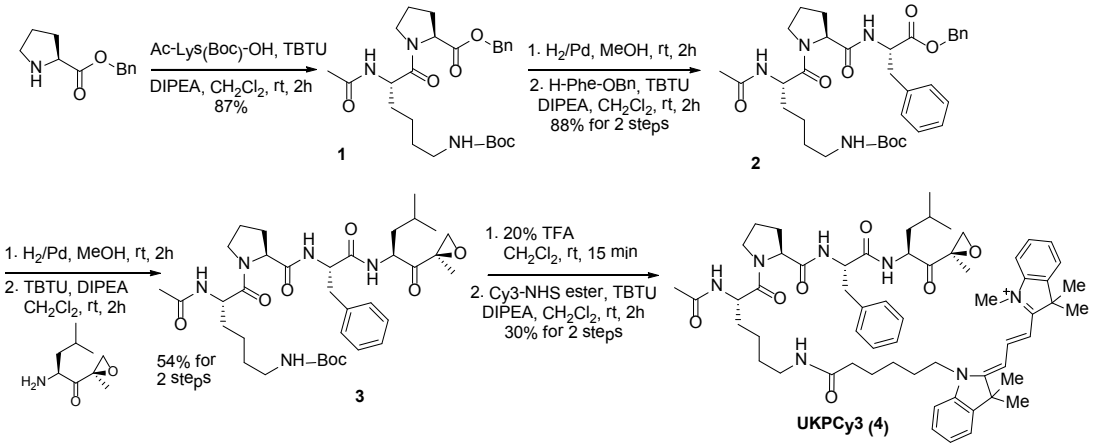
1. Synthesis of fluorescent probes

General Methods: All reactions were carried out in oven- or flame-dried glassware under an atmosphere of dry nitrogen, unless otherwise noted. Except as otherwise indicated, all reactions were magnetically stirred and monitored by analytical thin-layer chromatography using. Visualization was accomplished by fluorescence under ultra-violet light (254 nm) and by staining with a phosphomolybdic acid (PMA) solution. Column or flash chromatography (silica) was performed with the indicated solvents using silica gel (particle size 0.032-0.063 mm) purchased from Dynamic Adsorbents or MP Biomedicals. Chemical shift values (δ) are reported in ppm relative to residual chloroform (δ 7.26 ppm for ^{1}H; δ 77.23 ppm for ^{13}C). Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad).

Synthesis of UKPCy3:



Ac-Lys(Boc)-Pro-OBn (1): Ac-Lys(Boc)-OH (364 mg, 1.0 mmol), H-Pro-OBn (205 mg, 1.0 mmol), and TBTU (353 mg, 1.1 mmol) were dissolved in DCM (10 mL), and DIPEA (387 mg, 3.0 mmol) was added. The reaction mixture was stirred at room temperature for 2 hr. The resulting mixture was then concentrated under reduced pressure, and was subjected to flash column chromatography (Hexane/EtOAc 1/1) to yield compound **1** (400 mg, 87%); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.280-7.279 (m, 6H), 6.520 (s, 1H), 5.196-4.833 (m, 2H), 4,747-4.644 (m, 1H), 4.620-4.562 (m, 1H), 3.773-3.556 (m, 2H), 3.177-3.085 (m, 2H), 2.224-2.050 (m, 2H), 1.970-1.759 (m, 7H), 1.639-1.612 (m, 2H), 1.430 (m, 9H).

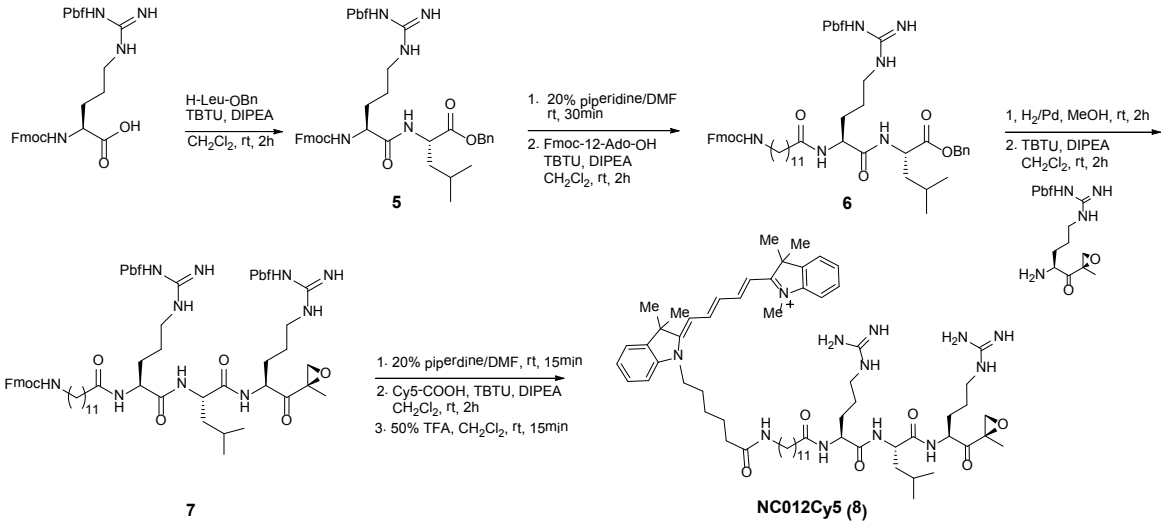
Ac-Lys(Boc)-Pro-Phe-OBn (2): Compound **1** (300 mg, 0.65 mmol) and a catalytic amount of Pd/C (5%) were dissolved in MeOH (5 mL). The solution was then stirred for 2 hr under a hydrogen atmosphere. The mixture was then filtered through celite and eluted with MeOH. The filtered solution was concentrated under reduced pressure, and used for the next step without further purification. The resulting crude product (250 mg), H-Phe-OBn (171 mg, 0.67 mmol) and TBTU (236 mg, 0.73 mmol) were dissolved in DCM (10 mL), and DIPEA (283 mg, 2.19 mmol) was added. The reaction mixture was stirred at room temperature for 2 hr. The resulting mixture was then concentrated under reduced pressure and was subjected to flash column chromatography (Hexane/EtOAc 1/4) to yield compound **2** (360 mg, 88 %); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.356-7.160 (m, 9H), 7.046 (m, 2H), 6.832 (s, 1H), 6.432 (s, 1H), 5.304 (s, 2H), 4.820 (m, 1H), 4.683 (m, 1H), 4.502 (m, 1H), 3.733-3.666 (m, 3H), 3.155- 3.045 (m, 6H), 1.998-1.853 (m, 6H), 1.604-1.436 (m, 11H).

Ac-Lys(Boc)-Pro-Phe-Leu-EK(Epoxyketone) (UKP, 3): Compound **2** (200 mg, 0.33 mmol) and Pd-C (5%) were dissolved in MeOH (5 mL). The solution was then stirred for 2 hr under a hydrogen atmosphere. After the reaction, the mixture was filtered using celite. The filtered solution was concentrated under reduced pressure and used for the following step without further purification. The resulting product (50 mg, 0.10 mmol), TBTU (35 mg, 0.53 mmol) and the previously synthesized H-Leu-EK (16 mg, 0.10 mmol) were dissolved in DCM (10 mL), followed by the addition of DIPEA (38 mg, 0.30 mmol). The reaction mixture was stirred at room temperature for 2 hr. The resulting mixture was then concentrated under reduced pressure and was subjected to flash column chromatography (Hexane/EtOAc 1/4) to yield compound **3** (64 mg, 54%); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.279-7.097 (m, 6H), 6.918 (s, 1H), 6.510 (m, 2H), 4.916-4.630 (m, 2H), 4,548-4.464 (m, 1H), 4.417-4.287 (m, 1H), 3.707-3.666 (m, 2H), 3.513-3.343 (m, 2H), 3.262-3.021 (m, 4H), 2.865-2.804 (m, 1H), 2.081-1.877 (m, 7H), 1.681-1.138 (m, 18H), 1.000-0.704 (m, 6H); MS (MALDI): m/z calcd. for $C_{33}H_{52}N_4O_7$ 671.3, found 672.3 [M+H], 694.3 [M +Na], 710.3 [M+K].

Ac-Lys(Cy3)-Pro-Phe-Leu-EK (UKPCy3, 4): TFA (20 μL, 0.170 mmol) was added to a solution of compound **3** (15 mg, 0.022 mmol) in DCM (1 mL). The reaction mixture was stirred at room temperature for 15 min. The product was concentrated under reduced pressure and dried under high vacuum. The final product was used for the next step without further purification. The resulting product (10 mg, 0.017 mmol), Cy3-CO_2H (8 mg, 0.017 mmol), and TBTU (6 mg, 0.019 mmol) were dissolved in DCM (10 mL), and DIPEA (7 mg, 0.051 mmol) was added. The reaction mixture was stirred at room temperature for 2 hr. The resulting mixture was then concentrated under reduced pressure and was subjected to flash column chromatography (DCM/MeOH 10/1) to yield compound **4** (5 mg, 30%); MS (MALDI): m/z calcd. for $C_{61}H_{82}N_7O_7$ 1024.63, found 1024.6 [M+H], 1048.63 [M+Na], 1063.63 [M+K].

Synthesis of UKPCy5: UKPCy5 was synthesized by following the same procedure used for UKPCy3, except Cy3 was replaced with Cy5; MS (MALDI): m/z calcd. for $C_{63}H_{84}N_7O_7$ 1050.64, found. 1051.0.

Synthesis of NC012Cy5:



Fmoc-Arg(Pbf)-Leu-OBn (5): Fmoc-Arg(Pbf)-OH (648 mg, 1.0 mmol), H-Leu-OBn (393 mg, 1.0 mmol), and TBTU (353 mg, 1.1 mmol) were dissolved in DCM (10 mL), and DIPEA (388 mg, 3.0 mmol) was added. After stirring at room temperature for 2 hr, the reaction mixture was concentrated under reduced pressure and was subjected to flash column chromatography (Hexane/EtOAc 1/2) to yield compound **5** (1.1 g, 92%); ^{1}H NMR (500 MHz, CDCl3): δ = 7.728-7.704 (m, 2H), 7.554-7.530 (m, 2H), 7.226 (m, 9H), 7.30 (m, 7H), 6.245 (s, 1H), 5.153 (s, 1H), 5.153-4.561 (m, 2H), 4.561 (m,1H), 4.353 (m, 1H), 4.291-4.152 (m, 2H), 4.067 (m, 1H), 2.884 (s, 2H), 2.804-2.723 (m, 11H), 2.559-2.480 (m, 5H), 2.113-1.904 (m, 3H), 1.430 (s, 6H), 0.879-0.827 (m, 6H).

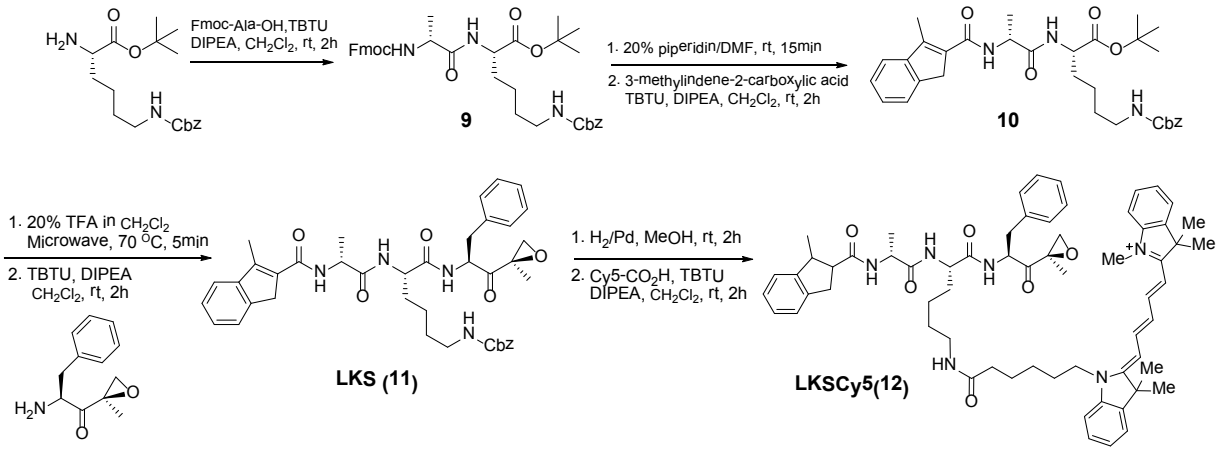
Fmoc-Ado-Arg(Pbf)-Leu-OBn (6): A solution of piperidine (20% solution in DMF, 3 mL) was added to compound **5** (620 mg, 0.73 mmol) and stirred for 30 min at room temperature. The reaction mixture was concentrated under high vacuum and used for the next reaction without further purification. The resulting crude product, Fmoc-12-Ado-OH (318 mg, 0.73 mmol), and TBTU (258 mg, 0.81 mmol) were dissolved in DCM (5 ml). DIPEA (283 mg, 2.19 mmol) was added, and the reaction mixture was stirred for 2h at room temperature. The resulting mixture was concentrated under reduced pressure. The crude product was subjected to flash column chromatography (Hexane/EtOAc 1/3) to obtain compound **6** (660 mg, 86%); ^{1}H NMR (500 MHz, CDCl3): δ = 7.708-7.664 (m, 4H), 7.589-7.375 (m, 2H), 7.347-7.080 (m, 11H), 5.092 (d, J =6.0 Hz, 1H), 4.985 (d, J =6.0 Hz,1H), 4.318-4.218 (m, 2H), 4.218 (m,1H), 4.097 (m, 1H), 3.977 (m, 1H), 2.290 (m, 2H), 1.727-1.623 (m, 6H), 1.609-1.455 (m, 13H), 1.271-1.243 (m, 8H), 0.744 (m, 6H).

Fmoc-Ado-Arg (Pbf)-Leu-Arg(Pbf)-EK (7): Compound **6** (100 mg, 0.10 mmol) and Pd-C (5%) were dissolved in MeOH (5 mL). The solution was then stirred for 2 hr under a hydrogen atmosphere. After the reaction, the mixture was filtered using celite, and the filtered solution was concentrated under reduced pressure, and used for the following step without further purification. The resulting crude product, TBTU (35 mg, 0.11 mmol) and the previously synthesized H-Arg(Pbf)-EK (47 mg, 0.10 mmol) were dissolved in DCM (10 mL), and DIPEA (38 mg, 0.30 mmol) was added. The reaction mixture was stirred at room temperature for 2 hr. The resulting mixture was concentrated under reduced pressure and was subjected to flash column chromatography (Hexane/EtOAc 1/4) to yield compound **7** (116 mg, 85%); ^{1}H NMR (400 MHz, CDCl3): δ = 7.799-7.780- (m, 2H), 7.589-7.543 (m, 2H), 7.409-7.397 (m, 2H), 6.444 (m, 3H), 4.500-4.410 (m, 3H), 4.298-4.211- (m, 1H), 4.035-3.988 (m, 1H), 3.705-3.664 (m, 1H), 3.267-2.955 (m, 12H), 2.880 (s, 12H), 2.513-2.453 (m, 6H), 2.112 (s, 6H), 1.623-1.533 (m, 6H), 1.543-1.355 (m, 21H), 1.211-1.199 (m, 17H), 0.801-0.744 (m, 6H)

NC012Cy5 (8): A solution of piperidine (20% solution in DMF, 3 mL) was added to compound **7** (30 mg, 0.02 mmol) and the reaction mixture was stirred at room temperature for 15 min. The reaction mixture was then concentrated under high vacuum and used for the following step without further purification. The resulting crude product, Cy5-CO_2H (10 mg, 0.02 mmol) and TBTU (7 mg, 0.02 mmol) were dissolved in DCM (5 mL), and DIPEA (7 mg, 0.06 mmol) was added. The reaction mixture was stirred at room temperature for 2 hr. The resulting mixture was then concentrated under reduced pressure and was subjected to flash column chromatography (DCM/MeOH 10/1) to yield a fully assembled Pbf-protected NC012Cy5. To deprotect the Pbf

groups, the Pbf-protected NC012Cy5 was dissolved in a solution of 50% TFA in DCM. After stirring for 15 min at room temperature, the reaction mixture was concentrated under high vacuum, followed by trituration with water. After filtration using a syringe filter, the resulting filtrate was lyophilized to yield NC012Cy5 (3 mg, 47%); MS (MALDI): m/z calcd. for $C_{66}H_{102}N_{11}O_6^+$ 1144.8, found 1144.

Synthesis of LKSCy5: LKS01 was synthesized by using the procedure previously reported by us.[1]



Fmoc-D-Ala-Lys(Z)-OtBu (9): Fmoc-D-Ala-OH (156 mg, 0.5 mmol), H-Lys(Z)-OtBu ·HCl (186 mg, 0.5 mmol), and TBTU (11mg, 0.5 mmol) were dissolved in DCM (10 mL), and DIPEA (18 mg, 1.5 mmol) was added. After stirring at room temperature for 2 hr, the reaction mixture was concentrated under reduced pressure and was subjected to flash column chromatography (Hexane/EtOAc 5/1) to give compound **9** (302 mg, 96%); ^{1}H NMR (500 MHz, CDCl3): δ = 7.74 (d, *J*=7.5 Hz, 2H), 7.57 (d, *J*=7.2 Hz, 2H), 7.38 (t, *J*=7.5 Hz, 2H), 7.30 (m, 7H), 6.74 (bs, 1H), 5.53 (d, *J*=7.0 Hz, 1H), 5.05 (m ,3H), 4.52 (q, *J*=7.0 Hz,1H), 4.38 (m ,2H), 4.29 (m ,1H), 4.18 (m, 1H), 3.14 (s, 2H), 2.79 (s, 2H), 1.83 (m, 1H), 1.64 (m, 1H), 1.49 (m, 2H), 1.43 (s, 9H), 1.38 (d, *J*=6.5Hz, 3H).

Ind-D-Ala-Lys(Z)-OtBu (10): A solution of piperidine (20% solution in DMF, 3 mL) was added to compound **10** (220 mg, 0.35 mmol) and stirred for 15 min at room temperature. The reaction mixture was concentrated under high vacuum and used for the next reaction without further purification. The resulting crude product, 3-methylindene-2-carboxylic acid (610 mg, 0.35 mmol), and TBTU (102 mg, 0.39 mmol) were dissolved in DCM (5 mL). DIPEA (18 mg, 1.05 mmol) was added, and the reaction mixture was stirred for 2 hr at room temperature. The reaction mixture was concentrated under reduced pressure, and the crude product was subjected to flash column chromatography (Hexane/EtOAc 5/1) to obtain compound **10** (142 mg, 95%); ^{1}H NMR (500 MHz, CDCl3): δ = 7.42 (t, *J*=7.0 Hz, 2H), 7.36-7.24 (m, 7H), 7.02 (d, *J*=8.0 Hz, 1H), 6.60 (d, *J*=7.0 Hz, 1H), 5.11 (m, 1H), 5.07 (s, 2H), 4.72 (m, 1H), 4.46 (m, 1H), 3.58 (s, 2H), 3.18 (m, 2H), 2.49 (s, 3H), 1.85 (m, 1H), 1.68 (m, 1H), 1.54 (m, 2H), 1.47 (d, *J*=7.0 Hz, 3H), 1.42 (s, 9H), 1.30 (m, 3H).

Ind-D-Ala-Lys(Z)-Phe-EK (LKS, 11) : TFA (40 µL, 0.34 mmol) was added to a solution of compound **10** (30 mg, 0.05 mmol) in DCM (1 mL) and the reaction mixture was heated at 70 °C for 5 min by using a microwave.

The reaction mixture was concentrated under reduced pressure and used without further purification in the next reaction. The resulting product, the previously synthesized H-Phe-EK (25 mg, 0.05 mmol) and TBTU (10 mg, 0.06 mmol) were dissolved in DCM (5 mL), and DIPEA (17 mg, 0.15 mmol) was added. The reaction was stirred for 2 hr at room temperature. The reaction mixture was then concentrated under reduced pressure, and the crude product was subjected to flash column chromatography (Hexane/EtOAc 2/1) to give compound **11** (23 mg, 82%); ^{1}H NMR (400 MHz, acetone-d6): δ = 7.79 (d, *J*=9.0 Hz, 1H), 7.57 (d, *J*=10.5, 1H), 7.49 (m, 2H), 7.42-7.20 (m, 13H), 6.83 (bs, 1H), 5.06 (s, 2H), 4.67 (m, 1H), 4.53 (m, 1H), 4.36 (m, 1H), 3.72 (m, 2H), 3.35 (d, *J*=6.0 Hz, 1H), 3.10 (m, 4H), 2.93 (d, *J*=6.5 Hz, 1H), 2.73 (s, 2H), 2.50 (t, *J*=3.0 Hz, 2H), 1.80 (m, 1H), 1.49 (m, 4H), 1.39 (m, 5H), 1.30 (m, 3H).

Ind-D-Ala-Lys(Cy5)-Phe-EK (LKSCy5,12) : LKS (**11**) (10 mg, 0.014 mmol) and Pd-C (5%) were dissolved in MeOH (3 mL) in a vessel capped with a rubber septum. The solution was then stirred for 2 hr under a hydrogen atmosphere. After the reaction, the mixture was filtered using celite. The filtrate was concentrated under reduced pressure, and used for the following step without further purification. To the resulting concentrate, Cy5-CO_2H (10 mg, 0.014 mmol), and TBTU (5 mg, 0.015 mmol) were added, followed by the addition of DCM (5 mL) and DIPEA (6 mg, 0.042 mmol). The reaction mixture was then stirred for 2 hr at room temperature and concentrated under reduced pressure. The resulting residue was subjected to flash column chromatography (DCM/MeOH 2/1) to give LKSCy5 (**12**) (3.8 mg, 30%); MS (MALDI): m/z calcd. for $C_{64}H_{79}N_6O_6$ 1027.61, found 1028.63 [M+H], 1050 [M+Na], 1066 [M+K].

Synthesis of LKSCy3: LKSCy3 was synthesized by following the same procedure used for LKSCy5, except Cy5 was replaced with Cy3; MS (MALDI): m/z calcd. for $C_{62}H_{77}N_6O_6$ 1001.59, found. 1002.

2. Fluorescence spectra of proteasomes treated with fluorescent probes

FRET probes (10 μM) were added to a buffer solution containing 2 μg of rabbit purified 20S constitutive proteasome (Boston Biochem). After incubation of the reaction mixture at room temperature for 1hr, excess FRET probes were removed using a Nanosep centrifugal device (300K Device, Pall Life Sciences). The recovered proteasome samples from the filter device were redissolved in 50 μL of 20S assay buffer (20 mM Tris/Cl pH 8.0, 0.5 mM EDTA, and 0.035 % SDS), and the fluorescence spectra were obtained after excitation at 540 nm using a fluorescence microplate reader (SpectraMax M5).

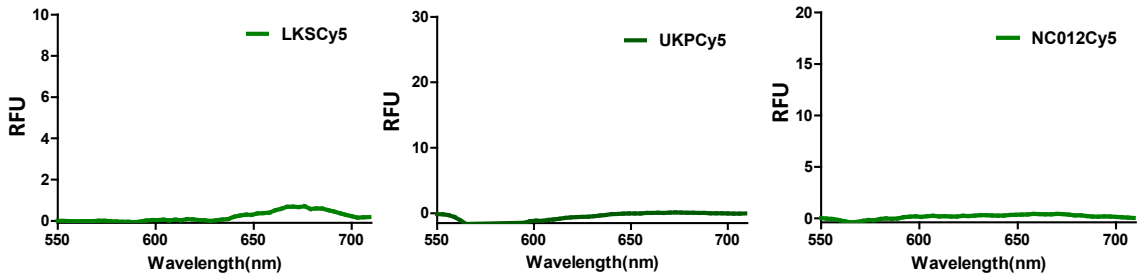


Figure 1. Emission spectra of the purified 20S proteasome treated with LKSCy5, UKPCy5 or NC012Cy5, following excitation at 540 nm.

3. Enzyme kinetics using purified proteasomes

Purified 20S human constitutive proteasome and immunoproteasome (R&D Systems) was used to assess the *in vitro* inhibition of proteasome catalytic activities by FRET probes. Briefly, reactions were setup in a 100 uL total volume in a 96-well format. 20S proteasomes at 0.5 ug/mL in assay buffer (20 mM Tris-HCl, 0.5 mM EDTA, 0.035% SDS except when using Boc-LRR-AMC) were mixed with FRET probes and substrates. The rate of substrate hydrolysis was measured by monitoring the fluorescence of liberated AMC (7-amino-4-methylcoumarin) over a period of 90 minutes once per minute at room temperature. AMC fluorescence was measured using excitation and emission wavelengths of 360 and 460 nm on a SpectraMax M5 fluorescence plate reader (Molecular Devices). The following substrates and concentrations were used: For β1 activity, 100 μM Ac-nLPnLD-AMC, for β2 and β2i activity, 200 μM Boc-LRR-AMC, β5 activity, 20 μM Ac-WLA-AMC, for β1i activity, 100 μM Ac-PAL-AMC, for β5i activity, 100 μM Ac-ANW-AMC.

4. In-gel fluorescence and immunoblotting assays

In-gel fluorescence and western blotting assays were performed by procedures similar to those previously reported by us.[1,2]

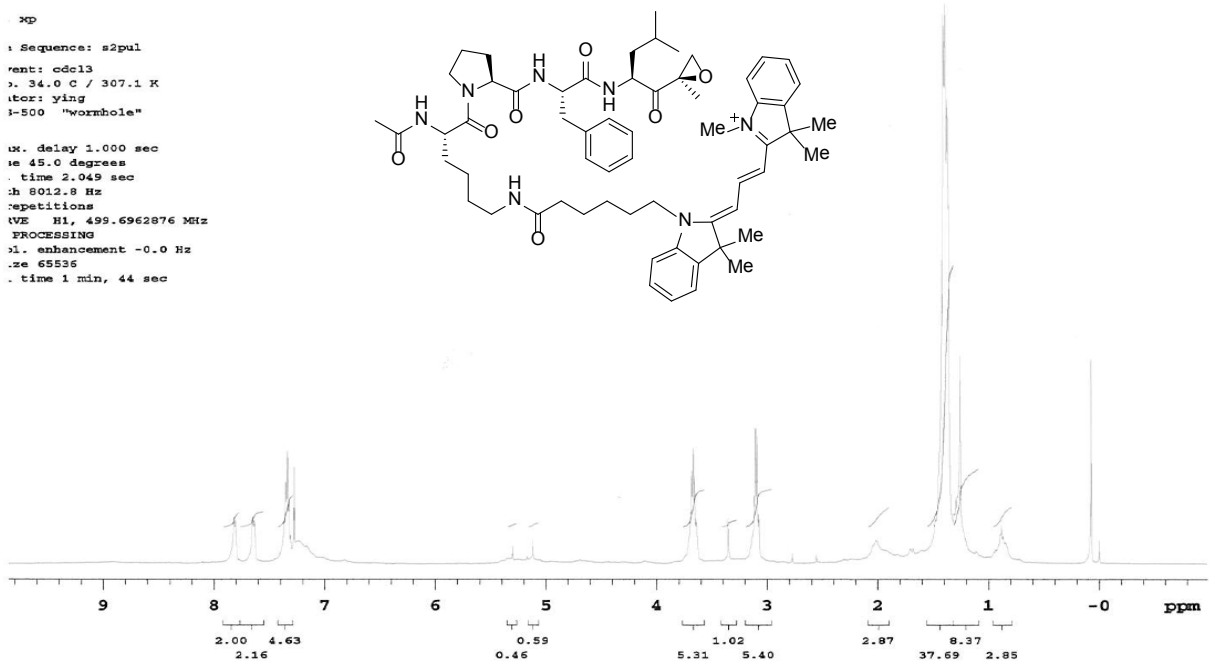
References

(1) Sharma, L. K.; Lee, N. R.; Jang, E. R.; Lei, B.; Zhan, C. G.; Lee, W.; Kim, K. B. *Chembiochem* 2012, *13*, 1899.

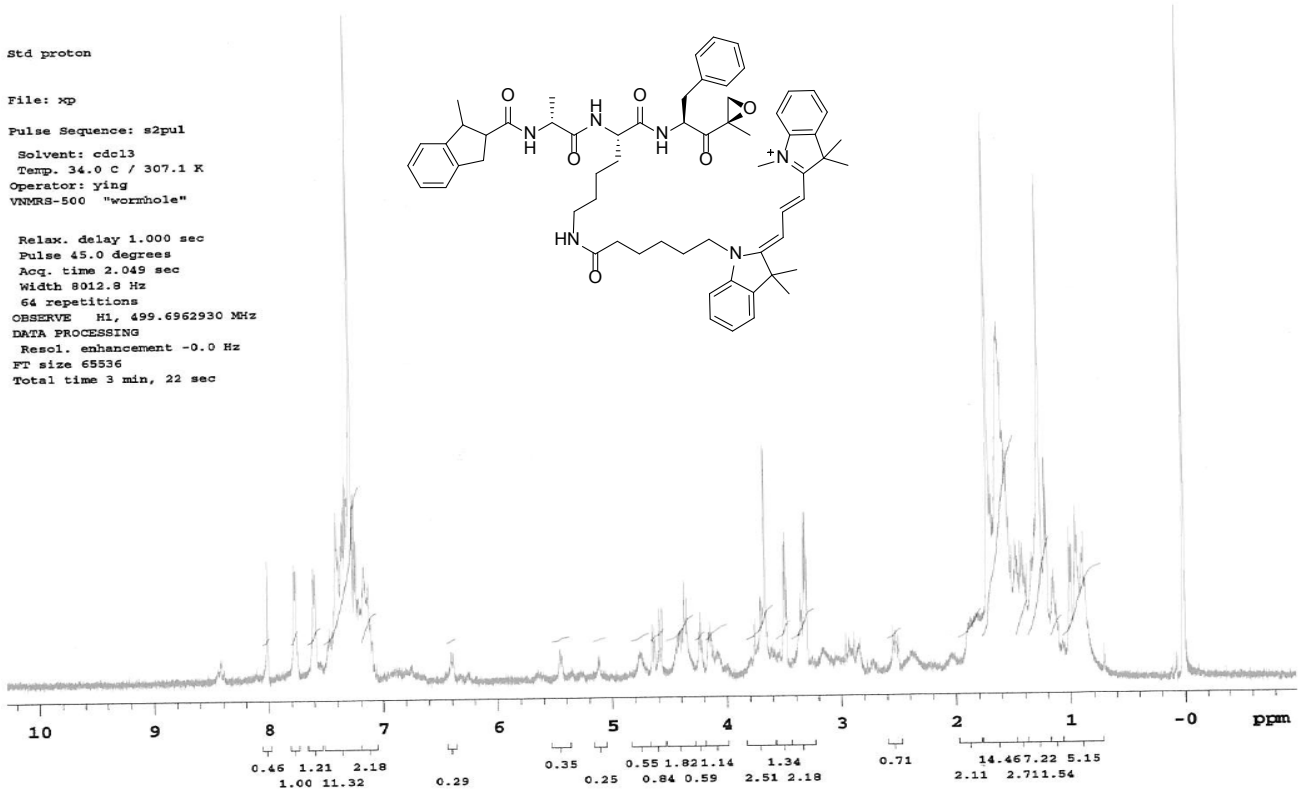
(2) Carmony, K. C.; Lee, D. M.; Wu, Y.; Lee, N. R.; Wehenkel, M.; Lee, J.; Lei, B.; Zhan, C. G.; Kim, K. B. *Bioorg Med Chem* 2012, *20*, 607.

5. NMR and Mass spectra of FRET probes

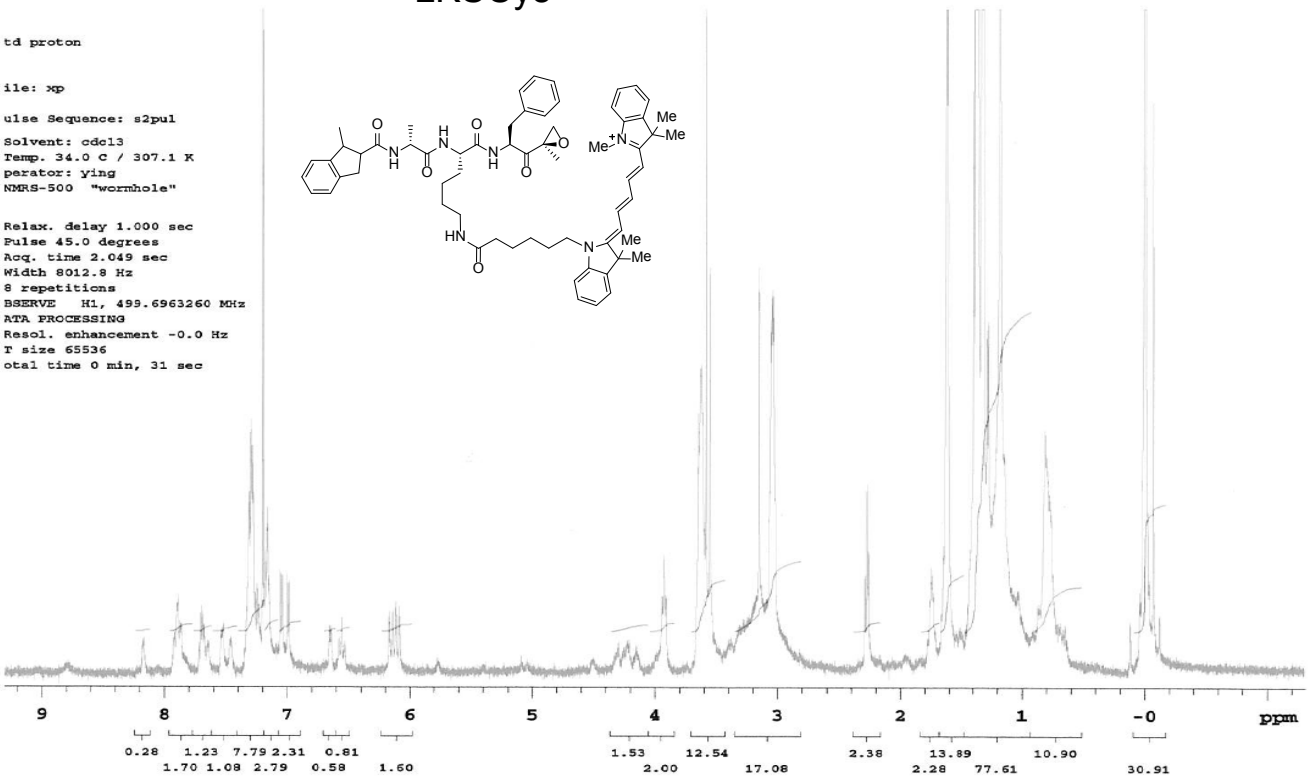
UKPCy3



LKSCy3



LKSCy5



NC012Cy5