Electronic Supplementary Information

An "OFF-ON-OFF" fluorescence protein-labeling probe for real-time visualization of the degradation of short-lived proteins in cellular systems

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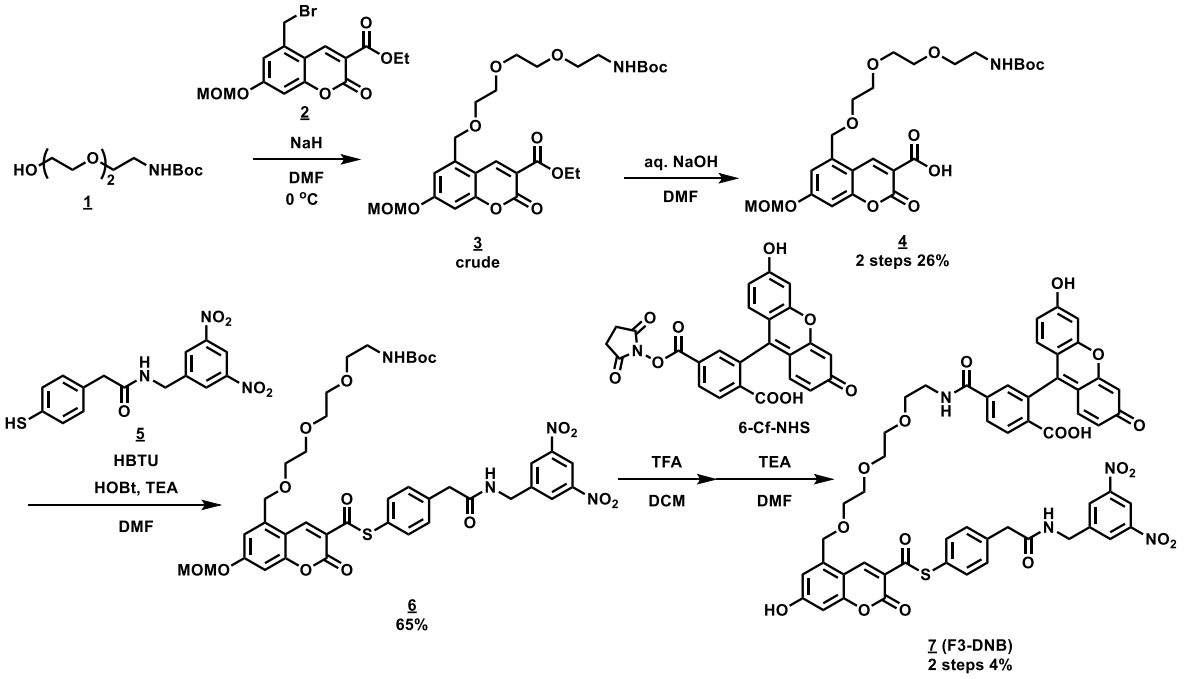
1. Synthetic Procedures

Materials and instruments

General chemicals were supplied by Tokyo Chemical Industries, Wako Pure Chemical Industries, Sigma-Aldrich Chemical Co. and Kishida Chemical Co. and used without further purification. Enzymes for subcloning experiments were purchased from Takara Bio, Inc. and New England Biolabs. Synthetic oligonucleotides were purchased from Invitrogen. Artificial genes were purchased from FASMAC. NMR spectra were recorded on a BRUKER Ascend^{TM} 500 instrument at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR spectra using tetramethylsilane as an internal standard. High-resolution mass spectra (HRMS) were recorded using a JEOL JMS-700. ESI-TOF MS and a Waters LCT-Premier XE spectrometer. Silica gel chromatography was performed using BW-300 silica (Fuji Silysia Chemical Ltd.) as a stationary phase. High-performance liquid chromatography (HPLC) purification was performed using an Inertsil ODS-3 column (4.6 or 10.0 mm × 250 mm, GL-Science, Inc.). Size exclusion chromatography was performed using a Superdex^{TM} 75 10/300 GL column (GE Healthcare Life Science) connected to an ÄKTA explorer system (GE Healthcare Life Science) or an NGC Chromatography System (Bio-Rad). Gel permeation chromatography (GPC) was performed using a JAIGEL-1H column connected to an UFLC SHIMADZU system using $CHCl_3$ as solvent. Fluorescence spectra were measured using a F7000 spectrometer (Hitachi) fitted with a photomultiplier with a voltage of 700 V. UV-vis absorption spectra were obtained using a V-650 spectrometer (Jasco). Fluorescence gel images were acquired using a Typhoon FLA 9500 imager (GE Healthcare Life Science). Fluorescence microscopic analyses were acquired using a confocal laser scanning microscope (Olympus, FLUOVIEW FV10i) equipped with a 60× lens.

1.1. Synthesis of F3-DNB 7



5-(14,14-dimethyl-12-oxo-2,5,8,13-tetraoxa-11-azapentadecyl)-7-(methoxymethoxy)-2-oxo-2*H*-chromene-3-carboxylic acid 4

Sodium hydride (169 mg, 4.2 mmol) was added to dry DMF (5 mL) and the mixture stirred for 30 min at 0 °C under a N_2 atmosphere. A solution of compound **1**[S1] (1.05 g, 4.2 mmol) and compound **2**[S2] (1.30 g, 3.5 mmol) in

dry DMF (145 mL) was then added dropwise and the resultant reaction mixture stirred at 0 °C for 2 h (to generate ester **3**). 2 M aq. NaOH solution (20 mL) was then added and the reaction mixture stirred at rt for 4 h. Solvent was then removed in vacuo and the residue then treated with 10% aq. citric acid to adjust the pH to 2.0. The reaction mixture was extracted with DCM, washed with brine, the combined organic layers dried (Na_2SO_4) and the solvent evaporated in vacuo. The crude residue was then purified by GPC ($CHCl_3$) to afford the title compound **4** (463 mg, 0.90 mmol) in 26% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.16 (s, 1H), 7.14 (s, 1H), 7.06 (d, *J* = 2.0 Hz, 1H), 5.30 (s, 2H), 5.04 (br, 1H), 4.84 (s, 2H), 3.77-3.33 (m, 12H), 3.50 (s, 3H), 1.43 (s, 9H).
^{13}C NMR (125 MHz, $CDCl_3$) δ 164.2, 163.2, 157.5, 156.0, 148.4, 140.5, 116.0, 111.7, 111.0, 102.9, 94.5, 79.2, 70.7, 70.4, 70.3, 70.2, 70.2, 56.7, 40.4, 28.4.
HRMS (FAB-) Calcd for $[M-H]^-$ 510.1975, found 510.1957

_S_-(4-(2-((3,5-dinitrobenzyl)amino)-2-oxoethyl)phenyl)-5-(14,14-dimethyl-12-oxo-2,5,8,13-tetraoxa-11-azapentadecyl)-7-(methoxymethoxy)-2-oxo-2*H*-chromene-3-carbothioate 6

Compound **4** (67.9 mg, 0.13 mmol), HBTU (60.9 mg, 0.16 mmol), HOBt (24.6 mg, 0.16 mmol) and TEA (22.5 µL, 0.16 mmol) were dissolved in dry DMF (8 mL) and stirred at rt under N_2 for 1 h. Compound **5**[S3] (56.2 mg, 0.16 mmol) was then added and the reaction mixture stirred at rt for 9 h. After removing the solvent in vacuo, the crude residue was dissolved in EtOAc and washed with sat. aq. $NaHCO_3$, 10% aq. citric acid and brine. The organic layer was dried (Na_2SO_4) and solvent evaporated in vacuo to afford a crude residue that was purified by silica gel column chromatography (EtOAc/Hexane = 90/10) to afford the title compound **6** (72.8 mg, 87 µmol) in 65% yield.

^{1}H NMR (500 MHz, Acetone-d_6): δ 8.88 (s, 1H), 8.83 (t, *J* = 2.0 Hz, 1H), 8.59 (d, *J* = 2.0 Hz, 2H), 8.12 (br, 1H), 7.48-7.44 (m, 4H), 7.16 (d, *J* = 2.5 Hz, 1H), 7.03 (d, *J* = 2.0 Hz, 1H), 5.82 (br, 1H), 5.42 (s, 2H), 4.92 (s, 2H), 4.71 (d, *J* = 6.0 Hz, 2H), 3.73 (s, 2H), 3.72-3.14 (m, 12H), 3.50 (s, 1H), 1.38 (s, 9H).

^{13}C NMR (125 MHz, Acetone-d_6): δ 185.9, 170.5, 162.6, 157.9, 157.8, 148.6, 144.8, 144.2, 141.2, 137.5, 135.0, 130.0, 127.6, 127.0, 118.8, 117.1, 115.0, 111.1, 102.0, 94.4, 77.7, 70.4, 70.3, 70.0, 69.9, 69.8, 69.7, 55.9, 42.5, 42.0, 40.2, 27.7.

HRMS (MALDI) Calcd for $[M+Na]^+$ 863.2422, found 863.2425.

Synthesis of F3-DNB 7

Compound **6** (32.1 mg, 38 µmol) and TFA (1.0 mL) were dissolved in DCM (4 mL) and the reaction mixture stirred at rt for 5 h, before the solvent was then removed in vacuo and the residue dried for 4 h under high vacuum. The crude residue was then dissolved in dry DMF (5 mL), 6-Cf-NHS (15.8 mg, 33 µmol) and dry TEA (45 µL) added and the reaction mixture then stirred overnight at rt under N_2. The solvent was removed in vacuo, the crude product extracted with EtOAc and the combined organic layers washed with brine, dried (Na_2SO_4) and the solvent evaporated in vacuo. The crude residue was then purified by reverse-phase HPLC to afford **F3-DNB 7** (1.6 mg, 1.5 µmol) in 4% yield.

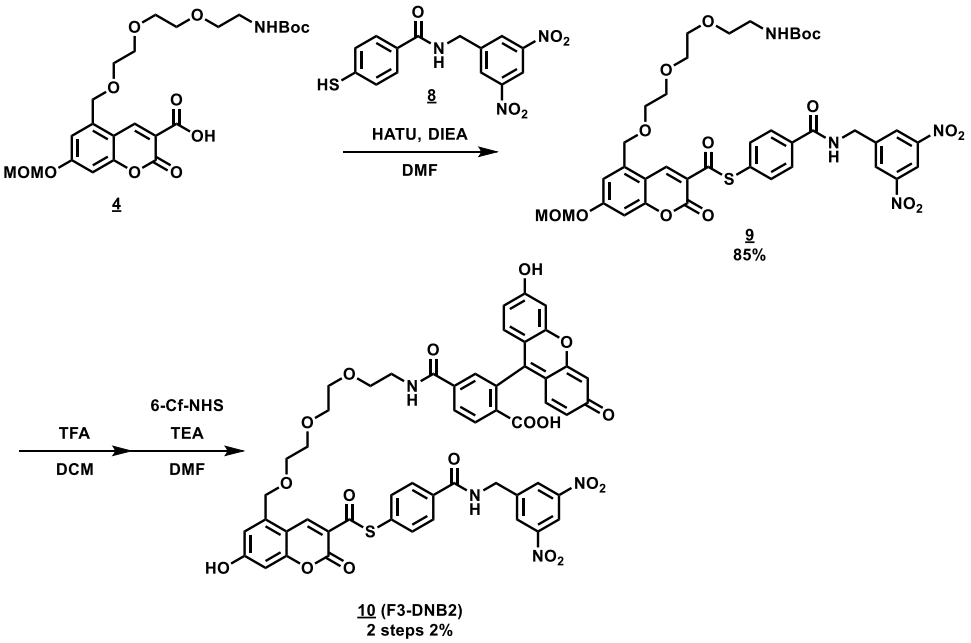
^{1}H NMR (500 MHz, Acetone-d_6): δ 8.81 (t, *J* = 2.0 Hz, 1H), 8.74 (s, 1H), 8.58 (d, *J* = 2.0 Hz, 2H), 8.18 (dd, *J* = 8.0 Hz, *J* = 1.5 Hz, 2H), 8.01 (d, *J* = 8.0 Hz, 1H), 7.94 (t, *J* = 5.0 Hz, 1H), 7.69 (s, 1H), 7.42-7.37 (m, 4H), 6.96 (d, *J* =

2.0 Hz, 1H), 6.75 (d, *J* = 2.5 Hz, 1H), 6.70 (d, *J* = 2.5 Hz, 2H), 6.67 (d, *J* = 8.5 Hz, 2H), 6.61 (dd, *J* = 9.0 Hz, *J* = 2.5 Hz, 2H), 4.73 (s, 2H), 4.69 (d, *J* = 6.5 Hz, 2H), 3.69 (s, 2H), 3.56-3.38 (m, 12H).

^{13}C NMR (125 MHz, Acetone-d_6): δ 186.2, 170.7, 168.0, 165.2, 164.6, 159.6, 158.6, 158.2, 153.2, 152.4, 148.6, 144.7, 144.4, 141.4, 141.2, 137.4, 135.0, 129.9, 129.4, 128.9, 127.6, 127.2, 124.6, 122.4, 117.1, 117.0, 115.0, 112.6, 110.2, 109.6, 102.5, 101.8, 83.1, 70.3, 70.2, 69.9, 69.9, 69.8, 69.0, 42.4, 42.4, 42.0, 41.9, 39.9, 39.7.

HRMS (MALDI) Calcd for $[M+Na]^+$ 1077.2113, found 1077.2097.

1.2. Synthesis of F3-DNB2 10



***S*-(4-((3,5-dinitrobenzyl)carbamoyl)phenyl)-5-(14,14-dimethyl-12-oxo-2,5,8,13-tetraoxa-11-azapentadecyl)-7-(methoxymethoxy)-2-oxo-2*H*-chromene-3-carbothioate 9**

Compound **4** (60.0 mg, 0.12 mmol) and HATU (67.1 mg, 0.18 mmol) and DIEA (62.0 µL, 0.36 mmol) were dissolved in dry DMF (5 mL) and the reaction mixture stirred for 30 min at 0 °C under N_2. Compound **8**[S3] (59.0 mg, 0.18 mmol) was then added and the reaction mixture stirred overnight at rt. After removing the solvent in vacuo, the crude residue was dissolved in EtOAc and the combined organic layers washed with water, brine, dried (Na_2SO_4) and the solvent evaporated in vacuo. The crude residue was then purified by silica gel chromatography (DCM/EtOAc = 70/30) to afford the title compound **9** (82.1 mg, 99 µmol) in 85% yield.

^{1}H NMR (500 MHz, Acetone-d_6): δ 8.91 (s, 1H), 8.86 (t, *J* = 2.0 Hz, 1H), 8.80 (t, *J* = 6.0 Hz, 1H), 8.72 (d, *J* = 2.5 Hz, 1H), 8.05 (d, *J* = 8.5 Hz, 2H), 7.64 (d, *J* = 8.5 Hz, 2H), 7.17 (d, *J* = 2.0 Hz, 2H), 7.04 (d, *J* = 2.0 Hz, 2H), 5.82 (br, 1H), 5.42 (s, 2H), 4.93 (d, *J* = 6.0 Hz, 2H), 4.92 (s, 2H), 3.72-3.16 (m, 10H), 3.51 (s, 3H), 1.37 (s, 9H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 186.0, 167.5, 162.7, 158.8, 158.0, 148.7, 144.9, 143.7, 140.1, 135.1, 134.5, 133.1, 128.0, 127.9, 127.7, 118.6, 117.9, 117.8, 115.7, 111.6, 102.9, 94.5, 79.6, 70.8, 70.5, 70.1, 60.4, 56.7, 43.0, 31.0, 28.4, 14.2.

HRMS (FAB+) Calcd for $[M+H]^+$ 827.2446, found 827.2463.

F3-DNB2 10

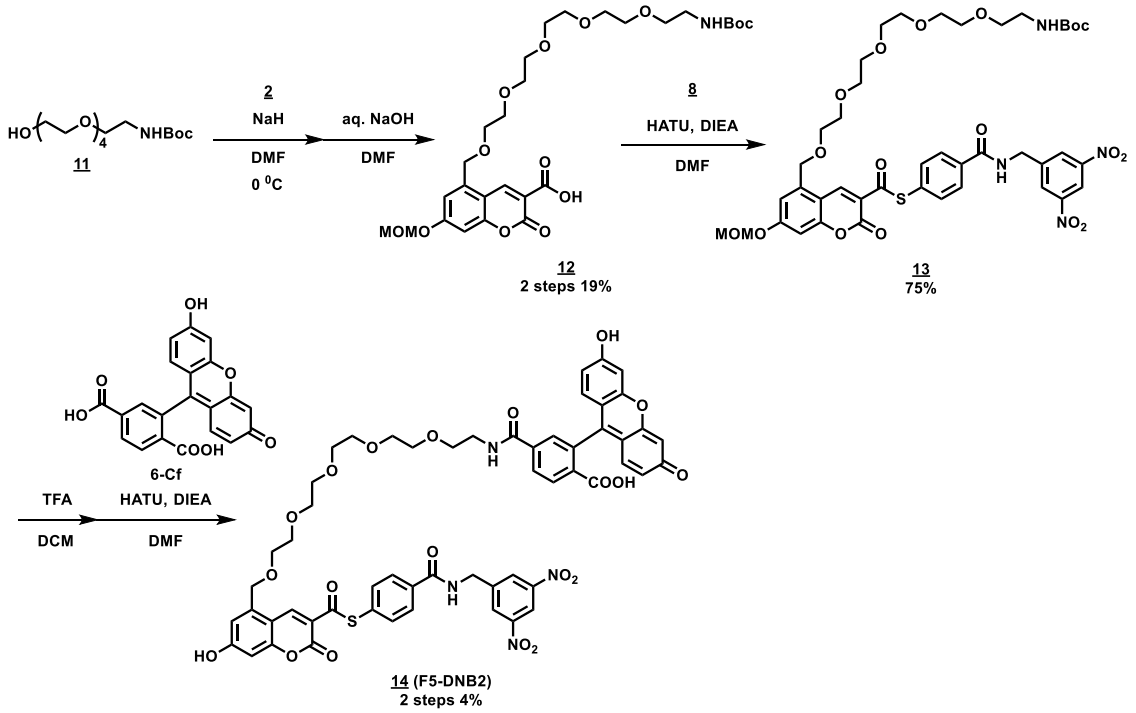
Compound **9** (58.0 mg, 70 μmol) and TFA (1.0 mL) were dissolved in DCM (4 mL) and stirred at rt for 3 h. The solvent was removed in vacuo and the residue dried overnight under high vacuum. The crude residue was dissolved in dry DMF (3 mL) and 6-Cf-NHS (36.6 mg, 77 μmol) and dry TEA (24.5 μL) added, with the reaction mixture then stirred overnight at rt under N_2. The solvent was removed in vacuo and the crude residue purified by reverse-phase HPLC to afford **F3-DNB2 10** (1.6 mg, 1.5 μmol) in 2% yield.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.43 (s, 1H), 10.15 (s, 2H), 9.43 (t, *J* = 5.5 Hz, 1H), 8.73 (t, *J* = 2 Hz, 1H), 8.72 (s, 1H), 8.69 (t, *J* = 5.5 Hz, 1H), 8.62 (d, *J* = 2 Hz, 2H), 8.13 (dd, *J* = 8.0 Hz, *J* = 1.0 Hz 1H), 8.04 (d, *J* = 8.0 Hz, 1H), 7.98 (d, *J* = 8.5 Hz, 2H), 7.66 (s, 1H), 7.61 (d, *J* = 8.5 Hz, 2H), 6.90 (d, *J* = 2.5 Hz, 1H), 6.76 (d, *J* = 2.0 Hz, 2H), 6.68 (d, *J* = 2 Hz, 2H), 6.59-6.53 (m, 4H), 4.74 (d, *J* = 5.5 Hz, 2H), 4.73 (s, 2H), 3.53-3.40 (m, 12H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 185.4, 168.5, 166.4, 166.4, 165.0, 160.0, 158.7, 158.5, 153.1, 152.3, 148.5, 145.4, 144.6, 141.8, 141.0, 135.2, 135.0, 132.6, 129.9, 129.7, 128.6, 128.4, 128.3, 125.3, 122.7, 117.7, 116.5, 115.2, 113.2, 109.6, 109.4, 102.7, 101.9, 83.7, 70.1, 70.1, 69.9, 69.8, 69.5, 69.1, 42.4, 42.3.

HRMS (FAB-) Calcd for $[M-H]^-$ 1039.1986, found 1039.1959.

1.3. Synthesis of F5-DNB2 14



5-(20,20-dimethyl-18-oxo-2,5,8,11,14,19-hexaoxa-17-azahenicosyl)-7-(methoxymethoxy)-2-oxo-2*H*-chromene-3-carboxylic acid 12

Sodium hydride (87.0 mg, 2.2 mmol) in dry DMF (10 mL) was stirred at 0 °C under N_2 for 20 min. Compound **11**[S4] (729 mg, 2.2 mmol) and compound **2** (668 mg, 1.8 mmol) in dry DMF (80 mL) was then added dropwise and

the resultant reaction mixture stirred at 0 °C for 2 h. 2 M aq. NaOH solution (15 mL) was then added and the reaction mixture stirred at rt for a further 5 h, before the solvent was removed in vacuo. The crude residue was then treated with 10% aq. citric to adjust the pH to 2.0. The crude product was then extracted with DCM, the combined organic layers washed with brine, dried (Na_2SO_4) and evaporated in vacuo to afford a crude residue that was purified by GPC ($CHCl_3$) to yield the title compound **12** (209 mg, 0.35 mmol) in 19% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.15 (s, 1H), 7.13 (d, *J* = 2.5 Hz, 1H), 7.05 (d, *J* = 2.5 Hz, 1H), 5.29 (s, 2H), 5.09 (br, 1H), 4.83 (s, 2H), 3.75-3.30 (m, 20H), 1.44 (s, 9H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 164.2, 163.2, 163.1, 157.5, 156.0, 148.4, 140.5, 116.0, 111.7, 111.1, 102.8, 94.5, 79.2, 70.7, 70.7, 70.6, 70.6, 70.5, 70.5, 70.4, 70.3, 70.2, 70.2, 56.7, 40.4, 28.4.

HRMS (FAB+) Calcd for $[M+Na]^+$ 622.2476, found 622.2483.

***S*-(4-((3,5-dinitrobenzyl)carbamoyl)phenyl)-5-(20,20-dimethyl-18-oxo-2,5,8,11,14,19-hexaoxa-17-azahenicosyl)-7-(methoxymethoxy)-2-oxo-2*H*-chromene-3-carbothioate 13**

Compound **12** (161 mg, 0.27 mmol), HATU (153 mg, 0.40 mmol) and DIEA (70.0 µL, 0.40 mmol) were dissolved in dry DMF (5 mL) and the reaction mixture stirred at 0 °C under N_2 for 1 h. Compound **8** (116 mg, 0.35 mmol) was then added and the resultant solution stirred at rt for 4 h. Solvent was then removed in vacuo to afford a crude residue that was dissolved in EtOAc, with the organic layer then washed with water, brine and dried (Na_2SO_4). The solvent was then evaporated in vacuo to afford a crude residue that was purified by silica gel chromatography (DCM/EtOAc = 70/30) to afford the title compound **13** (183 mg, 0.20 mmol) in 75% yield.

^{1}H NMR (500 MHz, Acetone-d_6): δ 8.91 (s, 1H), 8.86 (t, *J* = 2.0 Hz, 1H), 8.80 (t, *J* = 6.0 Hz, 1H), 8.72 (d, *J* = 2.0 Hz, 1H), 8.06 (d, *J* = 8.5 Hz, 2H), 7.64 (d, *J* = 8.5 Hz, 2H), 7.17 (d, *J* = 2.5 Hz, 2H), 7.04 (d, *J* = 2.0 Hz, 2H), 5.86 (br, 1H), 5.42 (s, 2H), 4.93 (d, *J* = 6.0 Hz, 2H), 4.92 (s, 2H), 3.72-3.17 (m, 20H), 1.39 (s, 9H).

^{13}C NMR (125 MHz, Acetone-d_6): δ 187.0, 168.2, 168.1, 164.6, 159.8, 159.8, 150.4, 146.5, 146.4, 143.2, 136.7, 136.7, 134.7, 129.8, 129.7, 120.3, 119.0, 116.8, 112.9, 103.8, 96.2, 79.5, 72.2, 72.1, 72.1, 71.8, 71.7, 71.6, 71.5, 57.7, 44.3, 44.2, 42.0, 41.9, 31.5, 29.5.

HRMS (FAB+) Calcd for $[M+Na]^+$ 937.2789, found 937.2783.

F5-DNB2 14

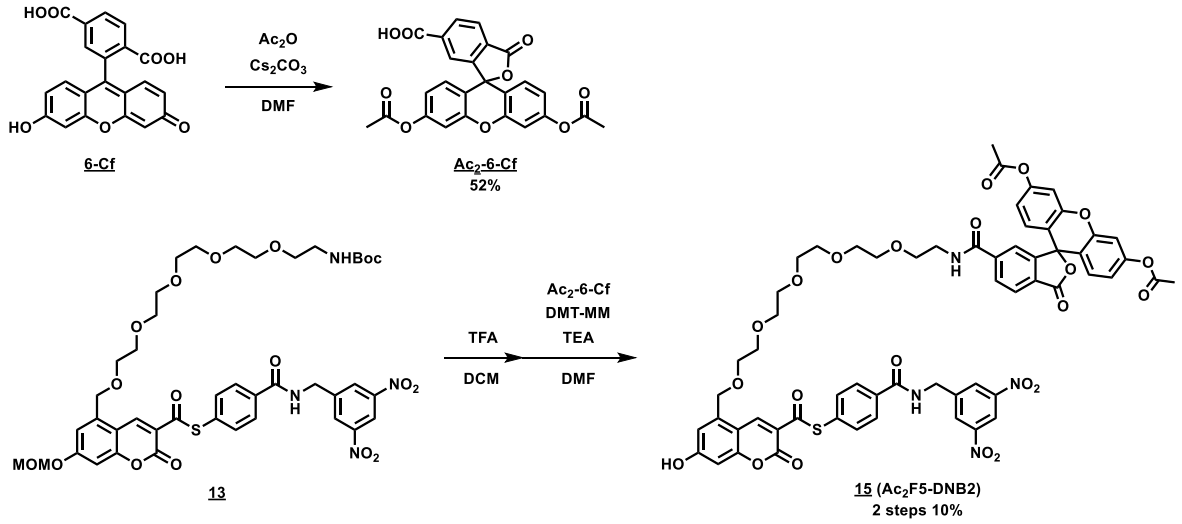
Compound **13** (40.7 mg, 45 µmol) and TFA (1.0 mL) were dissolved in DCM (4 mL) and the reaction mixture stirred at rt for 3 h. The solvent was removed in vacuo and the crude residue dried overnight under high vacuum. 6-Cf (20.2 mg, 54 µmol), HATU (40.7 mg, 0.11 mmol) and DIEA (31.0 µL) were dissolved in dry DMF (7 mL) and stirred at 0 °C under N_2 for 1 h. The crude residue from the TFA deprotection step was then added and the reaction stirred at rt under N_2 for 8 h. The solvent was removed under vacuo with the crude residue then purified by reverse-phase HPLC to afford the title compound **F5-DNB2 14** (2.0 mg, 1.8 µmol) in 4% yield.

^{1}H NMR (500 MHz, Acetone-d_6): δ 8.85 (t, *J* = 2.0 Hz, 1H), 8.83 (s, 1H), 8.81 (br, 1H), 8.71 (d, *J* = 2.0 Hz, 1H), 8.21 (dd, *J* = 8.0 Hz, *J* = 1.5 Hz, 1H), 8.04 - 8.02 (m, 3H), 7.76 (s, 1H), 7.62 (d, *J* = 8.0 Hz, 2H), 7.01 (d, *J* = 2.5 Hz, 1H), 6.77 (d, *J* = 2.5 Hz, 1H), 6.74 (d, *J* = 2.5 Hz, 2H), 6.67 (d, *J* = 9.0 Hz, 2H), 6.61 (dd, *J* = 9.0 Hz, *J* = 2.5 Hz, 2H), 4.91 (d, *J* = 5.5 Hz, 2H), 4.81 (s, 2H), 3.64-3.39 (m, 20H).

^{13}C NMR (125 MHz, Acetone-d_6): δ 187.0, 169.8, 168.3, 167.2, 166.2, 161.3, 160.4, 160.1, 155.2, 154.2, 150.4, 146.6, 146.4, 143.5, 143.0, 137.0, 136.6, 136.6, 134.9, 131.2, 131.1, 130.7, 129.8, 129.6, 126.5, 124.4, 119.0, 118.7, 116.6, 114.3, 112.1, 111.5, 104.3, 103.5, 84.8, 72.1, 72.0, 72.0, 71.9, 71.9, 71.7, 71.5, 71.1, 44.3, 41.7, 41.6.

HRMS (FAB+) Calcd for $[M+Na]^+$ 1151.2480, found 1151.2473.

1.4. Synthesis of Ac_2F5-DNB2 15



Ac_2F5-DNB2 15

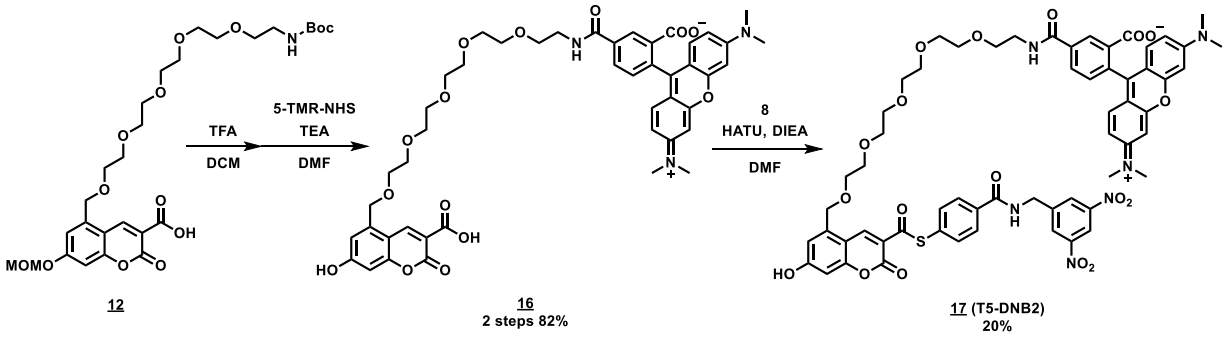
Compound **13** (30.6 mg, 33 μmol) and TFA (0.5 mL) were dissolved in DCM (2 mL) and stirred at rt for 3 h. The solvent was removed in vacuo to afford a crude residue that was dried overnight under high vacuum. Ac_2-6-Cf[S3] (17.2 mg, 37 μmol), DMT-MM (28.1 mg, 0.10 mmol) and TEA (16.3 μL) were dissolved in DMF (2.5 mL) and stirred at rt for 1 h. The crude residue from the initial TFA mediated *N*-Boc deprotection step of **13** was then added and the solution stirred overnight at rt. The solvent was then removed in vacuo to afford a crude residue that was purified by reverse-phase HPLC to afford the title compound **Ac_2F5-DNB2 15** (3.9 mg, 3.2 μmol) in 10% yield.

^{1}H NMR (500 MHz, Acetone-d_6): δ 8.84 (t, *J* = 2.0 Hz, 1H), 8.81 (s, 1H), 8.78 (t, *J* = 5.5 Hz, 1H), 8.71 (d, *J* = 1.5 Hz, 1H), 8.24 (d, *J* = 8.0 Hz, 1H), 8.08 (d, *J* = 8.0 Hz, 1H), 8.03 (d, *J* = 8.0 Hz, 2H), 7.88 (s, 1H, w), 7.62 (d, *J* = 8.0 Hz, 2H), 7.19 (d, *J* = 2.0 Hz, 2H), 6.99 (d, *J* = 1.5 Hz, 2H), 6.97 (d, *J* = 8.5 Hz, 2H), 6.92 (dd, *J* = 8.5 Hz, *J* = 1.5 Hz, 2H), 6.77 (d, *J* = 1.5 Hz, 2H), 4.90 (d, *J* = 6.0 Hz, 2H), 4.78 (s, 2H), 3.61-3.34 (m, 20H), 2.28 (s, 6H).

^{13}C NMR (125 MHz, Acetone-d_6): δ 187.0, 170.3, 169.5, 168.2, 165.9, 160.3, 160.0, 154.7, 154.5, 153.1, 150.4, 146.5, 146.4, 143.5, 143.4, 137.0, 136.7, 136.6, 134.9, 131.7, 131.1, 130.0, 129.8, 129.6, 126.8, 124.4, 120.2, 119.0, 118.9, 118.1, 116.4, 112.3, 111.5, 103.5, 83.0, 72.1, 72.0, 71.9, 70.9, 71.7, 71.6, 71.5, 70.9, 56.9, 44.3, 41.8, 41.7, 21.9.

HRMS (FAB-) Calcd for $[M-H]^-$ 1211.2720, found 1211.2725.

1.5. Synthesis of T5-DNB2 17



5-((1-(3-carboxy-7-hydroxy-2-oxo-2H-chromen-5-yl)-2,5,8,11,14-pentaoxahexadecan-16-yl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate 16

Compound **12** (50.0 mg, 83 µmol) and TFA (0.5 mL) were dissolved in DCM (2.0 mL) and stirred at rt for 7 h. The solvent was removed in vacuo to afford a crude residue that was dried overnight under high vacuum. 5-TMR-NHS (48.3 mg, 92 µmol), and TEA (58.0 µL) were dissolved in DMF (5 mL), the crude residue from the initial TFA mediated *N*-Boc deprotection step added and the solution stirred under N_2 for 11 h at rt. The solvent was then removed in vacuo to afford a crude residue that was purified by reverse-phase HPLC to afford the title compound **16** (59.4 mg, 68 µmol) in 82% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.93 (s, 1H), 8.67 (d, *J* = 1.5 Hz, 1H), 8.21 (dd, *J* = 8.0, 1.5 Hz, 1H), 7.83 (br, 1H), 7.24 (d, *J* = 8.0 Hz, 1H), 7.04 (d, *J* = 9.5 Hz, 2H), 7.04 (d, *J* = 2.0 Hz, 1H), 6.68 (dd, *J* = 9.5, 2.0 Hz, 2H), 6.61 (d, *J* = 2.0 Hz, 1H), 6.59 (d, *J* = 2.0 Hz, 2H), 4.64 (s, 2H), 3.72-3.62 (m, 20H), 3.15 (s, 12H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 168.9, 167.6, 166.4, 164.9, 164.2, 158.2, 156.2, 155.7, 148.2, 140.1, 136.1, 135.6, 131.0, 130.9, 128.0, 127.4, 116.9, 112.4, 111.8, 109.1, 107.4, 102.6, 96.9, 70.6, 70.6, 70.6, 70.5, 70.5, 70.5, 70.3, 70.0, 69.8, 69.8, 45.6, 40.6, 40.1, 29.7.

HRMS (FAB+) Calcd for $[M]^+$ 867.3215, found 867.3225.

T5-DNB2 17

Compound **16** (13.0 mg, 15 µmol), HATU (6.91 mg, 18 µmol), and DIEA (7.80 µL, 45 µmol) were dissolved in DMF (4 mL) and stirred at 0 °C under N_2 for 2 h. Compound **8** (10 mg, 30 µmol) was then added and the resultant solution stirred at rt for 3 h. The solvent was then removed in vacuo to afford a crude residue that was purified by reverse-phase HPLC to afford the title compound **T5-DNB2 17** (3.6 mg, 3 µmol) in 20% yield.

^{1}H NMR (500 MHz, CD_3OD): δ 8.90 (t, *J* = 2.5 Hz, 1H), 8.73 (d, *J* = 1.5 Hz, 1H), 8.70 (s, 1H), 8.63 (d, *J* = 2.5 Hz, 2H), 8.25 (dd, *J* = 7.5, 1.5 Hz, 1H), 7.90 (d, *J* = 8.5 Hz, 2H), 7.49 (d, *J* = 7.5 Hz, 1H), 7.49 (d, *J* = 8.5 Hz, 2H), 7.05 (d, *J* = 9.5 Hz, 2H), 6.97 (dd, *J* = 9.5, 2.5 Hz, 2H), 6.82 (d, *J* = 2.5 Hz, 2H), 6.81 (d, *J* = 2.0 Hz, 1H), 6.69 (d, *J* = 2.0 Hz, 1H), 4.78 (s, 2H), 4.58 (s, 2H), 3.70-3.49 (m, 20H), 3.26 (s, 12H).

^{13}C NMR (125 MHz, CD_3OD): δ 185.3, 168.0 166.7, 165.9, 164.7, 159.0, 158.8, 158.4, 157.4, 157.3, 148.6, 144.8, 143.7, 141.2, 136.6, 136.2, 134.6, 134.2, 133.3, 131.4, 131.0, 130.6, 130.5, 130.1, 127.5, 127.5, 117.1, 116.3, 114.8, 114.2, 113.2, 109.4, 101.4, 96.1, 70.2, 70.2, 70.2, 70.1, 70.1, 70.0, 69.8, 69.0, 42.3, 39.9, 39.5.

HRMS (FAB+) Calcd for $[M+H]^+$ 1183.3601, found 1183.3603.

2. Supplementary Figures

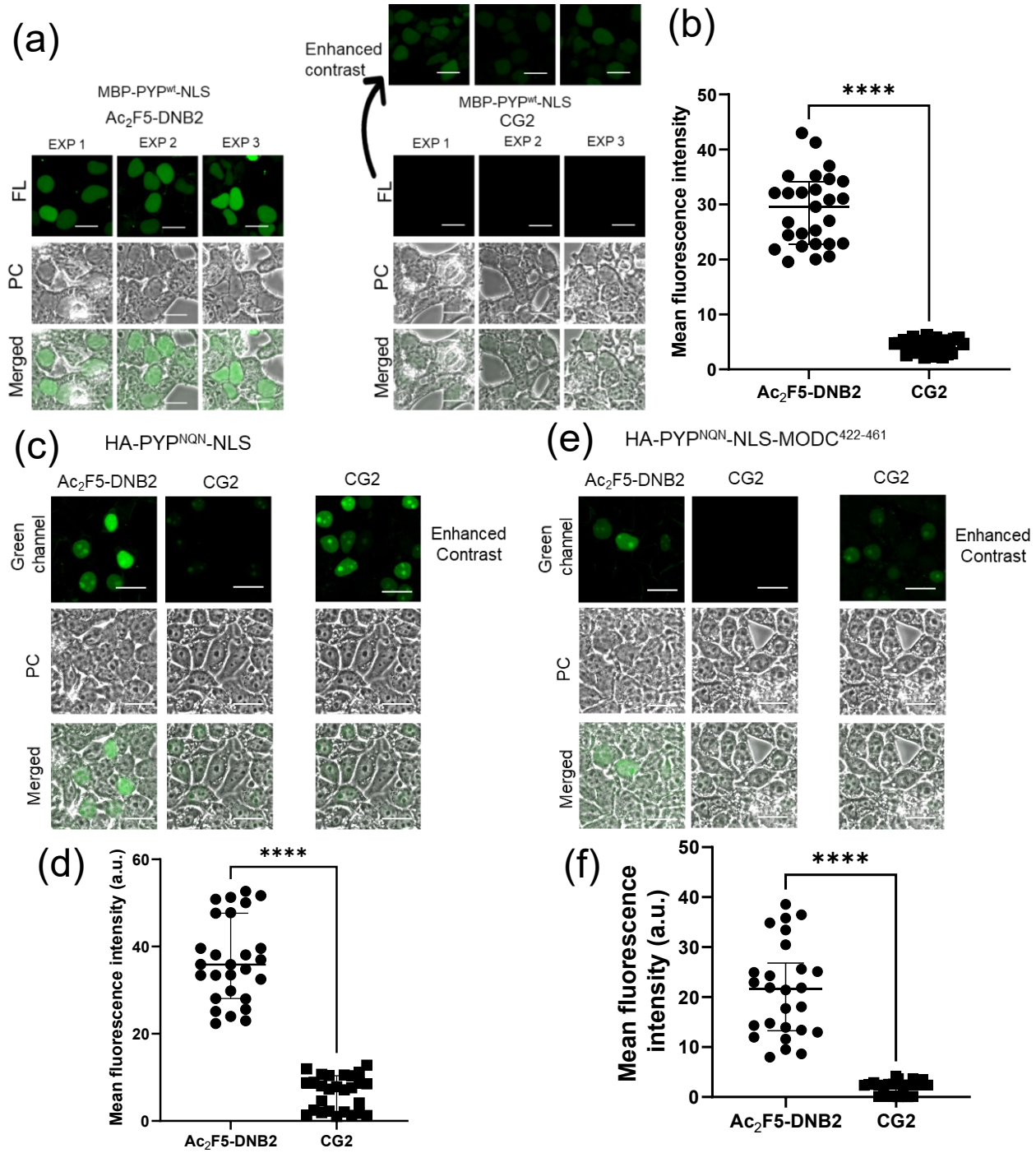


Figure S1. Comparison of mean fluorescence intensities of Ac_2F5-DNB2 and CG2[S1] bound to (a) MBP-PYP^{wt}-NLS, (c) HA-PYP^{NQN}-NLS and (e) HA-PYP^{NQN}-NLS-$MODC^{422-461}$ in HEK293T cells. Live-cell imaging was conducted by treating cells with Ac_2F5-DNB2 (1.0 μM) or CG2 (1.0 μM) with excitation at 473 nm. Cells were incubated until maximum fluorescence intensities were obtained (Ac_2F5-DNB2: 60 min; CG2: 5 min) and then washed twice with HBSS buffer to remove excess unreacted probe before images were then acquired. Enhanced-contrast images of CG2 treated cells are shown on the right-hand side of Figures (a), (c) and (e). Statistical analyses of fluorescence intensities of cells labeled with Ac_2F5-DNB2 and CG2 are shown in Figures (b), (d) and (f). Data correspond to mean fluorescence intensities of cell nuclei expressing genes for: (b) MBP-PYP^{wt}-NLS with medians ± interquartile range (N = 27 cells for Ac_2F5-DNB2 and CG2); (d) HA-PYP^{NQN}-NLS with medians ± interquartile range (N = 26 cells for Ac_2F5-DNB2 and N = 23 cells for CG2); and (f) HA-PYP^{NQN}-NLS-$MODC^{422-461}$ with medians ± interquartile range (N = 27 cells for Ac_2F5-DNB2 and CG2). Differences between mean fluorescence intensities of Ac_2F5-DNB2 and CG2 determined using an unpaired Student`s t-test and found to be statistically significant (****$P < 0.0001$) for data acquired from three independent experiments. Scale bar: 20 μm. PC = Phase Contrast. FL = Fluorescence.

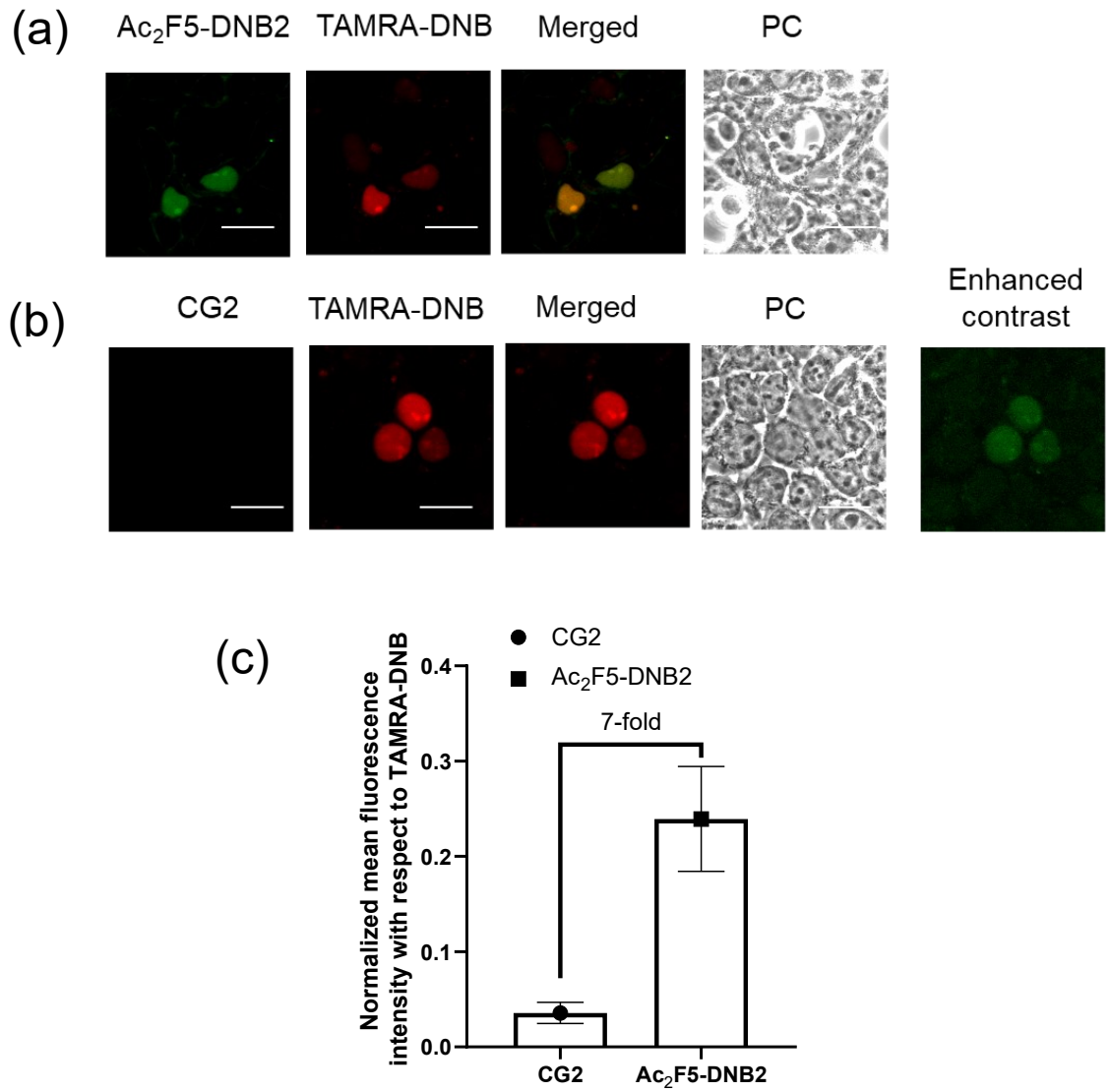


Figure S2.TAMRA-DNB-normalized fluorescence intensities of Ac_2F5-DNB2 and CG2 bound to HA-PYP^{NQN}-NLS-$MODC^{422-461}$ in living HEK293T cells. HEK293T cells expressing a gene for HA-PYP^{NQN}-NLS-$MODC^{422-461}$ were treated with TAMRA-DNB (1.0 μM) [3] and (a) Ac_2F5-DNB2 (1.0 μM) or (b) CG2 (1.0 μM). Cells were imaged using a no-cell washing protocol with excitation at 473 nm (Ac_2F5-DNB2 and CG2) and 559 nm (TAMRA-DNB), respectively. TAMRA-DNB-labeled cells were incubated with Ac_2F5-DNB2 or CG2 until maximal fluorescence intensities were obtained (Ac_2F5-DNB2: 60 min; CG2: 5 min). Enhanced-contrast images of CG2 treated cells are shown on the right-hand side of the figure. Scale bar: 20 μm. PC = Phase Contrast. FL = Fluorescence. (c) Quantitative analyses of fluorescence intensities of Ac_2F5-DNB2 and CG2-treated cells. Data correspond to mean fluorescence intensities of Ac_2F5-DNB2 and CG2 in cell nuclei, which are normalized with respect to TAMRA-DNB with mean ± S.D. (*N* = 7 cells).

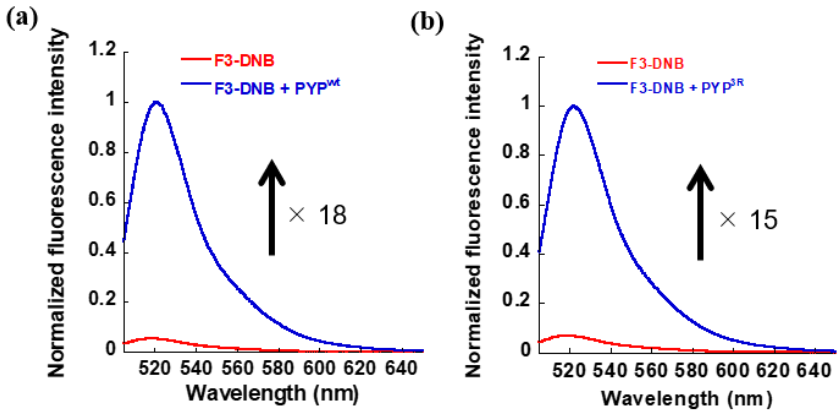


Figure S3. Fluorescence analyses of the labeling reactions of (a) PYP^{wt} and (b) PYP^{3R} with F3-DNB. Normalized fluorescence intensities of 6.0 μM F3-DNB in absence and presence of (a) 5.0 μM PYP^{wt}; (b) 5.0 μM PYP^{3R} in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, with incubation carried out for (a) 18 h and (b) 7 h, respectively. Excitation wavelength of 498 nm used for F3-DNB probe.

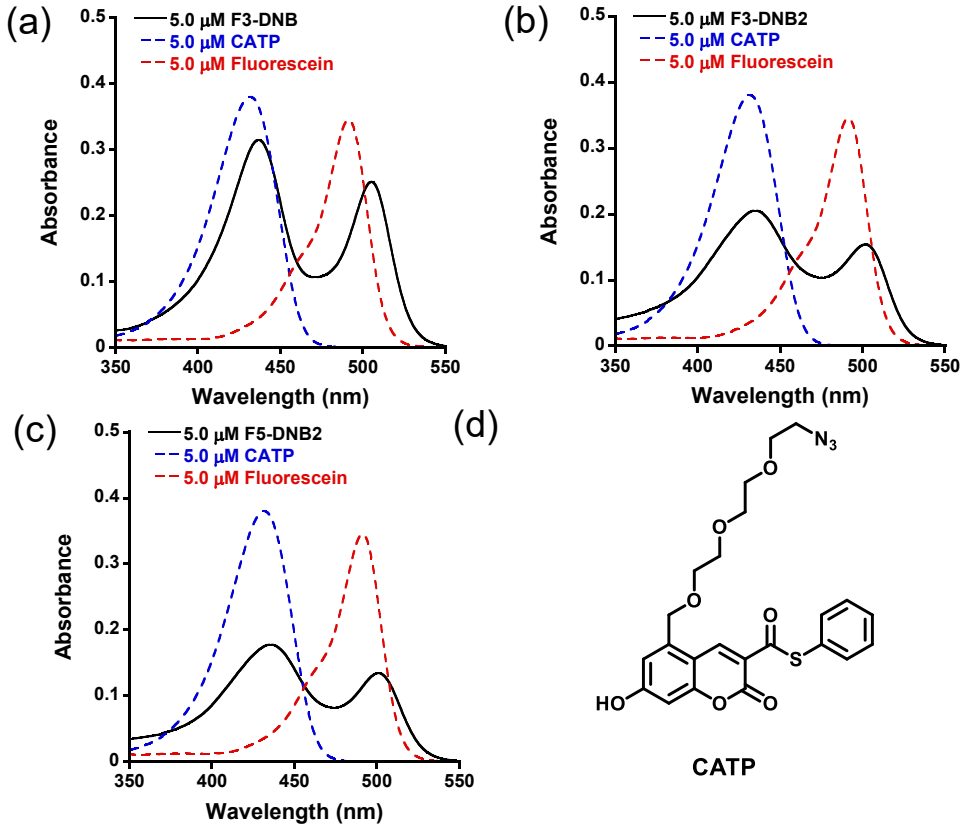


Figure S4. Comparison of absorption spectra of CATP, fluorescein and (a) F3-DNB, (b) F3-DNB2, (c) F5-DNB2. Spectra were recorded using CATP (5.0 μM), fluorescein (5.0 μM), F3-DNB (5.0 μM), F3-DNB2 (5.0 μM) and F5-DNB2 (5.0 μM) in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C. (d) Structure of CATP.

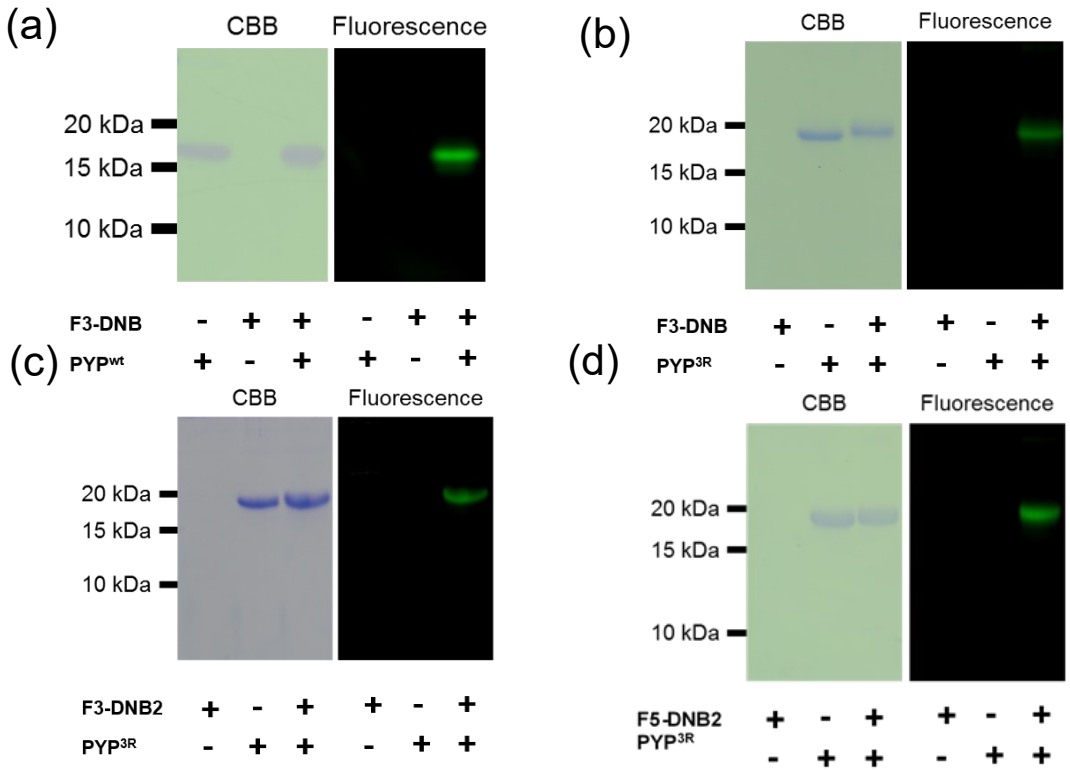


Figure S5. SDS-PAGE analysis of labeling reaction between (a) F3-DNB and PYP^{wt}, (b) F3-DNB and PYP^{3R}, (c) F3-DNB2 and PYP^{3R} and (d) F5-DNB2 and PYP^{3R}. (a) PYP^{wt} (20 μM) labeled with F3-DNB (25 μM), (b) PYP^{3R} (20 μM) labeled with F3-DNB (15 μM), (c) F3-DNB2 and PYP^{3R} and (d) F5-DNB2 and PYP^{3R} in HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C for 13.5 h, 7h, 4h and 2 h, respectively. CBB = Coomassie Brilliant Blue.

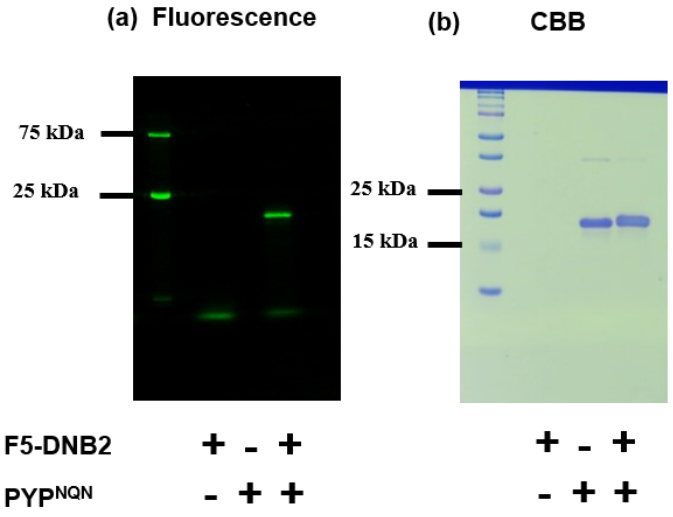


Figure S6. SDS-PAGE analysis of labeling reactions between F5-DNB2 and PYP^{NQN}. PYP^{NQN} (20 μM) was labeled with F5-DNB2 (15 μM) in HEPES buffer (pH 7.4), 150 mM NaCl; 1.0% DMSO at 37 °C for 2 h. Fluorescence and CBB-stained images are shown in (a) and (b), respectively. CBB = Coomassie Brilliant Blue.

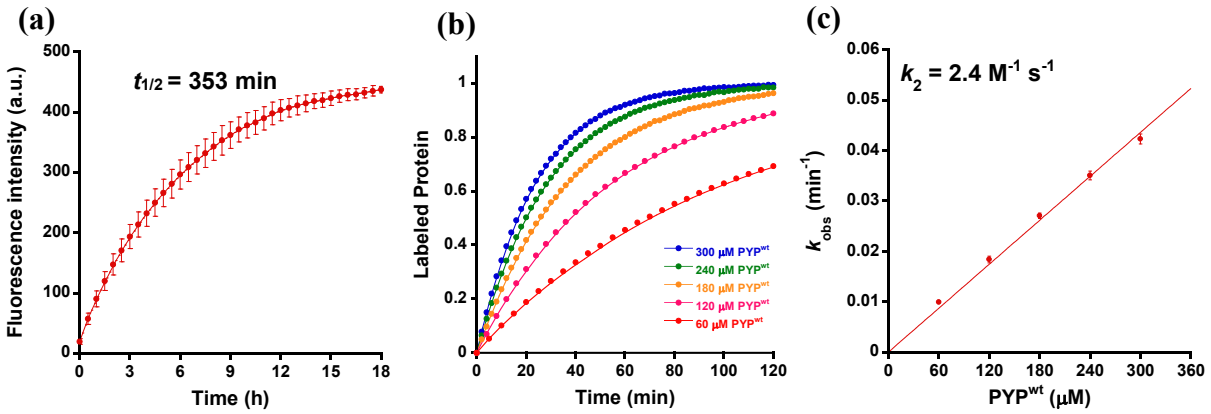


Figure S7. Kinetic analyses of labeling reactions between F3-DNB and PYP^{wt}. (a) Time-course of fluorescence intensities of F3-DNB (6.0 μM) after labeling with PYP^{wt} (5.0 μM) in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C. Excitation and emission wavelengths for F3-DNB were 498 and 521 nm, respectively. Error bars denote SD, n = 3. (b) Plots of labeled protein fraction against reaction time. F3-DNB (3.0 μM) reacted with 60-300 μM PYP^{wt} in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C. Excitation and emission wavelengths for F3-DNB probe were 498 and 521 nm, respectively. (c) Plot of pseudo-first-order rate constant (k_2) against protein concentration for F3-DNB. Error bars denote SD, n = 3.

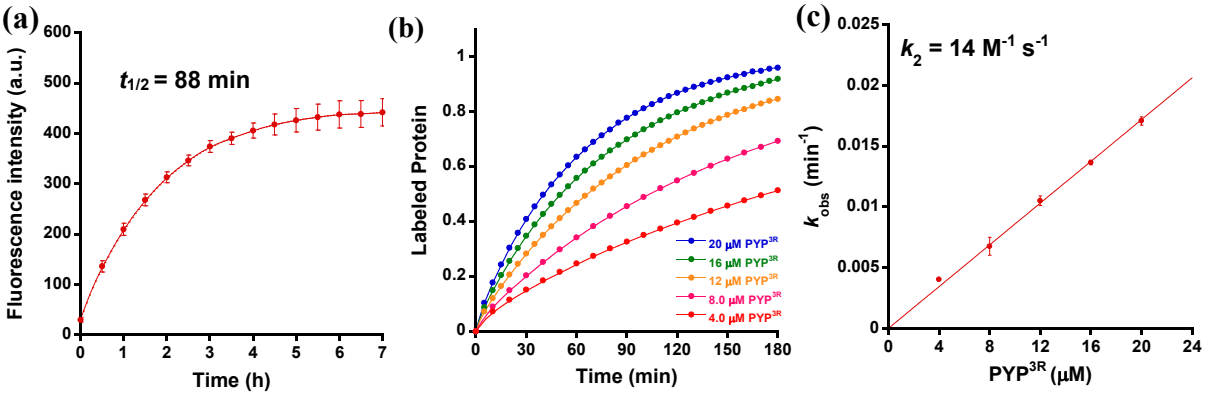


Figure S8. Kinetic analyses of labeling reactions between F3-DNB and PYP^{3R}. (a) Time-course of fluorescence intensity of F3-DNB (6.0 μM) in labeling reactions with PYP^{3R} (5.0 μM) in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C. Excitation and emission wavelengths for F3-DNB were 498 and 522 nm, respectively. Error bars denote SD, n = 3. (b) Plots of labeled protein fraction against reaction time. F3-DNB (0.20 μM) reacted with 4.0-20 μM PYP^{3R} in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C. Excitation and emission wavelengths for F3-DNB were 498 and 522 nm, respectively. (c) Plot of pseudo-first-order rate constant (k_2) against protein concentration for F3-DNB. Error bars denote SD, n = 3.

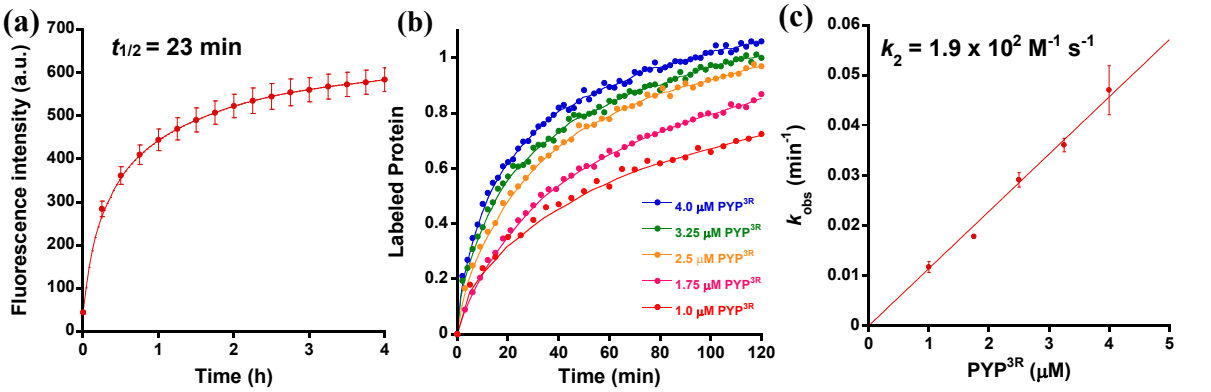


Figure S9. Kinetic analyses of labeling reactions between F3-DNB2 and PYP^{3R}. (a) Time-course of fluorescence intensity of F3-DNB2 (5.0 μM) for labeling reactions with PYP^{3R} (6.0 μM) in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C. Excitation and emission wavelengths for F3-DNB2 were 498 and 523 nm, respectively. Error bars denote SD, n = 3. (b) Plots of labeled protein fraction against reaction time. F3-DNB2 (50 nM) reacted with 1.0-4.0 μM PYP^{3R} in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C. Excitation and emission wavelengths for F3-DNB2 were 498 and 523 nm, respectively (c) Plot of pseudo-first-order rate constant (k_2) against protein concentration for F3-DNB2. Error bars denote SD, n = 3.

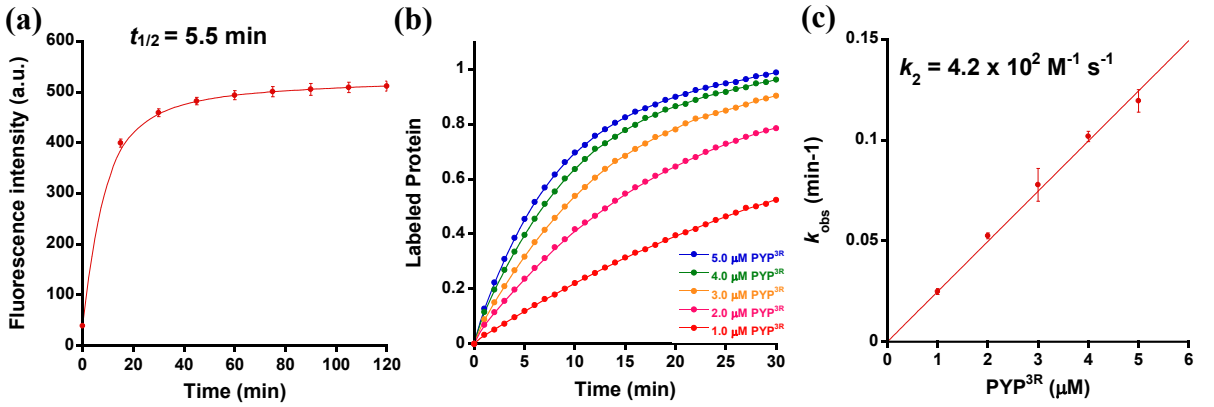


Figure S10. Kinetic analyses of labeling reactions between F5-DNB2 and PYP^{3R}. (a) Time-course of fluorescence intensity of F5-DNB2 (5.0 μM) for labeling reactions with PYP^{3R} (6.0 μM) in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C. Excitation and emission wavelengths for F5-DNB2 were 500 and 523 nm, respectively. Error bars denote SD, n = 3 (b) Plots of labeled protein fraction against reaction time. F5-DNB2 (50 nM) reacted with 1.0-5.0 μM PYP^{3R} in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C. Excitation and emission wavelengths for F5-DNB2 probe were 500 and 523 nm, respectively. (c) Plot of pseudo-first-order rate constant (k_2) against protein concentration for F5-DNB2. Error bars denote SD, n = 3.

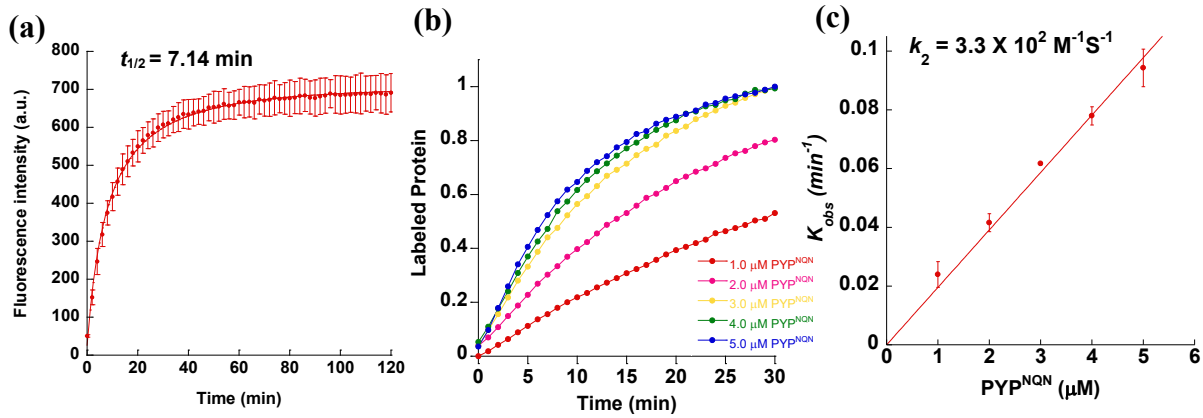


Figure S11. Kinetic analyses of labeling reactions between F5-DNB2 and PYP^{NQN}. (a) Time-course of fluorescence intensity of F5-DNB2 (5.0 μM) in labeling reactions with PYP^{NQN} (6.0 μM) in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C. Excitation and emission wavelengths for F5-DNB2 were 500 and 521 nm, respectively Error bars denote SD, n = 3. (b) Plots of labeled protein fraction against reaction time. F5-DNB2 (50 nM) reacted with 1.0-5.0 μM PYP^{NQN} in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C. Excitation and emission wavelengths of F5-DNB2 were 500 and 521 nm, respectively. (c) Plot of pseudo-first-order rate constant (k_2) against protein concentration for F5-DNB2. Error bars denote SD, n = 3.

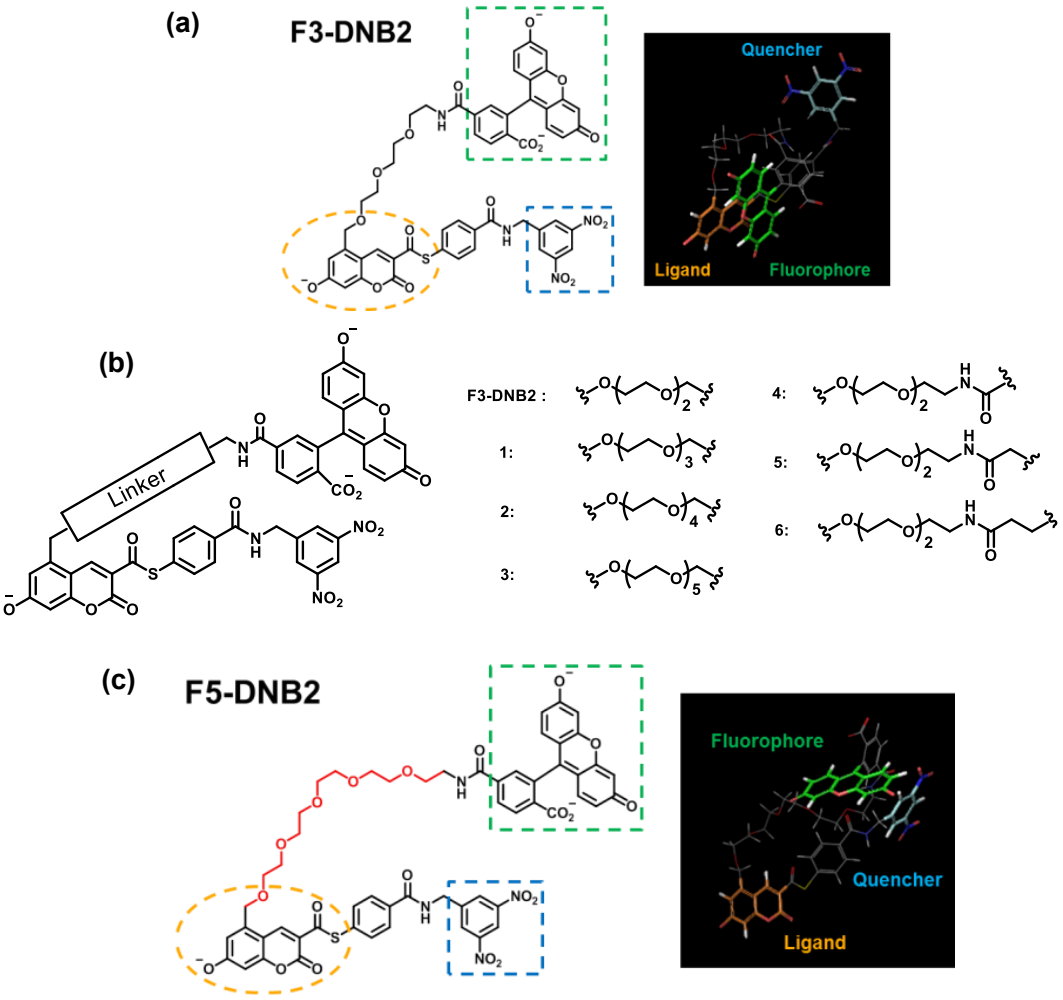


Figure S12. (a) Representative conformations of F3-DNB2 generated using MacroModel simulations. (b) Candidate structures identified as new probes predicted to have faster PYP-tag labeling properties. (c) Representative conformation of F5-DNB2 generated using MacroModel simulations.

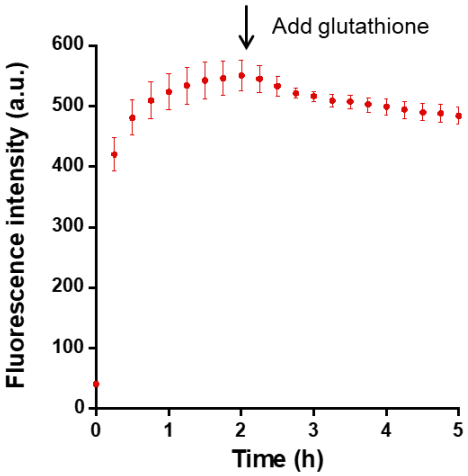


Figure S13. Time-course of fluorescence intensity of F5-DNB2 (5.0 μM) after labeling with PYP^{3R} (6.0 μM) followed by treatment with10 mM glutathione in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl, 1.0% DMSO at 37 °C. Excitation and emission wavelengths of F5-DNB2 probe were 500 and 523 nm, respectively. Error bars denote SD, *n* = 3.

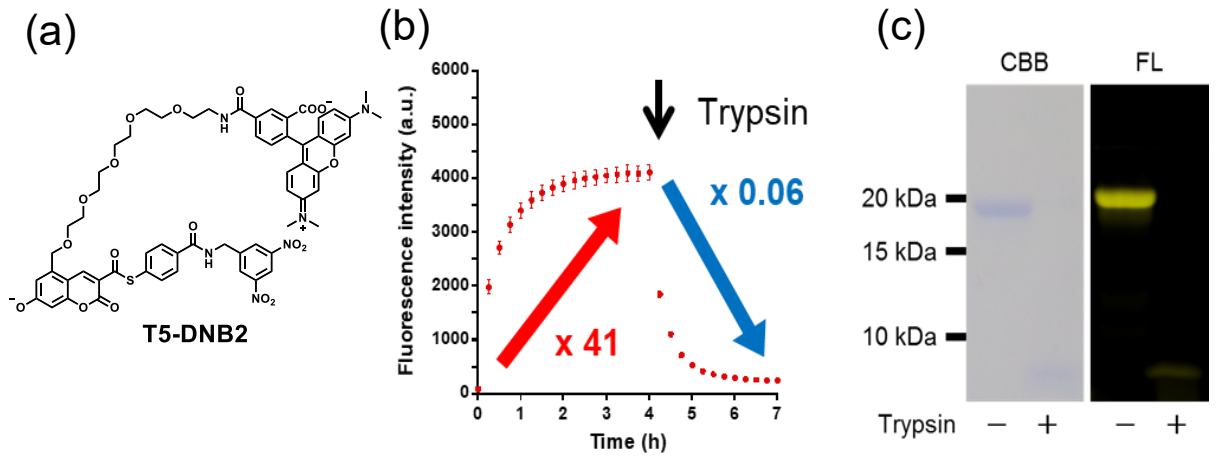


Figure S14. Protein labeling and degradation studies using "OFF-ON-OFF" fluorescence response of a T5-DNB2 probe. (a) Structure of T5-DNB2. (b) PYP^{wt} (6.0 μM) was incubated with T5-DNB2 (5.0 μM) for 240 min and then treated with Trypsin (0.3 μM) in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl and 1.0% DMSO at 37 ºC. Fluorescence intensities were recorded using excitation and emission wavelengths of 555 and 583 nm. Error bars denote SD, *n* = 3. (c) SDS-PAGE analysis of protein degradation. PYP^{wt} (20 μM) was labeled with T5-DNB2 (15 μM) for 120 min and then reacted with Trypsin (1.0 μM) in 20 mM HEPES buffer (pH 7.4) and 150 mM NaCl at 37 ºC for 180 min. CBB = Coomassie Brilliant Blue. FL = Fluorescence.

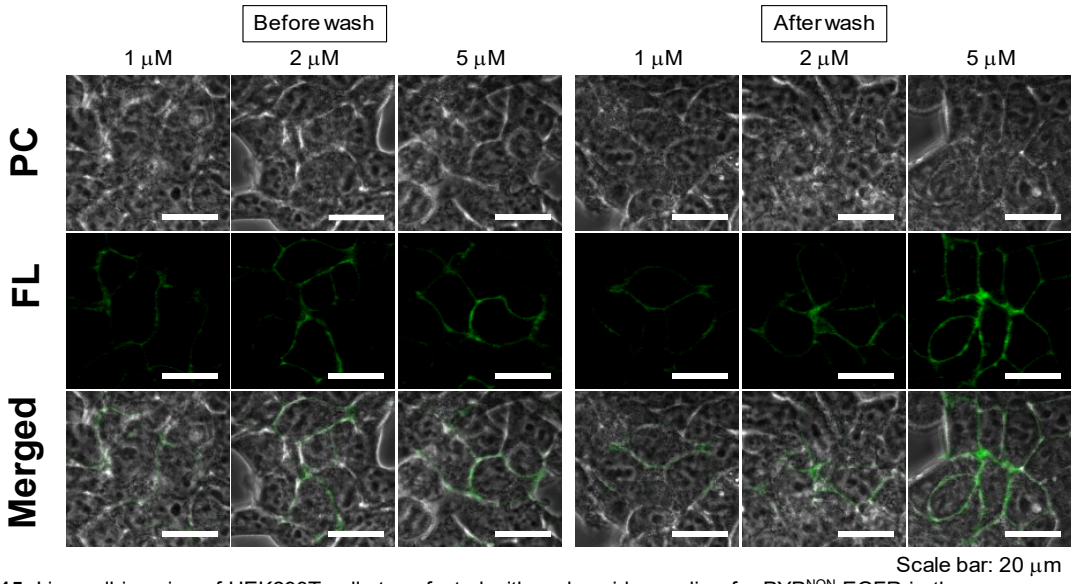


Figure S15. Live-cell imaging of HEK293T cells transfected with a plasmid encoding for PYP^{NQN}-EGFR in the presence of F5-DNB2 (1.0 2.0 or 5.0 μM) using excitation at 473 nm. Cells were incubated with F5-DNB2 (1.0 2.0 or 5.0 μM) for 60 min and images acquired before and after cell washing. Scale bar 20 μm.

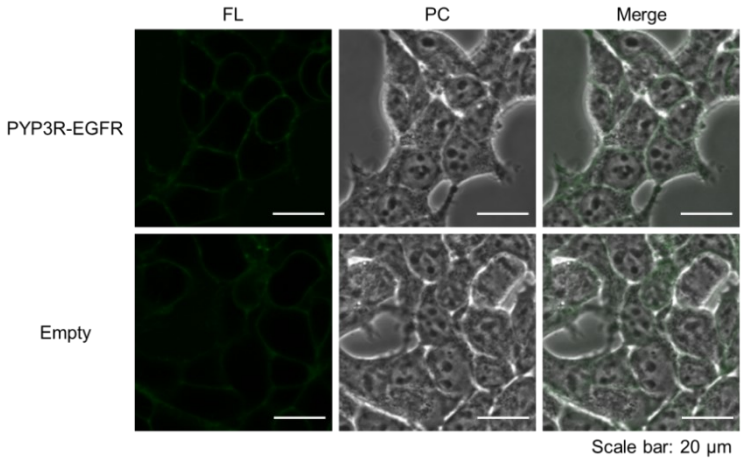


Figure S16. Live-cell imaging of HEK293T cells transfected with plasmids encoding for either PYP^{3R}-EGFR or an empty plasmid in the presence of F5-DNB2 (2.0 μM), with excitation carried out at 473 nm. Scale bar 20 μm.

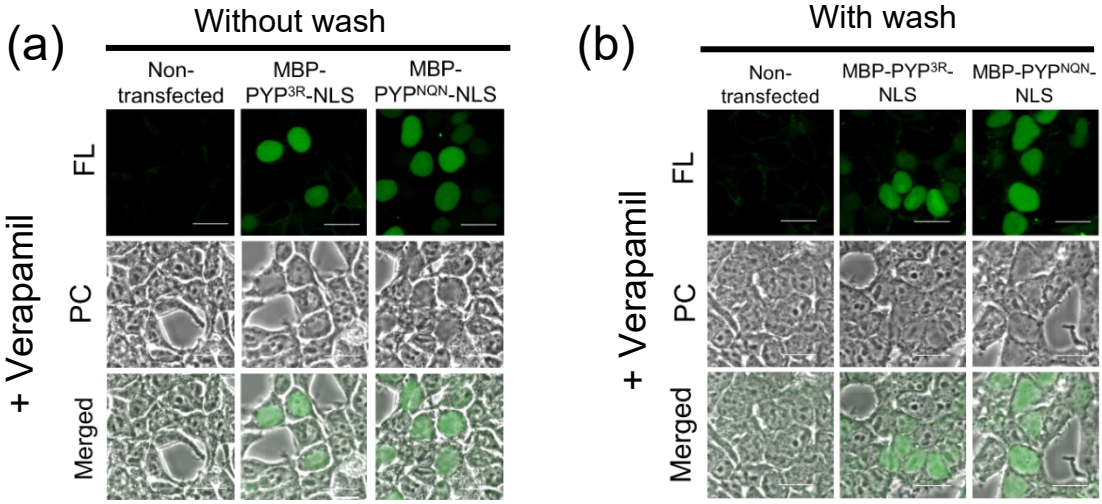


Figure S17. Live-cell imaging of HEK293T cells expressing gene for MBP-PYP^{3R}-NLS/MBP-PYP^{NQN}-NLS using (a) no-wash or (b) wash protocols. Cells were incubated with Ac_2F5-DNB2 (1.0 μM) in the presence of 10 μM Verapamil for 60 min and then imaged using excitation at 473 nm. (b) Cells were washed twice with HBSS buffer to remove unreacted probe before images were then acquired. Scale bar: 20 μm. PC = Phase Contrast. FL = Fluorescence.

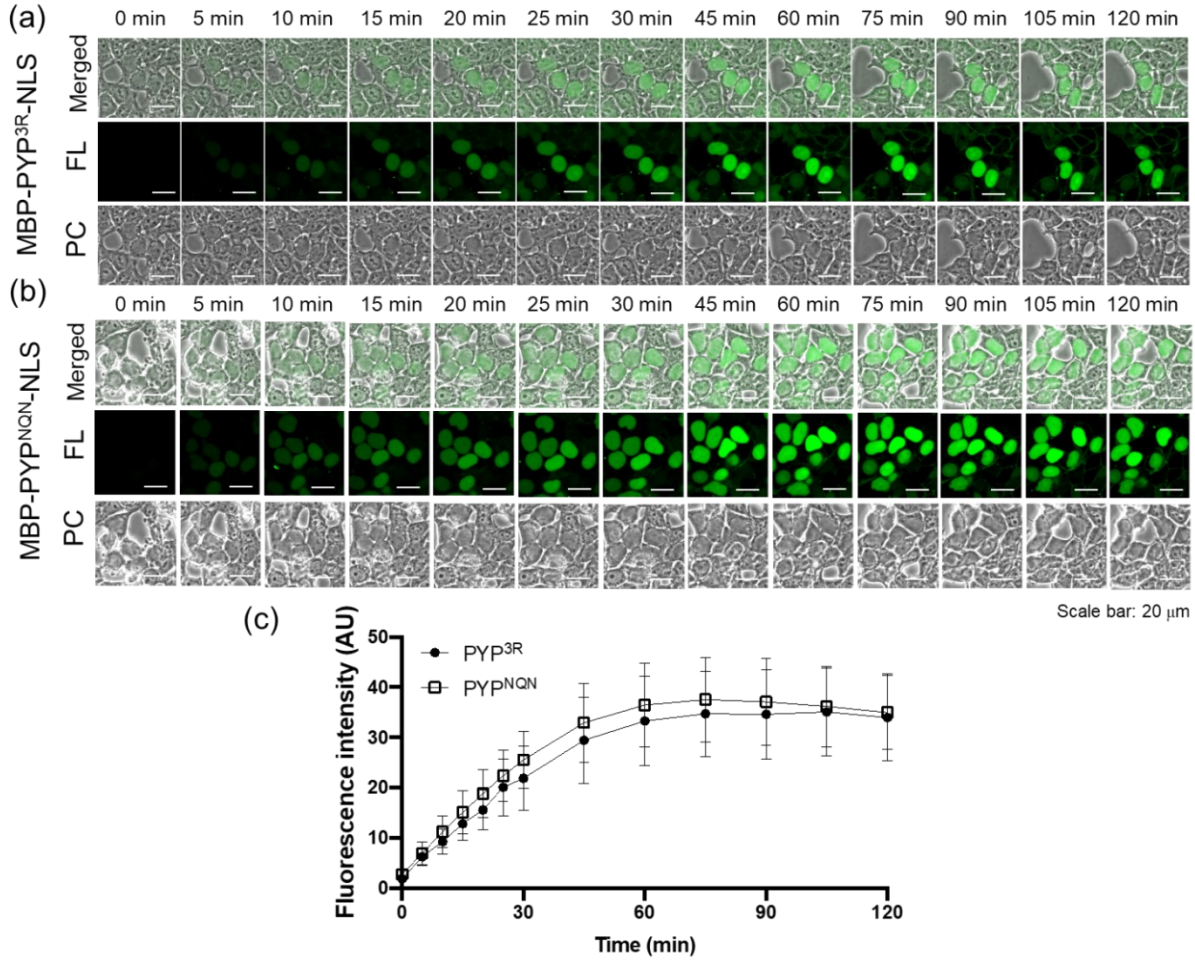


Figure S18. Time-lapse imaging of PYP-tagged proteins using Ac_2F5-DNB2. Fluorescence images of (a) MBP-PYP^{3R}-NLS or (b) MBP-PYP^{NQN}-NLS labeled with Ac_2F5-DNB2 (1.0 μM) using laser excitation at 473 nm. (c) Time course of fluorescence intensities quantified using imaging data shown in Figure 6a. Data are presented as mean fluorescence intensities with error bars representing standard deviation. (*N* = 27 cells). Imaging data obtained from three independent experiments. Scale bar denotes 20 μm. PC = Phase Contrast. FL = Fluorescence.

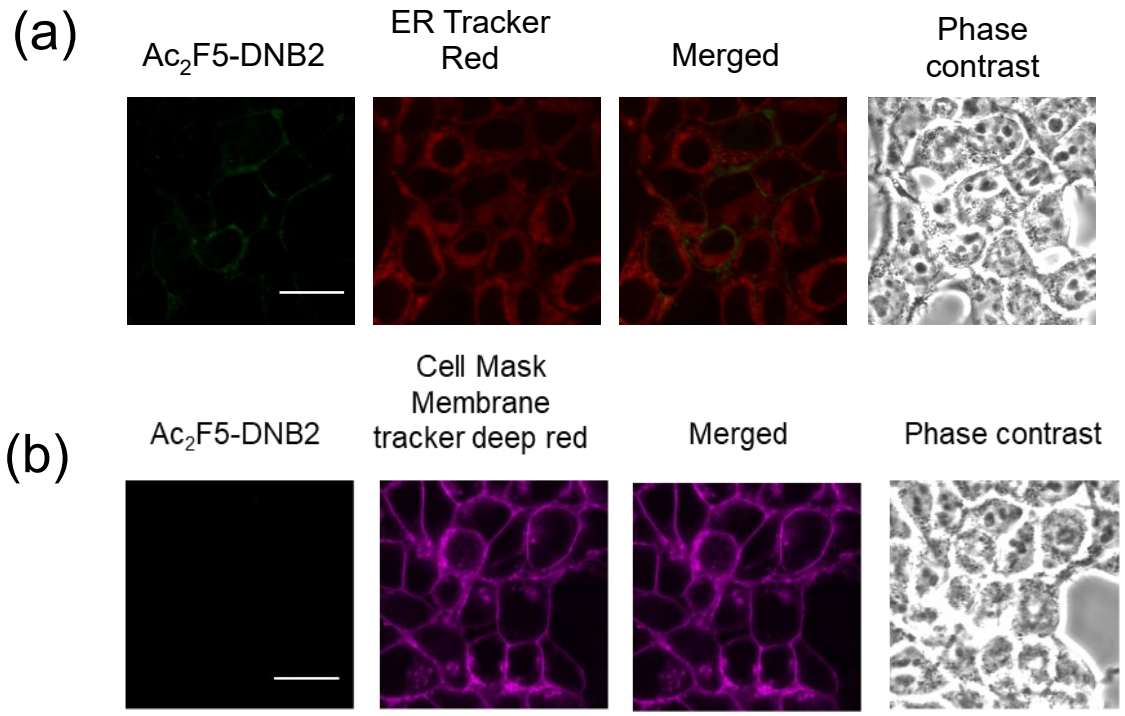


Figure S19. Live-cell imaging of HEK293T cells using Ac_2F5-DNB2 (1.0 μM) and ER-tracker red (0.2 μL/mL) or CellMask™ Deep red (0.2 μL/mL). Image acquisition conducted 60 min after addition of Ac_2F5-DNB2. Cell imaging carried out using excitation at 473 nm for Ac_2F5-DNB2, 559 nm for ER-tracker red) and 635 nm for CellMask™ Deep Red, respectively. Scale bar: 20 μm.

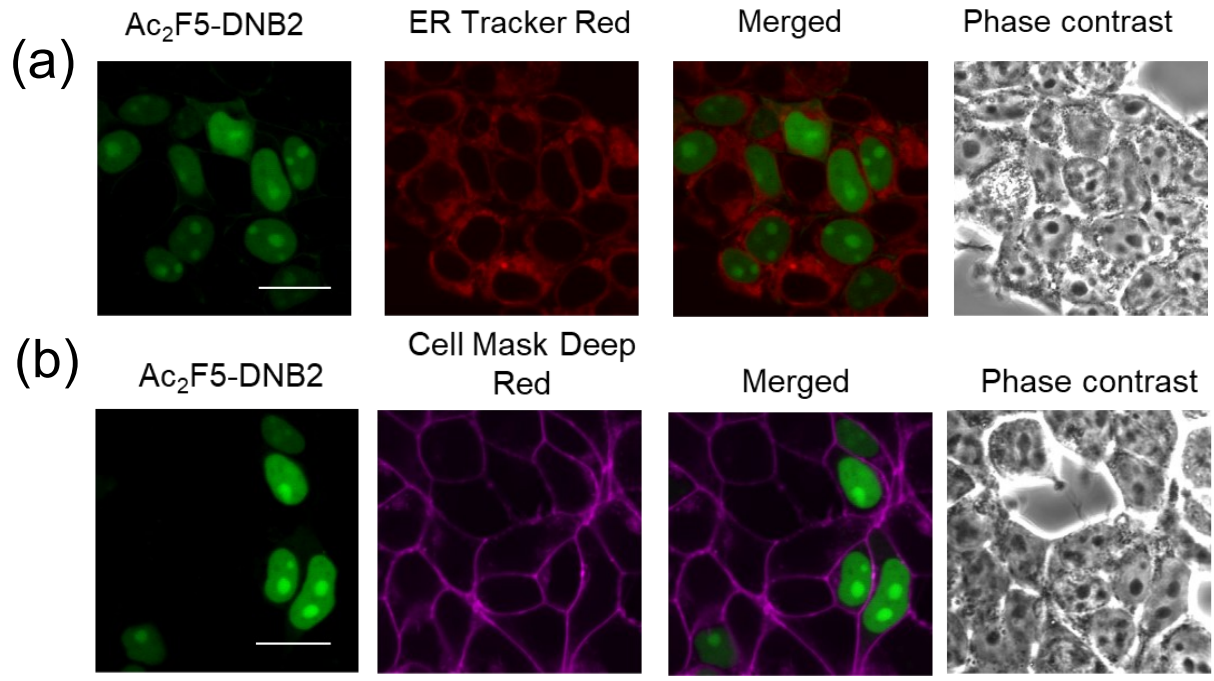


Figure S20. Live-cell imaging of HEK293T cells expressing a gene for HA-PYP^{NQN}-NLS using Ac_2F5-DNB2 (1.0 μM) in the presence of (a) 0.2 μL/mL ER-tracker red or (b) 0.2 μL/mL CellMask™ Deep Red in HEK293T cells. Image acquisition conducted 60 min after addition of Ac_2F5-DNB2. Cell imaging carried out using excitation at 473 nm for Ac_2F5-DNB2, 559 nm for ER-tracker red) and 635 nm for CellMask™ Deep Red, respectively. Scale bar: 20 μm.

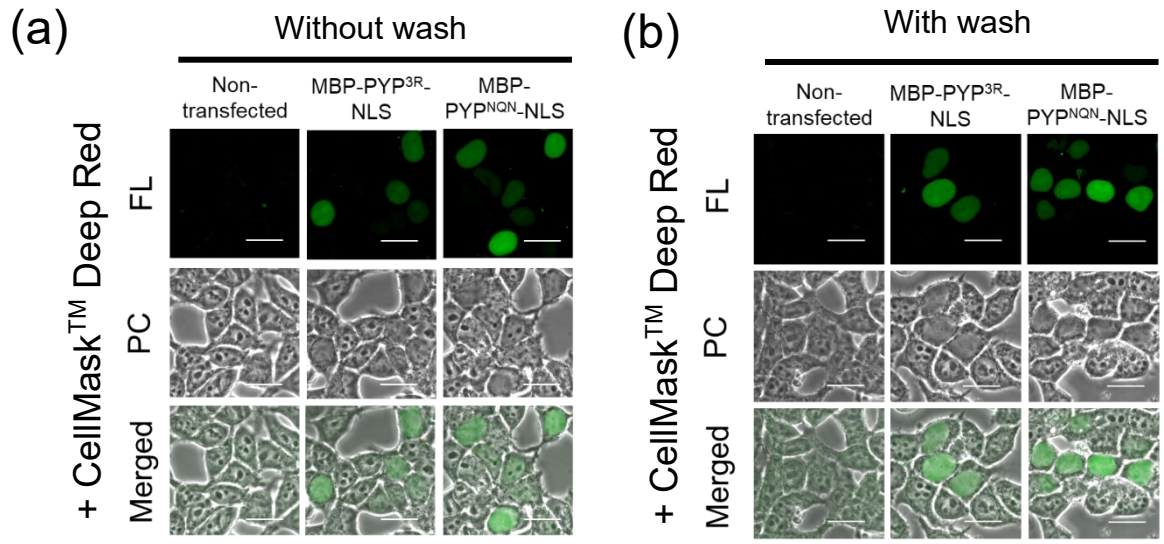


Figure S21. Live-cell imaging of HEK293T cells expressing a gene for MBP-PYP^{3R}-NLS/MBP-PYP^{NQN}-NLS using (a) no-wash or (b) wash protocols. Cells were incubated with Ac_2F5-DNB2 (1.0 μM) in the presence of 0.2 μL/mL of CellMask™ Deep Red for 60 min and then imaged using excitation at 473 nm. For (b) cells were washed twice with HBSS buffer to remove unreacted probe before images were then acquired. Scale bar: 20 μm. PC = Phase Contrast; FL = Fluorescence.

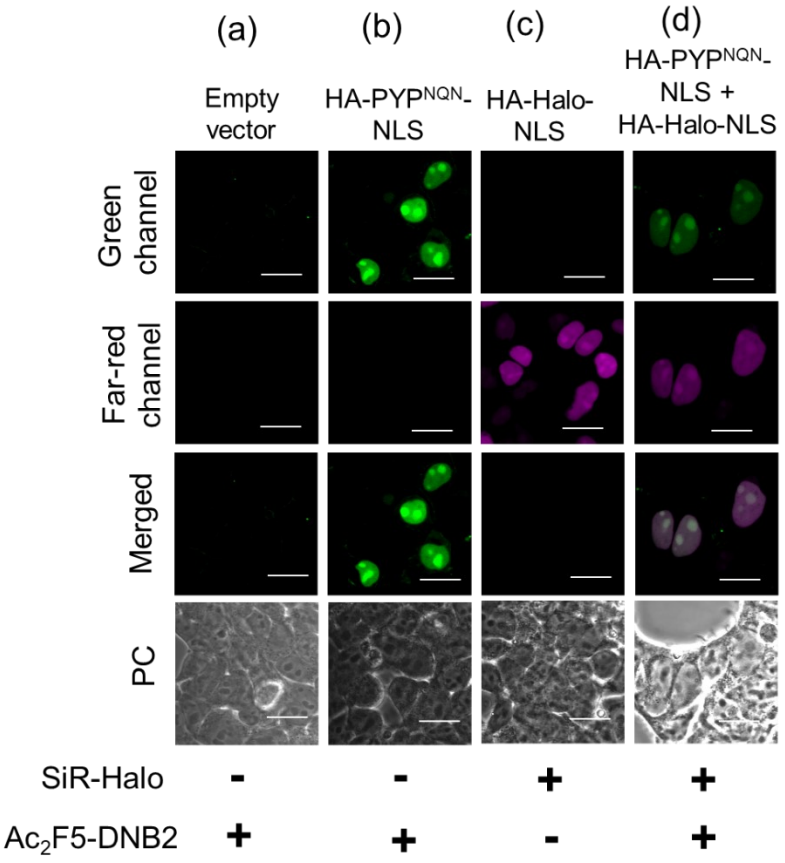


Figure S22. Live-cell imaging of HEK293T cells co-expressing genes for HA-PYP^{NQN}-NLS or/and HA-Halo-NLS using Ac_2F5-DNB2 (1.0 μM) or/and SiR-Halo (1.0 μM) with excitation at 473 and 635 nm, respectively. Image acquisition was conducted 60 min after addition of Ac_2F5-DNB2 or/and SiR-Halo, followed by washing twice with HBSS buffer. Cells were transfected with (a) empty vector, (b) HA-PYP^{NQN}-NLS, (c) HA-Halo-NLS and (d) HA-PYP^{NQN}-NLS and HA-Halo-NLS with transfected cells then incubated with (a,b) Ac_2F5-DNB2, (c) SiR-Halo, and (d) Ac_2F5-DNB2 and SiR-Halo, respectively. Scale bar: 20 μm. PC = Phase Contrast.

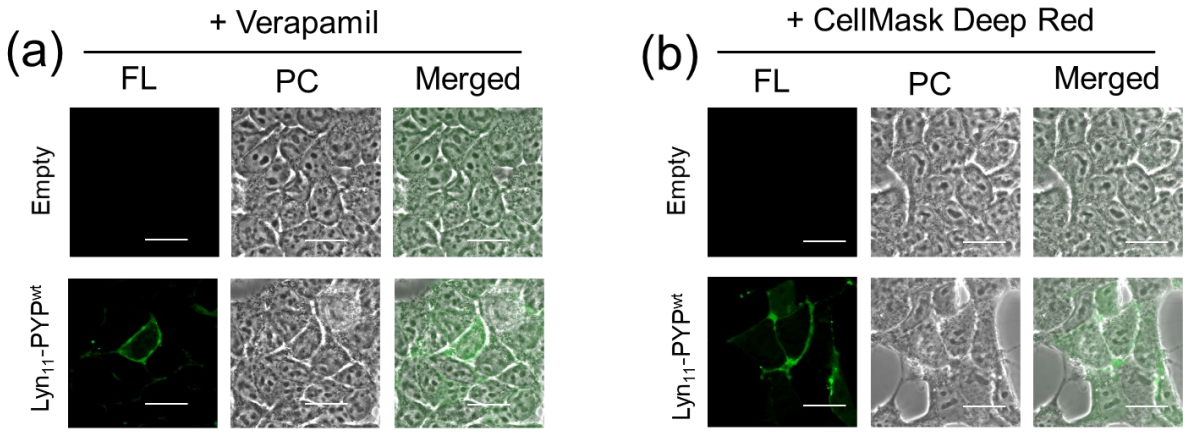


Figure S23. Live-cell imaging of HEK293T cells expressing an empty vector or a gene encoding Lyn_{11}-PYP^{wt}. Images were obtained in the presence of Ac_2F5-DNB2 (1.0 µM) and (a) 10 µM verapamil or (b) 0.2 µL/mL CellMask™ Deep Red using excitation at 473 nm (Ac_2F5-DNB2). Image acquisition was conducted 60 min after addition of Ac_2F5-DNB2 followed by washing twice with HBSS buffer. Scale bar: 20 µm. PC = Phase Contrast; FL = Fluorescence.

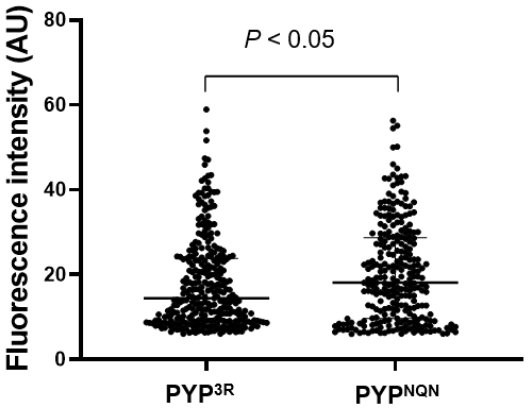


Figure S24. Statistical analyses of fluorescence intensities of F5-DNB2-labeled cells expressing PYP^{3R} and PYP^{NQN}. Data represent mean fluorescence intensities of cell nuclei with medians ± interquartile range after probes had been incubated with cells for 60 min. (N = 310 cells for PYP^{3R} and N = 265 cells for PYP^{NQN}). Differences between mean fluorescence intensities of PYP^{3R} and PYP^{NQN} were statistically significant as demonstrated using the Mann-Whitney test. In both cases, mean fluorescence intensities of cells of >6 were used for analysis.

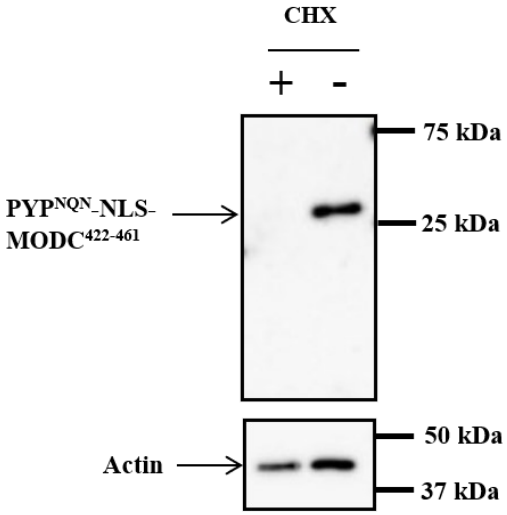


Figure S25. Degradation of HA-PYP^{NQN}-NLS-$MODC^{422-461}$ monitored by western blot analyses performed on extracts of cells that had been incubated with and without 100 µg/ml of CHX for 3 h before sampling. Anti-HA or anti-actin antibody was used as the 1st antibody. Exposure time for anti-HA and anti-Actin were 30 min and 30 sec, respectively.

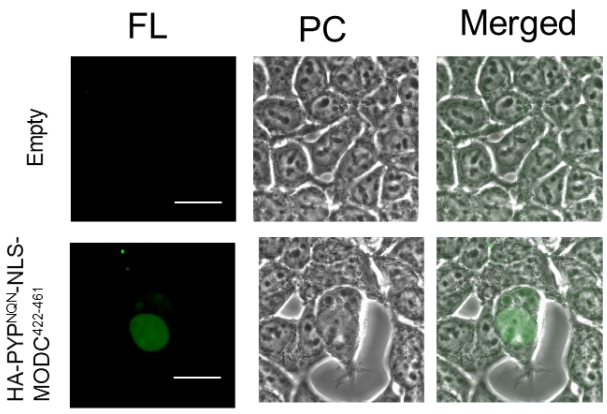


Figure S26. No-wash live-cell imaging of HEK293T cells expressing an empty vector or a gene for HA-PYP^{NQN}-NLS-MODC$^{422-461}$. Cell images were obtained in the presence of Ac_2F5-DNB2 (1.0 μM) and 0.2 μL/mL CellMask^{TM} Deep Red using excitation at 473 nm (Ac_2F5-DNB2). Image acquisition was conducted 60 min after addition of Ac_2F5-DNB2. Scale bar: 20 μm. PC = Phase Contrast. FL = Fluorescence.

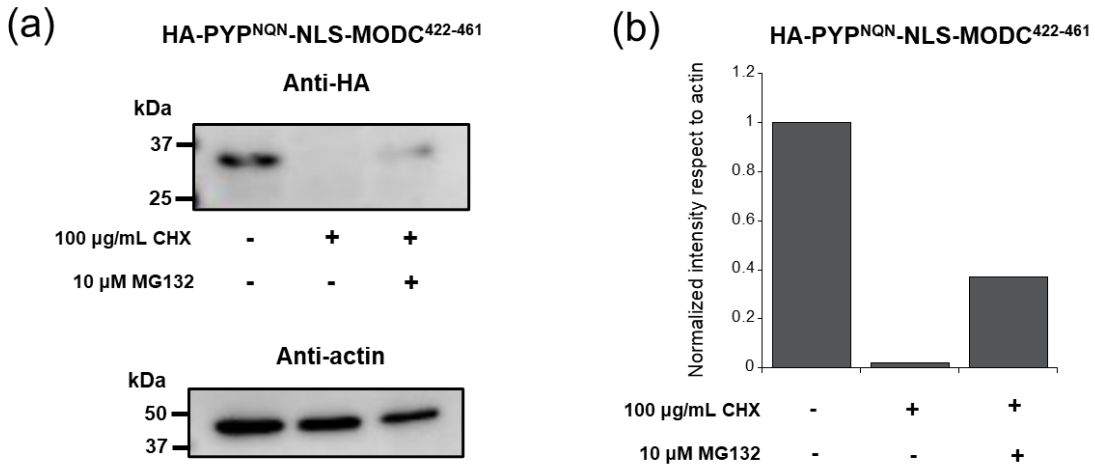


Figure S27. (a) Degradation of HA-PYP^{NQN}-NLS-MODC$^{422-461}$ monitored by western blot analyses. Cells were incubated with or without 10 μM MG132 (proteasome inhibitor). Anti-HA or anti-actin antibody were used as the 1st antibody. Exposure times for anti-HA and anti-actin were 30 min and 25 sec, respectively. (b) Analysis of band intensities are reported with respect to actin.

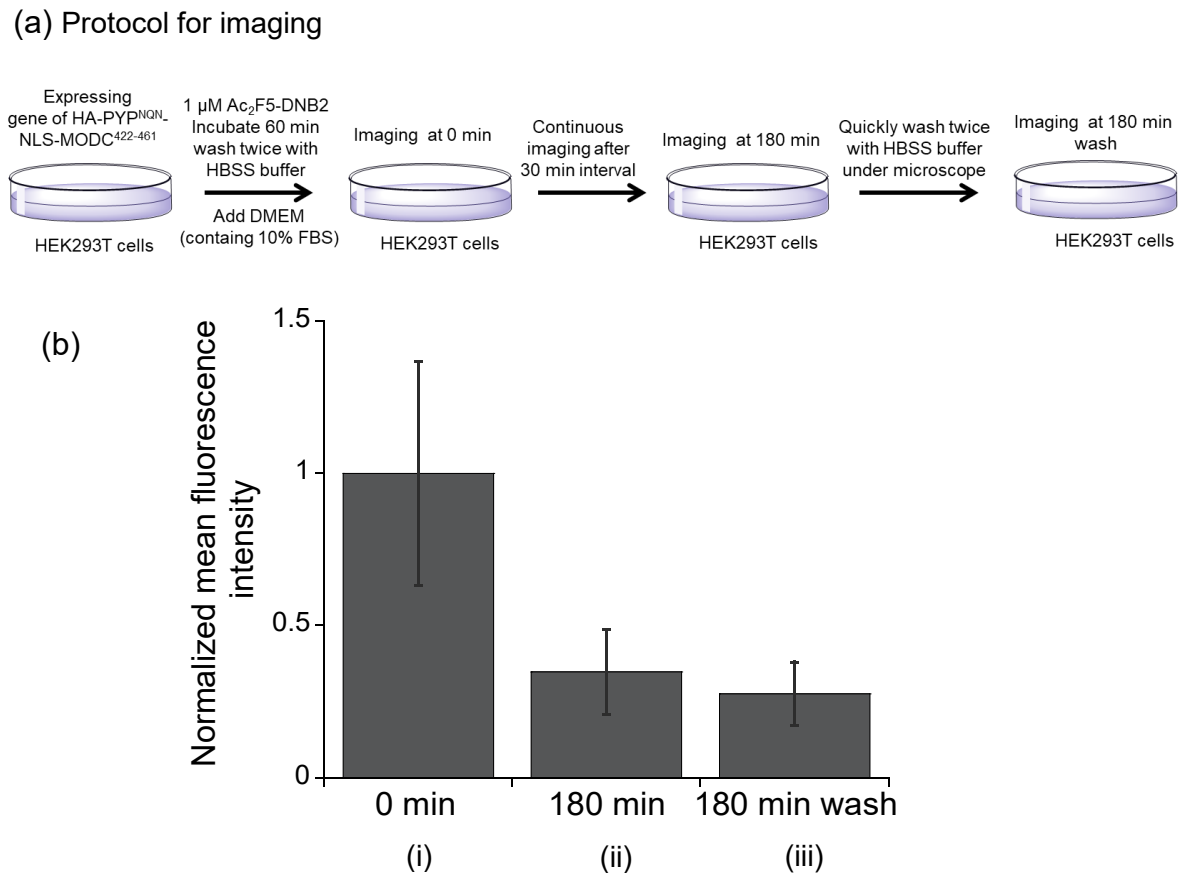


Figure S28: (a) Schematic representation of imaging protocol. (b) Normalized mean fluorescence intensities of HEK293T cells expressing genes for HA-PYP^{NQN}-NLS-MODC$^{422-461}$ treated with Ac_2F5-DNB2 (1.0 μM) after (i) 0 min, (ii) 180 mins and (ii) washing 180 min extract twice with HBSS buffer using excitation at 473 nm. Initial 0 min image acquisition was conducted 60 min after addition of Ac_2F5-DNB2 followed by washing twice with HBSS buffer. Data represent normalized mean fluorescence intensities of three independent experiments, with error bars representing standard deviation (*N* = 23 cells for each group).

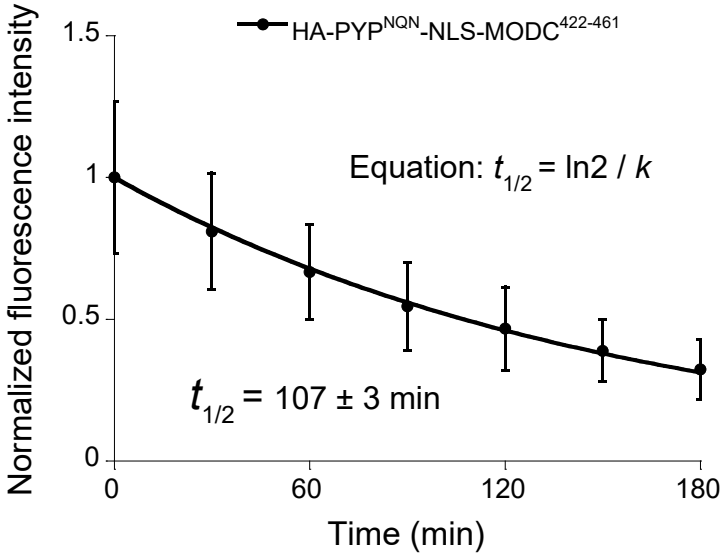


Figure S29. Time-course of degradation of HA-PYP^{NQN}-NLS-MODC$^{422-461}$ in the presence of Ac_2F5-DNB2. Half-life ($t_{1/2}$) of protein determined by fitting the fluorescence decay curve to a pseudo-first order rate equation.

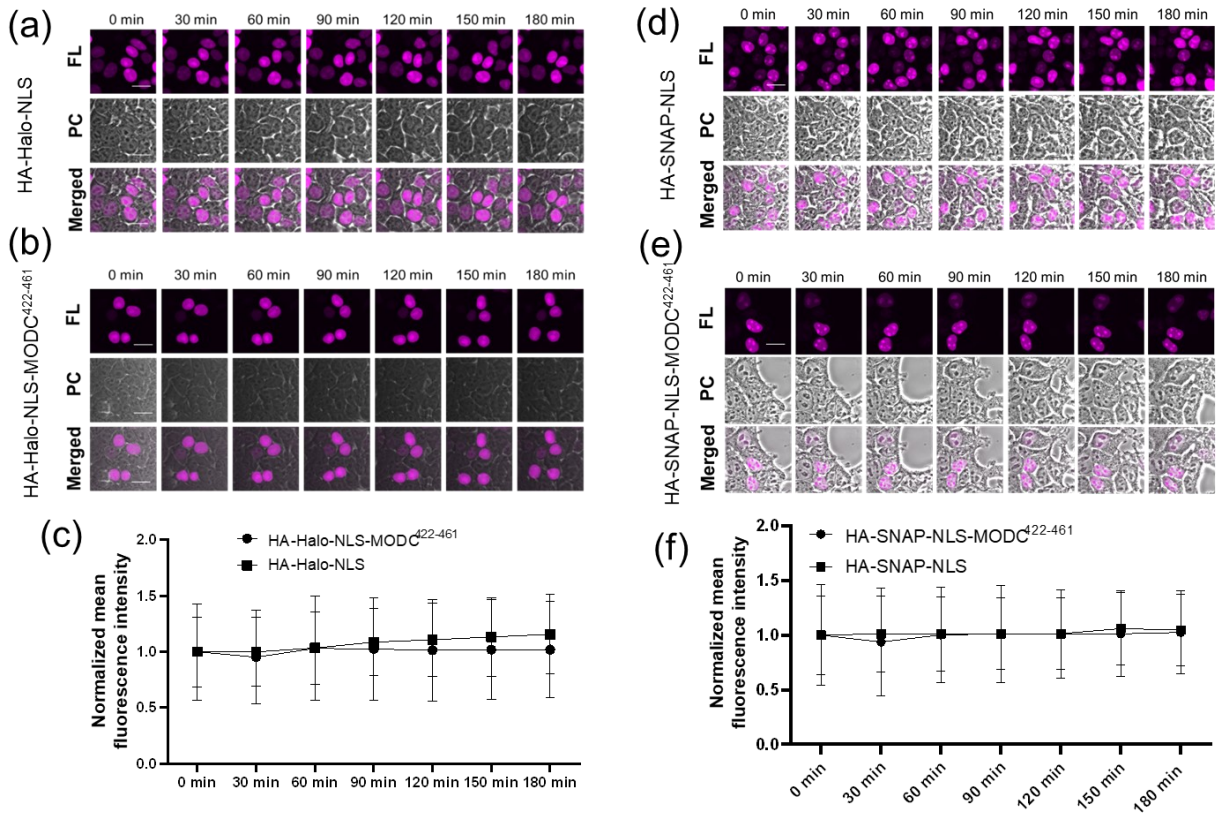


Figure S30. Time-lapse live-cell imaging of MODC-fused Halo and SNAP-tagged proteins. HEK293T cells were transfected with genes for (a) HA-Halo-NLS, (b) HA-Halo-NLS-MODC$^{422\text{-}461}$, (d) HA-SNAP-NLS or (e) HA-SNAP-NLS-MODC$^{422\text{-}461}$. Cells then incubated with (a, b) SiR-Halo (1.0 μM) or (d, e) SiR-SNAP (1.0 μM) for 30 min, respectively, followed by washing the cells twice with HBSS buffer with cell imaging carried out using excitation at 635 nm. (c, f) Quantitative analyses of time-lapse images produced in cell imaging experiments described in figures (a & b) and (d & e). Mean fluorescence intensities of cell nuclei obtained from three independent experiments with error bars representing standard deviation, (N = 24 cells) Scale bar: 20 μm. PC = Phase Contrast. FL = Fluorescence.

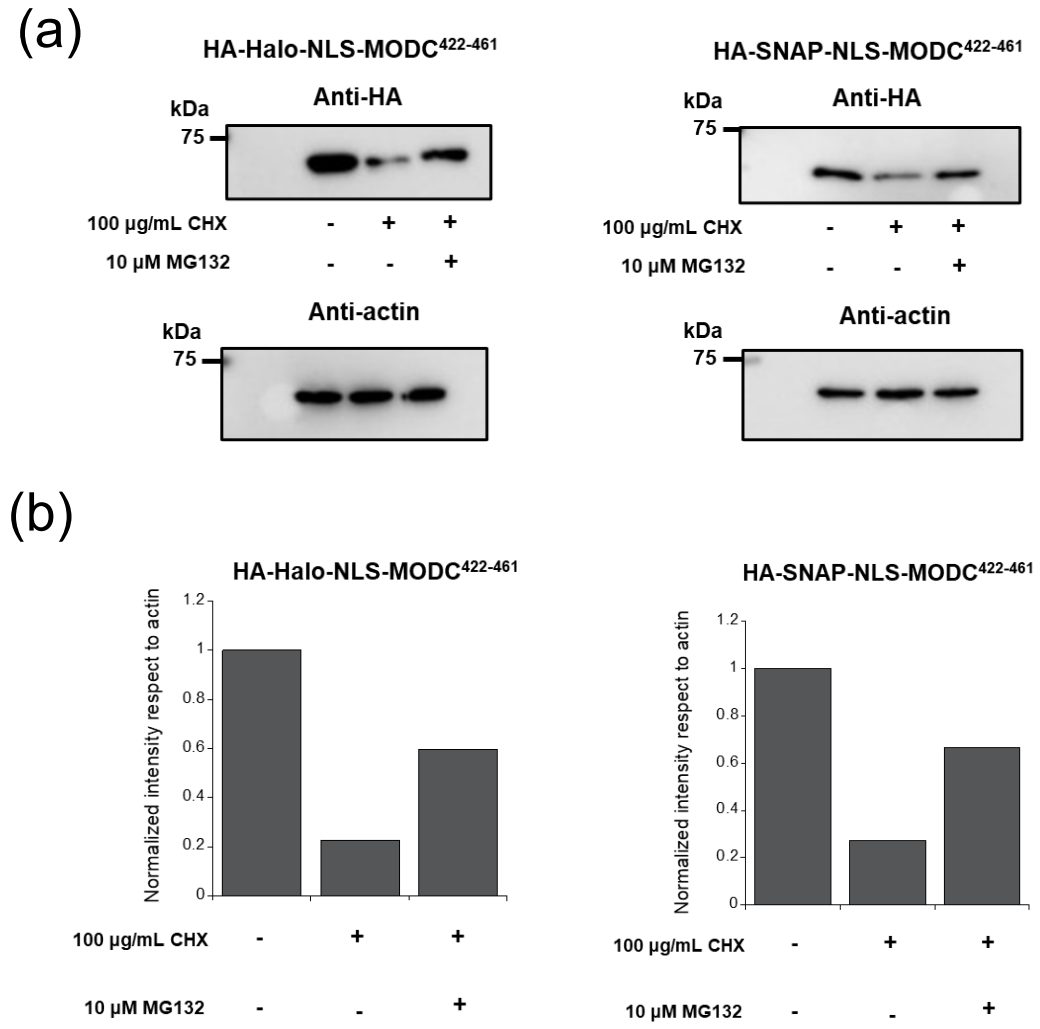


Figure S31. (a) Degradation of HA-Halo-NLS-MODC$^{422-461}$ and HA-SNAP-NLS-MODC$^{422-461}$ monitored by western blot analyses performed on cell extracts incubated with and without 10 µM MG132 (proteasome inhibitor). Anti-HA or anti-actin antibody used as 1st antibody. Exposure times for anti-HA (HA-Halo-NLS-MODC$^{422-461}$) and anti-Actin (HA-Halo-NLS-MODC$^{422-461}$) were 45 sec and 30 sec, respectively. Exposure times for anti-HA (HA-SNAP-NLS-MODC$^{422-461}$) and anti-Actin (HA-SNAP-NLS-MODC$^{422-461}$) were 5 min and 30 sec, respectively. (b) Band intensities reported with respect to actin.

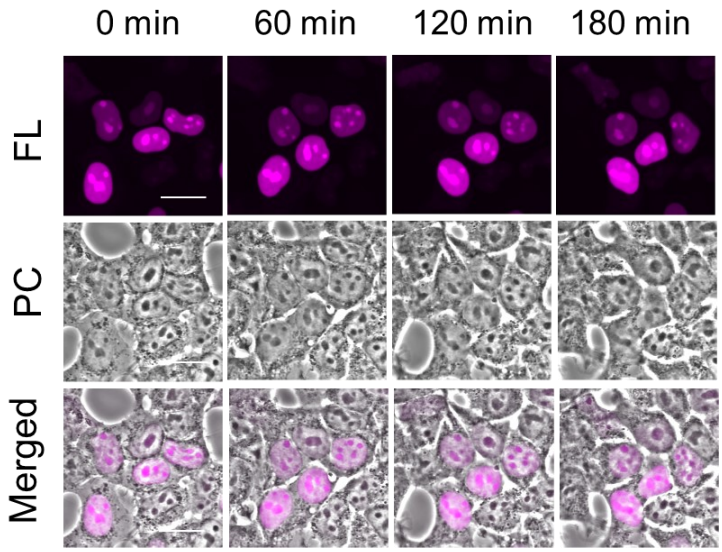


Figure S32. Live-cell imaging of HEK293T cells transfected with a gene for HA-SNAP-NLS-MODC$^{422-461}$ using a SiR-SNAP (500 nM) probe. Images acquired using a multiple washing protocol with excitation at 635 nm (SiR-SNAP). Image acquisition conducted after incubation of cells with probe for 30 min followed by multiple washing with HBSS buffer (see experimental procedure for details). Scale bar: 20 μm. PC = Phase Contrast. FL = Fluorescence.

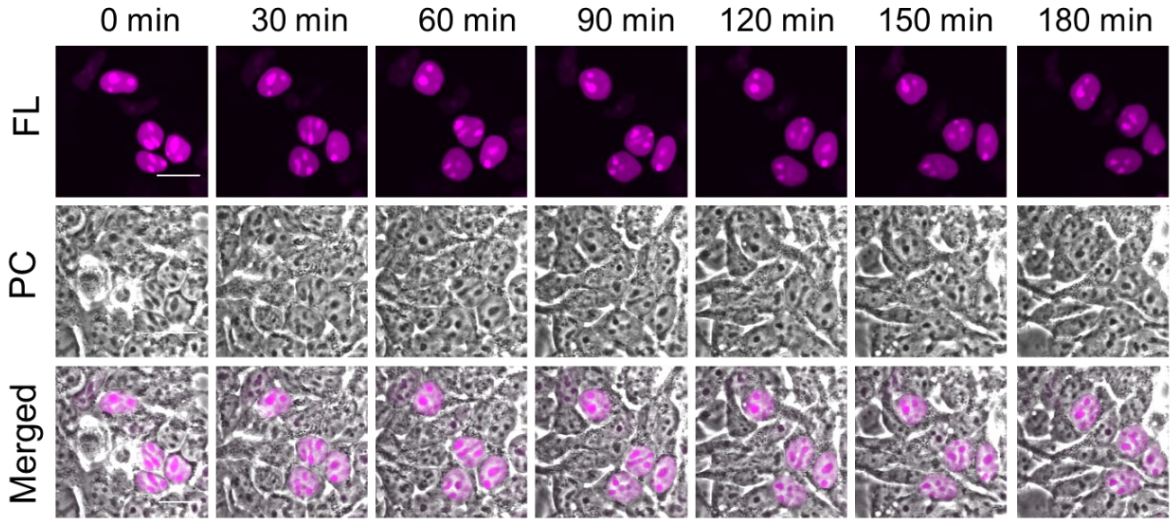


Figure S33. Live-cell imaging of HEK293T cells transfected with gene of HA-SNAP-NLS-MODC$^{422-461}$ using SiR-SNAP (500 nM) probe in presence of 100 μg/mL cyclohexamide with excitation at 635 nm. Image acquisition was conducted 30 min after incubation of SiR-SNAP followed by washing twice with HBSS buffer. Scale bar: 20 μm. PC = Phase Contrast; FL = Fluorescence.

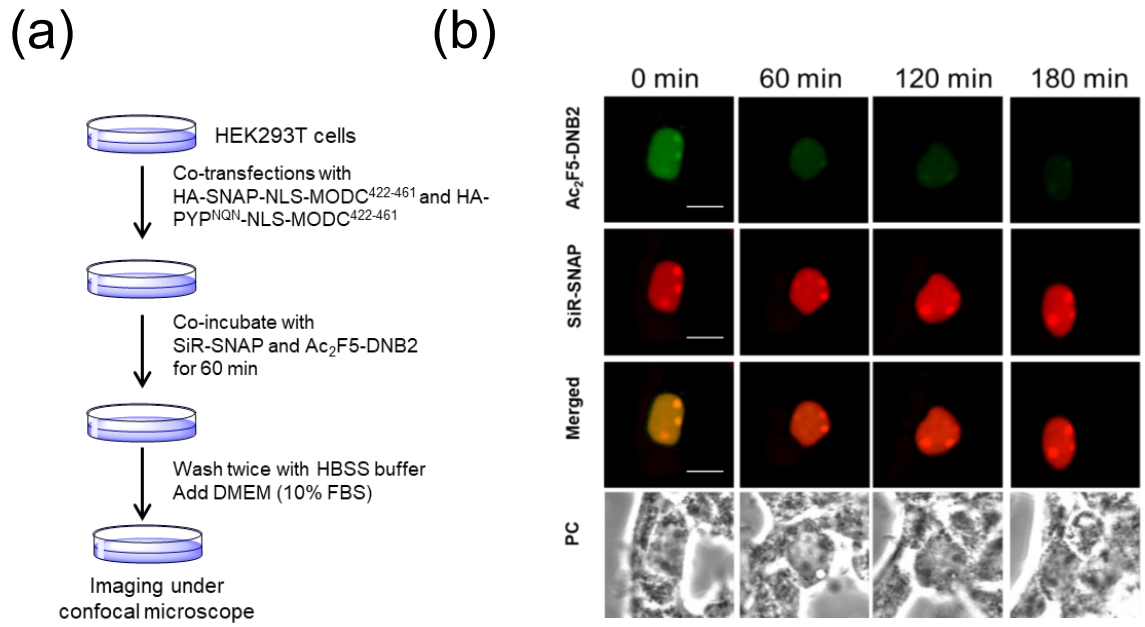


Figure S34. (a) Schematic representation of co-transfection and imaging protocol. (b) Live-cell imaging of HEK293T cells transfected with genes of HA-SNAP-NLS-MODC$^{422-461}$ and HA-PYP^{NQN}-NLS-MODC$^{422-461}$ using SiR-SNAP (1.0 μM) and Ac_2F5-DNB2 (1.0 μM) probes with excitation at 473 nm (Ac_2F5-DNB2) and 635 nm (SiR-SNAP), respectively. Image acquisition conducted after 60 min incubation for both probes, followed by washing twice with HBSS buffer. Images are a representation of two independent experiments. Scale bar: 10 μm. PC = Phase Contrast.

3. Supplementary text

Key role of dinitrobenzyl (DNB) quenching unit for carrying out fast probe labeling of PYP-tag proteins

The DNB moiety present in the OFF-ON-OFF probes used in these PYP-tag labeling studies serves as a non-sterically demanding quenching unit that modulates the probe's fluorescence response, whilst also ensuring that rapid labeling of PYP-tag can occur [S1-3]. For example, intramolecular association between the fluorophore and coumarin ligand in the 1st generation probe FCTP produces steric congestion in the thioester region that results in the probe undergoing a relatively slow trans-thioesterification reaction with the Cys69 thiol residue of PYP-tag (k_2 = 1.11 M^{-1} s^{-1}).[S2] The 2nd generation probe FCATP[S1] contains a cinnamic acid thioester ligand whose presence results in weaker intramolecular stacking interactions between its ligand and fluorophore units, which results in less steric congestion and faster PYP-tag labeling kinetics (k_2 = 11.1 M^{-1} s^{-1}). Finally, use of more extended NB or DNB quenching fragments of the 3rd generation probes FCANB[S1] or FC-DNB[S3], results in less steric hindrance in the vicinity of their reactive thiosester units, which results in much more rapid labeling of PYP-tag (FCANB: k_2 = 125 M^{-1} s^{-1}, FC-DNB: k_2 = 540 M^{-1} s^{-1}).

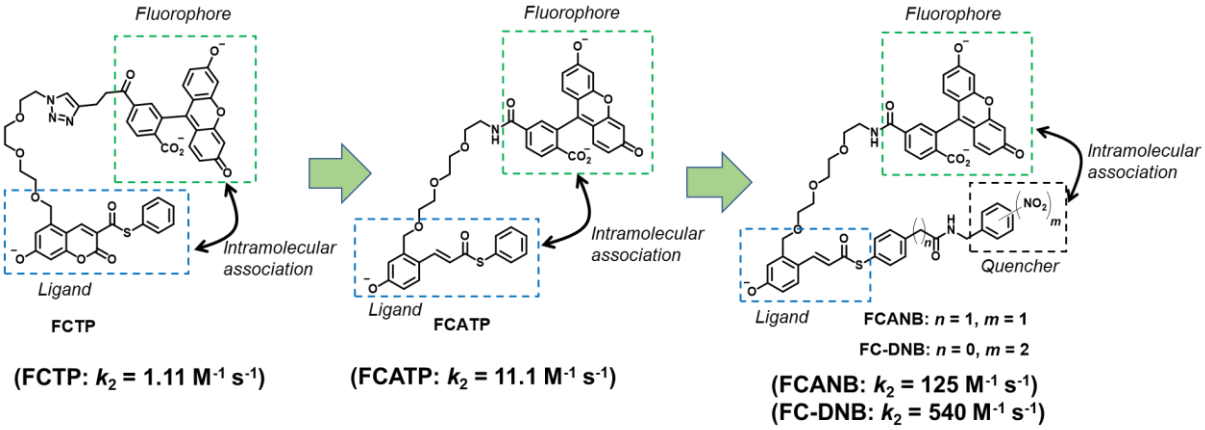


Figure S35: Chemical probe modification results in improved probe labeling kinetics for PYP-tag.

4. Supplementary tables

Table S1. Comparison of reactivities and fluorescence properties of F5-DNB2 and CG2 probes

	F5-DNB2	CG2
k_2 [M^{-1} s^{-1}]	4.2 x 10^2	4.8 x 10^3[b]
Φ_f	0.42[a]	0.17[b]
ε [M^{-1} cm^{-1}]	3.8 x 10^4[a]	3.57 x 10^4[b]
Brightness [ε x Φ_f]	15960	6069[b]
OFF-ON	14	64[c]
ON-OFF	0.24	0.02[c]

[a]Data obtained from labeling reactions of 6.0 μM PYP^{3R}-tag proteins with 5.0 μM F5-DNB2 for 2 h. [b]Data reported in reference [S6]. [C] Data reported in reference [S7].

Table S2. Photo-physical properties of different probes used for labeling PYP-tag proteins.

Probe	λ_{abs} [nm]	λ_{em} [nm]	ε [M^{-1} cm^{-1}]	Φ_f	k_2 [M^{-1} s^{-1}]
F3-DNB	506	520	4.4 x 10^4	0.02	-
F3-DNB2	504	520	2.7 x 10^4	0.07	-
F5-DNB2	503	521	2.4 x 10^4	0.07	-
F3-DNB/PYP^{wt}	499	521	8.6 x 10^4 [a]	0.67	2.4
F3-DNB/PYP^{3R}	500	522	7.6 x 10^4 [a]	0.52	14
F3-DNB2/PYP^{3R}	500	523	7.0 x 10^4 [a]	0.41	1.9 x 10^2
F5-DNB2/PYP^{3R}	503	523	3.8 x 10^4 [b]	0.42	4.2 x 10^2
F5-DNB2/PYP^{NQN}	499	521	3.7 x 10^4 [b]	0.41	3.3 x 10^2

[a]Data obtained after labeling reactions of PYP-tag proteins with probes were complete. [b]Data obtained after labeling reactions of 6.0 μM PYP-tag proteins with 5.0 μM F5-DNB2 for 2 h.

Table S3: MacroModel simulations of probe structures.

Candidate	Interaction between fluorophore and ligand	Interaction between fluorophore and quencher	Number of calculated conformations
(F3-DNB2)	84%	9%	421
1	63%	24%	200
2 (F5-DNB2)	< 1%	80%	312
3	11%	78%	315
4	4%	27%	443
5	2%	70%	534
6	4%	51%	469

*Simulation conducted using water and OPLS2005 as solvent and force field, respectively.

Table S4. OFF-ON and ON-OFF fluorescence ratios observed when DNB probes were used to label different PYP-tag proteins.

Probe	**OFF/ON ratio**	**ON/OFF ratio**
F3-DNB/PYP^{wt}	x 18	N.D.
F3-DNB/PYP^{3R}	x 15	N.D.
F3-DNB2/PYP^{3R}	x 10	N.D.
F5-DNB2/PYP^{3R}	x 14	x 0.24
F5-DNB2/PYP^{NQN}	x 14	x 0.32

N.D.; not determined.

5. Chemical Biology Procedures

Plasmid construction

pcDNA3.1(+)-PYP^{NQN}-EGFR

Construction of the plasmid was performed as described previously.[S8]

pcDNA3.1(+)-MBP-PYP^{wt}-NLS

Construction of the plasmid was performed as described previously.[S9]

pcDNA3.1(+)-MBP-PYP^{3R}-NLS

PCR was used to prepare the DNA fragment for PYP^{3R} from pET21b(+)-PYP^{3R} using the primers, 5′-GATGACAAGCTTATGGAACACGTAGCCTTC-3′ and 5′-AAGATCGGATCCGACGCGCTTGACGA-3′. PYP^{3R} and pcDNA3.1(+)-MBP-PYP^{wt}-NLS[S9] fragments were digested using *Hin*dIII and *Bam*HI and then ligated to produce pcDNA3.1(+)-MBP-PYP^{3R}-NLS.

pcDNA3.1(+)-MBP-PYP^{NQN}-NLS

Construction of the plasmid was performed as described previously.[S8]

pcDNA3.1(+)-HA-PYP^{3R}-NLS

PCR was used to prepare the DNA fragment for PYP^{3R} from pET21b(+)-PYP^{3R} using the primers, 5′-GATGACAAGCTTATGGAACACGTAGCCTTC-3′ and 5′-AAGATCGGATCCGACGCGCTTGACGA-3′. PYP^{3R} and pcDNA3.1(+)-HA-PYP^{wt}-NLS[10] fragments were digested using *Hin*dIII and *Bam*HI and then ligated to produce pcDNA3.1(+)-HA-PYP^{3R}-NLS.

pcDNA3.1(+)-HA-PYP^{NQN}-NLS

A plasmid encoding pcDNA3.1(+)-HA-PYP^{NRN}-NLS was created by incorporating R97N and R71N mutations into pcDNA3.1(+)-HA-PYP^{3R}-NLS using a Quikchange Lightning Multi Site-Directed Mutagenesis kit (Agilent Technologies) according to the manufacturer's protocol, using the primers: 5′-CGATGTTCGAGTACACCTTCAACTACCAAATGACGCCCACG-3′ and 5′-CGTGGGCGTCATTTGGTAGTTGAAGGTGTACTCGAACATCG-3′ for R97N; and 5′-GCCCCGTGCACTAACAGCCCGCGCTTCTACG-3′ and 5′-CGTAGAAGCGCGGGCTGTT-AGTGCACGGGGC-3′ for R71N. An objective plasmid, pcDNA3.1(+)-HA-PYP^{NQN}-NLS was created by incorporating R74Q mutations into pcDNA3.1(+)-HA-PYP^{NRN}-NLS, using the mutagenesis kit and the primer pairs: 5′-GCACTCGCAGCCCGCAGTTCTACGGCAAGTTCAAGG-3′ and 5′-CCTTGAACTTGCCGTAGAACTGCGGGCTGCGAGTGC-3′.

pcDNA3.1(+)-Lyn$_{11}$-PYP^{wt}

Construction of the plasmid was performed as described previously.[S11]

pcDNA3.1(+)-HA-PYP^{3R}-NLS-MODC$^{422-461}$

PCR was used to prepare the DNA fragment for PYP^{3R}-MODC$^{422-461}$ from pUCFa-PYP^{3R}-MODC$^{422-461}$ (purchased from Fasmac) using the primers, 5′-GATGACAAGCTTATGGAGCACGTTGCC-3′ and 5′-ACGCCCGAATTCTCATACATTGATCCTCGCACTG-3′. The PYP^{3R}-MODC$^{422-461}$ and pcDNA3.1(+)-HA-PYP^{3R}-NLS fragments were digested using *Hin*dIII and *EcoR*I, and then ligated to produce pcDNA3.1(+)-HA-PYP^{3R}-

MODC$^{422\text{-}461}$. The DNA fragment for PYP^{3R}-NLS was prepared from pcDNA3.1(+) HA-PYP^{3R}-NLS using the primers, 5′-GATGACAAGCTTATGGAACACGTAGCCT-TCGG-3′ and 5′-ACGCCCAGATCTTACCTTTCTCTTTTTCTAGGATCAACC-3′. The PYP^{3R}-NLS and pcDNA3.1(+)-HA-PYP^{3R}-MODC$^{422\text{-}461}$ fragments were digested using *Hin*dIII and *Bgl*II/*Bam*HI and then ligated to produce pcDNA3.1(+)-HA-PYP^{3R}-NLS-MODC$^{422\text{-}461}$.

pcDNA3.1(+)-HA-PYP^{NQN}-NLS-MODC$^{422\text{-}461}$

A plasmid encoding pcDNA3.1(+)-HA-PYP^{NRN}-NLS-MODC$^{422\text{-}461}$ was created by incorporating R97N and R71N mutations into pcDNA3.1(+)-HA-PYP^{3R}-NLS-MODC$^{422\text{-}461}$ using a Quikchange Lightning Multi Site-Directed Mutagenesis kit (Agilent Technologies) according to the manufacturer's protocol, using the primers: 5′-CGATGTTCGAGTACACCTTCAACTACCAAATGACGCCCACG-3′ and 5′-CGTGGGCGTCATTTGGTAGTTGAAGGTGTACTCGAACATCG-3′ for R97N; and 5′-GCCCCGTGCACTAACAGCCCGCGCTTCTACG-3′ and 5′-CGTAGAAGCGCGGGCTGTT-AGTGCACGGGGC-3′ for R71N. An objective plasmid, pcDNA3.1(+)-HA-PYP^{NQN}-NLS-MODC$^{422\text{-}461}$ was created by incorporating R74Q mutations into pcDNA3.1(+)-HA-PYP^{NRN}-NLS-MODC$^{422\text{-}461}$, using the mutagenesis kit and primer pairs: 5′-GCACTCGCAGCCCGCAGTTCTACGGCAAGTTCAAGG-3′ and 5′-CCTTGAACTTGCCGTAGAACTGCGGGCTGCGAGTGC-3′.

pcDNA3.1(+)-HA-Halo-NLS

PCR was used to prepare the DNA fragment for Halo from pcDNA3.1(+)-Halo using the primers, 5′-CGAGTCAAGCTTATGTCCGAAATCGGTACTG-3′ and 5′-GTATCCGGATCCACCGGAAATCTCCAGAGTAG-3′. The Halo and pcDNA3.1(+)-HA-PYP^{wt}-NLS fragments were digested using *Hin*dIII and *Bam*HI and then ligated to produce pcDNA3.1(+)-HA-Halo-NLS.

pcDNA3.1(+)-HA-SNAP-NLS

PCR was used to prepare the DNA fragment for SNAP from pcDNA3.1(+)-SNAP using the primers, 5′-CAGCACAAGCTTATGGACAAAGACTGCGAAATG-3′ and 5′-GCAGTCGGATCCCTCGAGTTTAAACGCG-3′. The SNAP and pcDNA3.1(+)-HA-PYP^{wt}-NLS fragments were digested using *Hin*dIII and *Bam*HI and then ligated to produce pcDNA3.1(+)-HA-SNAP-NLS.

pcDNA3.1(+)-HA-Halo-NLS-MODC$^{422\text{-}461}$/ pcDNA3.1(+)-HA-SNAP-NLS-MODC$^{422\text{-}461}$

PCR was used to prepare the DNA fragment for NLS-MODC$^{422\text{-}461}$ from pcDNA3.1(+)-HA-PYP^{NQN}-NLS-MODC$^{422\text{-}461}$ using the primers, 5′-GATTCTGGATCCGGAGGATCTGGAGG-3′ and 5′-CGGCAAGAATTCTCATACATTGATCCTCGCACTG-3′. The NLS-MODC$^{422\text{-}461}$ and pcDNA3.1(+)-HA-Halo-NLS/ pcDNA3.1(+)-HA-SNAP-NLS fragments were digested using *BamH*I and *EcoR*I and then ligated to produce pcDNA3.1(+)-HA-Halo-NLS-MODC$^{422\text{-}461}$/ pcDNA3.1(+)-HA-SNAP-NLS-MODC$^{422\text{-}461}$.

Preparation of recombinant proteins (PYP-tag)

E. coli cells [BL21 (DE3) (Novagen)] transformed with plasmids encoding PYP-tag (PYP^{wt}, PYP^{3R}, PYP^{NQN}) were cultivated in Luria-Bertani medium containing 100 μg/ml of ampicillin at 37 °C. The culture flask was incubated at 20 °C until the OD_{600} value of the culture medium reached 0.6-0.8, with addition of IPTG (Isopropyl β-D-1-thiogalactopyranoside) then inducing overnight protein expression to afford a final protein concentration of 100 μM.

The cells were harvested by centrifugation at 5000 rpm for 15 min, resuspended in Bind buffer (50 mM Sodium phosphate, 300 mM NaCl, 1 mM DTT, pH 8.0) and then lysed by sonication. The supernatant of the cell lysate was obtained by centrifugation at 15000 rpm for 20 min and then passed through a Ni column (Roche). The resin was then washed with Wash buffer (50 mM Sodium phosphate, 300 mM NaCl, 5 mM Imidazole, 1 mM DTT, pH 8.0) and eluted with a second buffer (50 mM Sodium phosphate, 300 mM NaCl, 250 mM Imidazole, 1 mM DTT, pH 8.0) in accordance with the manufacturer's protocol. The eluted fraction was further purified through size exclusion chromatography (Superdex^{TM} 75 10/300 GL, GE healthcare) using a running buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) and the purity and molecular weight of proteins assessed by SDS-PAGE. Purified protein was dissolved in the assay buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) and then flash-frozen with liquid nitrogen for storage at -80 °C.

Protein labeling and degradation *in vitro* by SDS-PAGE

Protein labeling was carried out by reacting PYP-tag (20 μM) with each DNB probe (15 μM; except F3-DNB/PYP^{wt} where 25 μM was used) in 20 mM HEPES buffer containing 150 mM NaCl (pH 7.4) at 37 °C and then incubated with Trypsin (1.0 or 1.5 μM). Reaction mixtures were then heated at 95 °C for 5 min and subsequently analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fluorescence images were obtained using a Typhoon FLA 9500 Biomolecular Imager with gels stained using Coomassie Brilliant Blue (CBB). The excitation wavelength used for fluorescence imaging was 473 nm.

Fluorescence and Absorption spectroscopy

Fluorescence spectra were measured after incubation of each probe (5 μM) with or without PYP-tag (6 μM) in 20 mM HEPES buffer containing 150 mM NaCl (pH 7.4) at 37 °C. Spectra were recorded using an excitation wavelength of 498 nm (F3-DNB/PYP^{wt}, F3-DNB/PYP^{3R} and F3-DNB2/PYP^{3R}) or 500 nm (F5-DNB2/PYP^{3R}/PYP^{NQN}) using a slit width of 2.5 nm for both excitation and emission. Relative fluorescence quantum yields were measured using fluorescein in 0.1 M NaOH aq. (λ_{ex} = 492 nm, F_f = 0.85 or λ_{ex} = 470 nm, F_f = 0.91)[S12,S13] as a reference. Protein degradation was induced by adding a solution of Trypsin (0.3 or 0.5 μM) to a solution of the probe (5 μM) and PYP-tag (6 μM) in 20 mM HEPES buffer containing 150 mM NaCl (pH 7.4) 120 min after the protein labeling reaction had finished (maximum fluorescence intensity). Absorption spectra of the probes (5 μM) were measured in 20 mM HEPES buffer containing 150 mM NaCl (pH 7.4) at 37 °C.

Kinetic analyses of protein labeling reactions

The second-order rate constant of the labeling reaction of each probe was obtained from time-course experiments. Each probe was reacted with an excess amount of PYP-tag and their fluorescence intensities then monitored over time. Experiments were carried out using the conditions described below; **F3-DNB/PYP^{wt}**: 3 μM F3-DNB, 60, 120, 180, 240 and 300 μM PYP^{wt}; **F3-DNB/PYP^{3R}**: 200 nM F3-DNB, 4, 8, 12, 16 and 20 μM PYP^{3R}; **F3-DNB2/PYP^{3R}**: 50 nM F3-DNB2, 1, 1.75, 2.5, 3.25 and 4 μM PYP^{3R}; **F5-DNB2/PYP^{3R}**: 50 nM F5-DNB2, 1, 2, 3, 4 and 5 μM PYP^{3R}; **F5-DNB2/PYP^{NQN}**: 50 nM F5-DNB2, 1, 2, 3, 4, and 5 μM PYP^{NQN}. Fluorescence data were converted into labeled fraction values using the following equation:

$$[\text{Labeled fraction}] = (F_t - F_0) / (F_{max} - F_0)$$

where, F_t, F_{max}, and F_0 represent the observed, maximum, and initial fluorescence intensities, respectively.

The pseudo-first-order rate constant, k_{obs}, was obtained by fitting fluorescence data to the following equation:

[Labeled fraction] = 1 − exp(−k_{obs} t)

This enabled the pseudo-first-order rate constant to be plotted against the protein concentration with the resultant plot then fitted to the equation: k_{obs} = k_2 [PYP-tag], which enabled the second-order rate constant, k_2 to be determined.

Cell cultures

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 0.1 mg/mL streptomycin). Transfected cells were maintained under a 5% CO_2 atmosphere at 37 °C throughout the experiments.

Preparation of MG132 solution

Proteasome inhibitor MG132 was purchased from Merck Millipore Corp. USA (Made in Germany) and prepared as a 10 mM stock solution in DMSO (analytical grade).

Preparation of SiR-SNAP and SiR-Halo solution

SiR-SNAP probe was purchased from Spirochrome (SiR650-BG) and used as received. SiR-Halo probe was purchased from Goryo Chemical (Japan) and used as received. Probe solutions (1 mM) were prepared using DMSO (analytical grade) as solvent according to the manufacturer's protocol.

Fluorescence imaging of short-lived proteins in living cells using CG2

Transfection of HEK293T cells with pcDNA3.1(+)-MBP-PYP^{wt}-NLS or pcDNA3.1(+)-HA-PYP^{NQN}-NLS or pcDNA3.1(+)-HA-PYP^{NQN}-NLS-MODC$^{422-461}$ was conducted using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's protocol. The resultant cells were incubated at 37 °C for 24 h and then washed twice with HBSS. The cells were then incubated with CG2 (1.0 μM) in DMEM (0.3% DMSO) for 5 min, washed twice with HBSS buffer and cells then imaged using a confocal laser-scanning microscope. All images were captured using an excitation frequency of 473 nm with an emission range of 490-590 nm using a 60 × lens (Olympus FLUOVIEW FV10i).

Fluorescence imaging of cell surface proteins in living cells in the presence of F5-DNB2

Transfection of HEK293T cells with pcDNA3.1(+)-PYP^{NQN}-EGFR or pcDNA3.1(+)-PYP^{3R}-EGFR was conducted using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer`s protocol. The resultant cells were incubated at 37 °C for 24 h and then washed twice with HBSS. The resultant cells were then incubated with F5-DNB2 (1.0, 2.0 or 5.0 μM) in DMEM (0.1, 0.2 or 0.5% DMSO) for 60 min. Cell images were acquired with (or without) a cell washing step (3 times with HBSS buffer) using a confocal laser-scanning microscope. All images were captured using an excitation frequency of 473 nm with an emission range of 490-590 nm using a 60 × lens (Olympus FLUOVIEW FV10i).

Fluorescence imaging of proteins in living cells using Ac_2F5-DNB2

Transfection of HEK293T cells with pcDNA3.1(+)-MBP-PYP^{wt}-NLS or pcDNA3.1(+)-MBP-PYP^{3R}-NLS or pcDNA3.1(+)-MBP-PYP^{NQN}-NLS or pcDNA3.1(+)-HA-PYP^{NQN}-NLS or pcDNA3.1(+)-HA-PYP^{NQN}-NLS-MODC$^{422-461}$ or pcDNA3.1(+)-Lyn$_{11}$-PYP^{wt} was conducted using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's protocol. The resultant cells were incubated at 37 ^{o}C for 24 h and then washed twice with HBSS and cells then co-incubated with Ac$_2$F5-DNB2 (1.0 μM) with (or without) verapamil (10 μM) in DMEM (0.3% DMSO) for 60 min. Use of Ac$_2$F5-DNB2 alone only resulted in weakly fluorescent intracellular proteins, which we reasoned was due to low intracellular probe concentration levels caused by extrusion of the probe from the cell by membrane efflux pumps. Consequently, cells were incubated with Ac$_2$F5-DNB2 in the presence of verapamil (a broad-spectrum efflux pump inhibitor) which resulted in a significant increase in intracellular protein fluorescence response.[S14] Imaging of no-wash co-incubated cells was carried out immediately using a confocal laser-scanning microscope. Imaging of washed cells was carried out by first treating the cells with HBSS buffer to remove excess probe, followed by adding fresh DMEM containing 10% FBS, with images then acquired using a confocal laser-scanning microscope. All images were captured employing an excitation wavelength of 473 nm and an emission range of 490-590 nm using a 60 × lens (Olympus FLUOVIEW FV10i).

Fluorescence imaging of proteins in living cells using Ac$_2$F5-DNB2 in the presence of CellMask^{TM} Deep Red or ER Tracker Red

Transfection of HEK293T cells with pcDNA3.1(+)-HA-PYP^{NQN}-NLS or pcDNA3.1(+) were conducted using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's protocol. Cells were incubated at 37 ^{o}C for 24 h and then washed twice with HBSS buffer. Cells were then co-incubated with Ac$_2$F5-DNB2 (1.0 μM) and verapamil (10 μM) in DMEM (0.3% DMSO) for 60 min. Imaging of cells was carried out by first treating them with HBSS buffer twice (2 X 2 mL) to remove excess probe, followed by addition of fresh DMEM containing 10% FBS (0.2 μL/mL CellMask^{TM} Deep Red or ER Tracker Red from Invitrogen^{TM}) Images of cells treated with CellMask^{TM} Deep Red were captured using excitation wavelengths of 473 and 635 nm with emission ranges of 490-540 nm and 660-760 nm using a 60 × lens (Olympus FLUOVIEW FV10i). Images of cells treated with ER Tracker Red were captured using excitation wavelengths of 473 and 559 nm with emission ranges of 490-540 and 570-620 nm using a 60 × lens (Olympus FLUOVIEW FV10i).

Fluorescence imaging of proteins in living cells using Ac$_2$F5-DNB2 in presence of CellMask^{TM} Deep Red

Transfection of HEK293T cells with pcDNA3.1(+)-MBP-PYP^{3R}-NLS or pcDNA3.1(+)-MBP-PYP^{NQN}-NLS or pcDNA3.1(+)-HA-PYP^{NQN}-NLS or pcDNA3.1(+)-HA-PYP^{NQN}-NLS-MODC$^{422-461}$ or pcDNA3.1(+)-Lyn$_{11}$-PYP^{wt} were conducted using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's protocol. The resultant cells were incubated at 37 ^{o}C for 24 h and washed twice with HBSS buffer with the cells then co-incubated with Ac$_2$F5-DNB2 (1.0 μM) and verapamil (10 μM) in DMEM (0.3% DMSO) for 60 min. Imaging of the cells was carried out by first treating them with HBSS buffer twice (2 X 2 mL) to remove excess probe, followed by addition of fresh DMEM containing 10% FBS (0.2 μL/mL CellMask^{TM} Deep Red from Invitrogen^{TM}). No-wash imaging of co-incubated cells with Ac$_2$F5-DNB2 (1.0 μM) (0.2 μL/mL CellMask^{TM} Deep Red from Invitrogen^{TM}) in DMEM (0.2% DMSO) was carried out using a confocal laser-scanning microscope. All images were captured employing an excitation laser at 473 nm, an emission filter of 490-590 nm or 490-540 nm using a 60 × lens (Olympus FLUOVIEW FV10i).

Fluorescence imaging of proteins in living cells using SiR-SNAP and SiR-Halo probes

Transfection of HEK293T cells with pcDNA3.1(+)-HA-SNAP-NLS or pcDNA3.1(+)-HA-Halo-NLS or pcDNA3.1(+)-HA-SNAP-NLS-MODC$^{422-461}$ or pcDNA3.1(+)-HA-Halo-NLS-MODC$^{422-461}$ were conducted using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's protocol. The resultant cells were incubated at 37 ^{o}C for 24 h and then washed twice with HBSS buffer, with the cells then incubated with SiR-SNAP or SiR-Halo (1.0 μM) in DMEM (0.1% DMSO) for 30 min. Imaging of cells was carried out by first treating the cells with HBSS buffer twice (2 X 2 mL) to remove excess probe, followed by addition of fresh DMEM containing 10% FBS, with images then acquired using a confocal laser-scanning microscope. All images were captured employing an excitation wavelength of 635 nm and an emission range of 660-760 nm using a 60 × lens (Olympus FLUOVIEW FV10i).

Fluorescence imaging of SNAP-tag proteins in living cells using SiR-SNAP using a multiple washing procedure

Transfection of HEK293T cells with pcDNA3.1(+)-HA-SNAP-NLS-MODC$^{422-461}$ was conducted using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's protocol, with the cells then incubated at 37 ^{o}C for 24 h before being washed twice with HBSS buffer. The cells were then incubated with SiR-SNAP (500 nM) in DMEM (0.1% DMSO) for 30 min. Imaging was carried out by first washing the cells with HBSS buffer 2 mL, followed by incubation of the cells under a 5% CO_2 atmosphere at 37 ^{o}C for 1 min, followed by washing with HBSS buffer (5 times) to remove excess probe. Fresh DMEM containing 10% FBS was then added, with cell images acquired using a confocal laser-scanning microscope. All images were captured using an excitation wavelength of 635 nm and an emission range of 660-760 nm using a 60 × lens (Olympus FLUOVIEW FV10i).

Fluorescence imaging of proteins in living cells using SiR-SNAP in presnce of cyclohexamide

Transfection of HEK293T cells with pcDNA3.1(+)-HA-SNAP-NLS-MODC$^{422-461}$ was conducted using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's protocol. The resultant cells were incubated at 37 ^{o}C for 24 h and then washed twice with HBSS and the cells then incubated with SiR-SNAP (500 nM) in DMEM (0.1% DMSO) for 30 min. Imaging of washed cells was carried out by first treating the cells with HBSS buffer two times (2 X 2 mL) to remove excess probe, followed by adding with fresh DMEM containing 10% FBS and 100 μg/mL cyclohexamide, with images then acquired using a confocal laser-scanning microscope. All images were captured employing an excitation wavelength of 635 nm and an emission range of 660-760 nm using a 60 × lens (Olympus FLUOVIEW FV10i).

Live-cell imaging of non-transfected HEK293T cells

Non-transfected HEK293T cells were co-incubated with Ac_2F5-DNB2 (1.0 μM) and verapamil (10 μM)/(0.2 μL/mL CellMask^{TM} Deep Red or 0.2 μL/mL ER tracker Red from Invitrogen^{TM}) in DMEM with images of probe-treated cells then recorded using a confocal laser-scanning microscope at excitation wavelengths of 473 nm, 473 and 559 nm or 473 and 635 nm and emission ranges of 490-590 nm, 490-540 and 570-620 nm, or 490-540 and 660-760 nm, using a 60 × lens (Olympus FLUOVIEW FV10i).

Fluorescence imaging of proteins in living cells using Ac_2F5-DNB2 in the presence of MG132

Transfection of HEK293T cells with pcDNA3.1(+)-HA-PYP^{NQN}-NLS-MODC$^{422-461}$ was conducted using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's protocol. The resultant cells were incubated

at 37 ºC for 24 h and then washed twice with HBSS buffer, with cells then incubated with Ac_2F5-DNB2 (1.0 μM) + 10 μM verapamil in DMEM (0.3% DMSO) for 60 min. Imaging was carried out by first washing the cells with HBSS buffer twice (2 X 2 mL) to remove excess probe, followed by addition of fresh DMEM containing 10% FBS (containing 10 μM MG132). Cell images were then captured using an excitation wavelength of 473 nm and an emission range of 490-590 nm, using a 60 × lens (Olympus FLUOVIEW FV10i).

Fluorescence imaging of proteins in living cells treated with a mixture of SiR-SNAP/Ac_2F5-DNB2 or SiR-Halo/Ac_2F5-DNB2

Co-transfection of HEK293T cells with pcDNA3.1(+)-HA-Halo-NLS and pcDNA3.1(+)-HA-PYP^{NQN}-NLS or pcDNA3.1(+)-HA-SNAP-NLS-$MODC^{422-461}$ and pcDNA3.1(+)-HA-PYP^{NQN}-NLS-$MODC^{422-461}$ were conducted using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's protocol. The resultant cells were incubated at 37 ºC for 24 h and then washed twice with HBSS buffer. The cells were then incubated with SiR-SNAP (1.0 μM) and Ac_2F5-DNB2 (1.0 μM), or SiR-Halo (1.0 μM) and Ac_2F5-DNB2 (1.0 μM) in the presence of 10 μM verapamil in DMEM (0.4% DMSO) for 60 min. Imaging of cells was carried out by first washing them with HBSS buffer (2 X 2 mL) to remove excess probe, followed by addition of fresh DMEM containing 10% FBS, with images acquired using a confocal laser-scanning microscope. Cell images were captured using excitation wavelengths of 473 and 635 nm and an emission range of 490-540 and 660-760 nm using a 60 × lens (Olympus FLUOVIEW FV10i).

Fluorescence imaging of proteins in living cells treated with TAMRA-DNB/Ac_2F5-DNB2 or TAMRA-DNB/CG2

Transfection of HEK293T cells with pcDNA3.1(+)-HA-PYP^{NQN}-NLS-$MODC^{422-461}$ was conducted using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's protocol. The resultant cells were incubated at 37 ºC for 24 h and then washed twice with HBSS buffer. These cells were then incubated with TAMRA-DNB[S3] (1.0 μM) in DMEM (0.1% DMSO) for 30 min and then washed with HBSS buffer twice (2 X 2 mL) to remove excess probe. Fresh DMEM containing 10% FBS was then added, the cells incubated for a further 30 min and then washed twice with HBSS buffer. The cells were then incubated with (i) Ac_2F5-DNB2 (1.0 μM) and 10 μM verapamil in DMEM (0.3% DMSO) for 60 min; or (ii) CG2 (1.0 μM) in DMEM (0.3% DMSO) for 5 min. No-wash images of the cells was carried out using a confocal laser-scanning microscope, with all images acquired using excitation wavelengths of 473 and 559 nm and an emission range of 490-540 and 570-620 nm using a 60 × lens (Olympus FLUOVIEW FV10i).

Monitoring protein degradation using western blot analyses

HEK293T cells were transfected with pcDNA3.1(+)-HA-PYP^{NQN}-NLS-$MODC^{422-461}$ using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. Transfected cells were incubated at 37 °C for 24 h and cells then incubated with 100 μg/ml of cycloheximide for 0 and 3 h, respectively. The cells were then lysed by adding buffer (0.5 M Tris-HCl pH 6.8, 5% SDS, 10% glycerol, 50 mM DTT and 0.5% bromophenol blue) followed by heating at 95 °C for 5 min. Protein extracts were separated by SDS-PAGE and subsequently blotted onto a polyvinylidene difluoride membrane. Specific detection of expressed protein targets was carried out by blocking the membrane with 3% non-fat powdered milk in TBS-Tween buffer (Tris-buffered saline 0.1% Tween; pH 7.6) at rt for 30 min, followed by incubation at rt for 1 h with anti-HA. The membrane was then washed with TBS-Tween buffer five times and then incubated with 3% non-fat powdered milk in TBS-Tween buffer containing ECL™ Peroxidase labeled anti-mouse antibody at rt for 1 h. The membrane was subsequently washed with TBS-Tween buffer three times and any chemiluminescence from target proteins detected using an Amersham™ ECL Prime Western Blotting Detection System (GE Healthcare) in accordance with the manufacturer's protocol.

Monitoring protein degradation using western blot analyses in the presence of MG132

HEK293T cells were transfected with pcDNA3.1(+)-HA-PYP^{NQN}-NLS-MODC$^{422-461}$ or pcDNA3.1(+)-HA-Halo-NLS-MODC$^{422-461}$ or pcDNA3.1(+)-HA-SNAP-NLS-MODC$^{422-461}$ using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. The cells were incubated at 37 °C for 24 h, washed and then incubated with or without 100 µg/ml of cycloheximide, and with or without 10 µM of MG132 for 3 h. The cells were then lysed by adding lysis buffer (20 mM HEPES, 150 mM NaCl, 1 mg/mL leupeptin hemisulfate salt, 1 mg/mL Pepstatin and 1% NP40) followed by heating at 95 °C for 5 min. Cell extracts were separated by SDS-PAGE with gels subsequently blotted onto a polyvinylidene difluoride membrane. Specific detection of protein targets was carried out by blocking the membrane with 3% non-fat powdered milk in TBS-Tween buffer (Tris-buffered saline 0.1% Tween; pH 7.6) at rt for 30 min, followed by incubation at rt for 1 h with anti-HA. The membrane was washed with TBS-Tween buffer five times and then incubated with 3% non-fat powdered milk in TBS-Tween buffer containing ECL™ Peroxidase labeled anti-mouse antibody at rt for 1 h. The membrane was subsequently washed with TBS-Tween buffer three times and any chemiluminescence from target proteins detected using an Amersham™ ECL Prime Western Blotting Detection System (GE Healthcare) in accordance with the manufacturer's protocol.

Determination of half-life ($t_{1/2}$) of PYP^{NQN}-NLS-MODC$^{422-461}$ using Ac$_2$F5-DNB2

The half-life for protein degradation, $t_{1/2}$, was estimated by monitoring the decrease in fluorescence intensity that originated from the Ac$_2$F5-DNB2 probe. The normalized fluorescence intensity, F_t / F_0, was plotted against time, where F_0 and F_t represent initial and observed fluorescence intensities during time-lapse imaging, respectively. Fluorescence decay curves are reported for a pseudo-first-order reaction according to the following equation:

$$[\text{Normalized fluorescence intensity}] = \exp(-kt)$$

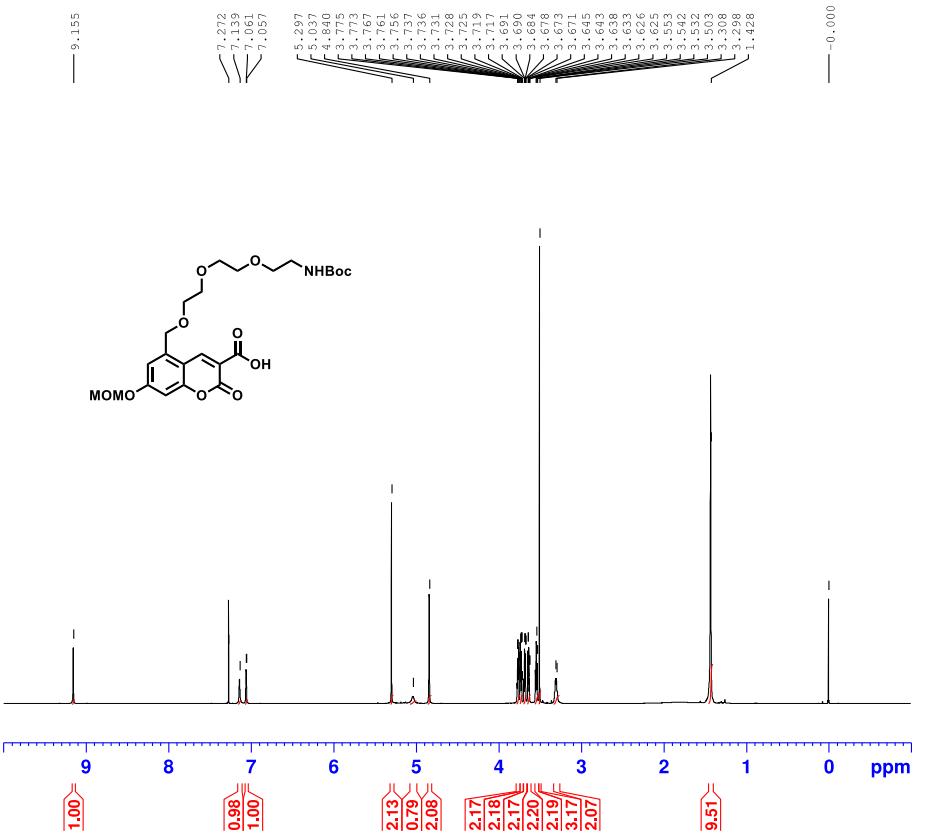
where, k represents the pseudo-first order rate constant determined from the plot, whose value enables the half-life of the protein to be determined using the equation:

$$t_{1/2} = \ln 2 / k$$

Statistical analyses of fluorescently labeled cells

Fluorescence intensities of cell nuclei were quantified using ImageJ (NIH) Software using data from three independent imaging experiments. Background mean fluorescence intensities were calculated for selected cytosolic regions that were then subtracted from mean intensities measured for fluorescent cell nuclei. Fluorescence data was plotted against time using GraphPad 8 or GraphPad 9 software, with error bars representing mean values ± interquartile range or mean values ± standard deviations. Statistical analyses were conducted using two-tailed unpaired Student`s *t*-tests to determine significant differences between the mean fluorescence intensities of each group. F5-DNB2 labeled PYP^{3R}-NLS and PYP^{NQN}-NLS cells that exhibited mean fluorescence intensities greater than 6 AU were used to carry out statistical analyses. Fluorescence data was plotted using GraphPad 8 software, with error bars representing mean values ± interquartile range. Statistical analyses were conducted using Mann-Whitney tests to determine significant differences between the brightness levels of PYP^{3R}-NLS and PYP^{NQN}-NLS cells.

6. ^{1}H and ^{13}C NMR spectra

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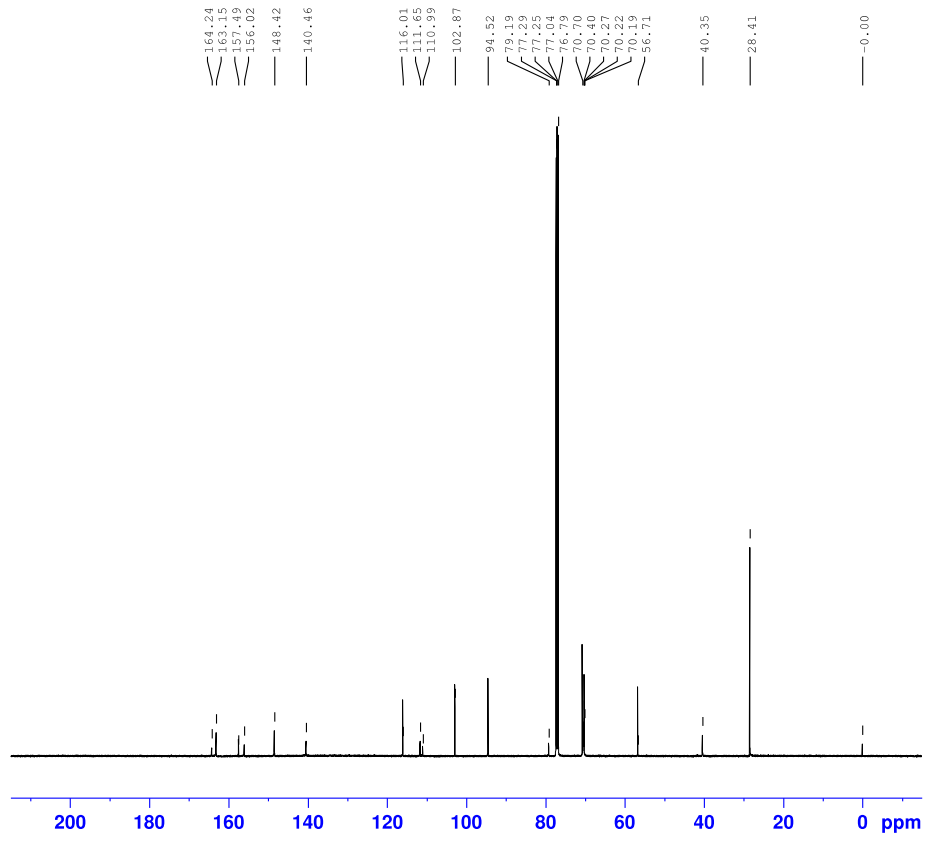
^{1}H NMR spectra of compound **4**

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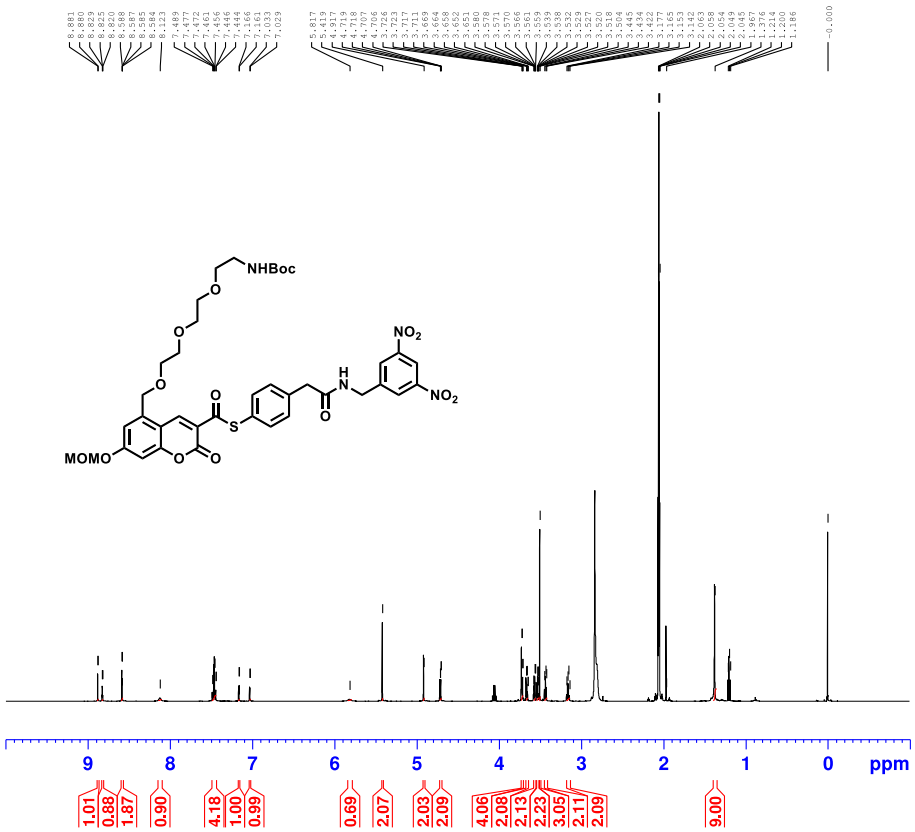
^{13}C NMR spectra of compound **4**



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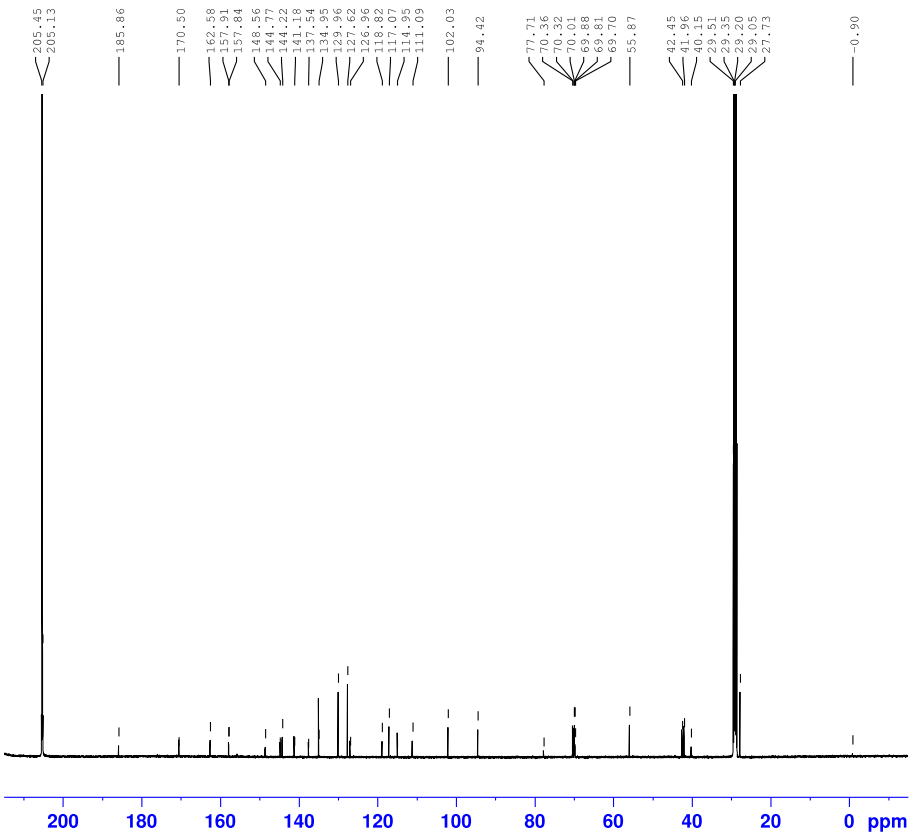
^{1}H NMR spectra of compound **6**



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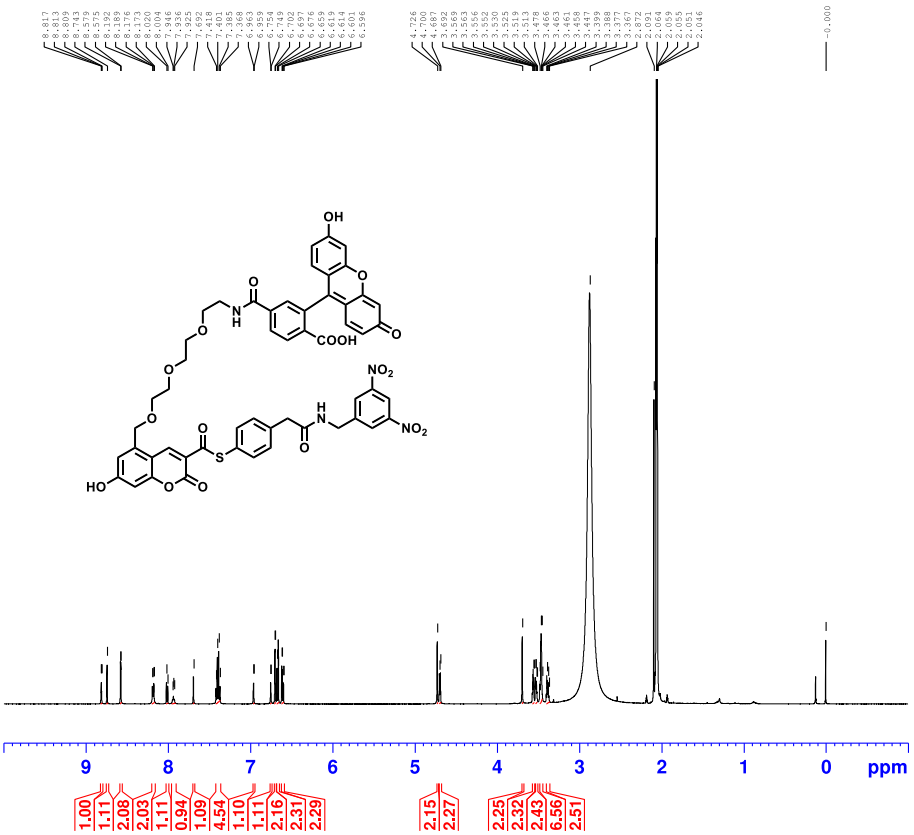
^{13}C NMR spectra of compound **6**

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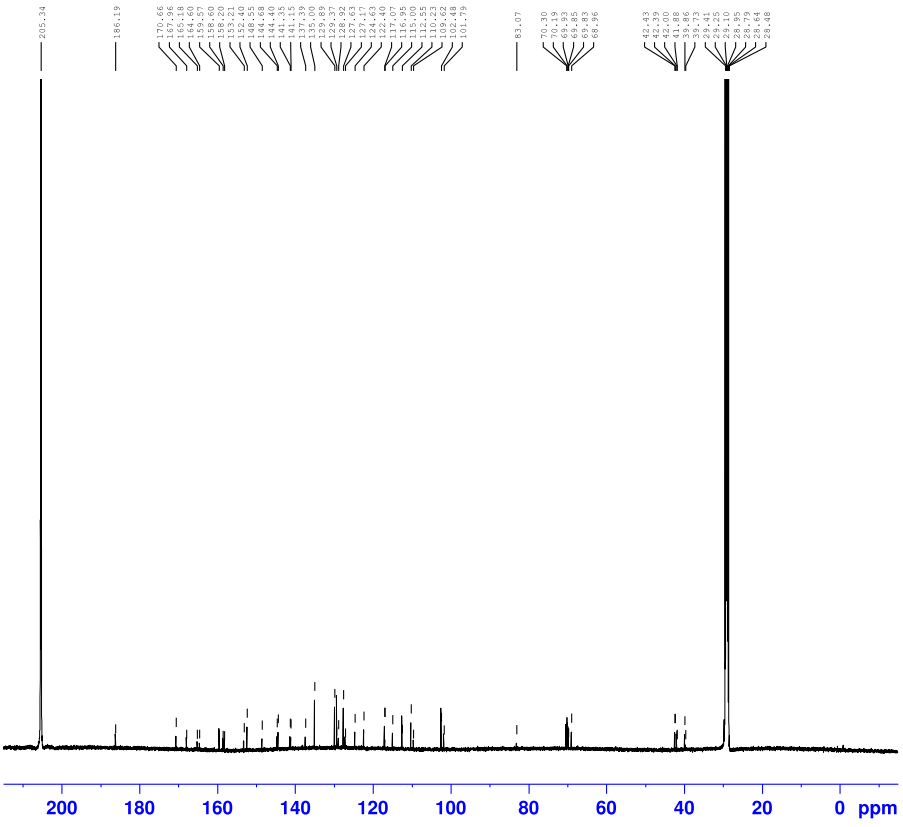
^{1}H NMR spectra of compound **7**

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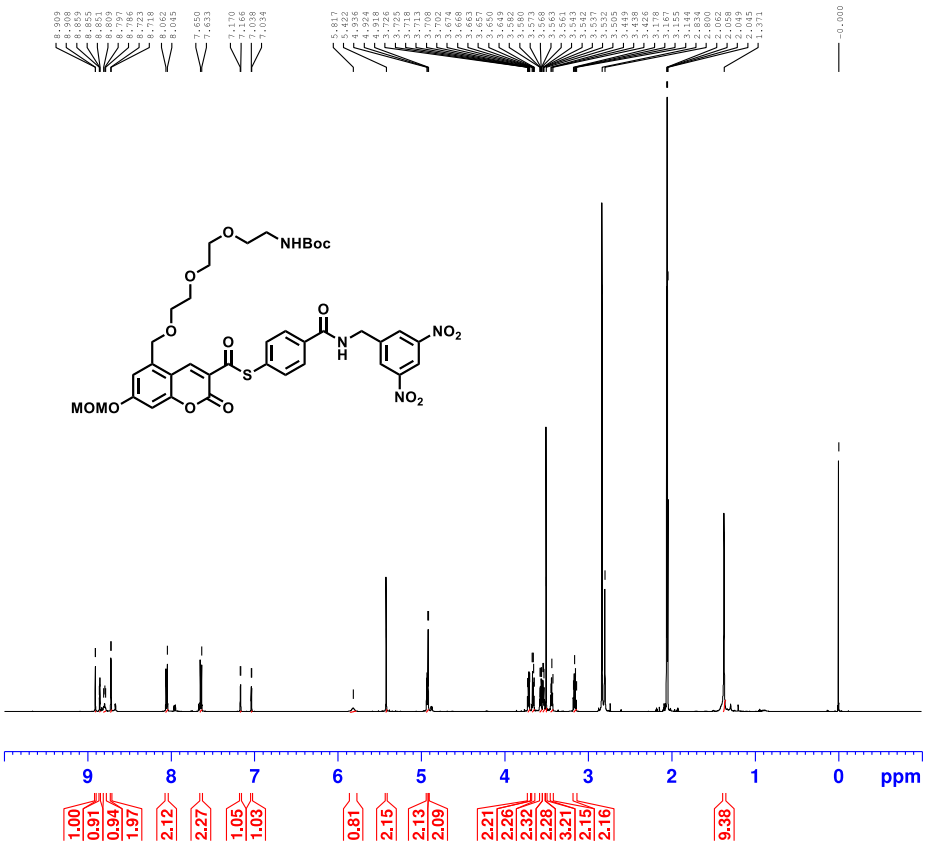
DS                    4
SWH           29761.904 Hz
FIDRES            0.454131 Hz
AQ             1.1010048 sec
RG                197.07
DW                16.800 usec
DE                18.00 usec
TE                298.2 K
D1             2.00000000 sec
D11            0.03000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1          125.7753932 MHz
NUC1                   13C
P1                 10.00 usec
PLW1          77.00000000 W

======== CHANNEL f2 ========
SFO2          500.1520006 MHz
NUC2                   1H
CPDPRG[2          waltz16
PCPD2              80.00 usec
PLW2          12.30000019 W
PLW12          0.43241999 W
PLW13          0.27675000 W

F2 - Processing parameters
SI                 32768
SF            125.7628175 MHz
WDW                   EM
SSB       0
LB                1.00 Hz
GB        0
PC                1.40
```

^{13}C NMR spectra of compound **7**



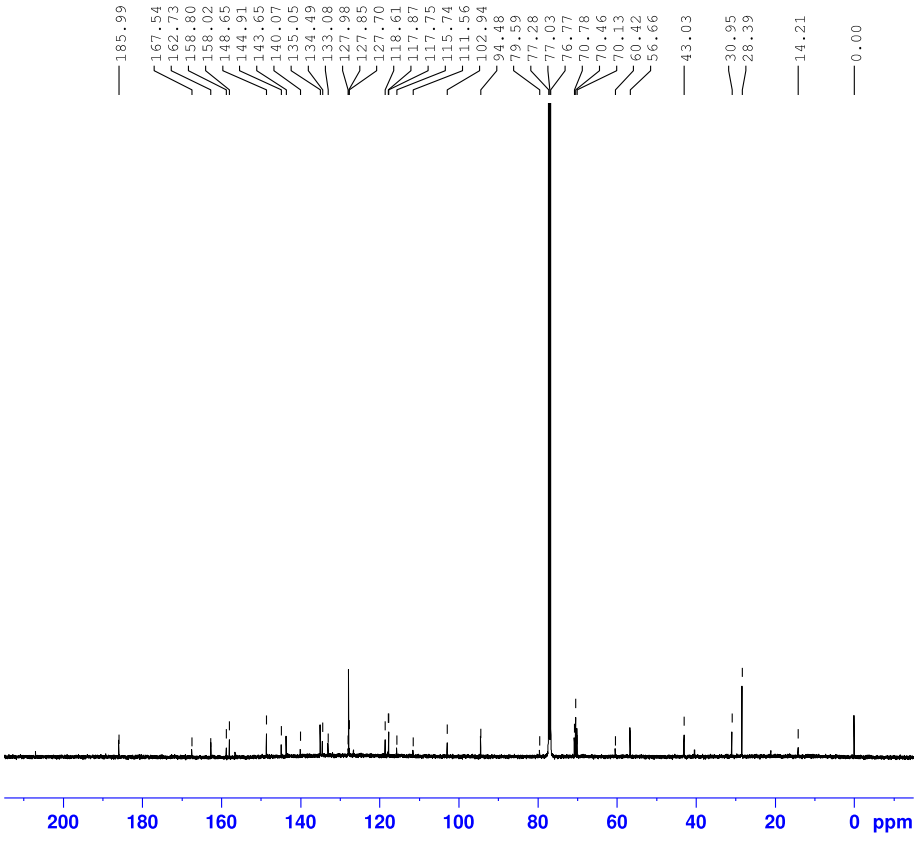

```
Current Data Parameters
NAME       161020 comp18
EXPNO               1
PROCNO              1

F2 - Acquisition Parameters
Date_           20161020
Time              14.47
INSTRUM           spect
PROBHD   5 mm CPPBBO BB
PULPROG           zg30
TD               65536
SOLVENT        Acetone
NS                  16
DS                   2
SWH           10000.000 Hz
FIDRES        0.152588 Hz
AQ            3.2767999 sec
RG                69.94
DW               50.000 usec
DE               10.00 usec
TE               298.1 K
D1           1.00000000 sec
TD0                 1

======== CHANNEL f1 ========
SFO1        500.1530886 MHz
NUC1               1H
P1              15.00 usec
PLW1        12.30000019 W

F2 - Processing parameters
SI                65536
SF          500.1500066 MHz
WDW                EM
SSB       0
LB              0.30 Hz
GB        0
PC              1.00
```

^{1}H NMR spectra of compound **9**




```
Current Data Parameters
NAME      161014 comp18 13C
EXPNO               1
PROCNO              1

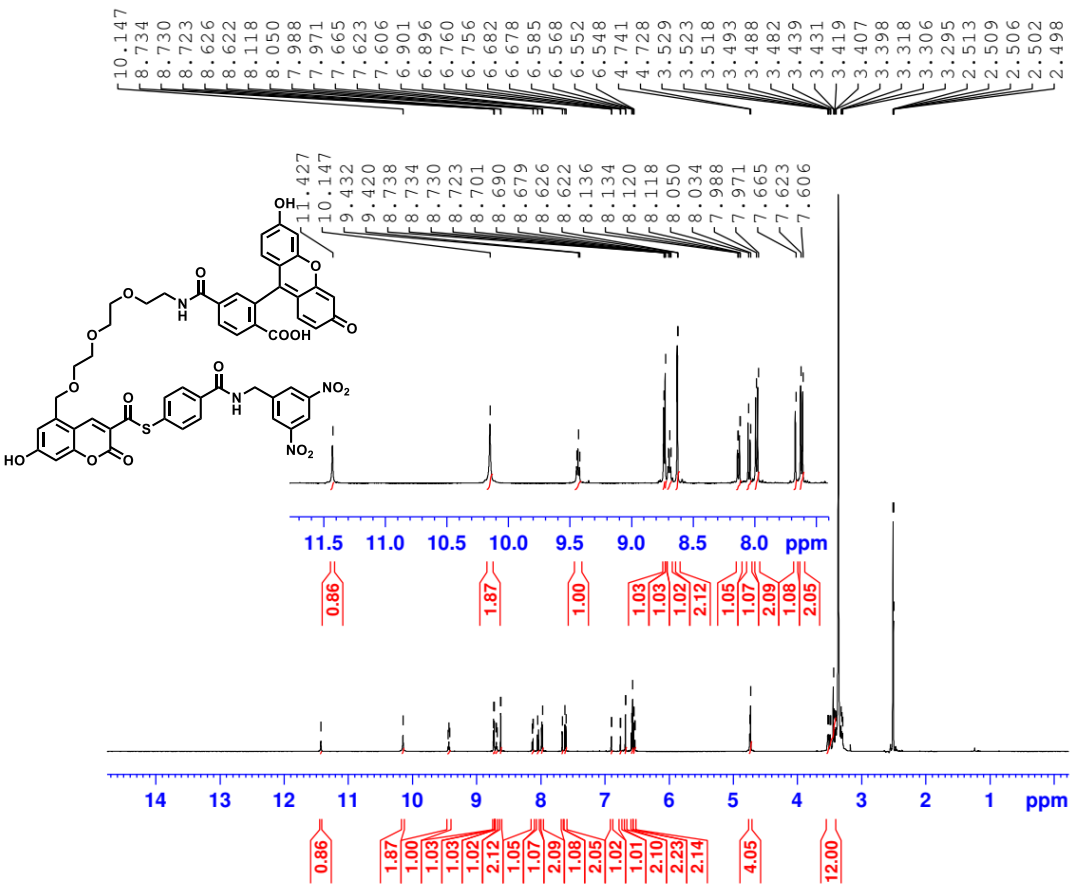
F2 - Acquisition Parameters
Date_           20161014
Time              16.46
INSTRUM           spect
PROBHD   5 mm CPPBBO BB
PULPROG          zgpg30
TD               65536
SOLVENT          CDCl3
NS                1024
DS                   4
SWH           29761.904 Hz
FIDRES        0.454131 Hz
AQ            1.1010048 sec
RG                197.07
DW               16.800 usec
DE               18.00 usec
TE               298.2 K
D1           2.00000000 sec
D11          0.03000000 sec
TD0                 1

======== CHANNEL f1 ========
SFO1        125.7753932 MHz
NUC1               13C
P1              10.00 usec
PLW1        77.00000000 W

======== CHANNEL f2 ========
SFO2        500.1520006 MHz
NUC2               1H
CPDPRG[2         waltz16
PCPD2           80.00 usec
PLW2        12.30000019 W
PLW12        0.43241999 W
PLW13        0.27675000 W

F2 - Processing parameters
SI                32768
SF          125.7628173 MHz
WDW                EM
SSB       0
LB              1.00 Hz
GB        0
PC              1.40
```

^{13}C NMR spectra of compound **9**



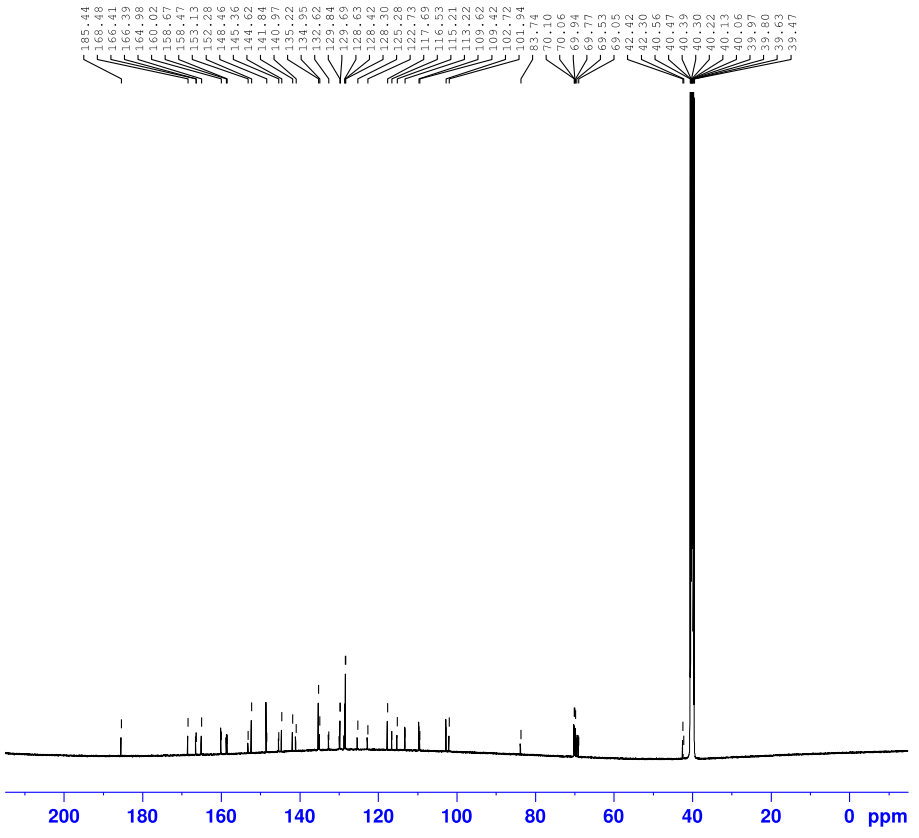
```
Current Data Parameters
NAME        180301 comp19
EXPNO                1
PROCNO               1

F2 - Acquisition Parameters
Date_          20180301
Time             13.09
INSTRUM          spect
PROBHD   5 mm CPPBBO BB
PULPROG          zg30
TD              65536
SOLVENT          DMSO
NS                 16
DS                  2
SWH         10000.000 Hz
FIDRES          0.152588 Hz
AQ           3.2767999 sec
RG                55.95
DW              50.000 usec
DE              10.00 usec
TE              298.2 K
D1           1.00000000 sec
TD0                 1

======== CHANNEL f1 ========
SFO1       500.1530886 MHz
NUC1              1H
P1              15.00 usec
PLW1       12.30000019 W

F2 - Processing parameters
SI              65536
SF         500.1500000 MHz
WDW                EM
SSB       0
LB              0.30 Hz
GB        0
PC              1.00
```

^{1}H NMR spectra of compound **10**



```
Current Data Parameters
NAME      180301 comp19 13C
EXPNO                1
PROCNO               1

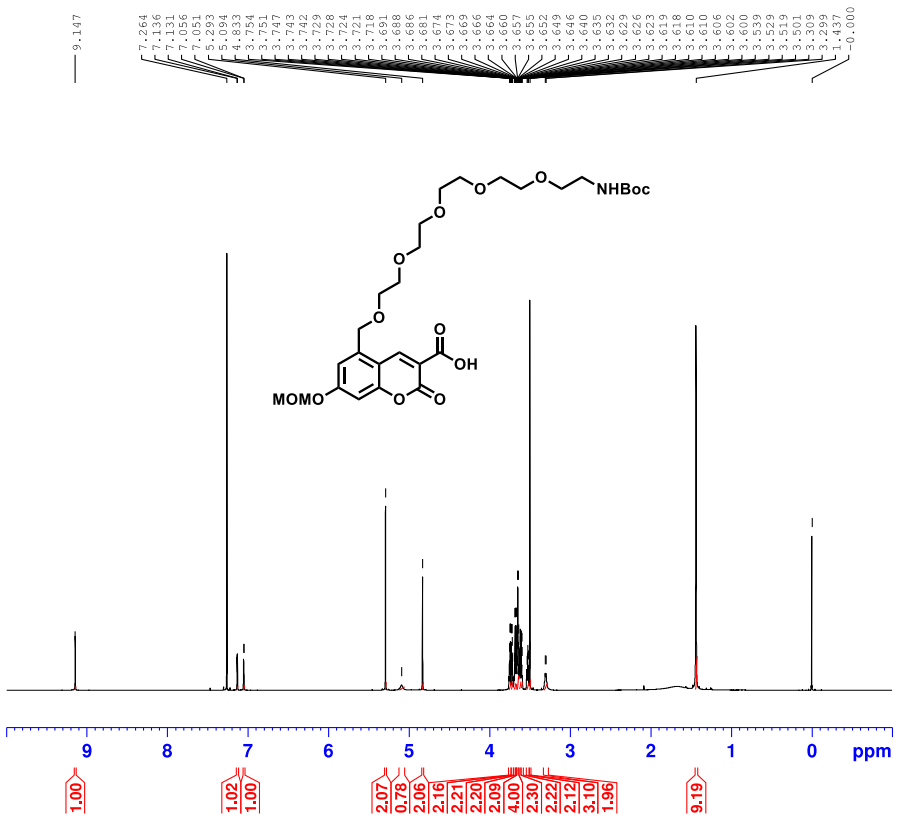
F2 - Acquisition Parameters
Date_          20180301
Time              8.14
INSTRUM          spect
PROBHD   5 mm CPPBBO BB
PULPROG         zgpg30
TD              65536
SOLVENT          DMSO
NS               8192
DS                  4
SWH         29761.904 Hz
FIDRES          0.454131 Hz
AQ           1.1010048 sec
RG              197.07
DW              16.800 usec
DE              18.00 usec
TE              298.1 K
D1           2.00000000 sec
D11          0.03000000 sec
TD0                 1

======== CHANNEL f1 ========
SFO1       125.7753932 MHz
NUC1              13C
P1              10.00 usec
PLW1        77.00000000 W

======== CHANNEL f2 ========
SFO2       500.1520006 MHz
NUC2              1H
CPDPRG[2        waltz16
PCPD2           80.00 usec
PLW2        12.30000019 W
PLW12        0.43241999 W
PLW13        0.27675000 W

F2 - Processing parameters
SI              32768
SF         125.7628175 MHz
WDW                EM
SSB       0
LB              1.00 Hz
GB        0
PC              1.40
```

^{13}C NMR spectra of compound **10**



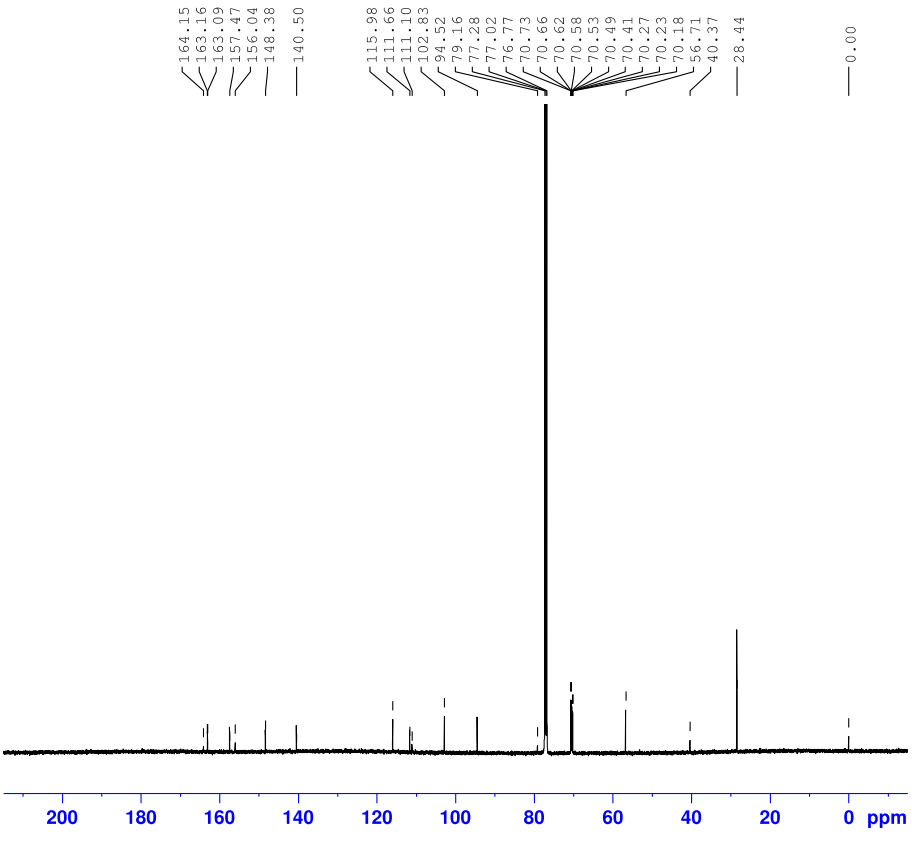

```
Current Data Parameters
NAME       161119 comp23
EXPNO                 1
PROCNO                1

F2 - Acquisition Parameters
Date_           20161119
Time              14.28
INSTRUM           spect
PROBHD   5 mm CPPBBO BB
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH           10000.000 Hz
FIDRES        0.152588 Hz
AQ            3.2767999 sec
RG                 62.19
DW                50.000 usec
DE                10.00 usec
TE                298.1 K
D1            1.00000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1         500.1530886 MHz
NUC1                   1H
P1                 15.00 usec
PLW1         12.30000019 W

F2 - Processing parameters
SI                 65536
SF            500.1500100 MHz
WDW                   EM
SSB       0
LB                 0.30 Hz
GB        0
PC                 1.00
```

^{1}H NMR spectra of compound **12**




```
Current Data Parameters
NAME      161119 comp23 13C
EXPNO                   1
PROCNO                  1

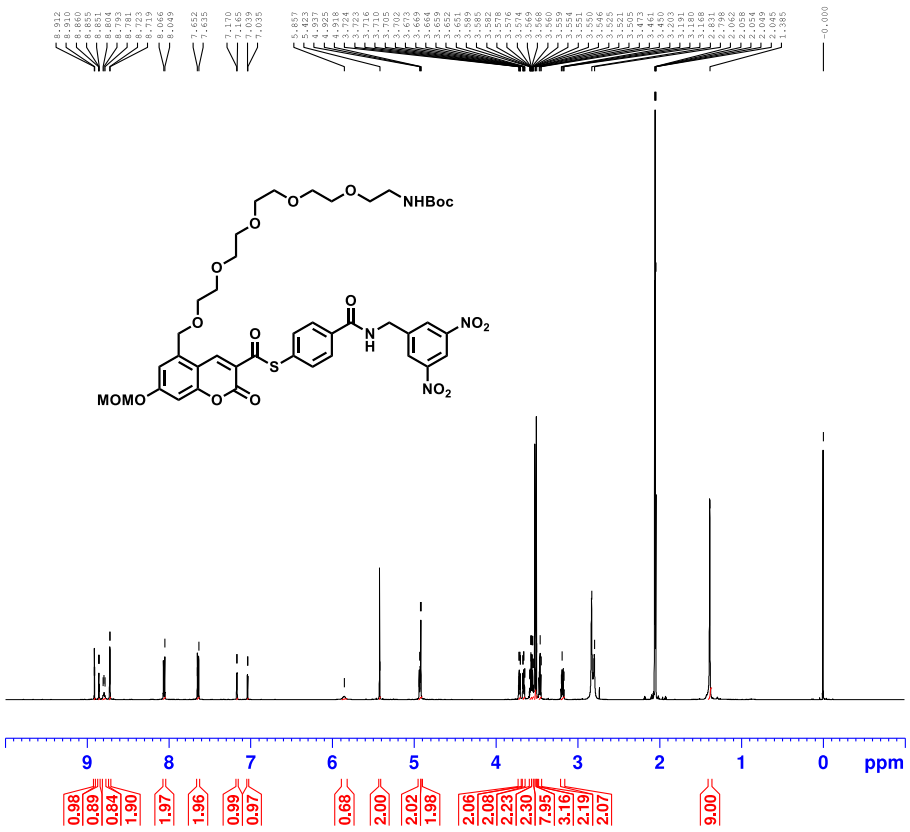
F2 - Acquisition Parameters
Date_            20161119
Time                17.52
INSTRUM             spect
PROBHD    5 mm CPPBBO BB
PULPROG            zgpg30
TD                  65536
SOLVENT             CDCl3
NS                   1024
DS                      4
SWH            29761.904 Hz
FIDRES          0.454131 Hz
AQ              1.1010048 sec
RG                197.07
DW                16.800 usec
DE                18.00 usec
TE                298.2 K
D1             2.00000000 sec
D11            0.03000000 sec
TD0                     1

======== CHANNEL f1 ========
SFO1          125.7753932 MHz
NUC1                    13C
P1                 10.00 usec
PLW1          77.00000000 W

======== CHANNEL f2 ========
SFO2          500.1520006 MHz
NUC2                    1H
CPDPRG[2           waltz16
PCPD2              80.00 usec
PLW2          12.30000019 W
PLW12          0.43241999 W
PLW13          0.27675000 W

F2 - Processing parameters
SI                  32768
SF            125.7628175 MHz
WDW                    EM
SSB       0
LB                 1.00 Hz
GB        0
PC                 1.40
```

^{13}C NMR spectra of compound **12**



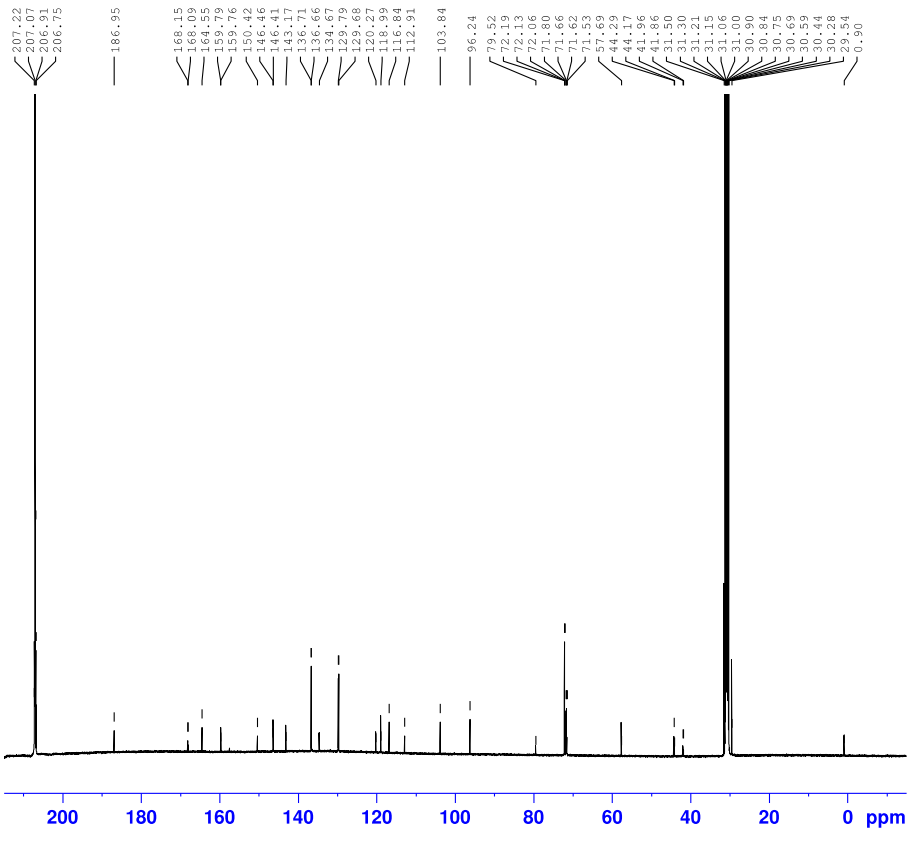
```
Current Data Parameters
NAME       161124 comp24
EXPNO                 1
PROCNO                1

F2 - Acquisition Parameters
Date_           20161124
Time               17.51
INSTRUM             spect
PROBHD   5 mm CPPBBO BB
PULPROG             zg30
TD                 65536
SOLVENT          Acetone
NS                    16
DS                     2
SWH            10000.000 Hz
FIDRES             0.152588 Hz
AQ              3.2767999 sec
RG                 62.19
DW                 50.000 usec
DE                 10.00 usec
TE                 298.2 K
D1              1.00000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1          500.1530886 MHz
NUC1                  1H
P1                 15.00 usec
PLW1          12.30000019 W

F2 - Processing parameters
SI                 65536
SF            500.1500067 MHz
WDW                   EM
SSB       0
LB                 0.30 Hz
GB        0
PC                 1.00
```

^{1}H NMR spectra of compound **13**



```
Current Data Parameters
NAME     161207 comp24 13C
EXPNO                 1
PROCNO                1

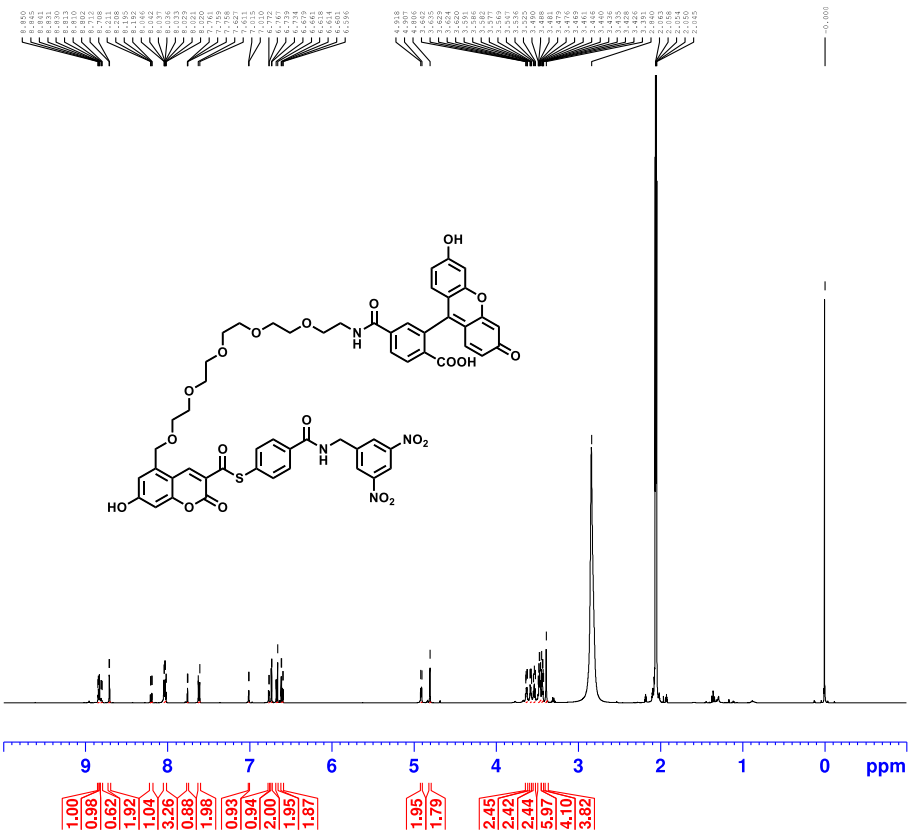
F2 - Acquisition Parameters
Date_           20161208
Time               7.14
INSTRUM             spect
PROBHD   5 mm CPPBBO BB
PULPROG          zgpg30
TD                 65536
SOLVENT          Acetone
NS                  8192
DS                     4
SWH            29761.904 Hz
FIDRES             0.454131 Hz
AQ              1.1010048 sec
RG                197.07
DW                 16.800 usec
DE                 18.00 usec
TE                 298.2 K
D1              2.00000000 sec
D11             0.03000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1          125.7753932 MHz
NUC1                 13C
P1                 10.00 usec
PLW1          77.00000000 W

======== CHANNEL f2 ========
SFO2          500.1520006 MHz
NUC2                  1H
CPDPRG[2          waltz16
PCPD2              80.00 usec
PLW2          12.30000019 W
PLW12          0.43241999 W
PLW13          0.27675000 W

F2 - Processing parameters
SI                 32768
SF            125.7625907 MHz
WDW                   EM
SSB       0
LB                 1.00 Hz
GB        0
PC                 1.40
```

^{13}C NMR spectra of compound **13**



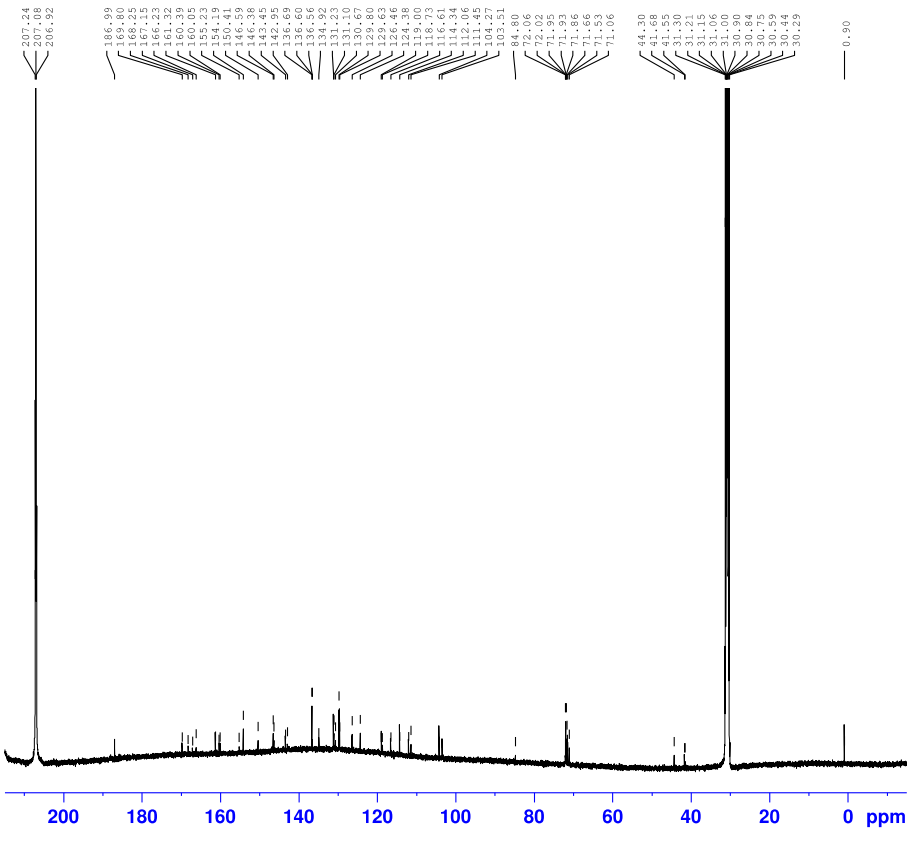

```
Current Data Parameters
NAME       161213 comp25
EXPNO               1
PROCNO              1

F2 - Acquisition Parameters
Date_           20161213
Time              18.53
INSTRUM           spect
PROBHD   5 mm CPPBBO BB
PULPROG           zg30
TD               65536
SOLVENT         Acetone
NS                512
DS                  2
SWH         10000.000 Hz
FIDRES          0.152588 Hz
AQ           3.2767999 sec
RG                69.94
DW              50.000 usec
DE              10.00 usec
TE              298.1 K
D1           1.00000000 sec
TD0                 1

======== CHANNEL f1 ========
SFO1        500.1530886 MHz
NUC1               1H
P1              15.00 usec
PLW1        12.30000019 W

F2 - Processing parameters
SI                65536
SF          500.1500065 MHz
WDW                EM
SSB       0
LB              0.30 Hz
GB        0
PC              1.00
```

^{1}H NMR spectra of compound **14**




```
Current Data Parameters
NAME      161216 comp25 13C
EXPNO                 1
PROCNO                1

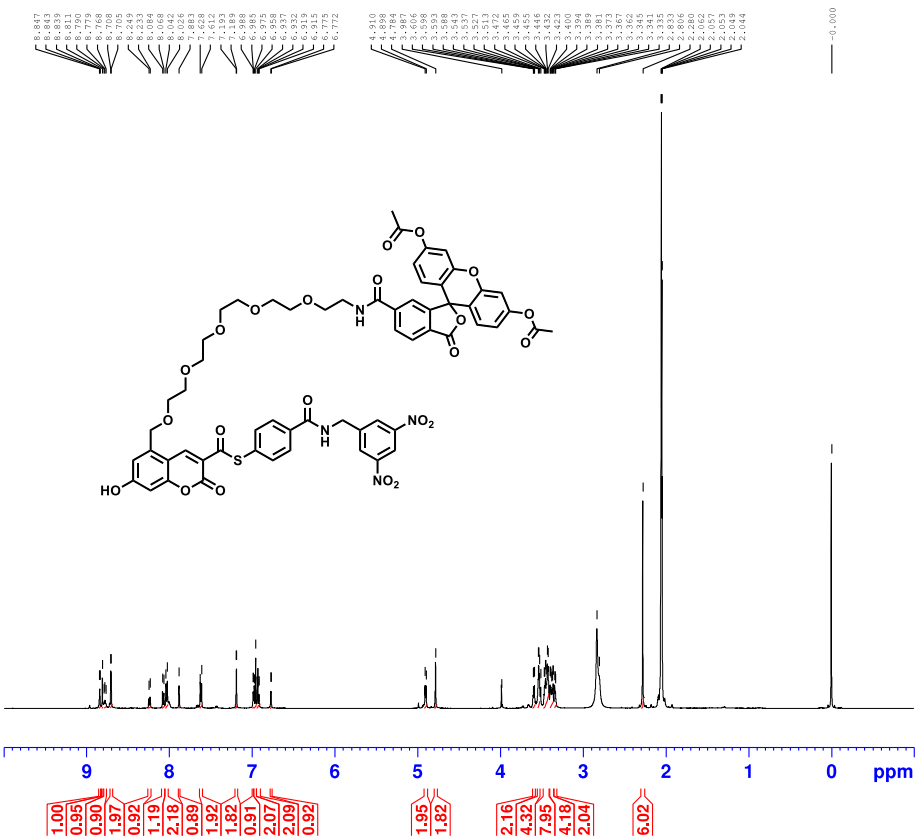
F2 - Acquisition Parameters
Date_           20161217
Time               7.14
INSTRUM           spect
PROBHD   5 mm CPPBBO BB
PULPROG          zgpg30
TD                65536
SOLVENT         Acetone
NS                 8192
DS                    4
SWH          29761.904 Hz
FIDRES         0.454131 Hz
AQ           1.1010048 sec
RG                197.07
DW              16.800 usec
DE              18.00 usec
TE              298.2 K
D1           2.00000000 sec
D11          0.03000000 sec
TD0                   1

======== CHANNEL f1 ========
SFO1        125.7753932 MHz
NUC1               13C
P1              10.00 usec
PLW1        77.00000000 W

======== CHANNEL f2 ========
SFO2        500.1520006 MHz
NUC2               1H
CPDPRG[2         waltz16
PCPD2           80.00 usec
PLW2        12.30000019 W
PLW12        0.43241999 W
PLW13        0.27675000 W

F2 - Processing parameters
SI                32768
SF          125.7625906 MHz
WDW                EM
SSB       0
LB              1.00 Hz
GB        0
PC              1.40
```

^{13}C NMR spectra of compound **14**



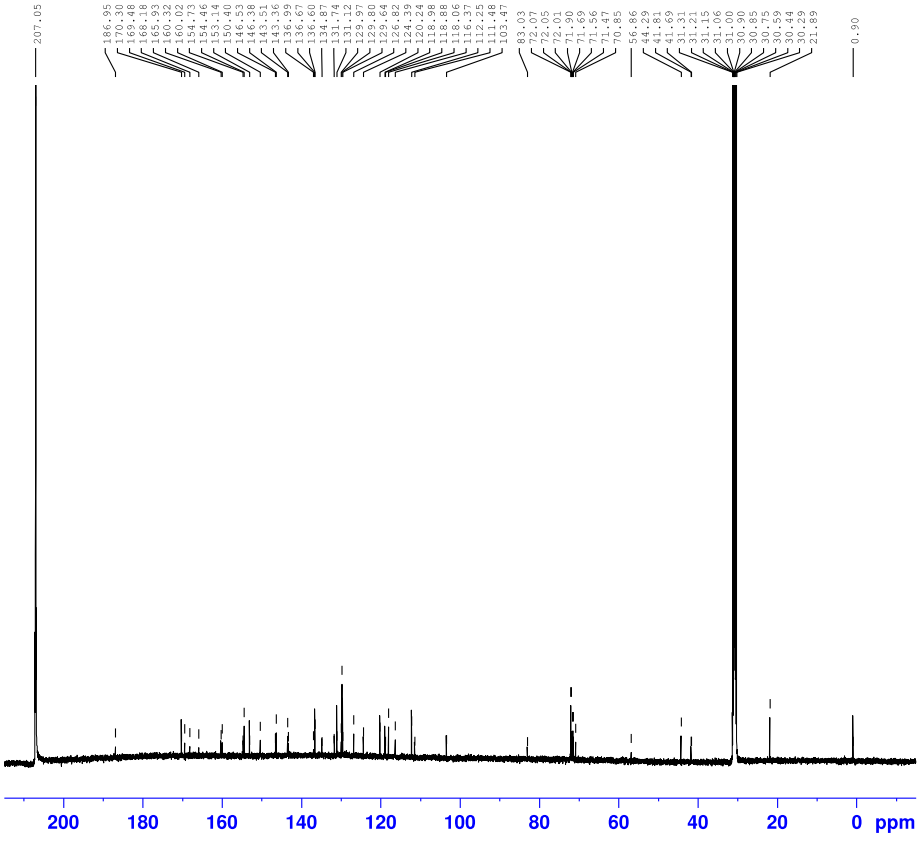
```
Current Data Parameters
NAME       170325 comp26
EXPNO                 1
PROCNO                1

F2 - Acquisition Parameters
Date_           20170325
Time               14.49
INSTRUM            spect
PROBHD   5 mm CPPBBO BB
PULPROG             zg30
TD                 65536
SOLVENT          Acetone
NS                    16
DS                     2
SWH            10000.000 Hz
FIDRES          0.152588 Hz
AQ             3.2767999 sec
RG                 55.95
DW                50.000 usec
DE                10.00 usec
TE                298.2 K
D1            1.00000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1          500.1530886 MHz
NUC1                    1H
P1                  15.00 usec
PLW1         12.30000019 W

F2 - Processing parameters
SI                 65536
SF            500.1500069 MHz
WDW                   EM
SSB       0
LB                 0.30 Hz
GB        0
PC                 1.00
```

^{1}H NMR spectra of compound **15**



```
Current Data Parameters
NAME      170325 comp26 13C
EXPNO                   2
PROCNO                  1

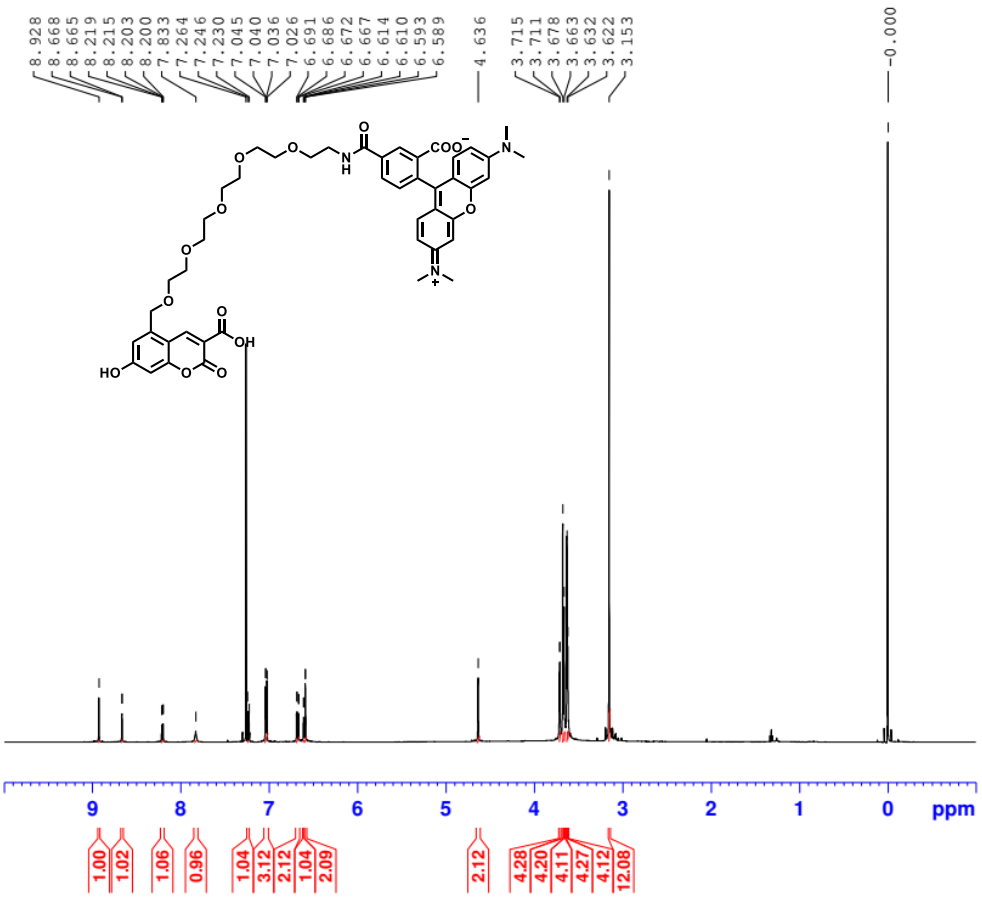
F2 - Acquisition Parameters
Date_           20170325
Time               16.34
INSTRUM            spect
PROBHD   5 mm CPPBBO BB
PULPROG          zgpg30
TD                 65536
SOLVENT          Acetone
NS                  1024
DS                     4
SWH            29761.904 Hz
FIDRES          0.454131 Hz
AQ             1.1010048 sec
RG                197.07
DW                16.800 usec
DE                18.00 usec
TE                298.2 K
D1            2.00000000 sec
D11           0.03000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1          125.7753932 MHz
NUC1                   13C
P1                  10.00 usec
PLW1         77.00000000 W

======== CHANNEL f2 ========
SFO2          500.1520006 MHz
NUC2                    1H
CPDPRG[2          waltz16
PCPD2              80.00 usec
PLW2         12.30000019 W
PLW12         0.43241999 W
PLW13         0.27675000 W

F2 - Processing parameters
SI                 32768
SF            125.7625909 MHz
WDW                   EM
SSB       0
LB                 1.00 Hz
GB        0
PC                 1.40
```

^{13}C NMR spectra of compound **15**



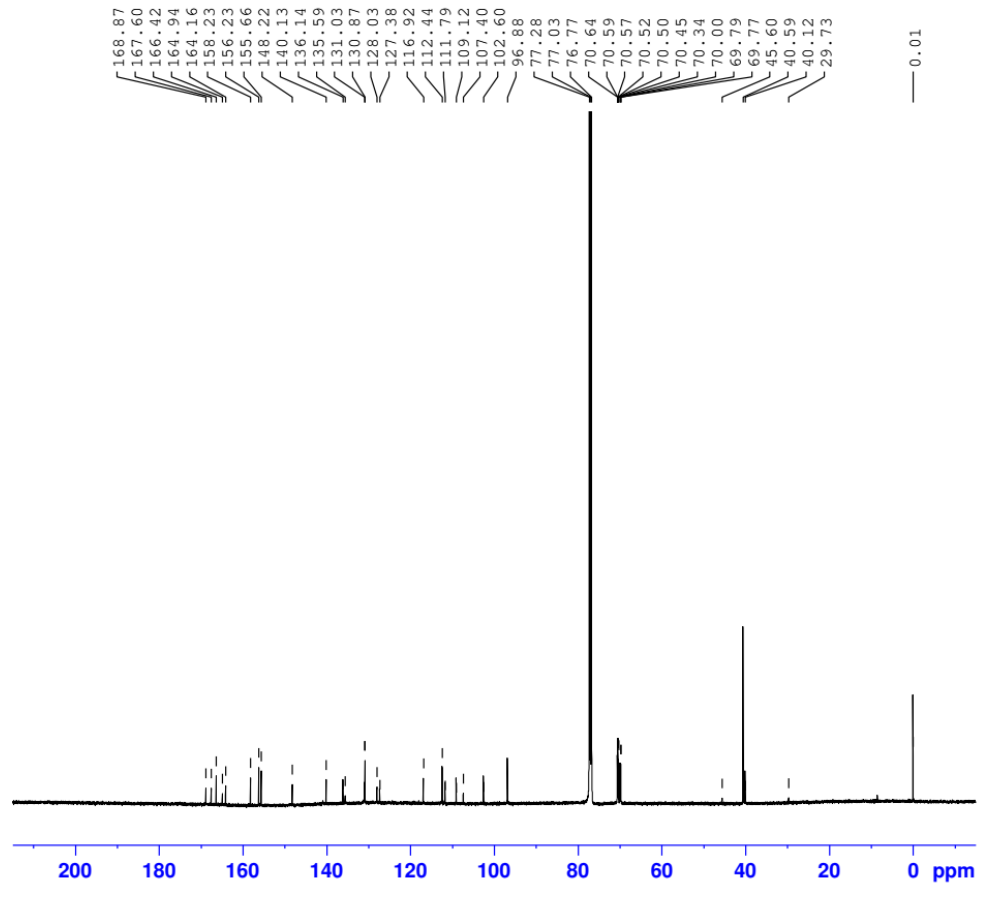
```
Current Data Parameters
NAME       171007 comp33
EXPNO               1
PROCNO              1

F2 - Acquisition Parameters
Date_           20171007
Time              14.36
INSTRUM           spect
PROBHD   5 mm CPPBBO BB
PULPROG           zg30
TD               65536
SOLVENT          CDCl3
NS                  16
DS                   2
SWH           10000.000 Hz
FIDRES            0.152588 Hz
AQ            3.2767999 sec
RG                69.94
DW               50.000 usec
DE               10.00 usec
TE                298.1 K
D1            1.00000000 sec
TD0                  1

======== CHANNEL f1 ========
SFO1         500.1530886 MHz
NUC1               1H
P1                15.00 usec
PLW1         12.30000019 W

F2 - Processing parameters
SI                65536
SF           500.1500094 MHz
WDW                EM
SSB       0
LB                0.30 Hz
GB        0
PC                1.00
```

^{1}H NMR spectra of compound **16**



```
Current Data Parameters
NAME     171010 comp33 13C
EXPNO               1
PROCNO              1

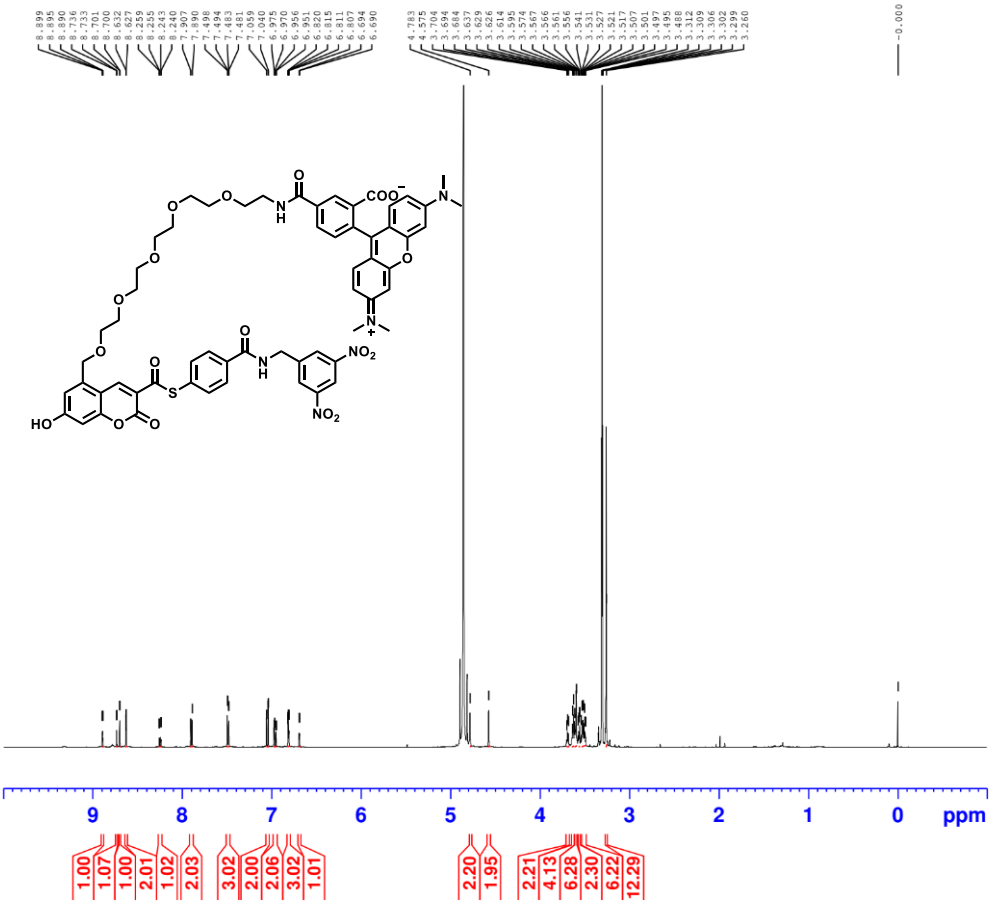
F2 - Acquisition Parameters
Date_           20171011
Time               6.58
INSTRUM           spect
PROBHD   5 mm CPPBBO BB
PULPROG          zgpg30
TD               65536
SOLVENT          CDCl3
NS                8192
DS                   4
SWH           29761.904 Hz
FIDRES            0.454131 Hz
AQ            1.1010048 sec
RG                197.07
DW               16.800 usec
DE               18.00 usec
TE                298.2 K
D1            2.00000000 sec
D11           0.03000000 sec
TD0                  1

======== CHANNEL f1 ========
SFO1         125.7753932 MHz
NUC1               13C
P1                10.00 usec
PLW1         77.00000000 W

======== CHANNEL f2 ========
SFO2         500.1520006 MHz
NUC2               1H
CPDPRG[2         waltz16
PCPD2             80.00 usec
PLW2         12.30000019 W
PLW12         0.43241999 W
PLW13         0.27675000 W

F2 - Processing parameters
SI                32768
SF           125.7628171 MHz
WDW                EM
SSB       0
LB                1.00 Hz
GB        0
PC                1.40
```

^{13}C NMR spectra of compound **16**



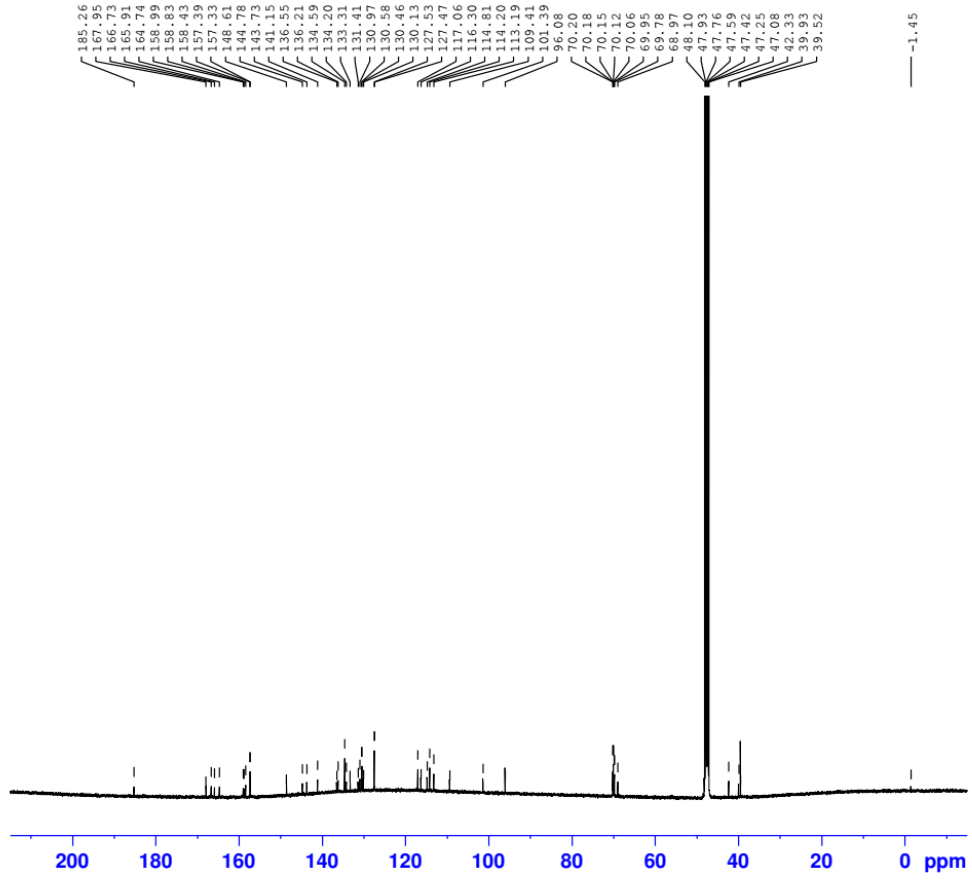

```
Current Data Parameters
NAME      171115 comp35
EXPNO                1
PROCNO               1

F2 - Acquisition Parameters
Date_          20171115
Time              10.44
INSTRUM           spect
PROBHD   5 mm CPPBBO BB
PULPROG           zg30
TD               65536
SOLVENT           MeOD
NS                  16
DS                   2
SWH           10000.000 Hz
FIDRES            0.152588 Hz
AQ             3.2767999 sec
RG                69.94
DW                50.000 usec
DE                10.00 usec
TE                298.1 K
D1             1.00000000 sec
TD0                  1

======== CHANNEL f1 ========
SFO1         500.1530886 MHz
NUC1               1H
P1                15.00 usec
PLW1         12.30000019 W

F2 - Processing parameters
SI                65536
SF           500.1500119 MHz
WDW                EM
SSB       0
LB                0.30 Hz
GB        0
PC                1.00
```

^{1}H NMR spectra of compound **17**




```
Current Data Parameters
NAME      171117 comp35 13C
EXPNO                1
PROCNO               1

F2 - Acquisition Parameters
Date_          20171117
Time               7.23
INSTRUM           spect
PROBHD   5 mm CPPBBO BB
PULPROG          zgpg30
TD               65536
SOLVENT           MeOD
NS                8192
DS                   4
SWH           29761.904 Hz
FIDRES            0.454131 Hz
AQ             1.1010048 sec
RG                197.07
DW                16.800 usec
DE                18.00 usec
TE                298.2 K
D1             2.00000000 sec
D11            0.03000000 sec
TD0                  1

======== CHANNEL f1 ========
SFO1         125.7753932 MHz
NUC1               13C
P1                10.00 usec
PLW1         77.00000000 W

======== CHANNEL f2 ========
SFO2         500.1520006 MHz
NUC2               1H
CPDPRG[2          waltz16
PCPD2             80.00 usec
PLW2         12.30000019 W
PLW12         0.43241999 W
PLW13         0.27675000 W

F2 - Processing parameters
SI                32768
SF           125.7628175 MHz
WDW                EM
SSB       0
LB                1.00 Hz
GB        0
PC                1.40
```

^{13}C NMR spectra of compound **17**

7. References

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