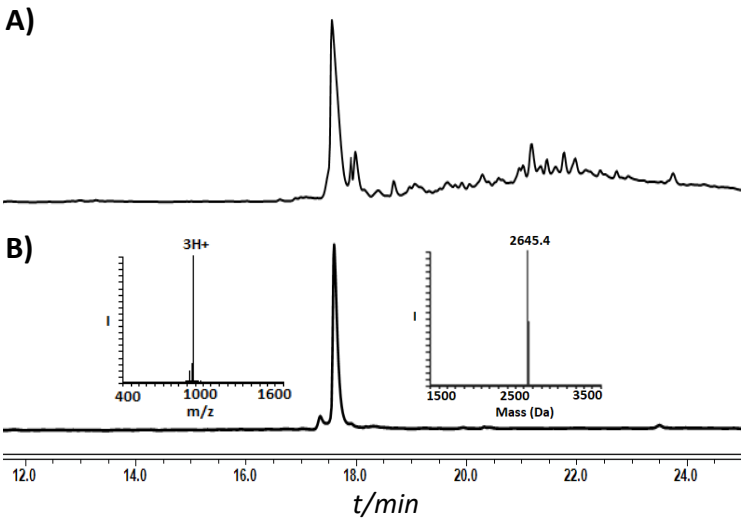


Figure S16. Synthesis of **cR10D11** peptides: (A) Analytical HPLC of crude **cR10D11**. (B) Analytical HPLC and mass analysis of purified **cR10D11** with the observed mass 2645.4 Da, calcd 2645.9 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

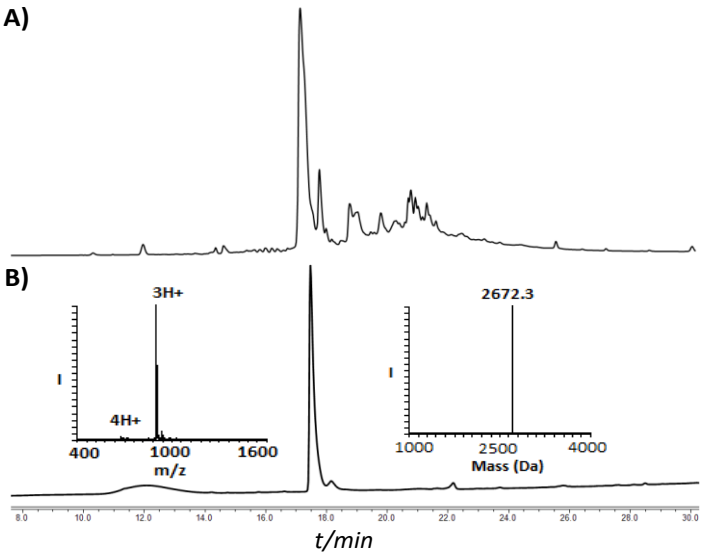


Figure S17. Synthesis of **cR10D12** peptides: (A) Analytical HPLC of crude **cR10D12**. (B) Analytical HPLC and mass analysis of purified **cR10D12** with the observed mass 2672.3 Da, calcd 2672 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

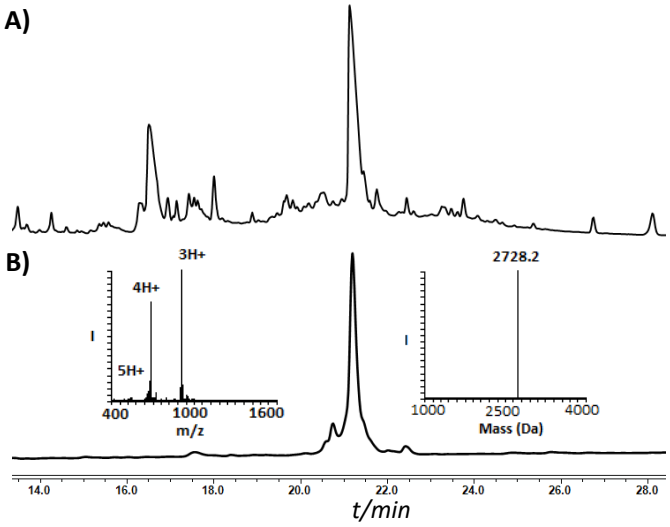
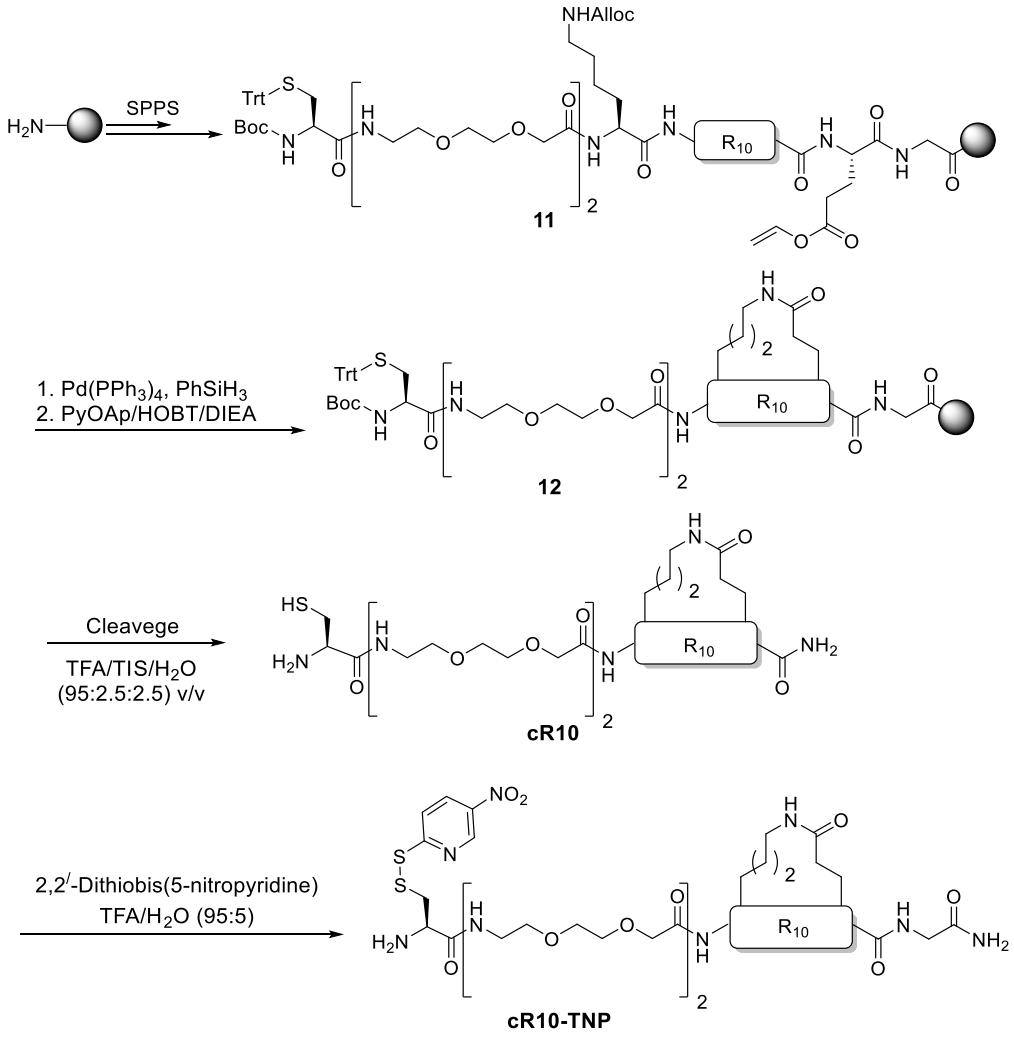


Figure S18. Synthesis of **cR10D13** peptides: (A) Analytical HPLC of crude **cR10D13**. (B) Analytical HPLC and mass analysis of purified **cR10D13** with the observed mass 2728.2 Da, calcd 2728 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

14. Synthesis of the activated form of cyclic deca-arginine, cR10-TNP:

cR10 sequence: CK(PEG)$_2$KRrRrRrRrRrEG; capital letters “R” and small letters “r” correspond to L-arginine and D-arginine, respectively.



Scheme S8: Synthesis of activated **cR10**.

For synthesizing the **cR10** peptide, all amino acids were manually double coupled on pre-swollen Rink amide resin (0.1 mmol) using 4 eq. amino acids, 4 eq. HATU and 8 eq. DIEA for 1 h. The linkers [2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid (abbreviated as PEG) were manually

coupled using 2 eq. Fmoc linker, 2 eq. HATU and 4 eq. DIEA for 2 h to give **11**. After completion of the sequence, Alloc and OAllyl protecting groups were simultaneously removed and the cyclization was performed on the solid support using 5 eq. of PyAOP, 5 eq. of HOBT, and 10 eq. of DIEA in DMF (8 ml for 0.1 mmol) for 90 min to give **12**. The peptide was then cleaved as mentioned above and purified by preparative HPLC using a C18 column with a gradient of 0-60% buffer B over 30 min to afford the corresponding cR10 peptide in ~25 % yield. The Cys residue of cR10 was then activated using 20 eq. DTNP in 95% TFA/H_2O for 1 h which upon purification gives cR10(TNP) 20 in 85% yield.

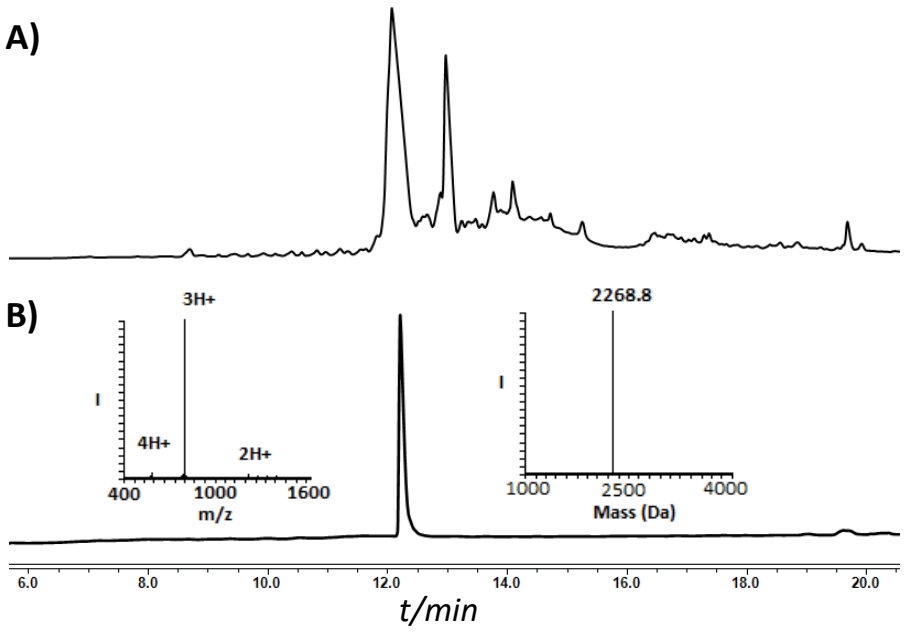


Figure S19. Synthesis of **cR10D14** peptides: (A) Analytical HPLC of crude **cR10D14**. (B) Analytical HPLC and mass analysis of purified **cR10D14** with the observed mass 2268.8 Da, calcd 2268 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

15. Stability of trans-azobenzene conjugated peptides under experimental conditions and sunlight:

To examine the stability of trans-azobenzenes, we have synthesized **D3** and **D11** coupled LAKAG by the previously described SPPS method using 4 eq. AA, 4 eq. HATU and 8 eq. DIEA. The peptide was then cleaved using TFA/TIS/H_2O/EDT (94.5:1.5:2.5:1.5) and purified by semi-preparative HPLC (C18 column) with a gradient of 0-60% buffer B over 30 min to afford the **D3-LAKAG** (**13**) and **D11-LAKAG** (**14**) with ~90% yield.

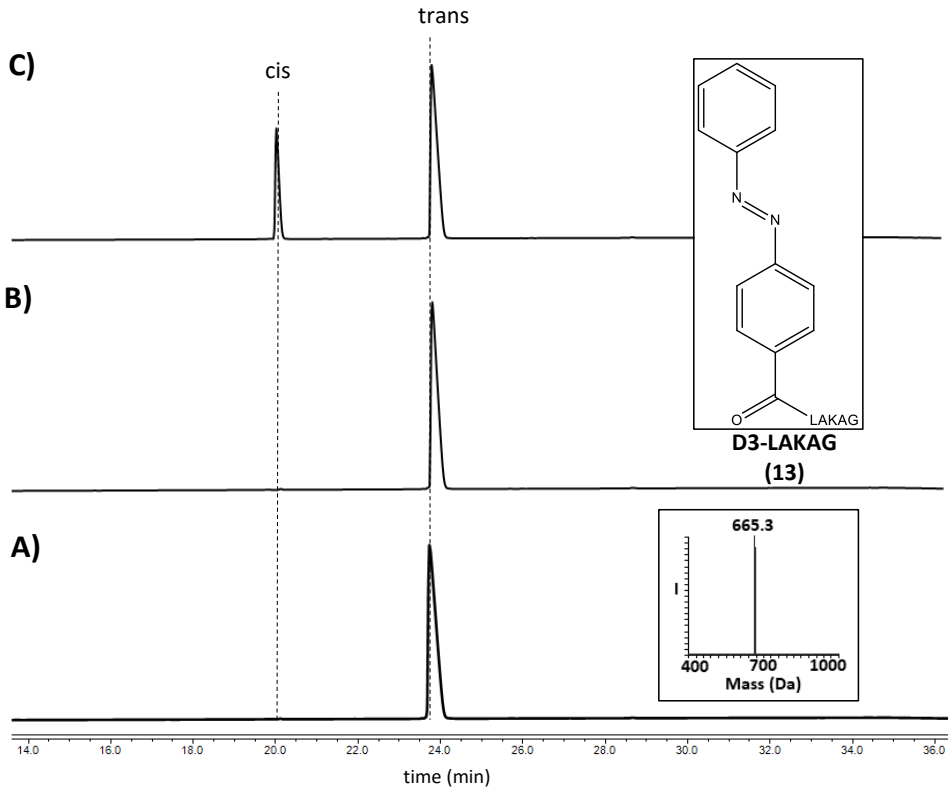


Figure S20. (A) Analytical HPLC and mass analysis of purified **13**. (B) Solution (50% ACN/water) of purified **13** was kept in dark under experimental condition for 2 h. (C) Under sunlight for 1 h (cis/trans: 26:74). The observed mass of **13** (cis or trans) was found 665.3 Da, calcd 665.1 Da (average isotopes) with the detection of HPLC by 214 nm.

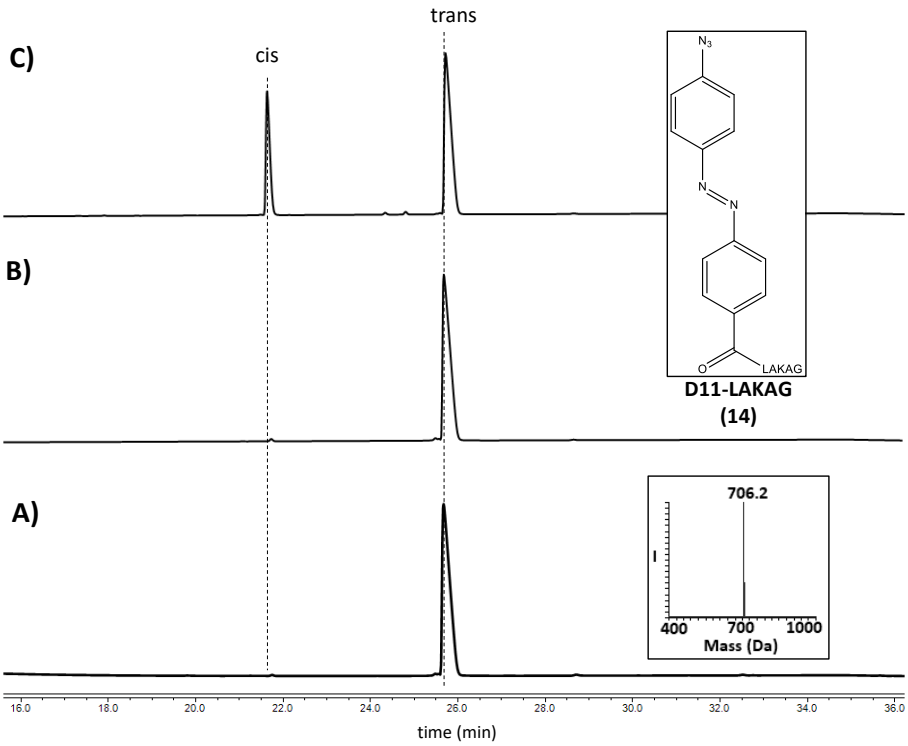
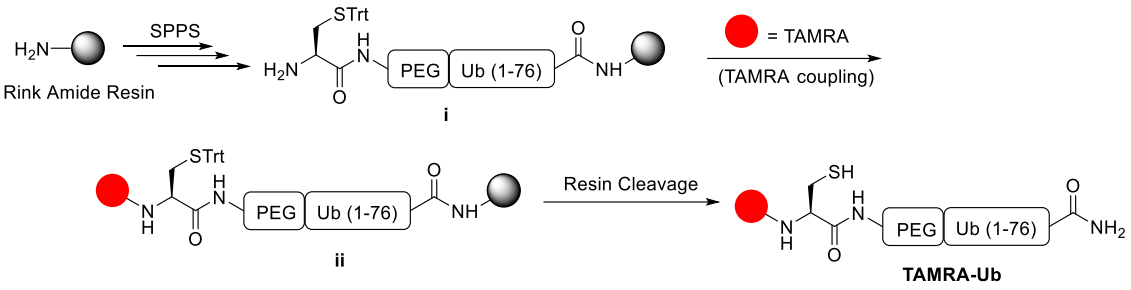


Figure S21. (A) Analytical HPLC and mass analysis of purified **14**. (B) Solution (50% ACN/water) of purified **14** was kept in dark under experimental condition for 2 h. (C) Under sunlight for 1 h (cis/trans: 32:68). The observed mass of **14** (cis or trans) was found 706.2 Da, calcd 706.5 Da (average isotopes) with the detection of HPLC by 214 nm.

16. Synthesis of TAMRA-Cys-PEG-Ub(1-76)-CONH$_2$ (TAMRA-Ub):



Scheme S9: Synthesis of **TAMRA-Ub**.

TAMRA-Cys-PEG-Ub(1-76)-CONH$_2$ (**TAMRA-Ub**), was prepared on Rink Amide resin (0.1 mmol). Fmoc amino acids were coupled on an automated peptide synthesizer using 4 equiv amino acids, 4 equiv HCTU, and 8 equiv of DIEA. Pseudoproline dipeptides Fmoc-Lue-Thr($\psi^{Me,\ Me}$Pro)-OH, Fmoc-Ile-Thr($\psi^{Me,\ Me}$Pro)-OH, Fmoc-Asp(O^{t}Bu)-(DMB)Gly-OH and Fmoc-Leu-Ser($\psi^{Me,\ Me}$Pro)-OH were coupled manually at positions Ile13–Thr14, Lue8–Thr9, Asp52-Gly53 and Leu56 -Ser57 using 2.5 eq. of the dipeptide, 2.5 eq. of HATU and 5 eq. of DIEA for 1.5 h and the linker [2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid (abbreviated as PEG) was coupled manually using 4 eq. amino acid, 4 eq. HATU and 8 eq. DIEA for 1.5 h to afford **i**. After completion of the sequence, the peptide resin was washed with DMF (3 × 5 mL) and TAMRA was coupled to the freed N-terminus using 1.5 eq. TAMRA, 1.5 eq. HATU and 3 eq. DIEA for 2 h to afford **ii.** Finally, the peptide was cleaved using TFA:TIS: water (95:2.5:2.5) cocktail (9 mL for 0.025 mmol peptide resin) for 2 h to give TAMRA-Cys-PEG-Ub(1-76)-CONH$_2$ (**TAMRA-Ub**). The cleavage mixture was filtered dropwise to a 10-fold volume of cold ether and centrifuged. The precipitated crude peptide was dissolved in acetonitrile-water (1:1) and lyophilized. The crude peptide **TAMRA-Ub** was purified by preparative HPLC using a C4 column with a gradient of 0-60% buffer B over 30 min with an isolated yield of ~25 % isolated yield.

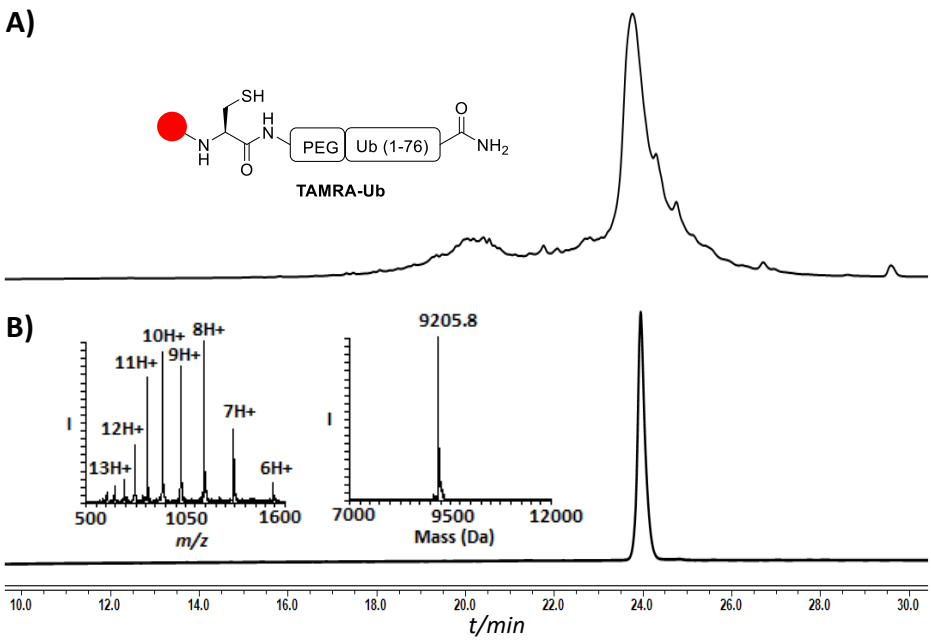
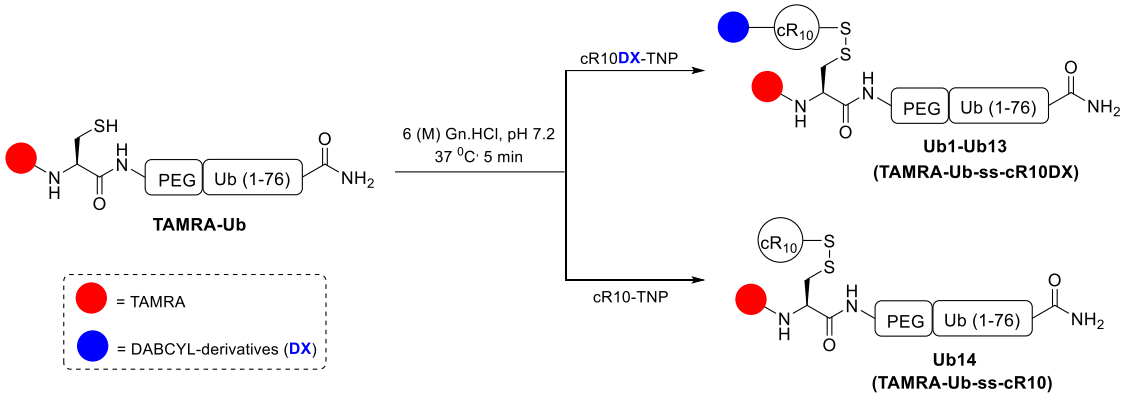


Figure S22. Synthesis of **TAMRA-Ub**: (A) Analytical HPLC of crude **TAMRA-Ub**. (b) Analytical HPLC and mass analysis of purified **TAMRA-Ub** with the observed mass 9205.8 Da, calcd 9206 Da (average isotopes). Detection of HPLC by 214 nm.

17. Synthesis of CPP-linked Ub conjugates (Ub1-Ub14):



Scheme S10: Synthesis of CPP-Ub conjugates; TAMRA-Ub-ss-cR10(D1-D13): **Ub1**-**Ub13**, and TAMRA-Ub-ss-cR10: **Ub14**.

To conjugate Cys functionalized CPPs [cR10DX(TNP) and cR10(TNP)] to **TAMRA-Ub**, DTNP-activated CPPs were dissolved in 6M Gn·HCl (pH=7.2) in a final concentration of 2 mM. The CPP-containing solution was added to the **TAMRA-Ub** solution and the mixture was kept at 37 °C for 5 min in dark. Next, the reaction mixture was purified by preparative reverse HPLC using a C4 column with a gradient of 0-60% buffer acetonitrile over 35 min to afford the corresponding **Ub1**-**Ub14** with an isolated yield of 40-45 %.

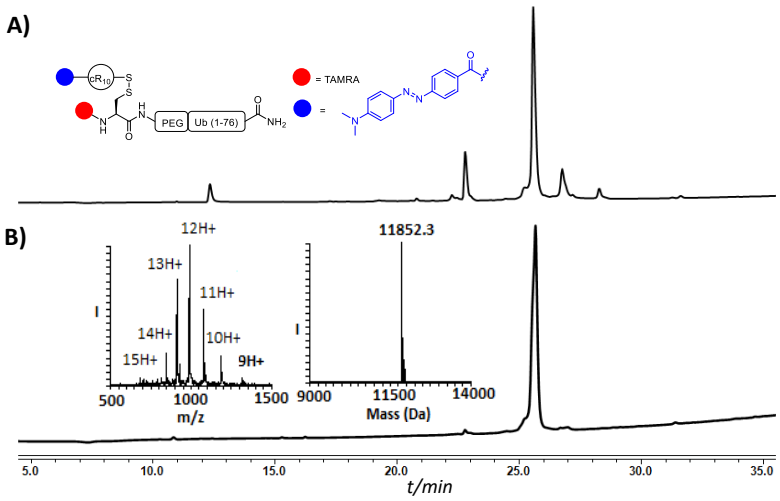


Figure S23. Synthesis of TAMRA-Ub-ss-cR10D1 **(Ub1)**: (A) Analytical HPLC of crude **Ub1**. (B) Analytical HPLC and mass analysis of purified **Ub1** with the observed mass 11852.2 Da, calcd 11852 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

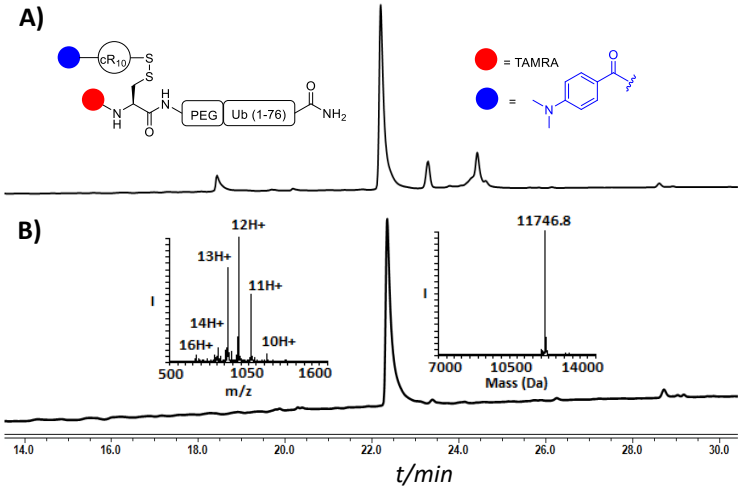


Figure S24. Synthesis of TAMRA-Ub-ss-cR10D2 **(Ub2)**: (A) Analytical HPLC of crude **Ub2**. (B) Analytical HPLC and mass analysis of purified **Ub2** with the observed mass 11746.8 Da, calcd 11747 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

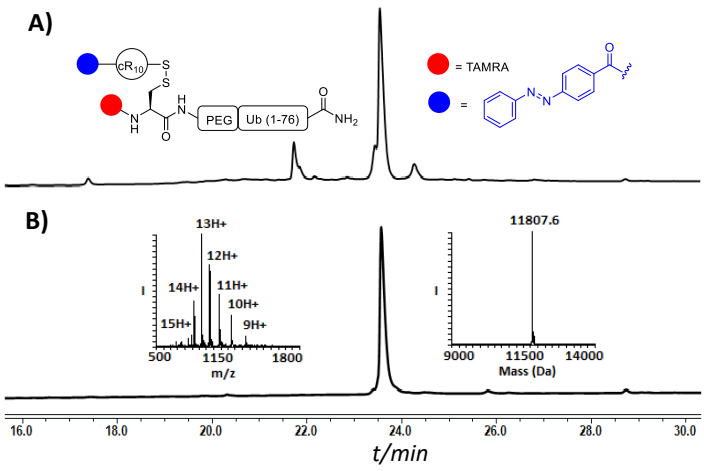


Figure S25. Synthesis of TAMRA-Ub-ss-cR10D3 **(Ub3)**: (A) Analytical HPLC of crude **Ub3**. (B) Analytical HPLC and mass analysis of purified **Ub3** with the observed mass 11807.6 Da, calcd 11808 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

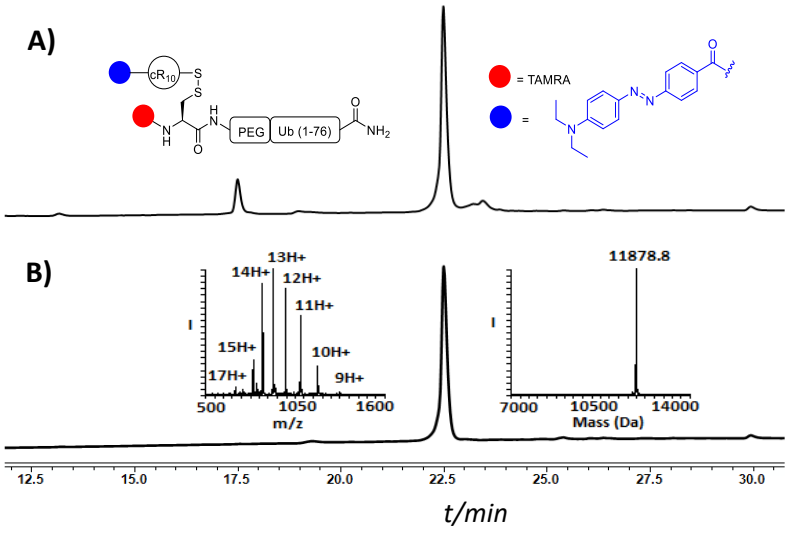


Figure S26. Synthesis of TAMRA-Ub-ss-cR10D4 **(Ub4)**: (A) Analytical HPLC of crude **Ub4**. (B) Analytical HPLC and mass analysis of purified **Ub4** with the observed mass 11878.8 Da, calcd 11880 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

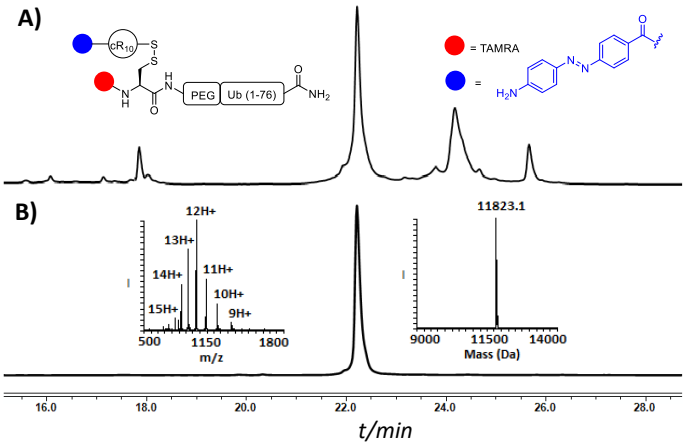


Figure S27. Synthesis of TAMRA-Ub-ss-cR10D5 **(Ub5)**: (A) Analytical HPLC of crude **Ub5**. (B) Analytical HPLC and mass analysis of purified **Ub5** with the observed mass 11823.1 Da, calcd 11824 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

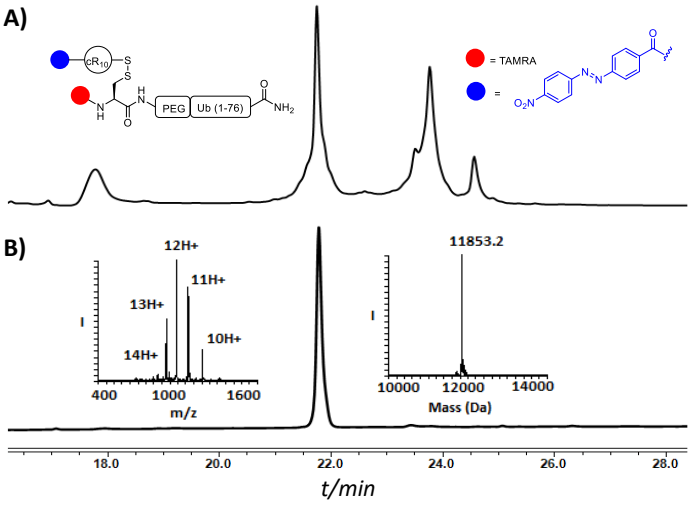


Figure S28. Synthesis of TAMRA-Ub-ss-cR10D6 **(Ub6)**: (A) Analytical HPLC of crude **Ub6**. (B) Analytical HPLC and mass analysis of purified **Ub6** with the observed mass 11853.2 Da, calcd 11854 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

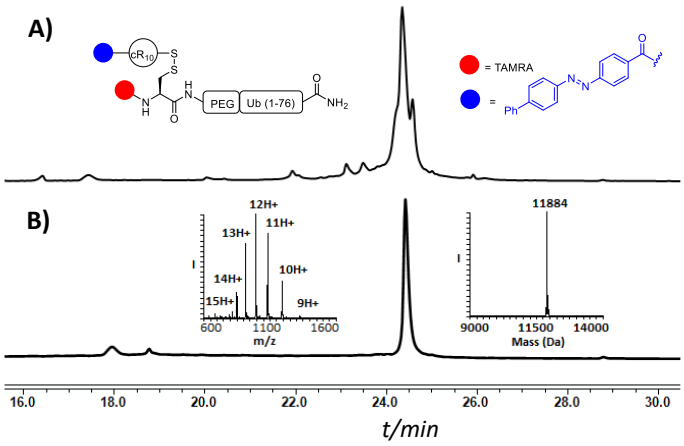


Figure S29. Synthesis of TAMRA-Ub-ss-cR10D7 **(Ub7)**: (A) Analytical HPLC of crude **Ub7**. (B) Analytical HPLC and mass analysis of purified **Ub7** with the observed mass 11884 Da, calcd 11885 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

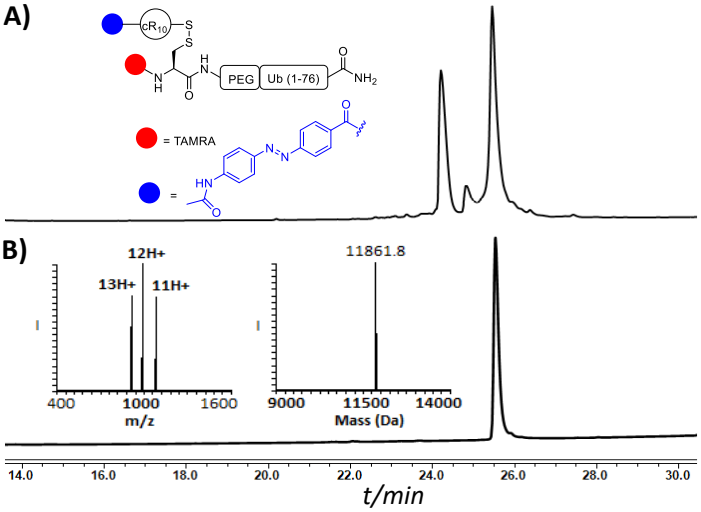


Figure S30. Synthesis of TAMRA-Ub-ss-cR10D8 **(Ub8)**: (A) Analytical HPLC of crude **Ub8**. (B) Analytical HPLC and mass analysis of purified **Ub8** with the observed mass 11861.8 Da, calcd 11864 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

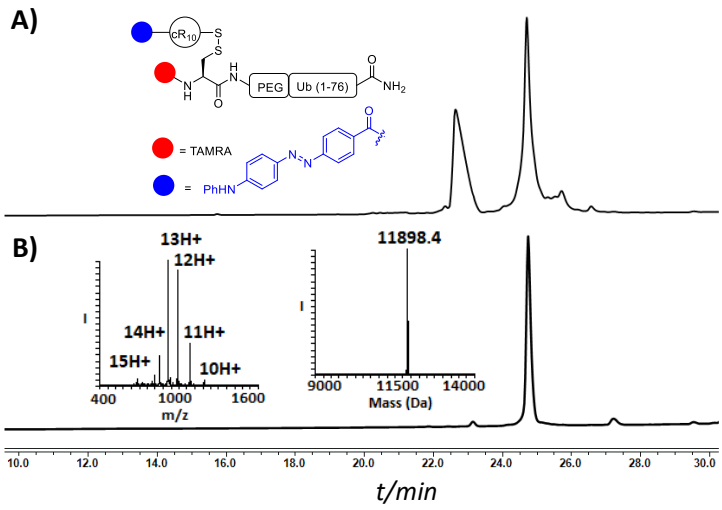


Figure S31. Synthesis of TAMRA-Ub-ss-cR10D8 **(Ub9)**: (A) Analytical HPLC of crude **Ub9**. (B) Analytical HPLC and mass analysis of purified **Ub9** with the observed mass 11898.4 Da, calcd 11899 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

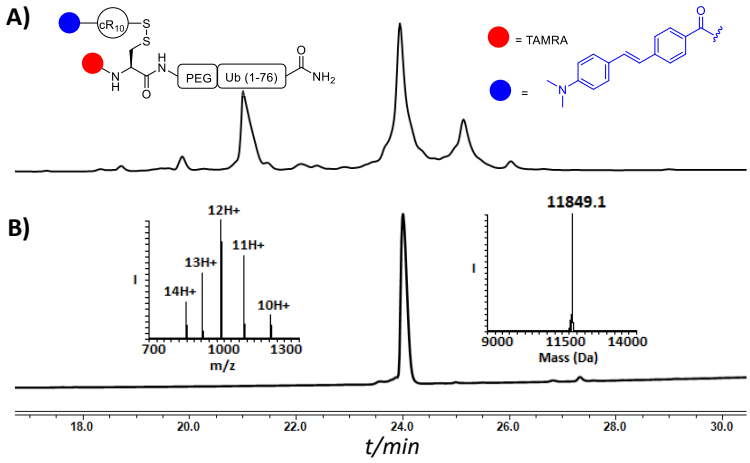


Figure S32. Synthesis of TAMRA-Ub-ss-cR10D10 **(Ub10)**: (A) Analytical HPLC of crude **Ub10**. (B) Analytical HPLC and mass analysis of purified **Ub10** with the observed mass 11849.1 Da, calcd 11850 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

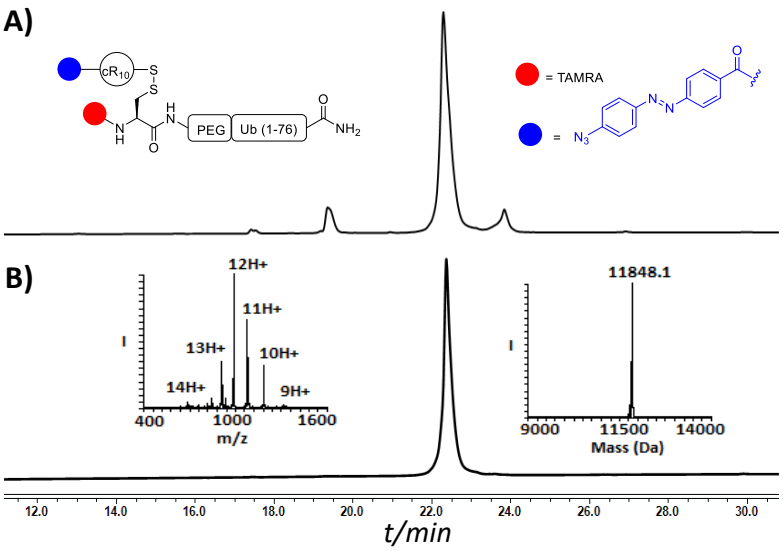


Figure S33. Synthesis of TAMRA-Ub-ss-cR10D11 **(Ub11)**: (A) Analytical HPLC of crude **Ub11**. (B) Analytical HPLC and mass analysis of purified **Ub11** with the observed mass 11848.1 Da, calcd 11849.9 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

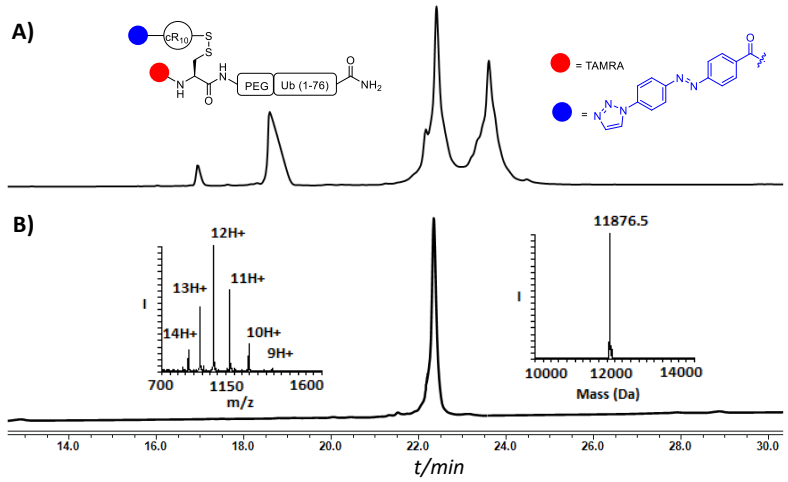


Figure S34. Synthesis of TAMRA-Ub-ss-cR10D12 **(Ub12)**: (A) Analytical HPLC of crude **Ub12**. (B) Analytical HPLC and mass analysis of purified **Ub12** with the observed mass 11876.5 Da, calcd 11876 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

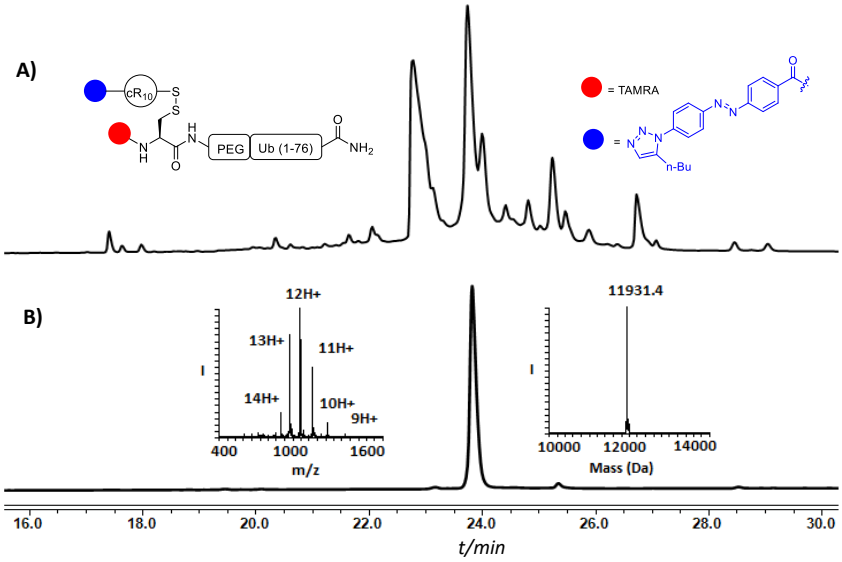


Figure S35. Synthesis of TAMRA-Ub-ss-cR10D13 **(Ub13)**: (A) Analytical HPLC of crude **Ub13**. (B) Analytical HPLC and mass analysis of purified **Ub13** with the observed mass 11931.4 Da, calcd 11932 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

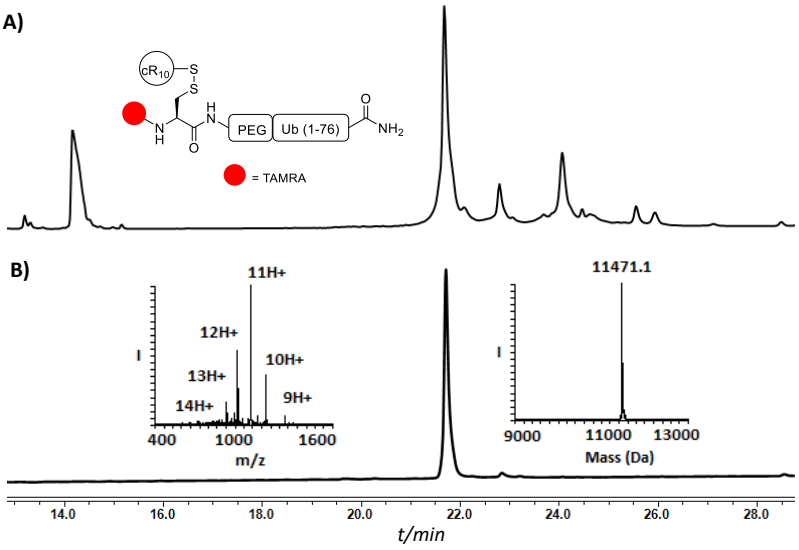


Figure S36. Synthesis of TAMRA-Ub-ss-cR10 **(Ub14)**: (A) Analytical HPLC of crude **Ub14**. (B) Analytical HPLC and mass analysis of purified **Ub14** with the observed mass 11471.1 Da, calcd 11472 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

18. Representative images of live U2OS cell delivery of CPP-Ub conjugates (Ub1-Ub13):

All the live-U2OS cell delivery experiments were carried out as mentioned in section 7 followed by CLSM imaging and analysis of over 150 cells for the cell delivery of each CPP-Ub conjugate as mentioned in section 8.

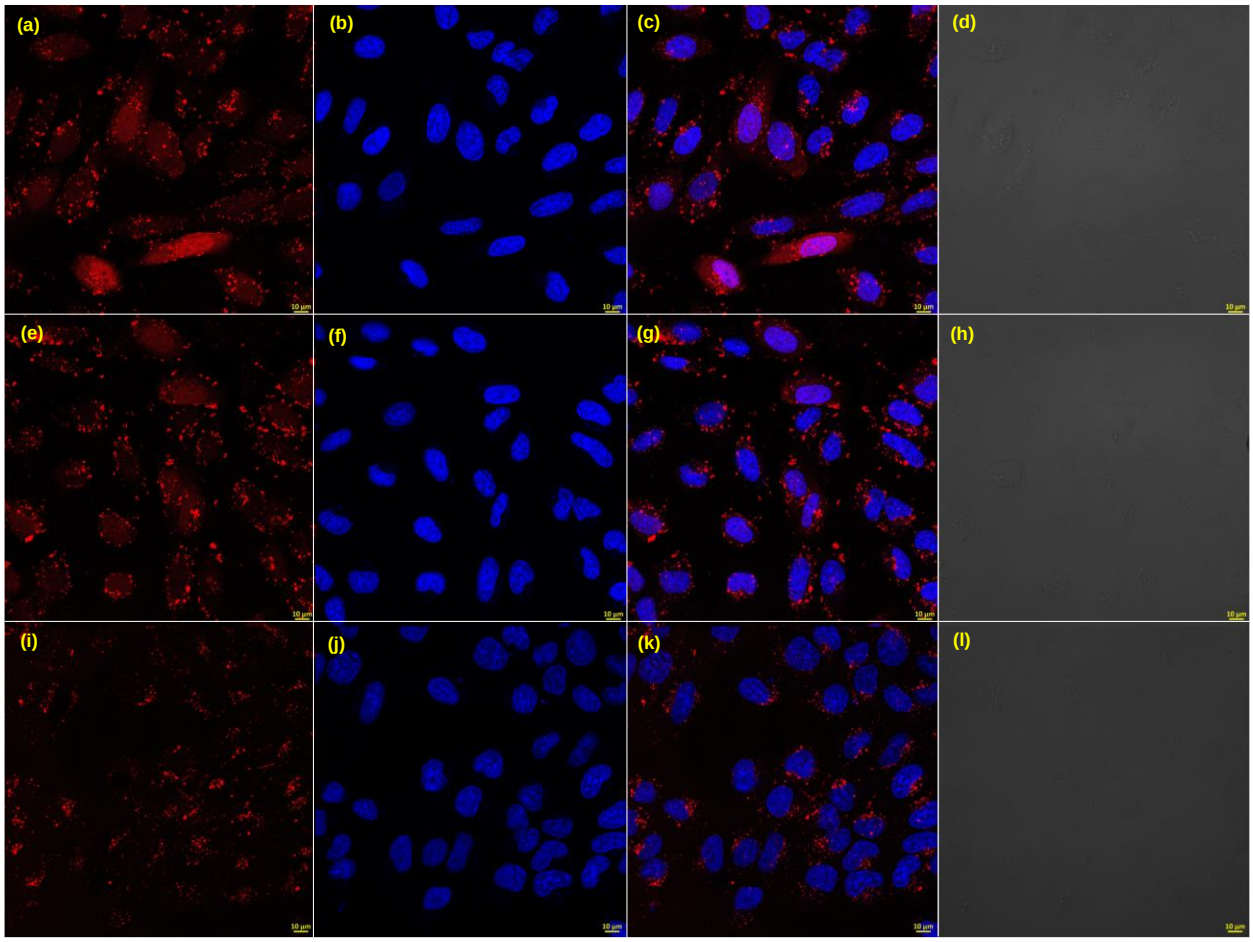


Figure S37: Representative images of cell delivery of **Ub1, Ub5,** and **Ub3** to live U2OS in 2 μM. (a,e,i) TAMRA from **Ub1, Ub5,** and **Ub3** (Red) respectively. (b,f,j) Hoechst (blue). (c,g,k) merge images of red (TAMRA channel) and blue (Hoechst channel). (d,h,l) Bright field. (Scale bars 10 μm).

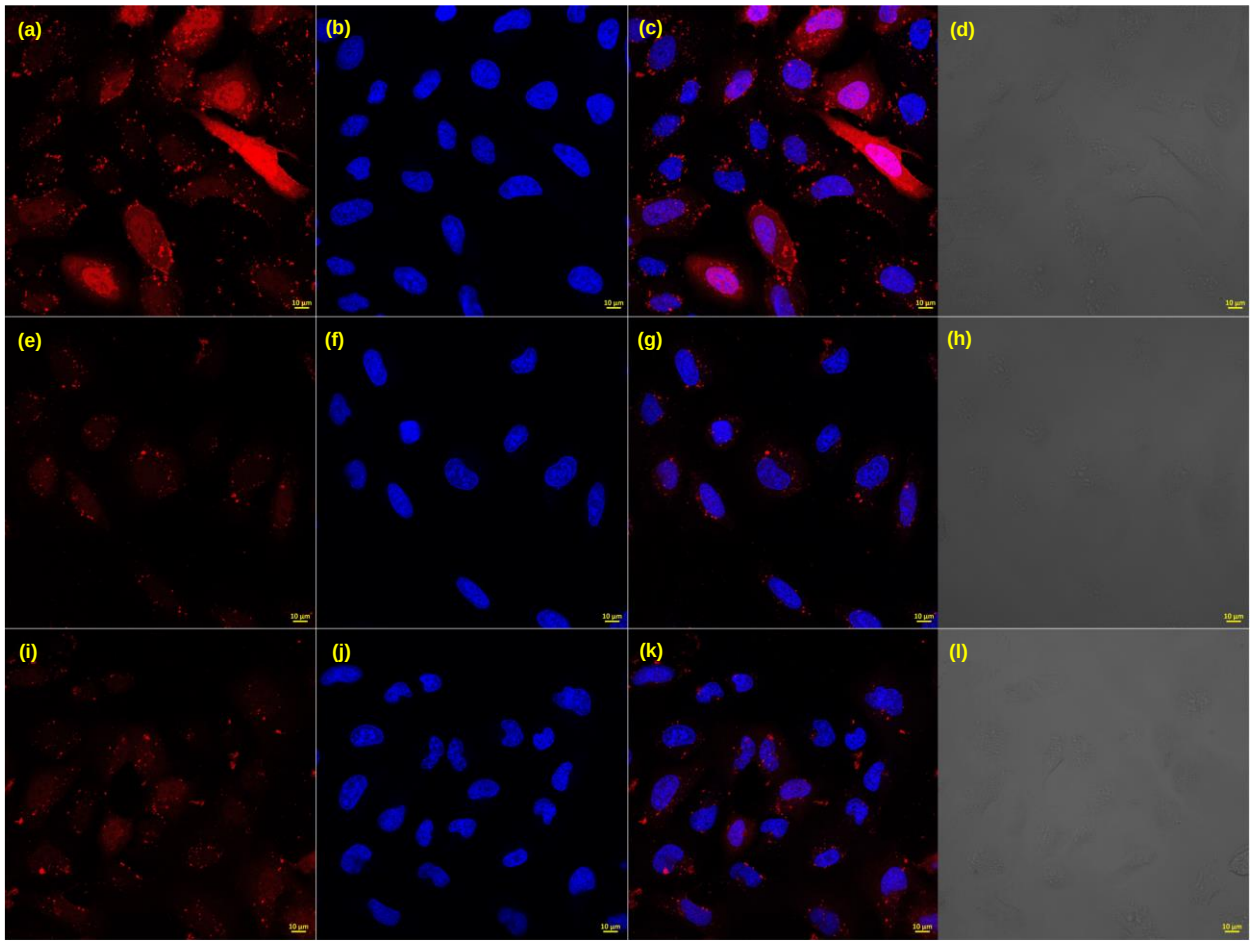


Figure S38: Representative images of cell delivery of **Ub1, Ub2,** and **Ub4** to live U2OS in 2 μM. (a,e,i) TAMRA from **Ub1, Ub2,** and **Ub4** (Red) respectively. (b,f,j) Hoechst (blue). (c,g,k) merge images of red (TAMRA channel) and blue (Hoechst channel). (d,h,l) Bright field. (Scale bars 10 μm).

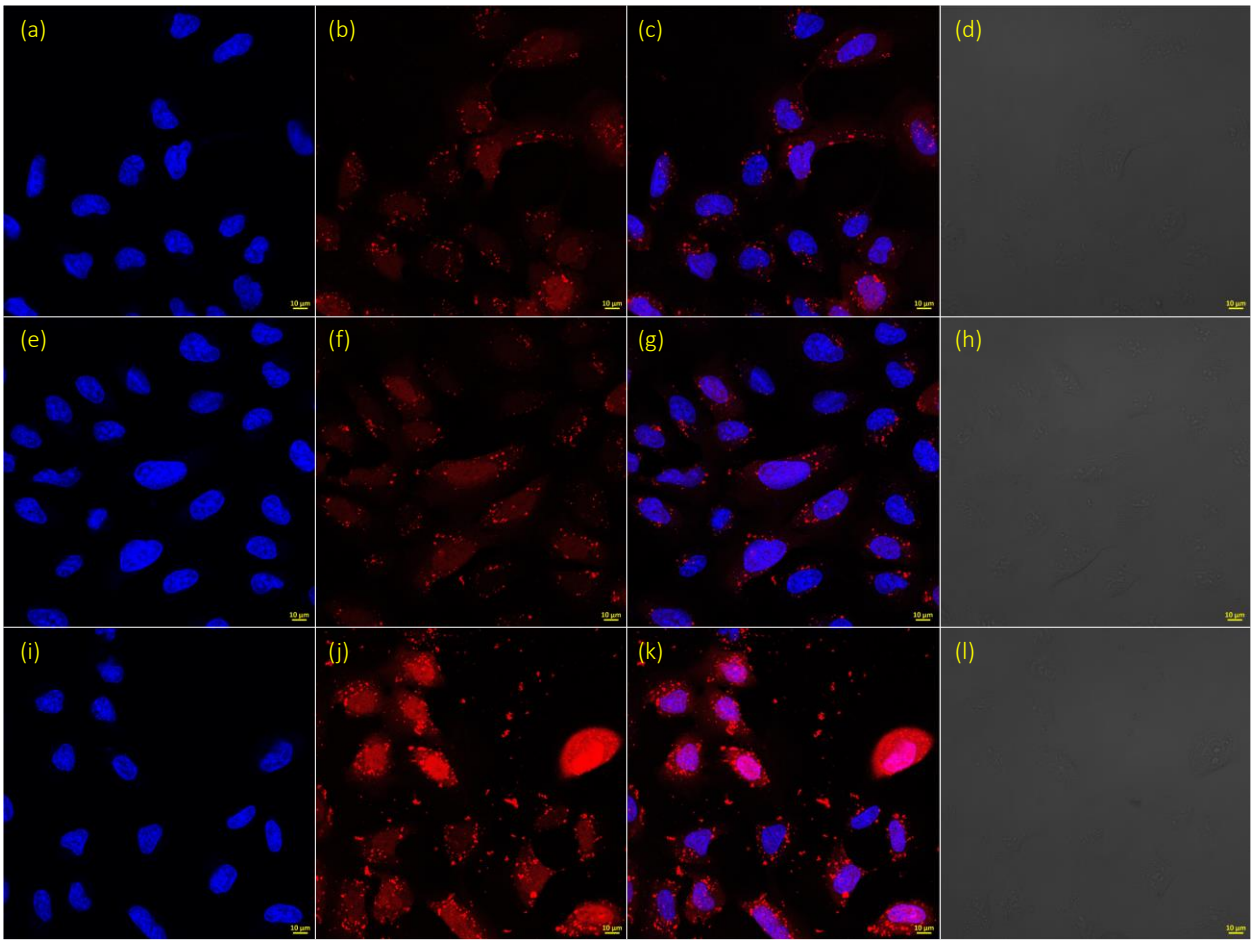


Figure S39: Representative images of cell delivery of **Ub1**, **Ub6**, and **Ub7** to live U2OS in 2 μM. (a,e,I) TAMRA from **Ub1**, **Ub6**, and **Ub7** (Red) respectively. (b,f,j) Hoechst (blue). (c,g,k) merge images of red (TAMRA channel) and blue (Hoechst channel). (d,h,l) Bright field. (Scale bars 10 μm).

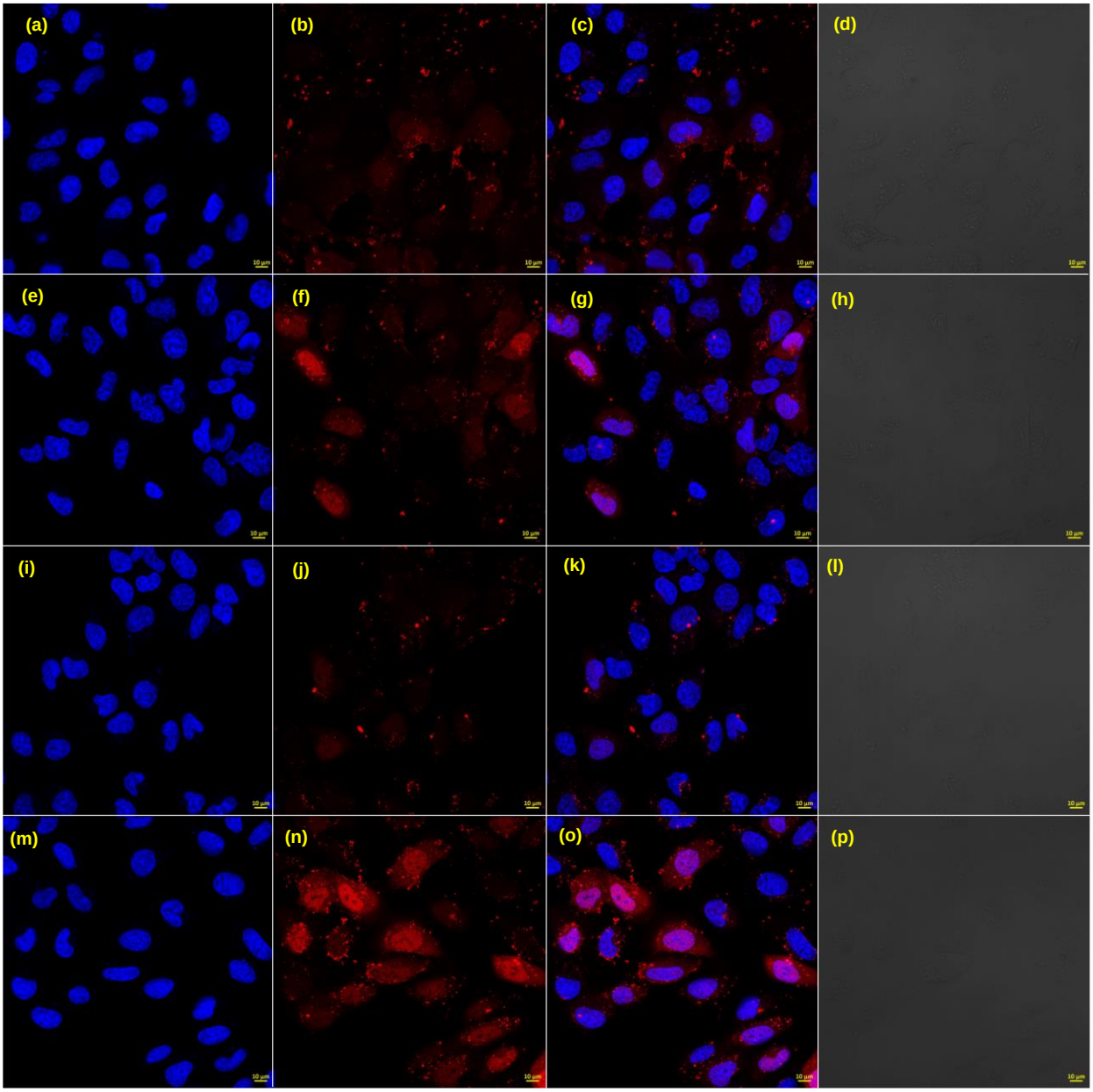


Figure S40: Representative images of cell delivery of **Ub1**, **Ub9**, **Ub8**, and **Ub11** to live U2OS in 2 μM. (a,e,l,m) TAMRA from **Ub1**, **Ub9**, **Ub8**, and **Ub11** (Red) respectively. (b,f,j,n) Hoechst (blue). (c,g,k,o) merge images of red (TAMRA channel) and blue (Hoechst channel). (d,h,l,p) Bright field. (Scale bars 10 μm).

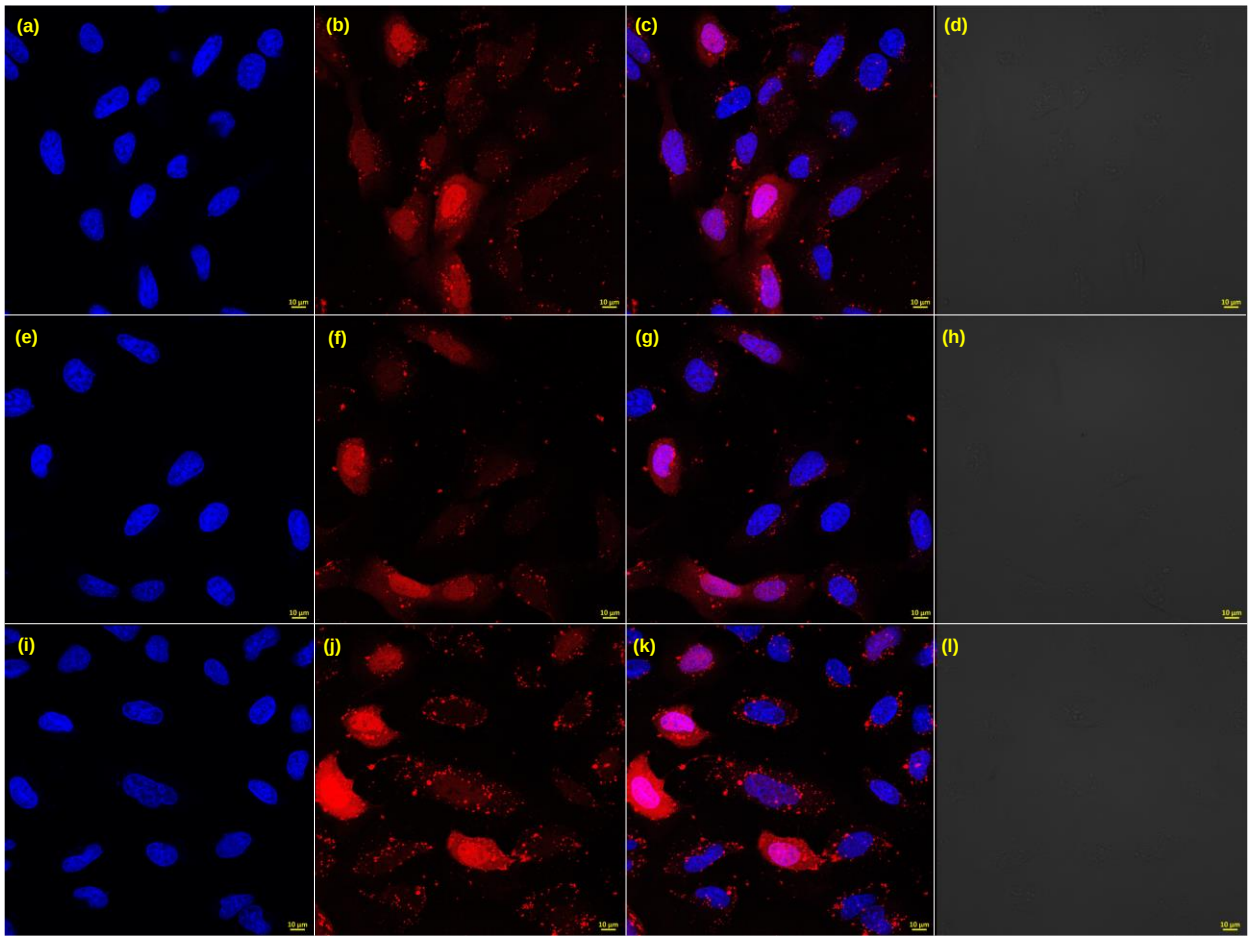


Figure S41: Representative images of cell delivery of **Ub11, Ub12,** and **Ub13** to live U2OS in 2 μM. (a,e,i) TAMRA from **Ub11, Ub12,** and **Ub13** (Red) respectively. (b,f,j) Hoechst (blue). (c,g,k) merge images of red (TAMRA channel) and blue (Hoechst channel). (d,h,l) Bright field. (Scale bars 10 μm).

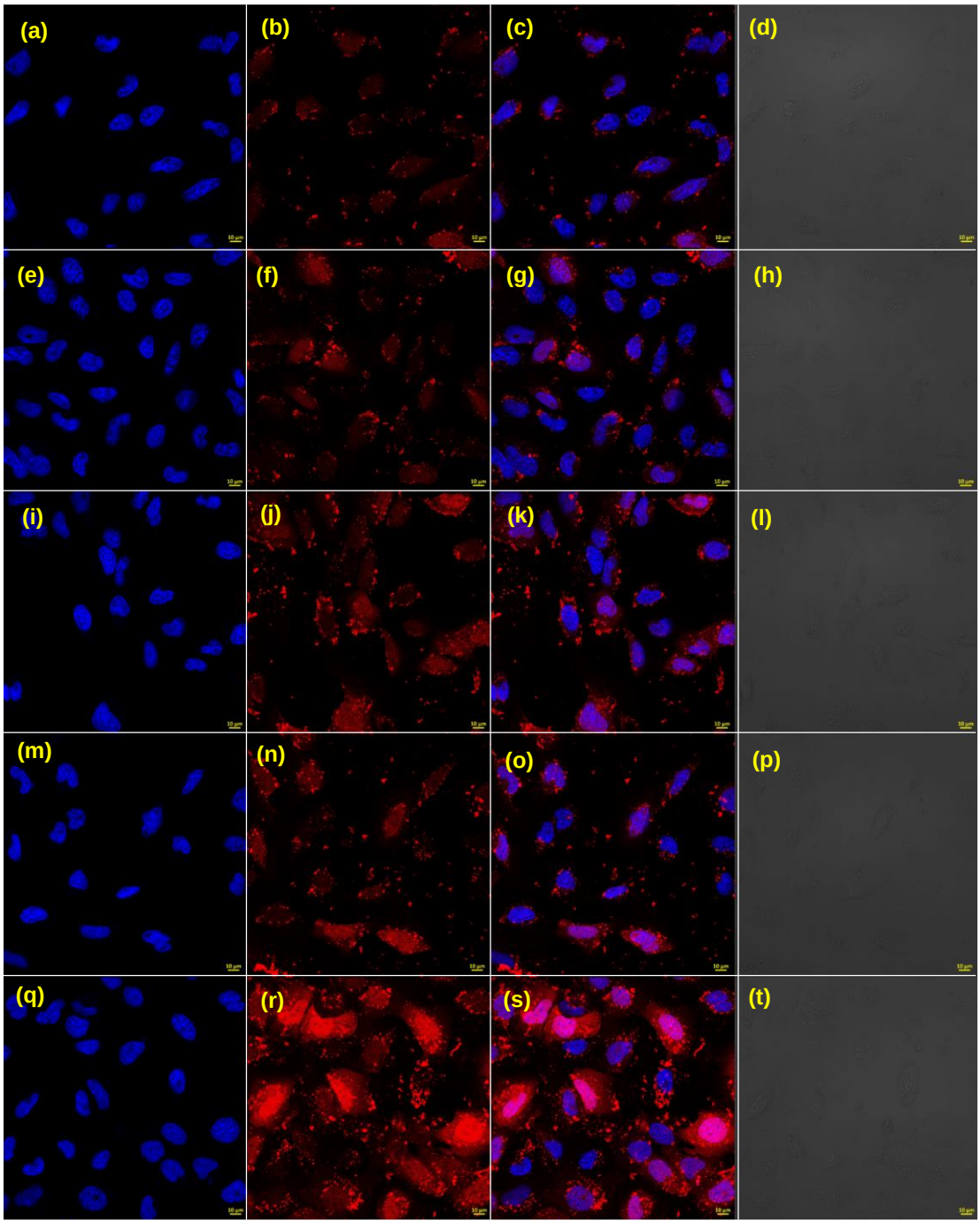


Figure S42: Comparisom among best five CPPs. Representative images of cell delivery of **Ub1**, **Ub4**, **Ub9**, **Ub7**, and **Ub11** to live U2OS in 2 μM. (a,e,l,m,q) TAMRA from **Ub1**, **Ub4**, **Ub9**, **Ub7**, and **Ub11** (Red) respectively. (b,f,j,n,r) Hoechst (blue). (c,g,k,o,s) merge images of red (TAMRA channel) and blue (Hoechst channel). (d,h,l,p,t) Bright field. (Scale bars 10 μm).

19. Statistical analysis of live-cell delivery of potent CPP-Ubs by Citation5 microscope:

Cytation 5 equipped with 4 lasers combines the high throughput microscopy with in-build Gen5 software allowing complete control over imaging and data processing. Hoechst and Cy3 (for TAMRA) filters were used for the analysis. U2OS cells were seeded in an 8-well plate and incubated at 37 °C with 5% CO_2 for 24h before experimentation. Following protein delivery, cells were washed with 1× PBS (twice) and then stained with Hoechst by incubating for ~10 minutes at 37 °C with 5% CO_2. Cells were then imaged in the Biospa incubator of Cytaion 5 at 37 °C with 5% CO_2, images were taken with 20x magnification. The average nuclear TAMRA fluorescence intensity was quantified according to the Hoechst masking by the high content microscopy and was repeated twice.

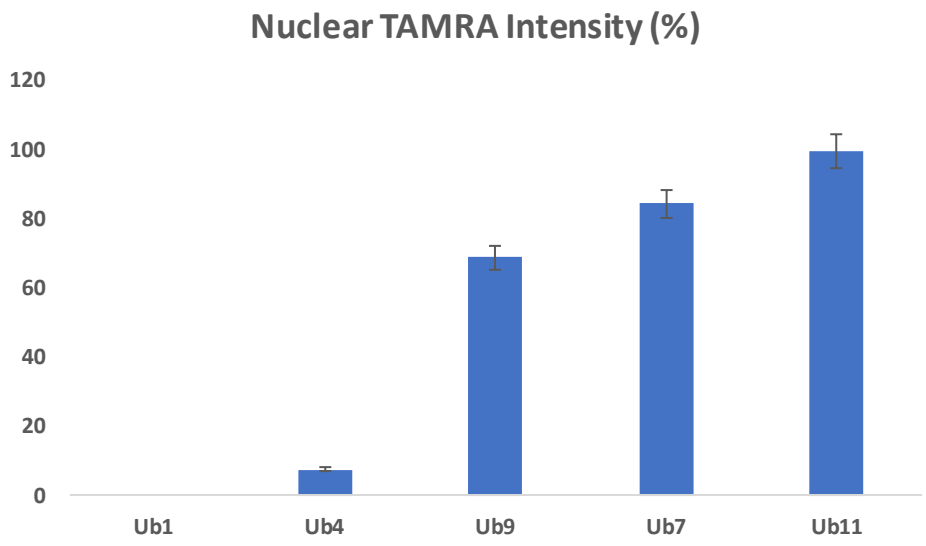


Figure S43. Quantification of relative cellular uptake. Average TAMRA intensities from high-content live-cell fluorescent microscopy with different CPPs.

20. Stability of Ub11 in cell delivery assay conditions:

The experiment was done similar to the protein delivery assay condition by following section 7. In brief, in the serum-free DMEM medium, the purified **Ub11** was incubated at 37 °C for 1 h in dark. Then the solution was diluted with ACN/water and performed HPLC-MS analysis.

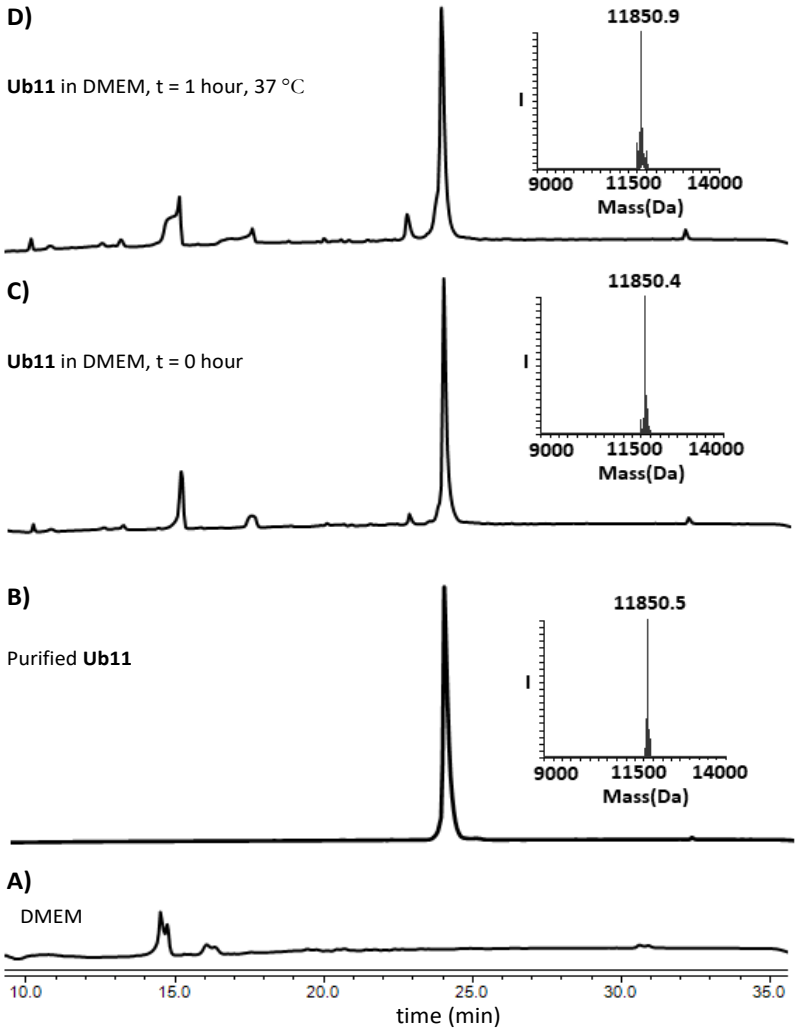


Figure S44. Stability of **Ub11** after at 37 °C in serum-free DMEM medium: (A) Analytical HPLC of serum-free DMEM. Analytical HPLC and mass analysis of **Ub11** (calcd 11849.8 Da, for average isotopes) for purified **Ub11** (B), before and after incubated at 37 °C for 1 h in dark (C-D). Detection of HPLC chromatogram at 214nm.

21. Cytosolic Ub delivery by the AzidoDABCYL CPP compared with the natural TAT CPP:

Natural HIV-1 (47-57) TAT peptide sequence: Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg

Cys-TAT sequence: Cys-Peg-Peg-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg

The synthesis of the **Cys-TAT** peptide was performed on Rink Amide resin (0.1 mmol) using SPPS method similarly as previous, in which Fmoc amino acids were coupled on an automated peptide synthesizer using 4 equiv amino acids, 4 equiv HATU, and 8 equiv of DIEA. The Cys residue of the peptide was then activated using 20 eq. DTNP in 95% TFA/H_2O for 1 h following DCM and cold ether washes to give TNP-linked CPPs, **TAT-TNP** (yield 80%) and used in the next step without further purification.

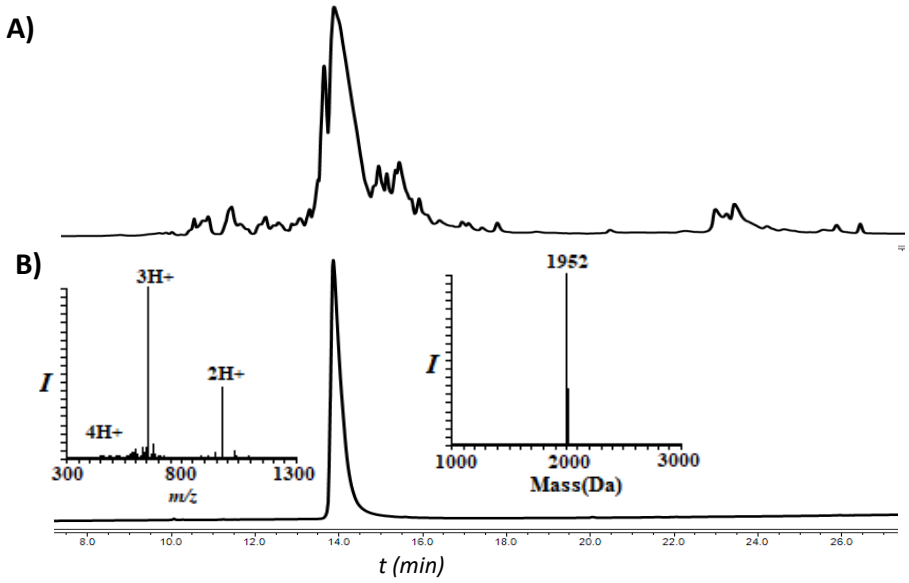


Figure S45. Synthesis of **Cys-TAT** peptides: (A) Analytical HPLC of crude **Cys-TAT**. (B) Analytical HPLC and mass analysis of purified **Cys-TAT** with the observed mass 1953.2 Da, calcd 1952 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

To conjugate Cys functionalized TAT to **TAMRA-Ub**, DTNP-activated CPPs were dissolved in 6M Gn·HCl (pH = 7.2) in a final concentration of 2 mM. The CPP-containing solution was added to the **TAMRA-Ub** solution and the mixture was kept at 37 °C for 5 min in dark. Next, the reaction mixture was purified by preparative reverse HPLC using a C4 column with a gradient of 0-60% buffer acetonitrile over 35 min to afford the corresponding TAT-Ub-conjugate (**Ub15**) with an isolated yield of 50-55 %.

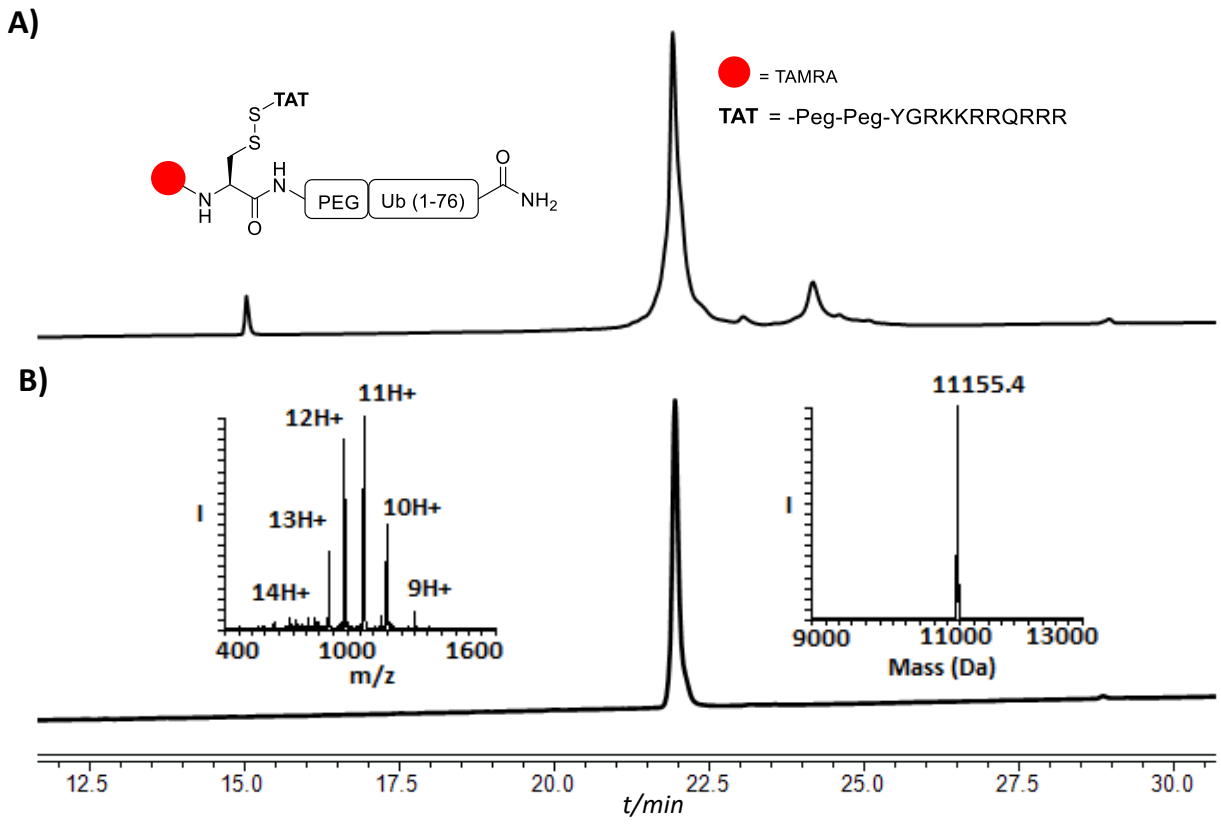


Figure S46. Synthesis of TAMRA-Ub-ss-TAT **(Ub15)**: (A) Analytical HPLC of crude **Ub15**. (B) Analytical HPLC and mass analysis of purified **Ub15** with the observed mass 11155.4 Da, calcd 11156.2 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

The live-U2OS cell delivery experiment was carried out as mentioned in section 7 followed by CLSM imaging and analysis of over 150 cells for the cell delivery of the TAT-Ub conjugate as mentioned in section 8.

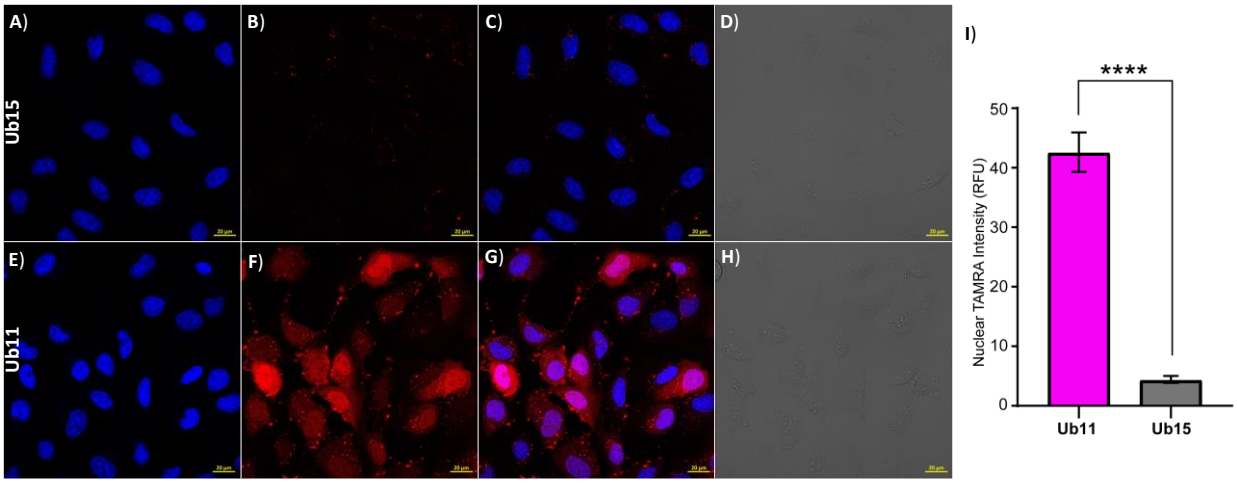


Figure S47. Representative images of delivery of Ub analogs **Ub15** (A–D) and **Ub11** (E–H) to live U2OS cells at 2 μM. A, E) **Ub15** and **Ub11** Hoechst (blue). B, F) (TAMRA, red). C, G) TAMRA and Hoechst channels combined. D, H) Bright field channel. Scale bars 20 μm. I) Quantification of nuclear TAMRA intensity of the cell images after delivery of **Ub15** and **Ub11** under Hoechst relative to the untreated cells. Data were plotted as mean ± SEM of four biological replicates (over 150 cells per analysis) and statistical significance was determined by unpaired t-test (****, $P<0.0001$).

22. Live-cell Delivery comparison study between Ub1 and Ub11 in presence of serum:

The live-U2OS cell delivery experiment was carried out as mentioned in section 7 the presence of 5% fetal bovine serum (FBS) in the treated medium. Then, followed by CLSM imaging and analysis of over 150 cells for the cell delivery of the CPP-Ub conjugate as mentioned in section 8.

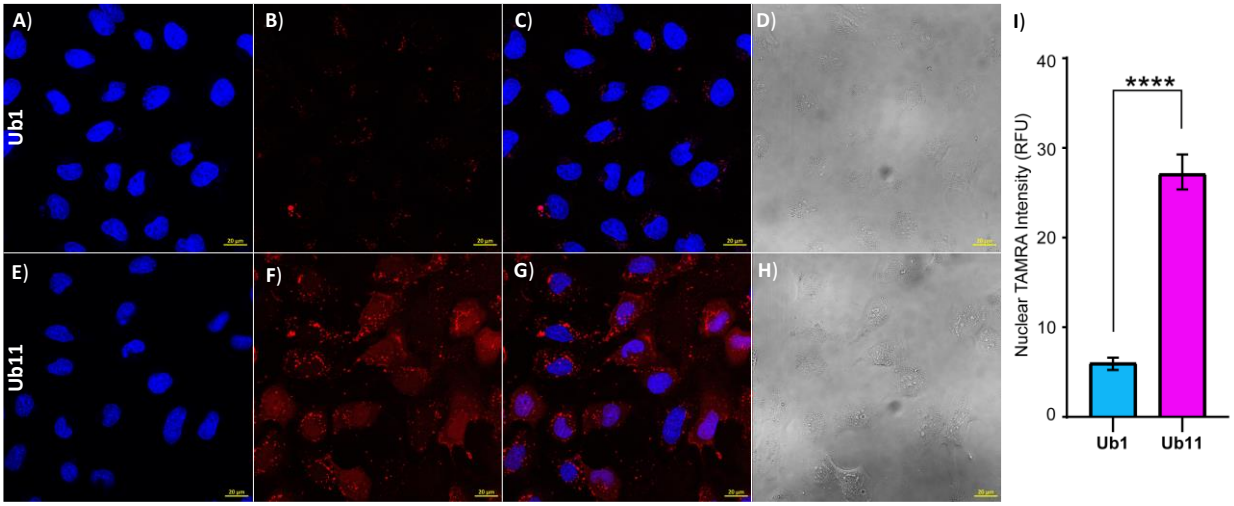


Figure S48. Representative images of delivery of Ub analogs **Ub1** (A–D) and **Ub11** (E–H) to live U2OS cells at 2 μM in the presence of 5% fetal bovine serum (FBS). A, E) **Ub1** and **Ub11** Hoechst (blue). B, F) (TAMRA, red). C, G) TAMRA and Hoechst channels combined. D, H) Bright field channel. Scale bars 20 μm. I) Quantification of nuclear TAMRA intensity of the cell images after delivery of **Ub1** and **Ub11** under Hoechst relative to the untreated cells. Data were plotted as mean ± SEM of four biological replicates (over 150 cells per analysis) and statistical significance was determined by unpaired t-test (****, P<0.0001).

23. Cell membrane integrity analysis using SYTOX™ Blue stain:

To assess the cell viability within the concentration range used for efficient live-cell delivery of synthetic Ub conjugates, we proceed similarly to the cell delivery experiment (Section 7) and stain the cells with SYTOX™ Blue (100 nM) instead of Hoechst. SYTOX™ Blue dead cell stain is a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes but will not cross uncompromised cell membranes. From this experiment, we get to know that the used concentration of Ub-CPP conjugate (up to 2 μM) in the cell-uptake experiment, was in the viable range and did not affect the cell membrane integrity during incubation.

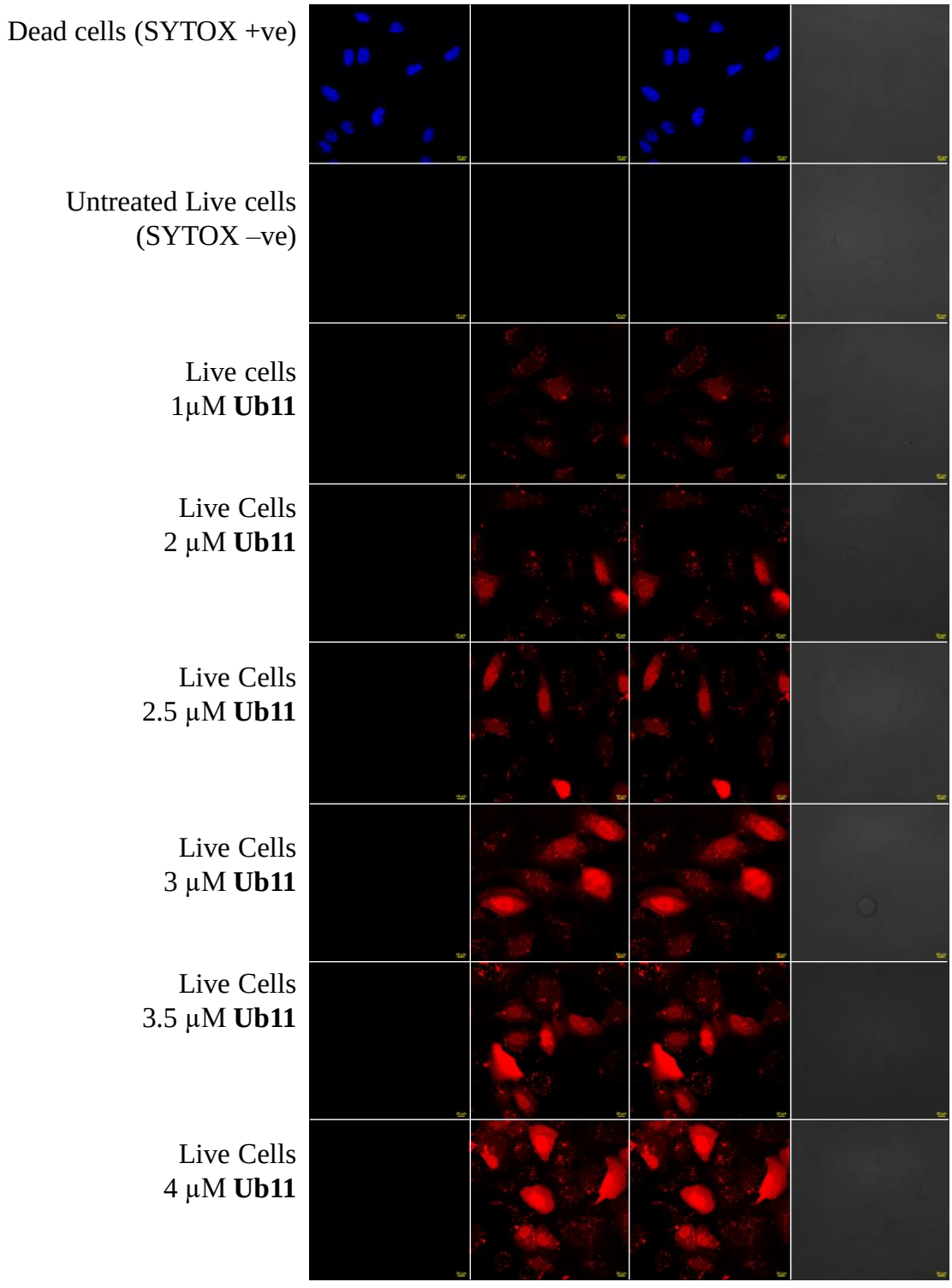


Figure S49: (a) Live U2OS cell delivery at various concentrations of **Ub11** with SYTOX Blue stain. Representative images are shown in the first column, for SYTOX blue (blue channel); second column: TAMRA (red channel); third column: merge channel for blue and red; and fourth column: brightfield. Scale bars 10 μm.

24. ^{1}H and ^{13}C NMR spectra:

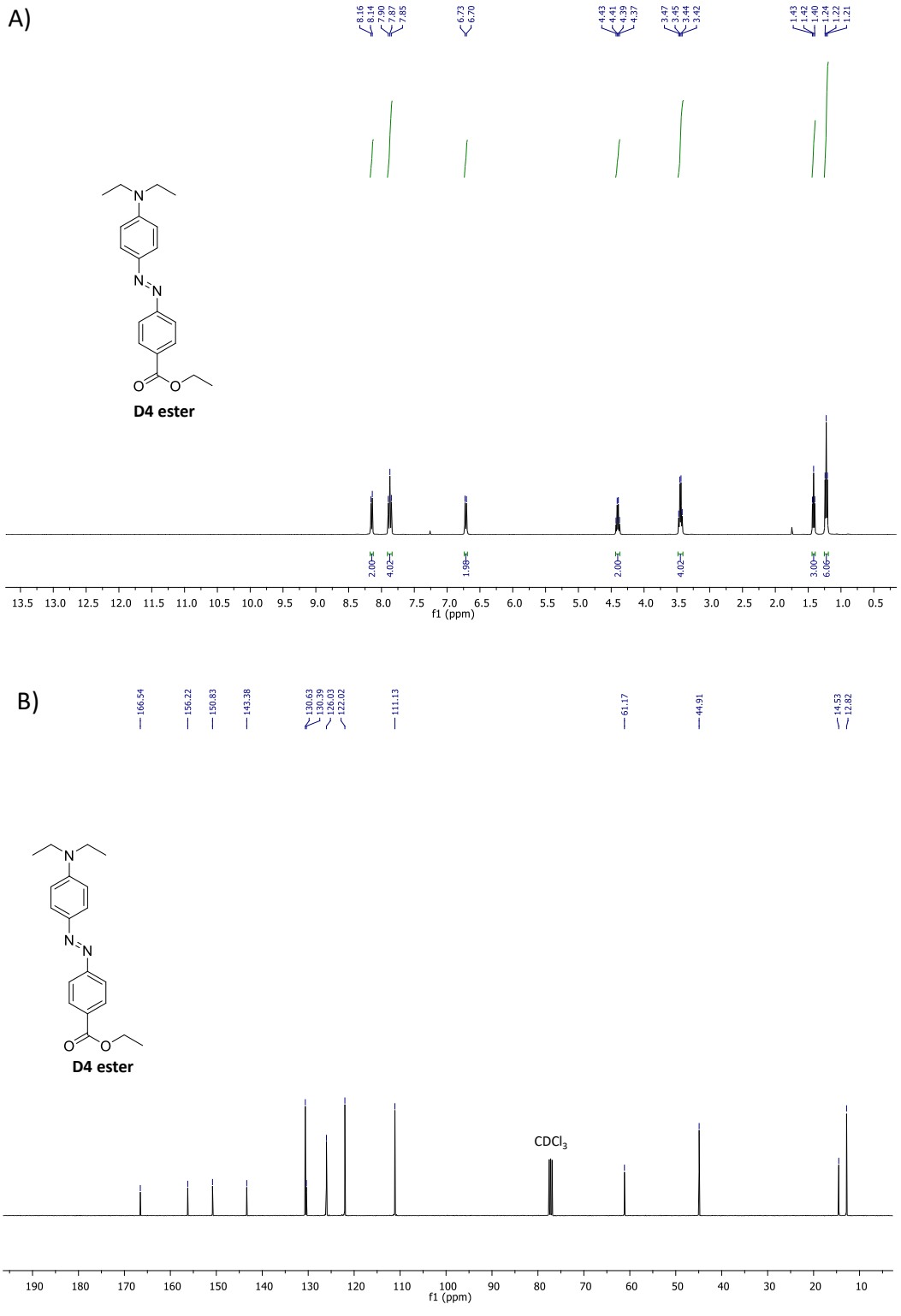


Figure S50: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D4 ester**.

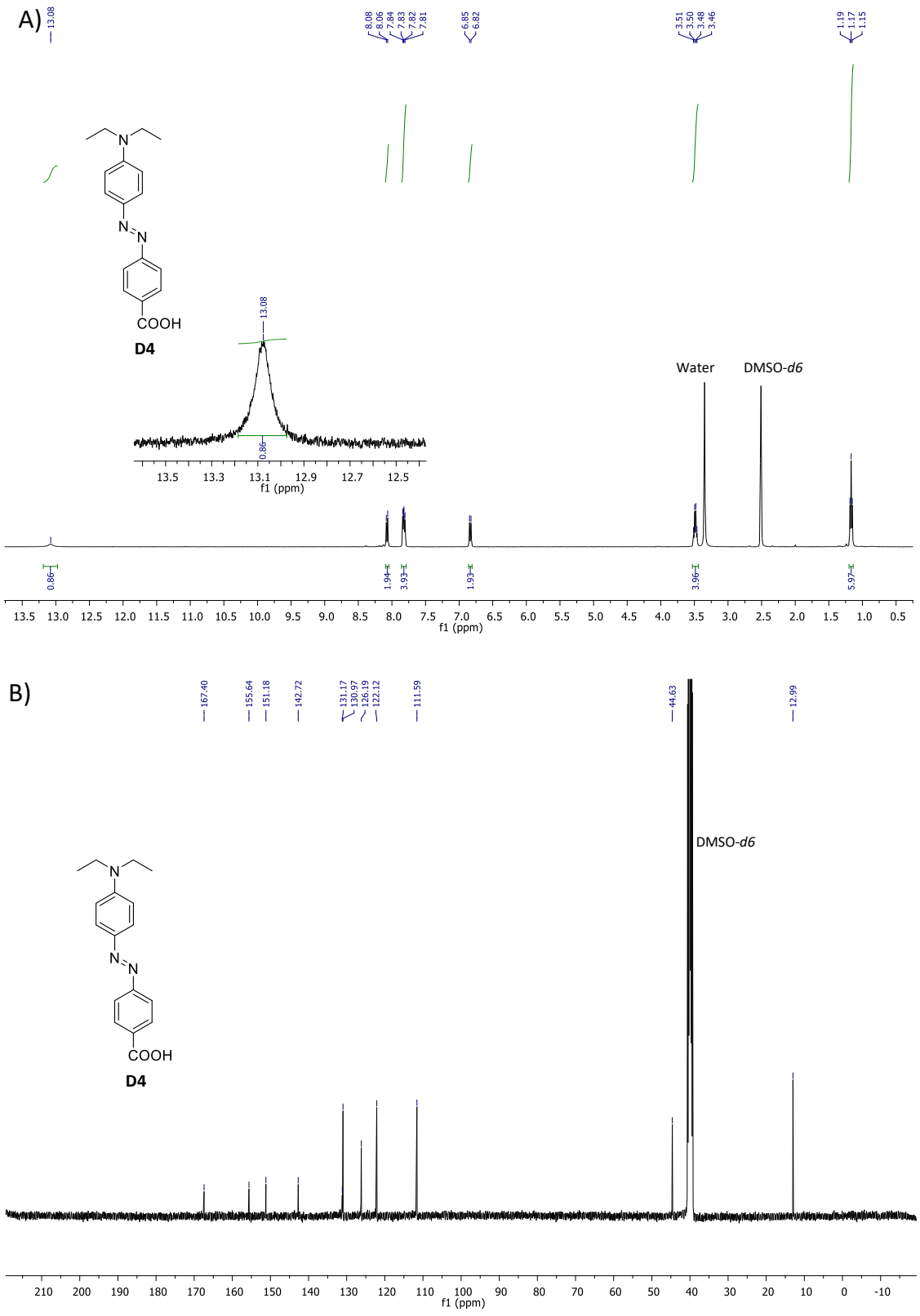


Figure S51: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D4**.

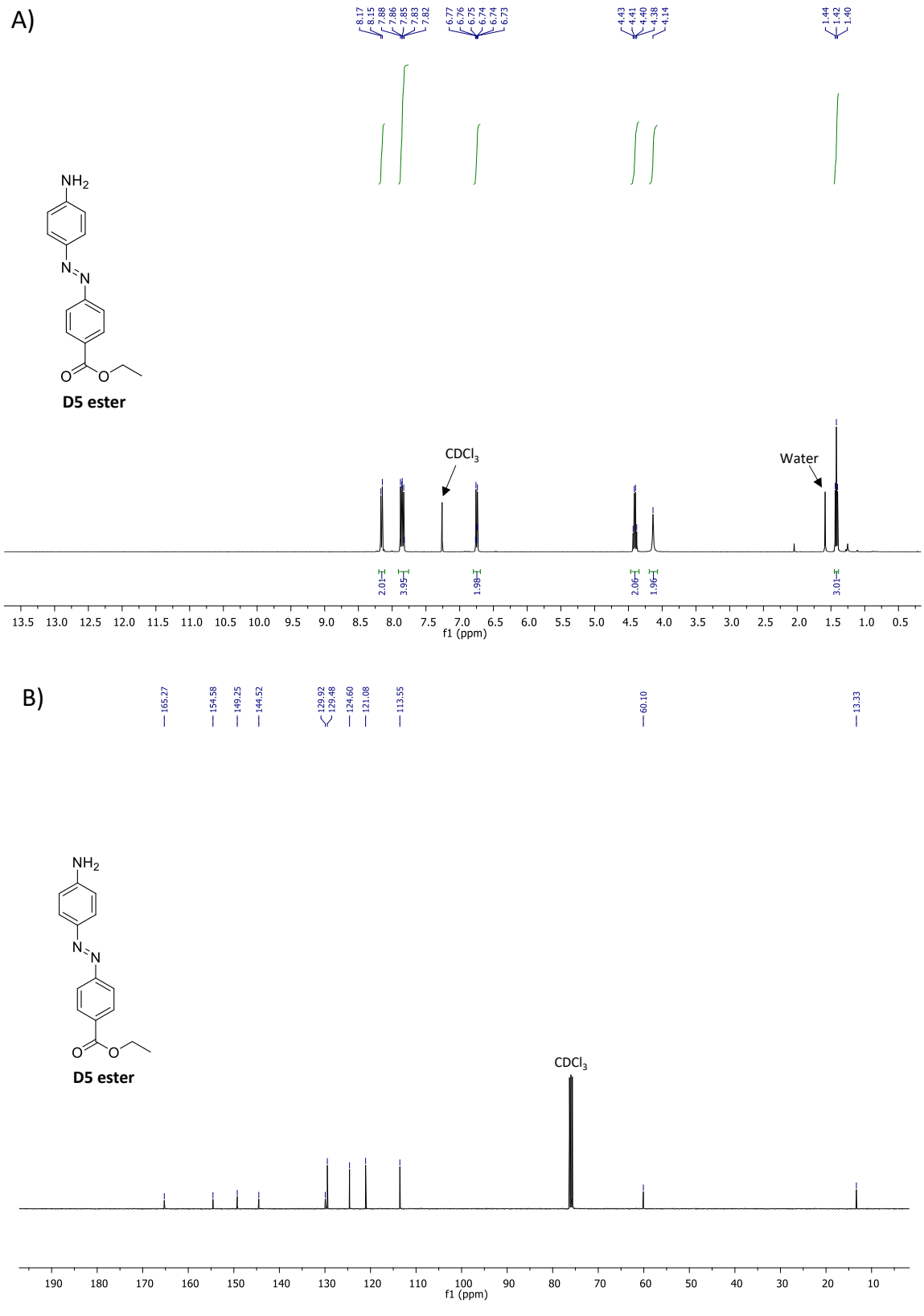


Figure S52: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D5 ester**.

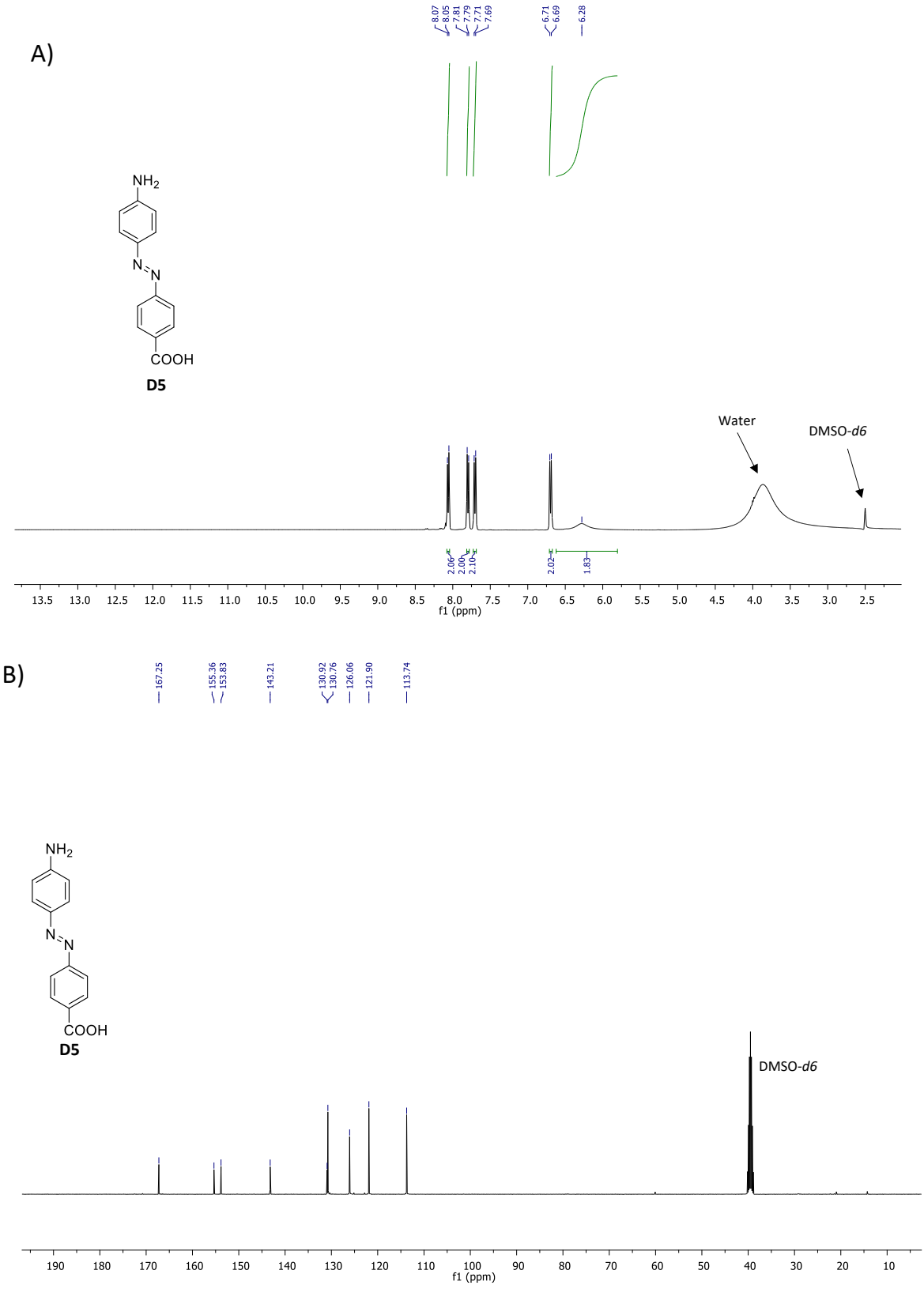


Figure S53: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D5**.

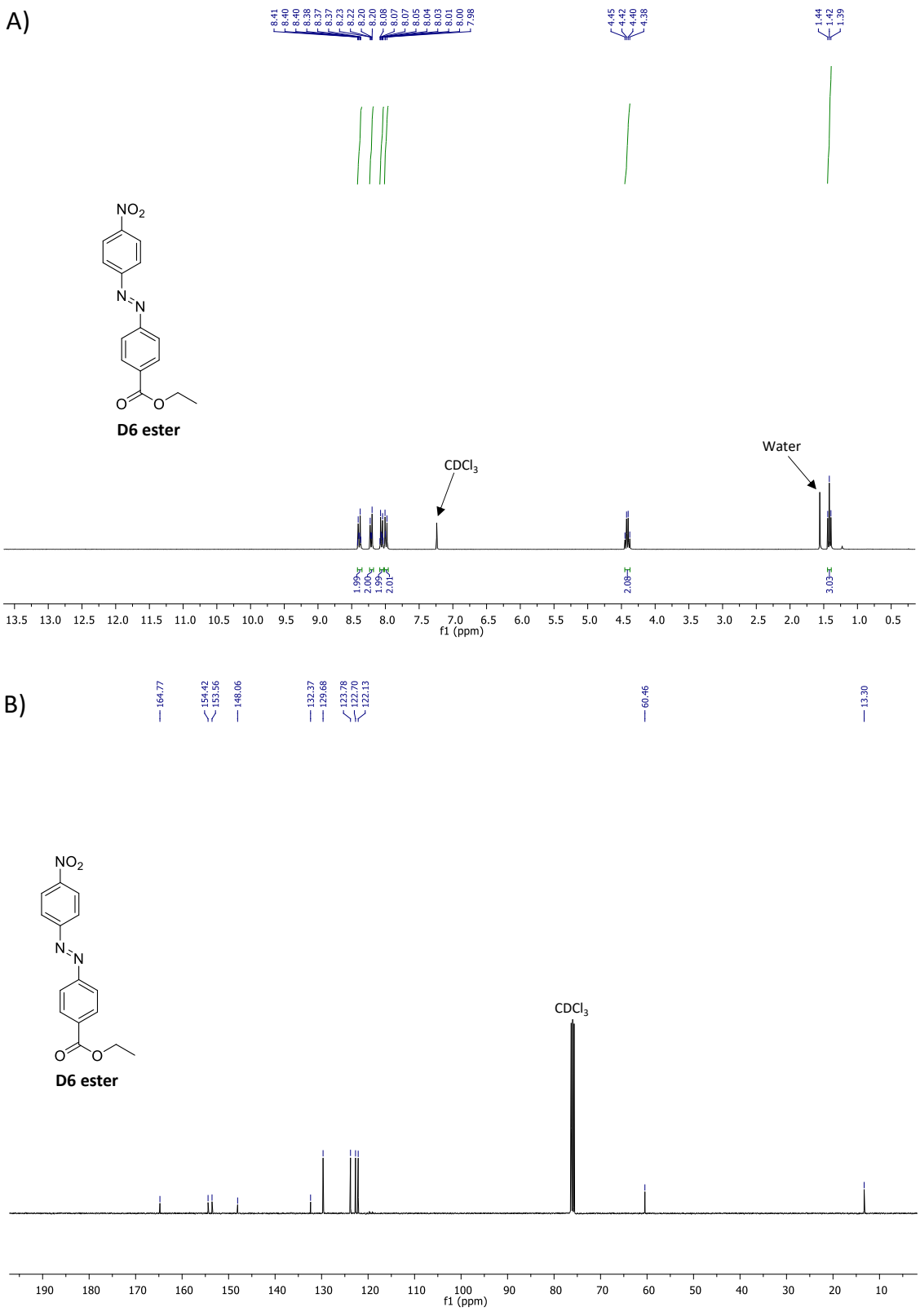


Figure S54: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D6 ester**.

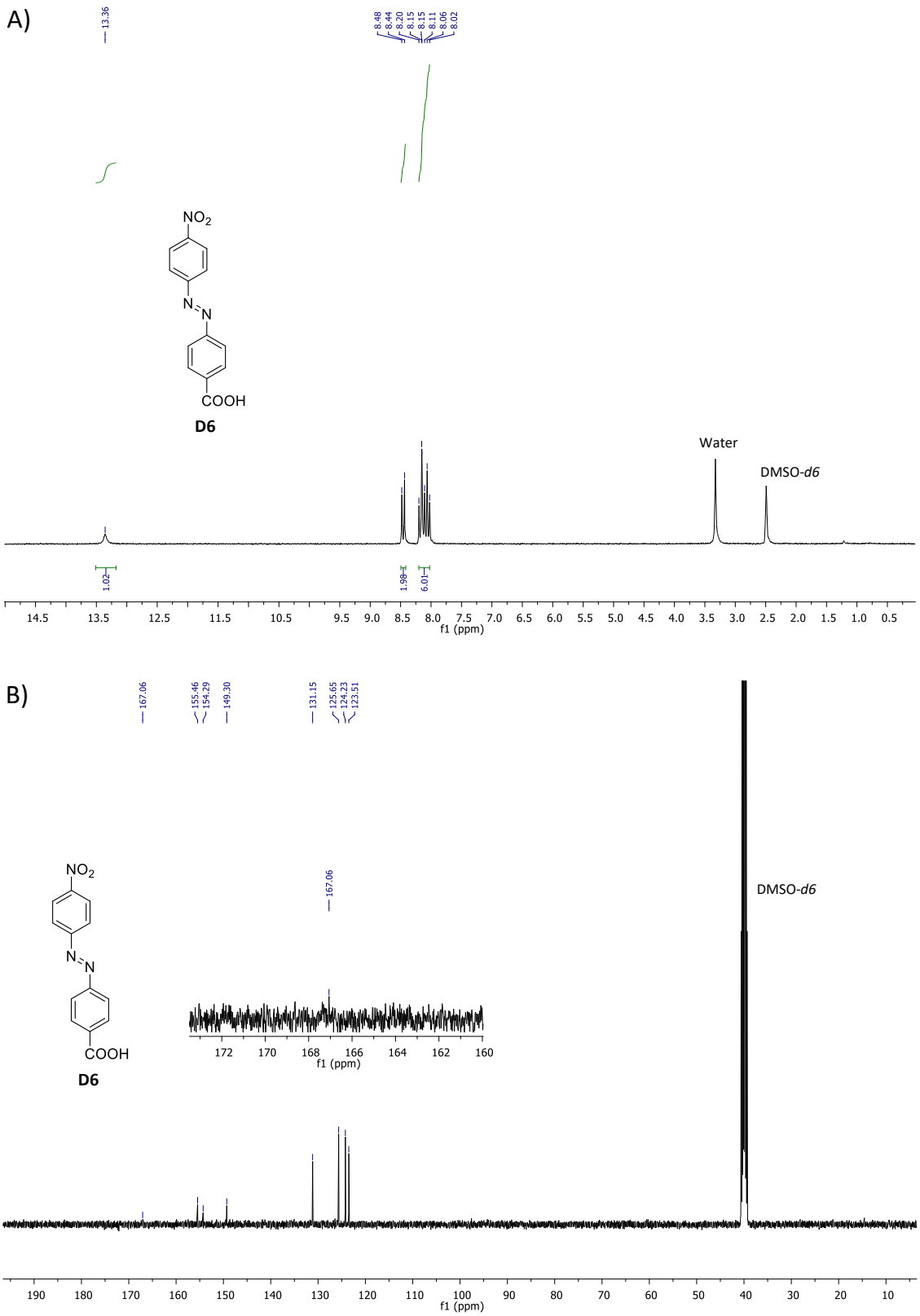


Figure S55: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D6**.

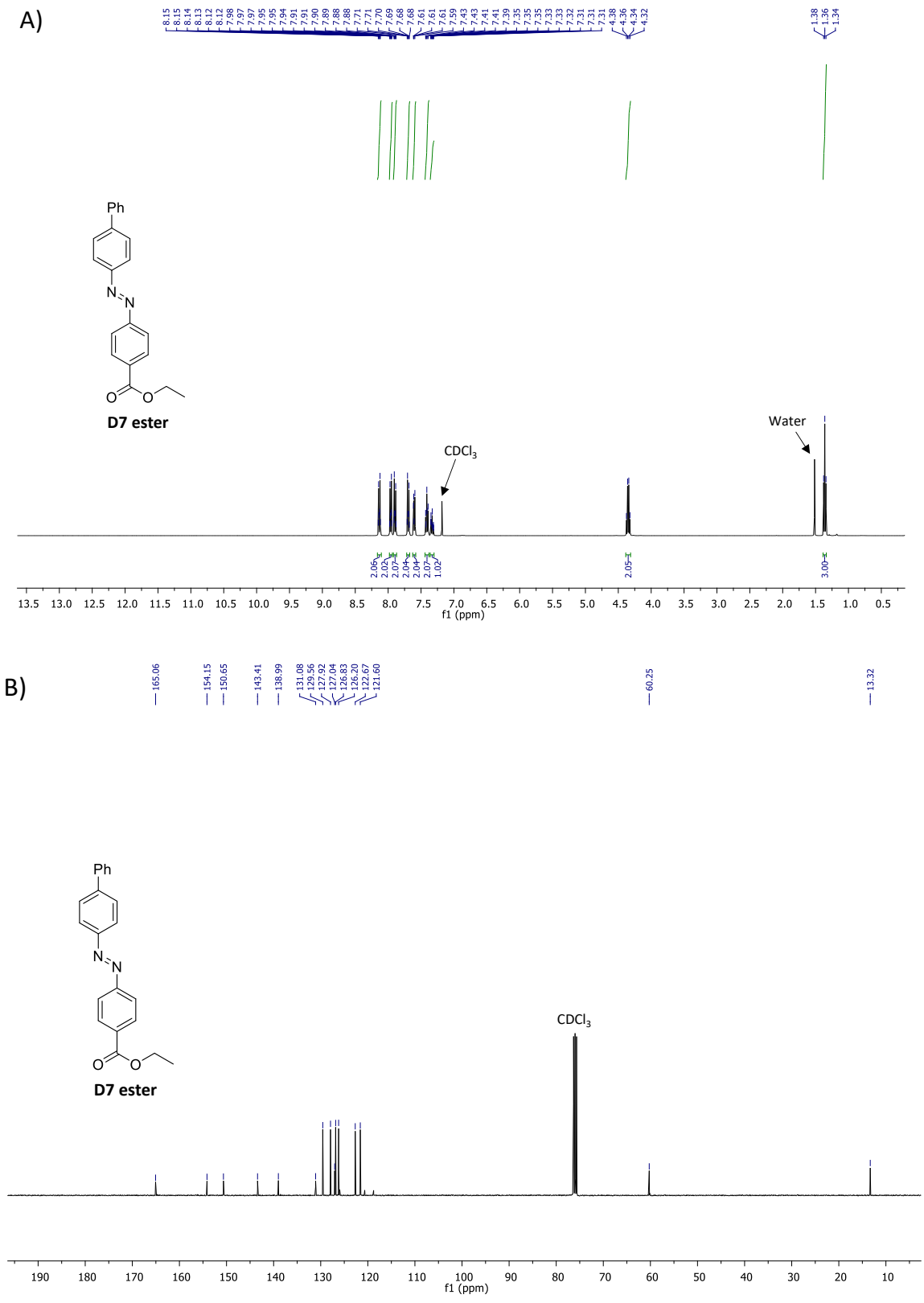


Figure S56: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D7 ester**.

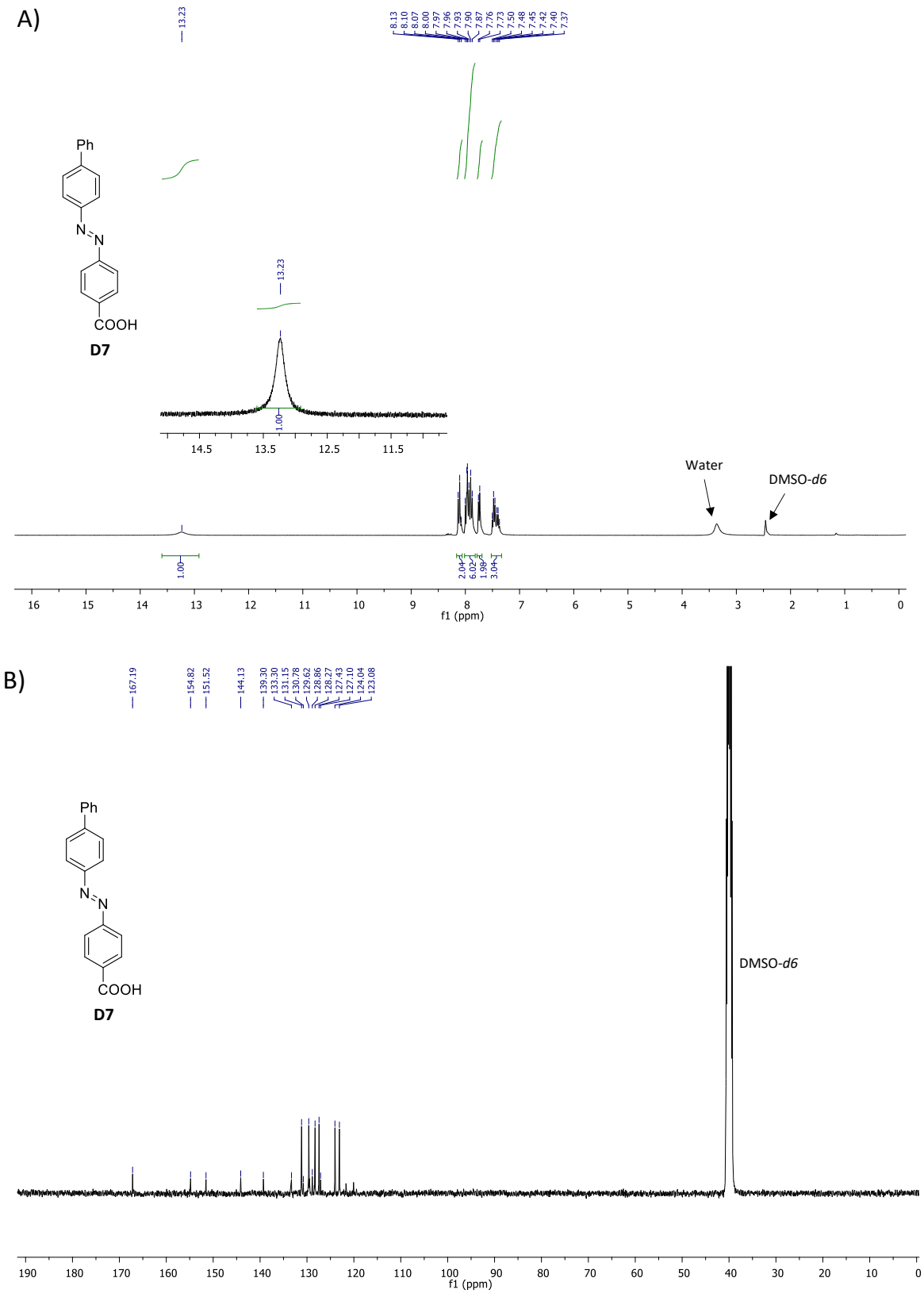


Figure S57: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D7**.

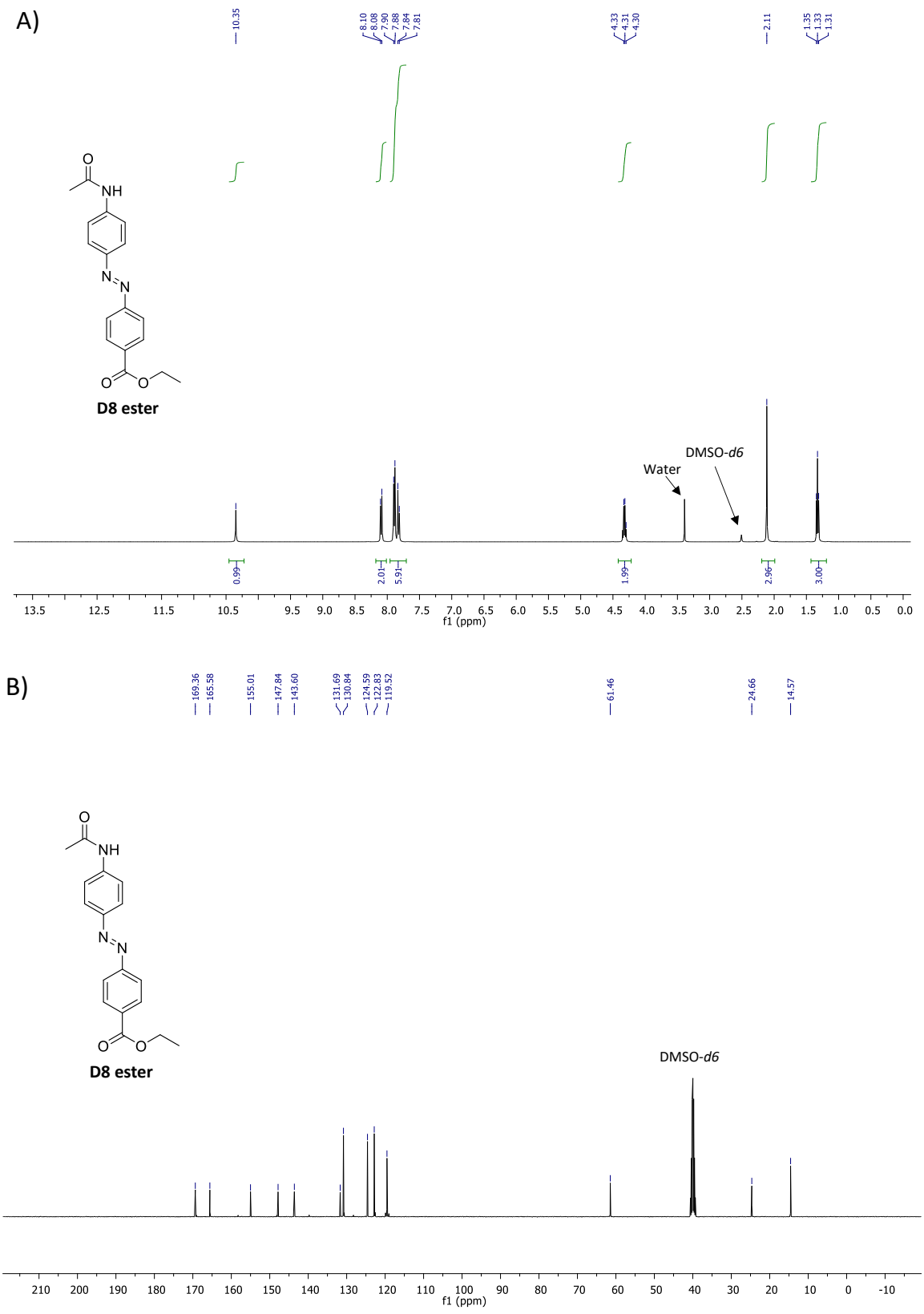


Figure S58: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D8 ester**.

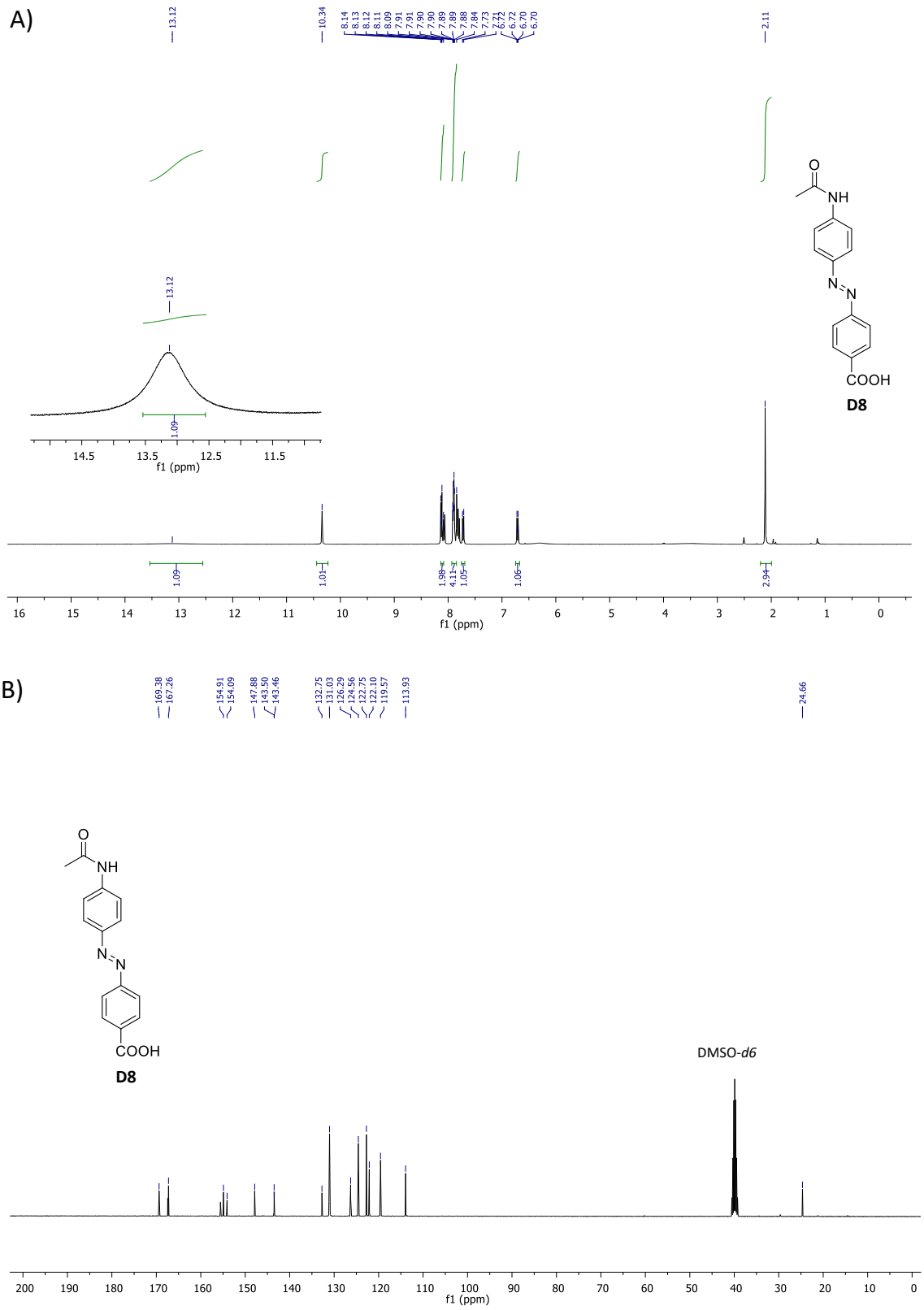


Figure S59: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D8**.

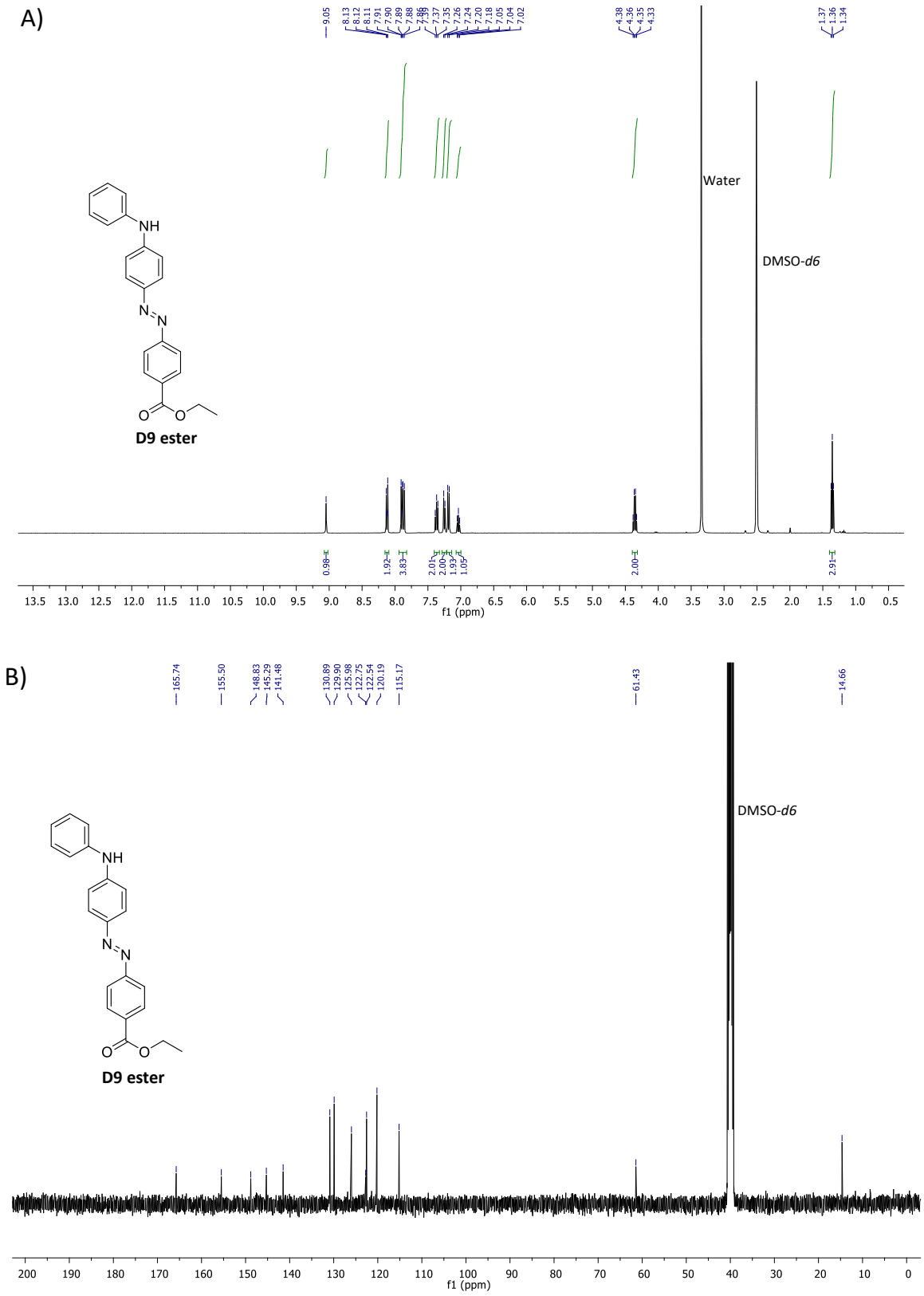


Figure S60: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D9 ester**.

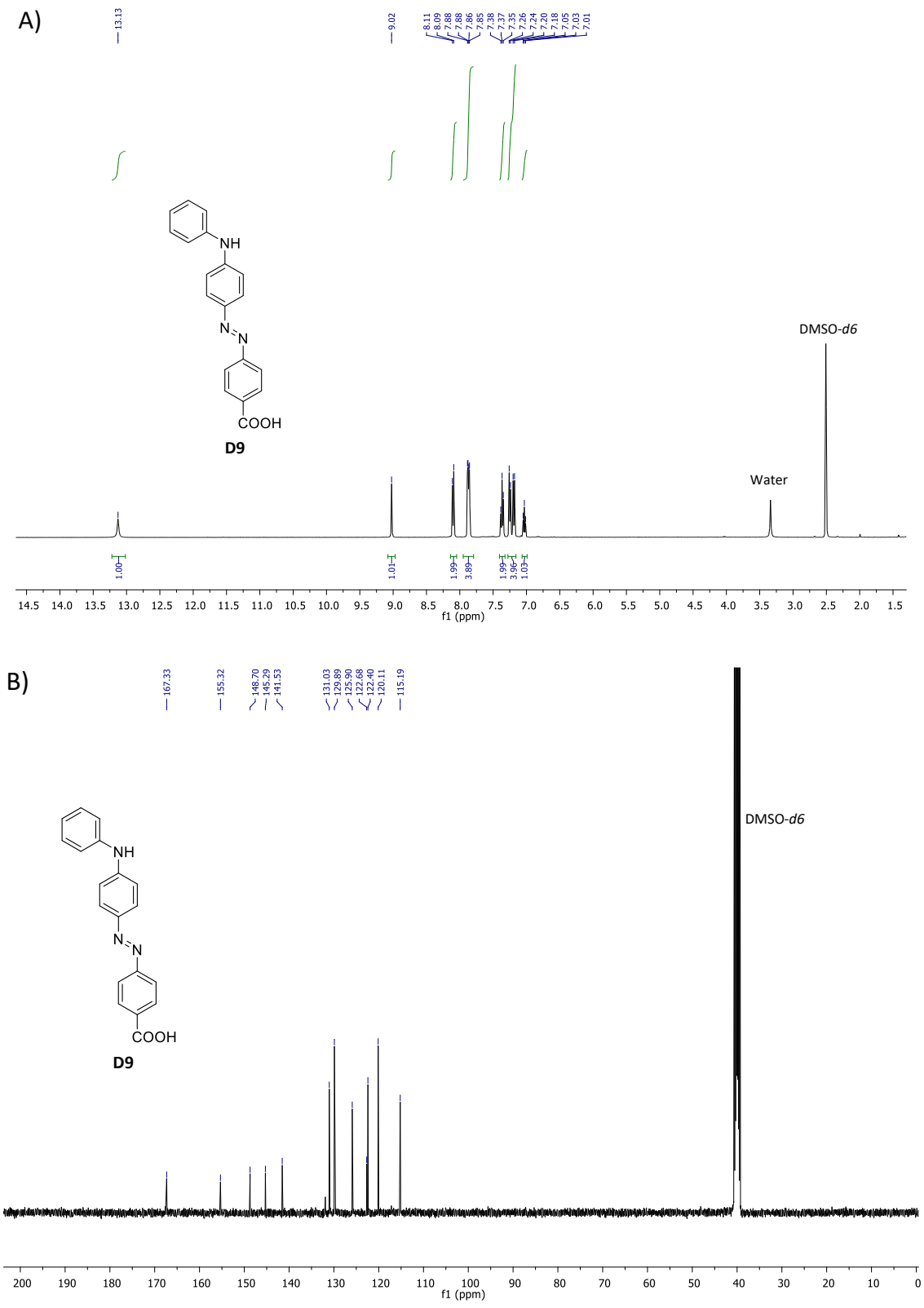


Figure S61: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D9**.

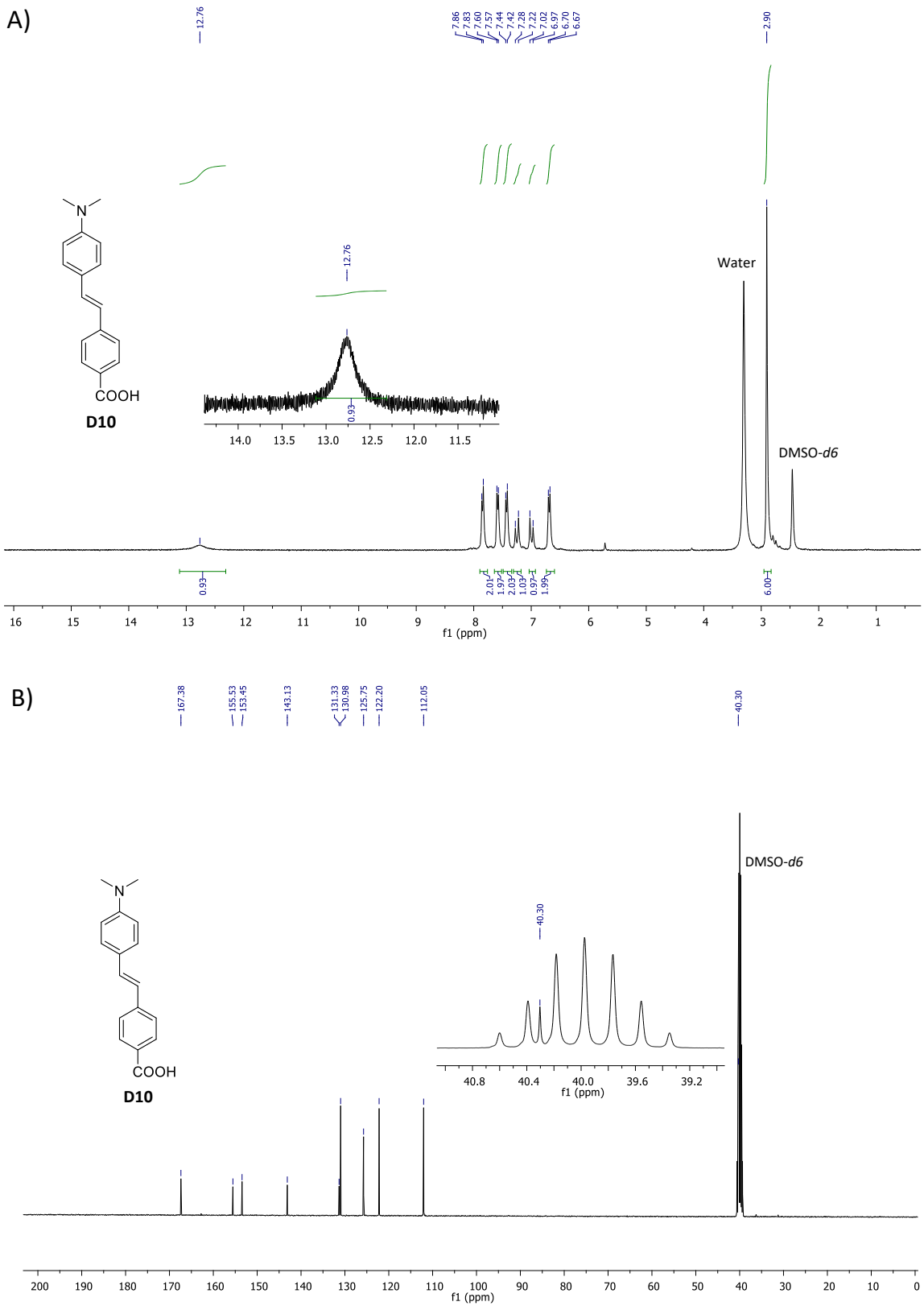


Figure S62: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D10**.

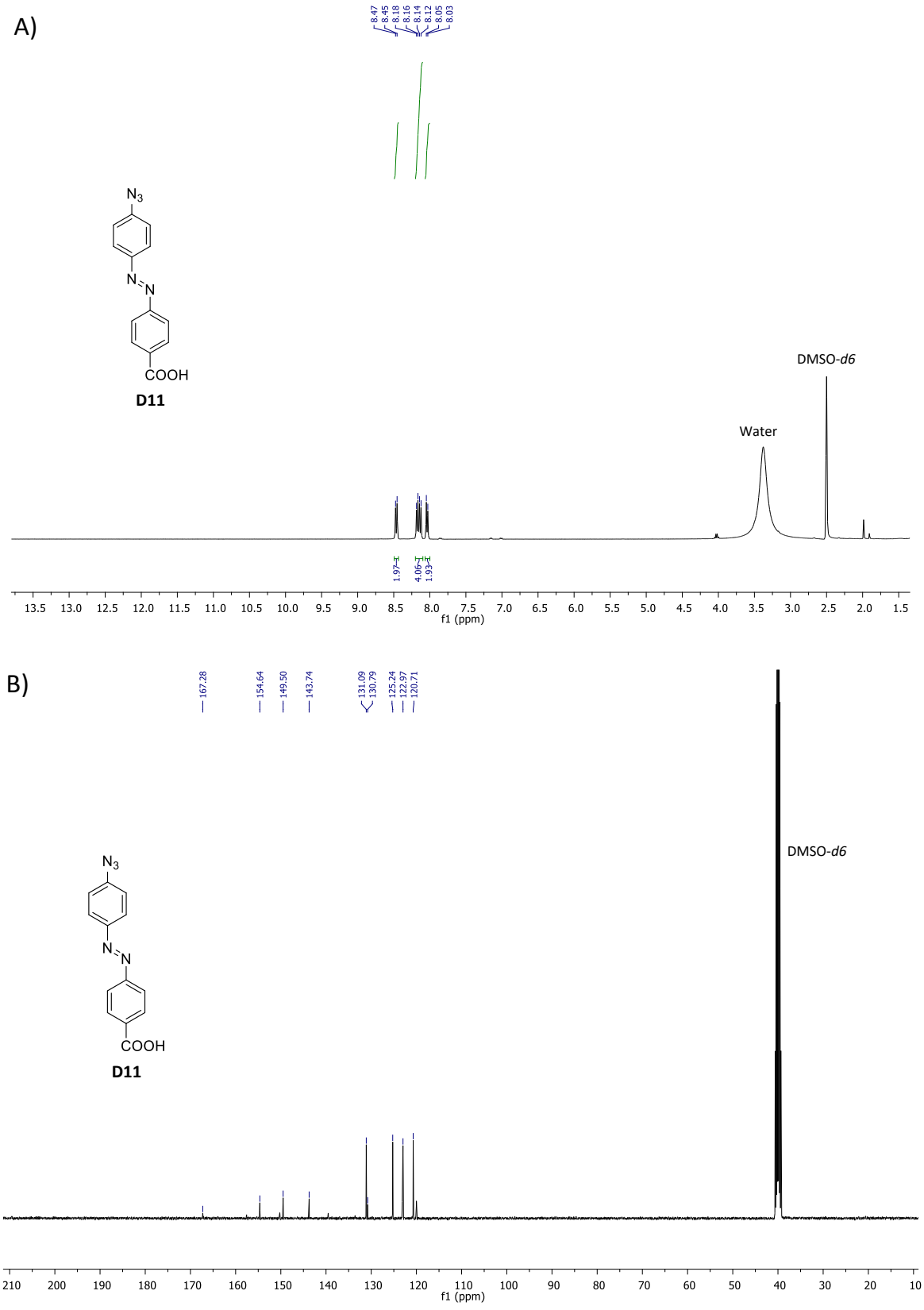


Figure S63: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D11**.

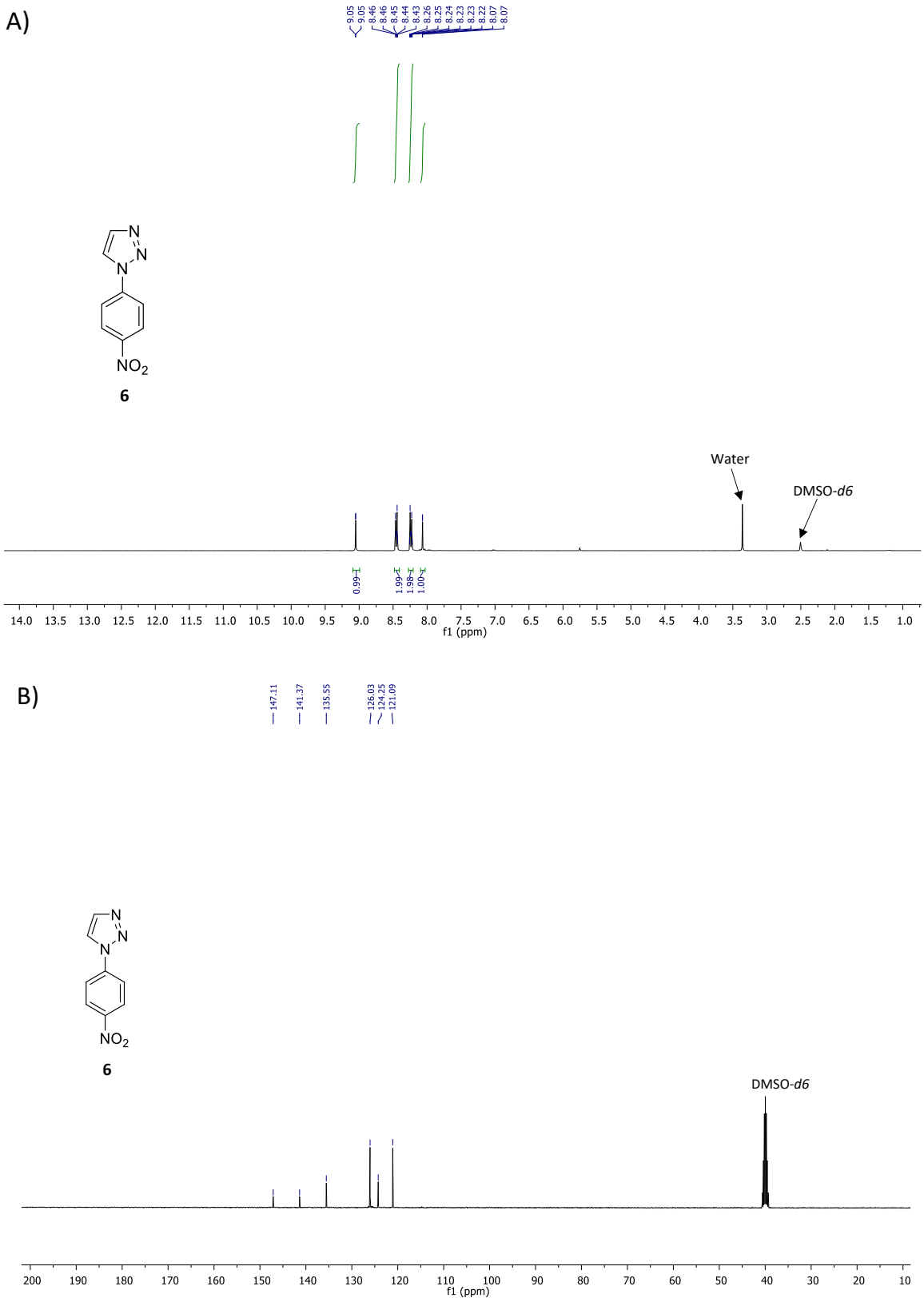


Figure S64: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **6**.

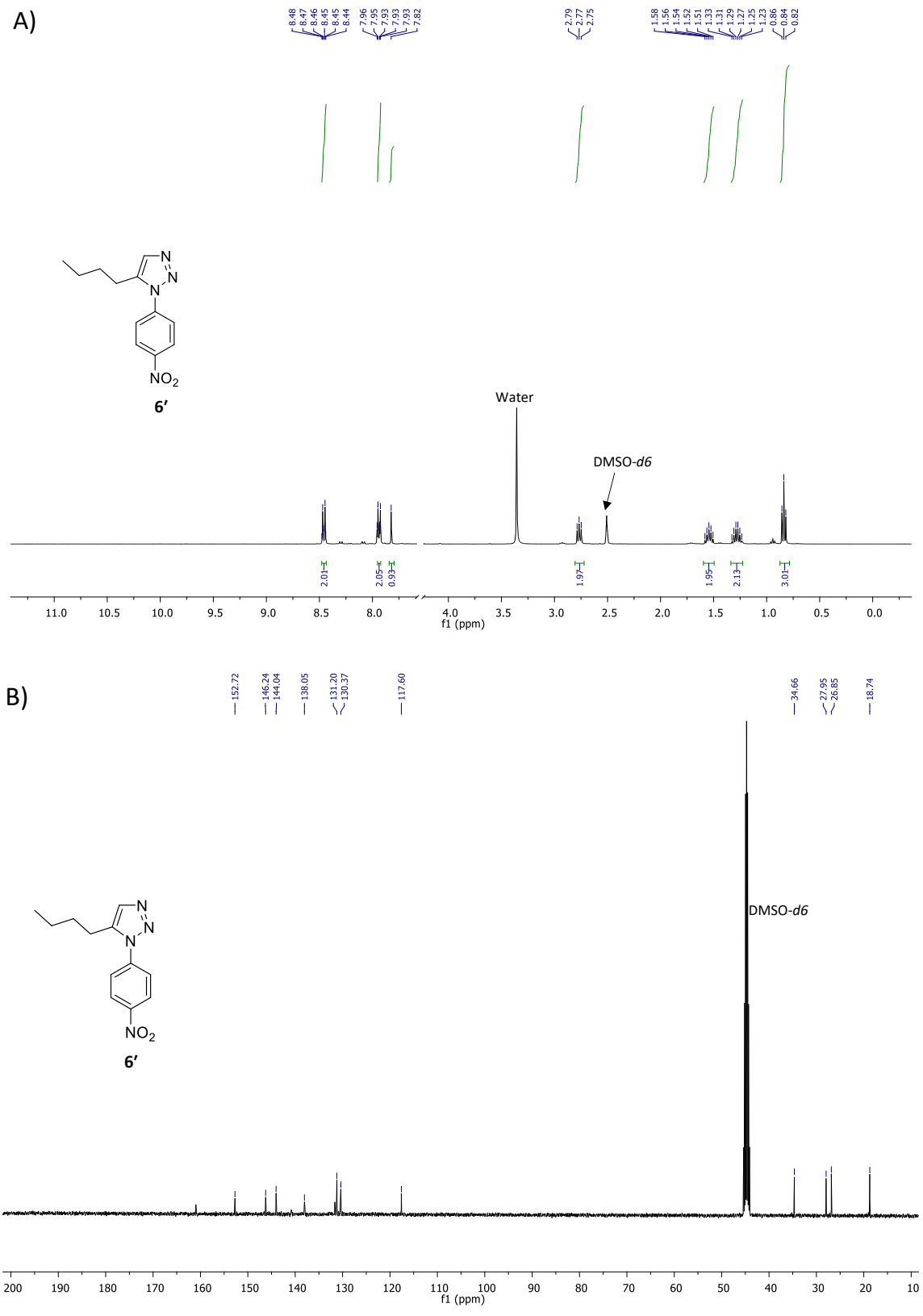


Figure S65: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **6′**.

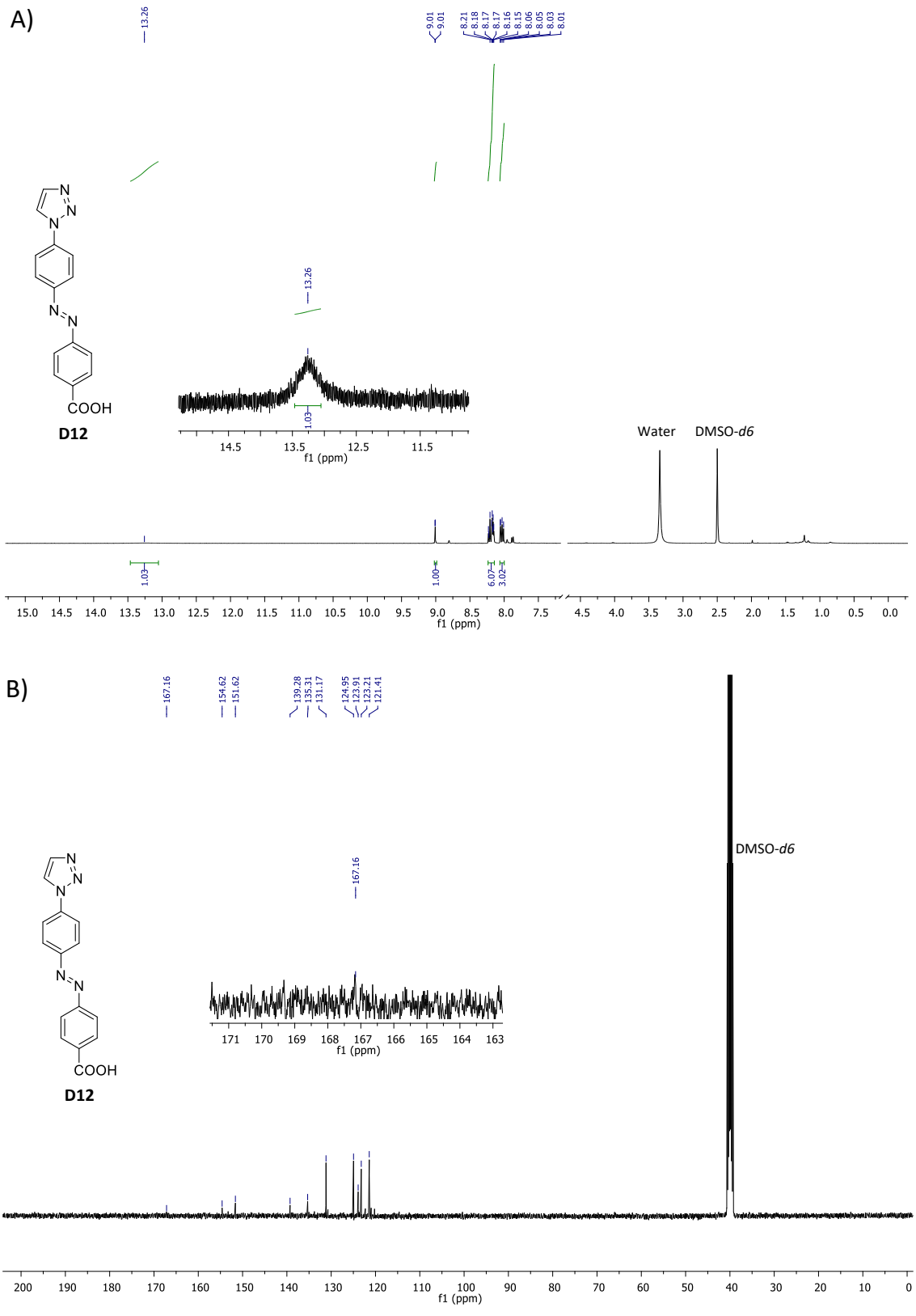


Figure S66: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D12**.

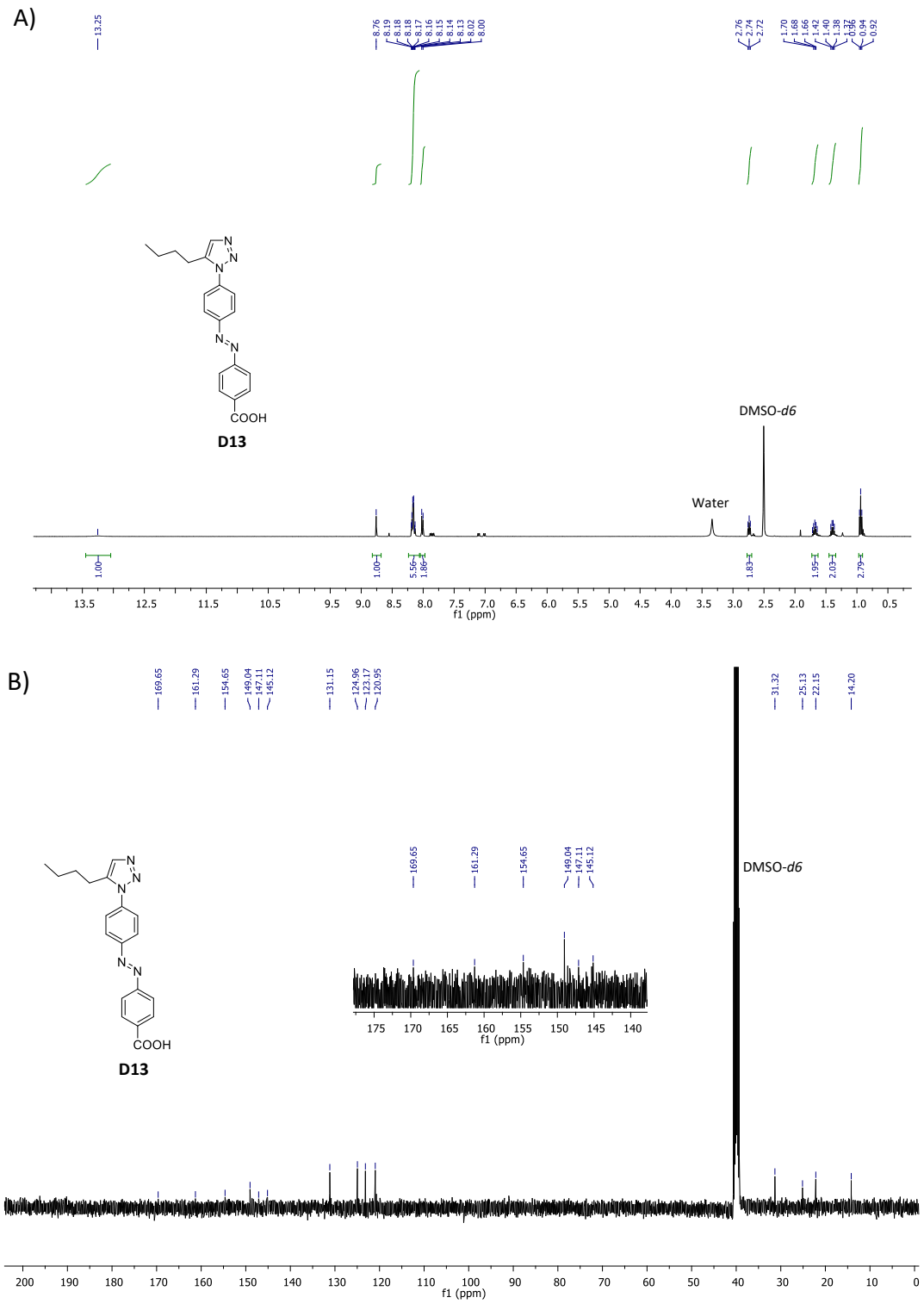


Figure S67: ^{1}H NMR (A) and ^{13}C NMR (B) of compound **D13**.

**** The source of all the water peaks was NMR solvents and not from any other sources.***

<u>HRMS spectra will be provided upon request.</u>

25. References:

1. Mandal, S., Mann, G., Satish, G. & Brik, A. Enhanced Live-Cell Delivery of Synthetic Proteins Assisted by Cell-Penetrating Peptides Fused to DABCYL. *Angew. Chemie Int. Ed.* **60**, 7333–7343 (2021).

2. Colom, A. *et al.* A fluorescent membrane tension probe. *Nat. Chem.* **10**, 1118–1125 (2018).

3. Galanti, A. *et al.* Electronic decoupling in C3-symmetrical light-responsive tris (azobenzene) scaffolds: self-assembly and multiphotochromism. *J. Am. Chem. Soc.* **140**, 16062–16070 (2018).

4. Hayashi, K. *et al.* Efficiency of lithium cations in hydrolysis reactions of esters in aqueous tetrahydrofuran. *Chem. Pharm. Bull.* **69**, 581–584 (2021).

5. Aksungur, T., Erol, F., Seferoğlu, N. & Seferoğlu, Z. Experimental and theoretical studies on monoazo dye including diphenylamine and N-methyldiphenylamine derivatives. *J. Mol. Struct.* **1217**, 128449 (2020).

6. Clement, B., Weide, M. & Ziegler, D. M. Inhibition of purified and membrane-bound flavin-containing monooxygenase 1 by (N, N-dimethylamino) stilbene carboxylates. *Chem. Res. Toxicol.* **9**, 599–604 (1996).

7. Habib, F. *et al.* Biofilm inhibition and DNA binding studies of isoxazole-triazole conjugates in the development of effective anti-bacterial agents. *J. Mol. Struct.* **1201**, 127144 (2020).

8. Kumar, A. S., Ghule, V. D., Subrahmanyam, S. & Sahoo, A. K. Synthesis of Thermally Stable Energetic 1, 2, 3-Triazole Derivatives. *Chem. Eur. J.* **19**, 509–518 (2013).

9. Bellamy, F. D. & Ou, K. Selective reduction of aromatic nitro compounds with stannous chloride in non acidic and non aqueous medium. *Tetrahedron Lett.* **25**, 839–842 (1984).

Author Contributions:

A.S. performed synthesis, purifications, and characterization of small molecules, cyclic peptides, proteins, and peptide-protein conjugates, also co-wrote the paper. S.M. carried out cell delivery assays and confocal analysis. J.G.G. performed the synthesis of Cy5-labeled cR10 and DABCYL-cR10. J.V.V.A. performed the time-lapse microscopy and fluorescence lifetime imaging microscopy experiments and analyzed the data with C.P.R.H. A.B. designed the project, supervised the entire project and the writing of the paper.