Supporting Information

Noncovalent Fluorescent Probes of Human Immuno- and Constitutive Proteasomes.

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Figure 1S.

Crystal structure of the yeast 20S proteasome in complex within the ChT-L active site with compound **3** (green).[1] Subunits β5 and β6 are respectively in grey and beige, H-bond network in black dotted lines, and the amino acids involved in the complex formation in stick forms.

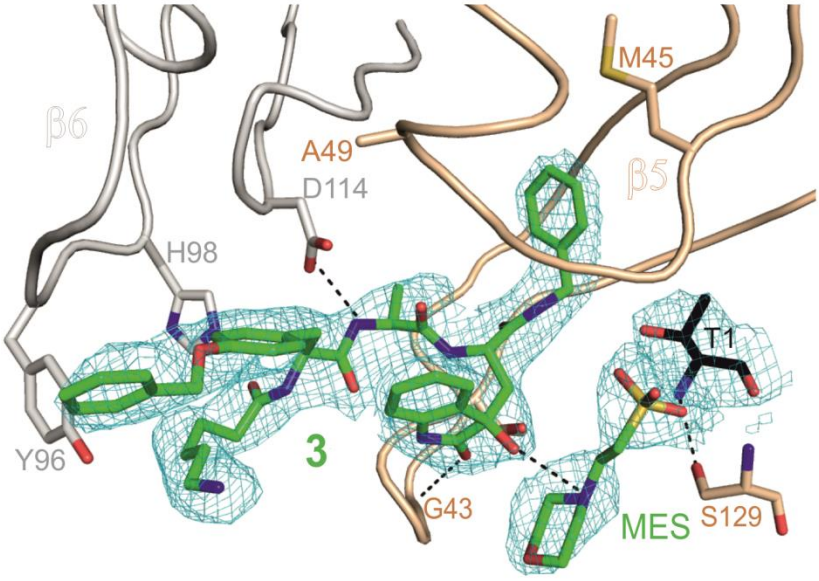


Figure 2S.

Inhibition profile for compounds **5** (ChT-L activity), **6b** (PA activity) and **6c** (T-L activity) of iPR (column A), cPR (column B) and cellular proteasome (column C) using in vitro assays (A, B) and cell-based proteasome Glo assays (C). In vitro assays: pH 8.0; 37 °C; [iPR] = [cPR] = 0.3 nM; [Suc-LLVY-AMC]$_0$ = 20 μM for ChT-L activity, for PA activity, [Z-LLE-βNA]$_0$ = 100 μM (iPR) and 50 μM (cPR), and for T-L activity, [Boc-LRR-AMC]$_0$ = 50 μM. Cellular assays: HEK-293 cells treated with the inhibitor for 1 h at 37 °C and chemoluminescence measurements using Suc-LLVY-Glo™, Z-LRR-Glo™ and Z-nLPnLD-Glo™ to detect ChT-L, T-L and PA activities, respectively. The experimental points were fitted to eq 1.

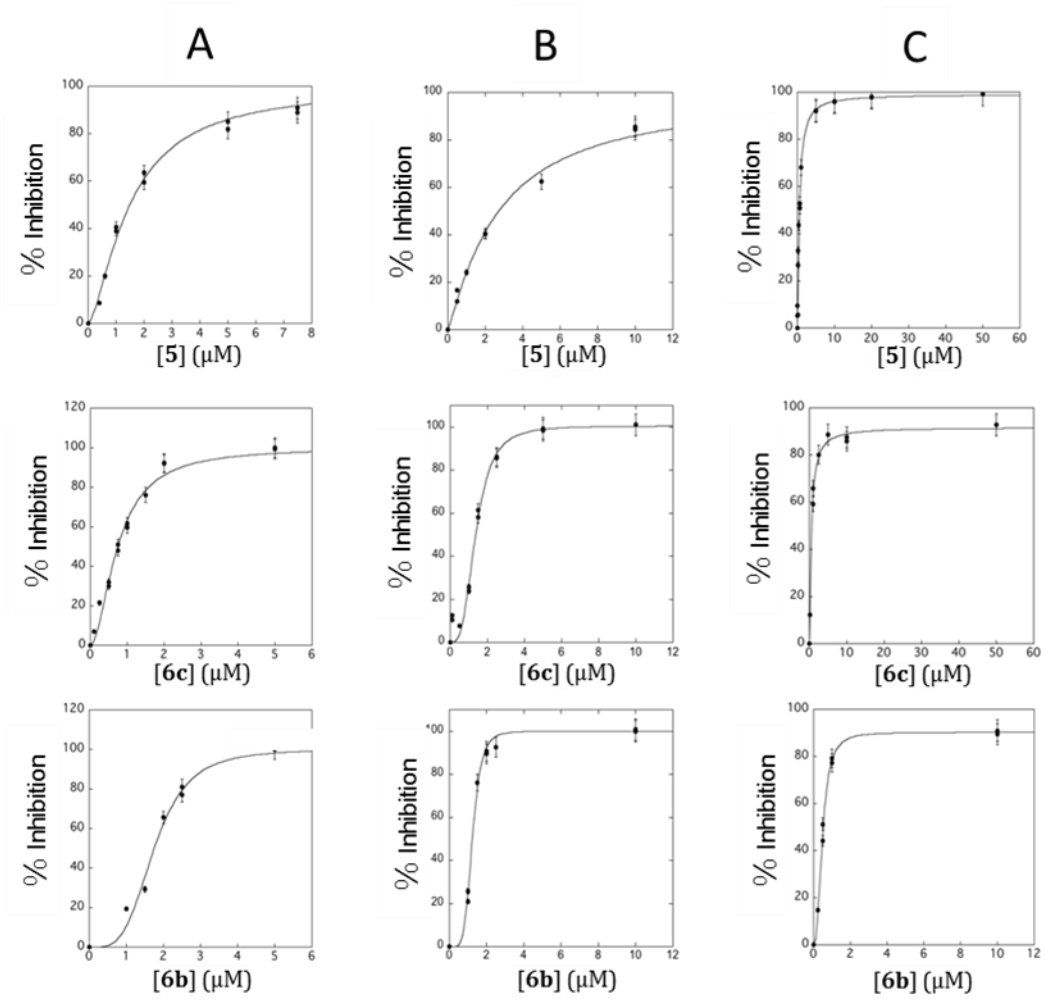
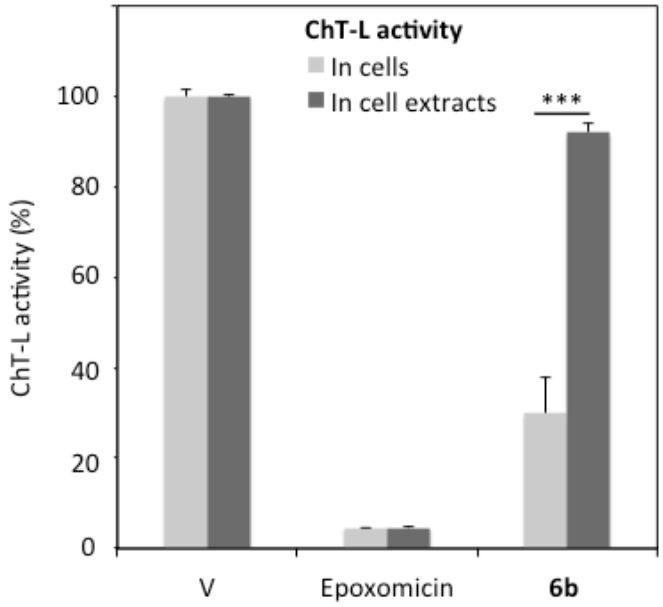


Figure 3S.

Recovery of ChT-L activity after incubation of HEK-293 cells with compound **6b** or epoxomicin. The ChT-L activity was measured directly in cells by proteasome Glo™ cell-based assay or in cell extracts using the fluorogenic substrate Suc-LLVY-AMC as described in the Experimental Section. In the two cases, HEK-193 cells were incubated 1 h at 37°C either with 3 µL DMSO [0,001% DMSO (v/v)] (vehicle control V) or by epoxomicin (1 µM) or compound **6b** (1 µM).
***T test: $p < 0.000005$



General methods

Enzymology: Purified human constitutive cPR and iPR were obtained from Boston Biochem. (Cambridge, USA). The fluorogenic substrates Suc-LLVY-AMC (ChT-L activity), Z-LLE-βNA (PA activity) and Boc-LRR-AMC (T-L activity) were obtained from Bachem (Weil am Rhein, Germany).

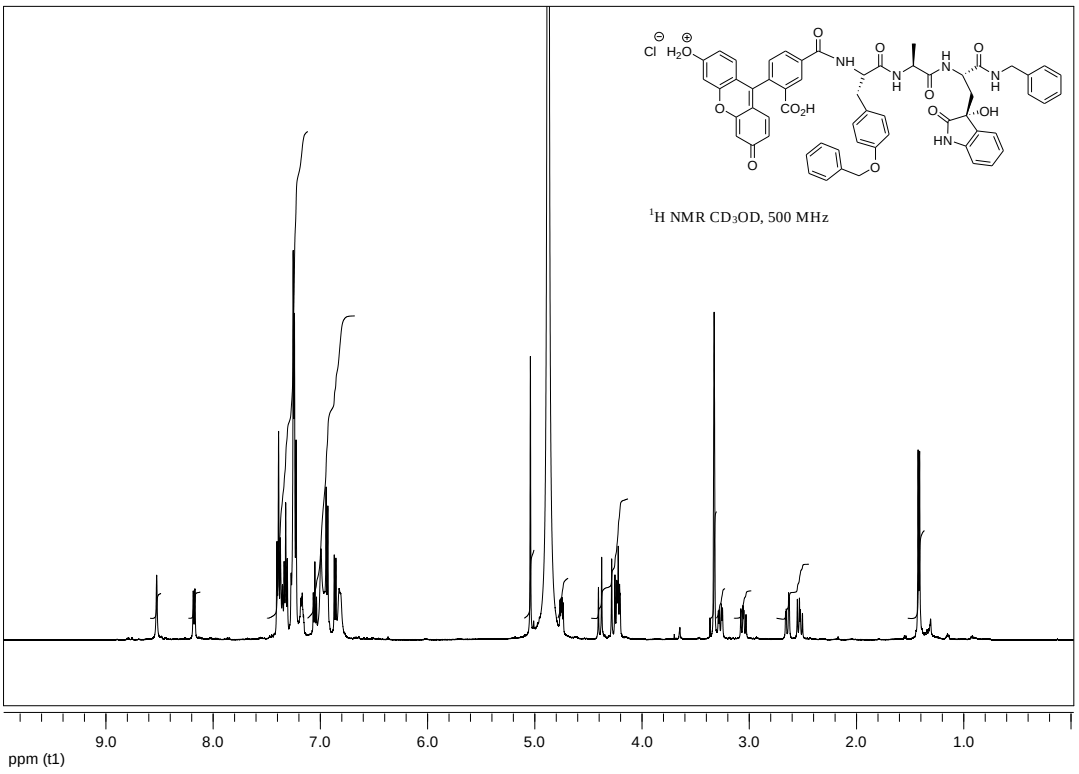
Chemistry: Commercially available reagents were used without further purification unless otherwise stated. Dry solvents were distilled from the appropriate drying reagents immediately before use. Yields refer to chromatographically and spectroscopically homogeneous materials, unless otherwise stated. Reactions were monitored by thin-layer chromatography carried out on silica gel aluminium sheets (60F-254) using UV light as a visualizing agent, 15% ethanolic phosphomolybdic acid and heat or 0.2% ethanolic ninhydrin and heat as developing agent. MPLC (medium pressure liquid chromatography) was performed on a Flashsmart two apparatus, using C18 silica flash column (AIT chromato, 40 -60 μm). ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature on a BRUCKER Avance 300 or Avance 500 spectrometers. Chemical shifts were reported in ppm (δ units) and residual non deuterated solvent was used as internal reference. High-resolution mass spectra were recorded on a Waters Q-TOF 2 or a Bruker MicrO-TOF Q II apparatus using an electrospray source (ESI). Microanalysis and mass spectra were performed by the Centre régional des mesures physiques de l'Ouest (CRMPO, Rennes, France).

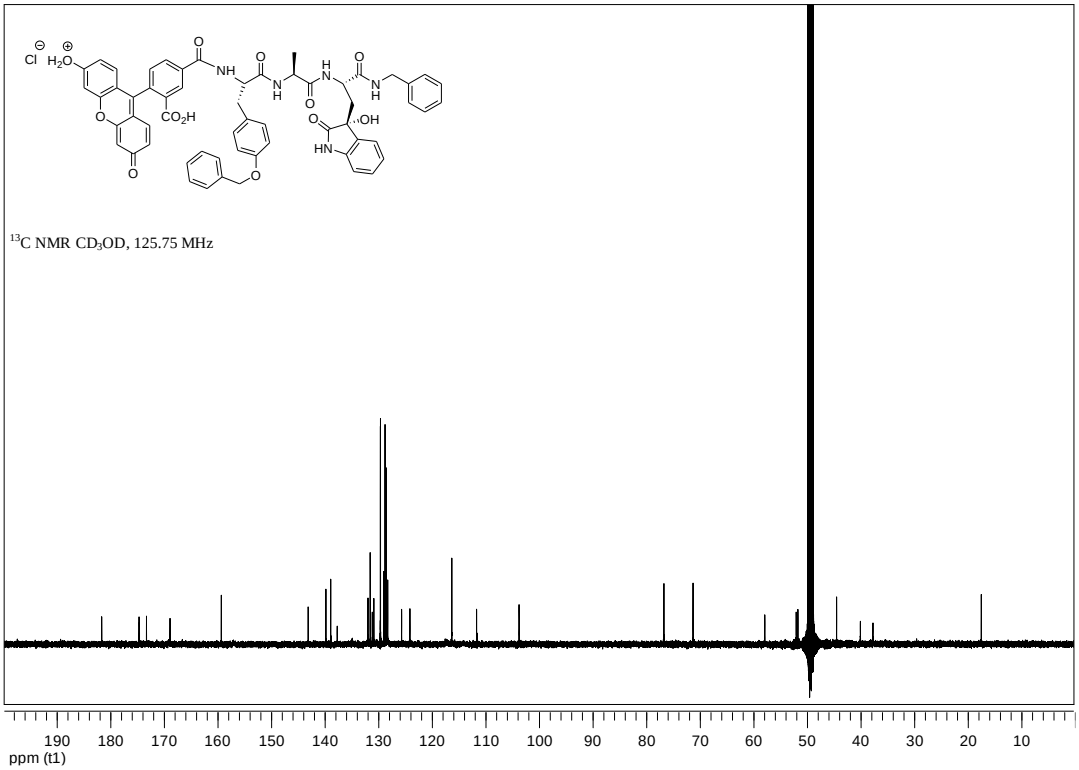
Reference tetramethylrhodamine derivative 7

To a solution of methyl aminohexanoate, hydrochloride **10b**[1] (111 mg, 0.61 mmol) in anhydrous DMF (10 mL) at 0 °C, was successively added triethylamine (250 μL, 1.8 mmol), carboxy tetramethylrhodamin **9**[2] (258 mg, 0.60 mmol), hydroxybenzotriazole (89 mg, 0.66 mmol) and EDC hydrochloride (126 mg, 0.66 mmol).The resulting mixture was stirred at room temperature for 16 h and then concentrated in vacuo (0.1 mbar). The residue was purified twice by reverse phase chromatography (MPLC) using C18 silica gel (5 g) and a 0.005 M aqueous HCl / acetonitrile gradient. Product **7** (220 mg, 65%) was obtained as a dark purple solid. ^{1}H NMR (CD_3OD, 300 MHz) δ 1.46 (m, 2H), 1.72 (m, 4H), 2.38 (m, 2H), 3.30 (s, 12H), 3.46 (m, 2H), 3.66 (s, 3H), 6.99-7.17 (m, 6H), 7.52 (d, *J* = 7.8 Hz, 1H), 8.24 (d, *J* = 7.8 Hz, 1H), 8.75 (s, 1H), 8.87 (br s, 1H). ^{13}C NMR (CD_3OD, 125.75 MHz) δ 25.72 (CH_2), 27.54 (CH_2), 30.13 (CH_2), 34.70 (CH_2), 40.85 (CH_3), 40.98 (CH_2), 52.01 (CH_3), 97.39 (CH), 114.79 (C), 115.29 (CH), 130.32 (CH), 130.84 (CH), 131.37 (CH), 132.20 (CH), 132.29 (C), 137.46 (C), 137.60 (C), 158.88 (C), 159.08 (C), 161.34 (C), 168.64 (C), 175.90 (C). HRMS (ESI, MeOH): calcd. $[M+H]^+$ $C_{32}H_{36}N_3O_6$ 558.2599, found 558.2600.

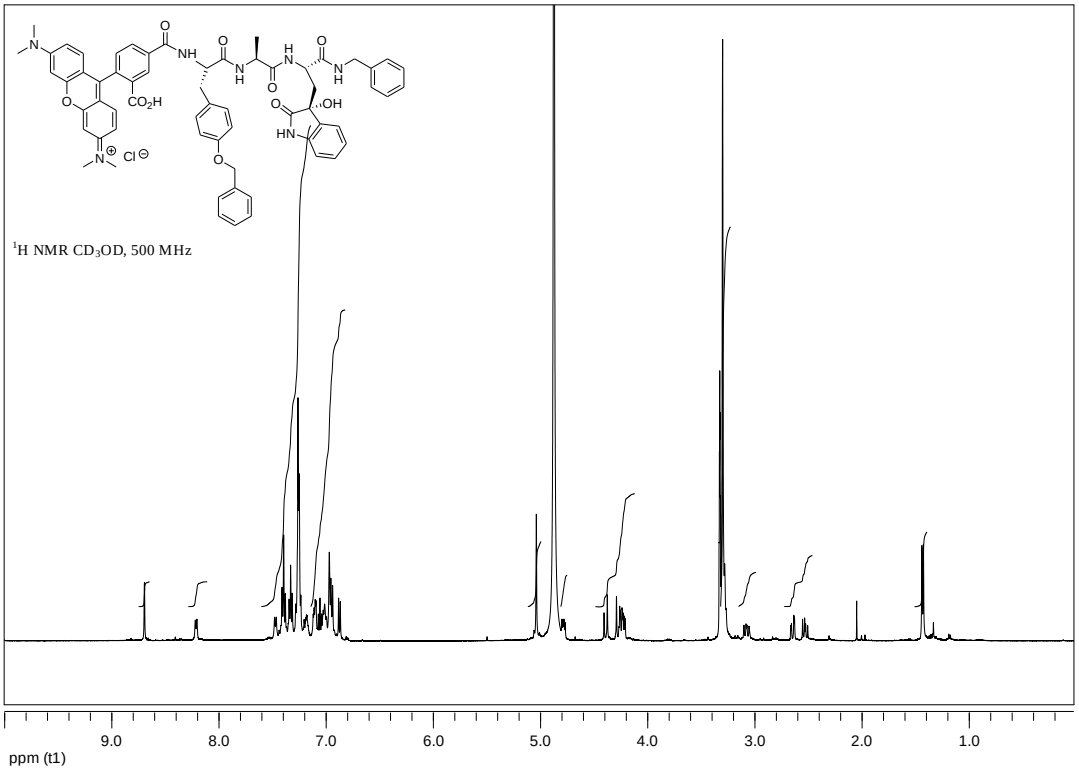
NMR spectra

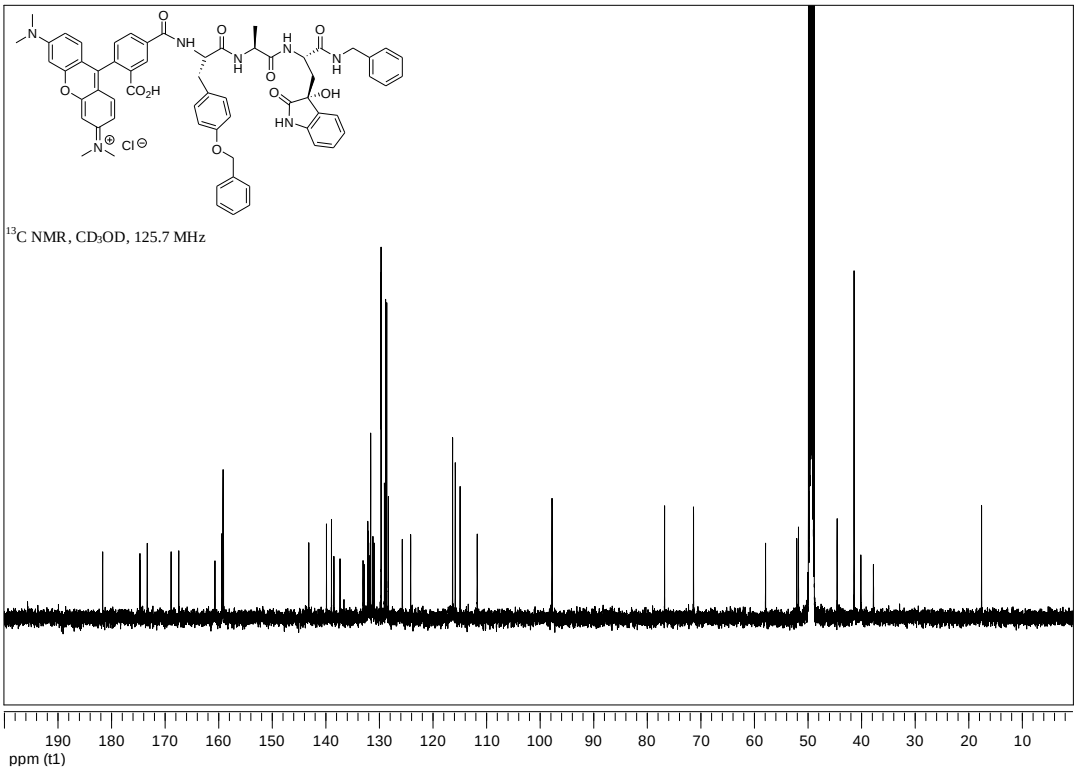
Fluorescein derivative **5**



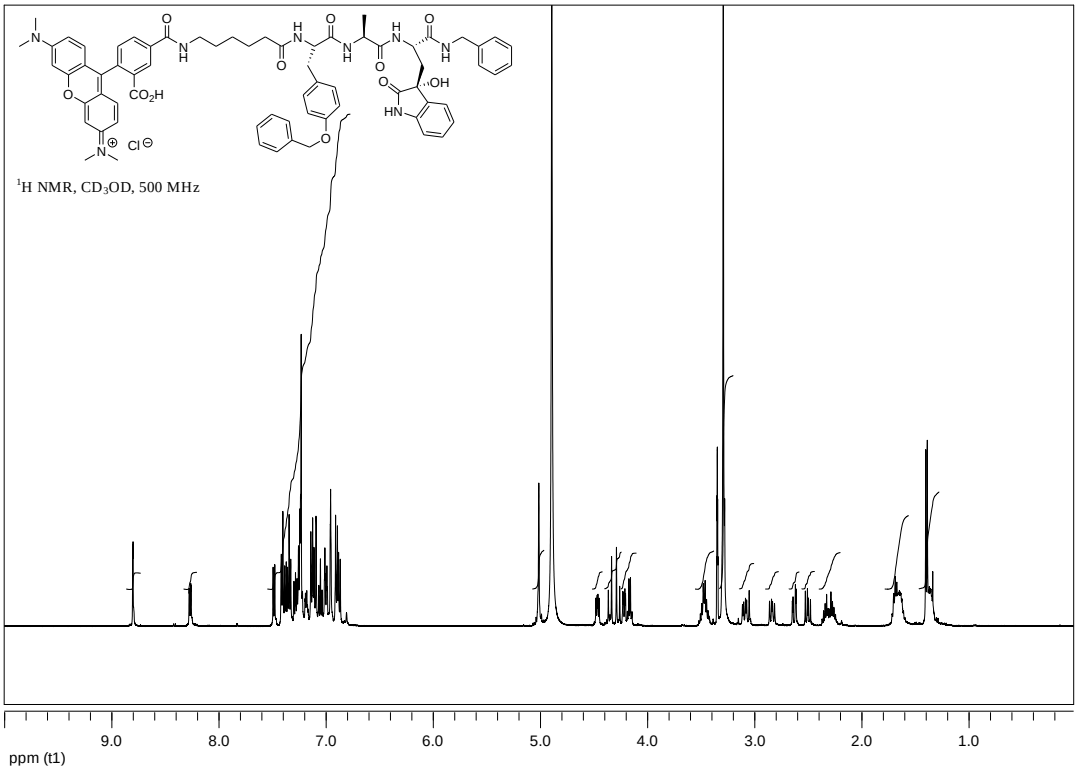


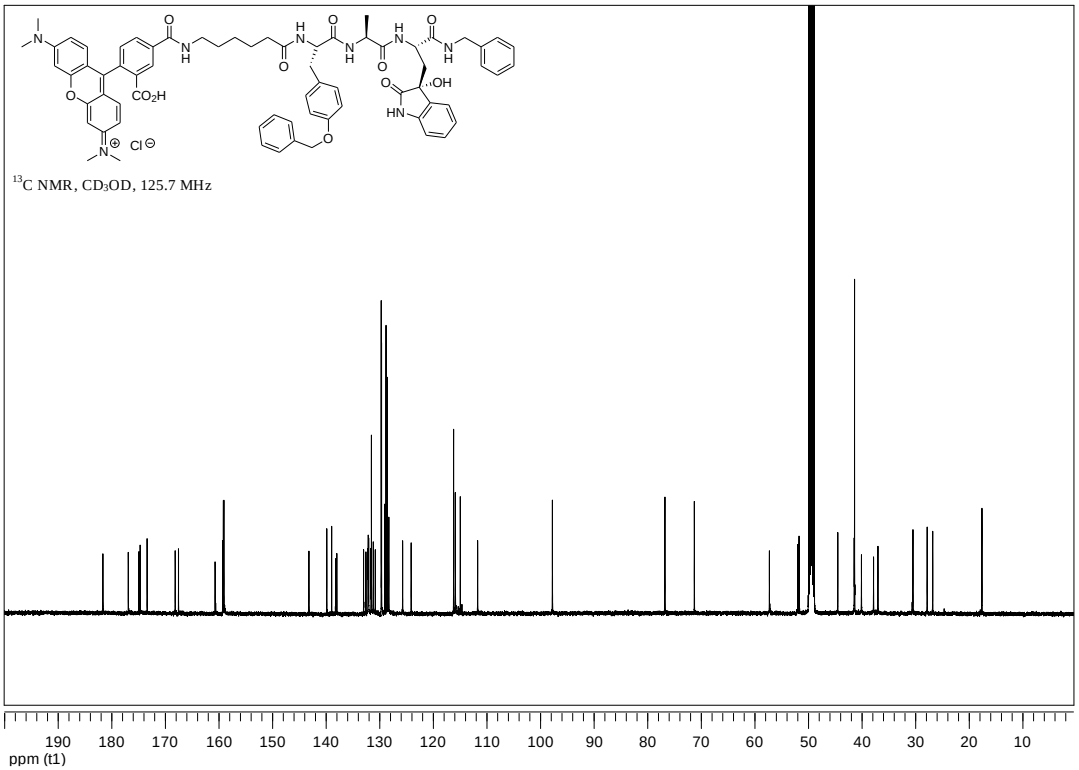
Tetramethylrhodamine derivative **6a**



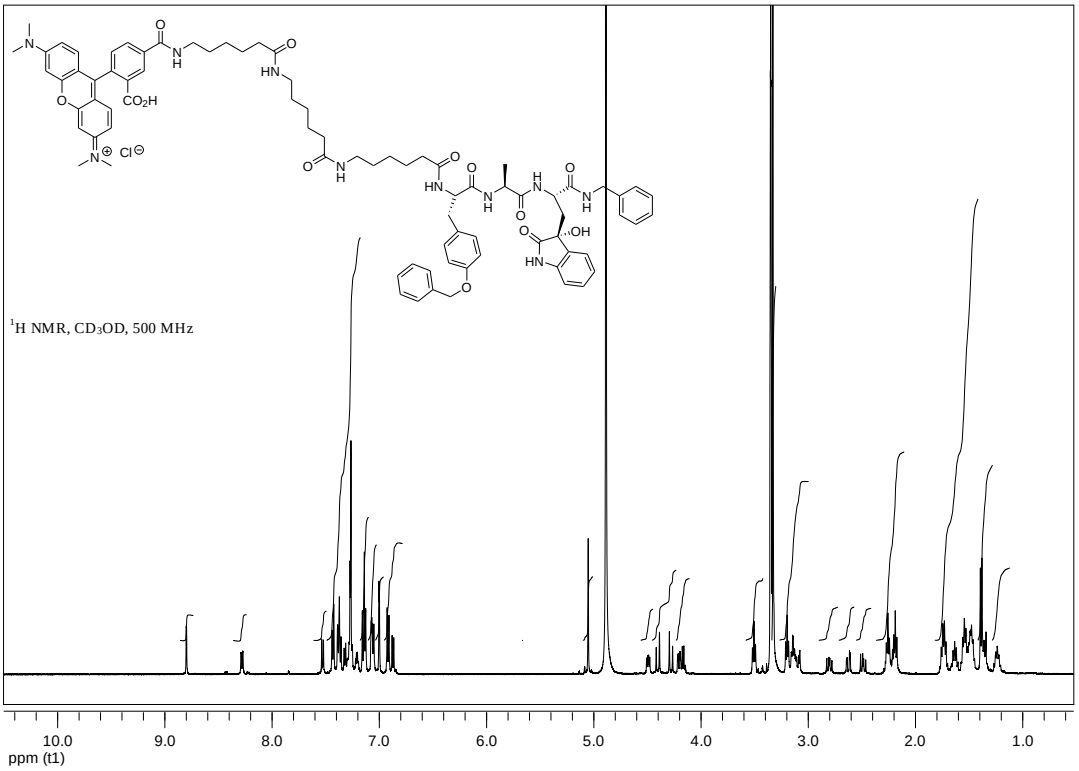


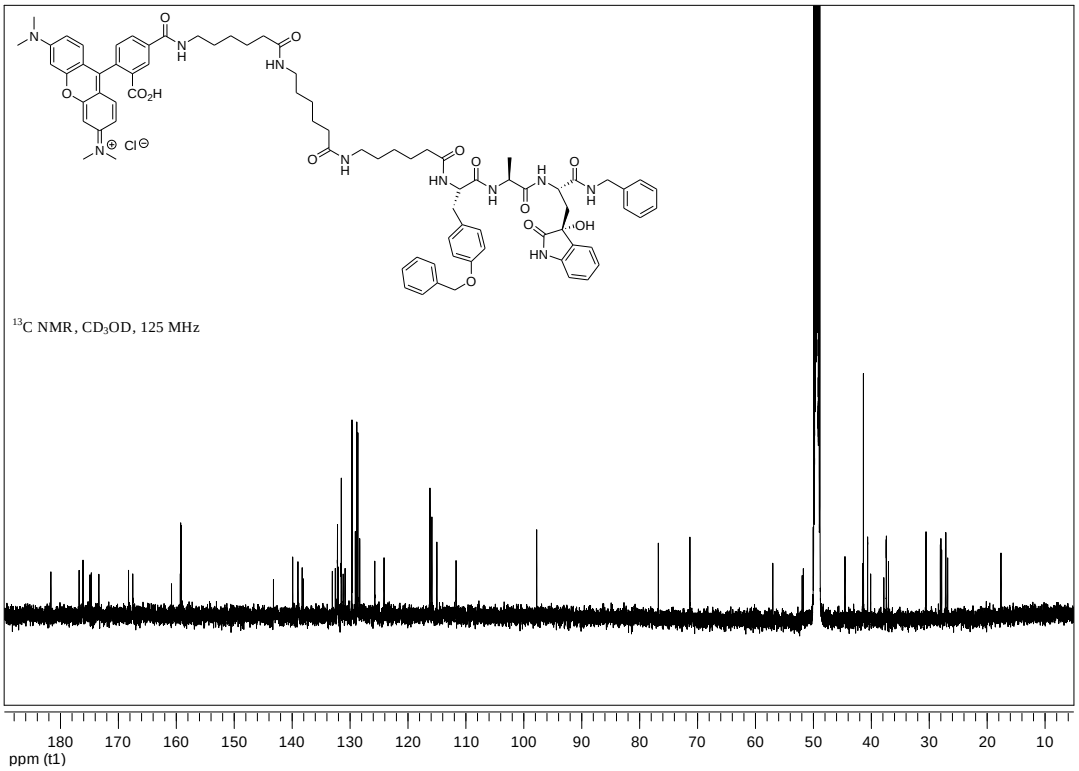
Tetramethylrhodamine derivative **6b**



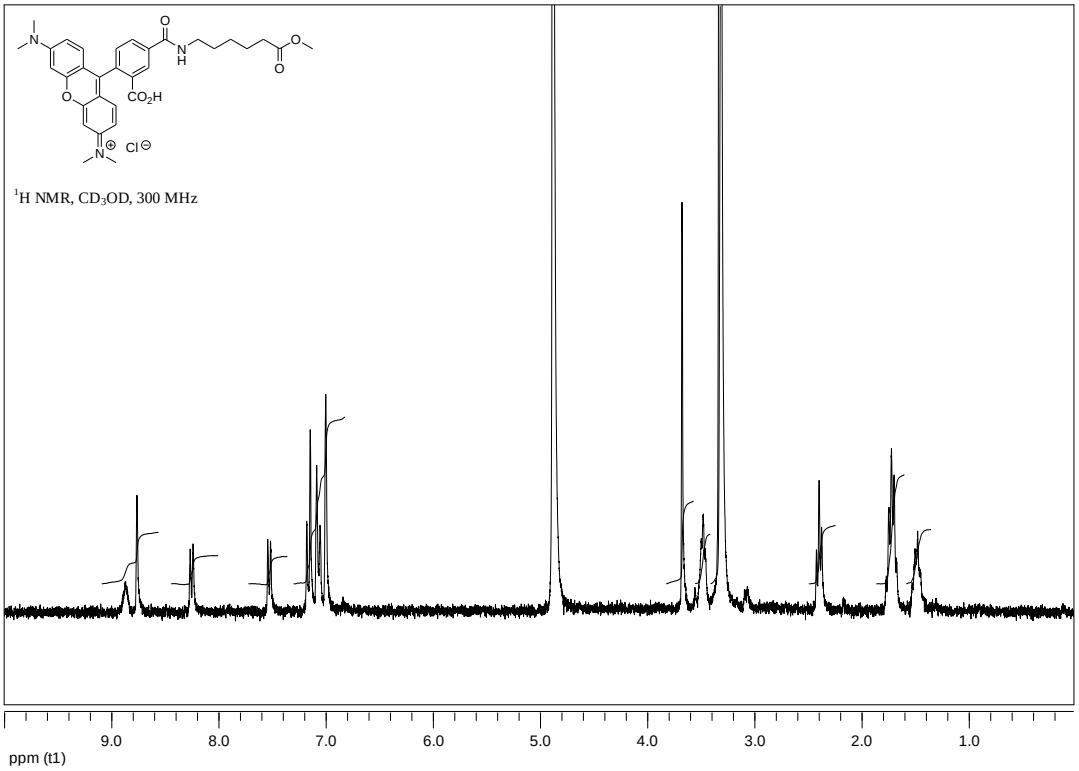


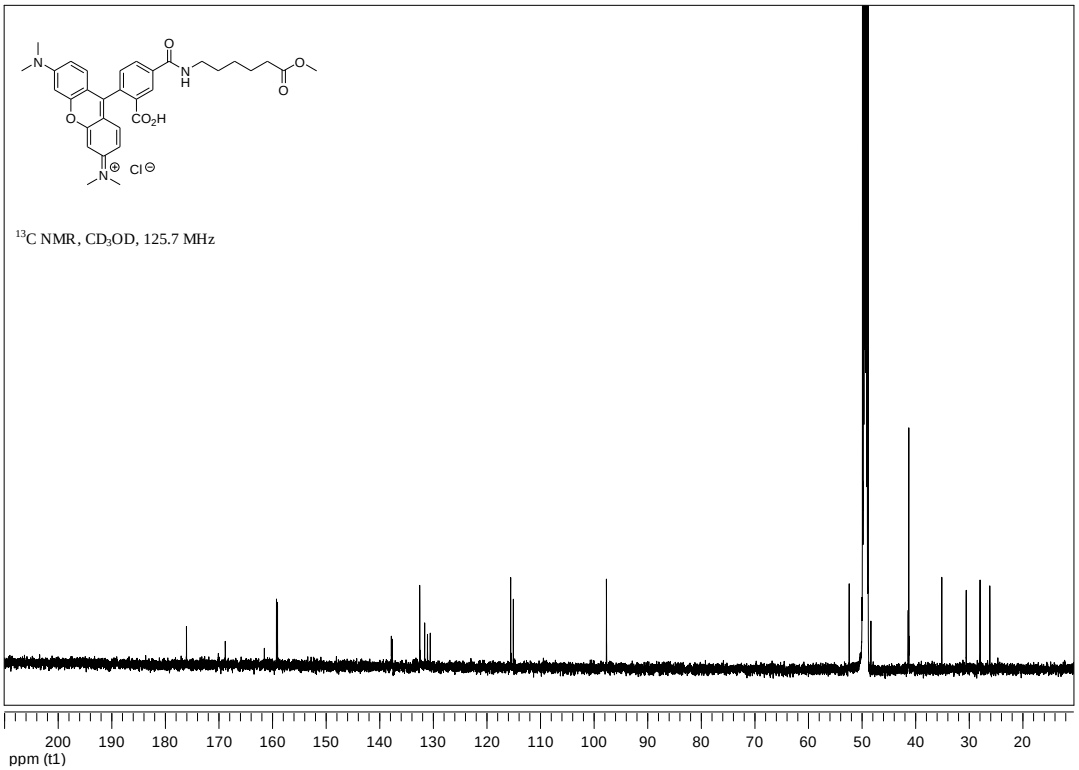
Tetramethylrhodamine derivative **6c**



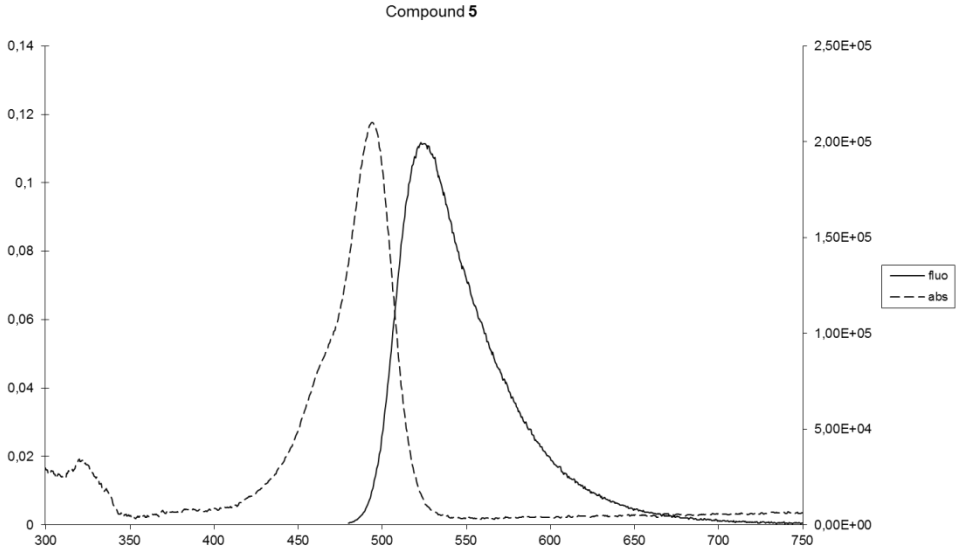


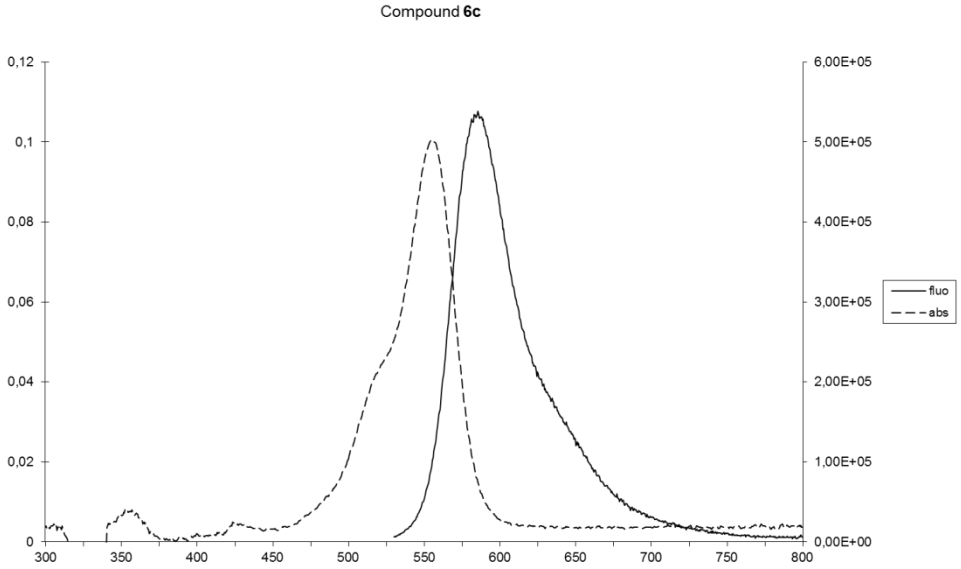
Tetramethylrhodamine derivative **7**





Absorption and emission spectra: compounds **5** and **6c** (water)





Data related to fluorophores released by proteasome substrates[3,4]

7-amino-4-methylcoumarin (water): λ_{max}(abs) = 342 nm, λ_{max}(em) = 443 nm

Kinetic of proteasome fluorogenic substrate hydrolysis releasing 7-amino-methylcoumarin was monitored at 460 nm, using $\lambda_{excitation}$ = 360 nm.

2-naphtylamine (water): λ_{max}(em) = 410 nm

Kinetic of proteasome fluorogenic substrate hydrolysis releasing 2-naphtylamine was monitored at 405 nm, using $\lambda_{excitation}$ = 330 nm.

No interference was observed between the fluorescence of compound **6** and luminescence due to reagents used in cell-based proteasome-Glo assay .

References

1. Desvergne, A.; Genin, E.; Maréchal, X.; Gallastegui, N.; Dufau, L.; Richy, N.; Groll, M.; Vidal, J.; Reboud-Ravaux, M., Dimerized Linear Mimics of a Natural Cyclopeptide (TMC-95A) Are Potent Noncovalent Inhibitors of the Eukaryotic 20S Proteasome. *J. Med. Chem.* **2013,** *56*, 3367-3378.

2. Kvach, M. V.; Stepanova, I. A.; Prokhorenko, I. A.; Stupak, A. P.; Bolibrukh, D. A.; Korshun, V. A.; Shmanai, V. V., Practical Synthesis of Isomerically Pure 5- and 6-Carboxytetramethylrhodamines, Useful Dyes for DNA Probes. *Bioconjugate Chem.* **2009,** *20*, 1673-1682.

3. Beynon, R.; Bond, J. S. (Editors), Proteolytic Enzymes: A Practical Approach, Second Edition, 2001, Oxford University Press

4. Johnson, I.D. Practical Considerations in the Selection and Application of Fluorescent Probes, in *Handbook of Biological Confocal Microscopy*, Editor: Pawley, J.B. Chapter 17, Springer, 2006, 3rd edition