Supporting Information

In Vivo Platelet Detection using a Glycoprotein IIb/IIIa-Targeted Near-Infrared Fluorescence Imaging Probe

Khanh Ha,[a,b,‡] Xiaoxin Zheng,[c,‡,#,°] Chase W. Kessinger,[a,c] Adam Mauskapf,[c] Wenzhu Li,[c] Makoto Orii,[c] Yoichiro Kawamura,[c] Scott A. Hilderbrand,[b,^] Farouc A. Jaffer,[c,d,*,#] Jason R. McCarthy[a,b,*,#]

[a]*Masonic Medical Research Institute, Utica, New York, USA*

[b]*Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA*

[c]*Cardiovascular Research Center, Cardiology Division, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA*

[d]*Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA*

‡These authors contributed equally to this work.

*Shared senior authorship

Correspondence:

Jason R. McCarthy, Masonic Medical Research Institute, 2150 Bleecker Street, Utica, NY 13501, jmccarthy@mmri.edu, Tel. (315) 624-7478, Fax. (315) 735-5648.

and

Farouc Jaffer, MGH CVRC, Simches Research Building, Room 3206, 185 Cambridge Street, Boston, MA 02114, fjaffer@mgh.harvard.edu, Tel. (617) 724-9353, Fax. (617) 643-3451.

and

Xiaoxin Zheng, Department of Cardiology, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuhan, Hubei 430060, R.R. China, xiaoxinzheng@whu.edu.cn, Tel. +86 (027)88041911-82213, Fax. +86 (027)88041237.

° Current address for Xiaoxin Zheng: Department of Cardiology, Renmin Hospital of Wuhan University, Cardiovascular Research Institute, Wuhan University, Hubei Key Laboratory of Cardiology, Wuhan, Hubei, China

^ Current address for Scott A. Hilderbrand: Dyne Therapeutics, Waltham, Massachusetts, USASupporting Information Figure 3. Determination of optimal imaging time after the injection of TIRO-CyAl5.5.

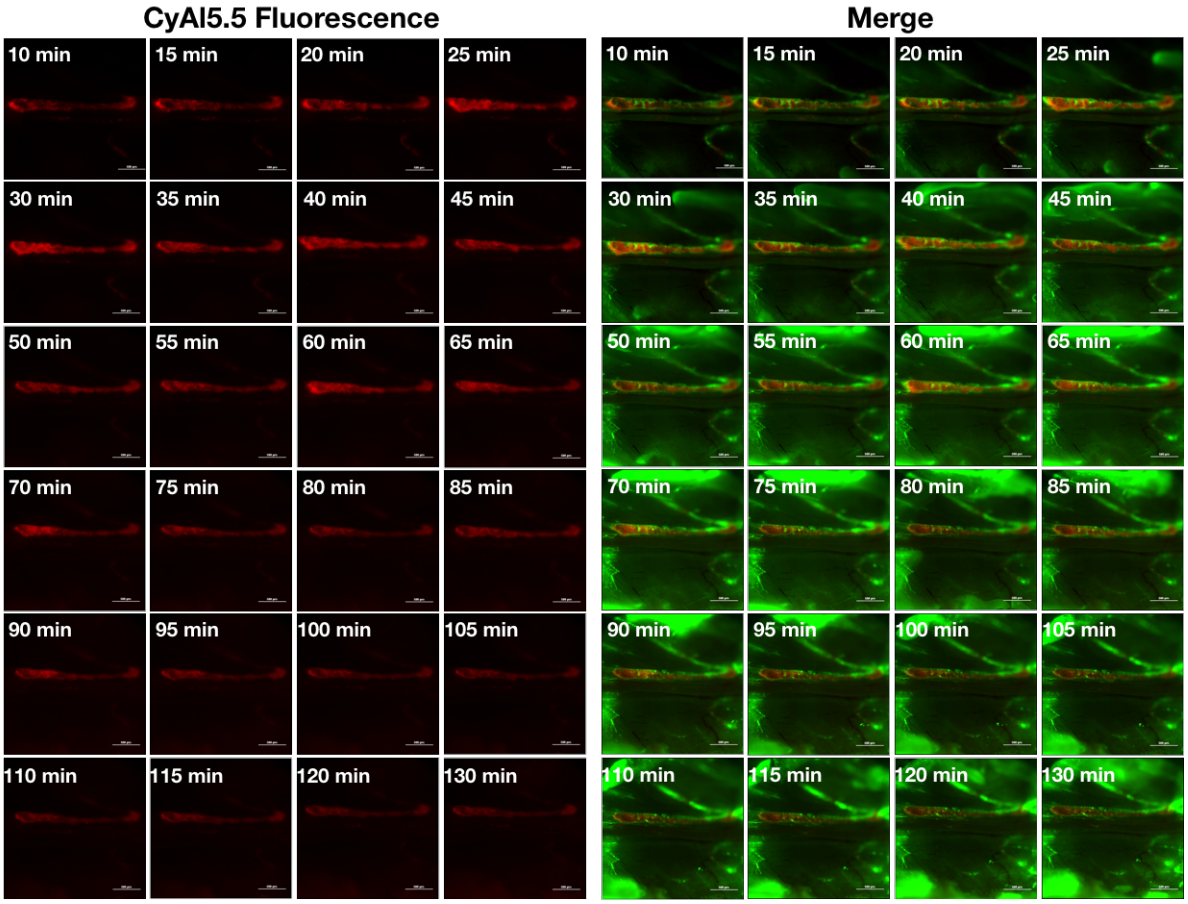


Fig. S1. Determination of optimal imaging time after the injection of TIRO-CyAl5.5. The imaging probe was injected into femoral thrombus-bearing mice and imaged every 5 minutes to examine the kinetics of binding to determine the optimal imaging time point for subsequent experiments. From these experiments and the blood half-life determined in Figure S1, 30 minutes appears ideal based on a high NIRF platelet thrombus signal. The left panels depict the accumulation of TIRO-CyAl5.5 within the thrombus (red pseudocolor), while the right panels are merged with vascular angiograms using fluorescein-labeled high molecular weight dextran (green pseudocolor). All images were windowed identically.

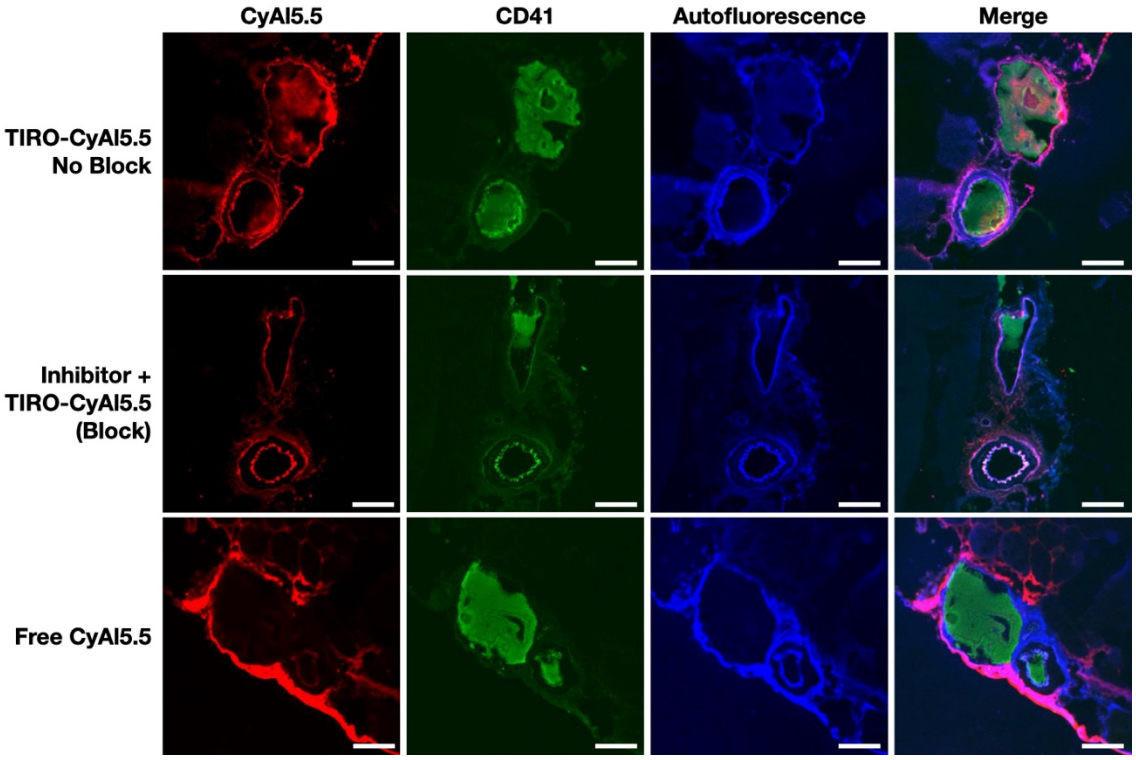


Fig. S2. Thrombus-specific binding of TIRO-CyAl5.5 in murine ferric chloride-induced venous thrombi. Each group shows representative fluorescence microscopy. CyAl5.5 (red), immunofluorescence (CD41, green), autofluorescence (blue), and merged image (CyAl5.5 + CD41 + autofluorescence). Significant autofluorescence co-localized with the non-specific staining in the CyAl5.5 channel. Tissue was resected 35 minutes after thrombus induction. Animal cohorts included mice receiving the imaging agent TIRO-CyAl5.5, a pre-block using the GPIIb/IIIa inhibitor (tirofiban) followed by injection of TIRO-CyAl5.5, and a group receiving the free dye (CyAl5.5). Scale bars = 100 μm.

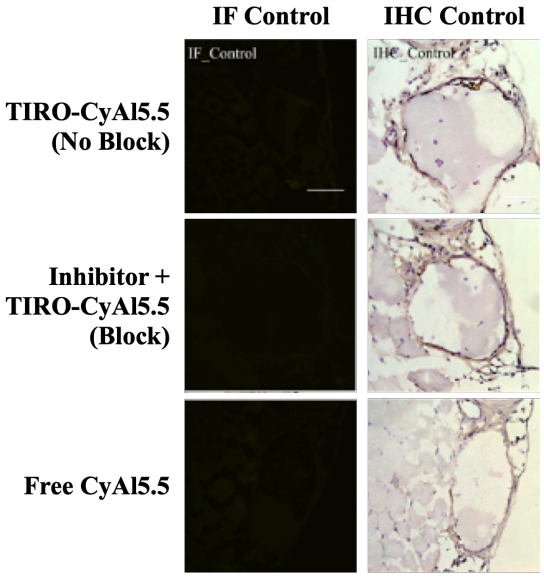


Figure S3. Immunofluorescence and immunohistochemistry control slides corresponding to the images in Figure 6. Primary antibody has been omitted in staining. Scale bars = 100 μm.

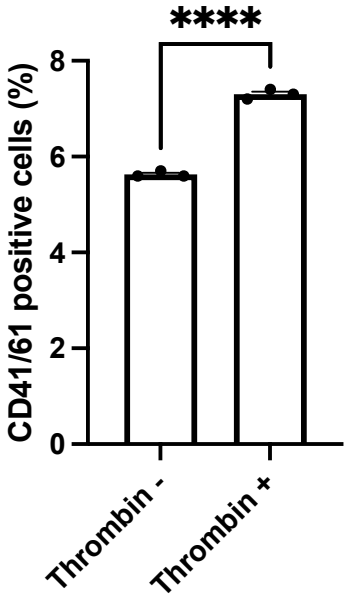


Fig. S4. Flow cytometric analysis of GPIIb/IIIa activation status. HEPES-buffered Tyrode-washed human platelets were incubated for 30 minutes with a FITC-labeled anti-CD41/61 antibody in the dark and then subject to flow cytometric analysis. **** $P < 0.0001$.

Probe synthesis:

General: Reagents were purchased from Sigma-Aldrich; deuterated solvents were from Cambridge Isotope Laboratories, Inc.; chemicals were used without further purification unless otherwise noted. Peptides were received on resin from the MGH Peptide/Protein Core Facility and were synthesized using Fmoc chemistries on rink amide resin. UV-vis spectra were recorded on a Varian Cary 50 UV-vis spectrophotometer (Palo Alto, CA, USA). Fluorescence data were collected with a Varian Cary Eclipse fluorescence spectrophotometer (Palo Alto, CA, USA). Silica gel (Sorbent Technologies, 60 Å, 40–63 μm, 230 × 400 mesh) was used for column chromatography.

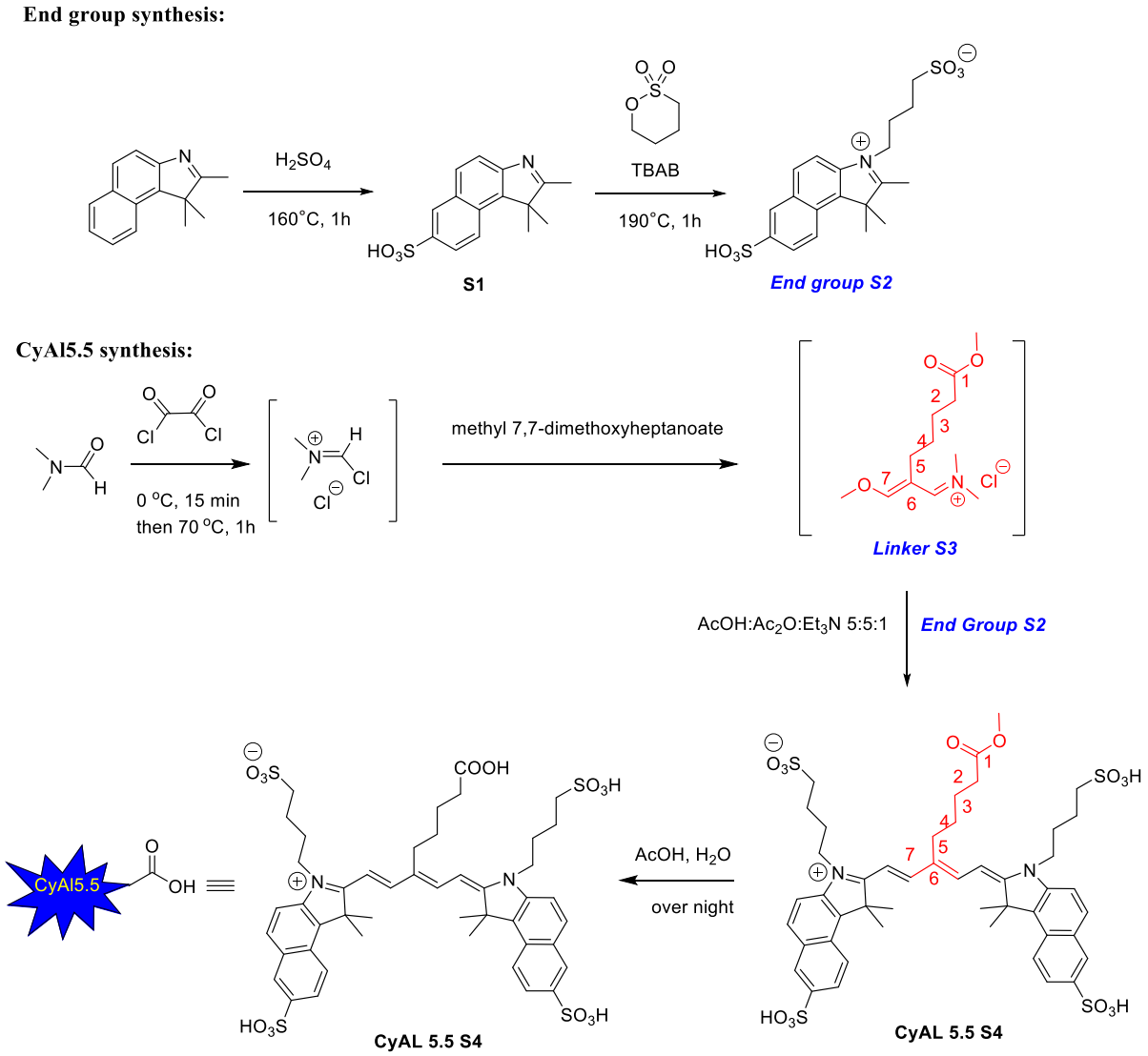
Chemical characterization: ^{1}H and ^{13}C NMR spectra were recorded at 23°C on a Bruker Avance III 400 MHz spectrometer. Chemical shifts were reported in parts per million (δ) and calibrated to the internal tetramethylsilane (TMS) standard or residual proton resonance and the natural abundance ^{13}C resonance of the solvent. Signal multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), m (multiplet), br (broad).

High-performance liquid chromatography-mass spectrometry analysis (HPLC/MS): HPLC/MS data were collected with a Waters 2695 high-performance liquid chromatography system (Milford, MA) equipped with a 2996 diode array detector, a Micromass ZQ4000 ESI-MS module, and an Agilent Pursuit XRS5 100×2.0 mm column at a flow rate of 0.3 mL/min. Gradients were run with buffer A (H_2O/0.1% TFA) and buffer B (90% acetonitrile/10% H_2O/0.1% TFA)

Preparative HPLC: Reverse phase HPLC purifications were performed using an Agilent Technologies, Inc., Pursuit XRS 10 C18 250 x 21.2 mm column. UV detection was accomplished at both 650 nm and 254 nm. A flow rate of 21 mL/min was used for all

purifications. Gradients were run with buffer A (H_2O/0.1% TFA) and buffer B (90% acetonitrile/10% H_2O/0.1% TFA) Elution gradients, as described below.

Synthesis of CyAl5.5



Scheme S1. Synthesis of CyAl5.5 dye.

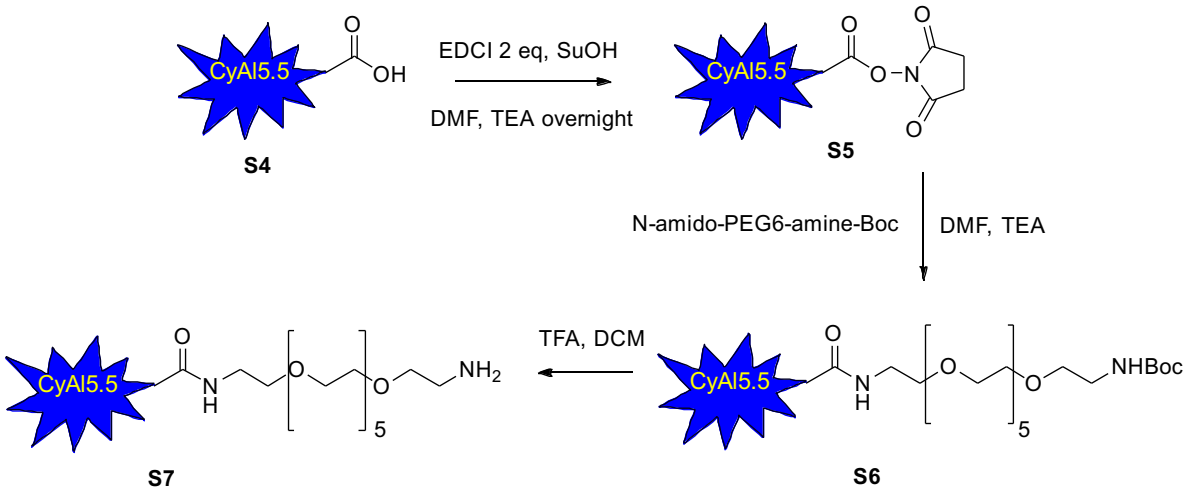
1,1,2-trimethyl-1H-benzo[e]indole-7-sulfonic acid, S1. To a 100 mL round bottom flask was added 1,1,2-trimethyl-1H-benz[e]indole (8.37g, 40mmol) and sulfuric acid (20mL). After stirring at 160°C for 1 h, the reaction mixture was allowed to cool to room temperature, and ice-cold deionized water (450 mL) was added. The formed precipitate was isolated by filtration

and washed with ice-cold deionized water (2 x 80 mL) and diethyl ether (3 x 120 mL). The resulting paste was dried to get a pale brown solid (7.2g, 62%). Compound **S1** has been previously reported, with the proton NMR from this improved synthesis matching reported values.[1]

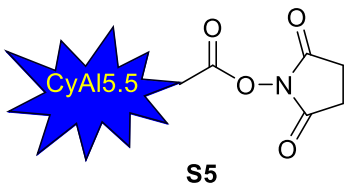
4-(1,1,2-trimethyl-7-sulfo-1H-benzo[e]indol-3-ium-3-yl)butane-1-sulfonate S2. 1,1,2-trimethyl-1H-benzo[e]indole-7-sulfonic acid (2.28 g, 7.9 x 10-3 mol) and tetra-n-butylammonium bromide (5.08 g, 1.5 x 10-2 mol) in butanesultone (5 mL) were stirred at 190° C for 1 h. The reaction mixture was allowed to cool to room temperature before the addition of acetone (150 mL in 25 mL portions) to affect precipitation. The precipitate was obtained by filtration. The isolated solid was then dissolved in deionized water (12 mL), precipitated with acetone (350 mL), and filtered to yield a light purple solid (3 g, 85% yield), that was used as is in subsequent reactions. Compound **S2** is also known, with a ^{1}H NMR matching reported values.[1]

Synthetic protocol for CyAl5.5 (S4). Into a heavy-walled pressure vessel containing oxalyl chloride (429 µL, 5 mmol, 1 equiv) was carefully added to DMF (385 µL, 5 mmol, 1 equiv) at 0°C (on ice) over 15 min (Scheme S1). Methyl 7,7-dimethoxyheptanoate (510 µL, 2.5 mml, 0.5 equiv) was then added. The vessel was sealed and the reaction mixture was stirred at 70°C for 1 h and then allowed to cool to room temperature. The end group S2 (2.15 g, 5 mmol) and a solution of acetic acid/acetic anhydride/triethylamine (6.25mL/6.25mL/1.25 mL) was then added to the vessel which was subsequently sealed and stirred at 160°C for 1 h. The reaction mixture was allowed to cool to room temperature and then concentrated under vacuum. The residue was dissolved in H_2O (50 mL) and stirred at room temperature overnight. The crude product was purified by preparative HPLC (gradient: 80% buffer A to 60% buffer A, 30 min), and all fractions containing the product combined and lyophilized, giving a dark blue-green solid (350 mg,15% yield). UV-*vis* (H_2O) λ_{max} (log ε): 677 nm (5.3); Fluorescence

(H_2O) λ_{max} (ϕ_{Fl}) 696 nm (0.12). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.27 (br s, 2H), 8.23 (d, J = 14.3 Hz, 2H), 8.20 (d, J = 8.9 Hz, 2H), 8.14 (d, J = 8.8 Hz, 2H), 7.86 (d, J = 8.7 Hz, 2H), 7.79 (d, J = 8.9 Hz, 2H), 6.26 (d, J = 14.2 Hz, 2H), 4.32 (t, J = 7.4 Hz, 4H), 2.75 – 2.61 (m, 6H), 2.35 (t, J = 7.1 Hz, 2H), 1.98 (br s, 12H), 1.93 – 1.85 (m, 4H), 1.84 – 1.74(q, 4H), 1.78 – 1.72 (m, 2H), 1.58 – 1.52 (m, 2H). 13C NMR (126 MHz, DMSO-d_6) δ 174.95, 174.19, 144.59, 140.68, 133.55, 131.43, 131.42, 130.93, 130.21, 128.33, 127.72, 126.47, 126.16, 122.22, 112.51, 99.88, 51.16, 51.12, 43.92, 38.00, 34.12, 27.08, 26.63, 25.26, 22.67. Yield, 0.60 g, 29%. LRMS-ESI $[M]^+$ m/z calcd. for $[C_{46}H_{54}N_2O_{14}S_4]^+$ 986.2, found 986.2.

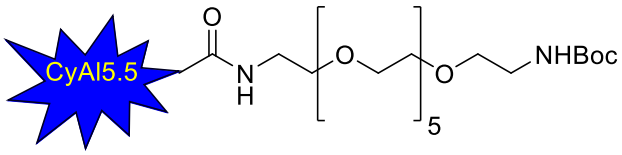


Scheme S2. Synthesis of CyAl5.5-N-amido-PEG_6-amine.



Synthesis of CyAl5.5-OSu (S5). N-(3-Dimethylaminopropyl)-N′-ethyl carbodiimide hydrochloride (EDCI) (191 mg, 1 mmol) was added to a stirred solution of CyAl5.5 (493 mg, 0.5 mmol) and N-hydroxysuccinimide (HOSu, 115 mg, 1 mmol) dissolved in 50 mL N,N-dimethylformamide (DMF) at 0 °C under an argon atmosphere. The reaction was allowed to proceed for 1 h at 0 °C, and was subsequently reacted for an additional 16 h at room temperature. DMF was removed under vacuum, with the residue being dissolved in buffer A and purified by HPLC (gradient: 80% buffer A to 60% buffer A, 30 min) to afford CyAl5.5-

OSu (**S5**) as a dark blue solid. Yield, 493 mg, 91%. LRMS-ESI $[M]^+$ m/z calcd. for $[C_{50}H_{57}N_3O_{16}S_4]^+$ 1083.3, found 1083.3.[3]



S6

Synthesis of CyAl5.5-N-amido-PEG$_6$-amine-Boc (S6). CyAl5.5-OSu (541.5 mg, 0.5 mmol, 1 eq.) was dissolved in anhydrous DMF (15 mL) and added dropwise to a stirred solution of amino-PEG$_6$-amine-Boc (233 mg, 0.55 mmol, 1.1 eq.) and N,N-diisopropylethylamine (0.1 mL, DIPEA, 0.6 mmol, 1.1 eq.) in anhydrous DMF (1 mL) which was cooled to 0 °C. The reaction was carried out under an argon atmosphere at 0 °C for 2 hours, at which point it was allowed to warm to room temperature and stir for an additional 16 h. The solvent was removed under vacuum, with the residue subsequently dissolved in 10 ml of buffer A and purified by HPLC (gradient: 80% buffer A to 60% buffer A, 30 min) to afford CyAL-5.5-amido-PEG$_6$-amine-Boc (S6) as a dark blue sticky solid. Yield, 918 mg, 95%. LRMS-ESI $[M+H]^+$ m/z calcd. for $[C_{65}H_{93}N_4O_{21}S_4]^+$ 1393.5, found 1393.6.

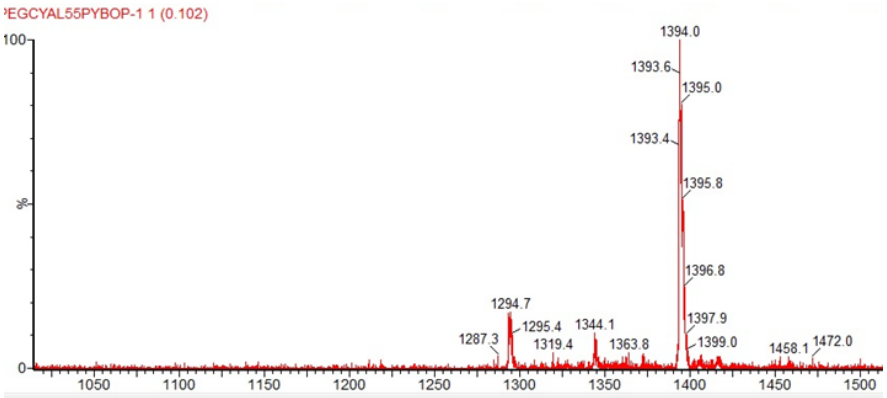
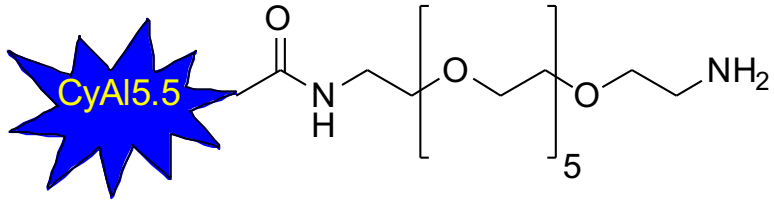


Figure S5. ESI-MS of CyAl5.5-N-amido-PEG$_6$-amine-Boc (S6)



S7

Synthesis of CyAl5.5-N-amido-PEG$_6$-amine (S7). CyAl5.5-N-amido-PEG$_6$-amine-Boc (697 mg, 0.5 mmol) was dissolved in anhydrous dichloromethane (DCM, 30 mL) and trifluoroacetic (3 mL) at 0 °C. The reaction was carried out under an argon atmosphere and proceeded for 2 hours. DCM and TFA were removed under vacuum, and the residue was then dissolved in 3 ml of buffer A and purified by HPLC (gradient: 80% buffer A to 60% buffer A, 30 min) to afford CyAl5.5-amido-PEG$_6$-amine (10) as a dark blue sticky solid. Yield, 633 mg, 98%. LRMS-ESI $[M+H]^+$ m/z calcd. for $[C_{60}H_{85}N_4O_{19}S_4]^+$ 1293.5, found 1293.9

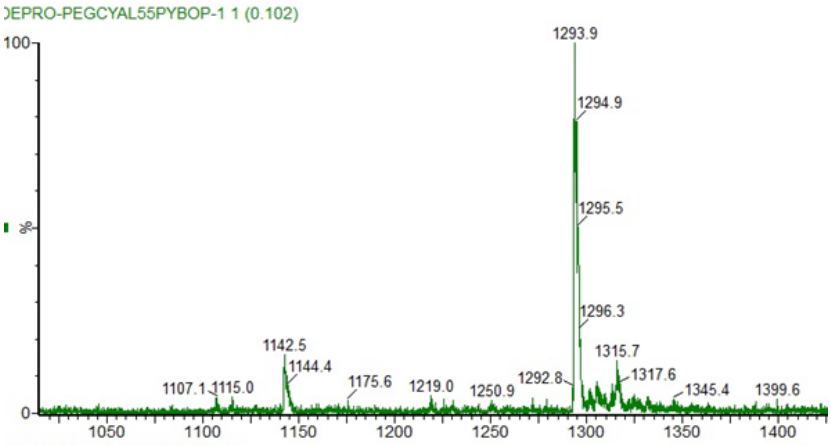
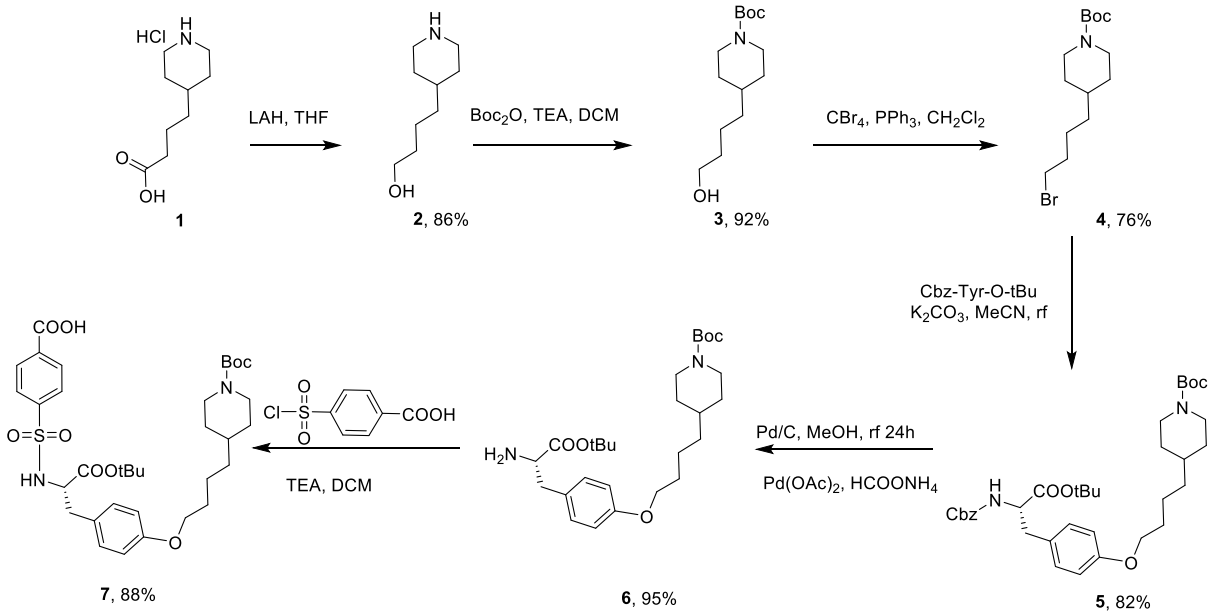
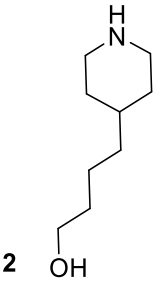


Figure S6. ESI-MS of CyAl5.5-N-amido-PEG$_6$-amine (S7)

The synthesis procedure of tirofiban derivative (5):

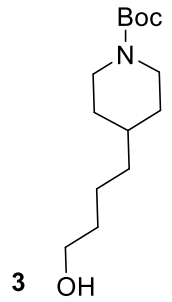


Scheme S3. Synthesis of Boc protected Tirofiban analog



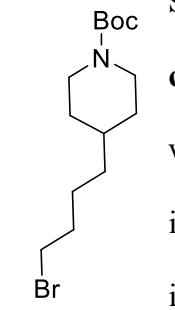
Procedures for preparation of 4-(Piperidin-4-yl)butan-1-ol (2). Under an atmosphere of argon, piperidine derivative **1** (400 mg, 2 mmol) was suspended in anhydrous THF (15 mL), and the mixture was cooled down in an ice-bath. $LiAlH_4$ (365 mg, 4.8 mmol) was added dropwise over a period of 10 min. The resulting suspension was stirred at 0 °C for 1 h and under reflux for 12 h. The mixture was cooled to rt, and a 20% aq NaOH solution (w/w, 10 mL) was added, with the resulting suspension vigorously stirred for 1 h. The solid material was removed by filtration through a pad of Celite, and the volatiles of the filtrate were removed under reduced pressure to yield product **2** as a pale-yellow oil (258 mg, 1.6 mmol, 86%), which was used without purification. Compound **2** is known with a ^{1}H NMR matching previously reported values (for carbon NMR, please see ref. N^o 5). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 3.68 (t, *J* = 6.4 Hz, 1H), 3.53 (t, *J* = 6.6 Hz, 2H), 3.06 – 2.98 (m, 2H), 2.52 (td, *J* = 12.4, 2.9 Hz, 2H), 1.82 – 1.74 (m, 1H), 1.67 – 1.57 (m, 2H), 1.47 (p, *J* = 6.9 Hz, 2H), 1.29 (dq, *J* = 10.2, 6.8, 5.8 Hz, 2H), 1.18 (dt, *J* = 12.3, 6.6 Hz, 2H), 1.07 (qd, *J* = 12.3, 4.2 Hz, 2H). LRMS (ESI): m/z $[M + H]^+$ calcd for $[C_9H_{20}NO]^+$

158.15, found 158.15.



3

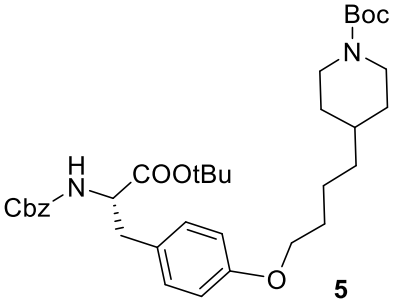
Synthesis of tert-Butyl 4-(4-hydroxybutyl)piperidine-1-carboxylate (3). To a solution of **2** (204 mg, 1.29 mmol) and 4-dimethylaminopyridine (237 mg, 1.94 mmol) in methylene chloride (15 mL) at 0 ˚C was added triethylamine (0.36 mL, 2.58 mmol) and di-*tert*-butyldicarbonate (564 mg, 2.58 mmol), and the reaction was warmed to room temperature and stirred for 6 h. After dilution with saturated ammonium chloride and extraction with ethyl acetate (3 x 30 mL), the combined organics were concentrated in vacuo. Flash chromatography (1:10 methanol/methylene chloride) gave product **3** as a colorless oil (305 mg, 92%). Compound **3** also is known and has a ^{1}H NMR matching reported values (for carbon NMR, please see ref. N^o 5). ^{1}H NMR (400 MHz, Methanol-*d*$_4$) δ 4.04 (d, *J* = 13.1 Hz, 2H), 3.54 (t, *J* = 6.5 Hz, 2H), 2.78 – 2.62 (m, 2H), 1.69 (d, *J* = 13.8 Hz, 2H), 1.56 – 1.42 (m, 3H), 1.44 (s, 9H), 1.42 – 1.32 (m, 2H), 1.31 – 1.23 (m, 2H), 1.03 (qd, *J* = 12.4, 4.1 Hz, 2H). LRMS (ESI): m/z $[M+H]^+$ calcd. for $[C_{14}H_{28}NO_3]^+$ 258.21, found: 258.22



4

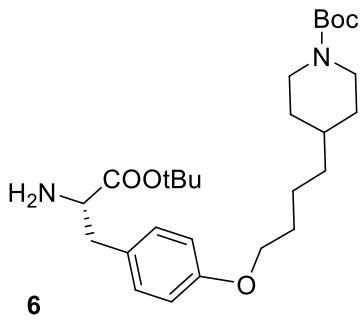
Synthesis procedure of tert-butyl 4-(4-bromobutyl)piperidine-1-carboxylate (4). Alcohol **3** (404 mg, 1.57 mmol) and PPh_3 (1.2 g, 4.57 mmol) were dissolved in CH_2Cl_2 (30 mL), and the solution was cooled to -5 ^{o} C (NaCl ice bath) under an atmosphere of argon. A solution of CBr_4 (3.4 g, 10.25 mmol) in CH_2Cl_2 (20 mL) was slowly dropped into the stirred mixture, thereby keeping the temperature of the mixture below 5^{o}C. After completed addition, stirring was continued at room temperature for 24 h. The solvent was removed under reduced pressure to give a brown residue, which was subjected to flash chromatography (eluent: light petroleum/acetone/25% aq NH_3 85:15:1 v/v/v) to afford compound **3** as a brown oil (250 mg, 50%). Compound **4** is known. The ^{1}H NMR matches with reported values (for carbon NMR, please see ref. N^o 5). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.00 (br s, 2H), 3.34 (t, *J* = 6.7 Hz, 2H), 2.60 (t, *J* = 12.9 Hz, 2H),

1.78 (p, *J* = 7.0 Hz, 2H), 1.64 – 1.55 (m, 2H), 1.52 – 1.49 (m, 2H), 1.38 (br s, 9H), 1.31 (ddd, *J* = 10.9, 7.3, 3.8 Hz, 1H), 1.22 – 1.13 (m, 2H), 1.01 (qd, *J* = 12.4, 4.4 Hz, 2H). LRMS (ESI): m/z $[M+H]^+$ calcd. for $[C_{14}H_{27}BrNO_2]^+$ 320.12, found: 320.15



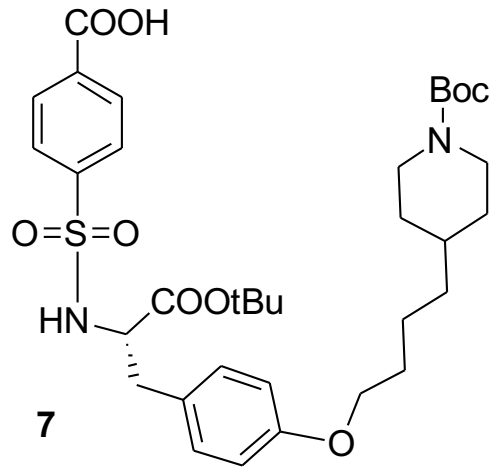
5

Synthesis procedure of tert-butyl (S)-4-(4-(4-(2-(((benzyloxy)carbonyl)amino)-3-(tert-butoxy)-3-oxopropyl)phenoxy)butyl)piperidine-1-carboxylate (5). A suspension of Cbz-Tyr-O-tBu (387 mg, 1 mmol, 1 equiv.), K_2CO_3 (690 mg, 5 mmol, 5 equiv.), NaI (74.5 mg, 0.5 mmol, 0.5 equiv.) and tert-butyl 4-(4-bromobutyl)piperidine-1-carboxylate (**4**) (320 mg, 1 mmol, 1 equiv.) in MeCN (200 mL) were refluxed for 24 h. After being cooled to room temperature, the reaction mixture was poured into water, extracted with EtOAc (4 × 75 mL) and washed with brine (2 × 50 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered to remove the solid, and evaporated to dryness. The crude products were purified by flash column chromatography on silica with n-hexanes/EtOAc (15:1) to give **5** as yellow oil (518 mg, 0.86 mmol, 86%).^{1}H NMR (500 MHz, Methanol-d_4) δ 7.48 – 7.25 (m, 5H), 7.13 (d, *J* = 8.6 Hz, 2H), 6.83 (d, *J* = 8.6 Hz, 2H), 5.17 – 4.98 (m, 2H), 4.28 (dd, *J* = 8.7, 6.2 Hz, 1H), 4.06 (dt, *J* = 13.4, 3.0 Hz, 2H), 3.97 (t, *J* = 6.3 Hz, 2H), 3.02 (dd, *J* = 13.8, 6.2 Hz, 1H), 2.87(dd, *J* = 13.8, 8.7 Hz, 1H), 2.78 – 2.62 (m, 2H), 1.77 (dt, *J* = 14.3, 6.7 Hz, 2H), 1.77– 1.64 (m, 2H), 1.62 – 1.48 (m, 2H), 1.47 (s, 9H), 1.42 (s, 9H), 1.38 – 1.24 (m, 3H), 1.07 (qd, *J* = 12.5, 4.3 Hz, 2H). ^{13}C NMR (126 MHz, MeOD) δ 171.49, 156.79, 156.00, 155.14, 136.80, 130.15, 128.16, 127.92, 127.66, 127.47, 114.97, 114.23, 81.48, 79.45, 67.55, 66.22, 60.24, 56.39, 36.80, 36.03, 35.67, 31.97, 29.25, 27.65, 27.08, 22.90, 19.78, 13.37. LRMS (ESI): m/z $[M + Na]^+$ calcd for $[C_{35}H_{50}N_2O_7Na]^+$ 633.35, found 633.36.[4,5]



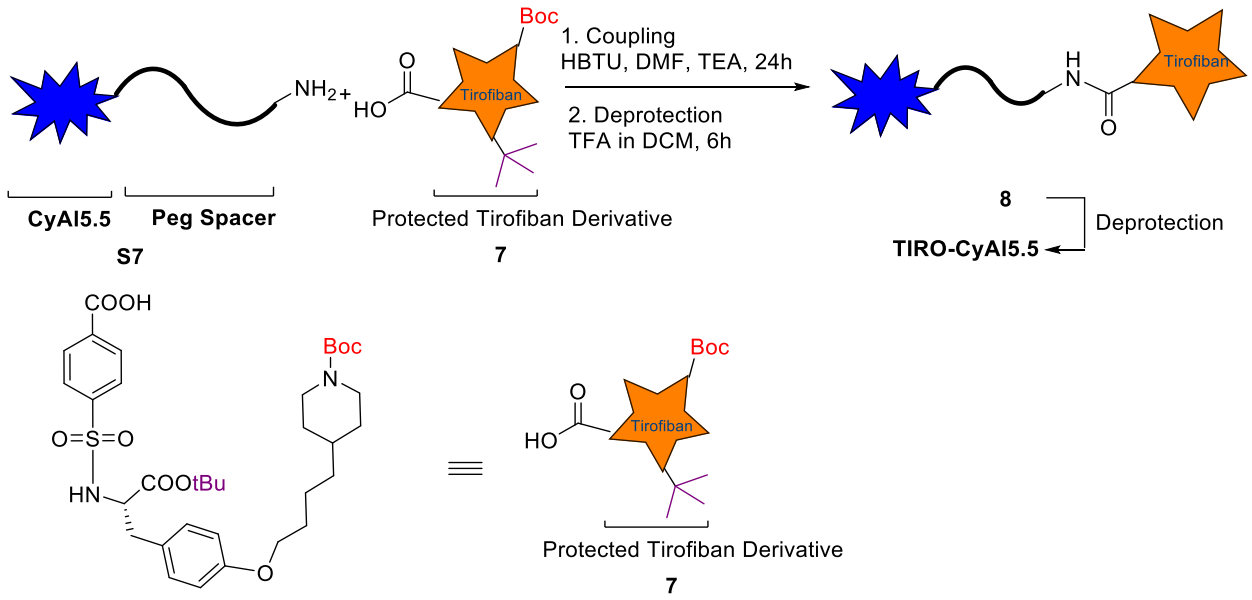
6

Synthesis procedure of tert-butyl (S)-4-(4-(4-(2-amino-3-(tert-butoxy)-3-oxopropyl)phenoxy)butyl)piperidine-1-carboxylate (6). tert-butyl (S)-4-(4-(4-(2-(((benzyloxy)carbonyl)amino)-3-(tert-butoxy)-3-oxopropyl)phenoxy)butyl)piperidine-1-carboxylate (**5**) (1.7 g, 2.8 mmol, 1 e equiv.) was dissolved in MeOH (20 mL). To the solution was added Pd/C (10%, 150 mg), $Pd(OAc)_2$ (235 mg, 0.42 mmol 15 mol%), and ammonium formate (882 mg, 14 mmol, 5 equiv.) in a round-bottomed flask. The atmosphere in the flask was replaced with hydrogen using a balloon filled with the gas, and the reaction was allowed to proceed for 3 h under hydrogen atmosphere while refluxing. Over the course of the reaction, a white precipitate was formed which was subsequently dissolved by the addition of methanol. The Pd/C was removed by filtration through Celite. The filtrate was concentrated under reduced pressure. The crude product was dissolved in EtOAc (100 mL) and washed with brine (2 × 50 mL) and HCl (2 × 20 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered to remove the drying agent, concentrated, and the crude products were purified by flash column chromatography on silica gel with n-hexanes/EtOAc (15:1) to give products **6** as yellow oil (1.27 g, 2.66 mmol, 95%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.11 (d, J = 8.2 Hz, 2H), 6.83 (d, J = 8.2 Hz, 2H), 4.04 (d, J = 13.1 Hz, 2H), 3.95 (t, J = 6.2 Hz, 2H), 3.54 (t, J = 6.7 Hz, 1H), 2.94 – 2.82 (m, 2H), 2.76 – 2.68 (m, 2H), 1.78 – 1.64 (m, 4H), 1.54 – 1.44 (m, 4H), 1.44 (s, 9H), 1.39 (s, 9H), 1.34 – 1.27 (m, 3H), 1.12 – 0.98 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 173.82, 158.09, 155.15, 130.10, 128.81, 114.12, 80.93, 79.42, 67.48, 55.89, 39.75, 35.98, 35.70, 31.96, 29.14, 27.33, 26.87, 22.79. LRMS (ESI): m/z $[M + H]^+$ calcd for $[C_{27}H_{45}N_2O_7]^+$ 477.34, found 477.364.[6]

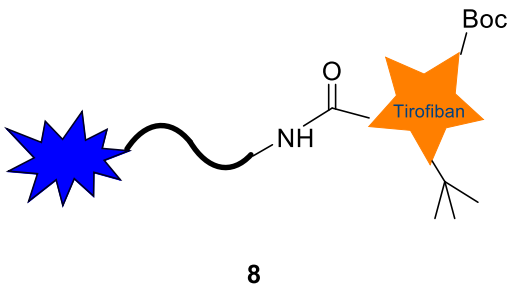


7

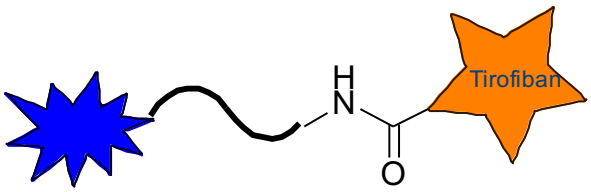
Procedures for preparation of (S)-4-(N-(1-(tert-butoxy)-3-(4-(4-(1-(tert-butoxycarbonyl)piperidin-4-yl)butoxy)phenyl)-1-oxopropan-2-yl)sulfamoyl)benzoic acid (7). To a solution of compound **6** (238 mg, 0.5 mmol) in dry dichloromethane (10 mL) at 0 °C was added triethylamine (0.086 mL, 0.55 mmol) and 4-(chlorosulfonyl)benzoic acid (110 mg, 0.50 mmol). The reaction mixture was stirred at room temperature overnight. The reaction mixture was then diluted with dichloromethane (75 mL) and washed with brine (2 × 50 mL) and HCl (2 × 20 mL). The solvent was removed under reduced pressure. The crude compound was purified by flash chromatography on silica gel, using ethyl acetate/hexane (3:5) as the eluent, to give product **7** (105.42 mg, 88%).^{1}H NMR (500 MHz, Methanol-d_4) δ 7.89 (d, J = 7.1 Hz, 2H), 7.80 (d, J = 8.5 Hz, 2H), 7.18 (d, J = 8.5 Hz, 2H), 6.84 (d, J = 7.2 Hz, 2H), 4.73 – 4.61 (m, 1H), 4.08 – 4.00 (m, 2H), 3.94 (dt, J = 7.8, 5.6 Hz, 2H), 3.17 (dd, J = 13.9, 6.2 Hz, 1H), 3.05 (dd, J = 8.8, 5.1 Hz, 1H), 2.74 – 2.68 (m, 2H), 1.80 – 1.66 (m, 4H), 1.53 – 1.50 (m, 1H), 1.45 (s, 9H), 1.44 (s, 9H), 1.34 – 1.30 (m, 2H), 1.26 – 1.24 (m, 4H). ^{13}C NMR (101 MHz, MeOD) δ 171.45, 158.18, 156.92, 155.16, 136.86, 129.97, 128.75, 128.02, 127.51, 127.29, 114.04, 81.41, 79.43, 67.46, 66.06, 56.38, 36.58, 35.98, 35.70, 31.95, 29.14, 27.32, 26.80, 22.80. LRMS (ESI): calcd for: $C_{34}H_{49}N_2O_9S$ $[M + H]^+$ = 661.32, found $[M + H]^+$ = 661.32.



Scheme S4. Final labelling and deprotection



Synthesis of compound 8. N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) (38 mg, 0.1 mmol) was added to a solution of protected tirofiban derivative **7** (66 mg, 0.1 mmol) and triethylamine (0.017 ml, 0.125 mmol) in 5 mL dimethylformamide (DMF) at 0 °C under an argon atmosphere. After 5 mins of CyAl5.5-N-amido-PEG$_6$-amine **S7** (129 mg, 0.1 mmol) was added to the reaction mixture. The reaction was then allowed to proceed for 1 h at 0 °C, followed by stirring overnight at rt. DMF was removed under vacuum. The crude product was purified by HPLC (gradient: 80% buffer A to 60% buffer A, 30 min) to afford compound **8** as a dark blue oil. Yield, 54 mg, 28%. LRMS-ESI $[M]^+$ m/z calcd. for $[C_{94}H_{131}N_6O_{27}S_5]^+$ 1935.7 found 1935.7.



TIRO-CyAl5.5

Synthesis of CyAl5.5-N-amido-PEG$_6$-Tirofiban (TIRO-CyAl5.5). CyAl5.5-N-amido-PEG$_6$-amine-tirofiban-Boc **8** (7.75 µmol, 15 mg, 0.5 mmol) was dissolved in a solution of TFA (2 mL) in DCM (2.5 mL) at 0 °C. The reaction was carried out under an argon atmosphere and proceeded for 6 hours. DCM and TFA were removed in vacuo, and the residue was dissolved in 0.5 ml of buffer A and purified HPLC (gradient: 80% buffer A to 60% buffer A, 30 min) to afford the product TIRO-CyAl5.5 as a dark blue sticky solid. Yield, 10 mg, 75%. LRMS-ESI $[M+H]^+$ m/z calcd. for $[C_{85}H_{116}N_6O_{25}S_5]^+$ 1780.6, found 1780.8.

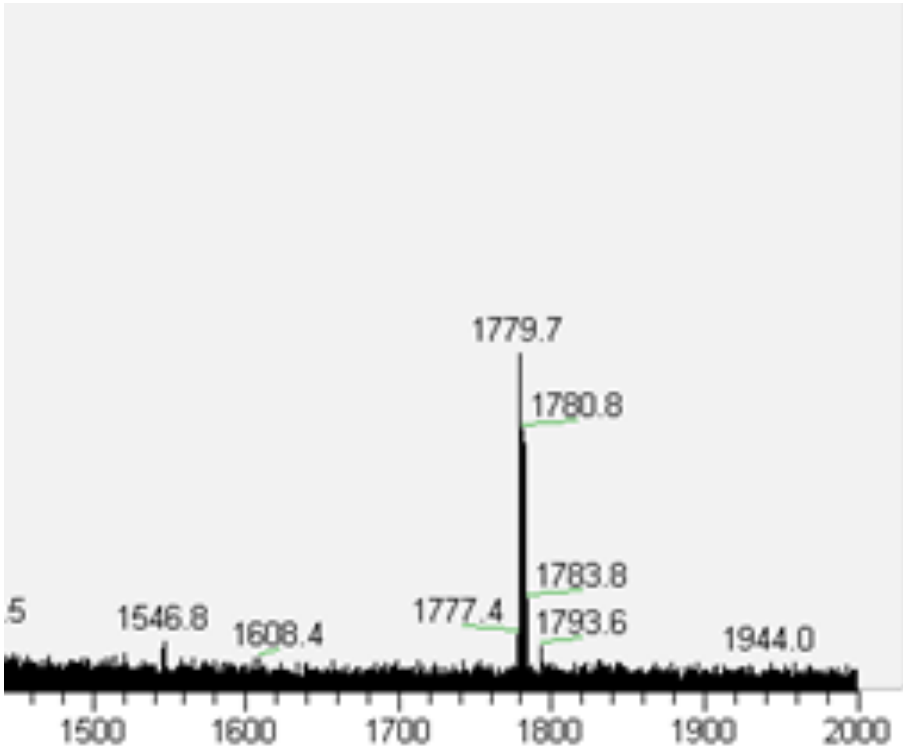


Figure S7. ESI-MS of TIRO-CyAl5.5

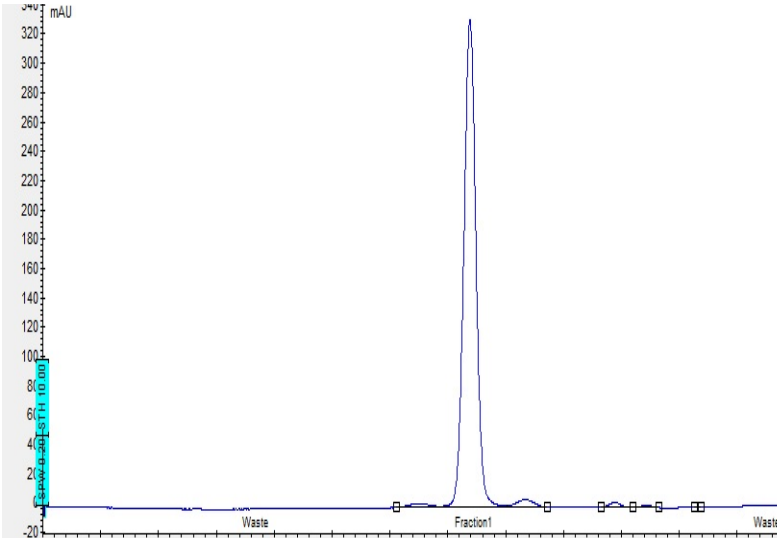


Figure S8. HPLC of TIRO-CyAl5.5

NMR Spectra

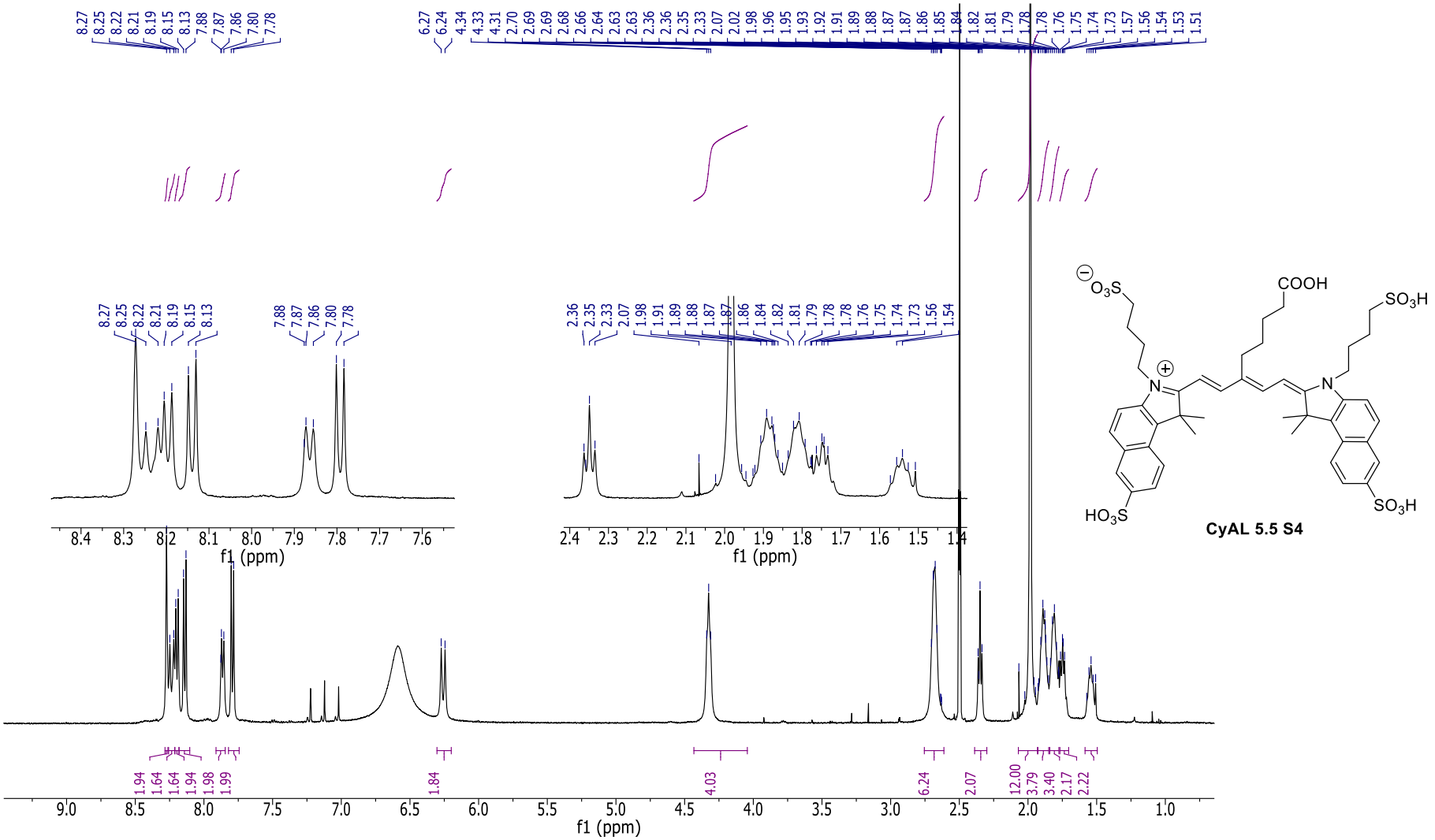


Figure S9. Proton NMR of CyAl5.5 S4.

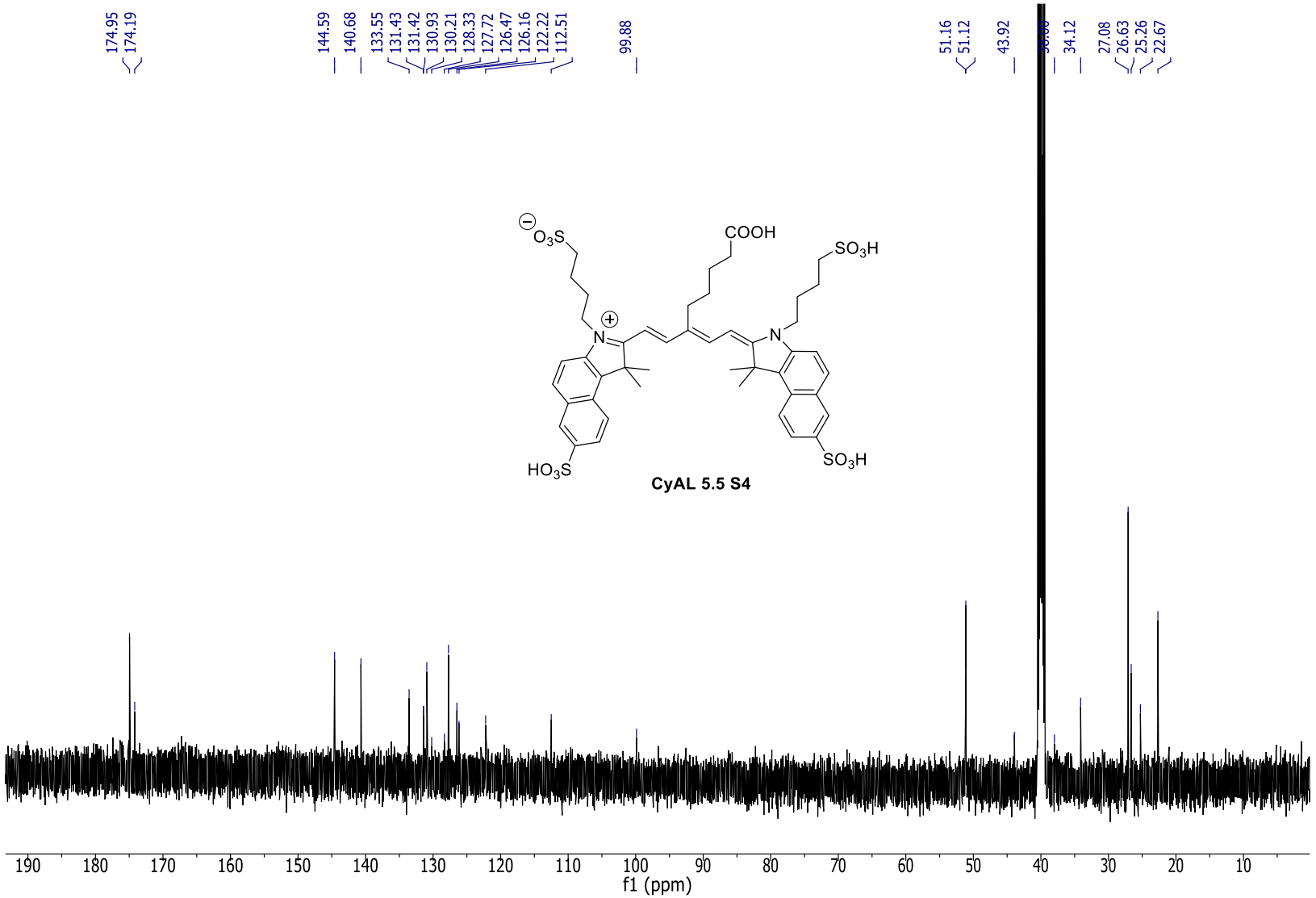


Figure S10. Carbon NMR of CyAl5.5 S4.

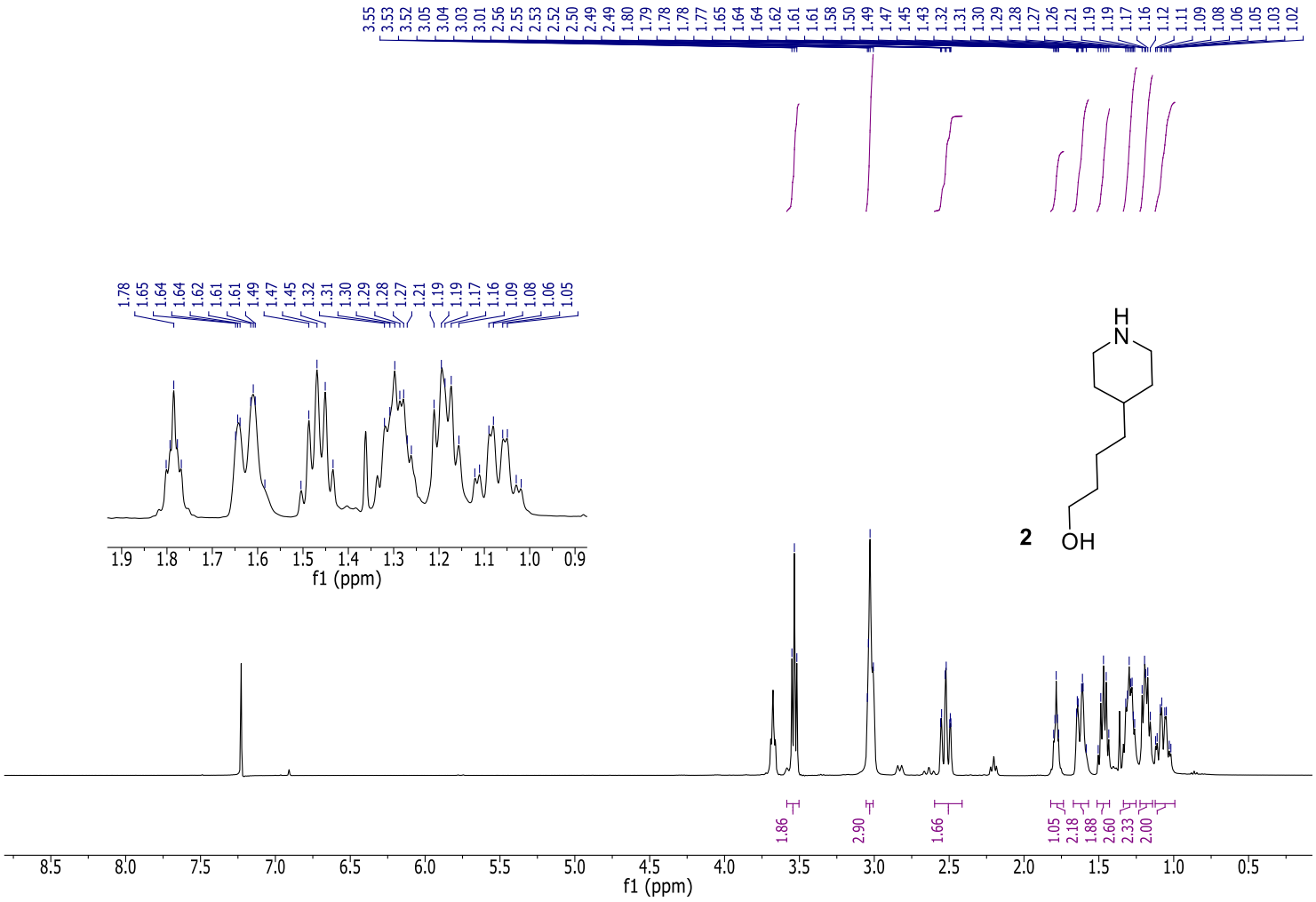


Figure S11. Proton NMR of compound 2

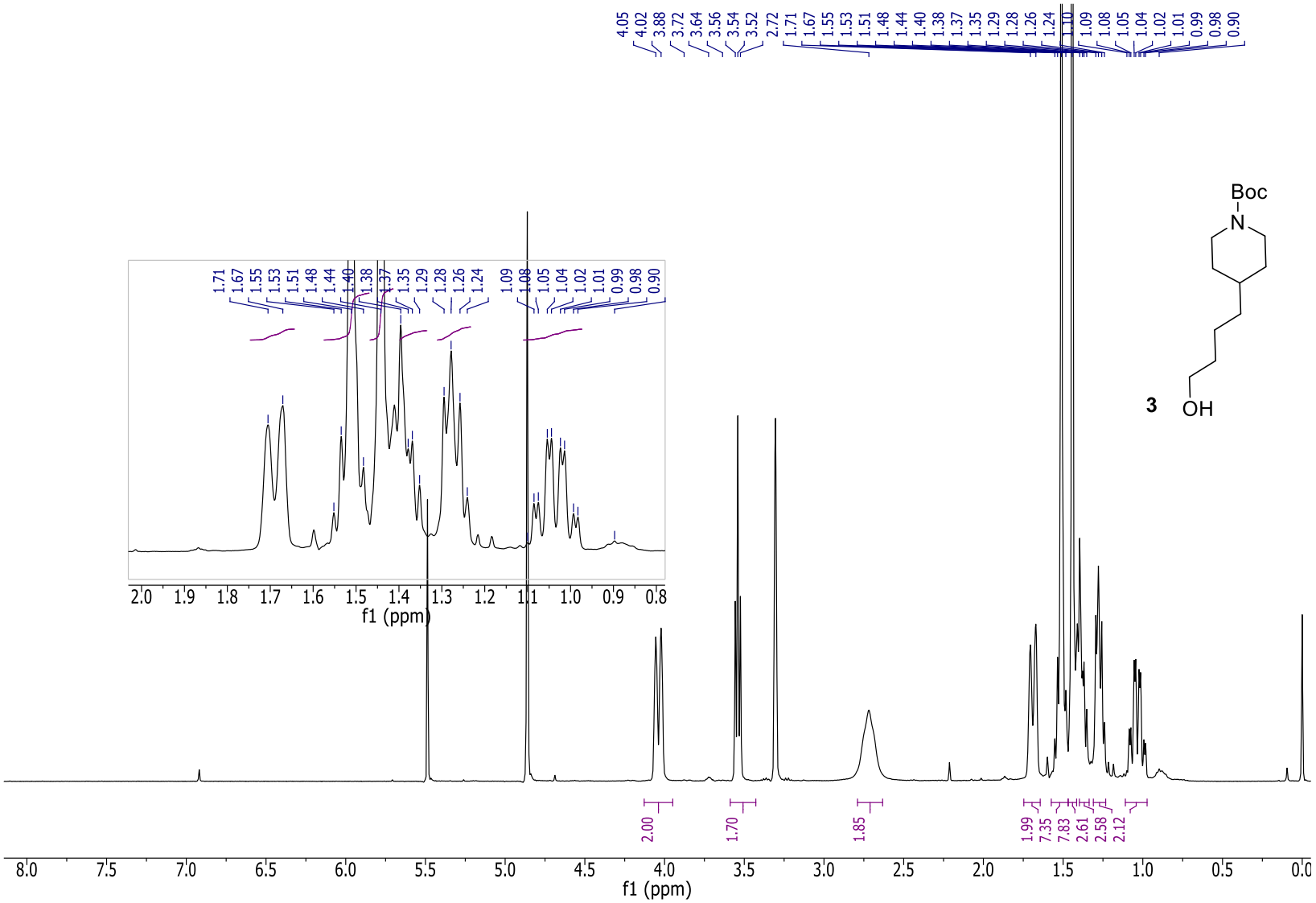


Figure S12. Proton NMR of compound 3

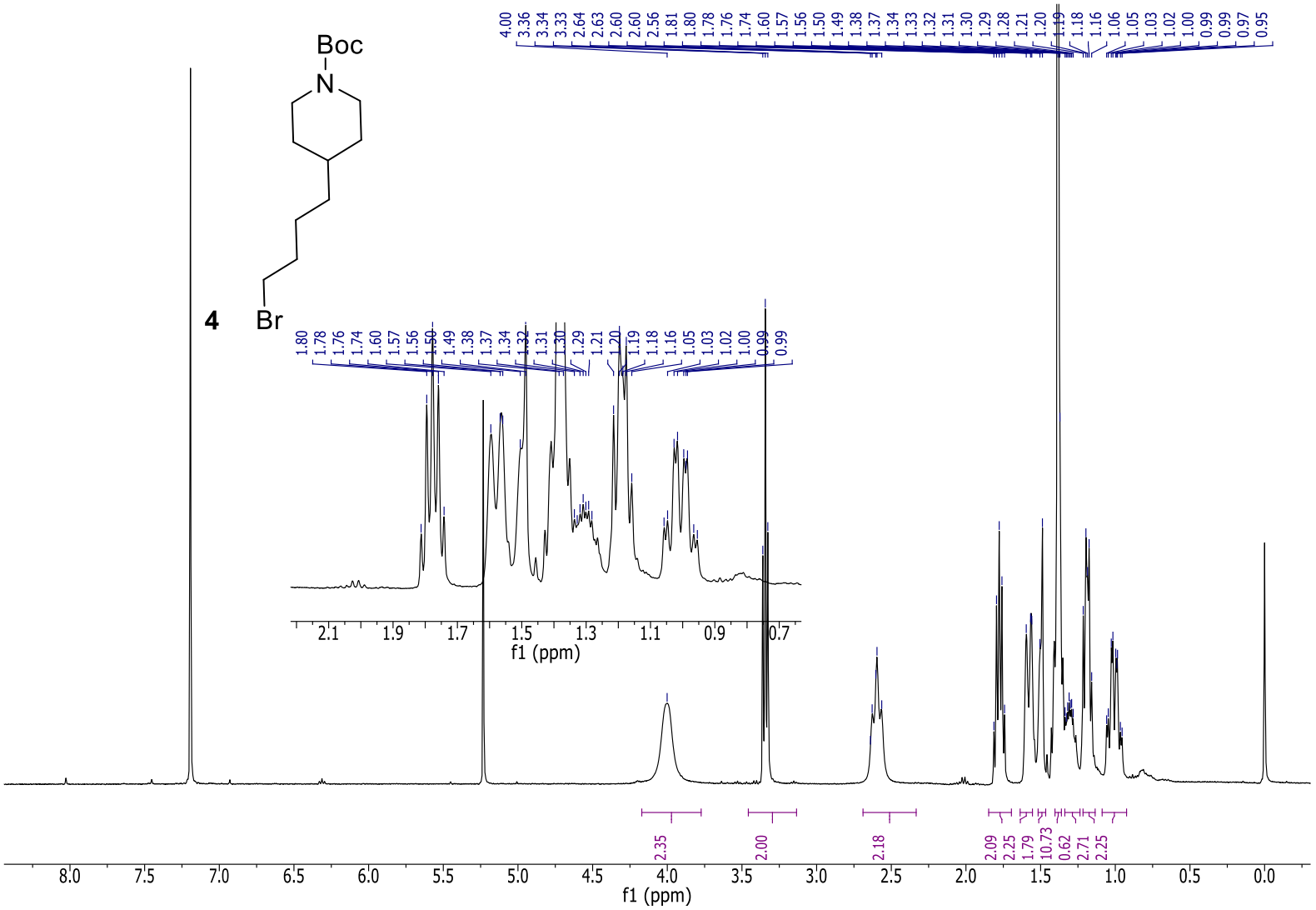


Figure S13. Proton NMR of compound 4

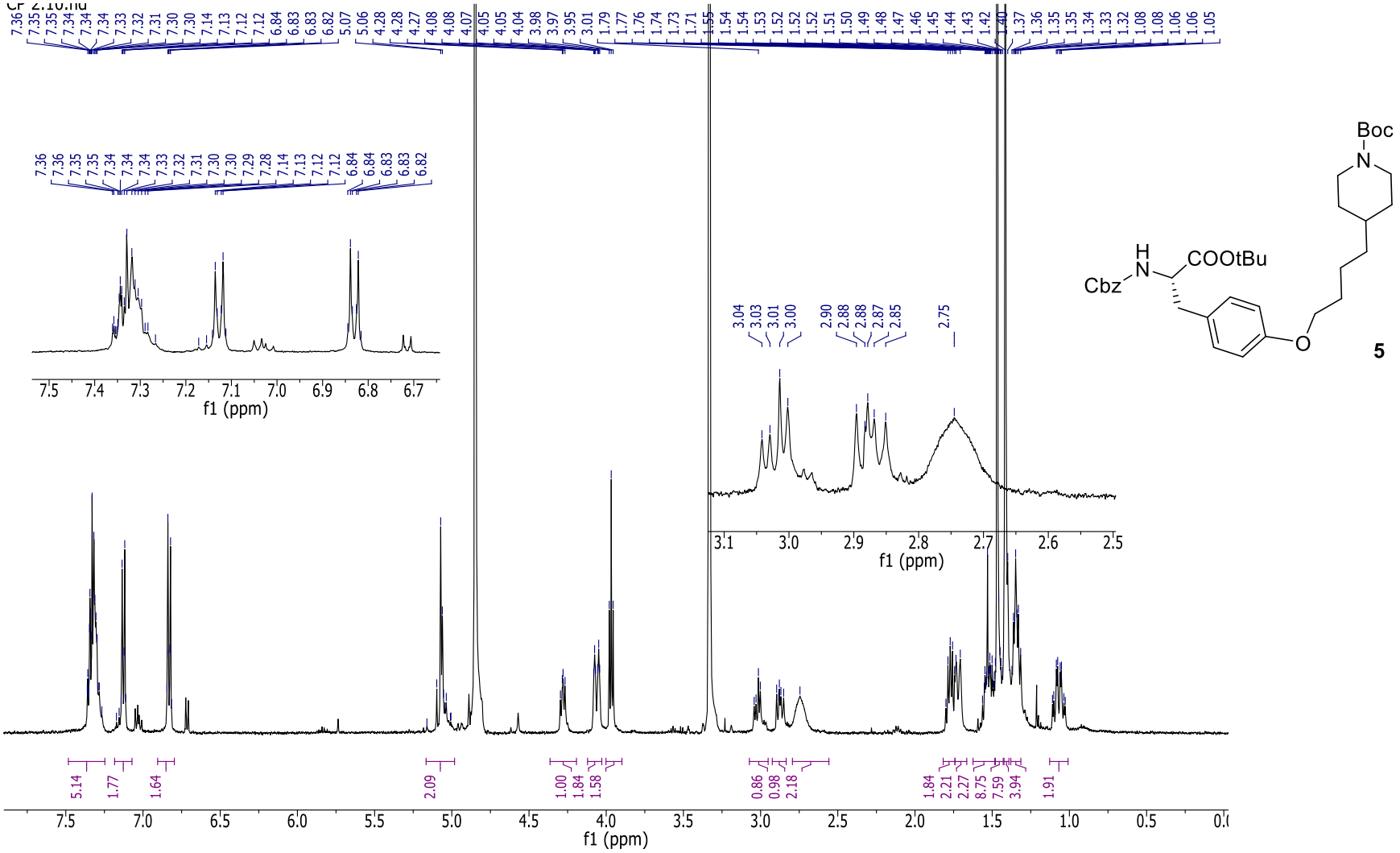


Figure S14. Proton NMR of compound 5

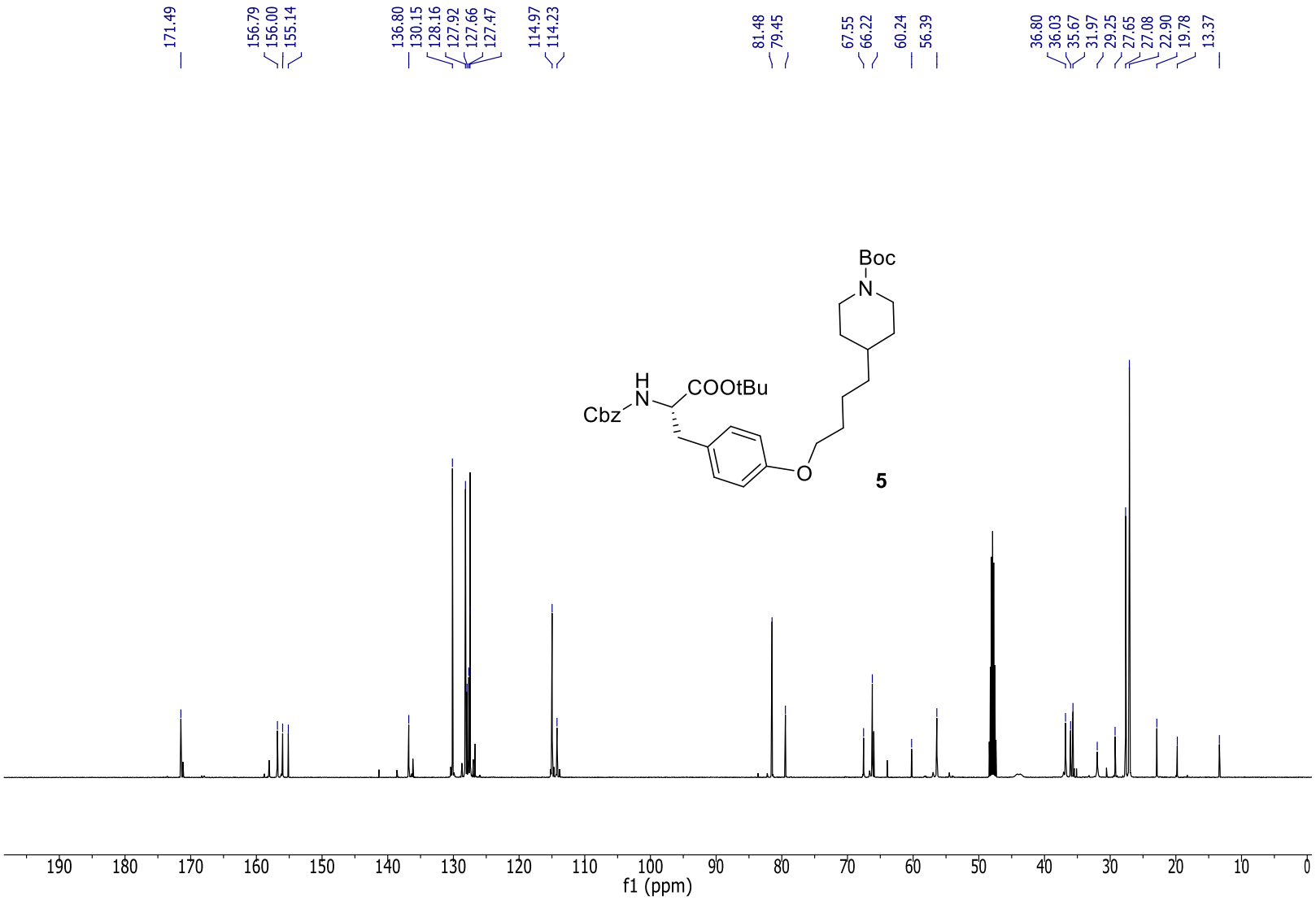


Figure S15. Carbon NMR of compound 5

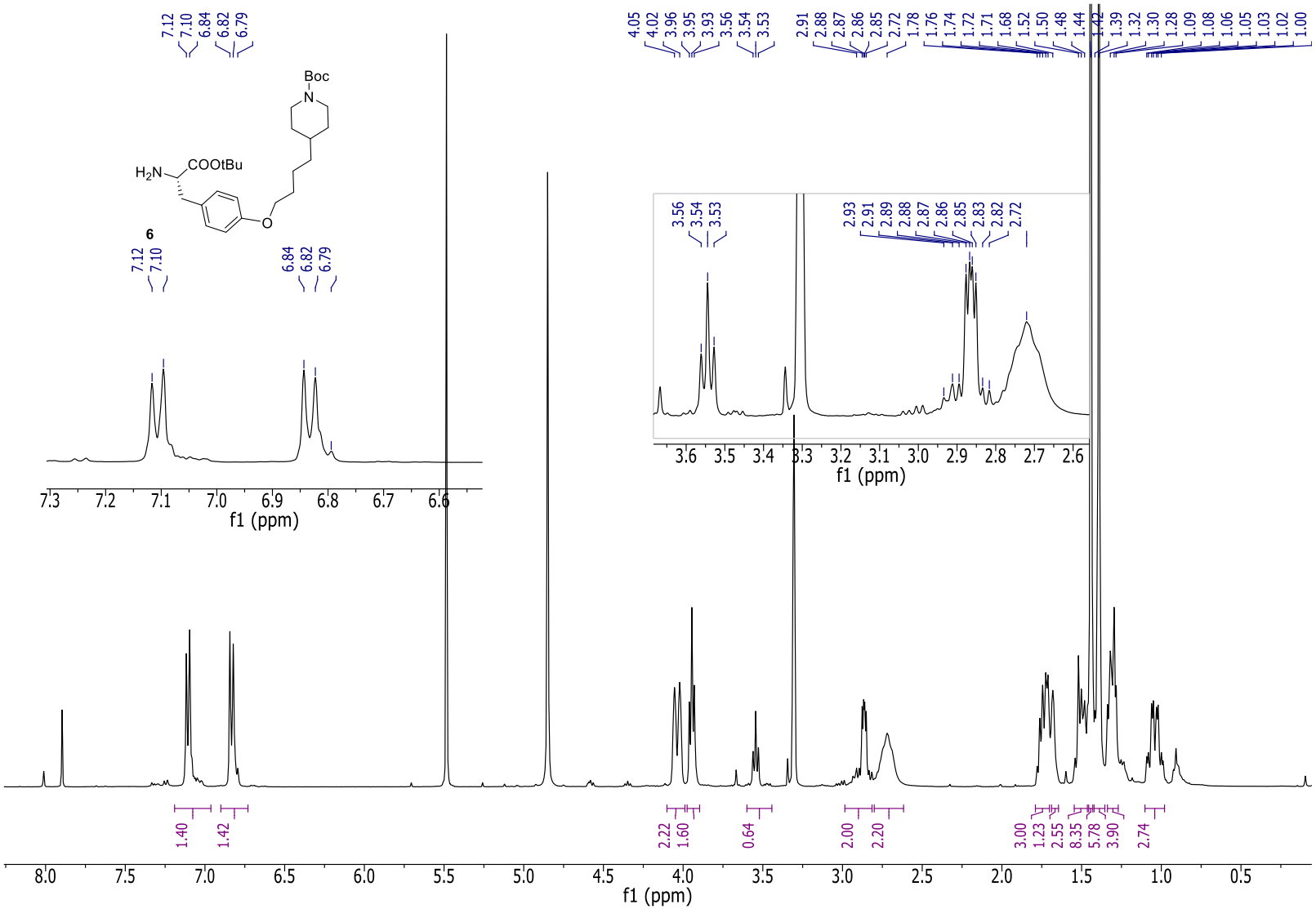


Figure S16. Proton NMR of compound 6

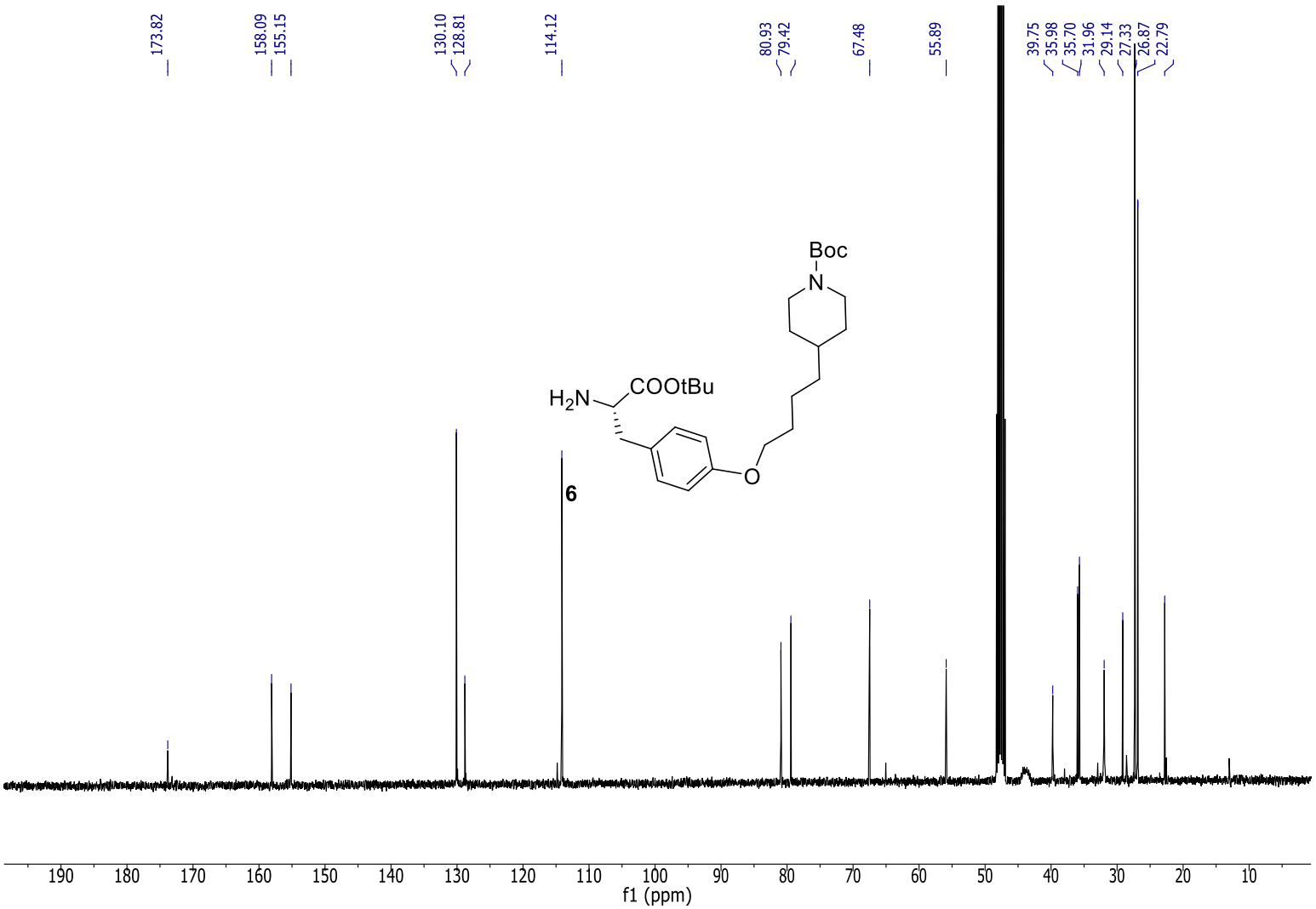


Figure S17. Proton NMR of compound 6

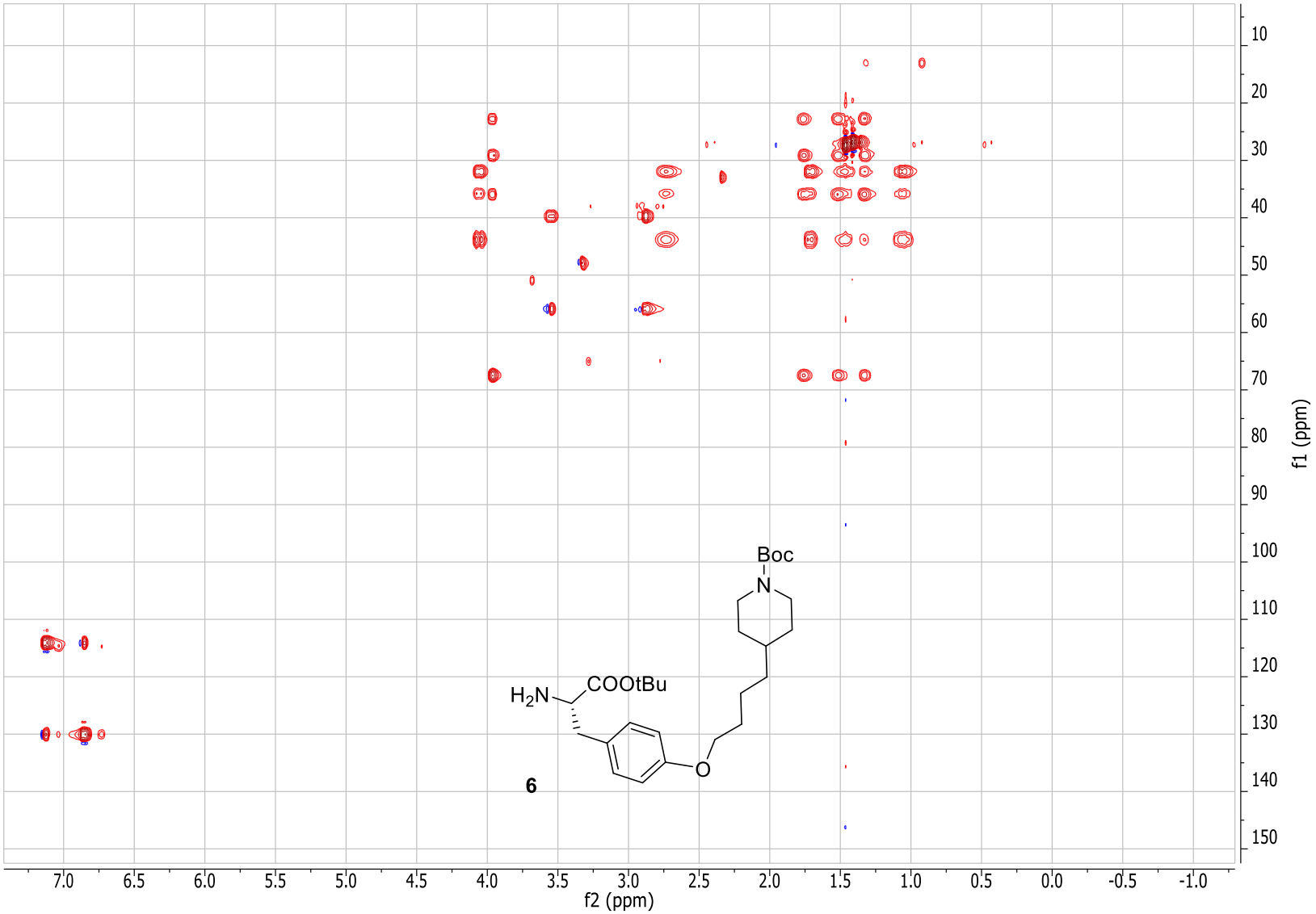


Figure S18. COSY NMR of compound 6

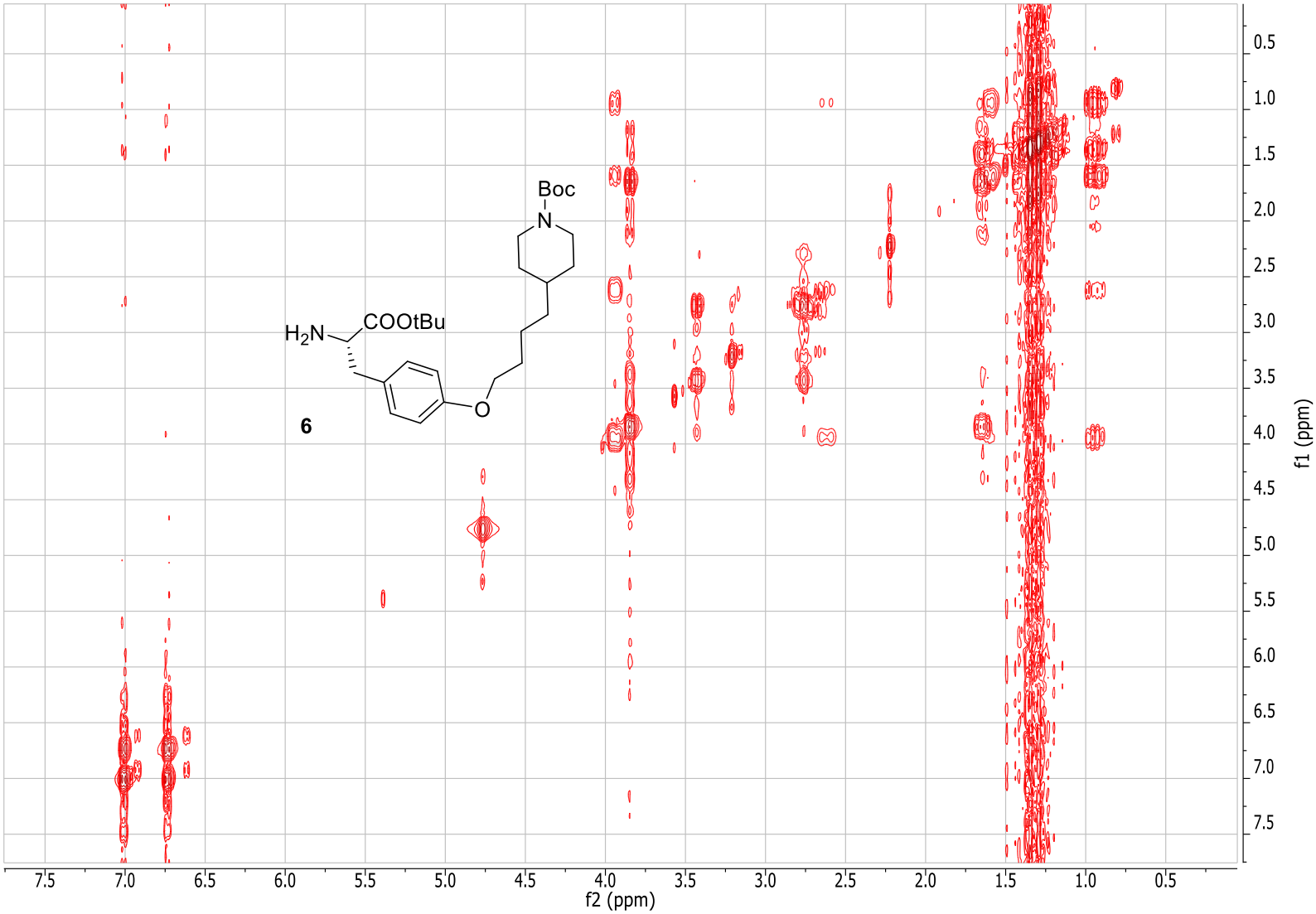


Figure S19. ROSY NMR of compound 6

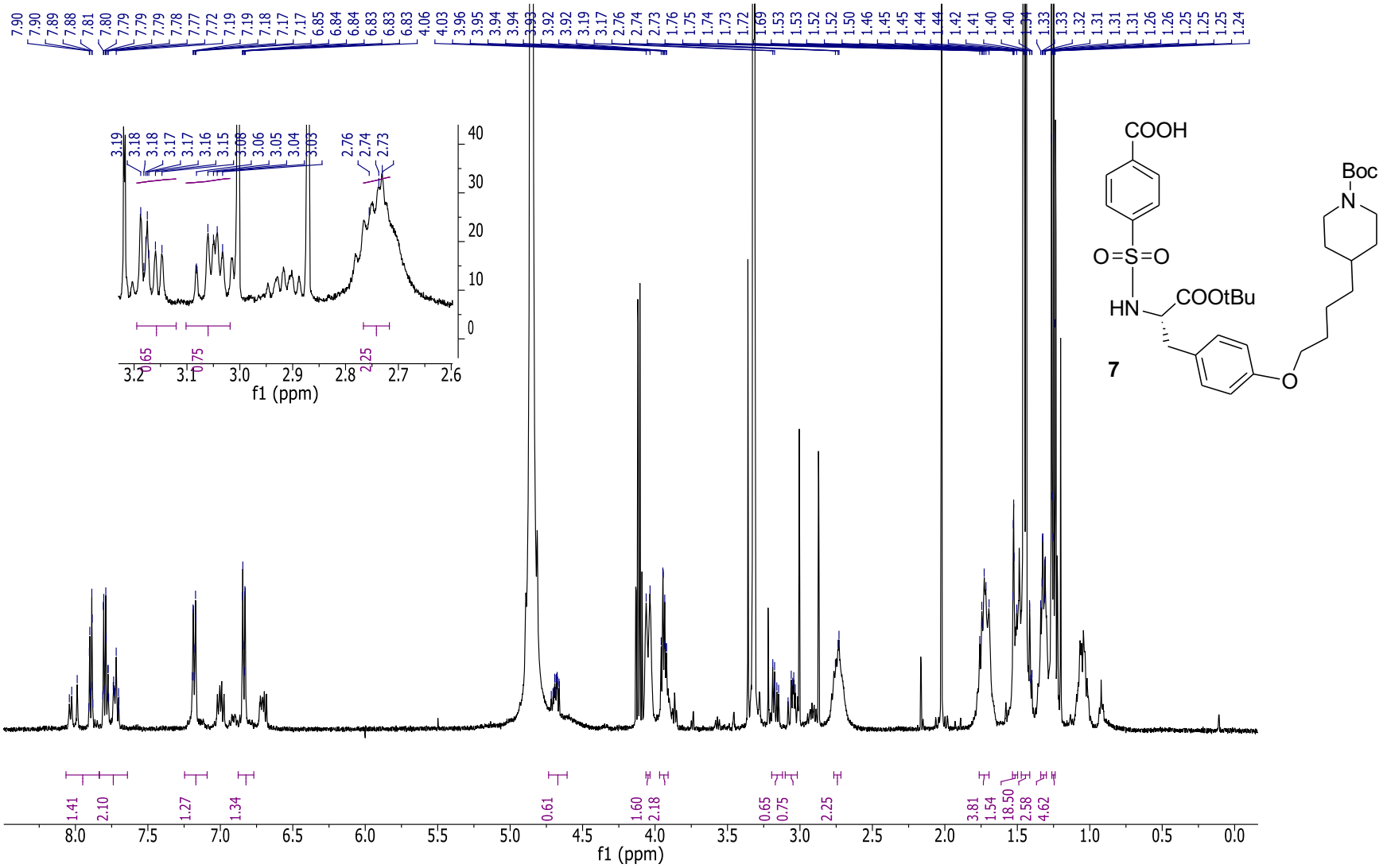


Figure S20. Proton NMR of compound 7

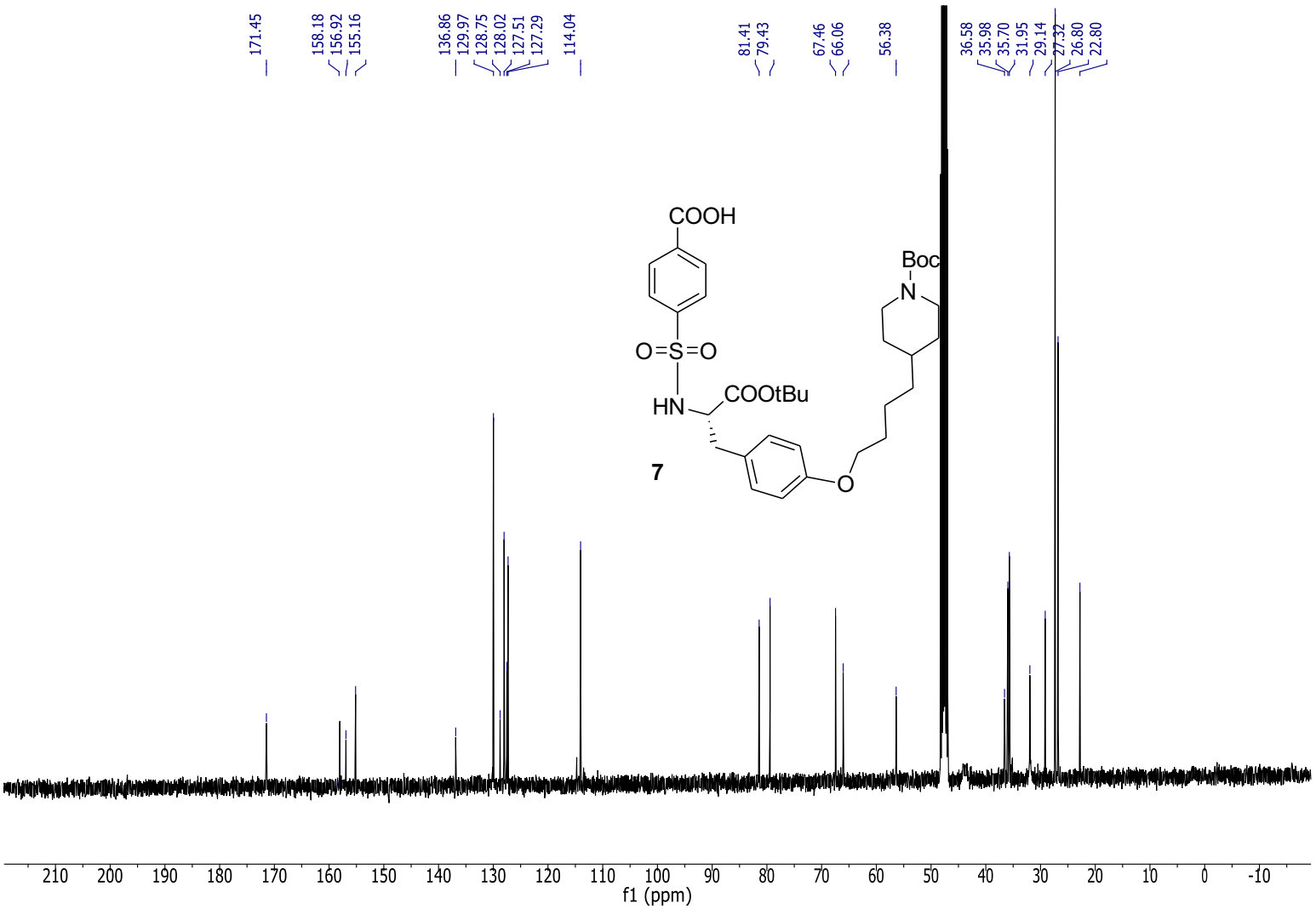


Figure S21. Carbon NMR of compound 7

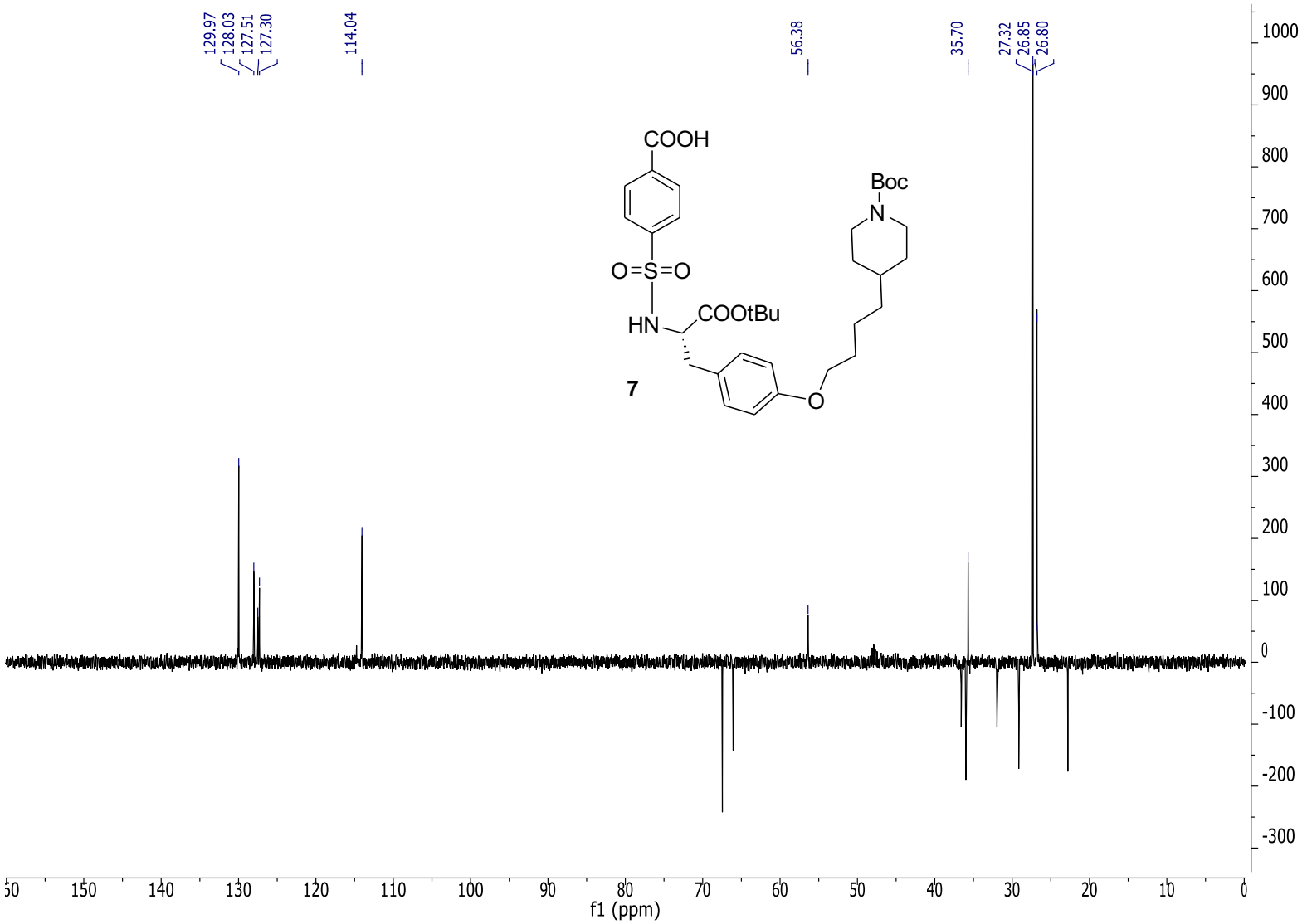


Figure S22. DEPT Carbon NMR of compound 7