Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

SUPPORTING INFORMATION

Design, Characterization and Cellular Uptake Studies of Fluorescence-Labeled Prototypic Cathepsin Inhibitors

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Inhibition Assays

Cathepsin L inhibition assay.[1] Human isolated cathepsin L (Enzo Life Sciences, Lörrach, Germany) was assayed spectrophotometrically at 405 nm and at 37 ºC. The reactions were followed over 20 min. Assay buffer was 100 mM sodium phosphate buffer pH 6.0, 100 mM NaCl, 5 mM EDTA, and 0.01% Brij 35. An enzyme stock solution of 135 μg/mL in 20 mM malonate buffer pH 5.5, 400 mM NaCl, and 1 mM EDTA was diluted 1:100 with assay buffer containing 5 mM DTT and incubated for 30 min at 37 ºC. A 10 mM stock solution of the chromogenic substrate Z-Phe-Arg-pNA was prepared in DMSO. The final concentration of DMSO was 2%, and the final concentration of the substrate was 100 μM (= 5.88 K_m). The assay was performed with a final concentration of 54 ng/mL of cathepsin L. Into a cuvette containing 940 μL assay buffer, inhibitor solution and DMSO in a total volume of 10 μL, and 10 μL of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 40 μL of the cathepsin L solution.

Cathepsin S inhibition assay.[2] Human recombinant cathepsin S (Enzo Life Sciences, Lörrach, Germany) was assayed fluorometrically. The wavelength for excitation was 360 nm and for emission 440 nm. The reactions were followed at 25 °C over 20 min. Assay buffer was 100 mM sodium phosphate buffer pH 6.0, 100 mM NaCl, 5 mM EDTA, and 0.01% Brij 35. An enzyme stock solution of 70 μg/mL in 100 mM MES buffer, pH 6.5, 1 mM EDTA, 50 mM L-cysteine, 10 mM dithiothreitol (DTT), 0.5% Triton X-100 and 30% glycerol was diluted 1:100 with a 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 2 mM EDTA, 0.01% Triton X-100 and 5 mM DTT and incubated for 60 min at 37 °C. A 10 mM stock solution of the fluorogenic substrate Z-Phe-Arg-AMC was prepared in DMSO. The assay was performed with a final substrate concentration of 40 μM (= 0.74 K_m), a final concentration of 42 ng/mL of cathepsin S, and a final DMSO concentration of 2%. Into a cuvette containing 920 μL assay buffer, inhibitor solution and DMSO in a total volume of 16 μL, and 4 μL of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 60 μL of the cathepsin S solution.

Cathepsin K inhibition assay.[1] Human recombinant cathepsin K (Enzo Life Sciences, Lörrach, Germany) was assayed fluorometrically. The wavelength for excitation was 360 nm and for emission 440 nm. The reactions were followed at 25 °C over 20 min. An enzyme stock solution of 23 μg/mL in 50 mM sodium acetate pH 5.5, 50 mM NaCl, 0.5 mM EDTA, 5 mM DTT was diluted 1:100 with assay buffer (100 mM sodium citrate pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01% CHAPS) containing 5 mM DTT and incubated for 30 min at 37 ºC. A 10 mM stock solution of the fluorogenic substrate Z-Leu-Arg-AMC was prepared in DMSO. The final concentration of DMSO was 2%, and the final concentration of the substrate was 40 μM (= 13.3 K_m). The assay was performed with a final concentration of 5 ng/mL of cathepsin K. Into a cuvette containing 960 μL assay buffer, inhibitor solution and DMSO in a total volume of 16 μL, and 4 μL of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 20 μL of the cathepsin K solution.

Cathepsin B inhibition assay.[1] Human isolated cathepsin B (Calbiochem, Darmstadt, Germany) was assayed spectrophotometrically at 405 nm and at 37 ºC. The reactions were followed over 20 min. Assay buffer was 100 mM sodium phosphate buffer pH 6.0, 100 mM NaCl, 5 mM EDTA, 0.01% Brij 35. An enzyme stock solution of 1.81 mg/mL in 20 mM sodium acetate buffer pH 5.0, 1 mM EDTA was diluted 1:500 with assay buffer containing 5 mM DTT and incubated for 30 min at 37 ºC. A 100 mM stock solution of the chromogenic substrate Z-Arg-Arg-pNA was prepared in DMSO. The final concentration of DMSO was 2% and the final concentration of the substrate was 500 μM (= 0.45 K_m). The assay was performed with a final concentration of 72 ng/mL of cathepsin B. Into a cuvette containing 960 μL assay buffer, inhibitor solution and DMSO in a total volume of 15 μL, and 5 μL of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 20 μL of the cathepsin B solution.

Molecular Docking

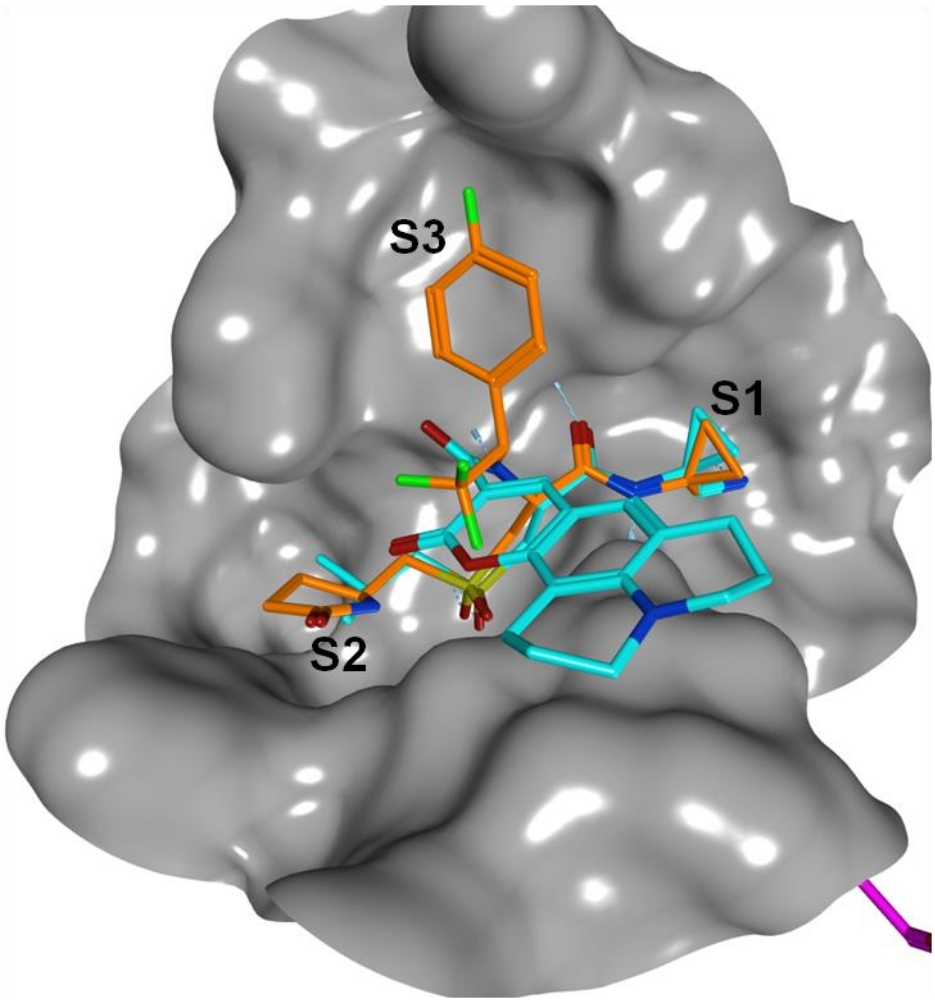


Figure S1. Overlay cathepsin S. Shown is an overlay of the predicted binding mode of probe **6** (cyan) and the co-crystallized nitrile inhibitor (orange) within the active site of cathepsin S (PDB ID 2FQ9)[3] in grey surface representation.

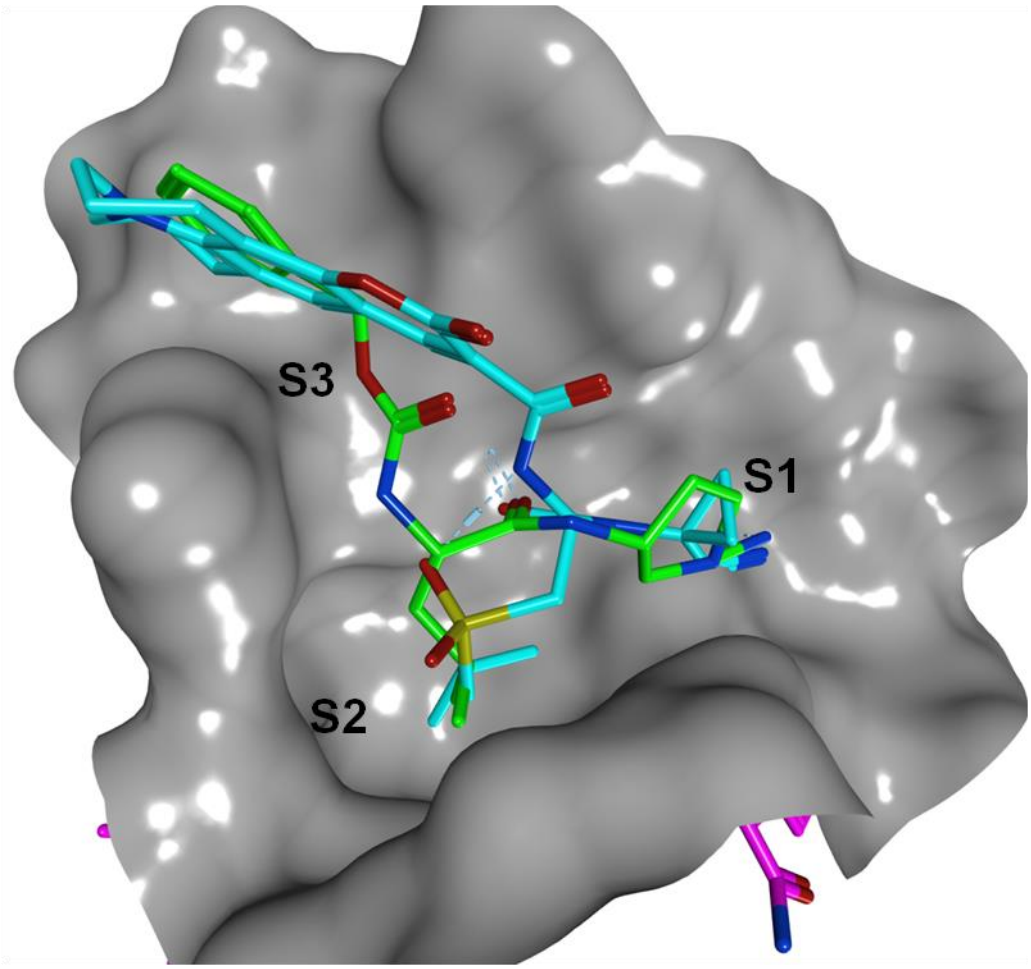


Figure S2. Overlay cathepsin K. An overlay of the predicted binding mode of probe **6** (cyan) and the crystallographic cyanamide inhibitor (green) within the active site of cathepsin K (PDB ID 1YK7)[4] in grey surface representation is depicted.

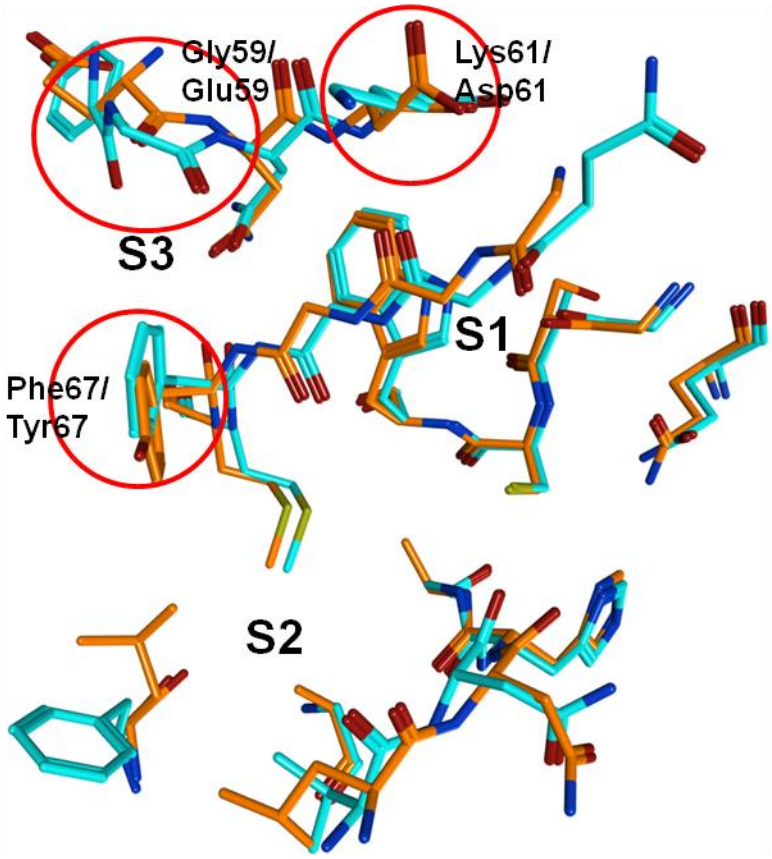


Figure S3. Active site comparison. An overlay of the active site residues of cathepsin S (cyan) and cathepsin K (orange) is depicted. Residues responsible for the different size of the S3 pockets are circled in red.

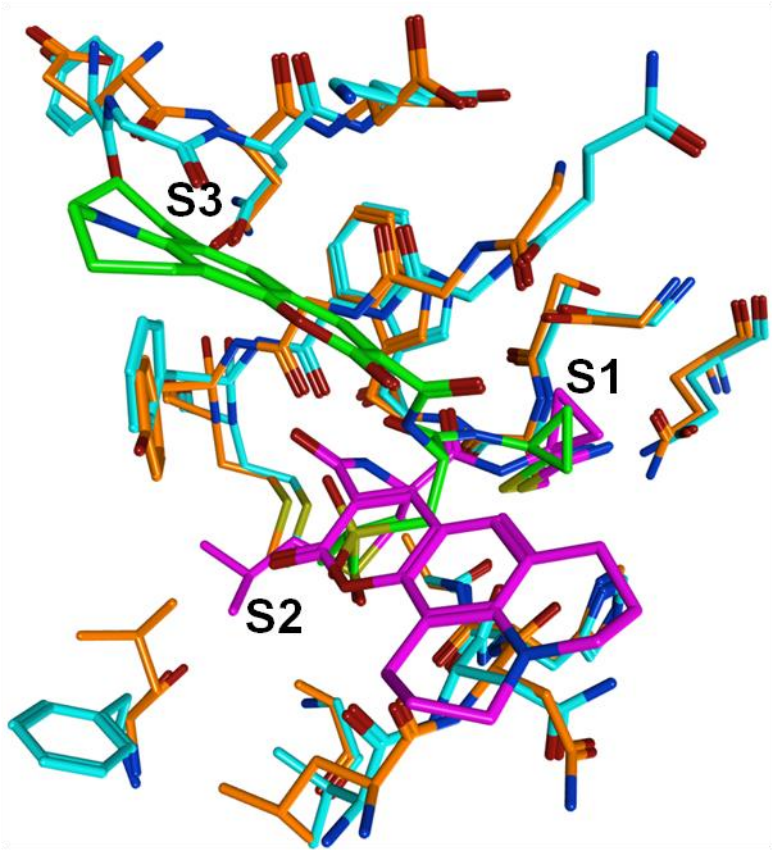


Figure S4. Active site comparison with inhibitor. An overlay of the active site residues of cathepsin S (cyan) and cathepsin K (orange) is depicted. Putative binding modes of probe **6** within the active sites of cathepsin S and K are shown in magenta and green, respectively.

UV and Fluorescence Spectra

The UV- and fluorescence spectra were recorded in CH_2Cl_2, MeOH, H_2O, (1% DMSO) and PBS (5% DMSO). A 10 mM stock solution of compound **6** in DMSO was diluted accordingly. The UV spectra were recorded at a final compound concentration of 10 µM on a Varian Cary 50 Bio apparatus. Fluorescence spectra were recorded on a flx-Xenius, Safas Monaco, spectrofluorometer at a final compound concentration of 1 µM.

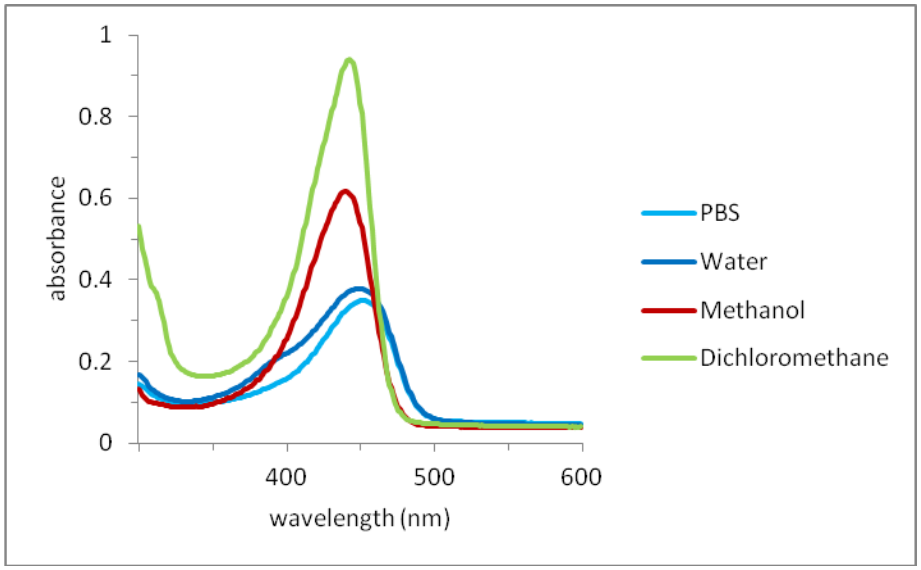


Figure S5. UV spectra of compound **6** (10 µM).

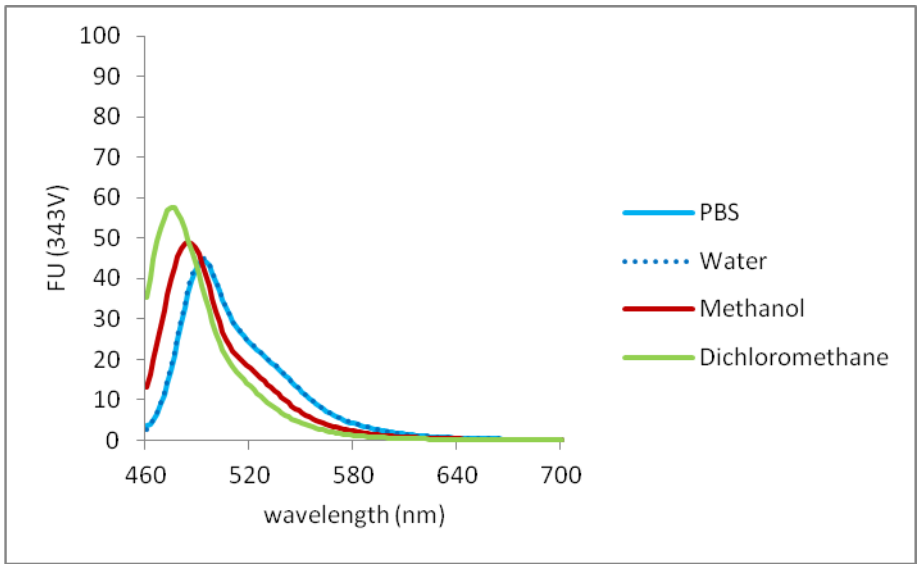


Figure S6. Fluorescence spectra of compound **6** (1 µM, λ_{ex} 450 nm). A PMT value of 343 V was adjusted.

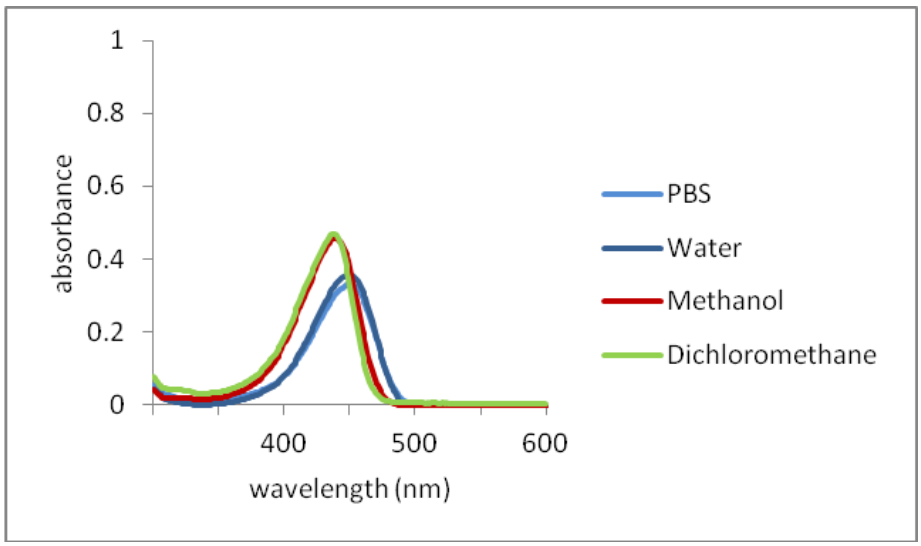


Figure S7. UV spectra of compound **7** (10 μM).

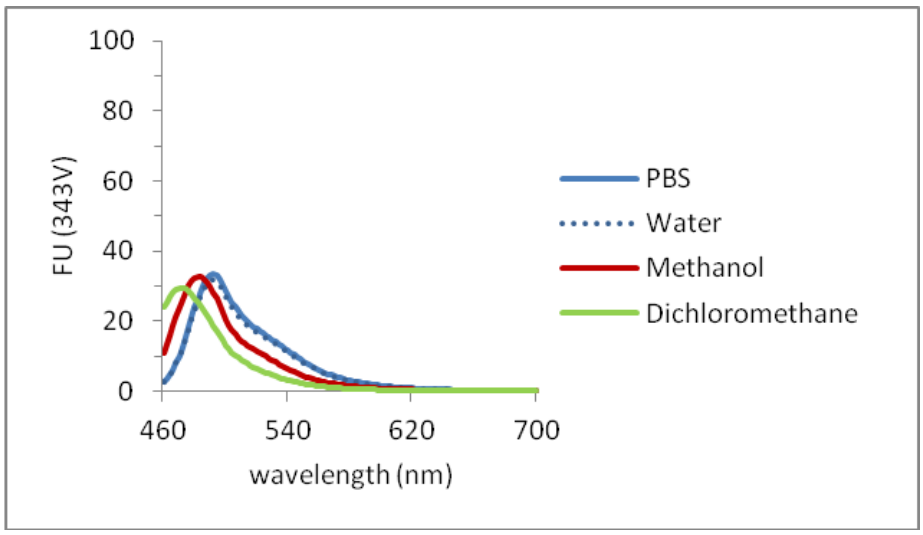


Figure S8. Fluorescence spectra of compound **7** (1 μM, λ_{ex} 450 nm). A PMT value of 343 V was adjusted.

Cell Viability after Treatment with Probe 7

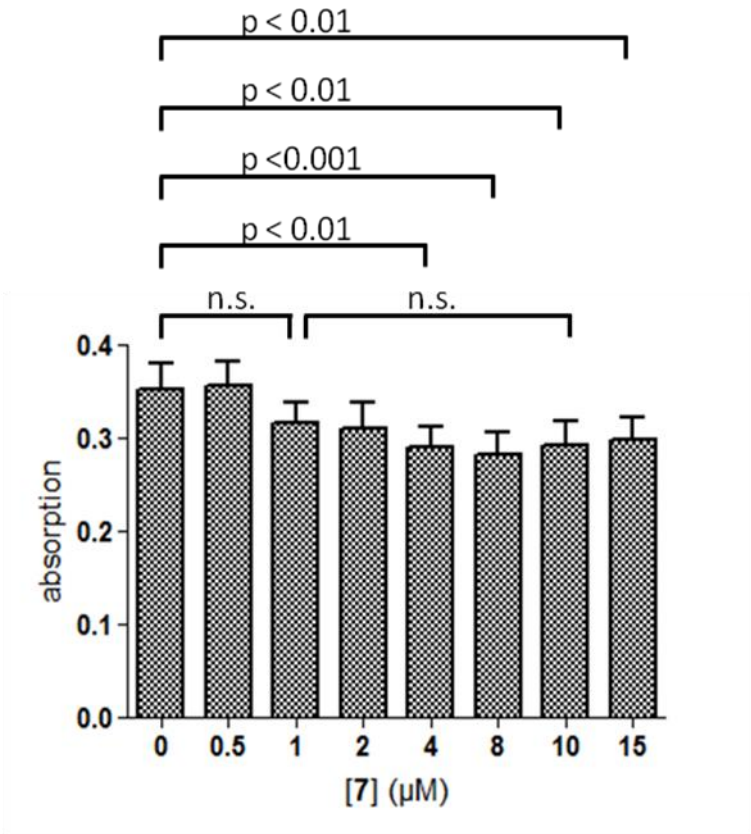


Figure S9. Results of the MTT assay for probe 7. HEK 293 cells were incubated for 3 h with compound **7** at different concentrations. Data of five independent experiments are presented as means ± SD. ANOVA revealed significant differences of the means between untreated cells and cells treated with 4, 8, 10 and 15 µM. The p values were calculated by using post-hoc analysis (Tukey´s test; 'n.s.' means 'not significant'). Only statistically significant differences are indicated except for compound concentrations of 1 µM and 10 µM, which have been used in cell-based uptake experiments.

Experimental HPLC Procedure for log $D_{7.4}$ Estimation

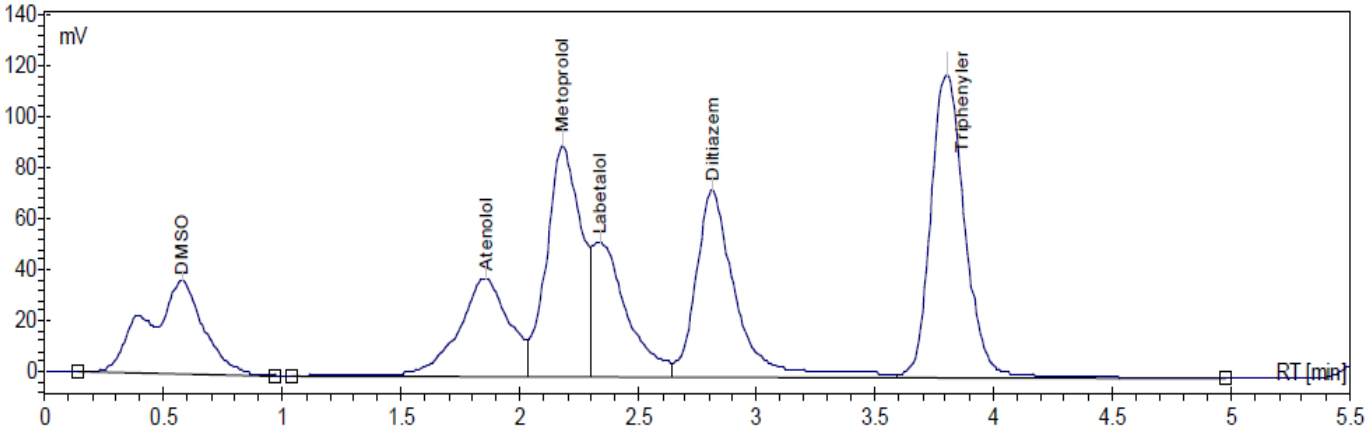


Figure S10. HPLC chromatogram of a mixture of five calibration compounds (atenolol 1.5 mg/mL, metoprolol tartrate 4.0 mg/mL, labetalol hydrochloride 0.12 mg/mL, diltiazem hydrochloride 0.075 mg/mL and triphenylene 0.01 mg/mL).

Table S1. Obtained t_R values of the calibration compounds and log $D_{7.4}$ values

compound	run a t_R (min)	run b t_R (min)	run c t_R (min)	mean t_R (min)	lit.[5] log $D_{7.4}$	log $D_{7.4}$ (from t_R)
atenolol	1.858	1.842	1.850	1.850	-1.38	-1.03
metoprolol	2.183	2.175	2.175	2.178	-0.06	0.11
labetalol	2.342	2.333	2.333	2.336	1.07	0.66
diltiazem	2.817	2.808	2.808	2.811	2.70	2.32
triphenylene	3.800	3.792	3.792	3.795	5.49	5.75

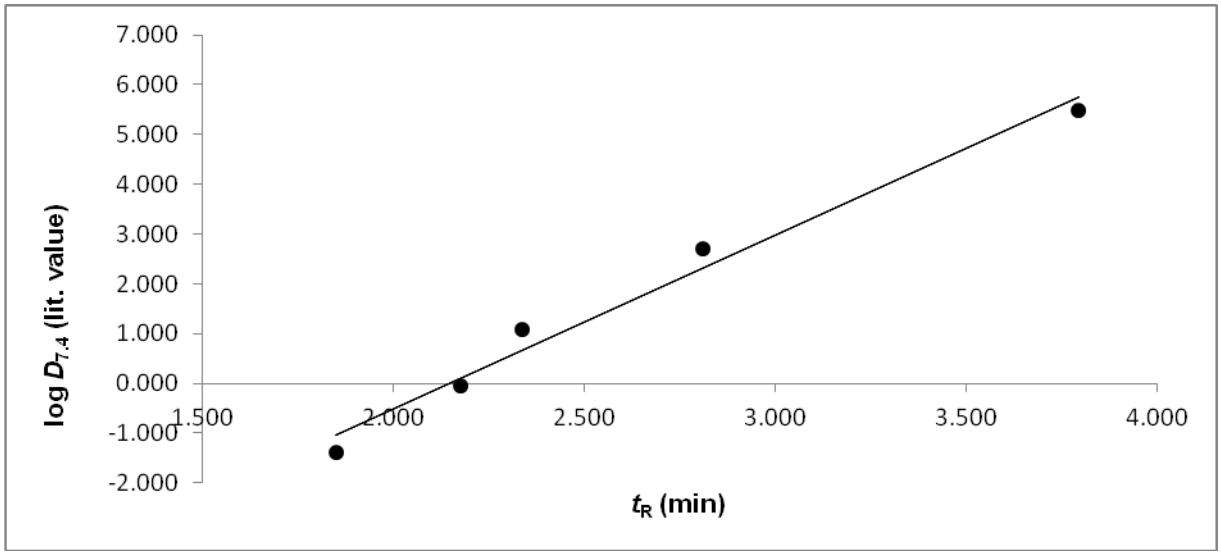


Figure S11. Calibration line using the standards listed in Table S1 for calculating log $D_{7.4}$. Linear regression gave the equation log $D_{7.4}$ = 3.485 t_R - 7.476 and R^2 = 0.981.

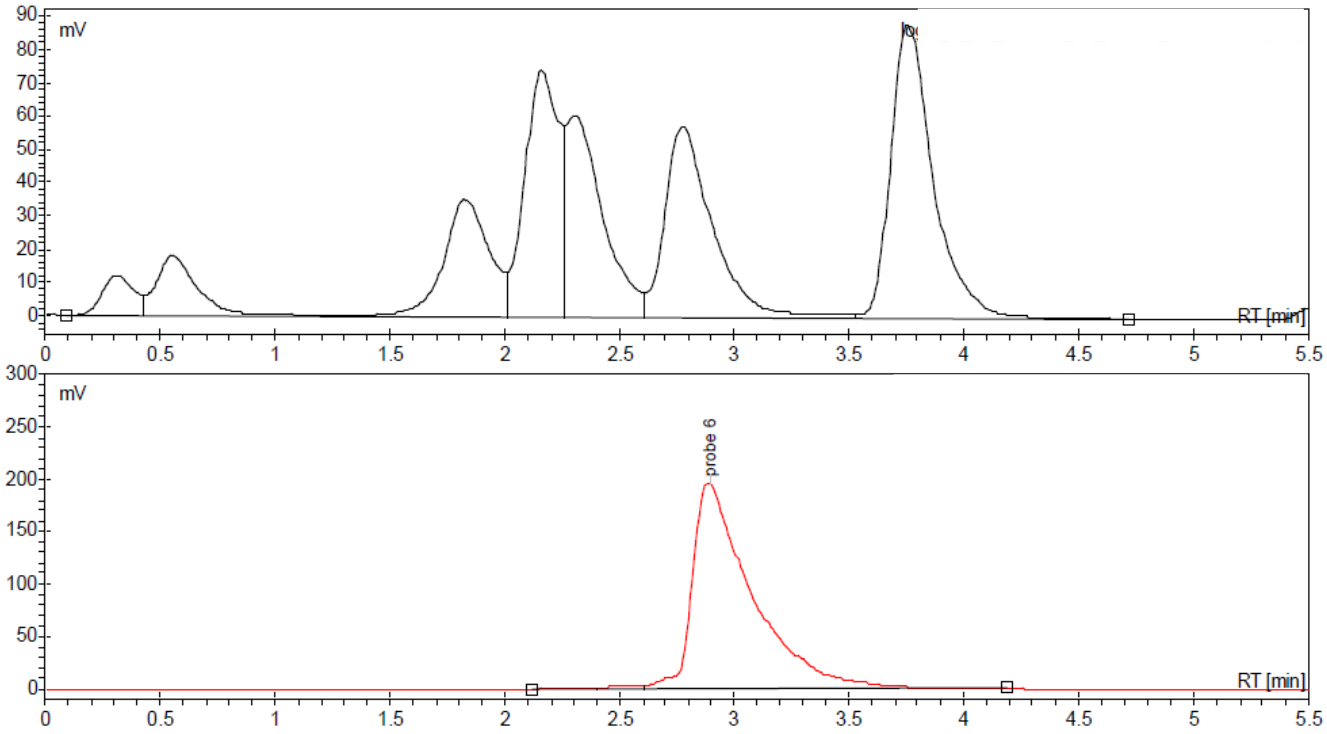


Figure S12. HPLC chromatogram of a mixture of five calibration compounds spiked with probe **6** (top: UV detection, bottom: fluorescence detection). Concentrations of the calibration compounds were as in Figure S10. The concentration of **6** was 0.014 mg/mL (25 µM). Probe **6** was detected by means of fluorescence detection (bottom). The mean retention time t_R was 2.906 min (three separate runs). According to the equation log $D_{7.4}$ = 3.485 t_R - 7.476, a log $D_{7.4}$ value of 2.65 was calculated.

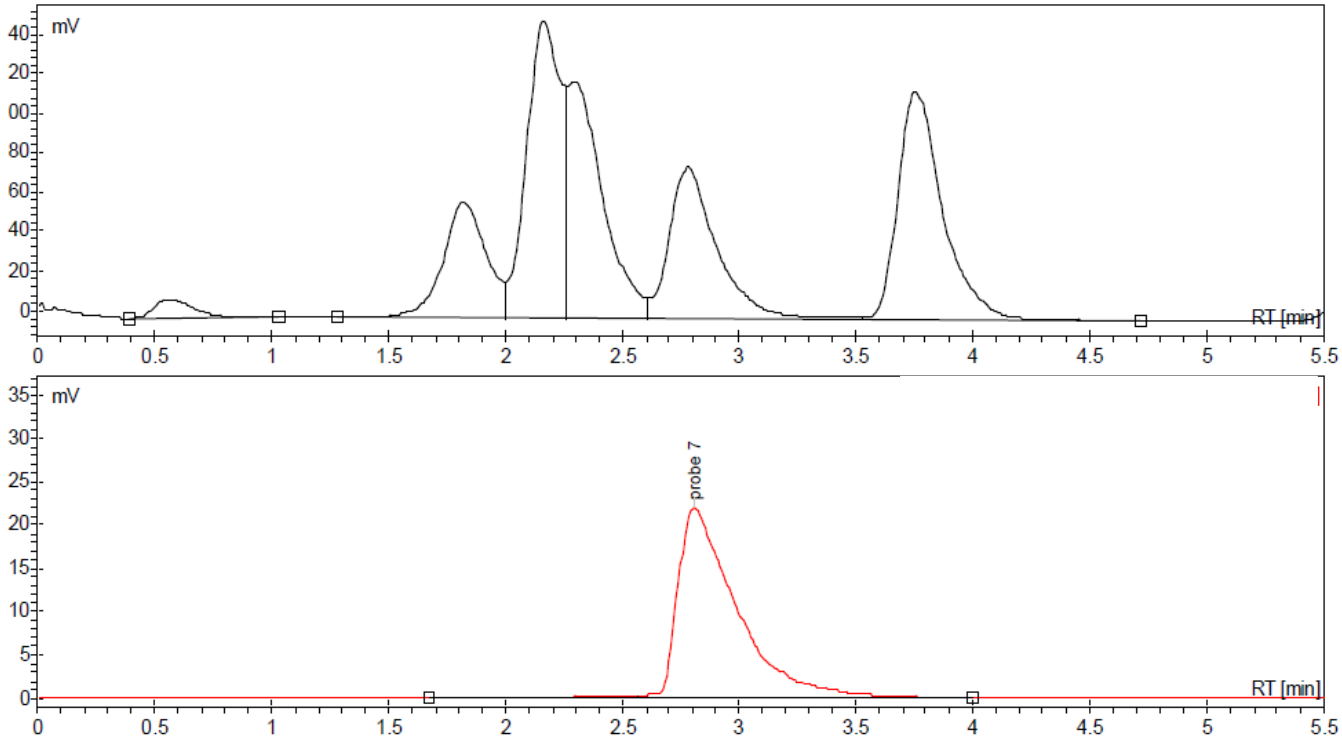


Figure S13. HPLC chromatogram of a mixture of five calibration compounds spiked with probe **7** (top: UV detection, bottom: fluorescence detection). Concentrations of the calibration compounds were as in Figure S10. The concentration of **7** was 0.0017 mg/mL (2.5 µM). Probe **7** was detected by means of fluorescence detection (bottom). The mean retention time t_R was 2.805 min (three separate runs). According to the equation $\log D_{7.4} = 3.485\ t_R - 7.476$, a $\log D_{7.4}$ value of 2.30 was calculated.

References

(1) Frizler, M.; Lohr, F.; Furtmann, N.; Kläs, J.; Gütschow, M. Structural optimization of azadipeptide nitriles strongly increases association rates and allows the development of selective cathepsin inhibitors. *J. Med. Chem.* **2011**, *54*, 396-400.

(2) Mertens, M. D.; Schmitz, J.; Horn, M.; Furtmann, N.; Bajorath, J.; Mareš, M.; Gütschow, M. A coumarin-labeled vinyl sulfone as tripeptidomimetic activity-based probe for cysteine cathepsins. *ChemBioChem* **2014**, *15*, 955-959.

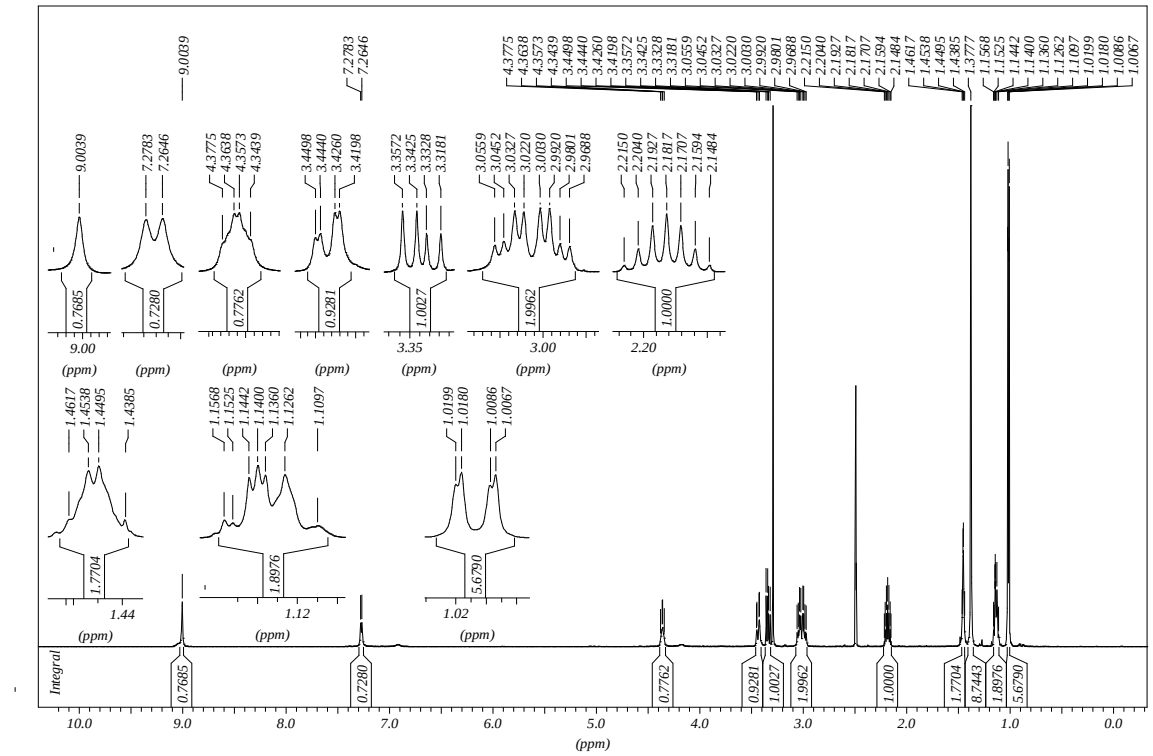
(3) Somoza, J. R. Cathepsin S with nitrile inhibitor. Unpublished, RCSB Protein Data Bank release March 21, 2006; PDB-ID 2FQ9.

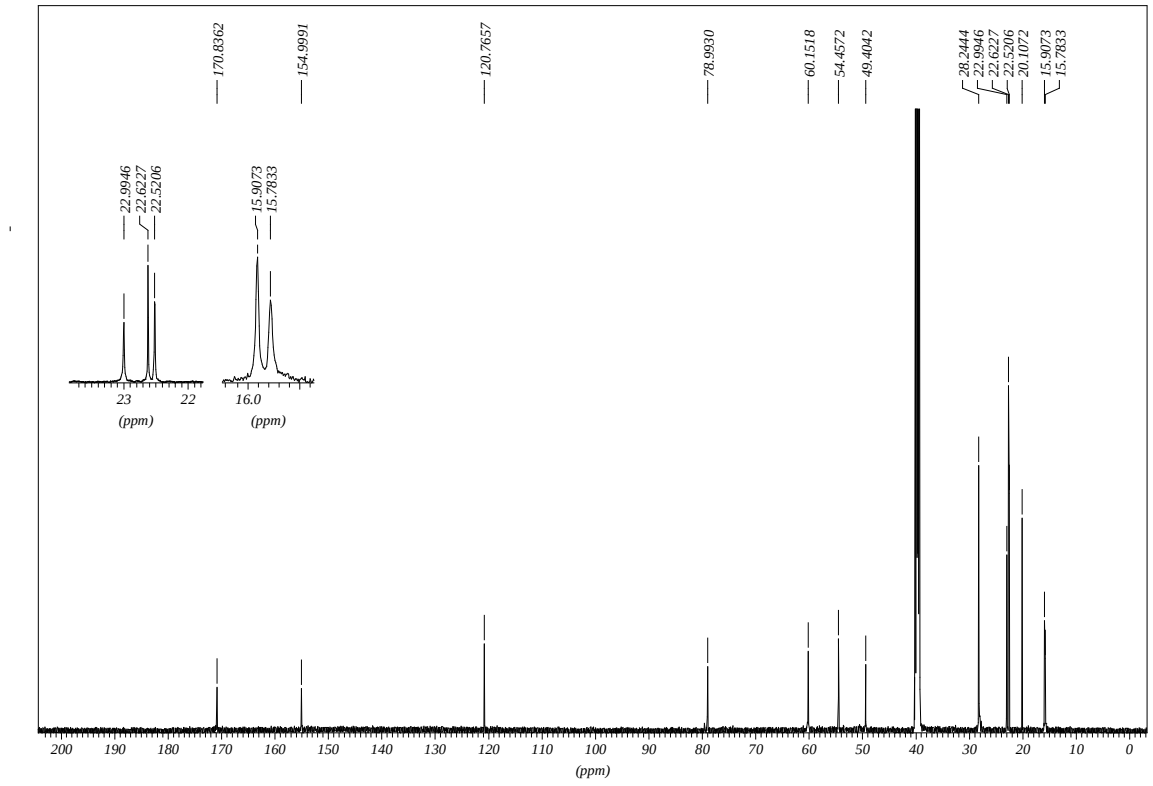
(4) Deaton, D. N.; Hassell, A. M.; McFadyen, R. B.; Miller, A. B.; Miller, L. R.; Shewchuck, L. M.; Tavares, F. X.; Willard, D. H.; Wrigt, L. L. Novel and potent cyclic cyanamide-based cathepsin K inhibitors. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1815-1819.

(5) Kerns, E. H.; Di, L.; Petusky, S.; Kleintop, T.; Huryn, D.; McConnell, O.; Carter, G. Pharmaceutical profiling method for lipophilicity and integrity using liquid chromatography-mass spectrometry. *J. Chromatogr. B, Analyt. Technol. Biomed. Life Sci.* **2003**, *791*, 381-388.

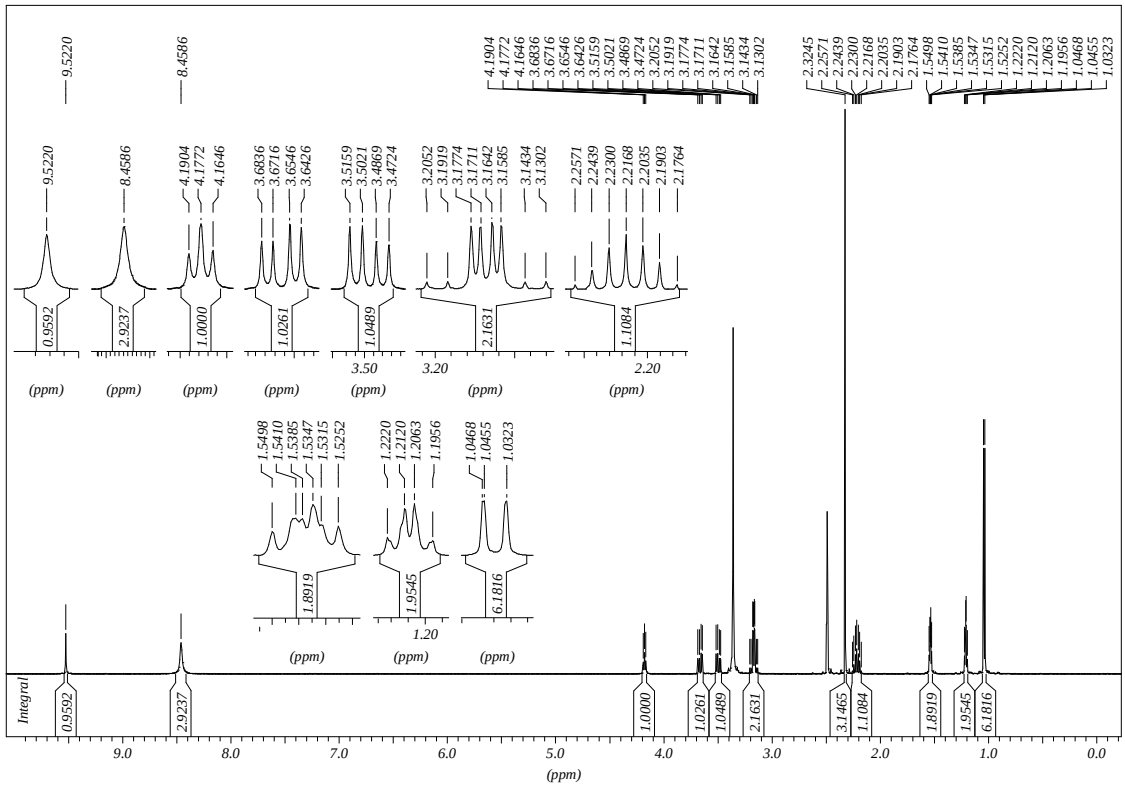
^{13}C and ^{1}H NMR Spectra

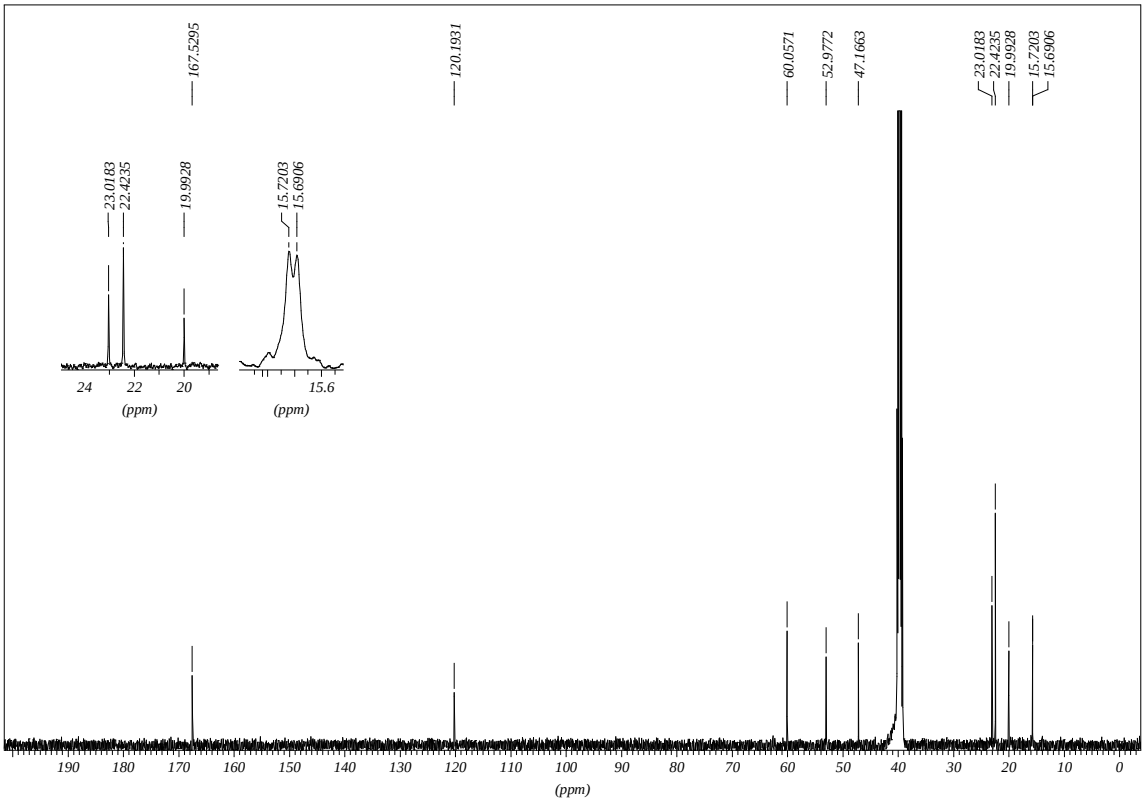
(*R*)-*N*-(*tert*-Butyloxycarbonyl)-*S*-(isobutyl)cysteinylsulfone-1-aminocyclopropanecarbonitrile (11)



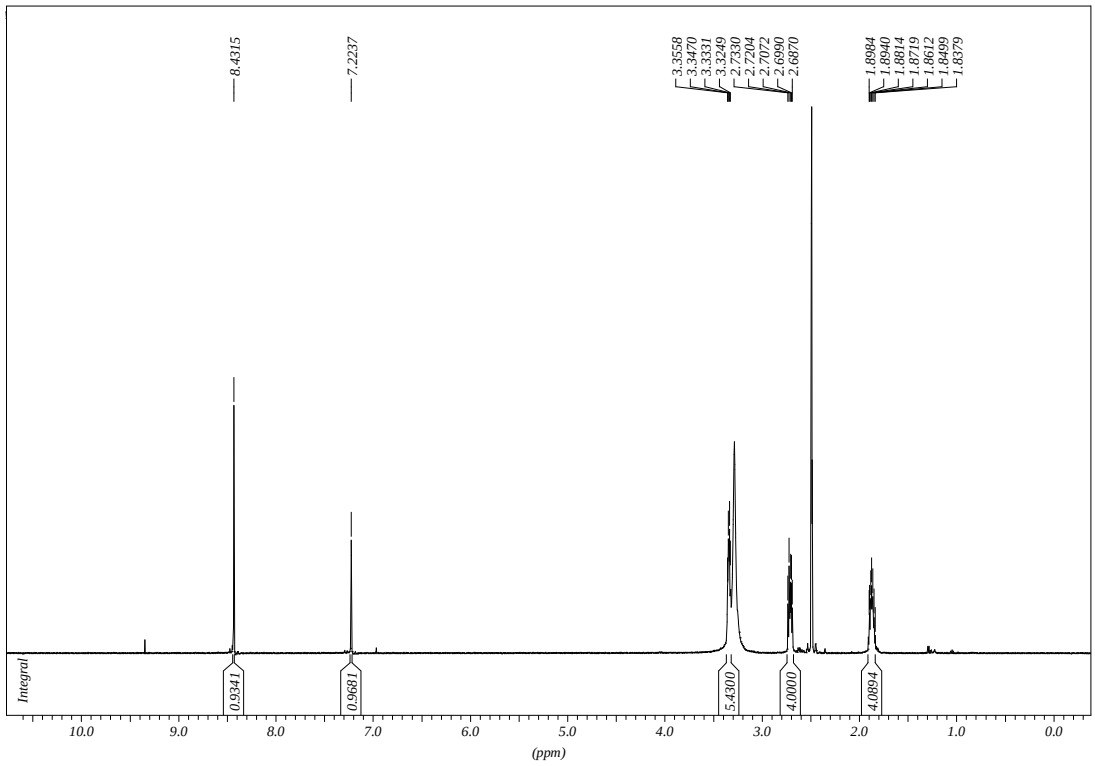


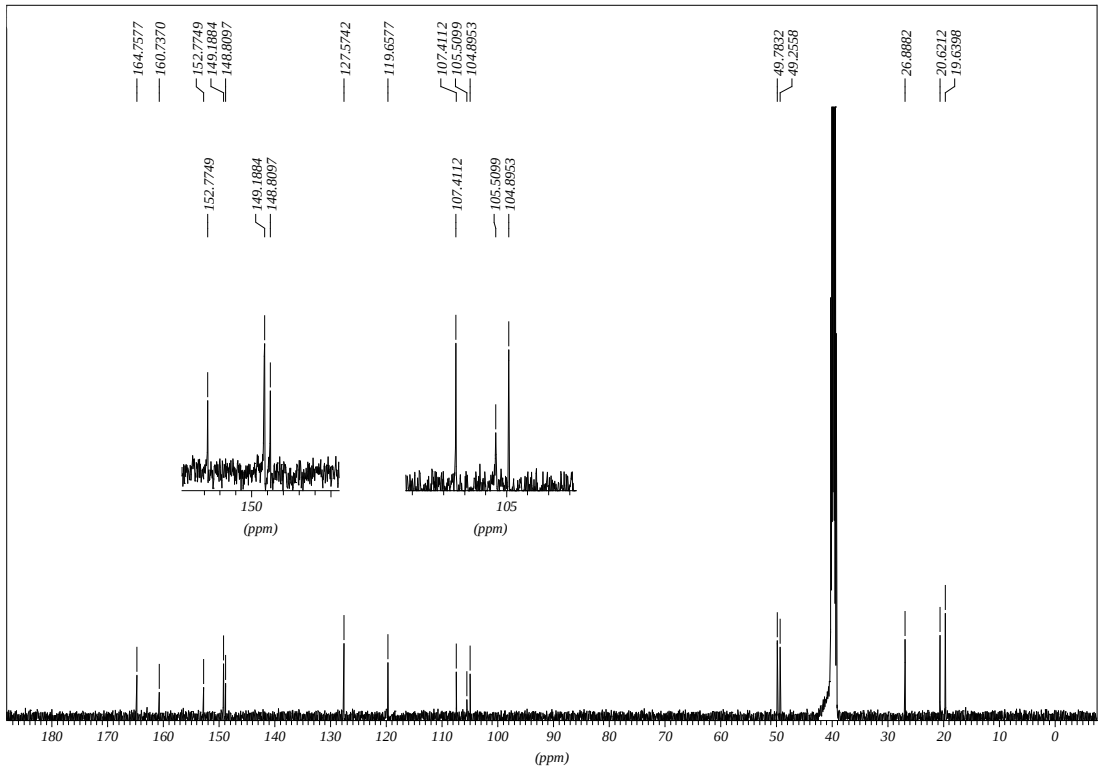
(*R*)-*S*-(Isobutyl)cysteinylsulfone-1-aminocyclopropanecarbonitrile methanesulfonate (12)



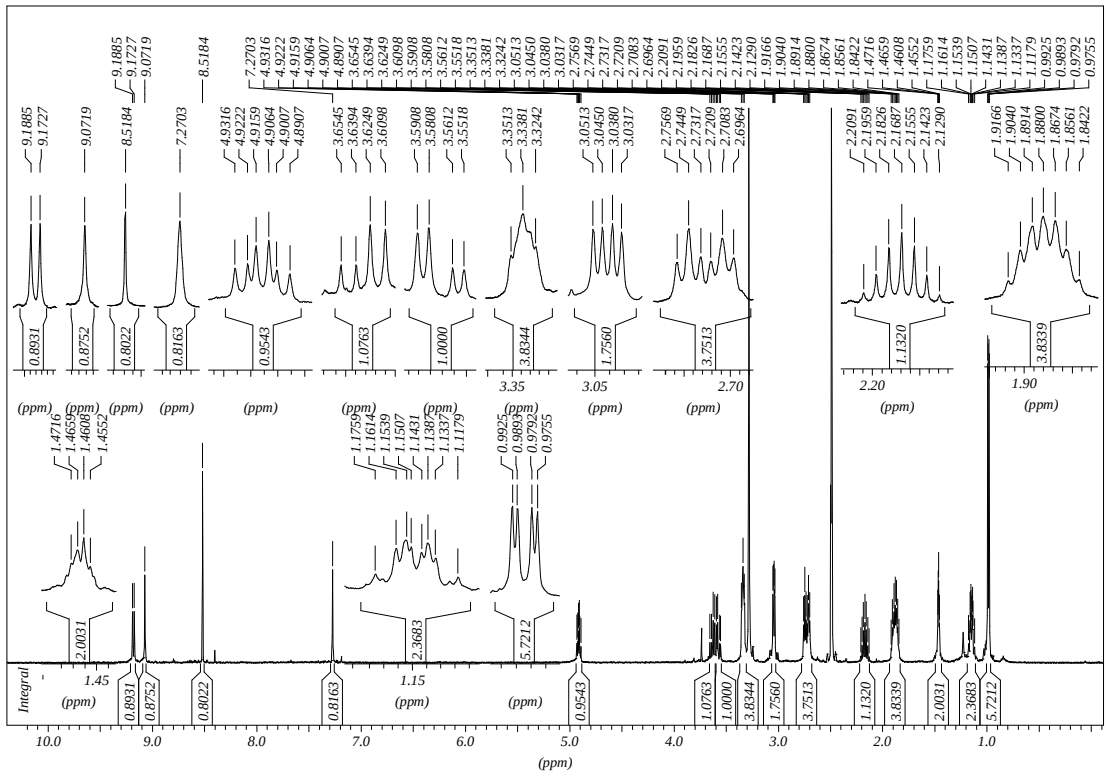


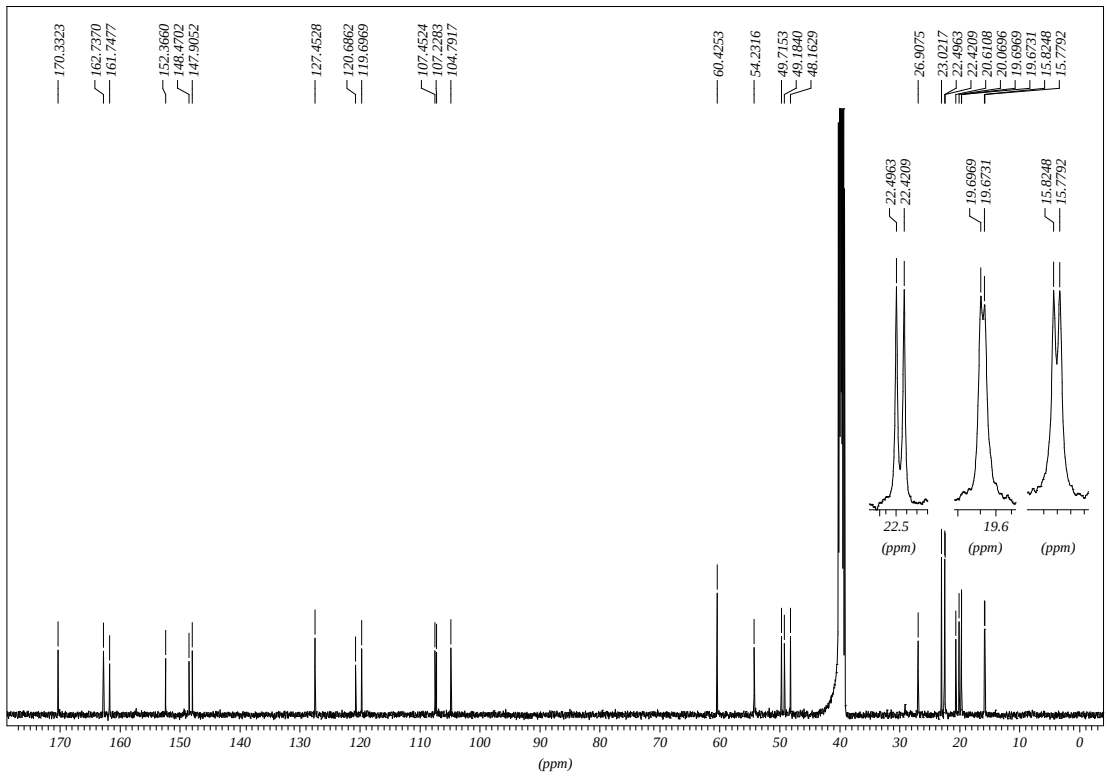
2,3,6,7-Tetrahydro-11-oxo-1*H*,5*H*,11*H*-[1]benzopyrano-[6,7,8-*ij*]quinolizine-10-carboxylic acid (14)



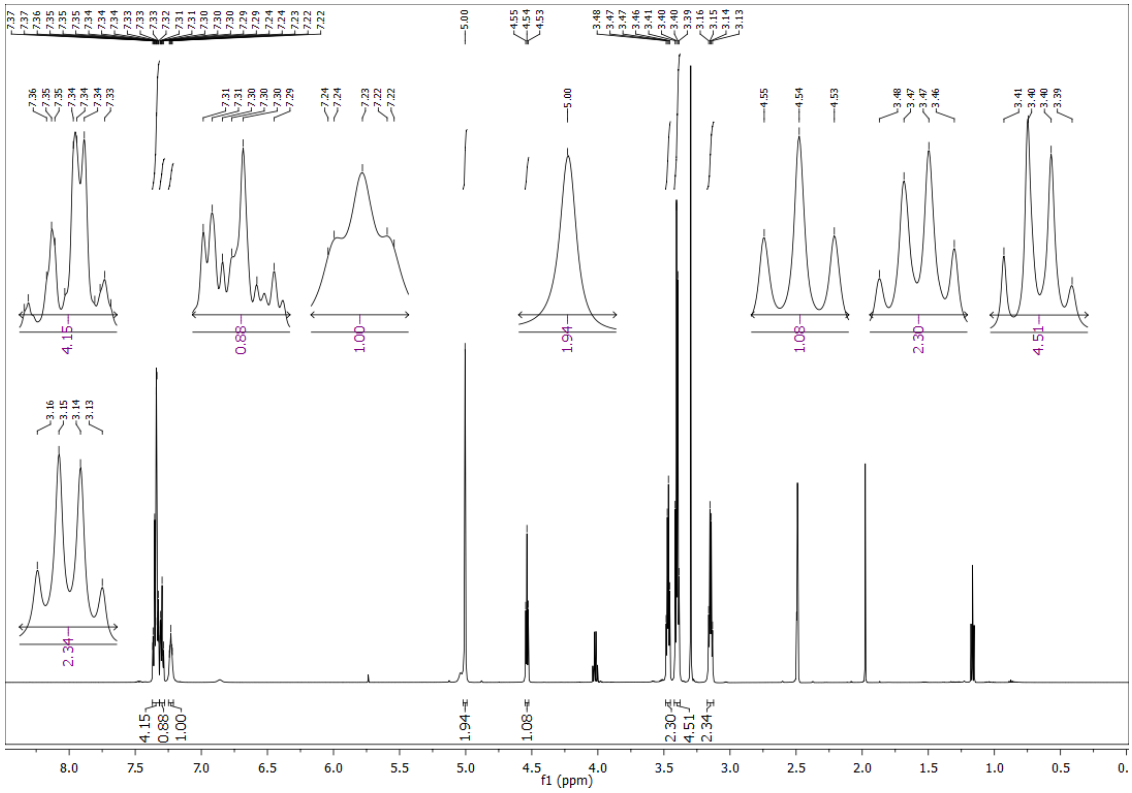


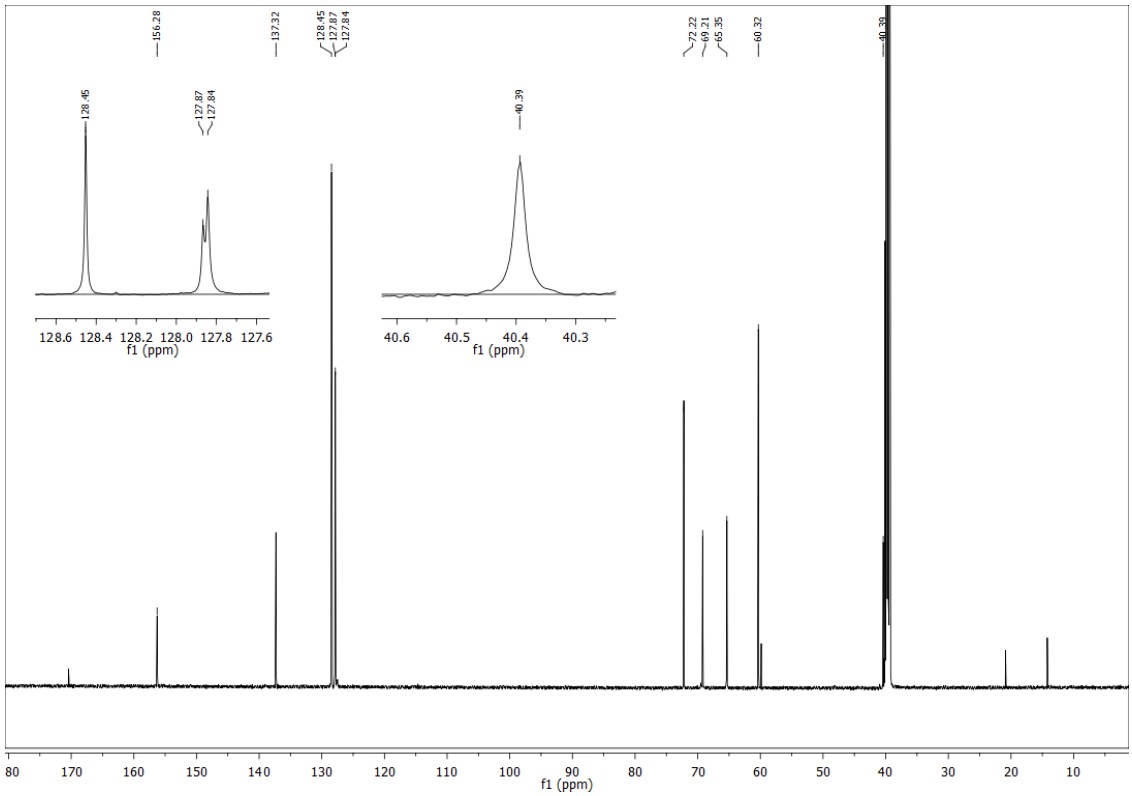
(*R*)-*N*-[(2,3,6,7-Tetrahydro-11-oxo-1*H*,5*H*,11*H*-[1]benzopyrano-[6,7,8-ij]quinolizin-10-yl)carbonyl]-*S*-(isobutyl)cysteinylsulfone-1-aminocyclopropanecarbonitrile (6)



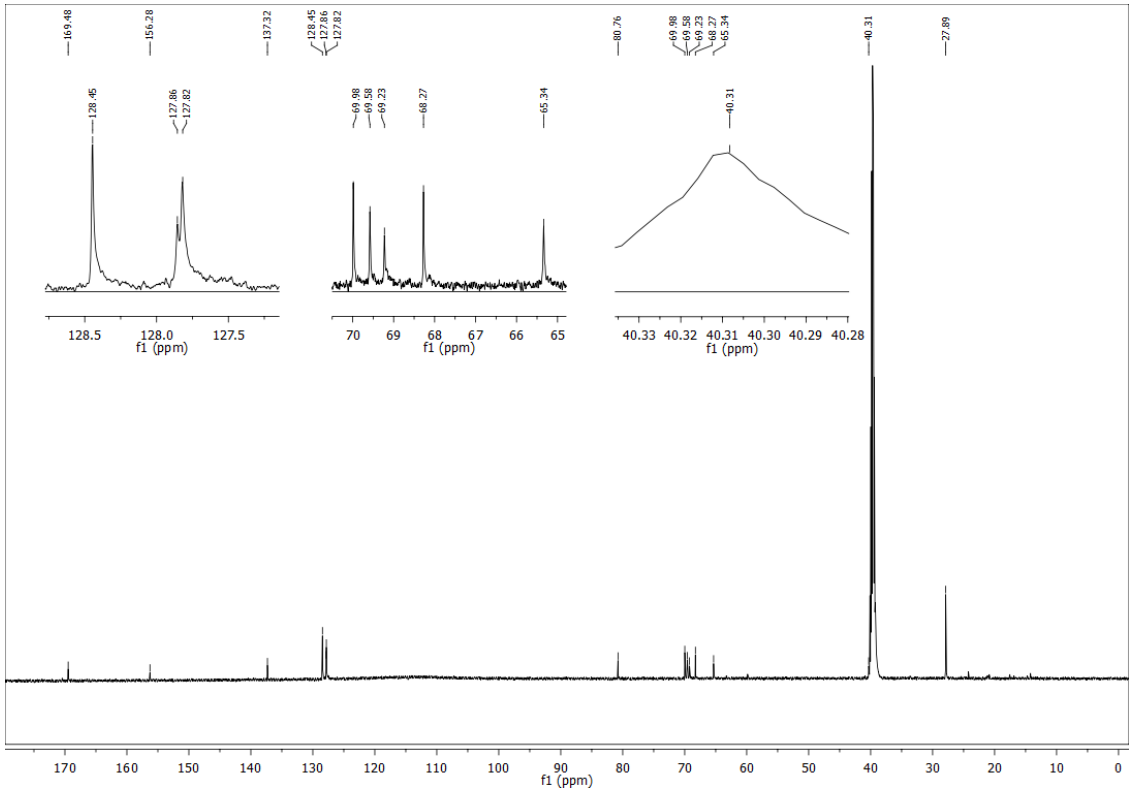


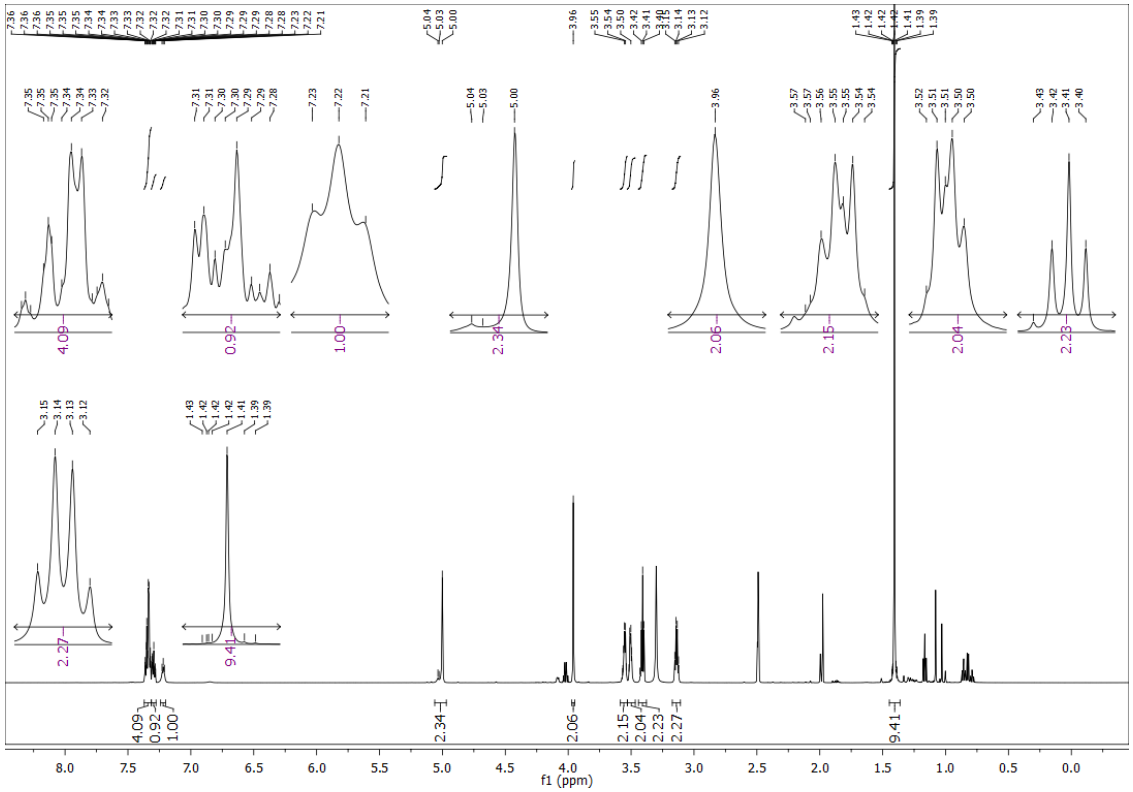
Benzyl 2-(2-hydroxyethoxy)ethylcarbamate (16)



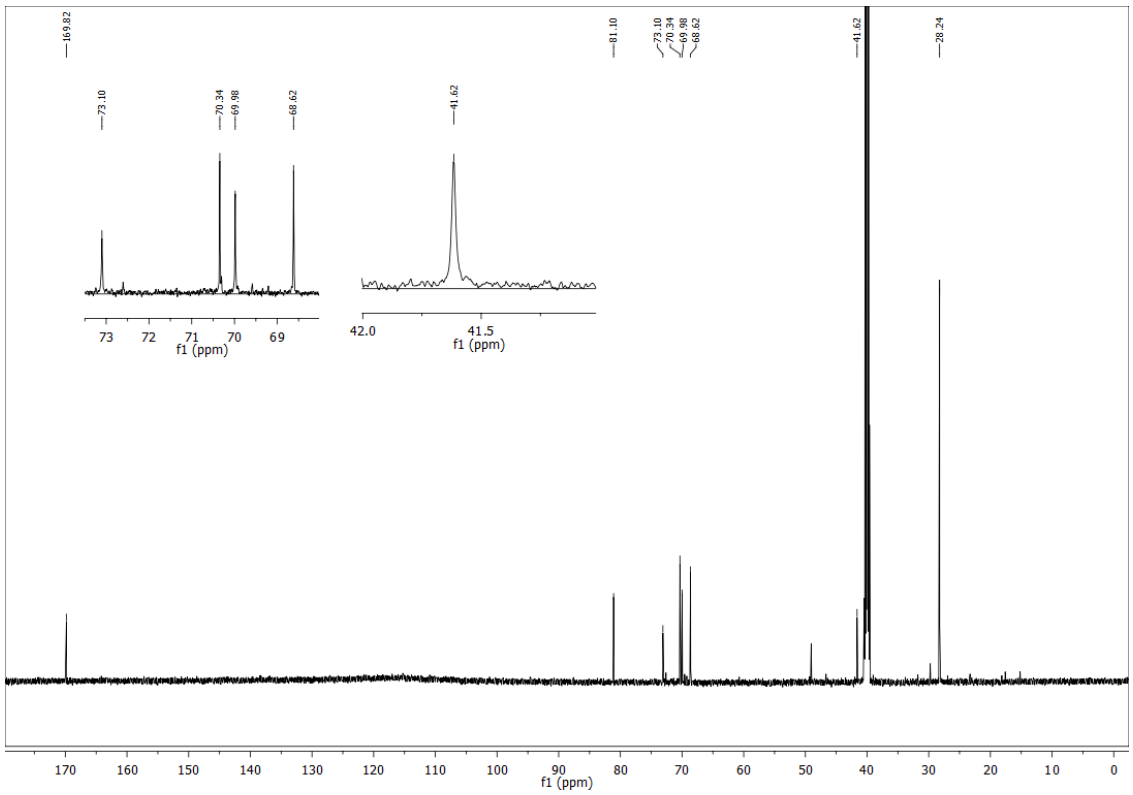


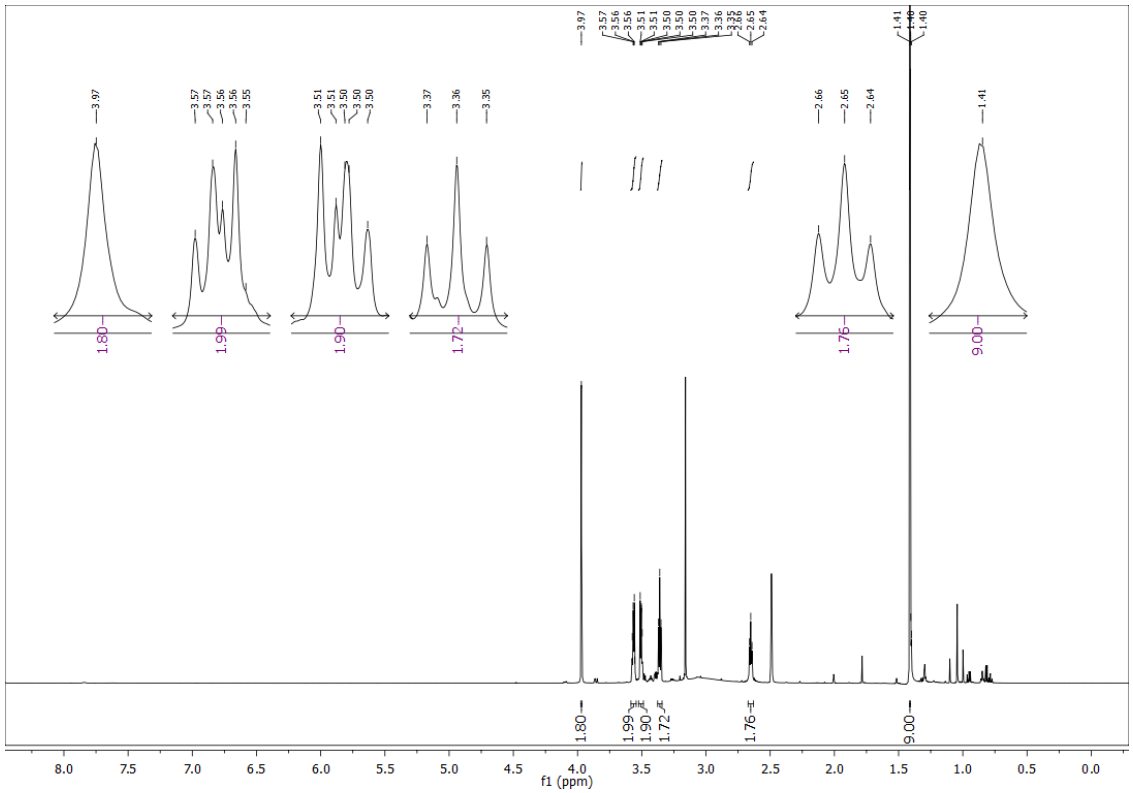
_N_-(Benzyloxycarbonyl)-2-(2-(2-aminoethoxy)ethoxy)acetate _tert_-butyl ester (17)



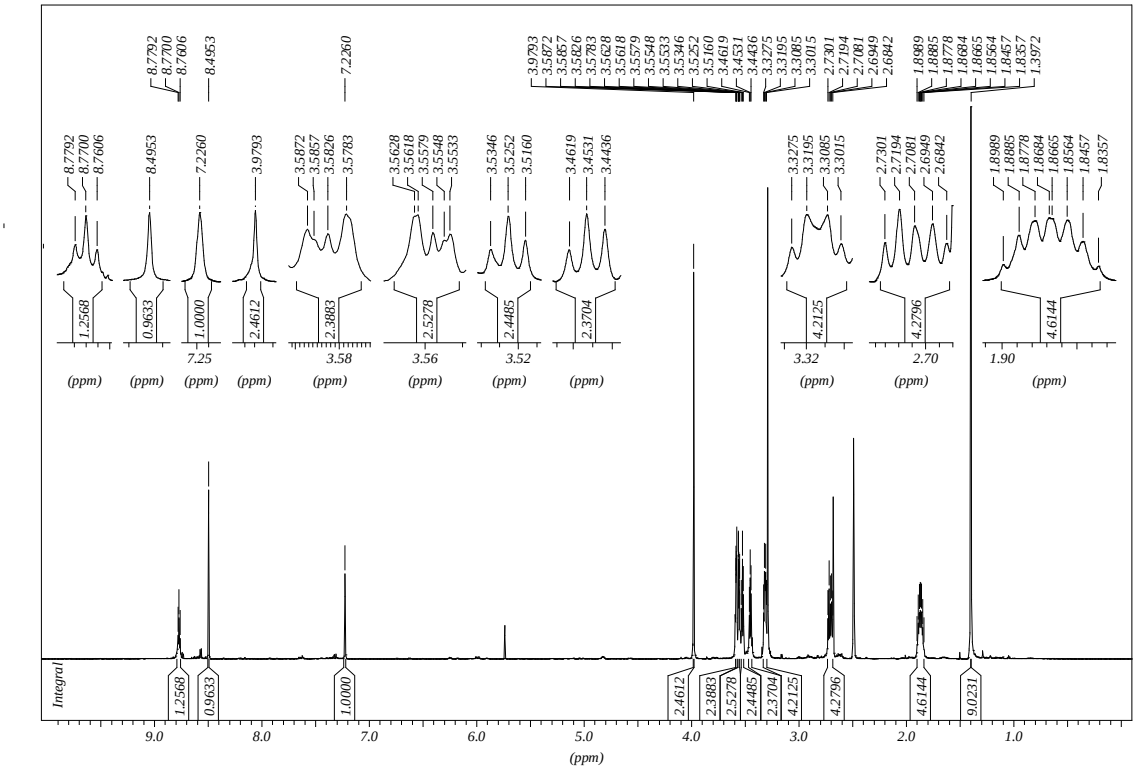


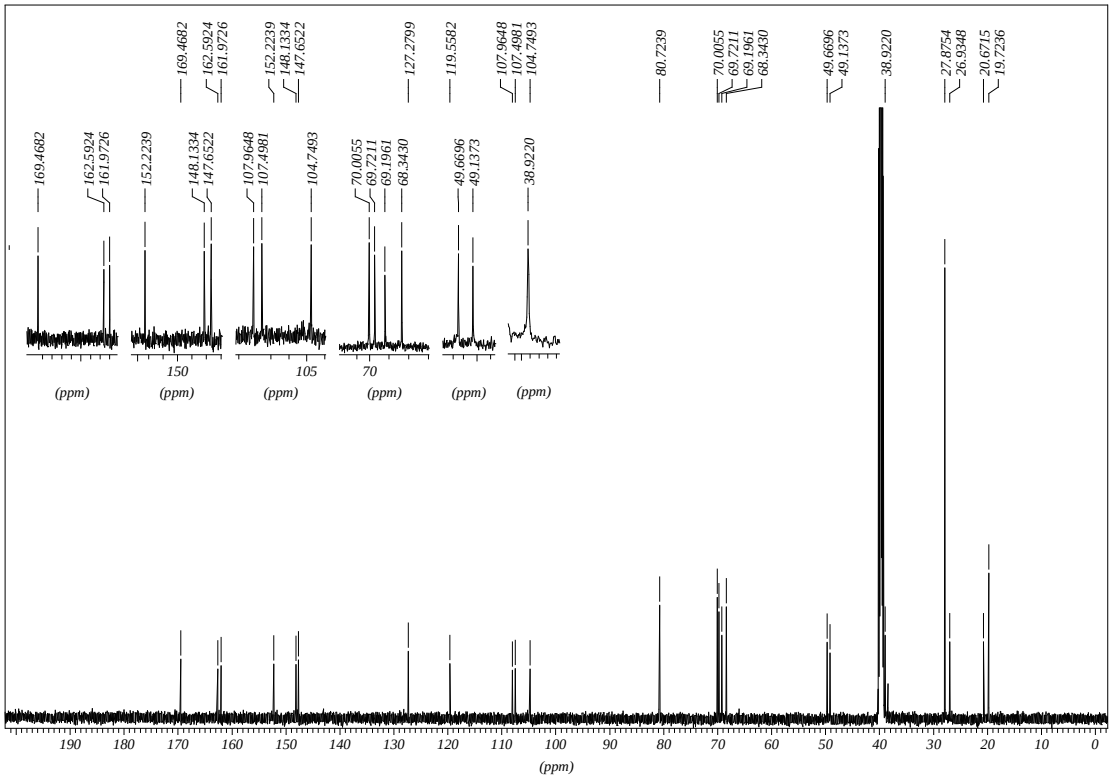
tert-Butyl 2-(2-(2-aminoethoxy)ethoxy)acetate (18)





tert-Butyl _N_-[(2,3,6,7-tetrahydro-11-oxo-1_H_,5_H_,11_H_-[1]benzopyrano-[6,7,8-ij]quinolizin-10-yl)carbonyl]-2-(2-(2-aminoethoxy)ethoxy)acetate _tert_-butyl ester (19)





***(R)*-*N*-[*N*-[(2,3,6,7-Tetrahydro-11-oxo-1*H*,5*H*,11*H*-[1]benzopyrano-[6,7,8-ij]quinolizin-10-yl)carbonyl]-2-(2-(2-aminoethoxy)ethoxy)acetyl]-*S*-(isobutyl)cysteinylsulfone-1-aminocyclopropanecarbonitrile (7)**