Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

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Selective Activity Based Probes for Cysteine Cathepsins

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Synthesis and Characterization of Activity Based Probes 2, 4 and 5

Abbreviations:

arom. = aromatic

Boc = *tert*-butyloxycarbonyl

DIPEA = diisopropyl-ethyl amine

DMF = dimethylformamide

ESI-MS = electrospray ionisation mass spectrometry

equiv. = equivalents

Fmoc = 9-fluorenylmethoxycarbonyl

HBTU = O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate

HOBt = 1-hydroxybenzotriazol

HPLC = high performance liquid chromatography

LC-MS = liquid chromatography mass spectrometry

NMR = nuclear magnetic resonance

RT = room temperature

TFA = trifluoro-acetic acid

General methods

Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification. All solvents used were of HPLC grade. Reactions were analyzed by thin-layer chromatography on Merck 50x100 mm silica gel 60 aluminium sheets with fluorescent indicator or LC-MS. Column chromatography was carried out with Merck silica gel 60 (0,040-0,063 mm). Reverse-phase HPLC was performed on a C18 column (Sun Fire 50x100 mm, Waters or XBridge^{TM}Prep C18, 5 μm, 10x100mm, Waters). LC/MS data were acquired using a HP-Agilent 1100 MSD system. NMR-data were recorded on a Bruker DRX-400 system in d_6-DMSO. Fluorescence assays was measured with a Tecan SAFIRE II spectrometer.

General Procedures for Solid-Phase Peptide Synthesis

Probes **2** and **4** were synthesized using standard solid-phase peptide synthesis. The 2-chlorotrityl-chloride resin (Novabiochem, loading 1.4 mmol/g) was used as solid support. For loading of the resin (100 mg, 0.14 mmol) 2 equiv. Fmoc-protected amino acid and 3 equiv. DIPEA (74 μl, 0.42 mmol) were dissolved in 2 ml CH_2Cl_2 and the reaction mixture was added to the resin. The reaction mixture was shaken overnight at room temperature. The resin was washed three times with 2 ml CH_2Cl_2 and 2 ml DMF. For Fmoc-deprotection the resin was treated two times for 15 min. with 2 ml 30% piperidine/DMF. A standard protocol was used for solid phase peptide synthesis: 4 equiv. Fmoc-protected amino acid, 4 equiv. HBTU (212 mg, 0.56 mmol), 4 equiv. HOBt (76 mg, 0.56 mmol) and 8 equiv. DIPEA (196 μl, 1.12 mmol) were dissolved in 2 ml CH_2Cl_2/DMF (1/1; v/v). The reaction mixture was stirred 20 min. at room temperature and then added to the resin. The reaction mixture was shaken for 2 hours at room temperature.

For the cleavage of the peptide from the solid-phase the resin was treated two times for 15 min. with 2 ml 2% TFA/CH_2Cl_2 (v/v). The solvent was co-evaporated with toluene under reduced pressure and the product was purified by preparative HPLC (H_2O+0.1% TFA; 10-95% CH_3CN, 15 min, 120 ml/min, column: Sun Fire 50x100 mm, Waters).

Compound **2**

The compound was prepared on solid support (100 mg of 2-chlorotrityl-chloride resin, loading: 1.4 mmol/g) according to the general procedure and purified by HPLC (H_2O+0.1% TFA; 10-95% CH_3CN, 15 min, 120 ml//min, column: Sun Fire 50x100 mm, Waters). Yield: 70 mg (0.063 mmol), 45%. ^{1}H-NMR (400 MHz, d_6-DMSO): δ = 9.35 (bs, 1H, CO_2H), 8.71 (m, 1H, H_{arom}), 8.27 (d, *J* = 8.8 Hz, 1H, H_{arom}), 8.27-8.08 (m, 3H, H_{arom}), 7.96-7.93 (dd, J_1 = 9.6 Hz, J_2 = 2.8 Hz, 3H, H_{arom}), 7.87 (d, *J* = 8.8 Hz, 2H, H_{arom}), 7.76 (d, *J* = 8.0 Hz, 1H, H_{arom}), 7.43 (t, *J* = 8.0 Hz, 1H, H_{arom}), 7.37 (t, *J* = 8.4 Hz, 1H, H_{arom}), 6.97 (d, *J* = 9.6 Hz, 2H, H_{arom}), 6.92 (d, *J* = 9.2 Hz, 2H, H_{arom}), 6.65 (d, *J* = 8.0 Hz, 1H, H_{arom}), 4.35 (m, 1H, CαH), 4.2 (m, 1H, CαH), 3,75 (m, 2H, N(CH_2)$_2$), 3,52 (m, 2H, N(CH_2)$_2$), 3.45-3.29 (m, 6H), 3.22-3.20 (m, 2H), 3.13 (s, 6H, N(CH_3)$_2$), 3.0 (m, 2H), 2.82 (m, 2H), 2.25-2.10 (m, 4H), 2.02 (m, 2H), 1.80-1.65 (m, 4H), 1.54 (m, 7H), 1.37 (m, 2H), 1.25 (t, *J* = 7.2 Hz, 7H). ESI-MS: Calculated: $[M/2]^+$ = 558.7, found: $[M/2]^+$ = 558.8.

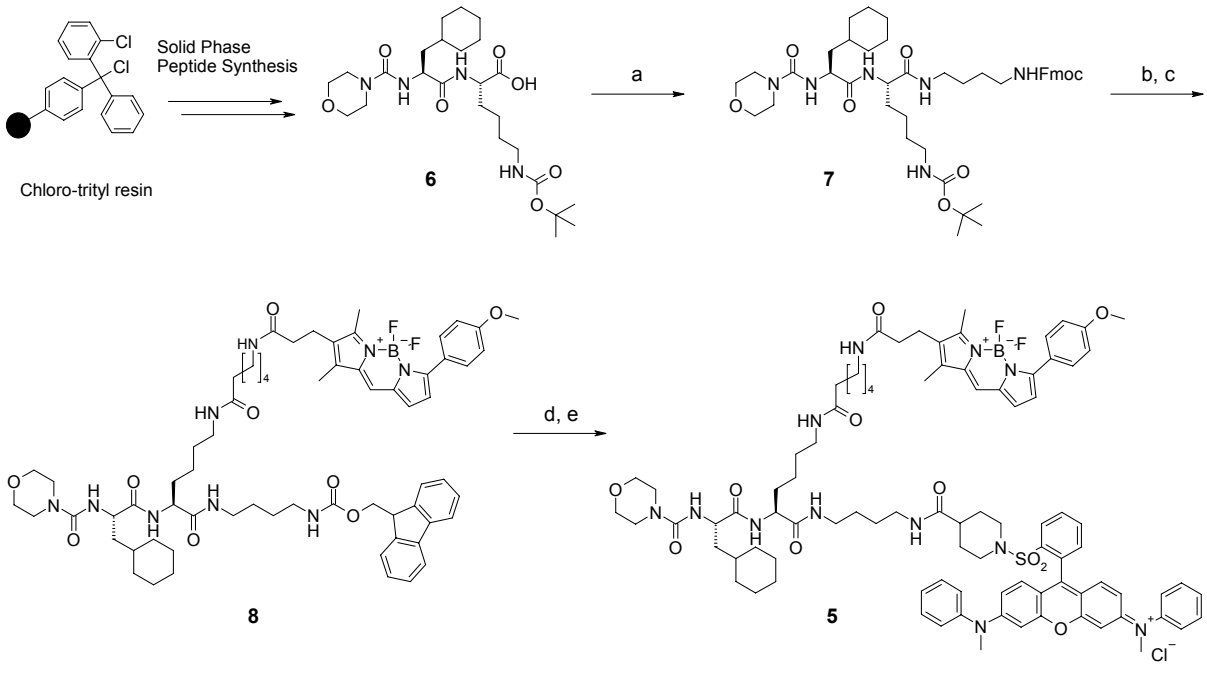
Compound **4**

The compound was prepared on solid support (100 mg of 2-chlorotrityl-chloride resin, loading: 1.4 mmol/g) according to the general procedure and purified by HPLC (H_2O+0.1% TFA; 10-95% CH_3CN, 15 min, 120 ml//min, column: Sun Fire 50x100 mm, Waters). Yield: 120 mg (0.115 mmol), 82%. ^{1}H-NMR (400 MHz, d_6-DMSO): δ = 8.71 (m, 1H, H_{arom}), 8.47 (d, *J* = 8.8 Hz, 1H, H_{arom}), 8.20 (t, *J* = 7.2 Hz, 1H, H_{arom}), 8.15-8.09 (m, 2H, H_{arom}), 8.04 (d, *J* = 8.0

Hz, 1H, H_{arom}), 8.03-7.92 (m, 3H, H_{arom}), 7.52 (t, *J* = 8.0 Hz, 1H, H_{arom}), 7.46 (t, *J* = 8.4 Hz, 1H, H_{arom}), 6.95 (m, 3H, H_{arom}), 4.35 (m, 1H, Cα*H*), 4.26-4.18 (m, 2H, Cα*H*), 3,65 (m, 3H, O(C*H*$_2$)$_2$), 3.55 (m, 1H, O(C*H*$_2$)$_2$), 3.37-3.30 (m, 8H), 3.12 (s, 6H, N(C*H*$_3$)$_2$), 2.21 (m, 2H), 2.00 (m, 1H), 1.18 (m, 2H), 1.70-1.60 (m, 5H), 1.58-1.50 (m, 5H), 1.40-1.30 (m, 4H), 1.15 (m, 4H), 0.85 (m, 2H). ESI-MS: Calculated: $[M/2]^+$ = 521.2, found: $[M/2]^+$ = 521.3.

Combination of Solid-Phase and Solution-Phase Synthesis

Compound **5** was synthesized using a combination of solid support and solution-phase synthesis (Scheme S1). Tripeptide **6** was prepared on the solid support using the chloro-trityl resin. Compound **6** was coupled to N-Fmoc-butane-1,4-diamine hydrochloride to yield compound **7**, which was further modified in solution. After removal of the Boc-group from the lysine side chain of compound **7** BodipyTMR X-OSu was coupled to the peptide under standard conditions and product **8** was purified by preparative HPLC. Following removal of the C-terminal Fmoc-group of compound **8** QSY7-OSu was coupled to the peptide and the final product **5** was purified by preparative HPLC.



Supporting Information Scheme S1: Synthesis of compound **5** using a combination of solid support and solution-phase synthesis: a) 1.2 equiv. HOBt, 1.3 equiv. HBTU, 2 equiv. N-Fmoc-butane-1,4-diamine hydrochloride, 3 equiv. DIPEA, DCM/DMF (1/1), 12 h; b) 50% TFA/DCM, 10 min.; c) 1 equiv. BodipyTMR X-OSu, 6 equiv. DIPEA, DMF, 12 h; d) Et_2NH/DMF (1/4), 30 min., RT; e. 1 equiv. QSY7-OSu, 6 equiv. DIPEA, DMF, 12 h.

Compound **6**

The compound was prepared on solid support (100 mg of 2-chlorotrityl-chloride resin, loading: 1,4 mmo/g) according to the general procedure and purified by HPLC (H_2O+0.1% TFA; 10-95% CH_3CN, 15 min, 120 ml//min, column: Sun Fire 50x100 mm, Waters). Yield: 710 mg (1.385 mmol), 99%. ^{1}H-NMR (400 MHz, d_6-DMSO): δ = 7.89 (d, *J* = 6.4 Hz, 1H, N*H*), 6.74 (m, 1H, N*H*), 6.44 (d, *J* = 6.8 Hz, 1H, N*H*), 4.22-4.17 (m, 1H, Cα*H*), 4.12 (m, 1H, Cα*H*), 3.54-3.51 (m, 4H, O(C*H*$_2$)$_2$), 3.32-3.25 (m, 6H, N(C*H*$_2$)$_2$, C*H*$_2$NHC(O)), 2.89-2.86 (m, 2H, CαHC*H*$_2$), 1.70-1.55 (m, 8H), 1.47 (t, *J* = 5.6 Hz, 2H), 1.37 (s, 9H, C(C*H*$_3$)$_3$), 1.34 (m, 2H), 1.13 (m, 3H), 0.87 (m, 2H). ^{13}C-NMR (100 MHz, d_6-DMSO): δ = 173.7, 172.7, 158.0, 157.7, 157.3, 155.4, 118.7, 115.7, 77.2, 65.9, 52.2, 51.9, 44.0, 33.5, 33.2, 31.8, 31.2, 29.2, 28.2, 26.0, 25.7, 25.6, 22.4. ESI-MS: Calculated: $[M+H]^+$ = 513.6, found: $[M+H]^+$ = 513.3.

Compound **7**

Compound **6** (718 mg, 1.4 mmol), 1.2 equiv. HOBt (227 mg, 1.68 mmol), 1.3 equiv. HBTU (690 mg, 1.82 mmol) and 2 equiv. DIPEA (489 µl mg, 2.8 mmol) were solved in 2 ml CH_2Cl_2/DMF (1/1; v/v) and stirred for 20 min. at room temperature. Subsequently 2 equiv. (869 mg, 2.8 mmol) N-Fmoc-butane-1,4-diamine hydrochloride and 1.5 equiv. DIPEA (367 µl, 2.1 mmol) were added to the reaction mixture, which was stirred over night. The solvent was removed and the remaining residue was purified on silica gel (gradient: CH_2Cl_2/1-5% MeOH (v/v)). Yield: 620 mg (0.770 mmol), 55%. ^{1}H-NMR (400 MHz, d_6-DMSO): δ = 7.98 (d, *J* = 8.0 Hz, 2H, Fmoc), 7.89 (m, 1H, N*H*), 7.77 (d, *J* = 7.6 Hz, 2H, Fmoc), 7.49 (t, *J* = 7.6 Hz, 2H, Fmoc), 7.41 (t, *J* = 6.8 Hz, 2H, Fmoc), 6.80 (m, 1H, N*H*), 6.63 (d, *J* = 7.6 Hz, 1H, N*H*), 4.34 (d, *J* = 7.6 Hz, 2H, Fmoc), 4.30 (m, 1H, Fmoc), 4.27-4.25 (m, 2H, Cα*H*), 3.62-3.52 (m, 4H, O(C*H*$_2$)$_2$), 3.42 (m, 4H, N(C*H*$_2$)$_2$), 3.36-3.32 (m, 4H, 2 x C*H*$_2$NHC(O)), 3.0 (m, 3H), 2.89-2.86 (m, 2H, CαHC*H*$_2$), 1.70-1.60 (m, 8H), 1.50 (m, 4H), 1.36 (s, 9H, C(C*H*$_3$)$_3$), 1.25-1.10 (m, 6H), 0.80-0.90 (m, 2H). ^{13}C-NMR (100 MHz, d_6-DMSO): δ = 173.0, 171.2, 158.6, 158.3, 158.0, 157.7, 157.5, 156.0, 155.5, 143.9, 140.7, 127.5, 127.0, 125.1, 120.0, 77.2, 65.9, 65.1, 52.5, 52.3, 48.5, 46.7, 44.0, 38.7, 38.2, 33.6, 33.1, 31.8, 31.6, 29.1, 28.2, 26.7, 26.2, 26.0, 25.7, 25.6, 22.6. ESI-MS: Calculated: $[M+H]^+$ = 805.0, found: $[M+H]^+$ = 805.4.

Compound **8**

For removal of the Boc-group compound **7** (6.6 mg, 8.218 µmol) was dissolved in 1 ml 50% TFA/CH_2Cl_2 and the reaction mixture was stirred for 10 min. at room temperature. The solvent was co-evaporated with toluene and the crude material was dissolved in 1 ml DMF. Subsequently 1 equiv. BodipyTMR X-OSu (5 mg, 8.218 µmol) and 6 equiv. DIPEA (8.6 µl, 49.3 µmol) were added to the reaction mixture, which was stirred for 12 h at room temperature. The solvent was removed under reduced pressure and the final product was purified by preparative HPLC (H_2O+0.05% TFA; 4-95% CH_3CN, 18 min, 4 ml/min, column:

XBridge^{TM}Prep C18, 5 μm (10x100mm), Waters). Yield: 6.8 mg (8.2 μmol), quantitative. ESI-MS: Calculated: $[M+Na]^+$ = 1221.3, found: $[M+Na]^+$ = 1221.6.

Compound **5**

For removal of the Fmoc-group compound **8** (6.8 mg, 8.2 μmol) was dissolved in 1 ml Et_2NH/DMF (1/4) and the reaction mixture was stirred for 30 min. at room temperature. The solvent was removed under reduced pressure and the crude material was dissolved in 1 ml DMF, followed by the addition of 1 equiv. QSY7-OSu (6.5 mg, 8.2 μmol) and 6 equiv. DIPEA (8.6 μl, 49.2 μmol). The reaction mixture was stirred at room temperature for 12 h. The solvent was removed under reduced pressure and the product was purified by preparative HPLC (H_2O+0.05% TFA; 4-95% CH_3CN, 18 min, 4 ml/min, column: XBridge^{TM}Prep C18, 5 μm (10x100mm), Waters). Yield: 13.3 mg (8.2 μmol), quantitative. ESI-MS: Calculated: $[M/2]^+$ = 808.4, found: $[M/2]^+$ = 808.5.

Methods and Materials for *in vitro* and cellular-based assays

Activity assays for probes 2, 4 and 5 against cysteine cathepsins S, K, B and L

For the in vitro enzyme activity assay the active cysteine proteases were dissolved in AHNP-buffer (150 mM Acetate/HEPES pH 6.5, 300 mM NaCl; 0.001% Pluronic; 5 – 100 mM cysteine depending on the enzyme) at a final concentration of 10 nM. The DMSO dissolved substrates 2, 4 and 5 were added at concentrations of 98 μM (probe 2) and 24 μM (probe 4 and 5) and fluorescence was measured with a Tecan SAFIRE II spectrometer. The final DMSO concentration in the assay did not exceed 1% (v/v). Steady-state kinetics were fitted by non-linear least squares regression using $v=[A]V/(K_M+(1+([A]/K_{si}))[A])$, where v is the initial velocity, V the maximal rate, K_M the Michaelis Menten constant and K_{si} the constant for substrateinhibition.

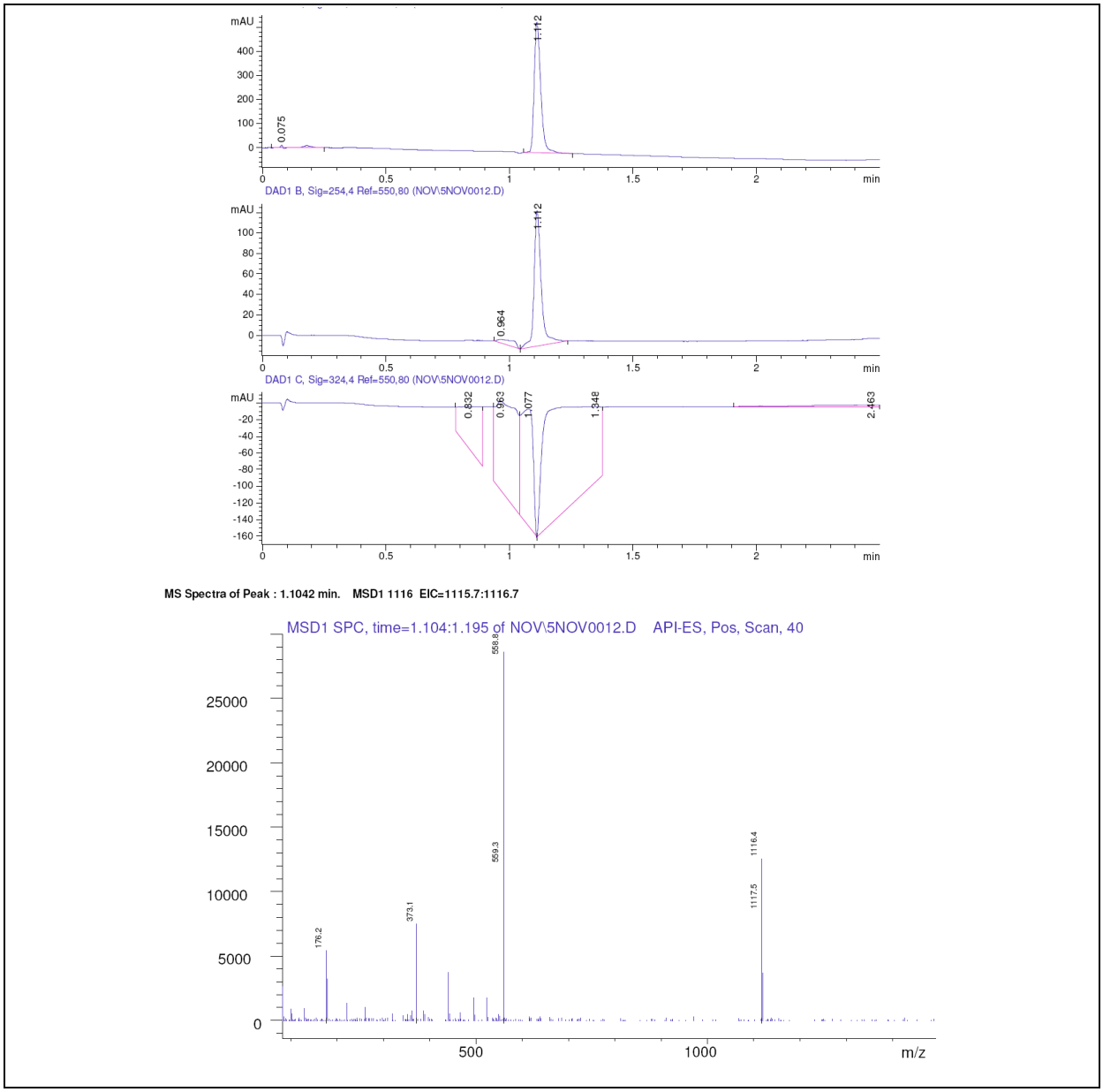
Cellular assay for ABP 5 in HaCaT cells

The HaCaT cell line was purchased from American Type Culture Collection and was maintained in DMEM growth medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin, 100 IU/ml and streptomycin, 100 μg/ml and cultivated in humidified atmosphere containing 5 % CO_2 at 37 °C.

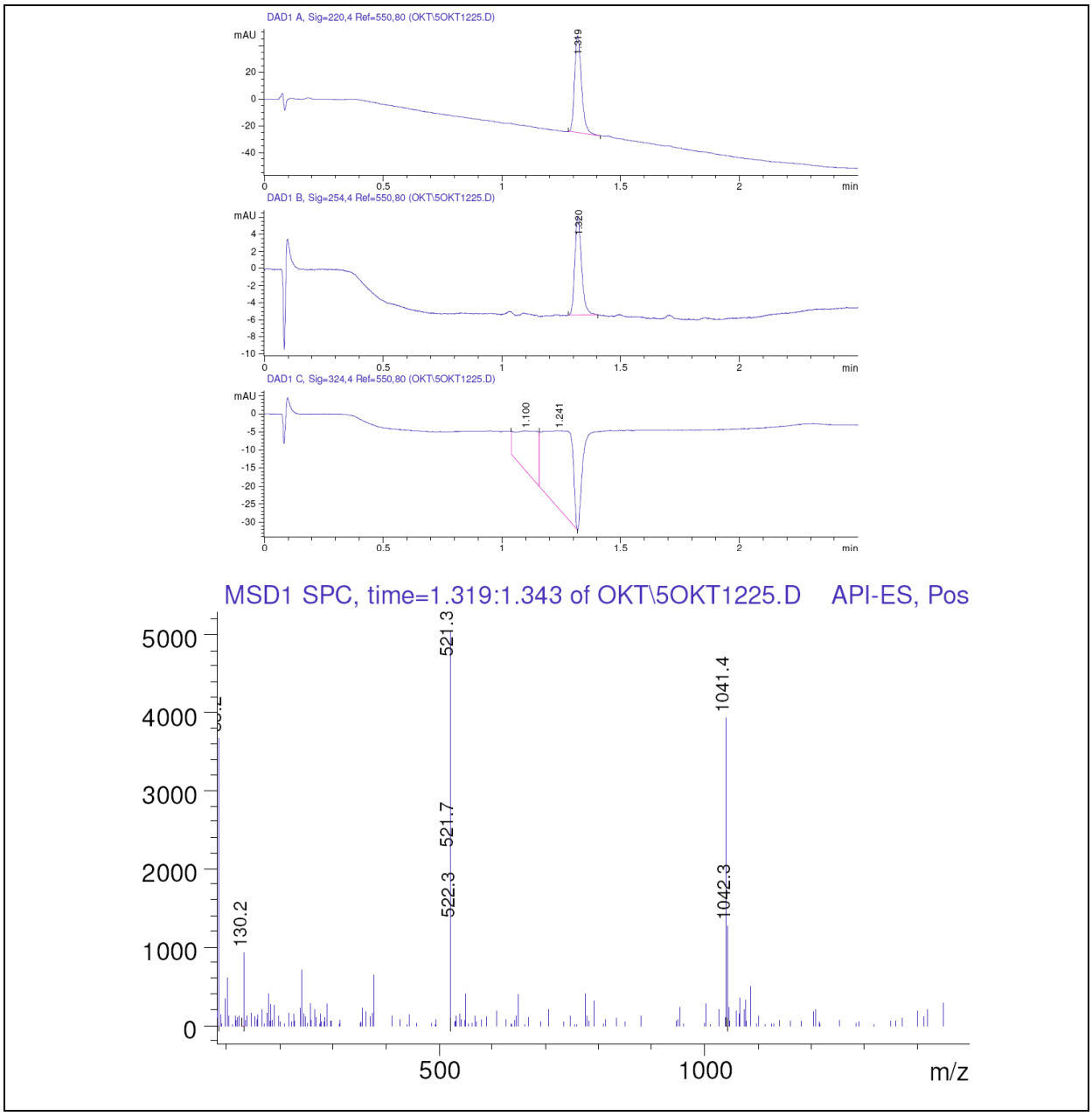
3×10^5 cells were seeded into a chambered glass well and cultivated overnight. The growth medium was exchanged in the morning, and cells were pretreated with 15 μM E-64d, 15 μM specific cathepsin S/L inhibitor or DMSO. After 2 hours a solution of compound **5** in DMSO in a negligible volume was added to all wells to a final concentration of 8 μM. Fluorescence in

living cells was observed and digital images acquired after 3 hours using an Olympus IX 71 epifluorescent microscope equipped with a M41002 filter cube.

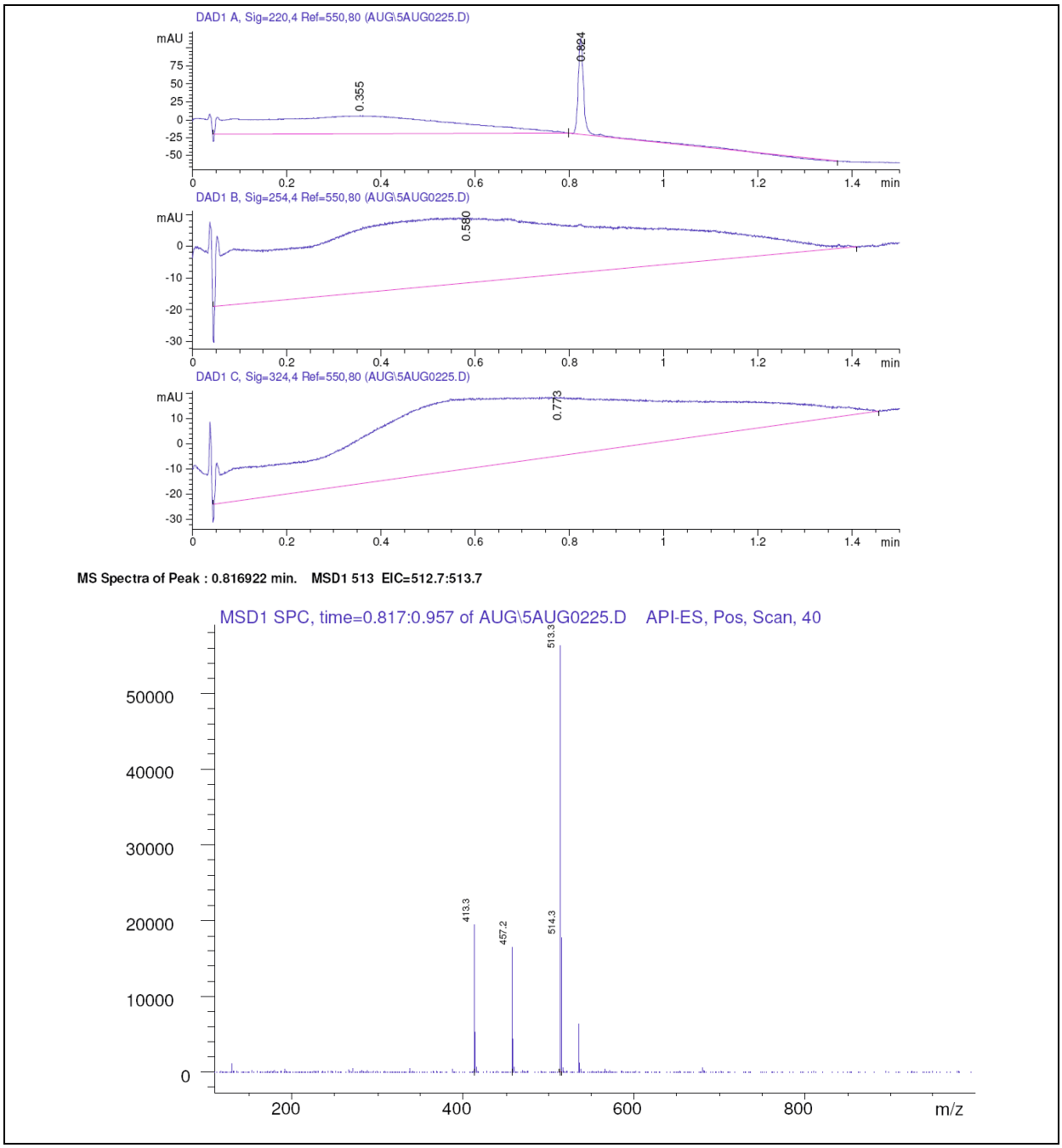
LC-MS spectra and NMR spectra



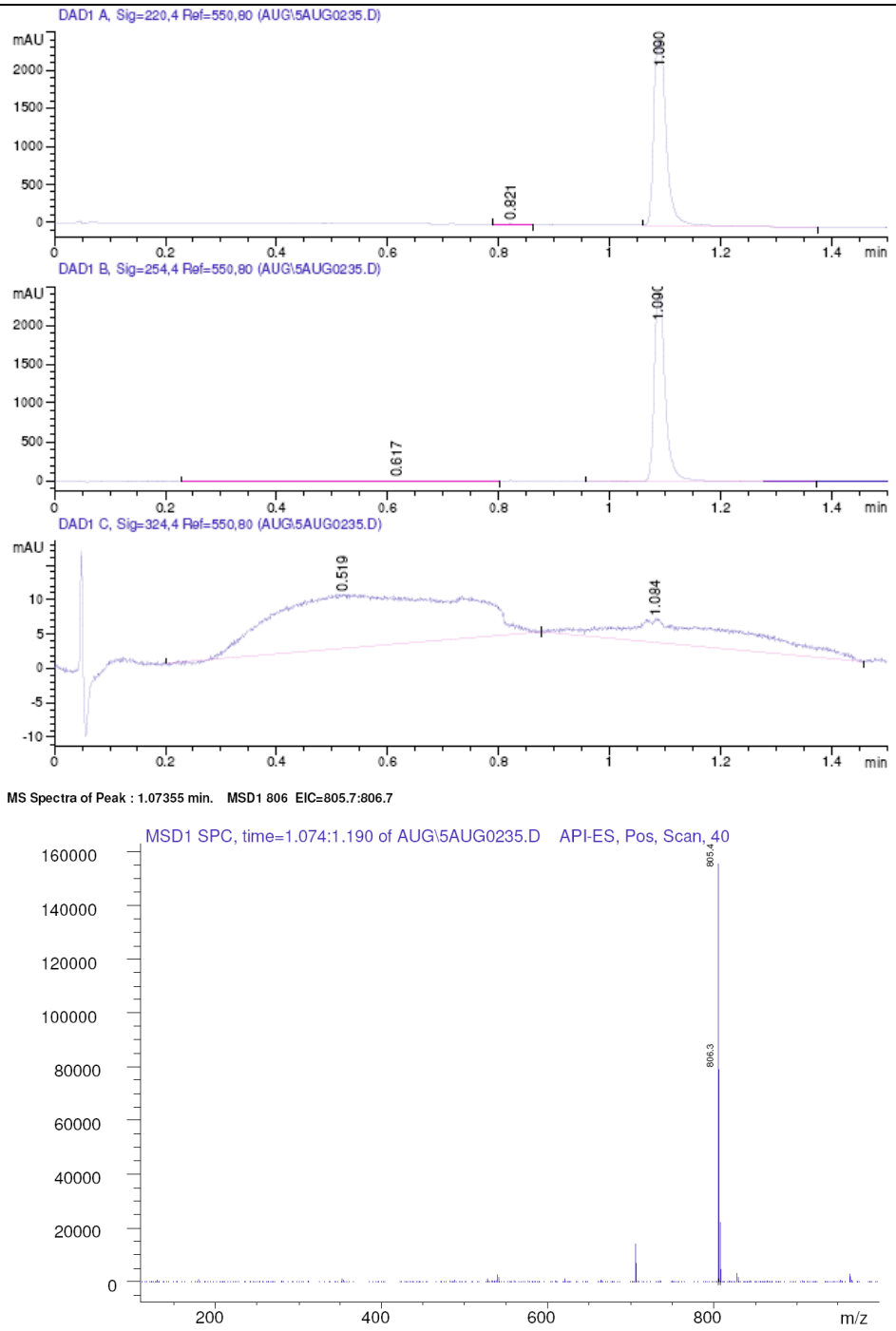
Supporting Information Figure S1: LC-MS of compound **2**.



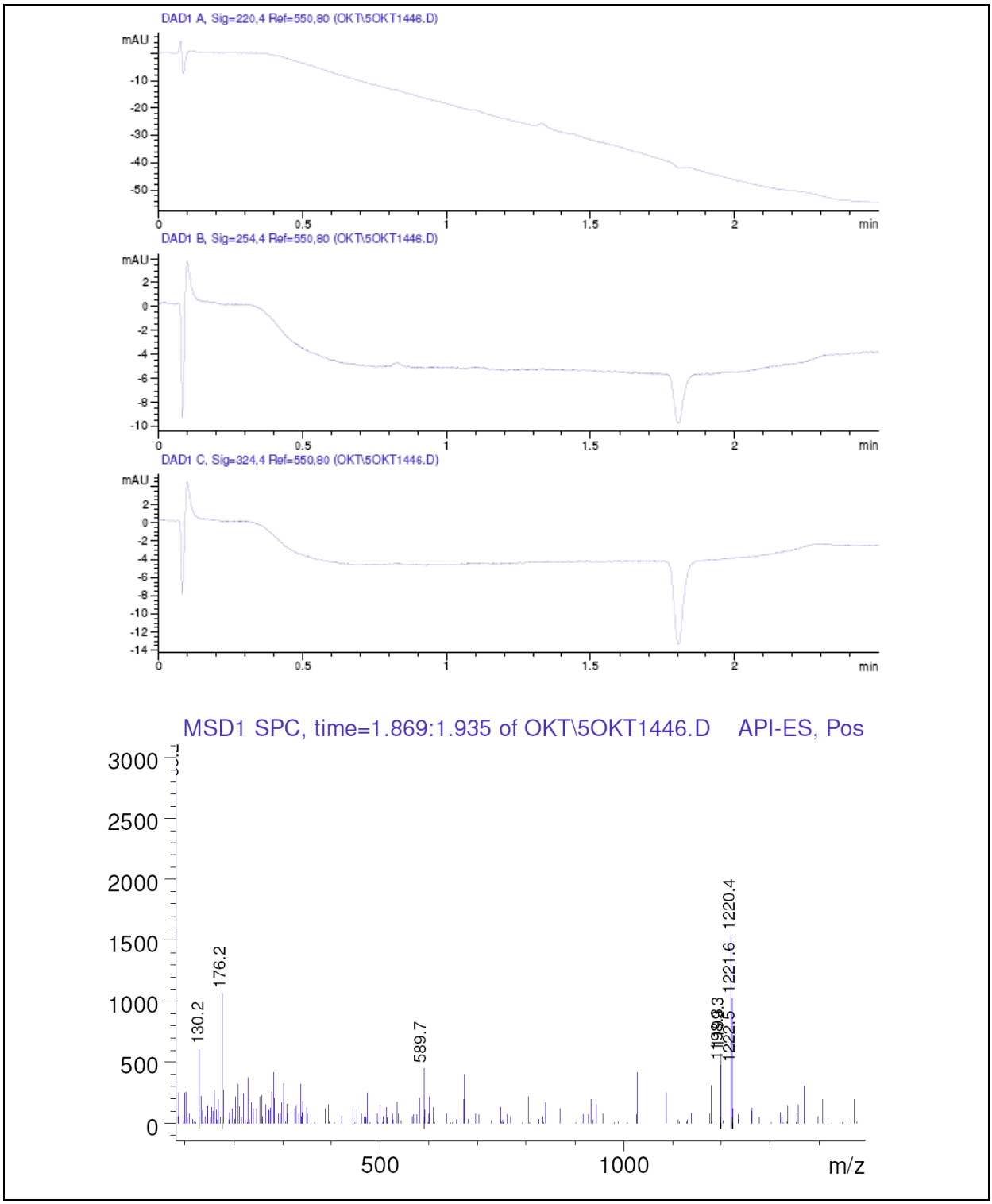
Supporting Information Figure S2: LC-MS of compound **4**.



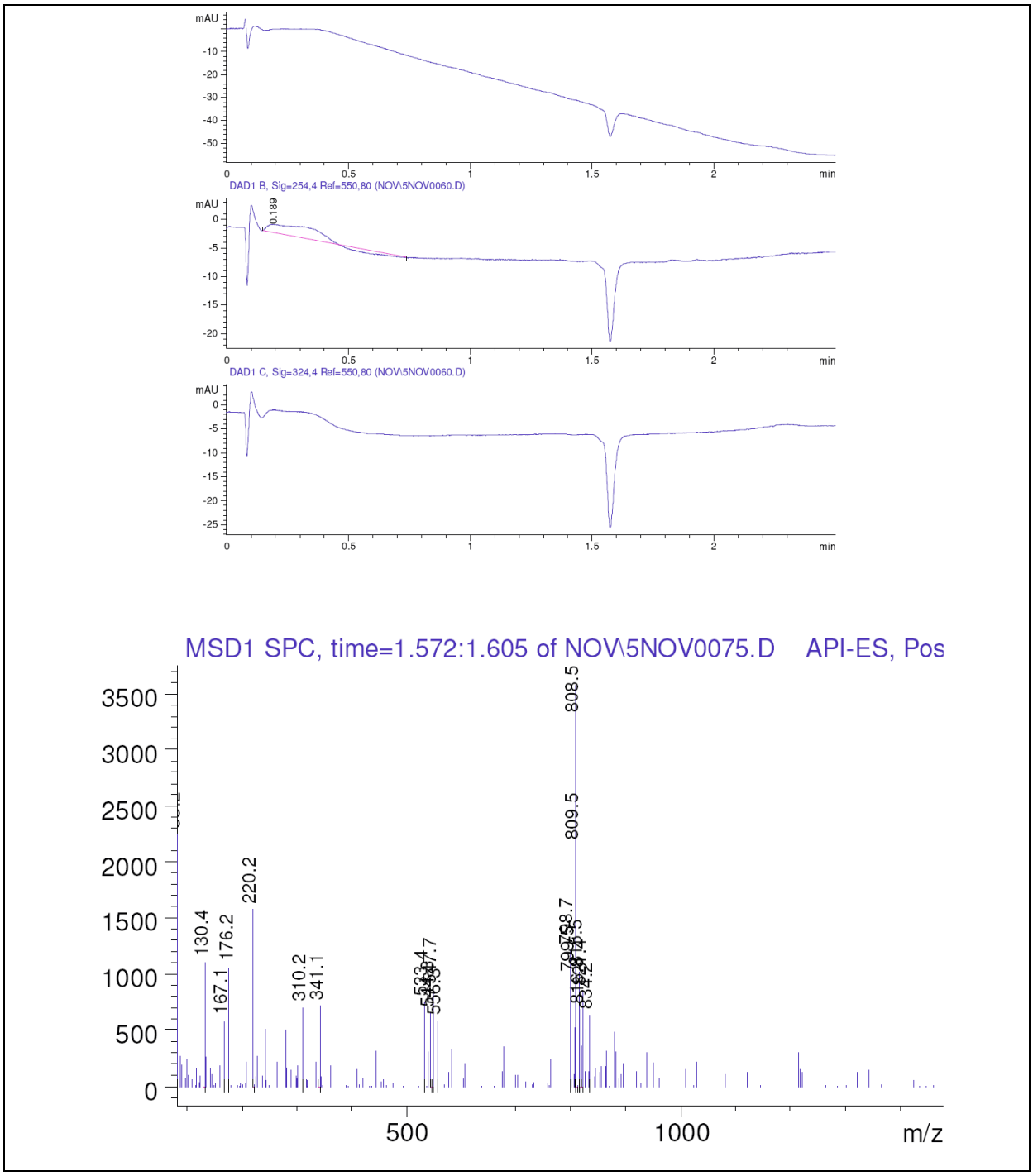
Supporting Information Figure S3: LC-MS of compound **6**.



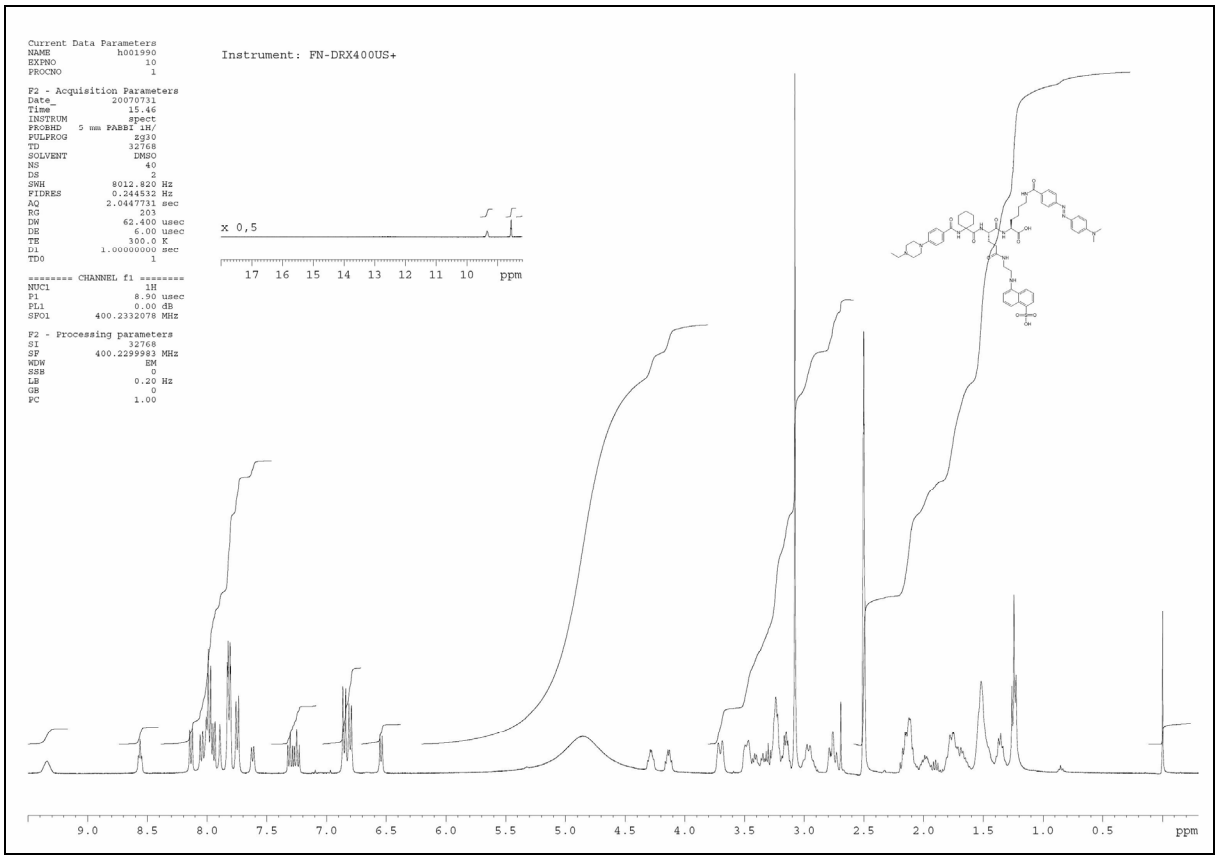
Supporting Information Figure S4: LC-MS of compound **7**.



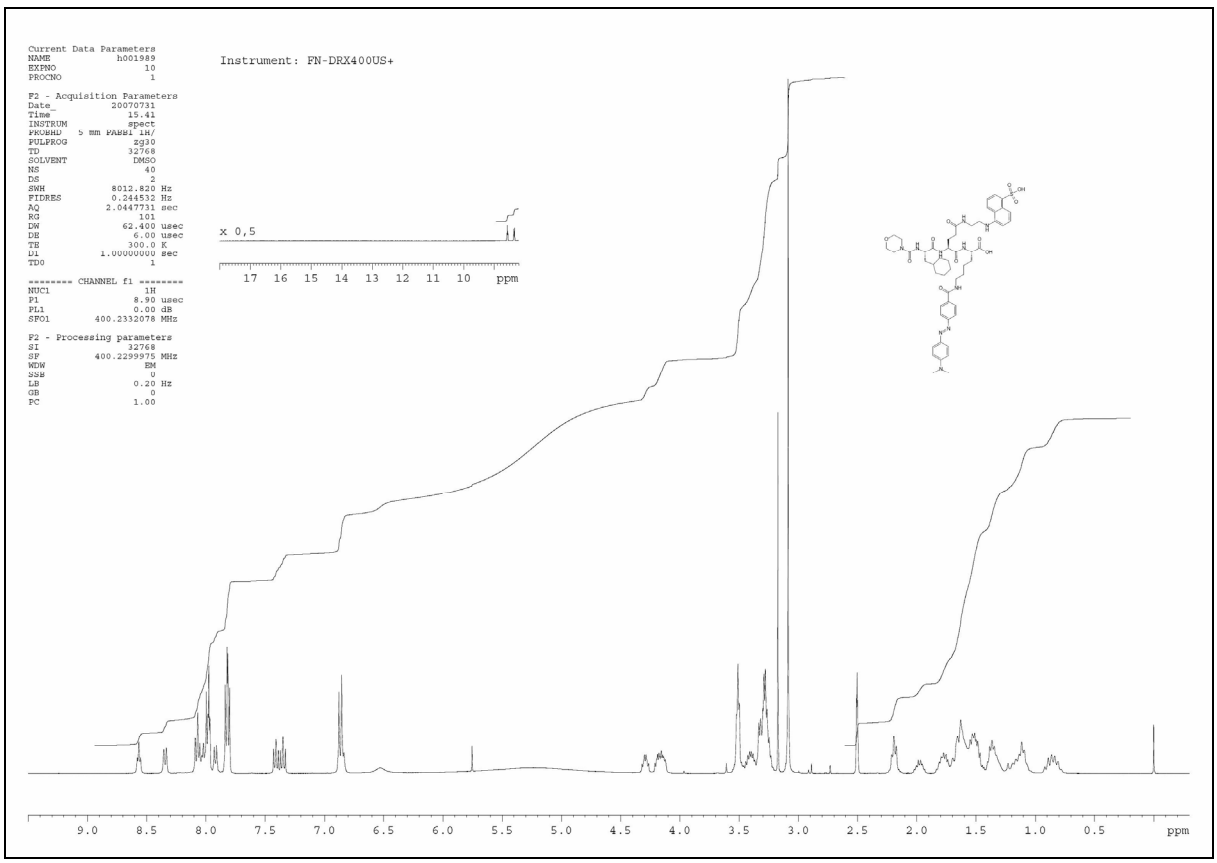
Supporting Information Figure S5: LC-MS of compound **8**.



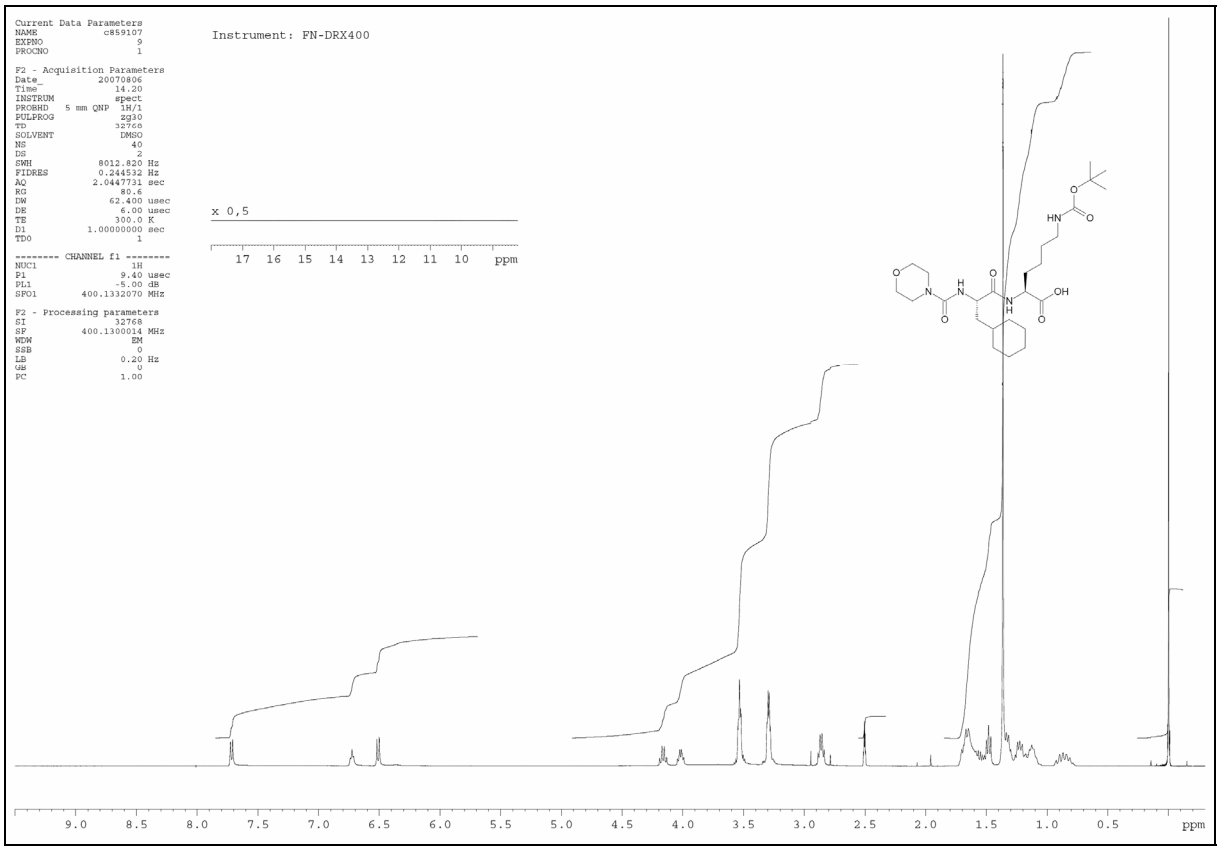
Supporting Information Figure S6: LC-MS of compound **5**.



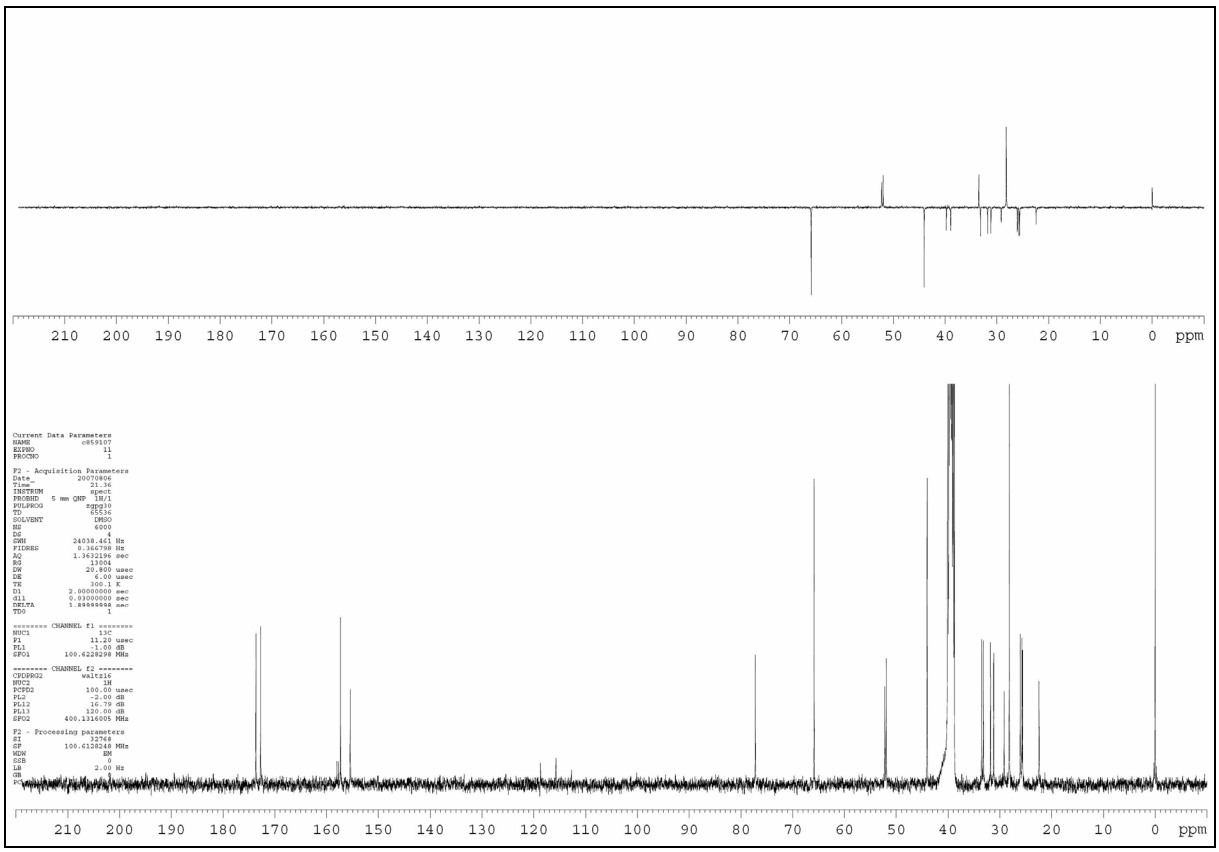
Supporting Information Figure S7: ^{1}H-NMR of compound **2**.



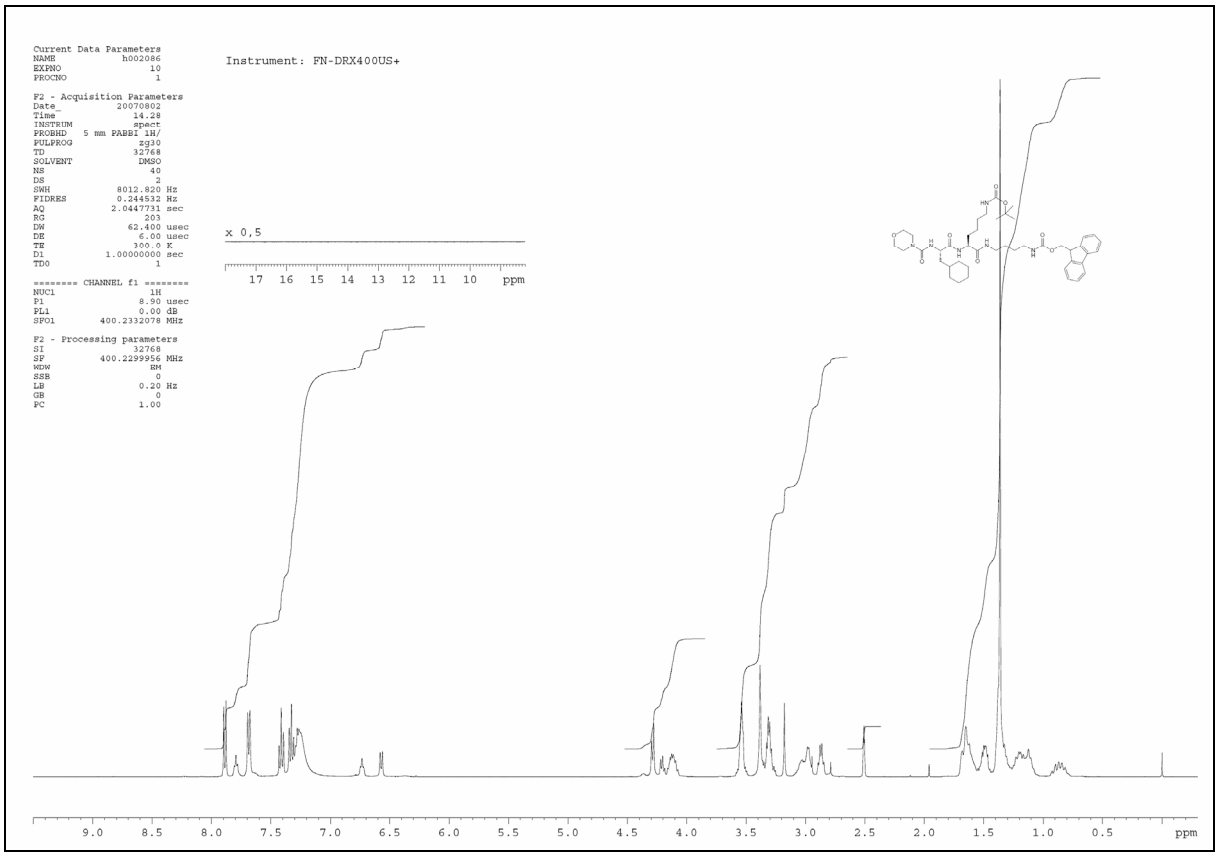
Supporting Information Figure S8: ^{1}H-NMR of compound **4**.



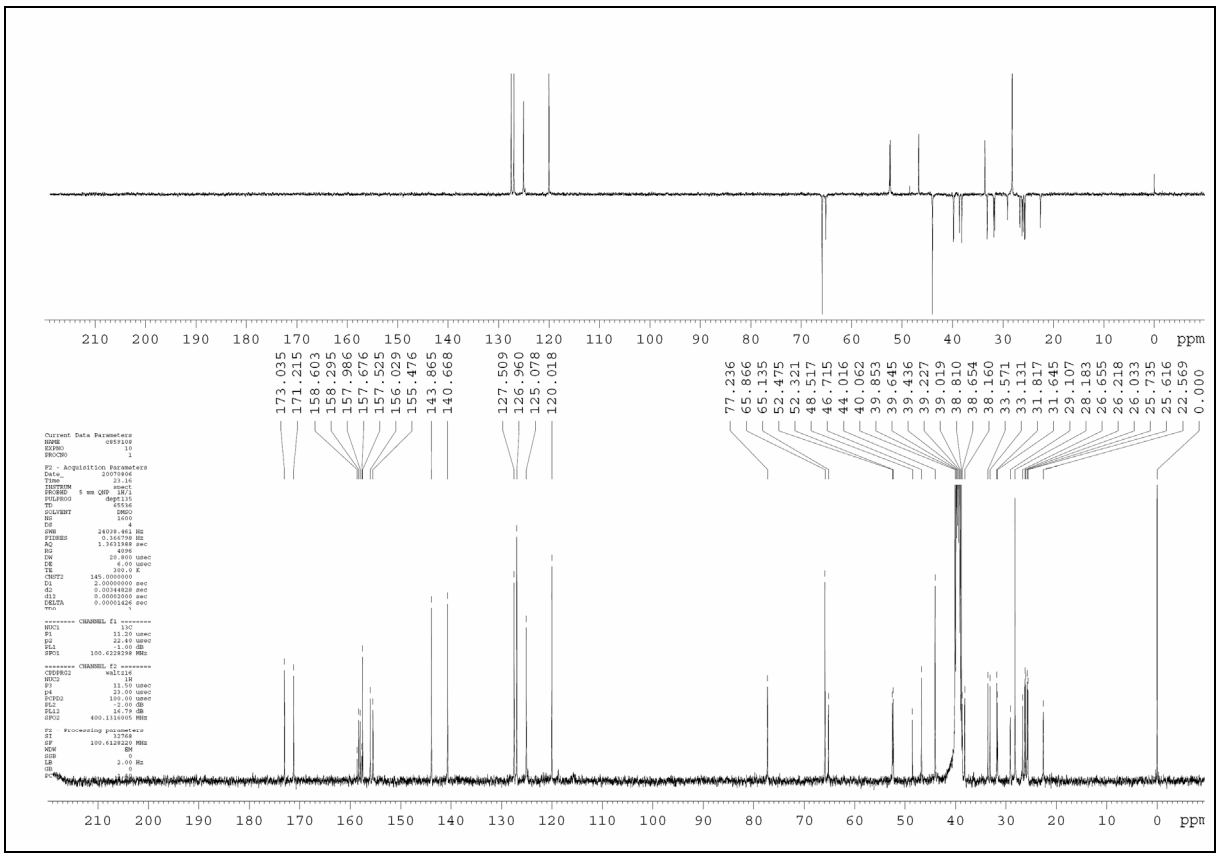
Supporting Information Figure S9: ^{1}H-NMR of compound **6**.



Supporting Information Figure S10: ^{13}C-NMR of compound **6**.



Supporting Information Figure S11: ^{1}H-NMR of compound **7**.



Supporting Information Figure S12: ^{13}C-NMR of compound **7**.