Supporting Information

Nitrodibenzofuran: a One- and Two-Photon Sensitive Protecting Group that is Superior to Brominated Hydroxycoumarin for Thiol Caging in Peptides

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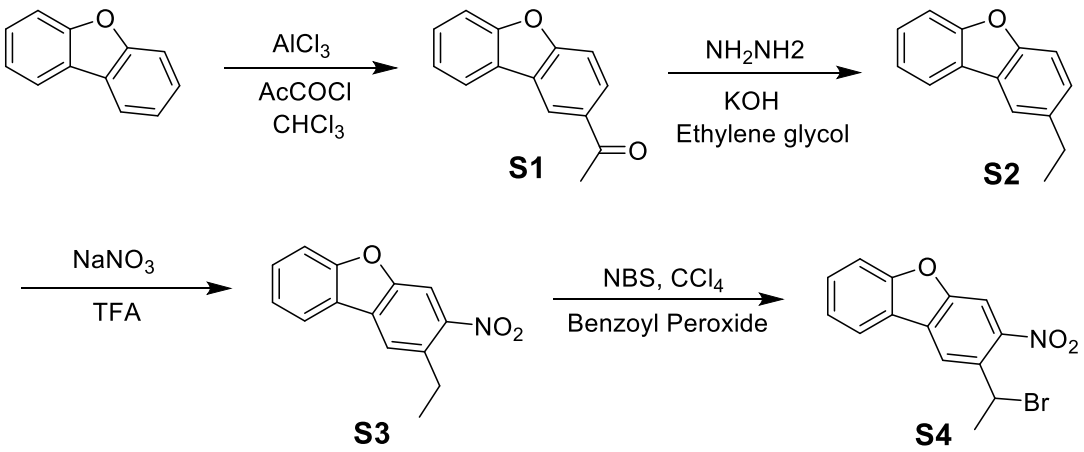
SYNTHESIS OF NDBF-BR

Scheme S1. Synthesis of NDBF-Br starting from dibenzofuran.



Synthesis of 2-acetyldibenzofuran (S1). Nitrodibenzofuran (2.5 g, 14.9 mmol) was dissolved in 30 mL of $CHCl_3$ in a round bottom flask equipped with a stir bar. In a separate 100 mL flask, 2.4 g (17.8 mmol) of freshly ground $AlCl_3$ was quickly added to 20 mL $CHCl_3$ (to avoid minimize moisture absorbtion), followed by addition of 1.4 mL (19.3 mmol) of acetyl chloride. The mixture was allowed to stir vigorously for about 10-15 min until most of the solid $AlCl_3$ was

dissolved. The resulting solution was slowly added to the solution of nitrodibenzofuran prepared above while stirring, which led to formation of a suspension. That mixture was allowed to stir for 2 h until the reaction reached almost complete conversion as judged by TLC (CH_2Cl_2). The mixture was poured into ice water and then diluted with 100 mL of 1.0 M $NaOH_{(aq)}$ solution followed by addition of 150 mL CH_2Cl_2. The two phases where stirred for 30 min until a clear organic phase was obtained. The organic layer was separated using a separatory funnel, and the aqueous phase was extracted with additional 100 mL of CH_2Cl_2. The combined organic layers were washed with brine and dried over Na_2SO_4. The solvent was evaporated *in vacuo*. The crude product was purified by silica gel coloumn chromatography using CH_2Cl_2 as the eluent. Solvent was evaporated *in vacuo* to afford 2.3 g (10.9 mmol) of compound **S1** as a white solid (73 % yield). In some cases, the final compound contains an impurity (less than 5% of 3-acetyldibenzofuran) which can be separated in further steps. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.60 (1H, s), 8.12 (1H, dd, J = 2 , 10 Hz), 8.01 (1H, d, J = 8.5 Hz), 7.59-7.62 (2H, m), 7.52 (1H, t, J = 7 Hz), 7.41 (1H, s), 2.73 (3H, s).

Synthesis of 2-ethyl nitrodibenzofuran (S2). In a round bottom flask, compound **S1** (2.1 g, 10.0 mmol) was combined with 40 mL of anhydrous ethylene glycol (40 mL), followed by addition of 1.4 g (25.0 mmol) of ground KOH and 0.78 mL (25 mmol) of hydrazine. The mixture was refluxed for 6 h until it was judged to be complete by TLC (Hexanes:EtOAc, 10:1, v/v). The reaction mixture was poured into ice water and then combined with 50 mL of 0.5 M aqueous HCl. The aqueous mixture was extracted with 3 × 100 mL CH_2Cl_2 (efficient extraction is critical for obtaining higher yield). The organic phase was washed with brine, dried over Na_2SO_4 and the solvent was evaporated *in vacuo.* The crude mixture was purified via silica gel column chromatography using a step gradient of solvents (Hexanes:EtOAc) starting from 10:1 (v/v)

going to 10:3 to afford 1.4 g of product **S2** (7.1 mmol) as a colorless oil (71 % yield). The column purification can usually be skipped if all starting material is consumed, since the resulting impurities with very low R_f values can be purified in later steps. However, in this case all of the residual ethylene glycol must be evaporated using a high vacuum pump. ^{1}H NMR (500MHz, $CDCl_3$) δ 7.95 (1H, d, J = 7 Hz), 7.79 (1H, s), 7.56 (1H, d, J = 8.5 Hz), 7.49 (1H, d, J = 8 Hz), 7.45 (1H, t, J = 7.5 Hz), 7.29-7.36 (2H, m), 2.82 (2H, q, J = 7.5 Hz), 1.34 (3H, t, J = 7.5 Hz).

Synthesis of 3-nitro-2-ethyldibenzofuran (NDBF, S3). Compound **S2** (1.0 g, 5.1 mmol) was mixed with 30 mL of CF_3CO_2H in a round bottom flask equipped with a stir bar. 434 mg of $NaNO_3$ (5.1 mmol) was added to the mixture while stirring, which caused the solution to turn dark (adding excess $NaNO_3$ can cause formation of a dinitro byproduct which is hard to separate from the product, hence, adding one equivalent, or slighly less, of $NaNO_3$ is safer). The resulting mixture was stirred for 2 h and judged completed by TLC (Hexanes:CH_2Cl_2, 2:1, v/v). The mixture was poured into ice water and the resulting mixture was partitioned between 50 mL of 0.5 M $NaOH_{(aq)}$ and 100 mL CH_2Cl_2. The organic layer was separated, washed with brine and dried over Na_2SO_4. Solvent was evaporated *in vacuo* and the crude mixture was purified via silica column chromatography using a step gradient of solvent (Hexanes: CH_2Cl_2) starting from 4:1 (v/v) going to 2:1 to afford 0.85 g of product **S3** (3.5 mmol) as a pale yellow oil (68.6 % yield). ^{1}H NMR (500MHz, $CDCl_3$) δ 8.15 (1H, s), 8.01 (1H, d, J = 7.5 Hz), 7.89 (1H, s), 7.62 (1H, d, J = 8 Hz), 7.57 (1H, t, J = 8 Hz), 7.42(1H, t, J = 7.5 Hz), 3.08 (2H, q, J = 7 Hz), 1.39 (3H, t, J = 7.5 Hz).

Synthesis of 3-nitro-2-bromoethyldibenzofuran (NDBF-Br, S4). Compound **S3** (0.70 g, 2.9 mmol), 0.52 g (2.9 mmol) of N-bromosuccinimide and 20 mg of benzoylperoxide was dissolved

in 20 mL of CCl_4 and refluxed for 4 h. The mixture was cooled to room temperature, diluted with 50 mL of CH_2Cl_2, washed with 0.1 % $NaHCO_{3(aq)}$, brine and then dried over Na_2SO_4. The solvent was evaporated *in vacuo* and the crude mixture was purified by silica column chromatography (EtOAc:Hexanes, 1:5, v/v) to afford 0.70 g of **S4** as a pale yellow solid (75 % yield). Characterization data has been previously reported by Davies and coworkers.[1]

ADDITIONAL FIGURES AND TABLES

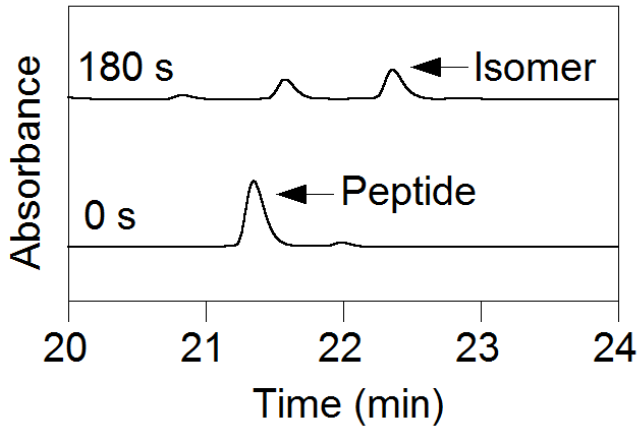


Figure S1. HPLC analysis of photolysis of 5-Fam-KKKSKTKC(Bhc)VIM. Formation of photo-isomer is apparent by the appearance of a new peak at higher retention time (22.25 min).

Ion	Observed Mass	Calcd Mass	Ion	Observed Mass	Calcd Mass
$[M + 3H]^+$	635.2597	635.2644	C_4^+	847.3990	847.4049
A_1^+	459.1556	459.1644	C_4^{2+}	424.2034	424.2072
A_1^{2+}	230.0817	230.1513	C_5^+	975.4940	975.6281
A_2^+	587.2505	587.2528	C_5^{2+}	488.2509	488.1607
A_3^{2+}	358.1767	358.0726	C_6^+	1076.5417	1076.4476
A_4^+	802.3776	802.2346	C_6^{2+}	538.7748	538.5560
A_5^+	930.4725	930.3101	X_1^+	176.0382	176.1213
A_6^{2+}	516.2640	516.1948	X_4^+	743.1420	743.3497
A_7^{2+}	580.3115	580.2455	X_4^{2+}	372.0750	372.1735
A_8^{2+}	757.7872	757.8033	X_5^+	871.2370	871.3129
A_9^{2+}	807.3214	807.3227	X_8^+	1187.4117	1187.6305
B_1^+	487.1505	487.1560	X_8^{2+}	594.2089	594.3130
B_2^+	615.2455	615.2582	X_{10}^+	722.3047	722.2593
B_2^{2+}	308.1267	308.1277	Y_1^+	150.0589	150.0580
B_3^+	743.3405	743.3497	Y_2^+	263.1430	263.1445
B_3^{2+}	308.1267	308.1277	Y_4^+	717.1628	717.1660
B_4^+	830.3725	830.3805	Y_4^{2+}	359.0853	359.0583
B_4^{2+}	415.6902	415.6909	Y_5^+	845.2578	845.2841
B_5^+	958.4674	958.4872	Y_6^+	946.3054	946.3098
B_5^{2+}	479.7377	479.7498	Y_7^+	1074.4004	1074.3961
B_6^+	1059.5151	1059.5208	Y_8^+	1161.4324	1161.4236
B_6^{2+}	530.2615	530.2635	Y_8^{2+}	581.2201	581.2468
B_7^+	1187.6101	1187.6305	Y_9^{2+}	645.2676	645.2709
B_7^{2+}	594.3080	594.3130	Y_{10}^{2+}	709.3151	709.3033
B_8^{2+}	771.7846	771.7882	Z_1^+	133.0324	133.0310
B_9^{2+}	821.3189	821.3290	Z_4^+	700.1362	700.2449
C_1^+	504.1771	504.1789	Z_5^+	828.2312	828.3145
C_2^+	632.2720	632.2689	Z_9^{2+}	636.7543	636.5992
C_3^+	760.3670	760.3704			

Table S1. Calculated and observed MS/MS fragments of the photoisomer derived from irradiation of 5-Fam-KKKSKTKC(Bhc)VIM.

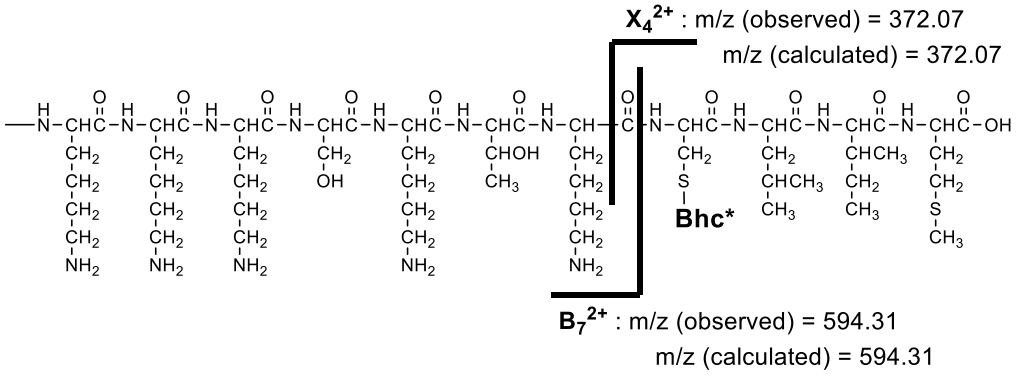


Figure S2. Two key MS/MS fragments of the photo-isomeric product of compound **5**, revealing that Bhc has not migrated to other residues within the peptide.

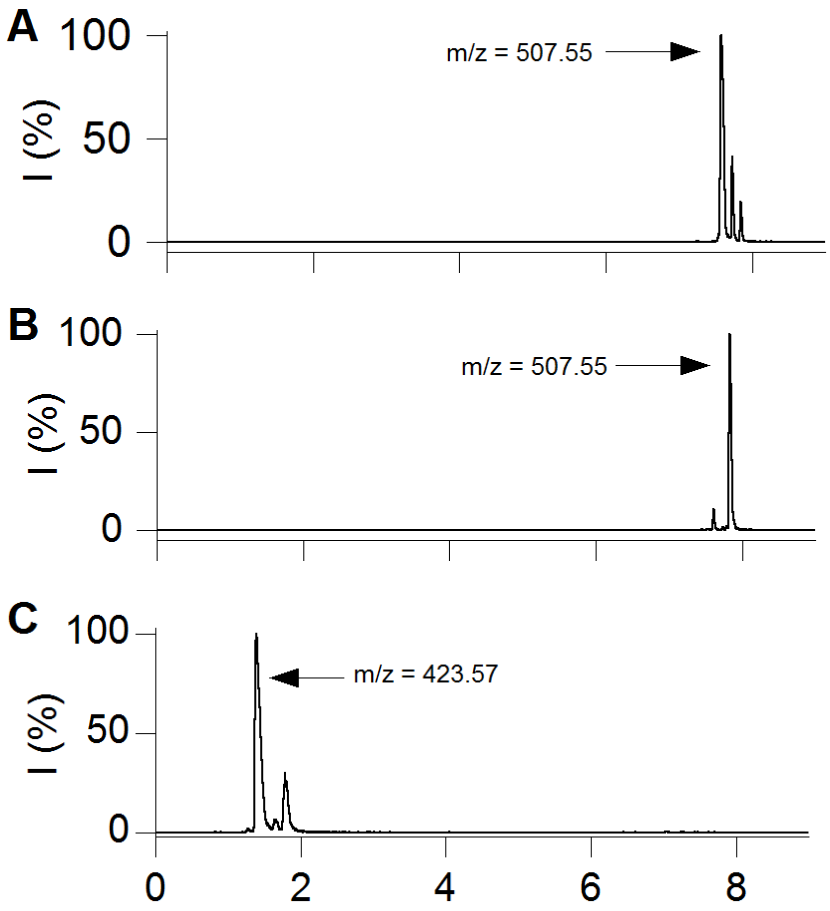


Figure S3. LC/MS analysis of photolysis reactions containing KKKSKTKC(Bhc)CVIM. (A) EIC chromatogram (m/z = 507.55) of a sample of KKKSKTKC(Bhc)CVIM prior to exposure to UV light; (B) EIC chromatogram (m/z = 507.55) of a photolyzed sample of KKKSKTKCC(Bhc)IM which clearly indicates formation of a new species with the same mass; (C) EIC chromatogram (m/z = 423.57) of a photolyzed sample showing that free peptide has been generated upon photolysis as well.

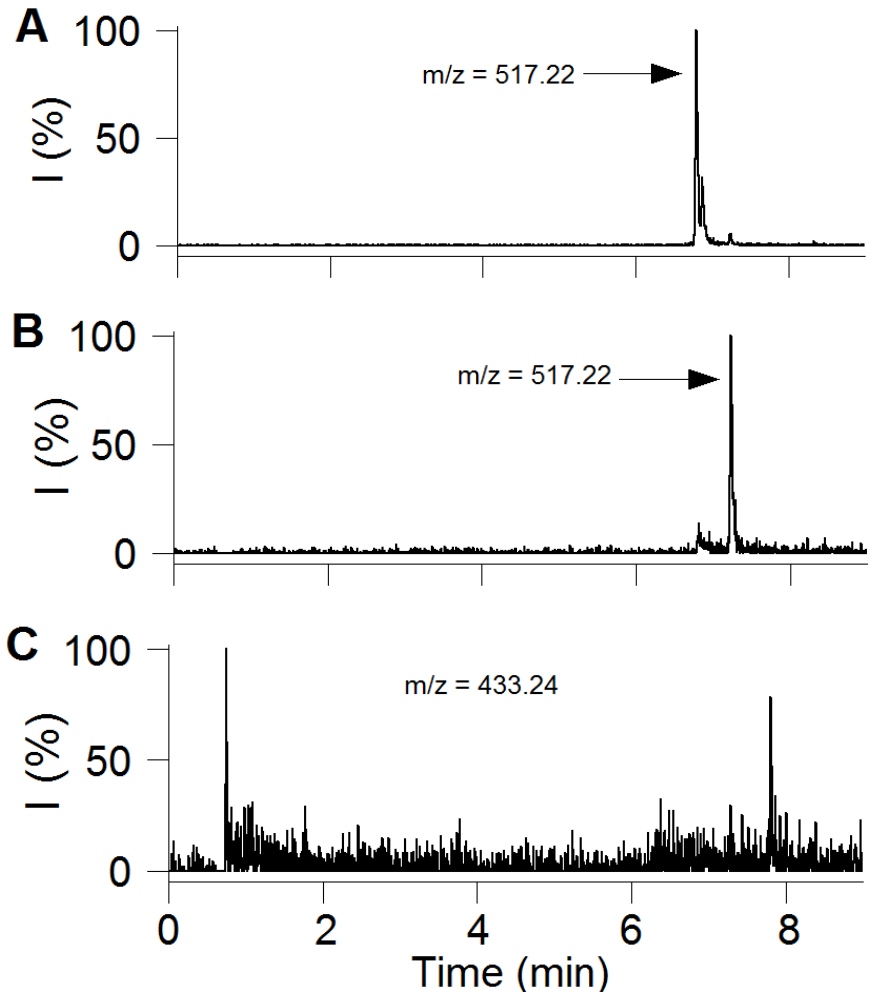


Figure S4. LC/MS analysis of photolysis reactions containing KKKSKTKCC(Bhc)IM. (A) EIC chromatogram (m/z = 517.22) of a sample of KKKSKTKCC(Bhc)IM without any exposure to UV light; (B) EIC chromatogram (m/z = 517.22) of a photolyzed sample of KKKSKTC(Bhc)CVIM which clearly indicates formation of a new species with the same mass; (C) EIC chromatogram (m/z = 433.24) of a photolyzed sample of KKKSKTC(Bhc)CVIM showing that no significant level of free peptide has been generated upon photolysis.

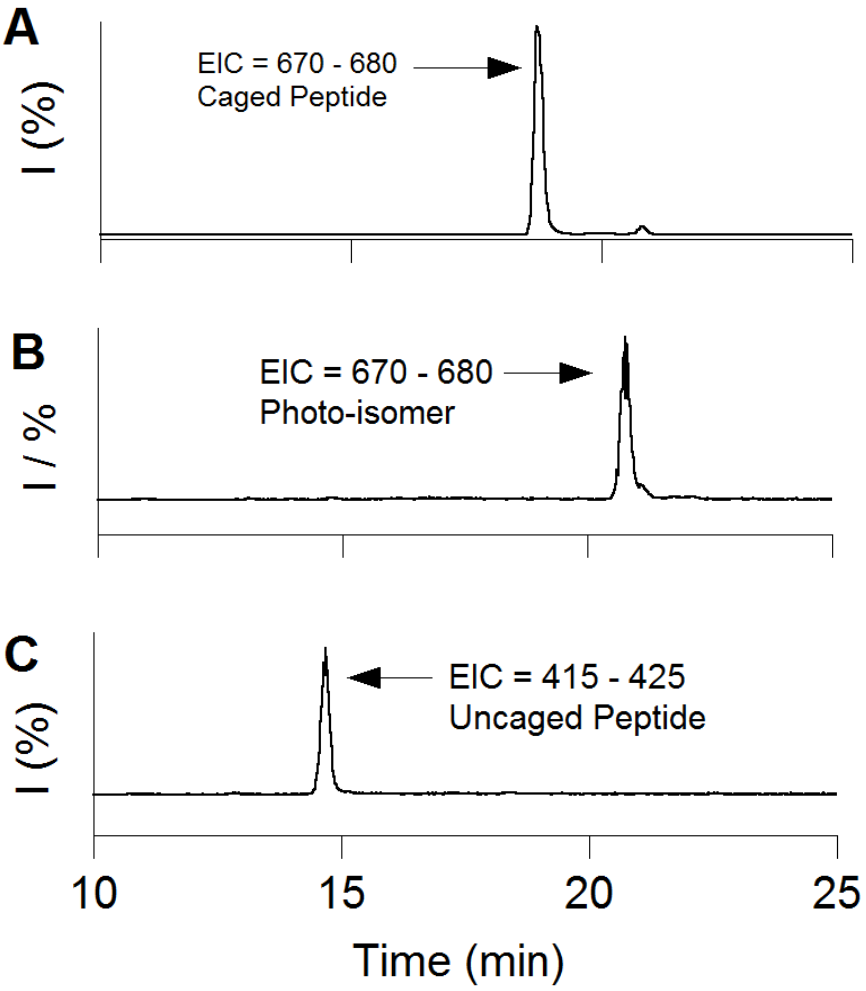


Figure S5. LC/MS analysis of photolysis reactions containing C(Bhc)VLS. (A) EIC chromatogram (m/z = 607 - 680) of a sample of C(Bhc)VLS without any exposure to UV light, (B) EIC chromatogram of a photolyzed sample of C(Bhc)VLS indicating formation of photo-isomer (m/z = 607 - 680) and (C) free peptide (415 – 425).

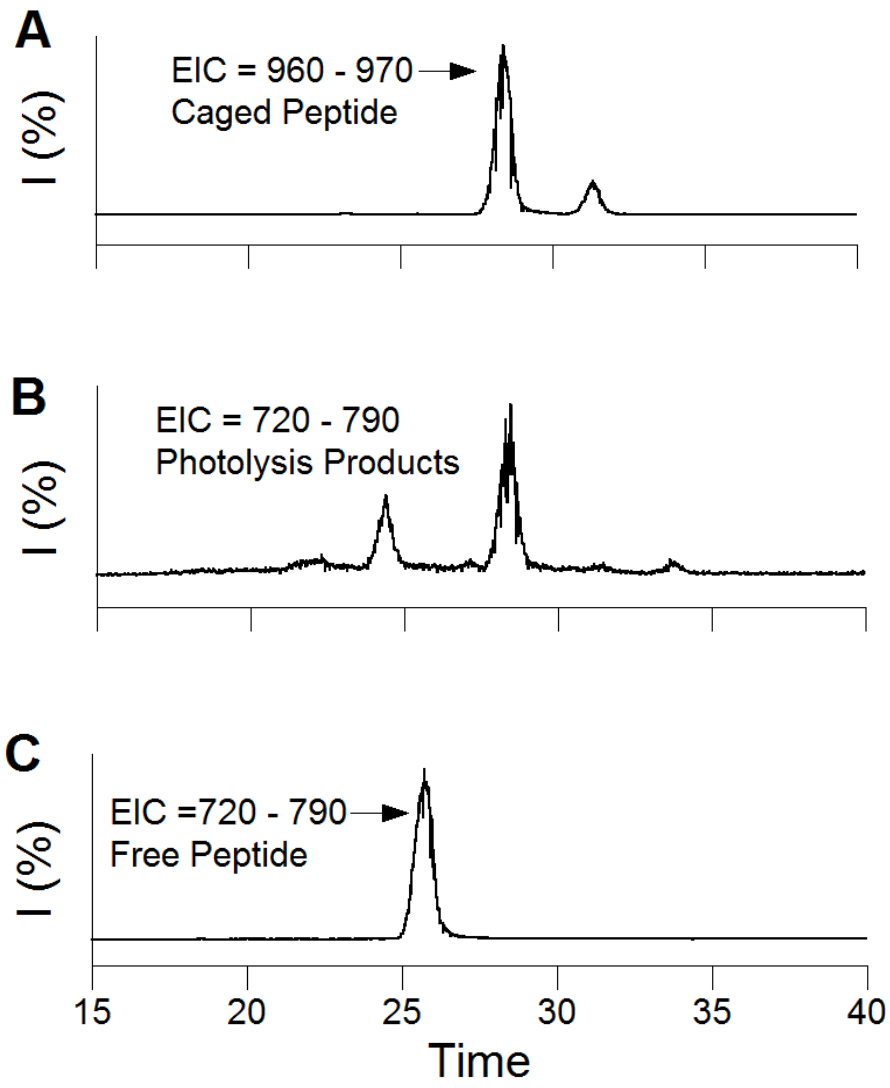


Figure S6. LC/MS analysis of photolysis reactions containing dansyl-GC(Bhc)VLS. (A) EIC chromatogram (m/z = 960 - 970) of a sample of dansyl-GC(Bhc)VLS without any exposure to UV light; (B) EIC chromatogram (m/z = 720 – 790) of a photolyzed sample of dansyl-GC(Bhc)VLS; (C) EIC chromatogram (m/z = 720 – 790) of standard sample of dansyl-GCVLS peptide. The absence of any ions related to the free peptide in the photolyzed sample (panel B) indicates that photolysis of dansyl-GC(Bhc)VLS does not lead to the production of free peptide.

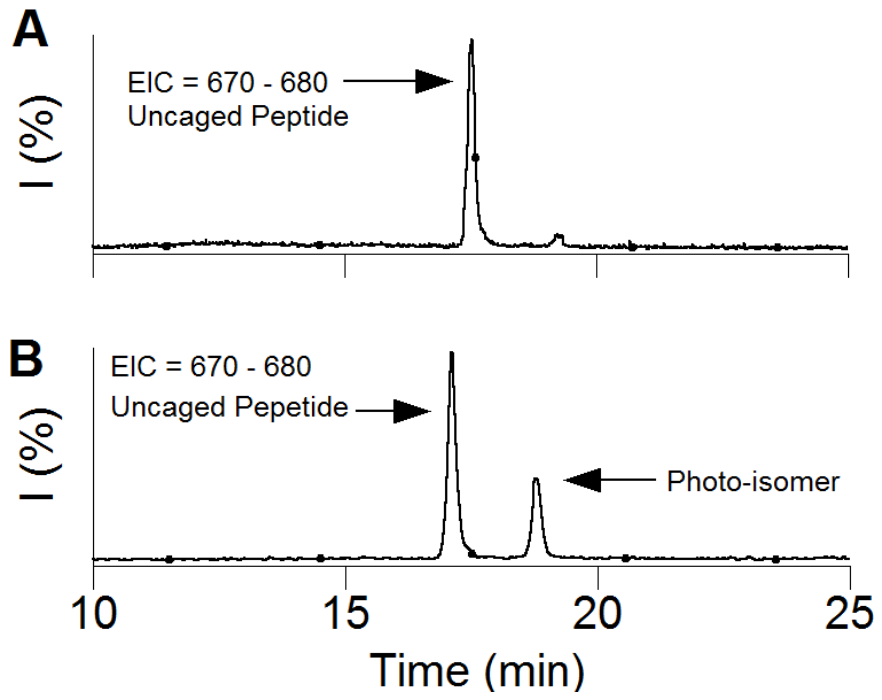


Figure S7. LC/MS analysis of two-photon photolysis of C(Bhc)VLS leading to generation of a photo-isomer. (A) EIC chromatogram (m/z = 607 - 680) of a sample of C(Bhc)VLS without any exposure to TP irradiation, (B) EIC chromatogram (m/z = 607 – 680) of a TP irradiated sample of C(Bhc)VLS which clearly indicates the formation a photo-isomer as evidenced by appearance of a peak at higher retention time having identical mass as starting caged peptide.

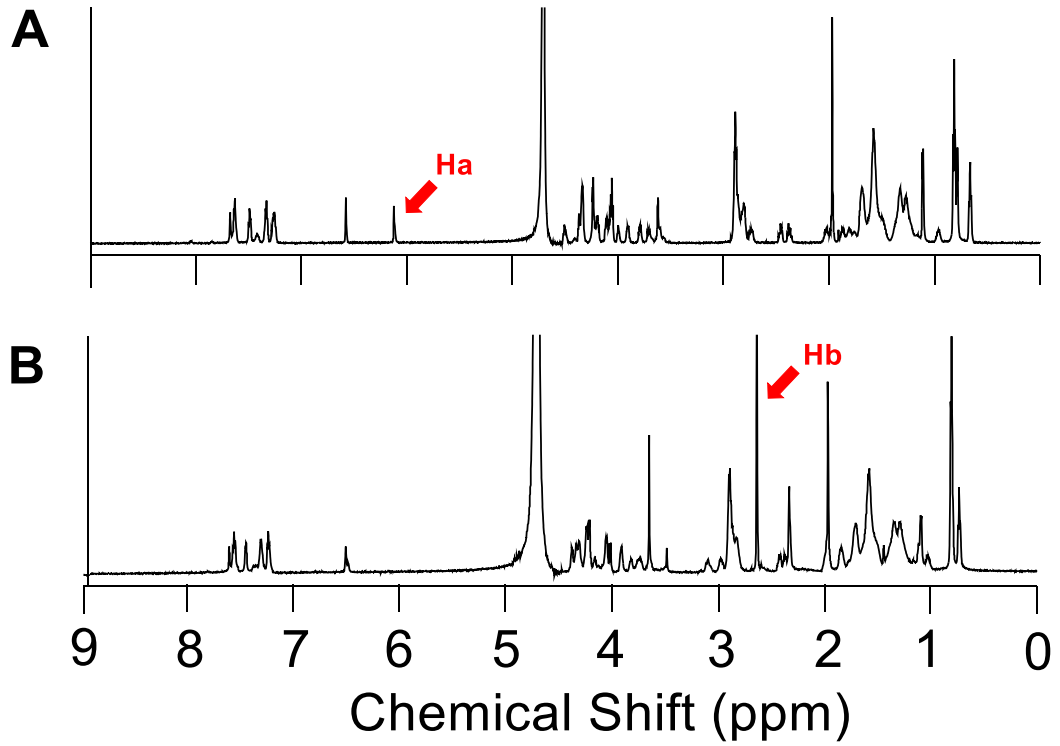


Figure S8. (A) ^{1}H NMR spectrum of a sample of caged peptide **5**, (B) and its photo-isomerized version.

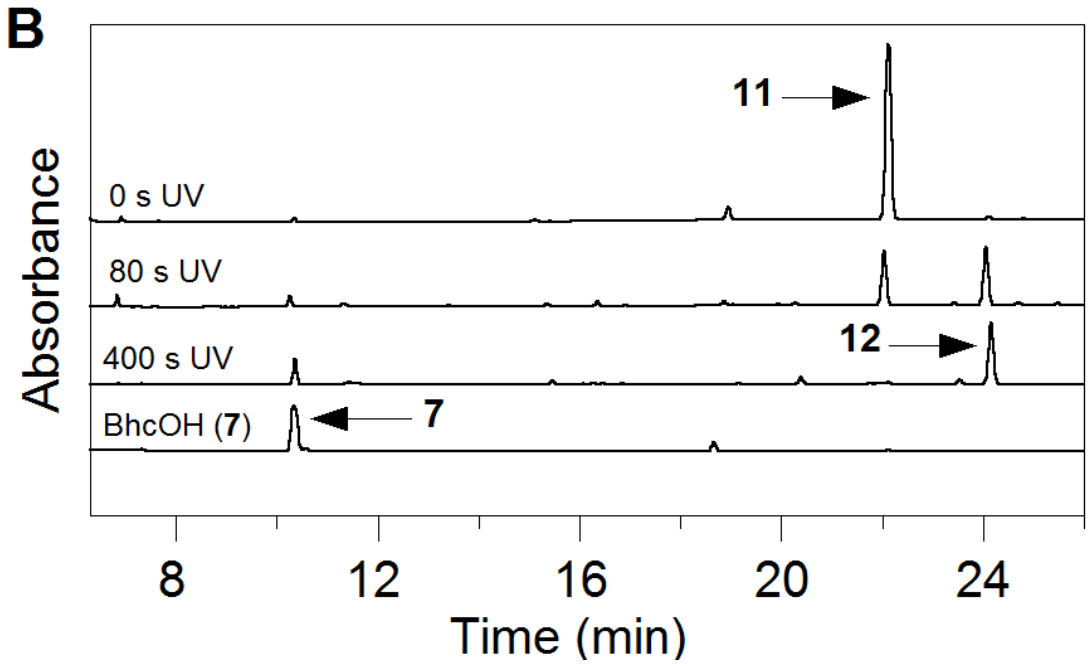


Figure S9. HPLC chromatograms representing time-course of photolysis of Bhc protected Boc-cysteamine (**11**).

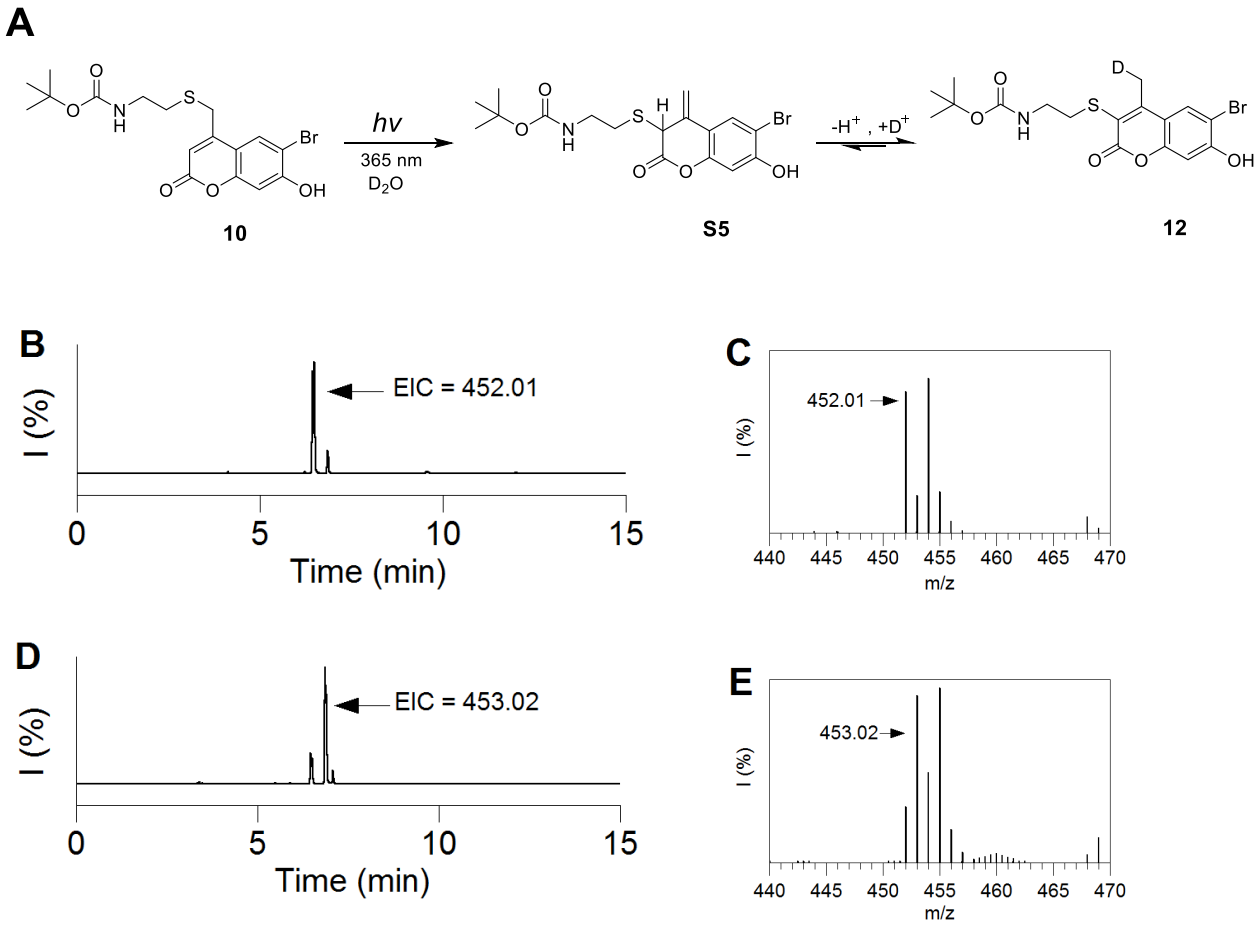


Figure S10. (A) Mechanism of photo-rearrangement of Bhc protected Boc-cysteamine (**10**) in D_2O. A 200 µM solution of **10** was irradiated for 2 min in deuterated photolysis buffer and subjected to LC-MS analysis. (B) EIC chromatogram of non-irradiated sample of **10** (calculated value for $[M + Na]^+$ = 452.01), and (C) the corresponding mass spectrum. (D) EIC chromatogram of irradiated sample of **10** (in the presence of D_2O) which shows formation of the photo-isomer (**12**) with one unit increase in mass relative to non-irradiated compound (calculated value for $[M + Na]^+$ is 453.02), and (E) is the corresponding mass spectrum. This data is consistent with the formation of **S5** as a photolysis intermediate and its subsequent tautomerization to form **12**.

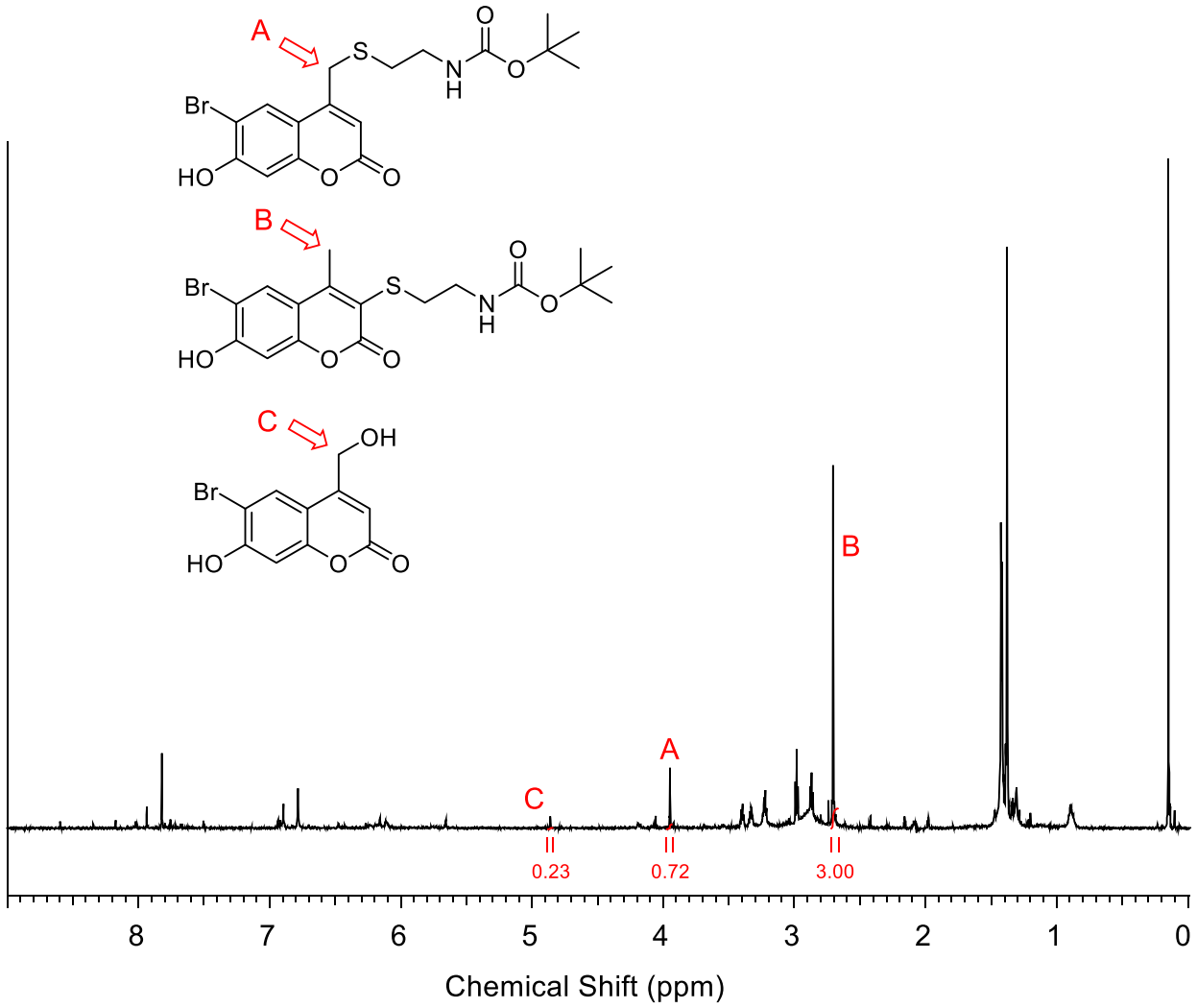


Figure S11. Crude NMR spectrum of a photolyzed sample of Boc-Bhc-cysteamine, which indicates the formation of a mixture of photo-isomer and uncaged product with a ratio of 8.7:1.0 (or 3/3H:0.23/2H). A 300 µM solution of Boc-Bhc-cysteamine and 1 mM DTT in 50 mM Tris buffer (pH = 7.2) was irradiated at 365 nm, lyophilized and the resulting powder was disolved in acetone-d6, and subjected to NMR analysis (Bruker Advance III 700 MHz spectrometer with 1.7 mm TCI cryoprobe instrument).

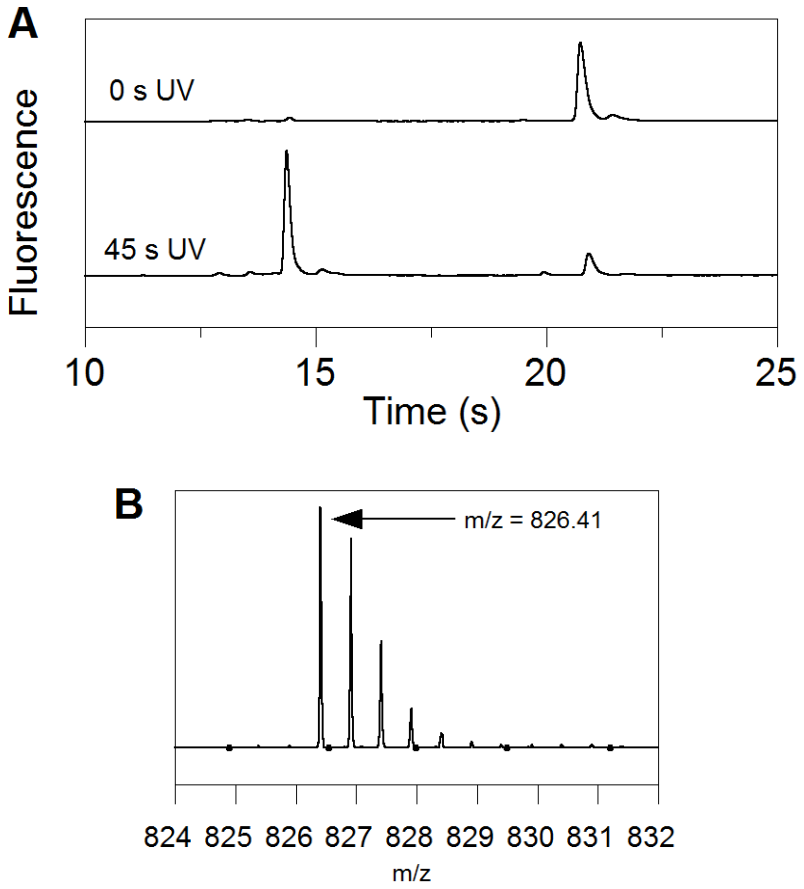


Figure S12. (A) HPLC traces demonstrating the clean photo-uncaging of a 100 μM solution of **17b** after 45 s irradiation at 365 nm. (B) mass spectrum of the free peptide (**6b**) (calculated for $[M+2H]^{2+}$ = 826.41) formed upon uncaging of compound **17b**.

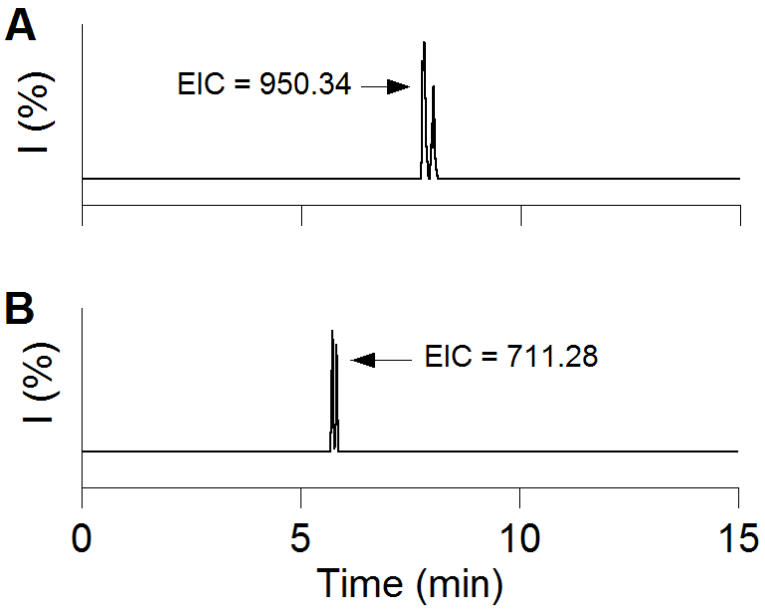


Figure S13. (A) EIC chromatogram (m/z = 950.34) of a sample of dansyl-GC(NDBF)VLS prior to exposure to UV light; (B) EIC chromatogram (m/z = 711.28) of a photolyzed (2 min at 365 nm) sample of dansyl-GC(NDBF)VLS indicating the formation of free peptide (dansyl-GCVLS). MS/MS fragmentation analysis also confirms the sequence of the two peptides (data not shown).

Ion	Observed Mass	Calcd Mass	Ion	Observed Mass	Calcd Mass
$[M + 2H]^+$	945.9450	94.9444	B_8^{2+}	765.3291	765.3427
A_1^+	459.1574	459.1556	B_9^{2+}	814.8596	814.8769
A_8^{2+}	751.3171	751.3452	B_{10}^{2+}	871.3977	871.4189
A_9^{2+}	800.8800	800.8794	B_{11}^{2+}	936.9163	936.9391
A_{10}^{2+}	857.4200	857.4214	C_1^+	504.1795	504.1771
B_1^+	487.1533	487.1505	C_2^+	632.2553	632.2720
B_2^+	615.2461	615.2423	C_4^+	847.3880	847.3990
B_3^+	743.3411	743.3275	C_6^+	1076.5411	1076.5417
B_4^+	830.3443	830.3725	C_6^{2+}	538.7741	538.7747
B_6^+	1059.4834	1059.5151	Y_1^+	150.0759	150.0589
B_7^+	1187.5613	1187.6101	Y_2^+	263.1576	263.1430
B_3^{2+}	372.1808	372.1742	Y_5^+	832.3501	832.3737
B_5^{2+}	479.7377	479.7460	Y_8^+	1148.5490	1148.5481
B_5^{2+}	530.2571	530.2615	Y_9^+	1276.6200	1276.6433
B_7^{2+}	594.3157	594.3090	Y_{10}^+	1404.6934	1404.7384

Table S2. Calculated and observed MS/MS fragments of 5-Fam-KKKSKTKC(NDBF)VIM.

Ion	Observed Mass	Calcd Mass	Ion	Observed Mass	Calcd Mass
$[M + 2H]^+$	826.4029	826.4148	B_6^{2+}	530.2672	530.2615
A_1^+	459.1574	459.1556	B_7^{2+}	594.3051	594.3090
A_8^{2+}	631.8154	631.8161	B_8^{2+}	645.8139	645.8136
A_{10}^{2+}	737.8743	737.8923	B_9^{2+}	695.3403	695.3478
B_1^+	487.1533	487.1505	B_{10}^{2+}	751.8809	751.8898
B_2^+	615.2423	615.2455	C_1^+	504.1795	504.1771
B_3^+	743.3394	743.3405	C_2^+	632.2665	632.2720
B_4^+	830.3569	830.3730	C_6^{2+}	538.7639	538.7748
B_6^+	1059.4691	1059.5151	Y_8^+	909.4658	909.4902
B_7^+	1187.5764	1187.6101	Y_9^+	1037.5529	1037.5852
B_2^{2+}	308.1409	308.1267	Y_{10}^+	1165.6354	1165.6801
B_3^{2+}	372.1808	372.1742	Z_{11}^{2+}	817.8973	817.9021
B_5^{2+}	479.7460	479.7377			

Table S3. Calculated and observed MS/MS fragments of 5-Fam-KKKSKTKCVIM produced upon uncaging of the corresponding NDBF caged peptide.

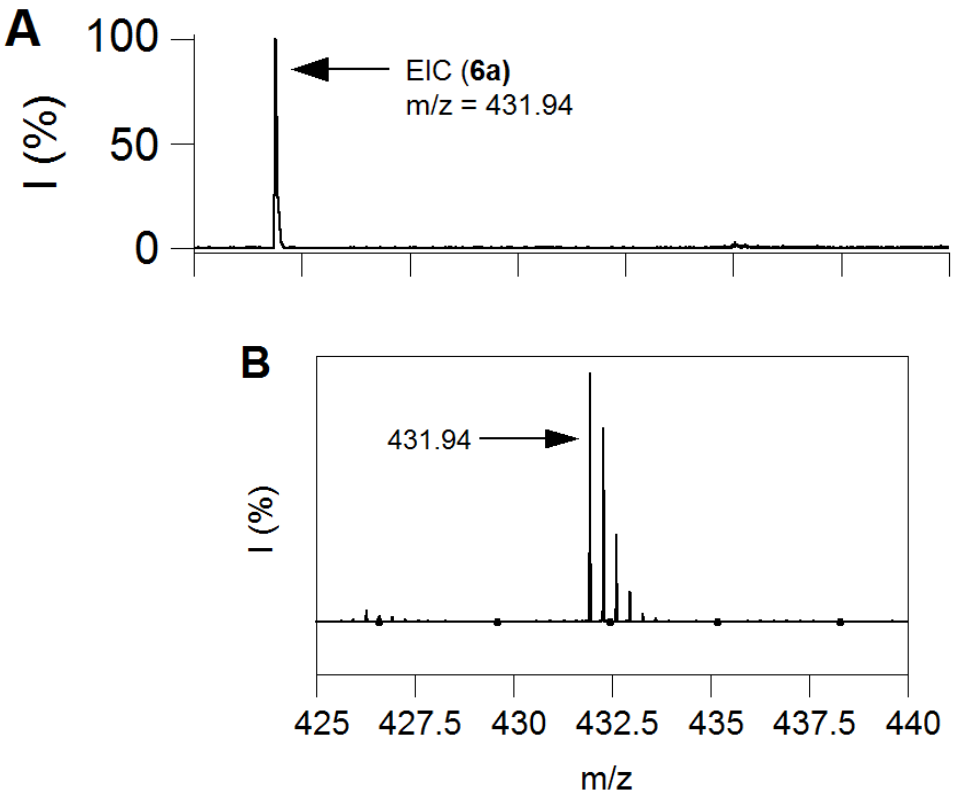


Figure S14. (A) EIC chromatogram of TP irradiated (2.5 min) sample of **17a** indicating the formation of free peptide **6a** as a result of uncaging (calculated mass for $[M+3H]^{3+}$ = 431.93), and (B) is the corresponding mass spectrum.

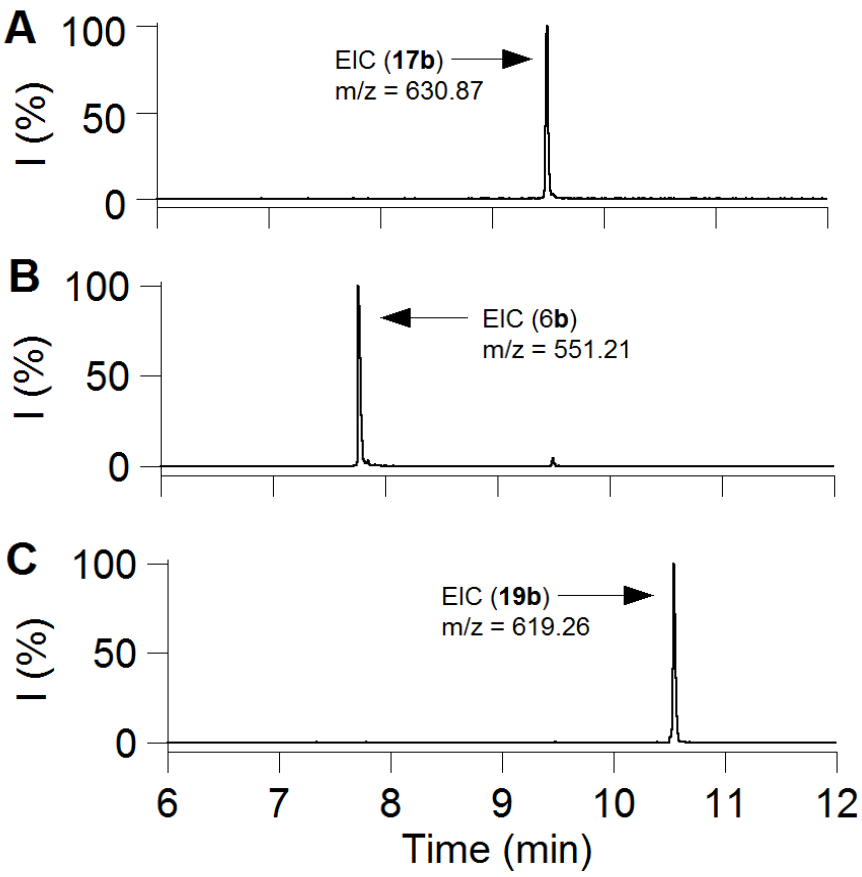


Figure S15. (A) EIC chromatogram (m/z = 630.30-630.31) of a 7.5 μM solution of **17b**, in prenylation buffer contining PFTase without irradiation, (B) EIC chromatogram (m/z = 551.00-552.00) of a solution of **17b**, after 60 s UV irradiation at 365 nm (Rayonet Photoreactor) in prenylation buffer without PFTase, (C) EIC chromatogram (m/z = 619.00-620.00) of **17b** after 60 s irradiation at 365 nm (Ti:Saphire laser, 170 mw, 100 fs) in presence of PFTase showing the formation of farnesylated peptide (**19b**).

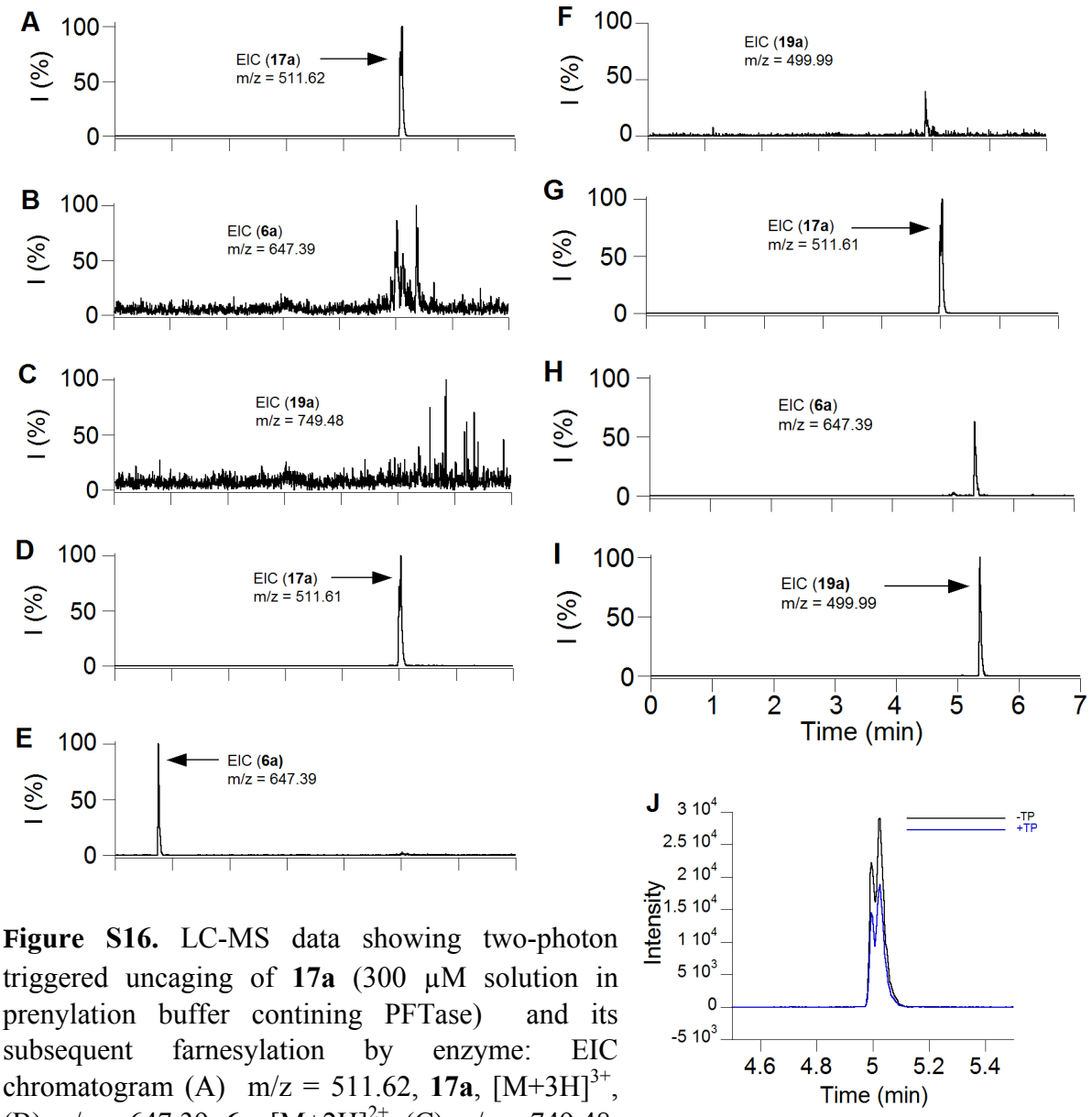


Figure S16. LC-MS data showing two-photon triggered uncaging of **17a** (300 µM solution in prenylation buffer contining PFTase) and its subsequent farnesylation by enzyme: EIC chromatogram (A) m/z = 511.62, **17a**, $[M+3H]^{3+}$, (B) m/z = 647.39, **6a**, $[M+2H]^{2+}$, (C) m/z = 749.48, **19a**, $[M+2H]^{2+}$ of a 300 µM solution of **17a**, in prenylation buffer containing PFTase without irradiation, neither uncaged nor the farnesylated peptide has formed; EIC chromatogram (D) m/z = 511.62, **17a**, $[M+3H]^{3+}$, (E) m/z = 647.39, **6a**, $[M+2H]^{2+}$, (F) m/z = 749.48, **19a**, $[M+2H]^{2+}$ of a solution of **17a**, after 2.5 min irradiation at 800 nm (Ti:Saphire laser, 170 mw, 90 fs) in prenylation buffer without PFTase showing the formation of undaged peptide **6a**; EIC chromatogram (G) m/z = 511.62, **17a**, $[M+3H]^{3+}$, (H) m/z = 647.39, **6a**, $[M+2H]^{2+}$, (I) m/z = 749.48, **19a**, $[M+2H]^{2+}$ of **17a** after 2.5 min irradiation at 800 nm (Ti:Saphire laser, 210 mw, 90 fs) in presence of PFTase showing the formation of farnesylated peptide (**19a**); (J) Analysis of **17a** by LC-MS before and after two-photon irradiation showing 30 % uncaging occurred.

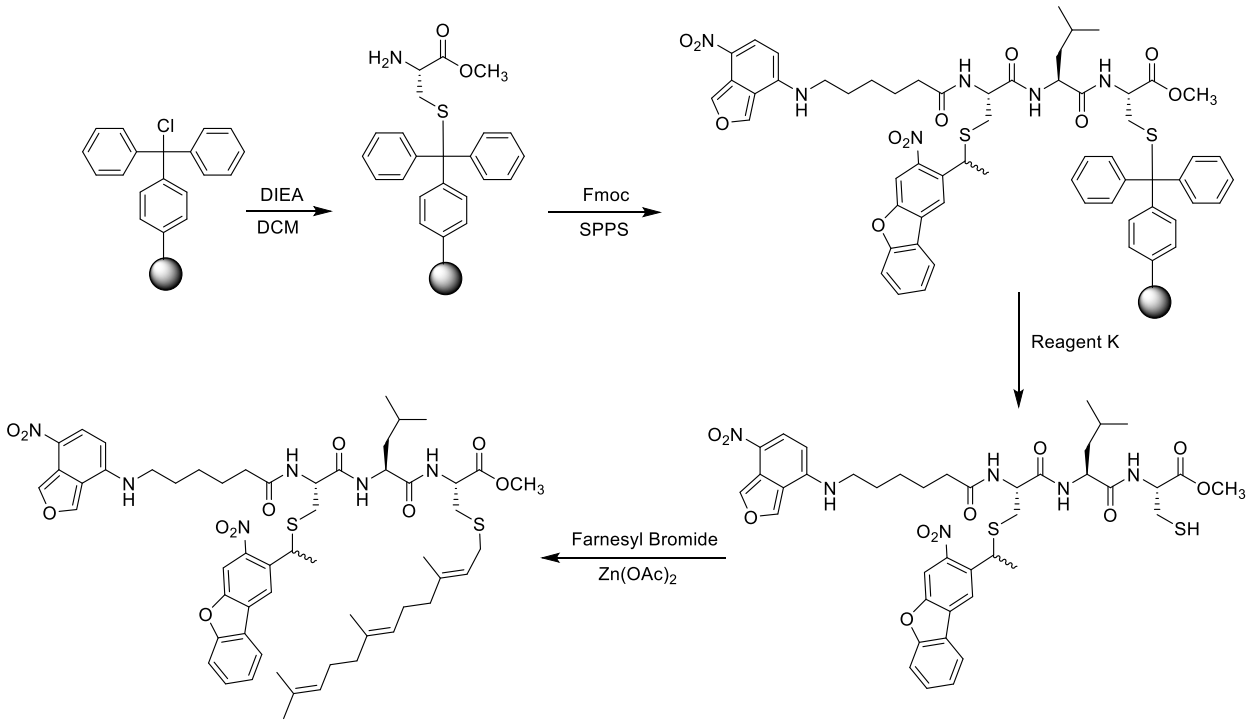


Figure S17. Schematic representation of synthesis of NBD-HecC(NDBF)LC(farnesyl)OMe (**20**) via cysteine anchoring method.

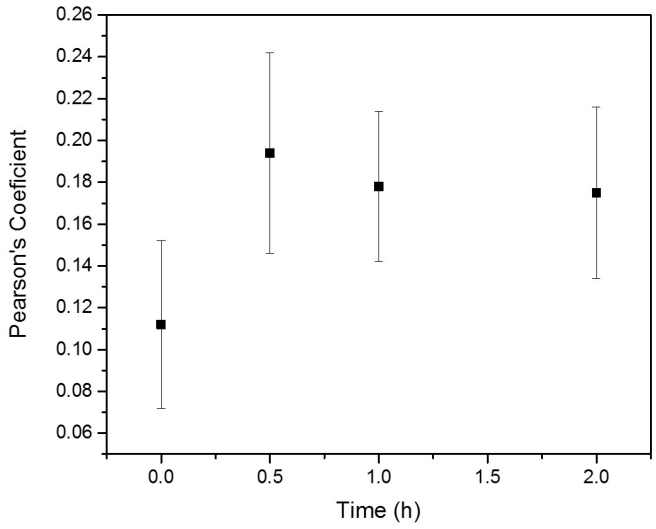


Figure S18. Kinetic analysis of peptide colocalization with a plasma membrane marker after uncaging. Live cell experiments in which SKOV-3 cells were treated with **20**, irradiated and the membrane localization of peptide was monitored by fluorescence microscopy over time, and

quantified by measuring the fluorescence co-localization of the peptide and a plasma membrane marker.

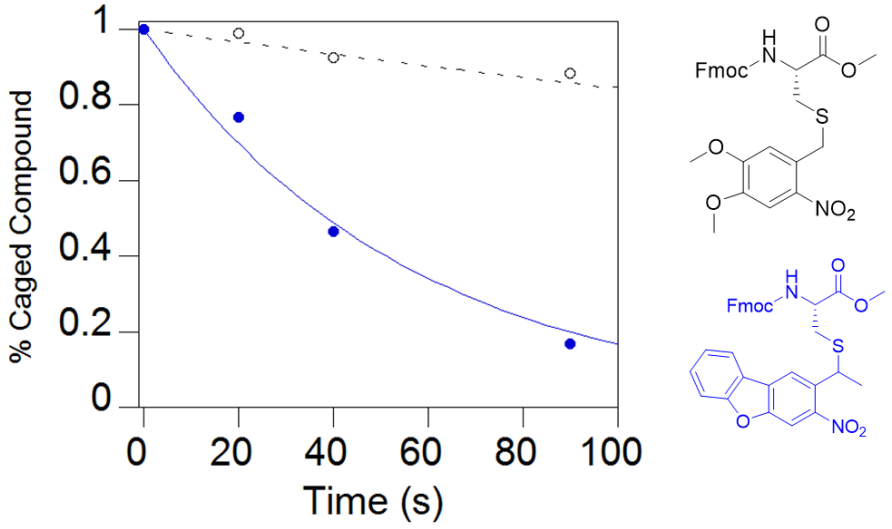


Figure S19. Uncaging efficiency of NDBF-protected versus NV- protected Fmoc-Cys-OMe upon UV irradiation (365 nm, Rayonet photo-reactor) obtained by RP-HPLC.

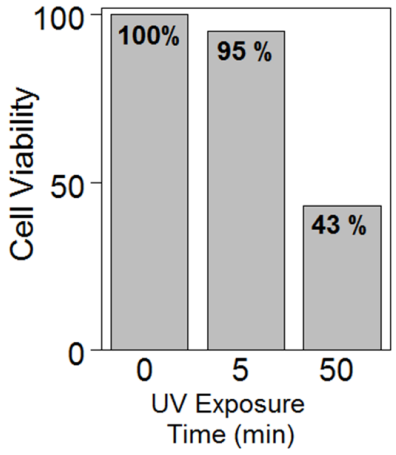
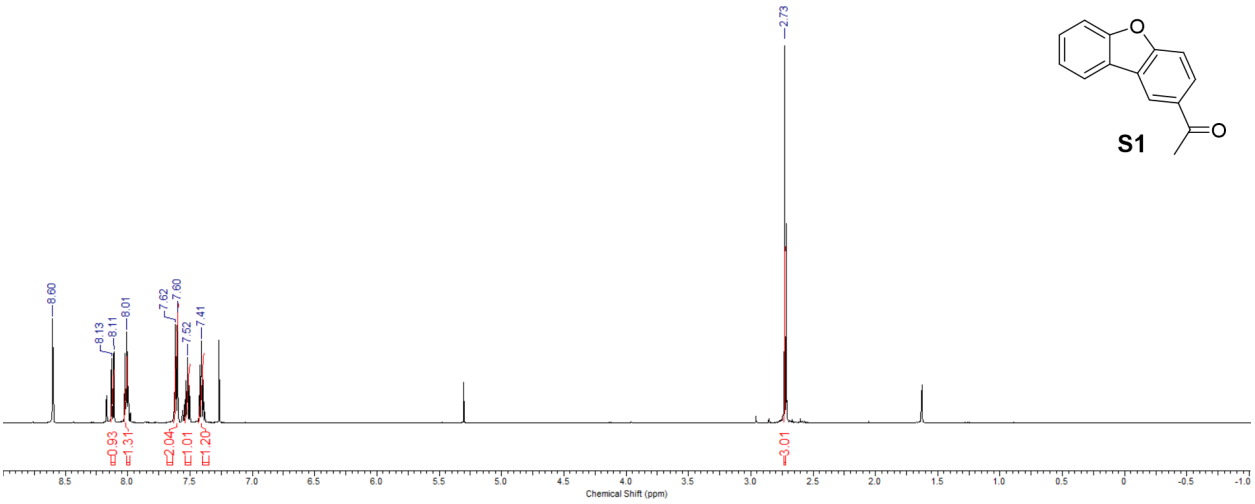


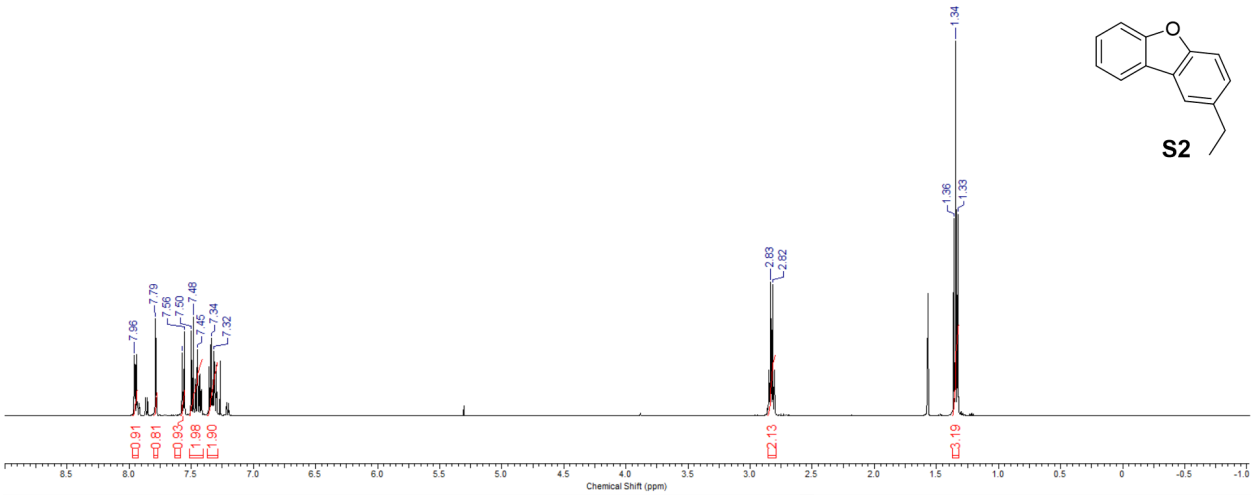
Figure S20. Cell viability data obtained via MTT assay, showing how long UV exposure results in major photo-toxicity and cell death. This result highlights the advantages of using highly sensitive NDBF as a caging group for biological studies.

References

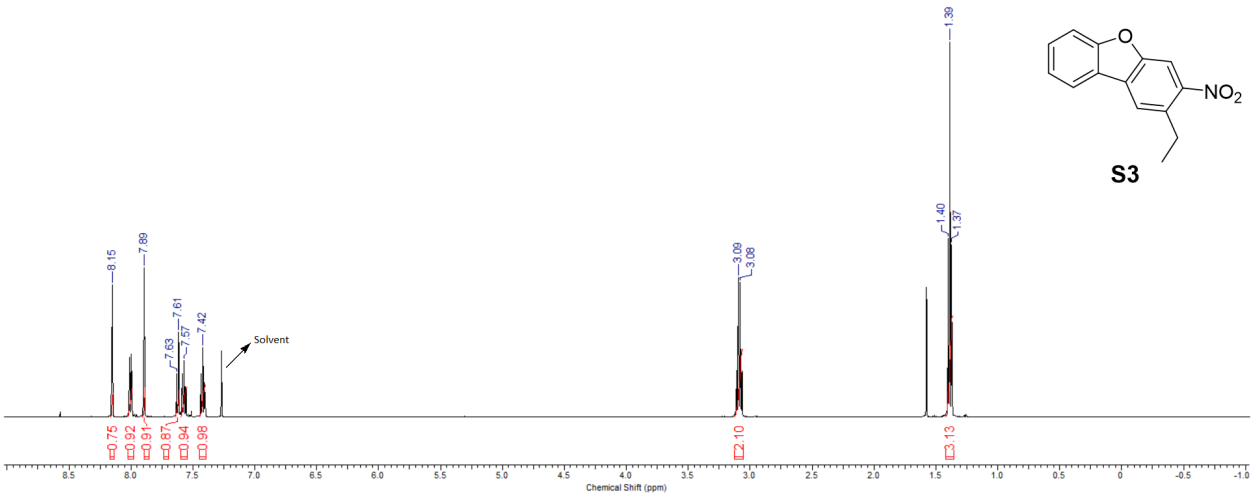
(1) Momotake, A.; Lindegger, N.; Niggli, E.; Barsotti, R. J.; Ellis-Davies, G. C. R. *Nat. Methods* **2006**, *3*, 35.



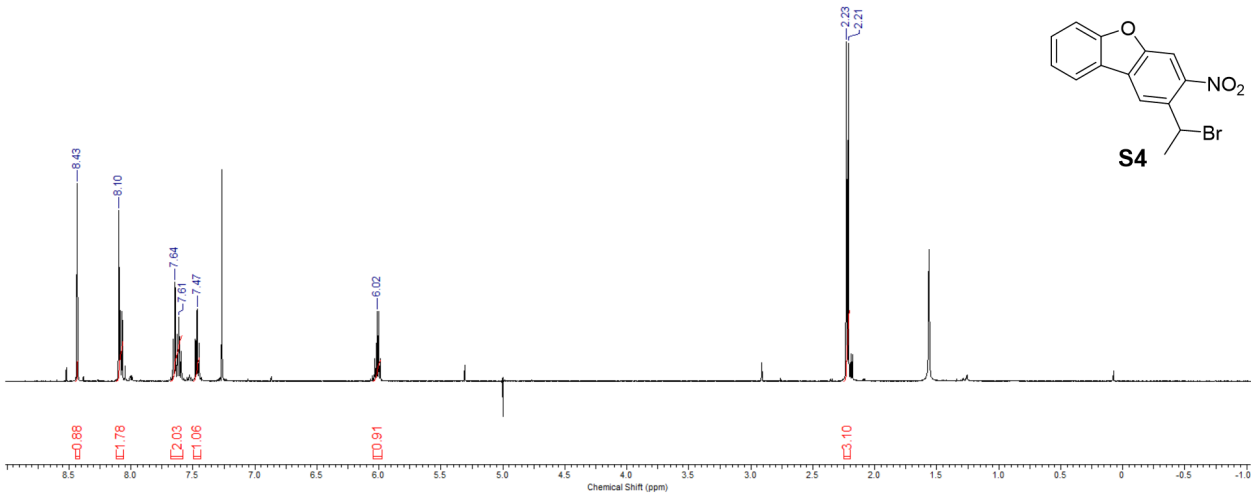
^{1}H NMR of Compound **S1** recorded at 500 MHz on a Varian Instrument at 25 °C in $CDCl_3$.



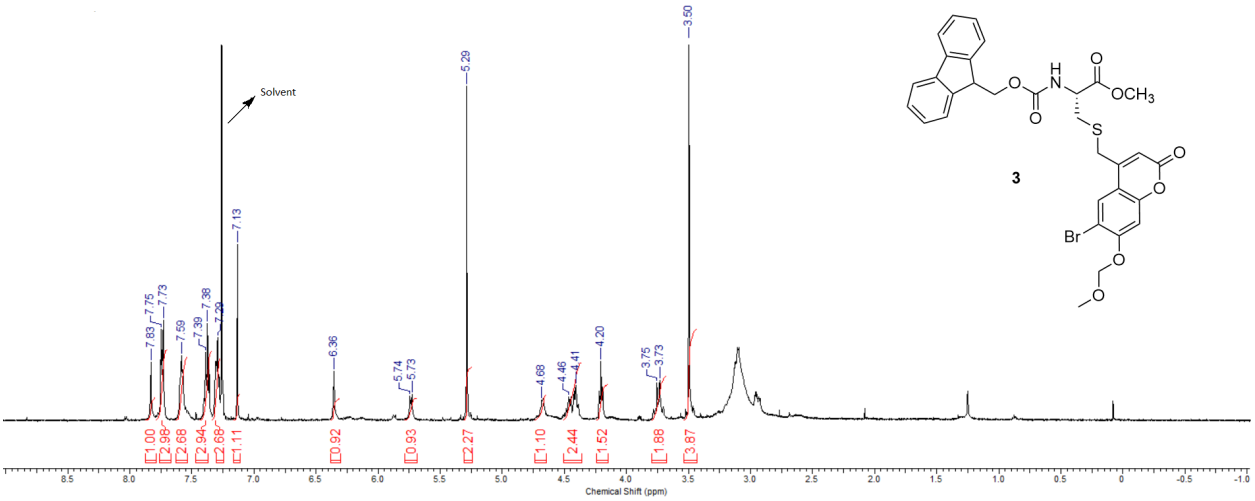
^{1}H NMR of Compound **S2** recorded at 500 MHz on a Varian Instrument at 25 °C in $CDCl_3$.



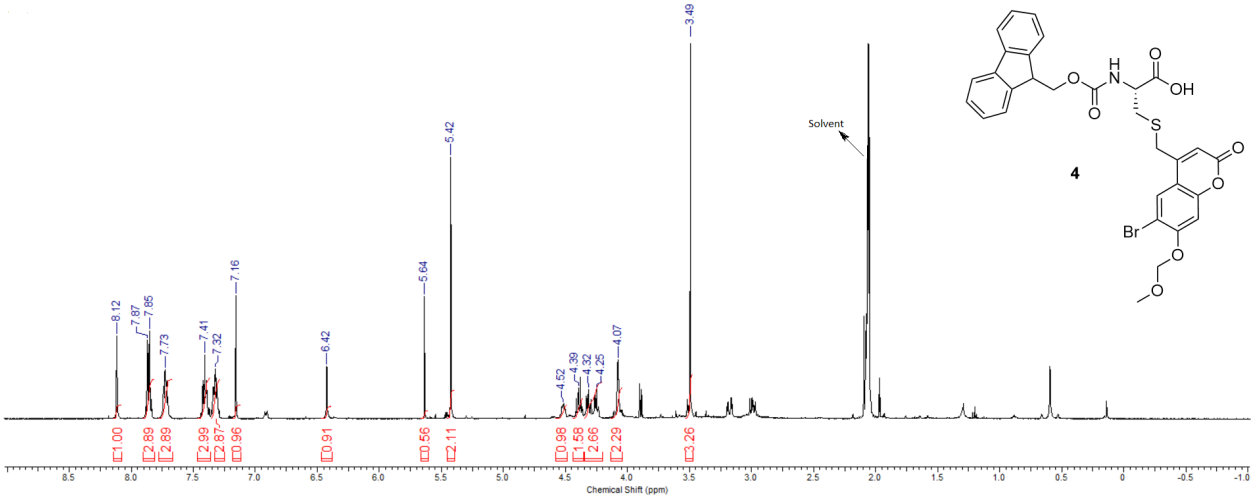
^{1}H NMR of Compound **S3** recorded at 500 MHz on a Varian Instrument at 25 °C in $CDCl_3$.



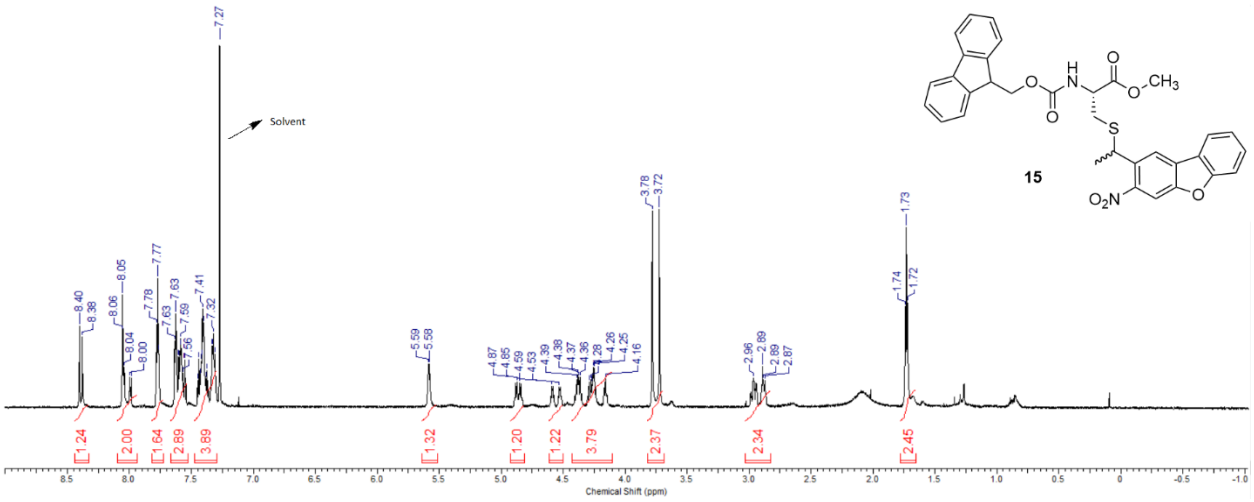
^{1}H NMR of Compound **S4** recorded at 500 MHz on a Varian Instrument at 25 °C in $CDCl_3$.



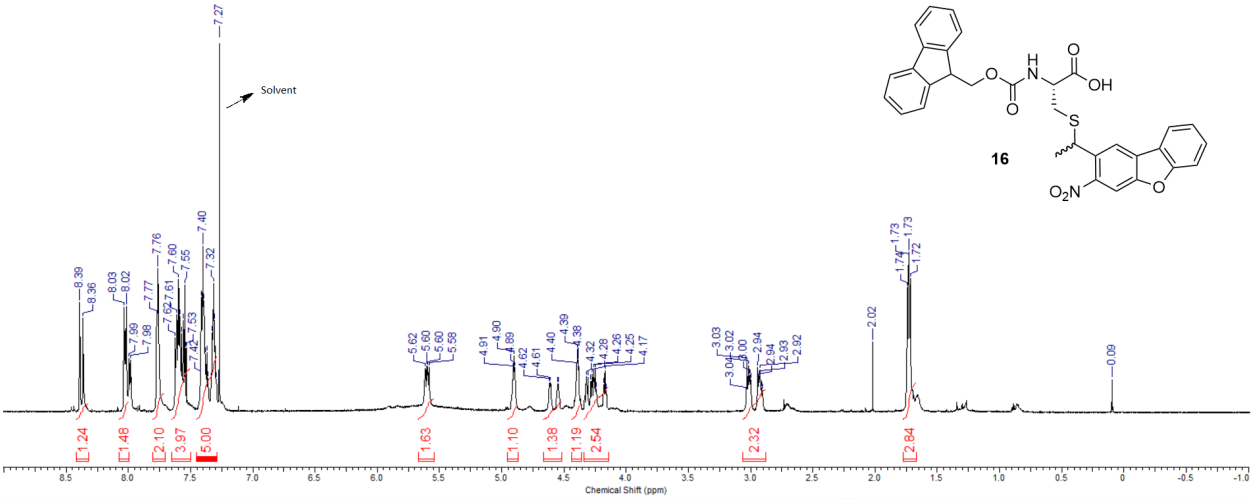
^{1}H NMR of Compound **3** recorded at 500 MHz on a Varian Instrument at 25 °C in $CDCl_3$.



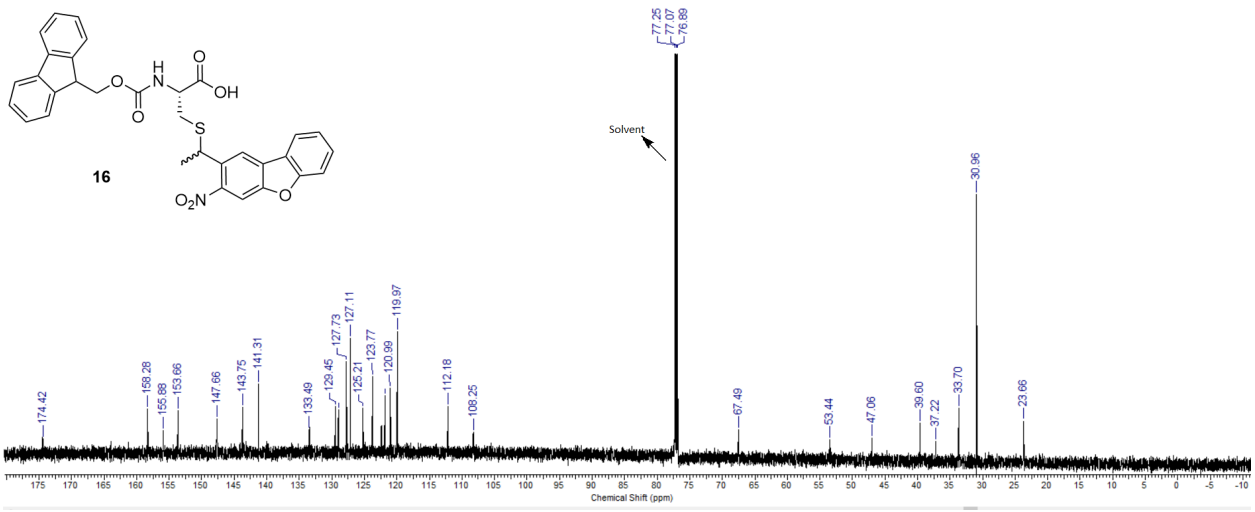
^{1}H NMR of Compound **4** recorded at 500 MHz on a Varian Instrument at 25 °C in d_6-acetone.



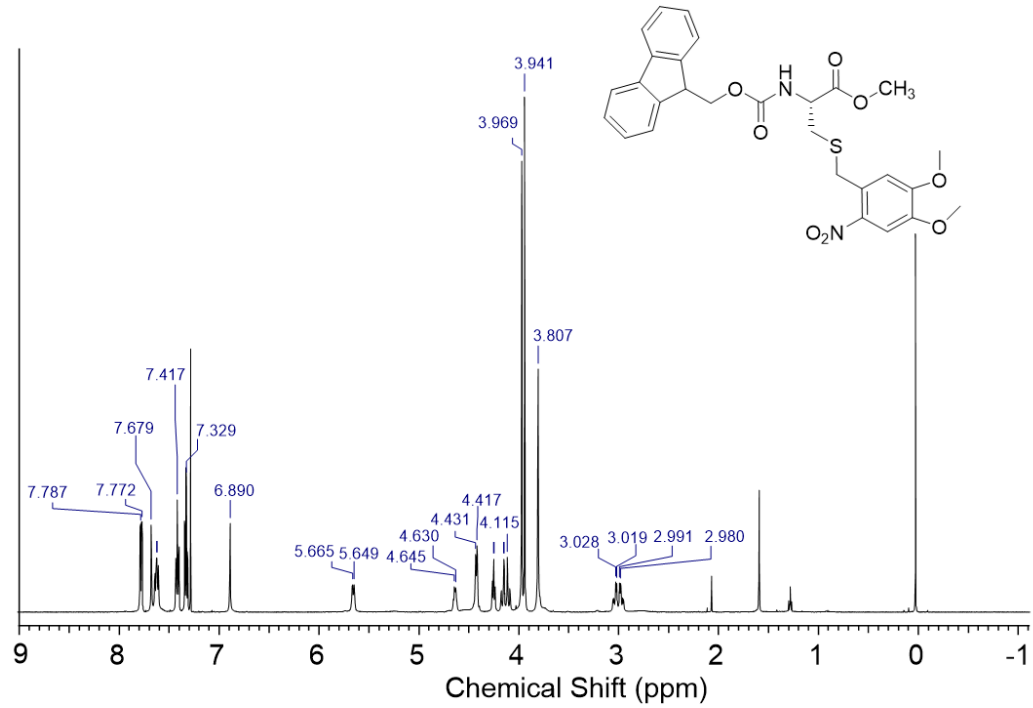
^{1}H NMR of Compound **15** recorded at 500 MHz on a Bruker Advance III 700 MHz spectrometer with 1.7 mm TCI cryoprobe at 25 °C in $CDCl_3$.



^{1}H NMR of Compound **16** recorded at 500 MHz on a Bruker Advance III 700 MHz spectrometer with 1.7 mm TCI cryoprobe at 25 °C in $CDCl_3$.



^{13}C NMR of Compound **16** recorded at 175 MHz on a Bruker Advance III 700 MHz spectrometer with 1.7 mm TCI cryoprobe at 25 °C in $CDCl_3$.



^{1}H NMR of Fmoc-Cys(NV)-OMe recorded at 500 MHz on a Bruker Advance III 700 MHz spectrometer with 1.7 mm TCI cryoprobe at 25 °C in $CDCl_3$.