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SUPPORTING INFORMATION

A fluorogenic probe targeting two spatially separated enzymes for selective imaging of cancer cells

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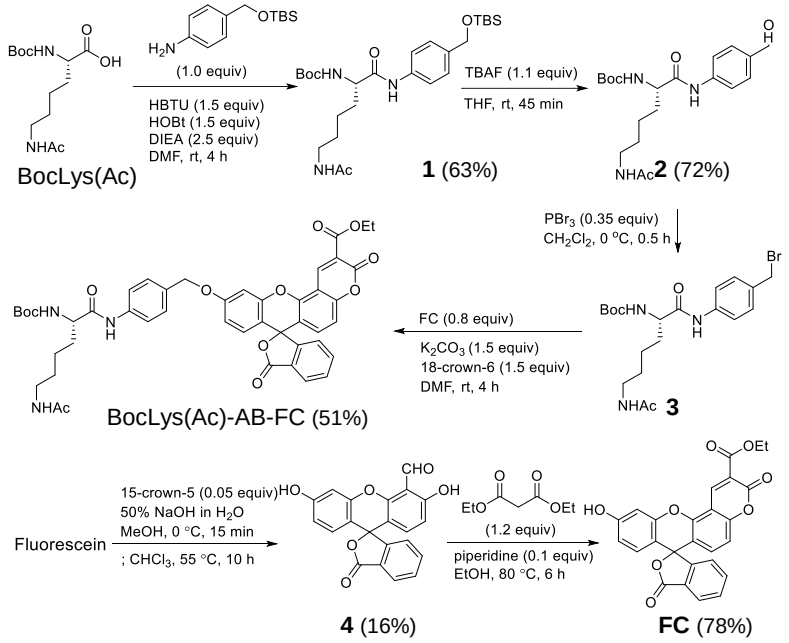
CONTENTS

Experimental details

Synthesis

General. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F254 glass plates (Merck Millipore). Compound spots were visualized by UV light (254 nm) and/or by staining with 10 wt% phosphomolybdic acid in ethanol. Flash column chromatography was conducted using silica gel 60 (230–400 mesh, Merck Millipore). NMR spectra were recorded on Bruker Avance lll HD 400 and Avance II 400 instruments. Mass spectra were obtained using a Waters 3100 LC/MS System. High resolution mass spectra were obtained using an Ultimate 3000 RS-Q-Exactive Orbitrap Plus. Chemical reagents used in this study were purchased from Sigma-Aldrich, TCI and Acrose, and human cathepsin L from Sino Biological.



Scheme S1. Synthesis of BocLys(Ac)-AB-FC.

Compound 1. To a stirred solution of BocLys(Ac) (1 g, 3.5 mmol), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 1.97 g, 5.2 mmol) and hydroxybenzotriazole (HOBt, 703 mg, 5.2 mmol) in DMF (12 mL) was added DIEA (1.5 mL, 1.12 g, 8.7 mmol) at room temperature. After stirring for 10 min, 4-(TBS-O-methyl)aniline (823 mg, 3.5 mmol) was added to the mixture. After stirring for 3 h, the mixture was diluted with EtOAc, washed with brine and water, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2 : EtOAc = 30:1) to give **1** as a white solid in 63% yield (1.11 g): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.88 (s, 1 H), 7.52 (d, 2 H, *J* = 8.4 Hz), 7.24 (d, 2 H, *J* = 8.4 Hz), 6.37 (s, 1 H), 5.53 (s, 1H), 4.69 (s, 2H), 4.27 (br s, 1 H), 3.23-3.28 (m, 2 H), 2.00 (s, 3 H), 1.89-1.96 (m, 1 H), 1.67-1.74 (m, 1 H), 1.53-1.60 (m, 2 H), 1.42-1.48 (m, 11 H), 0.93 (s, 9 H), 0.09 (s, 6 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.1, 170.9, 156.3, 137.4, 136.8, 126.7, 120.0, 80.1, 64.7, 55.0, 39.1, 32.1, 28.9, 28.4, 26.0, 23.1, 22.8, 18.5, -5.2; ESI-MS calcd for $C_{26}H_{45}N_3O_5Si$ $[M + H]^+$ = 508.3, found 508.9.

Compound 2. To a stirred solution of **1** (800 mg, 1.58 mmol) in THF (4 mL) was added 1 M *n*-tetrabutylammonium fluoride (TBAF, 1.73 mL, 1.73 mmol) in THF at 0 °C. The mixture was warmed to room temperature. After stirring for 45 min, the reaction mixture was diluted with EtOAc and washed with NH_4Cl. The aqueous layer was extracted with EtOAc three times. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2 : EtOAc = 20:1) to afford **2** as a white solid in 72% yield (448 mg): ^{1}H NMR (400 MHz, CD_3OD) δ 7.54 (d, 2 H, *J* = 8.3 Hz), 7.30 (d, 2 H, *J* = 8.3 Hz), 4.56 (s, 2 H), 4.13-4.17 (m, 1 H), 3.16 (t, 2 H, *J* = 6.7 Hz), 1.90 (s, 3 H), 1.66-1.81 (m, 2 H), 1.50-1.55 (m, 2 H), 1.35-1.48 (m, 11 H); ^{13}C NMR (100 MHz, CD_3OD) δ 173.6, 173.2, 157.9, 138.7, 138.6, 128.6, 121.3, 80.6, 64.8, 56.6, 40.2, 33.2, 30.0, 28.7, 24.3, 22.6; ESI-MS calcd for $C_{20}H_{31}N_3O_5$ $[M + H]^+$ 394.2, found 394.7.

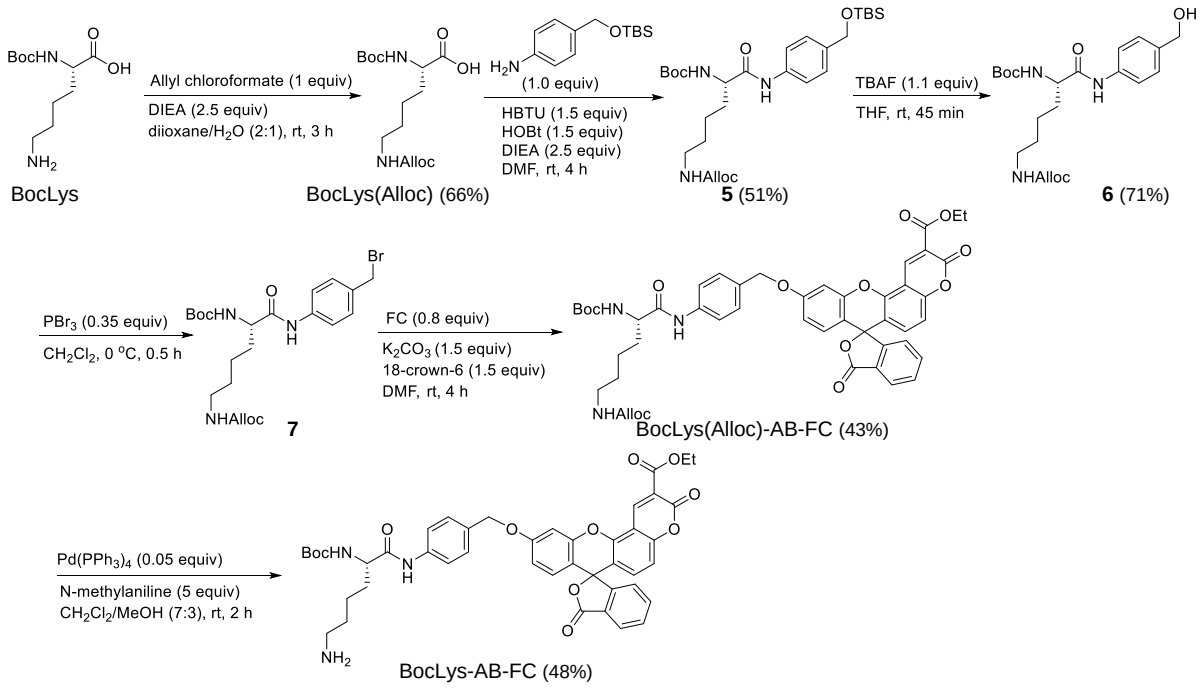
BocLys(Ac)-AB-FC. To a stirred solution of **2** (400 mg, 1.02 mmol) in anhydrous CH_2Cl_2 (3 mL) was added dropwise PBr_3 (360 μL of 1 M solution in CH_2Cl_2) at 0 °C for 10 min under N_2 atmosphere. After stirring for 30 min at the same temperature, the reaction mixture was quenched by addition of 5% $NaHCO_3$. The mixture was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give **3**. This compound was used for the next reaction without further purification.: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.17 (s, 1 H), 7.52 (d, 2 H, *J* = 7.8 Hz), 7.28 (d, 2 H, *J* = 7.8 Hz), 6.38 (s, 1 H), 5.59 (s, 1 H), 4.46 (s, 2 H), 4.28 (s, 1 H), 3.16-3.31 (m, 2 H), 1.97 (s, 3 H), 1.85-1.93 (m, 1 H), 1.66-1.75 (m, 1 H), 1.50-1.59 (m, 1 H), 1.36-1.49 (m, 11 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.2, 171.0, 156.4, 138.3, 133.5, 129.8, 120.2, 80.4, 55.1, 39.1, 33.6, 31.8, 28.9, 28.5, 23.2, 22.8; ESI-MS calcd for $C_{20}H_{30}BrN_3O_4$ $[M + H]^+$ 456.1, 458.1, found 456.5, 458.5.

To a stirred solution of **FC** (269 mg, 0.59 mmol) and K_2CO_3 (136.9 mg, 0.99 mmol) in anhydrous DMF (4 mL) was added 18-crown-6 (262 mg, 0.99 mmol) in anhydrous DMF (1 mL) at room temperature under N_2 atmosphere. After stirring for 30 min, compound **3** (300 mg, 0.66 mmol) was added to the mixture. After stirring for 4 h, the reaction mixture was diluted with EtOAc and washed with water and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane : EtOAc = 5:1) to afford BocLys(Ac)-AB-FC as a pale yellow solid in 51% yield (280 mg): ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.13 (s, 1 H), 9.01 (s, 1 H), 8.05 (d, 1 H, *J* = 7.0 Hz), 7.67-6.73 (m, 2 H), 7.61 (d, 2 H, *J* = 8.2 Hz), 7.35 (d, 2 H, *J* = 8.2 Hz), 7.15 (d, 1 H, *J* = 7.5 Hz), 7.00-7.06 (m, 2 H), 6.99 (s, 1 H), 6.71-6.77 (m, 2 H), 6.56 (s, 1 H), 5.51 (s, 1 H), 5.07 (s, 2 H), 4.48 (q, 2 H, *J* = 7.1 Hz), 4.27 (s, 1 H), 3.20-3.30 (m, 2 H), 2.01 (s, 3 H), 1.91-1.97 (m, 1 H), 1.69-1.75 (m, 1 H), 1.54-1.59 (m, 2 H), 1.40-1.49 (m, 14 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.2, 171.0, 168.9, 163.1, 160.7, 156.2, 156.1, 152.5, 151.3, 148.3, 142.8, 138.1, 135.5, 133.8, 131.6, 130.3, 129.2, 128.2, 126.4, 125.4, 123.9, 120.1, 117.8, 114.7, 113.7, 112.3, 110.9, 107.8, 102.1, 81.6, 80.3, 70.7, 62.3, 55.0, 44.0, 39.0, 31.6, 29.7, 28.6, 28.3, 22.9, 22.6, 14.3; HR ESI-MS calcd for $C_{46}H_{45}N_3O_{12}$ $[M + H]^+$ 832.3082, found 832.3078.

Compound 4 (ref 1)**.** To a stirred solution of fluorescein (2 g, 6.2 mmol) in MeOH (7 mL) was added dropwise a mixture of 50% NaOH in water (12 mL) and 15-crown-5 (68.4 mg, 0.31 mmol) at 0 °C. After stirring for 10 min, the mixture was warmed to 55 °C in a bath. To the mixture was added dropwise $CHCl_3$ (5 mL) while the reaction temperature was maintained at 55 °C. After stirring for 15 h at the same temperature, the mixture was cooled to room temperature. The mixture was acidified with 5 M HCl to precipitate the product. The solid was

filtered and dried. The crude product was purified by flash column chromatography (CH_2Cl_2 : EtOAc = 17:3) to afford **4** as a yellow solid in 16% yield (357 mg): ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.9 (s, 1 H), 10.6 (s, 1 H), 10.3 (s, 1 H), 8.01 (d, 1 H, J = 7.5 Hz), 7.80 (td, 1 H, J = 7.6, 1.1 Hz), 7.72 (td, 1 H, J = 7.5, 0.8 Hz), 7.30 (d, 1 H, J = 7.6 Hz), 6.94 (d, 1 H, J = 8.9 Hz), 6.86 (d, 1 H, J = 1.8 Hz), 6.70 (d, 1 H, J = 8.9 Hz), 6.65-6.57 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 193.0, 168.7, 163.1, 159.7, 152.5, 152.3, 151.0, 136.6, 135.9, 130.4, 129.1, 126.0, 124.9, 124.1, 113.6, 113.5, 109.8, 109.3, 109.2, 102.8, 81.9; ESI-MS calcd for $C_{21}H_{12}O_6$ $[M + H]^+$ 361.1, found 361.4.

FC (ref 1)**.** To a stirred solution of **4** (500 mg, 1.39 mmol) and diethyl malonate (267 mg, 1.67 mmol) in EtOH (5 mL) were added piperidine (11.8 mg, 0.14 mmol) at room temperature. The resulting mixture was heated at reflux with vigorous stirring for 9 h. The mixture was cooled to room temperature to precipitate the product. The solid was filtered to obtain a product as a light yellow solid in 78% yield (494 mg): ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.3 (s, 1 H), 9.01 (s, 1 H), 8.05 (d, 1 H, J = 7.4 Hz), 7.81 (t, 1 H, J = 7.2 Hz), 7.75 (t, 1 H, J = 7.4 Hz), 7.32 (d, 1 H, J = 7.5 Hz), 7.16-7.11 (m, 2 H), 6.95 (s, 1 H), 6.65 (s, 2 H), 4.36 (q, 2 H, J = 7.1 Hz), 1.36 (t, 3 H, J = 7.1 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.5, 162.5, 159.8, 155.6, 155.4, 152.3, 150.8, 147.6, 141.7, 135.9, 133.8, 130.5, 129.1, 125.6, 124.9, 124.0, 117.7, 114.3, 113.8, 112.2, 109.0, 107.4, 102.7, 81.4, 61.5, 14.0; ESI-MS calcd for $C_{26}H_{16}O_8$ $[M + H]^+$ 457.1, found 457.6.



Scheme S2. Synthesis of BocLys-AB-FC.

BocLys(Alloc). To a stirred solution of allyl chloroformate (979 mg, 8.12 mmol) in a mixture of dioxane and water (21 mL, 2:1) was added BocLys (2 g, 8.12 mmol) and DIEA (2.62 g, 20.3 mmol) at room temperature. After stirring for 4 h, the reaction mixture was diluted with CH_2Cl_2 and isopropanol (2:1), and washed with water and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified

by flash column chromatography (CH_2Cl_2 : EtOAc : MeOH = 10:2:1) to afford BocLys(Alloc) in 66% yield (1.77 g): ^{1}H NMR (400 MHz, CD_3OD) δ 5.84-6.00 (m, 1 H), 5.29 (dd, 1 H, *J* = 17.2, 1.5 Hz), 5.17 (dd, 1 H, *J* = 10.5, 1.1 Hz), 4.51 (d, 2 H, *J* = 5.4 Hz), 4.01-4.11 (m, 1 H), 3.10 (t, 2 H, *J* = 6.5 Hz), 1.75-1.86 (m, 1 H), 1.60-1.71 (m, 1 H), 1.48-1.55 (m, 2 H), 1.39-1.47 (m, 11 H); ^{13}C NMR (100 MHz, CD_3OD) δ 176.2, 158.7, 158.0, 134.5, 117.4, 80.4, 66.2, 54.7, 41.3, 32.3, 30.4, 28.7, 24.0; ESI-MS calcd for $C_{15}H_{26}N_2O_6$ $[M + H]^+$ 331.2, found 331.5.

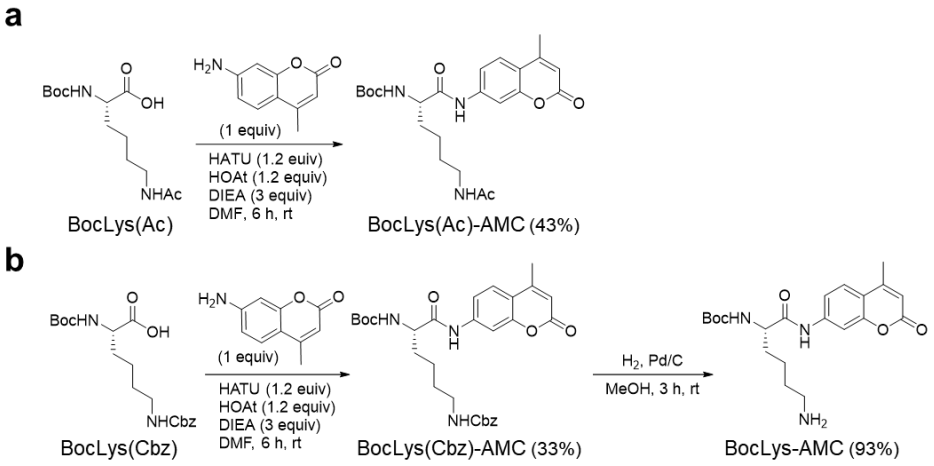
Compound 5. To a stirred solution of BocLys(Alloc) (1 g, 3.03 mmol), HBTU (1.72 g, 4.55 mmol) and HOBt (615 mg, 4.55 mmol) in DMF (10 mL) was added DIEA (1.32 mL, 979 mg, 7.58 mmol) at room temperature. After stirring for 10 min, 4-(TBS-O-methyl)aniline (719 mg, 3.03 mmol) was added to the mixture. After stirring for 3 h, the mixture was diluted with EtOAc and washed with brine and water, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2 : EtOAc = 30:1) to give **5** as a white solid in 51% yield (850 mg): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (s, 1 H), 7.48 (d, 2 H, *J* = 8.2 Hz), 7.21 (d, 2 H, *J* = 7.6 Hz), 5.83-6.00 (m, 1 H), 5.61 (s, 1 H), 5.28 (d, 1 H, *J* = 17.1 Hz), 5.18 (d, 1 H, *J* = 10.3 Hz), 5.10 (s, 1 H), 4.67 (s, 2H), 4.55 (s, 2 H), 4.30 (s, 1 H), 3.16 (s, 2 H), 1.85-1.95 (m, 1 H), 1.65-1.78 (m, 1 H), 1.42-1.48 (m, 13 H); ^{13}C NMR 100 MHz, $CDCl_3$) δ 171.2, 156.6, 156.3, 137.2, 136.7, 132.9, 126.5, 119.8, 117.5, 80.1, 65.4, 64.6, 64.2, 55.1, 40.3, 40.1, 32.0, 29.4, 28.3, 25.9, 22.7, 18.4, -5.2; ESI-MS calcd for $C_{28}H_{47}N_3O_6Si$ $[M + H]^+$ 550.3, found 550.9.

Compound 6. To a stirred solution of **5** (800 mg, 1.46 mmol) in THF (4 mL) was added 1 M *n*-tetrabutylammonium fluoride (1.60 mL, 1.60 mmol) in THF at 0 °C. The mixture was warmed to room temperature. After stirring for 45 min, the reaction mixture was diluted with EtOAc and washed with NH_4Cl. The aqueous layer was extracted with EtOAc three times. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2 : EtOAc = 20:1) to afford **6** as a white solid in 71% yield (452 mg): ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.07 (s, 1 H), 7.43 (d, 2 H, *J* = 7.6 Hz), 7.17 (d, 2 H, *J* = 7.6 Hz), 5.85-5.97 (m, 1 H), 5.65 (s, 1 H), 5.29 (dd, 1 H, *J* = 17.2, 1.4 Hz), 5.20 (dd, 1 H, *J* = 10.4, 1.0 Hz), 4.58 (s, 2 H), 4.55 (d, 2 H, *J* = 5.4 Hz), 4.31 (s, 1 H), 3.16 (t, 2 H, *J* = 6.3 Hz), 1.82-1.93 (m, 1 H), 1.66-1.78 (m, 1 H), 1.50-1.56 (m, 2 H), 1.40-1.48 (m, 11 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 156.7, 156.6, 137.2, 136.8, 133.0, 127.7, 120.0, 117.7, 80.4, 65.6, 64.6, 55.2, 40.5, 32.2, 29.6, 28.4, 22.8; ESI-MS calcd for $C_{22}H_{33}N_3O_6$ $[M + H]^+$ 436.2, found 436.7.

BocLys(Alloc)-AB-FC. To a stirred solution of **6** (400 mg, 0.92 mmol) in anhydrous CH_2Cl_2 (3 mL) was added dropwise PBr_3 (320 μL of 1 M solution in CH_2Cl_2) at 0 °C for 10 min under N_2 atmosphere. After stirring for 30 min at the same temperature, the reaction mixture was quenched by addition of 5% $NaHCO_3$. The mixture was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give **7**. This compound was used for the next reaction without further purification: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.10 (s, 1 H), 7.49 (d, 2 H, *J* = 7.5 Hz), 7.23 (d, 2 H, *J* = 7.5 Hz), 5.84-5.98 (m, 1 H), 5.63 (s, 1 H), 5.29 (dd, 1 H, *J* = 17.2, 1.4 Hz), 5.20 (dd, 1 H, *J* = 10.4, 1.0 Hz), 5.05 (s, 1 H), 4.43-4.63 (m, 4 H), 4.32 (s, 1 H), 3.17 (t, 2 H, *J* = 6.3 Hz), 1.82-1.97 (m, 1 H), 1.64-1.80 (m, 1 H), 1.49-1.60 (m, 2 H), 1.36-1.49 (m, 11 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.2, 156.7, 156.5, 138.1, 132.9, 129.8, 129.3, 120.0, 117.7, 80.5, 65.6, 55.2, 40.3, 33.6, 31.8, 29.6, 28.4, 22.7; ESI-MS calcd for $C_{22}H_{32}BrN_3O_5$ $[M + H]^+$ 498.2, 500.2, found 498.7, 500.7.

To a stirred solution of **FC** (247 mg, 0.54 mmol) and K_2CO_3 (125 mg, 0.90 mmol) in anhydrous DMF (2 mL) was added 18-crown-6 (238 mg, 0.90 mmol) in anhydrous DMF (1 mL) at room temperature under N_2 atmosphere. After stirring for 30 min, compound **7** (300 mg, 0.60 mmol) was added to the mixture. After stirring for 4 h, the reaction mixture was diluted with EtOAc and washed with water and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane : EtOAc = 5:1) to afford BocLys(Alloc)-AB-FC as a pale yellow solid in 43% yield (226 mg): ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.15 (s, 1 H), 8.67 (s, 1 H), 8.07 (d, 1 H, *J* = 7.6 Hz), 7.64-6.75 (m, 2 H), 7.59 (d, 2 H, *J* = 8.3 Hz), 7.38 (d, 2 H, *J* = 8.3 Hz), 7.16 (d, 1 H, *J* = 7.1 Hz), 7.02-7.07 (m, 2 H), 7.00 (s, 1 H), 6.72-6.80 (m, 2 H), 5.86-5.98 (m, 1 H), 5.30 (dd, 1 H, *J* = 17.3, 1.5 Hz), 5.21 (dd, 1 H, *J* = 10.4, 1.2 Hz), 5.09 (s, 2 H), 4.93 (s, 1 H), 4.57 (d, 2 H, *J* = 5.1 Hz), 4.50 (q, 2 H, *J* = 7.1 Hz), 4.22 (s, 1 H), 3.21 (t, 2 H, *J* = 5.7 Hz), 1.90-2.02 (m, 1 H), 1.67-1.78 (m, 1 H), 1.54-1.60 (m, 2 H), 1.43-1.50 (m, 14 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8, 169.0, 163.2, 160.8, 156.8, 156.4, 156.3, 156.2, 152.6, 151.4, 148.4, 142.9, 138.1, 135.6, 134.0, 132.9, 131.7, 130.4, 129.3, 128.4, 126.4, 125.5, 123.9, 120.1, 117.9, 117.8, 114.8, 113.8, 112.4, 111.0, 107.9, 102.1, 81.7, 80.6, 70.1, 65.7, 62.4, 55.1, 40.2, 31.4, 29.5, 28.4, 22.6, 14.4; ESI-MS calcd for $C_{48}H_{47}N_3O_{13}$ $[M + H]^+$ 874.3, found 875.2.

BocLys-AB-FC. To a stirred solution of BocLys(Alloc)-AB-FC (100 mg, 0.11 mmol) and $Pd(PPh_3)_4$ (6.4 mg, 0.0055 mmol) in a mixture of CH_2Cl_2 and MeOH (2 mL, 7:3) was added N-methylaniline (62 μL, 61.3 mg, 0.57 mmol) in a mixture of CH_2Cl_2 and MeOH (1 mL, 7:3) at room temperature under argon atmosphere. After stirring for 2 h, the volatile material was removed under reduced pressure. The residue was purified by preparative HPLC to afford BocLys-AB-FC as a light orange solid in 48% yield (41.7 mg): ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.1 (s, 1 H), 9.04 (s, 1 H), 8.06 (d, 1 H, *J* = 7.4 Hz), 7.73-7.84 (m, 2 H), 7.65 (d, 2 H, *J* = 8.3 Hz), 7.42 (d, 2 H, *J* = 8.3 Hz), 7.36 (d, 1 H, *J* = 1.7 Hz), 7.33 (d, 1 H, *J* = 7.5 Hz), 7.17 (s, 2 H), 7.06 (d, 1 H, *J* = 7.6 Hz), 6.86 (dd, 1 H, *J* = 8.8, 1.7 Hz), 6.76 (d, 1 H, *J* = 8.8 Hz), 5.15 (s, 2 H), 4.36 (q, 2 H, *J* = 7.0 Hz), 3.99-4.11 (m, 1 H), 2.75 (t, 2 H, *J* = 7.2 Hz), 1.48-1.73 (m, 4 H), 1.15-1.47 (m, 14 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.5, 168.6, 162.6, 160.4, 155.8, 155.6, 155.5, 152.3, 150.9, 147.6, 141.9, 139.0, 136.1, 133.9, 131.0, 130.7, 129.1, 128.7, 125.6, 125.1, 124.1, 119.2, 117.8, 114.2, 113.9, 112.4, 110.6, 107.6, 102.2, 81.2, 78.2, 79.7, 61.7, 55.1, 38.6, 31.2, 28.3, 26.7, 22.6, 14.3; HR ESI-MS calcd for $C_{44}H_{43}N_3O_{11}$ $[M + H]^+$ 790.2975, found 790.2965.



Scheme S3. Synthesis of (a) BocLys(Ac)-AMC and (b) BocLys-AMC.

BocLys(Ac)-AMC. To a solution of 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU, 79 mg, 0.21 mmol) and 1-hydroxy-7-azabenzotriazole (HOAt, 28 mg, 0.21 mmol) in DMF (1.7 mL) were added DIEA (67 mg, 0.52 mmol) and BocLys(Ac) (50 mg, 0.17 mmol) at room temperature. After stirring for 15 min, 7-amino-4-methylcoumarin (30 mg, 0.17 mmol) was added to the mixture. After stirring for 6 h, the volatile material was removed under reduced pressure. The residue was dissolved into EtOAc and the organic layer was washed with 1 M HCl, aqueous saturated $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2 : MeOH = 39:1) to give BocLys(Ac)-AMC as a white solid in 43% yield (33 mg): ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.72 (br s, 1 H), 7.72 (s, 1 H), 7.41-7.43 (m, 2 H), 7.10 (br s, 1 H), 6.13 (s, 1 H), 5.67 (br s, 1 H), 4.36 (br s, 1 H), 3.28 (br s, 2 H), 2.37 (s, 3 H), 2.07 (s, 3 H), 1.94 (br s, 1 H), 1.74 (br s, 1 H), 1.61 (br s, 2 H), 1.48 (br s, 2 H), 1.43 (s, 9 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.0, 171.1, 161.5, 156.5, 154.0, 152.9, 141.8, 125.1, 115.8, 113.1, 107.2, 80.5, 55.2, 39.1, 32.0, 28.9, 28.4, 23.2, 22.9, 18.6; HR ESI-MS calcd for $C_{23}H_{31}N_3O_6$ $[M + H]^+$ 446.2291, found 446.2282**.**

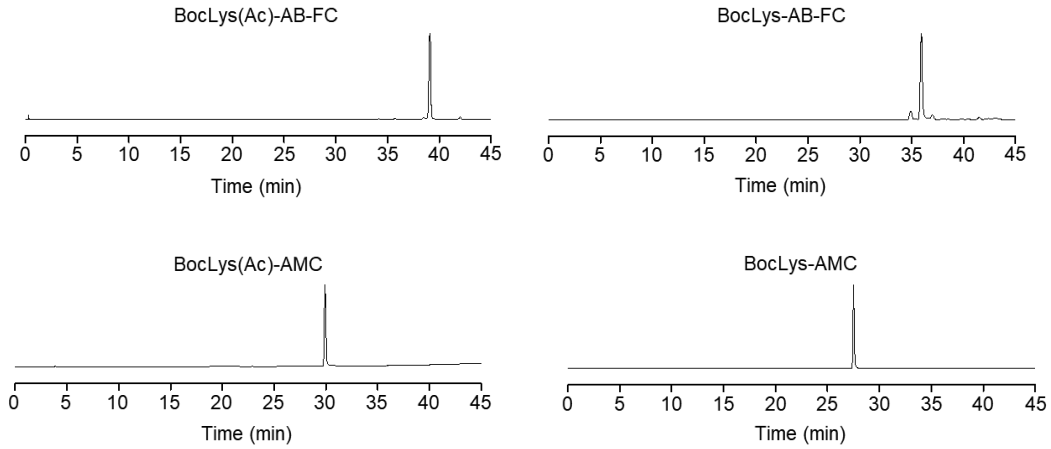
BocLys(Cbz)-AMC. To a solution of HATU (1.20 g, 3.15 mmol) and HOAt (429 mg, 3.15 mmol) in DMF (12 mL) were added DIEA (1.37 mL, 1.02 g, 7.89 mmol) and BocLys(Cbz) (1.00 g, 2.63 mmol) at room temperature. After stirring for 15 min, 7-amino-4-methylcoumarin (461 mg, 2.63 mmol) was added to the mixture. After stirring for 6 h, the volatile material was removed under reduced pressure. The residue was dissolved into EtOAc and the organic layer was washed with 1 M HCl, aqueous saturated $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2 : EtOAc = 3:1) to give BocLys(Cbz)-AMC as a white solid in 33% yield (462 mg): ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.40 (br s, 1 H), 7.70 (d, 1 H, J = 1.4 Hz), 7.38 (d, 1 H, J = 8.5 Hz), 7.30-7.33 (m, 5 H), 7.17 (d, 1 H, J = 7.5 Hz), 6.09 (br s, 1 H), 5.54 (d, 1 H, J = 7.5 Hz), 5.07 (br s, 2 H), 5.04 (br s, 1 H), 4.32-4.33 (m, 1 H), 3.18-3.19 (m, 2 H), 2.34 (s, 3 H), 1.87-1.89 (m, 1 H), 1.71-1.73 (m, 1 H), 1.50-1.54 (m, 4 H), 1.45 (s, 9 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.7, 161.3, 156.8, 154.0, 152.7, 141.5, 136.6, 128.6, 128.2,

128.1, 125.0, 115.8, 115.6, 113.2, 107.2, 80.8, 66.8, 55.4, 40.3, 31.6, 29.6, 28.5, 22.8, 18.6; ESI-MS calcd for $C_{29}H_{35}N_3O_7$ $[M + H]^+$ 538.2, found 538.8.

BocLys-AMC. A solution of BocLys(Z)-AMC (212 mg, 0.394 mmol), 10% Pd/C (21 mg, 10 wt % of the substrate) in MeOH (16 mL) was stirred for 3 h under H_2 atmosphere at room temperature. The reaction mixture was filtered through Celite 545, and the solution was concentrated under reduced pressure to obtain a white solid in 93% yield (148 mg). The product was used for the next reaction without further purification.: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.81 (br s, 1 H), 7.67 (s, 1 H), 7.37 (d, 1 H, *J* = 8.4 Hz), 7.22 (d, 1 H, *J* = 7.7 Hz), 6.06 (s, 1 H), 5.66 (br s, 1 H), 4.36 (s, 1 H), 2.81 (br s, 2 H), 2.74 (s, 2 H), 2.32 (s, 3 H), 1.86-1.87 (m, 1 H), 1.69-1.71 (m, 1 H), 1.53 (br s, 4 H), 1.43 (s, 9 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.9, 161.2, 156.6, 153.9, 152.7, 141.5, 124.9, 115.6, 115.5, 113.0, 107.1, 80.6, 55.5, 41.5, 32.5, 32.4, 28.4, 23.1, 18.5; HR ESI-MS calcd for $C_{21}H_{29}N_3O_5$ $[M+H]^+$ 404.2180, found 404.2176.

HPLC profiles of synthesized fluorogenic probes

Analytic RP-HPLC (C18 column, 250 x 4.6 mm; pore size, 5 μM) with a gradient of 5-100% CH_3CN in water (0.1% TFA) over 45 min (a flow rate; 1 mL/min, detection at 350 nm).



Measurements of cathepsin L activity. Human cathepsin L (Sino Biological) was dissolved in 120 μL of 400 mM sodium acetate containing 4 mM EDTA and 8 mM DTT (pH 5.5) and then activated at 37 ℃ for 1 min. After activation, a solution of 1.2 μL of 1 mM BocLys(Ac)-AB-FC in DMSO was added to above solution. Release of FC was detected using a fluorometer (JASCO, FT-8500) with 488 nm excitation filter and 520 nm emission filter. The reaction was run for 1 h with readings taken every 30 sec.

Cell Study

Cell culture. HeLa (cervical cancer cells), A549 (lung carcinoma epithelial cells), HT29 (colon cancer cells), MDA-MB-231 (breast cancer cells), HepG2 (liver cancer cells), As-Pc-1 (pancreatic cancer cells), DU145 (prostate cancer cells), AGS (gastric cancer cells), MRC-5 (fibroblast cell line derived from normal lung tissue), MEF (mouse embryonic fibroblasts), C2C12 (mouse myoblasts), and NRK (derived from normal rat kidney) cells were cultured in RPMI 1640 (Invitrogen), DMEM (Invitrogen) or MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin and 50 units/mL streptomycin at 37 ºC with 5% CO_2 atmosphere.

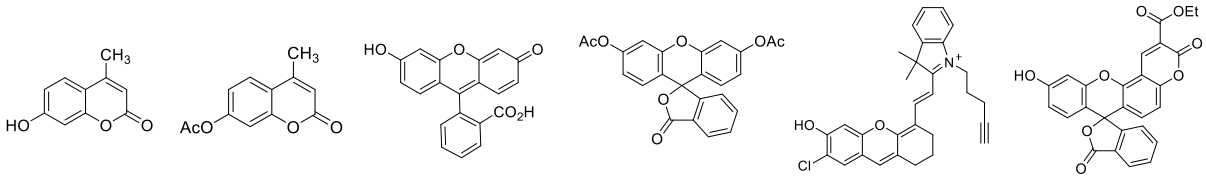
Imaging of cancer and normal cells using BocLys(Ac)-AB-FC. Cancer or normal cells in culture media were incubated with 25 μM BocLys(Ac)-AB-FC for 9 h. In addition, they were pre-incubated with 5 μM TSA, 50 μM SAHA or 20 μM Z-FF-FMK for 2 h followed by treatment with 25 μM BocLys(Ac)-AB-FC for 9 h. The nucleus of treated cells was stained with either Hoechst 33342 or NucRed 647. Cell images were obtained by using confocal fluorescence microscopy (Hoechst 33342: λ_{ex} = 405 nm, λ_{em} = 410–480 nm: FC; λ_{ex} = 488 nm, λ_{em} = 500-610 nm: NucRed 647; λ_{ex} = 640 nm, λ_{em} = 650-730 nm).

Detection of activities of HDACs and cathepsin L in cell lysates. Cancer and normal cells were lysed with Proprep lysis buffer (Intron). Cell lysates were placed into a 96-well plate. The lysates were incubated with 25 μM BocLys(Ac)-AB-FC or 10 μM BocLys(Ac)-AMC for 2 h. In addition, they were pre-incubated with 5 μM TSA, 50 μM SAHA or 20 μM Z-FF-FMK for 2 h followed by treatment with 25 μM BocLys(Ac)-AB-FC or 10 μM BocLys(Ac)-AMC for 2 h. The enzyme-catalyzed release of FC was monitored by using an Infinite® 200 PRO multimode microplate reader (BocLys(Ac)-AB-FC; λ_{ex} = 488 nm, λ_{em} = 530 nm; BocLys(Ac)-AMC; λ_{ex} = 405 nm, λ_{em} = 480 nm).

HPLC analysis of cell lysates treated with a probe. HeLa cells were incubated with 30 μM BocLys(Ac)-AB-FC for 16 h. After washing cells with Dulbecco's phosphate-buffered saline (DPBS) three times, they were detached from the surface with a cell scrapper using 500 μL of the lysis buffer (5% DMSO/95% 50 mM Tris buffer at pH 7.5 (v/v)). Cell suspensions were lysed with a sonicator (BANDELIN) with a pulse sequence of 20 sec on and 40 sec off with an amplitude of 20% on ice. The lysed samples were centrifuged at 15,000 g at 4 ºC for 15 min and the supernatant was collected. The supernatant was diluted with MeOH and centrifuged at 10,000 g for 5 min. After removal of volatile solvent under reduced pressure, the residue was analyzed by using analytical RP-HPLC with a gradient of 30-100% acetonitrile (0.1% TFA) in water (0.1% TFA) over 30 min.

Supplementary References

1. L. Hu, J. Liu, J. Zhang, H. Zhang, P. Xu, Z. Chen and E. Xiao, *RSC. Adv.*, 2019, **9**, 39532-39535.


CM
CM(OAc)
FI
FI(OAc)2
NIR dye
FC

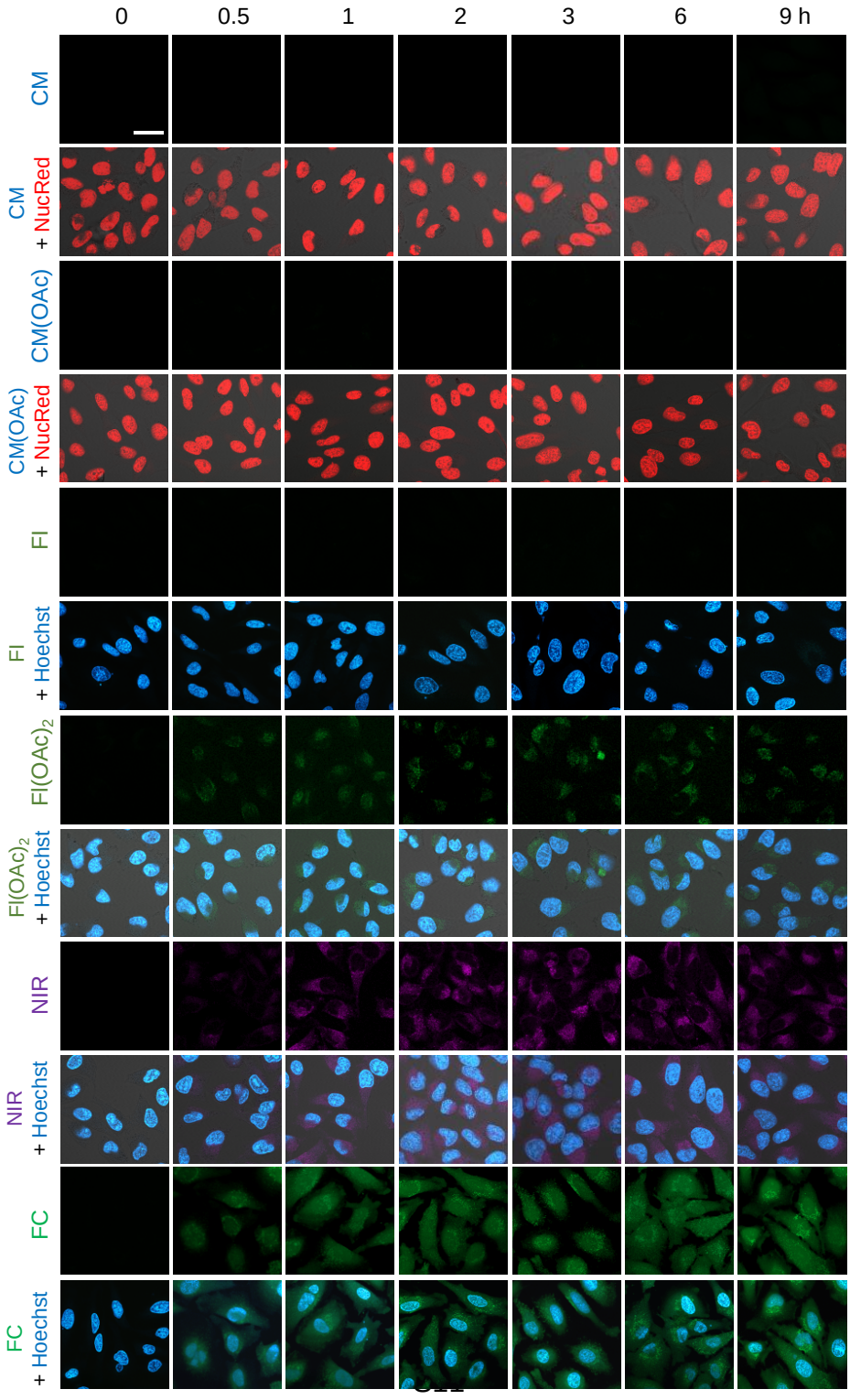

0
0.5
1
2
3
6
9 h
CM
CM + NucRed
CM(OAc)
CM(OAc) + NucRed
FI
FI + Hoechst
FI(OAc)2
FI(OAc)2 + Hoechst
NIR
NIR + Hoechst
FC
FC + Hoechst

Fig. S1 HeLa cells were incubated with 10 μM of CM, CM(OAc), FI, FI$(OAc)_2$, NIR or FC dye for indicated times. The nucleus of treated cells was stained with either Hoechst 33342 or NucRed 647. Cell images were obtained by using confocal fluorescence microscopy (scale bar = 10 μm). CM, CM(OAc) and Hoechst 33342; λ_{ex} = 405 nm, λ_{em} = 410–480 nm: Fl, Fl$(OAc)_2$ and FC; λ_{ex} = 488 nm, λ_{em} = 500-610 nm: NucRed 647; λ_{ex} = 640 nm, λ_{em} = 650-730 nm: NIR: λ_{ex} = 640 nm, λ_{em} = 650-820 nm.

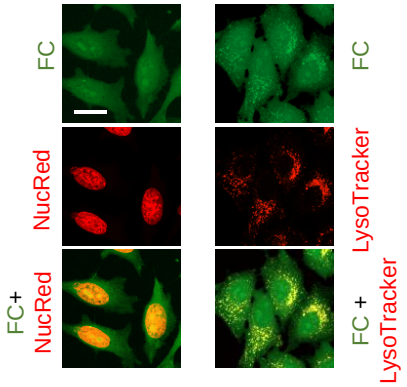


Fig. S2 HeLa cells were incubated with 10 μM FC for 9 h followed by treatment with LysoTracker Red for staining lysosomes or NucRed 647 for staining nuclei. Cell images were obtained by using confocal fluorescence microscopy (scale bar = 10 μm).

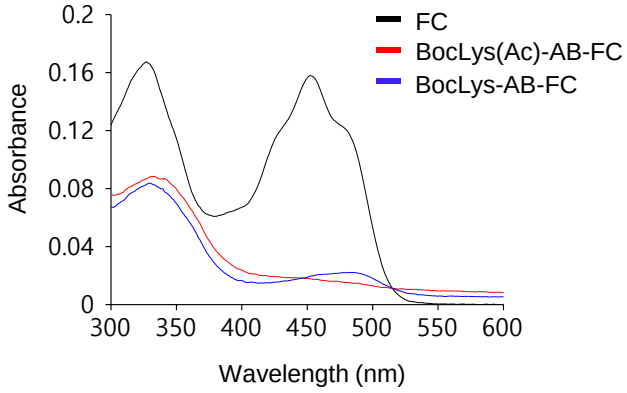


Fig. S3 UV spectra of FC, BocLys-AB-FC and BocLys(Ac)-AB-FC (10 μM) in 10 mM phosphate buffer (pH 7.4) containing 1% DMSO.

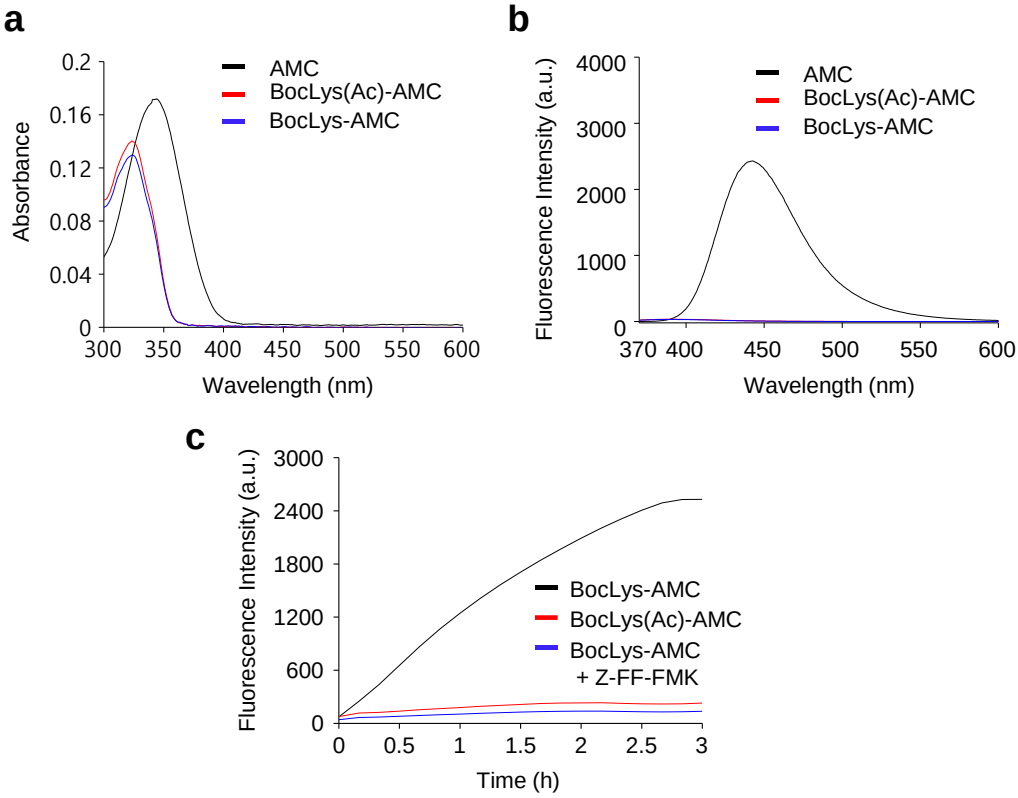


Fig. S4 (a) UV and (b) fluorescence spectra of AMC, BocLys-AMC and BocLys(Ac)-AMC (10 μM) in 400 mM sodium acetate buffer (pH 5.5) containing 1% DMSO. Fluorescence excitation wavelength is 360 nm. (c) The time course of the fluorescence intensity arising from BocLys(Ac)-AMC and BocLys-AMC in absence or presence of Z-FF-FMK by cathepsin L (λ_{ex}= 360 nm/ λ_{em}= 450 nm).

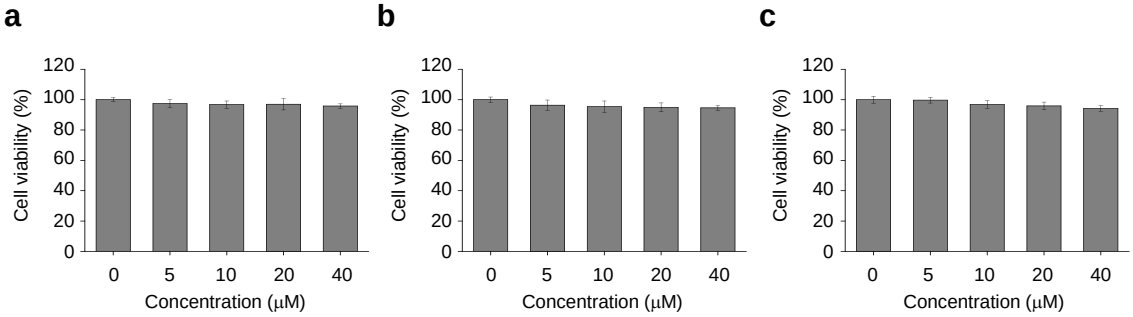


Figure S5. HeLa cells were incubated with indicated concentrations of (a) BocLys(Ac)-AB-FC, (b) BocLys-AB-FC, and (c) BocLys(Ac)-AMC for 24 h. Cell death was measured by means of a MTT assay (mean ± s.d., n = 3).

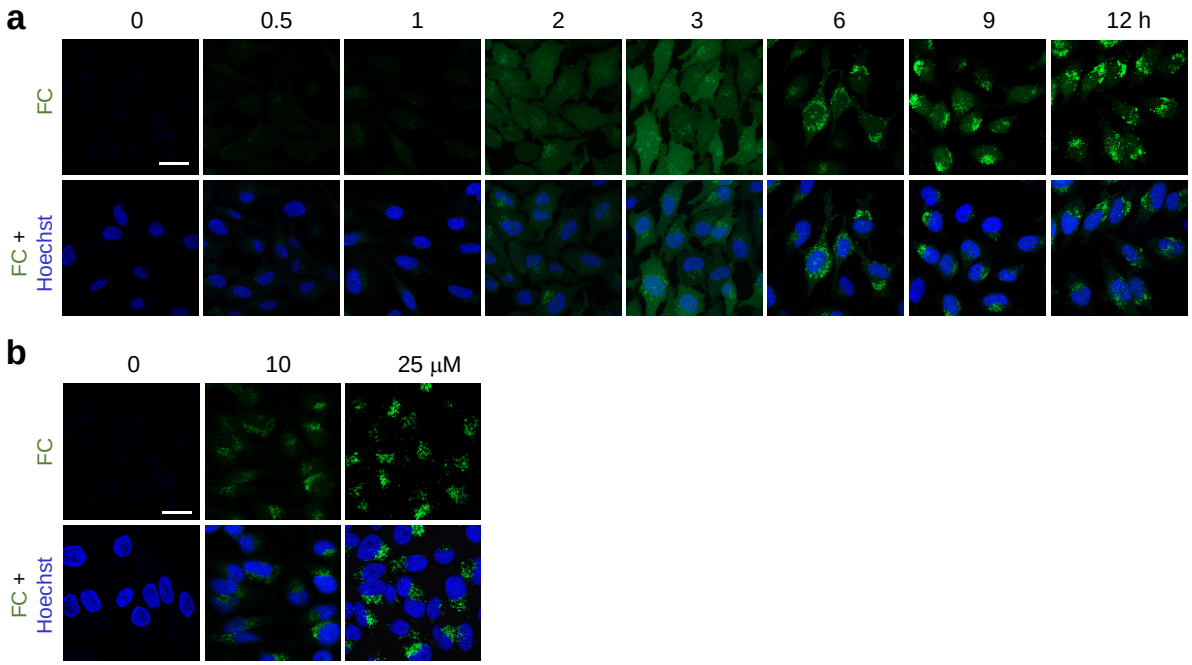


Figure S6. Time- and concentration-dependent detection of cancer cells using BocLys(Ac)-AB-FC. (a) HeLa cells were incubated with 25 μM BocLys(Ac)-AB-FC for indicated times followed by staining with Hoechst 33342. (b) HeLa cells were exposed to several concentrations of BocLys(Ac)-AB-FC followed by staining with Hoechst 33342. Images of treated cells were obtained by using confocal fluorescence microscopy (scale bar = 10 μm).

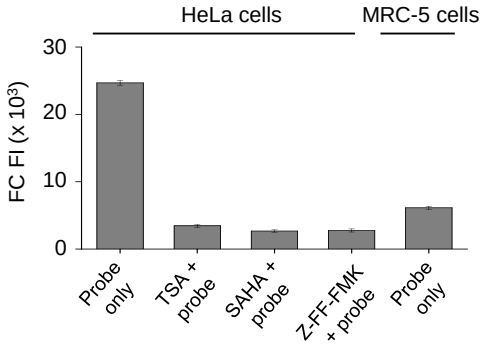


Figure S7. HeLa cells pre-incubated with 5 μM TSA, 50 μM SAHA or 20 μM Z-FF-FMK for 2 h were treated with 25 μM BocLys(Ac)-AB-FC for 9 h. In addition, MRC5 cells were exposed to 25 μM BocLys(Ac)-AB-FC for 9. After washing treated cells with DPBS, the fluorescence intensity was measured by using a microplate reader (mean ± s.d., n = 3).

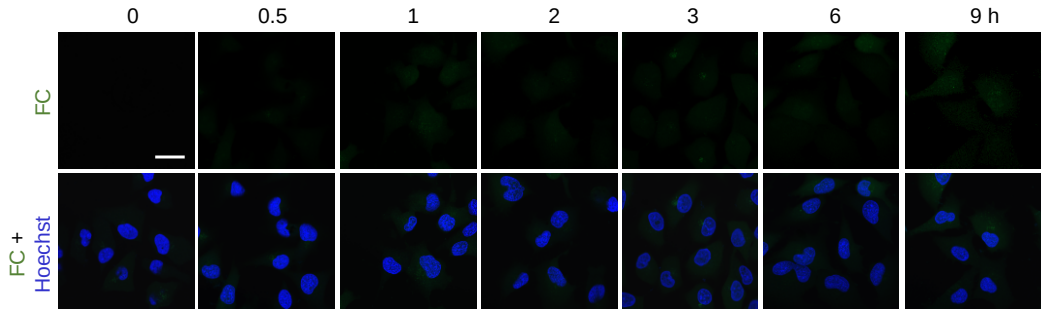


Figure S8. MRC-5 cells were incubated with 25 μM BocLys(Ac)-AB-FC for indicated times followed by staining with Hoechst 33342. Images of treated cells were obtained by using confocal fluorescence microscopy (scale bar = 10 μm).

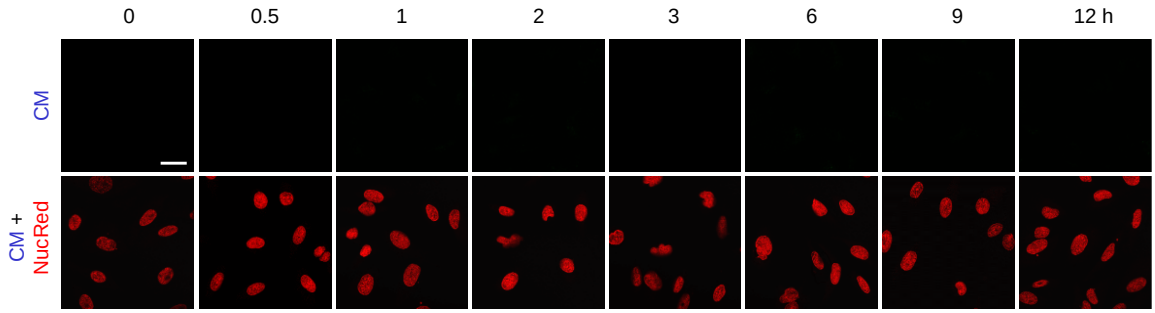
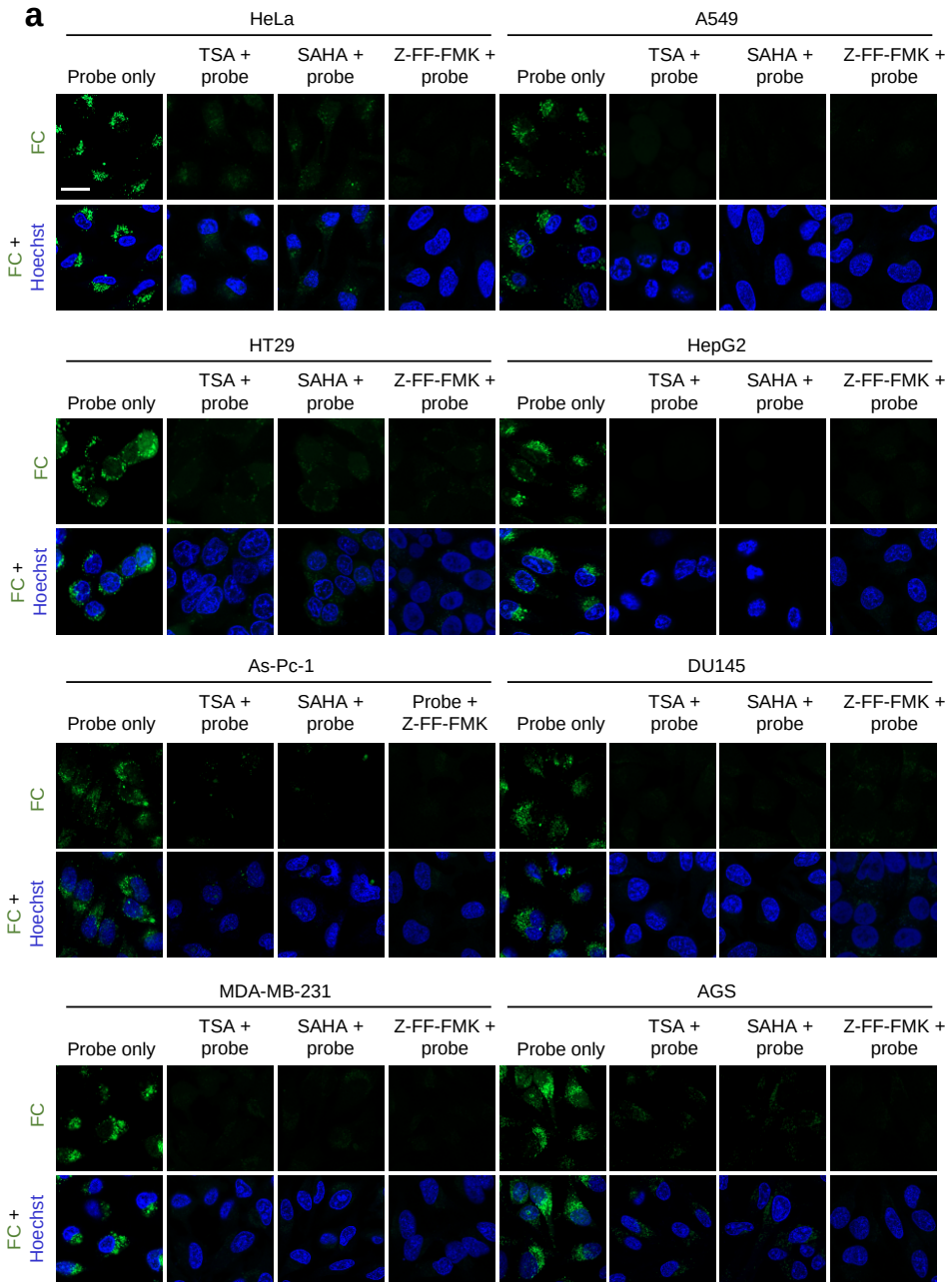


Figure S9. HeLa cells were incubated with 25 μM BocLys(Ac)-AMC for 9 h and then stained with NucRed 647. Cell images were obtained by using confocal fluorescence microscopy (scale bar = 10 μm).


a
HeLa
A549
HT29
HepG2
As-Pc-1
DU145
MDA-MB-231
AGS
Probe only
TSA + probe
SAHA + probe
Z-FF-FMK + probe
Probe + Z-FF-FMK
FC
FC + Hoechst

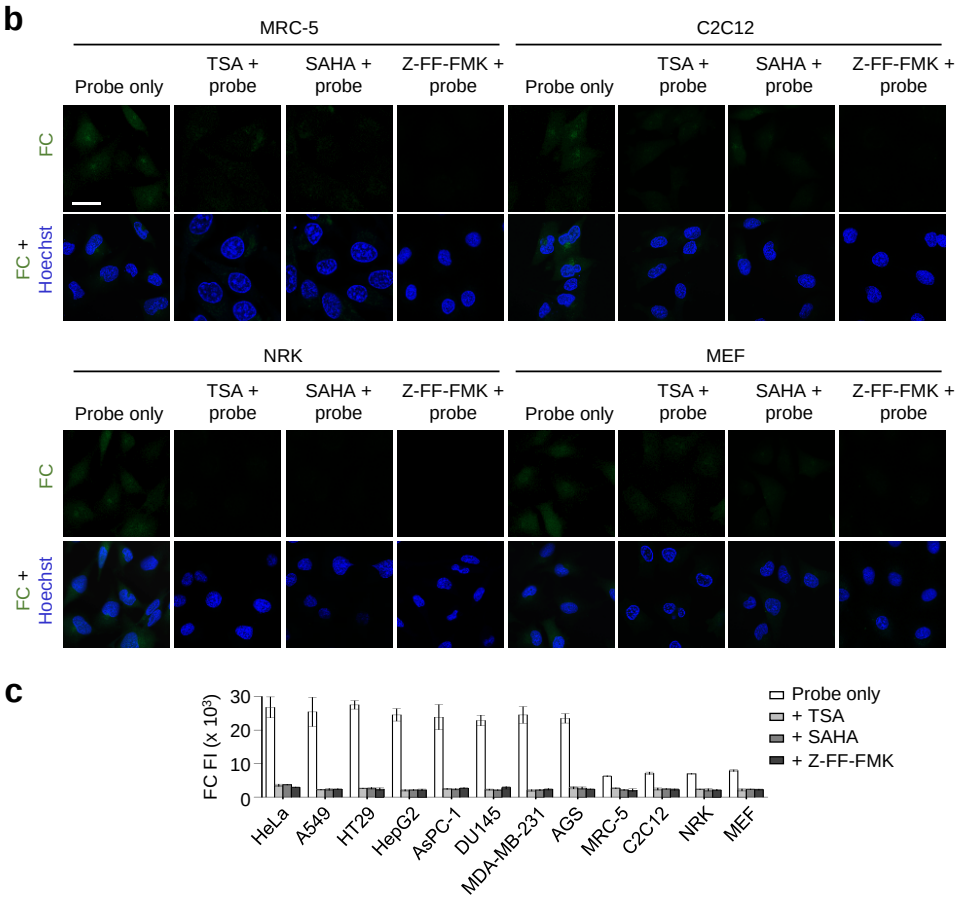


Figure S10. Detection of cancer and normal cells using BocLys(Ac)-AB-FC. (a) Cancer and (b) normal cells were incubated with 25 μM BocLys(Ac)-AB-FC for 9 h. In addition, they were pre-incubated with 5 μM TSA, 50 μM SAHA or 20 μM Z-FF-FMK for 2 h followed by treatment with 25 μM BocLys(Ac)-AB-FC for 9 h. Cell images were obtained by using confocal fluorescence microscopy (scale bar = 10 μm). (c) Graph shows the fluorescence intensity (FI) of FC in (a) and (b) (mean ± s.d., n = 3).

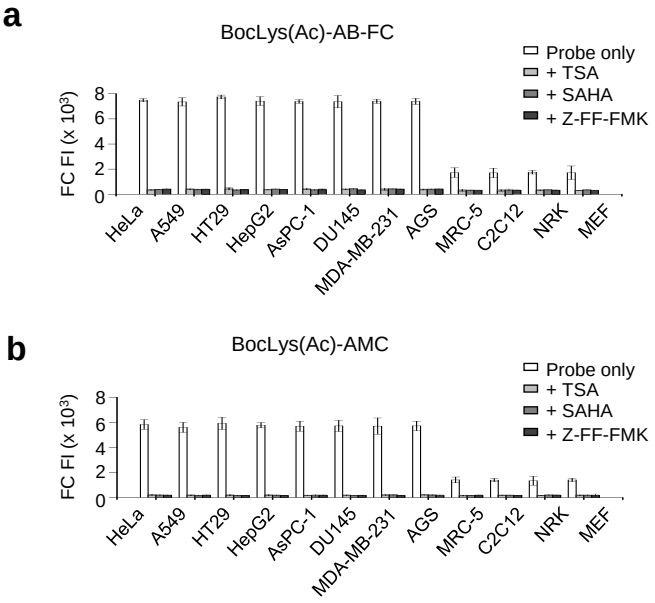
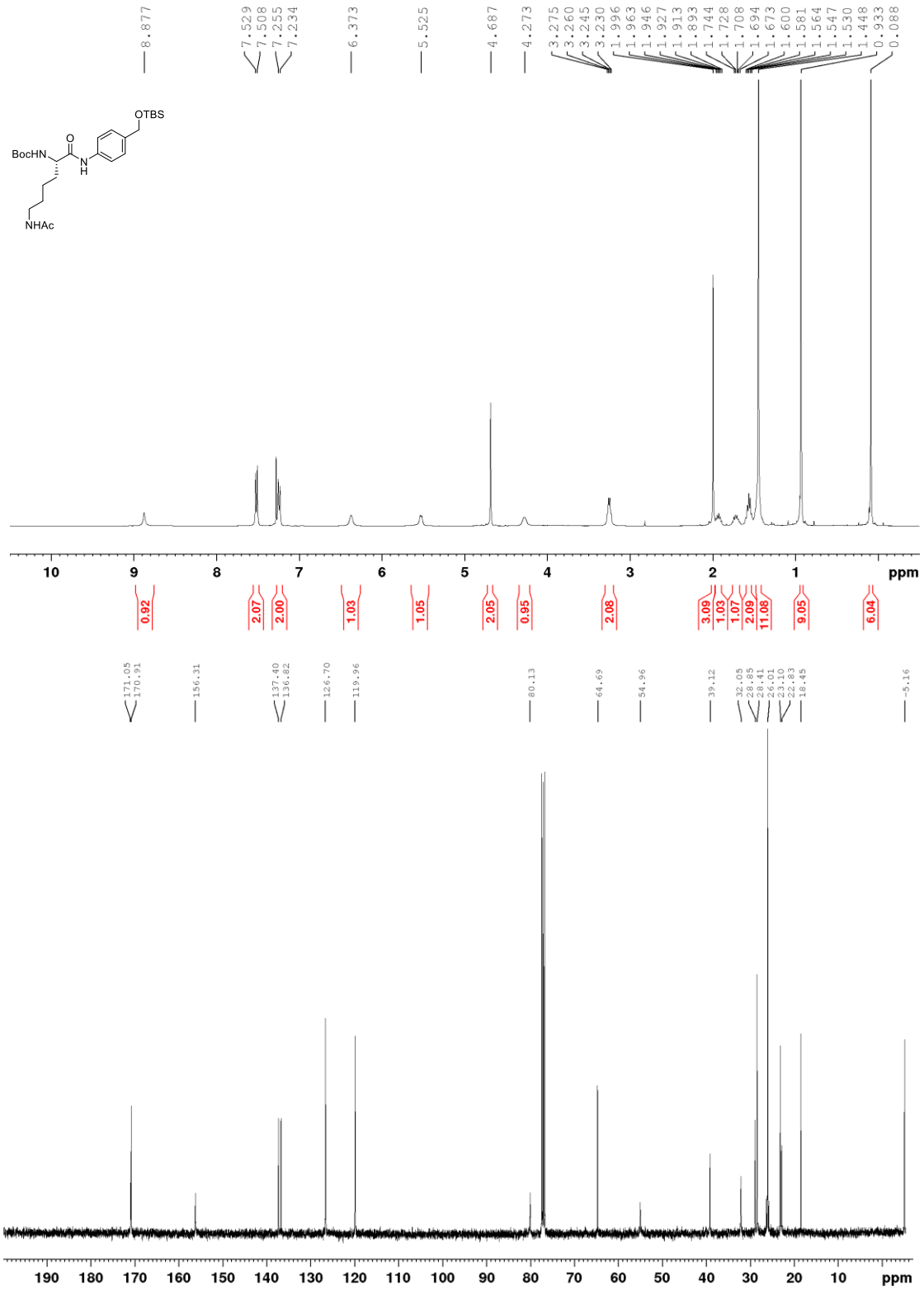


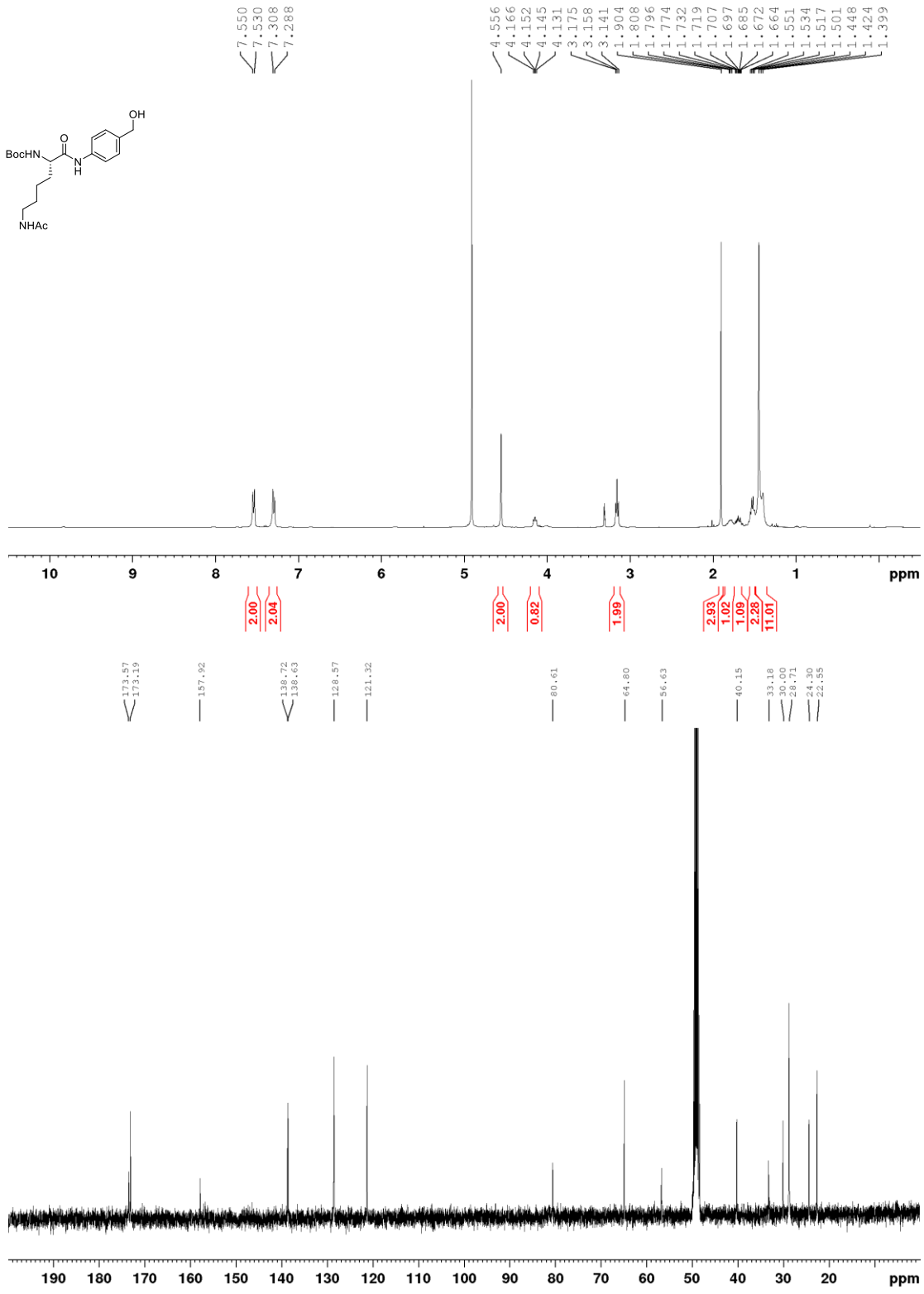
Figure S11. Indicated cell lysates were incubated with (a) 25 μM BocLys(Ac)-AB-FC and (b) 10 μM BocLys(Ac)-AMC for 2 h. In addition, they were pre-incubated with 5 μM TSA, 50 μM SAHA or 20 μM Z-FF-FMK for 2 h and then treated with (a) 25 μM BocLys(Ac)-AB-FC and (b) 10 μM BocLys(Ac)-AMC for 2 h. The fluorescence intensities of FC and AMC were determined by using a microplate reader (mean ± s.d., n = 3).

<NMR spectra>

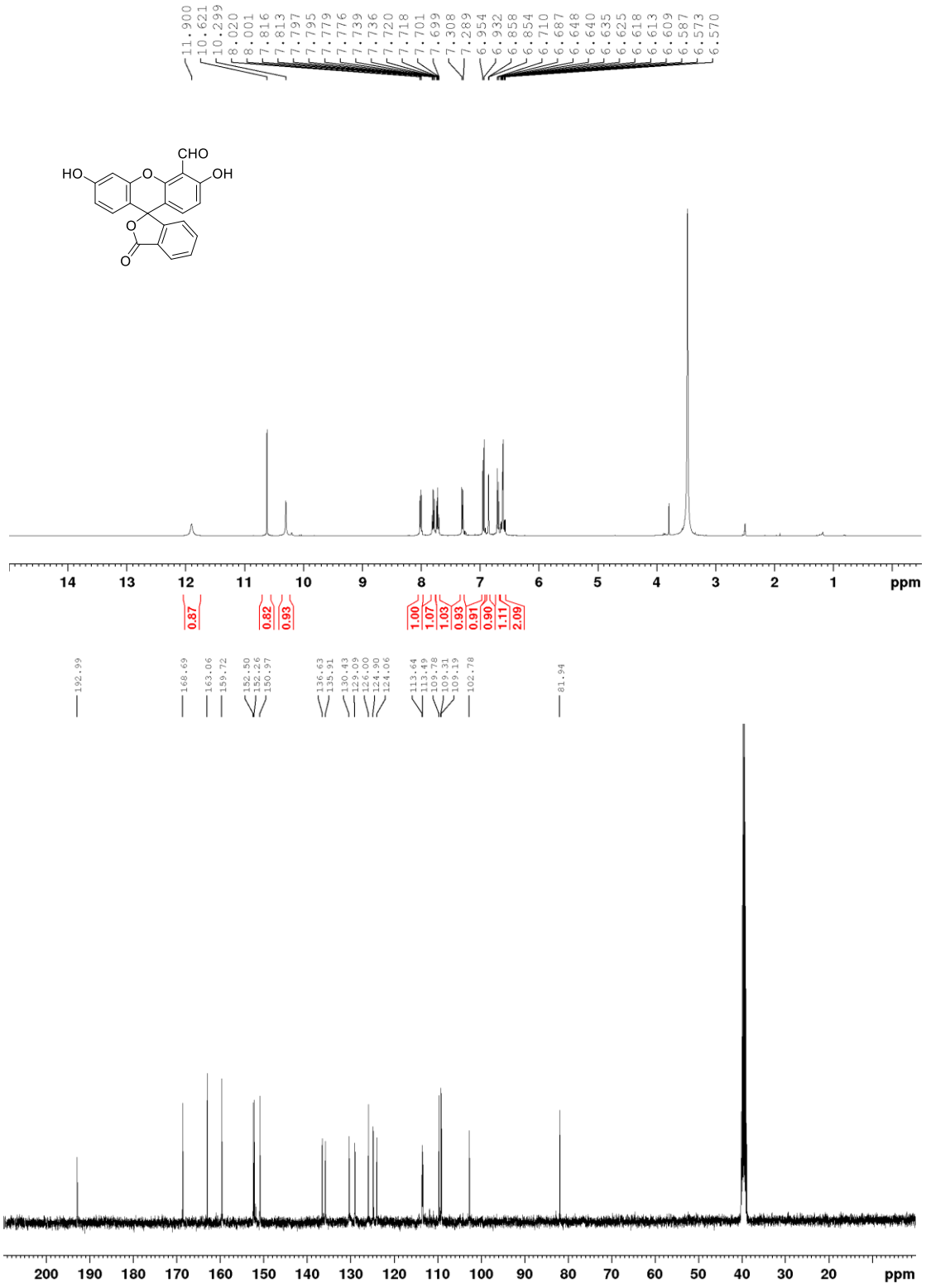
Compound 1 ($CDCl_3$, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)



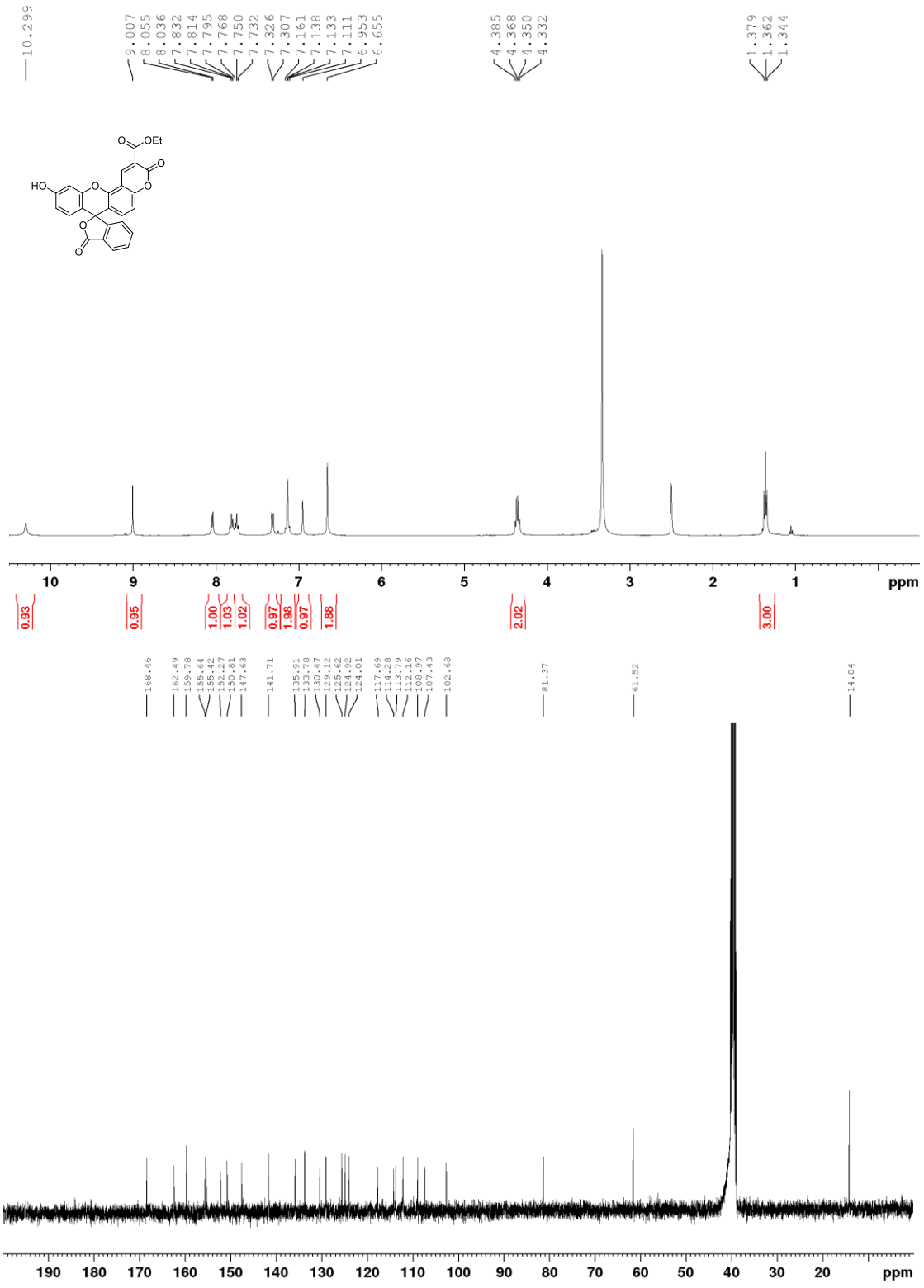
Compound 2 (CD_3OD, 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR)



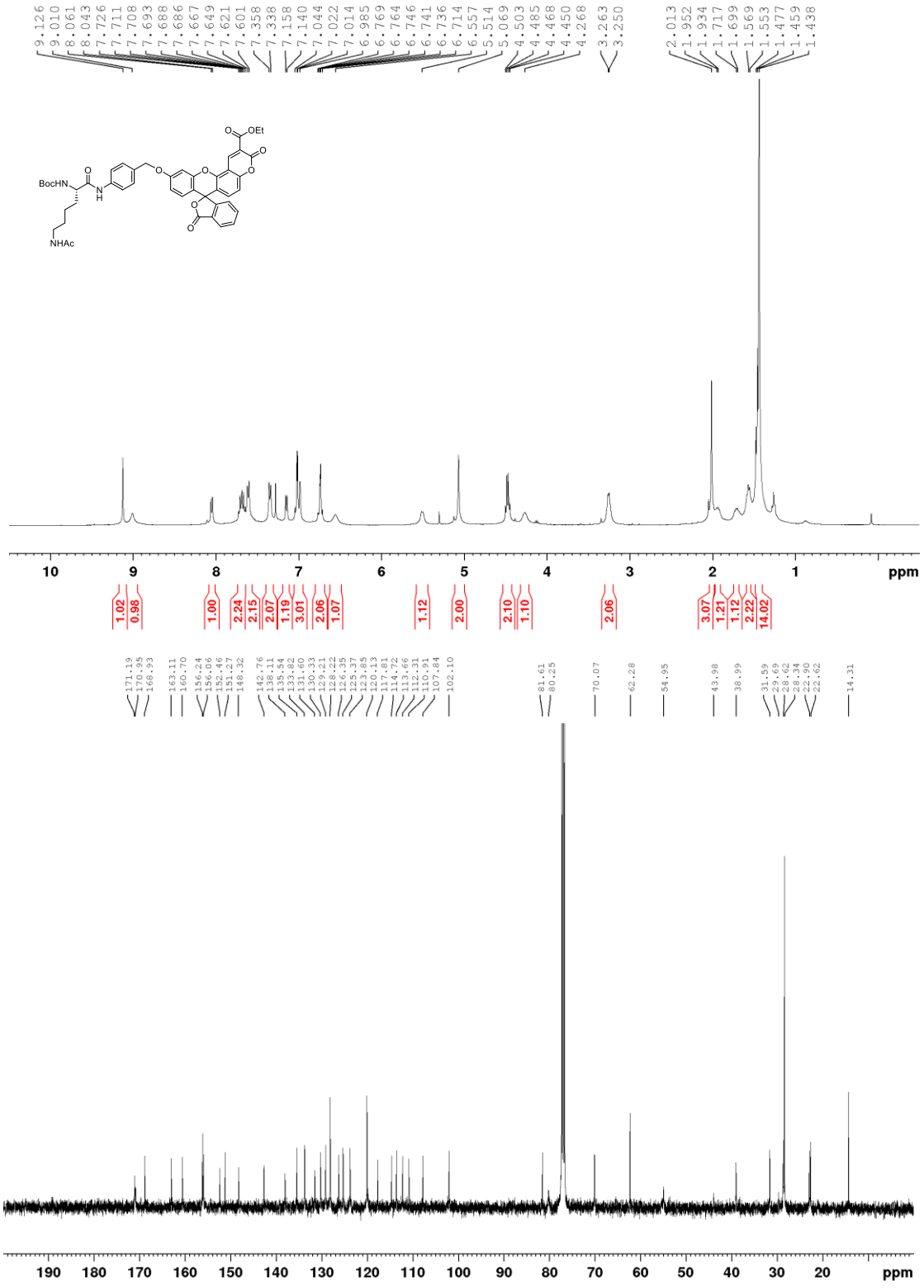
Compound 4 (DMSO-d_6, 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR)



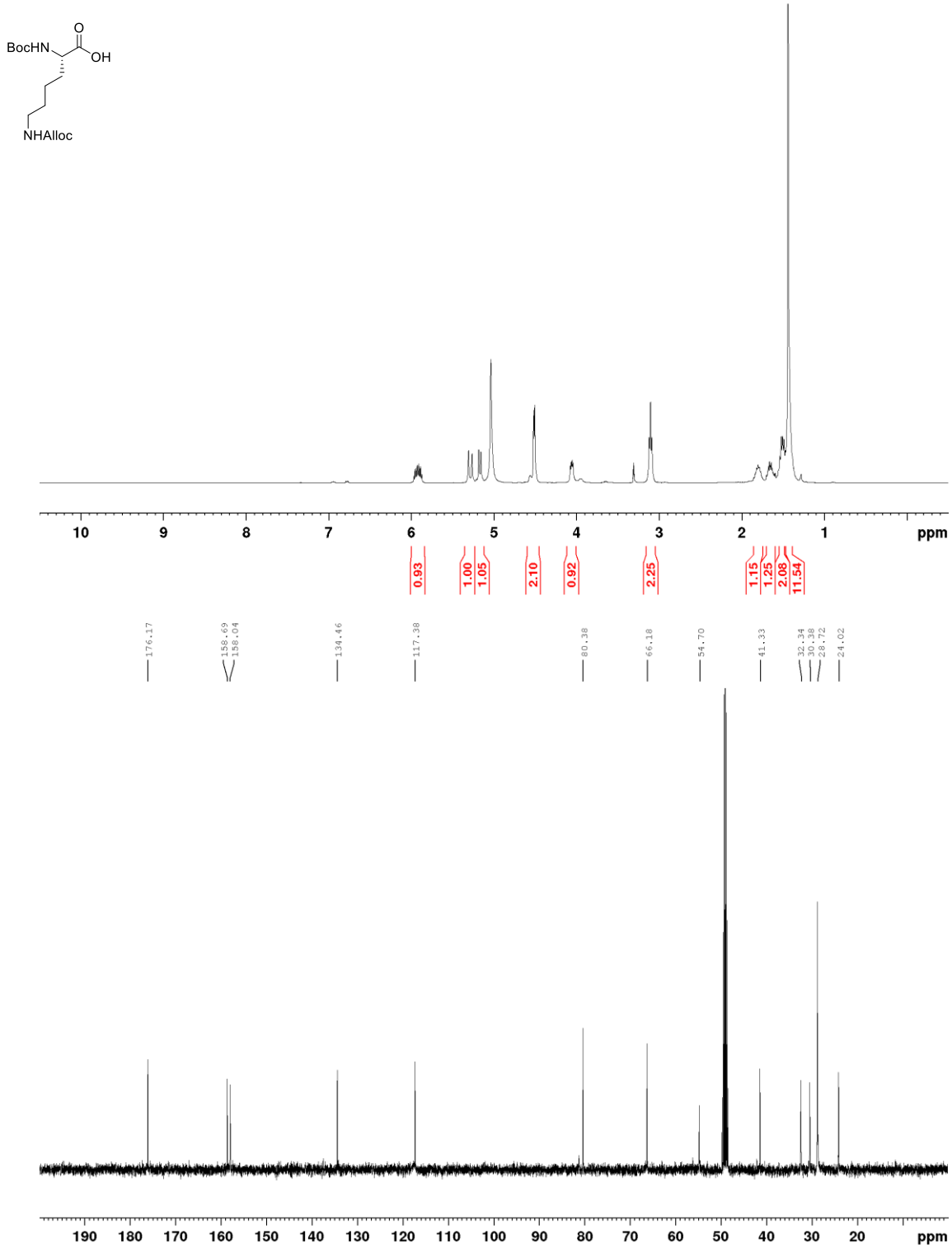
FC (DMSO-d_6, 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR)



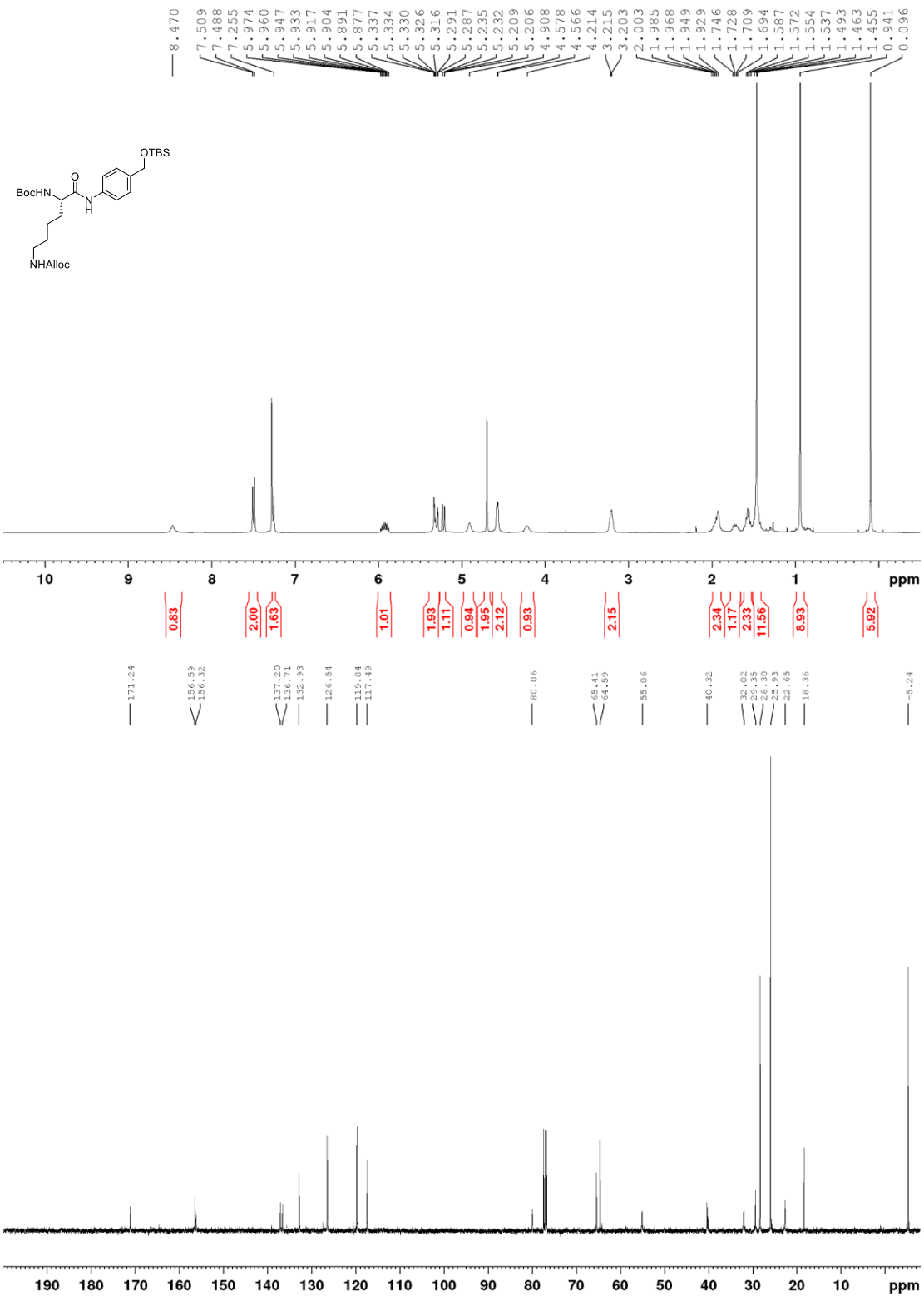
BocLys(Ac)-AB-FC ($CDCl_3$, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)



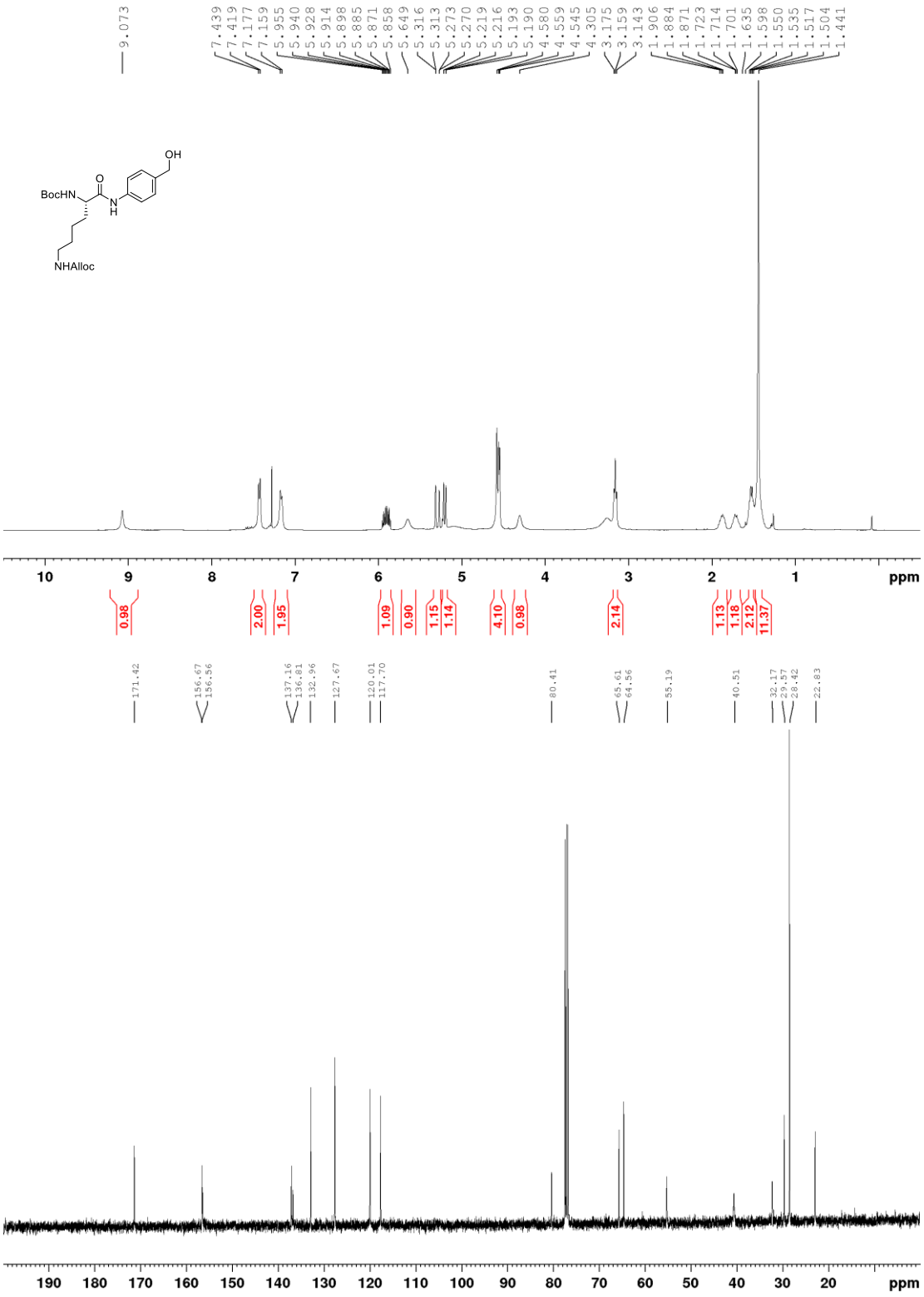
BocLys(Alloc) (CD_3OD, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)



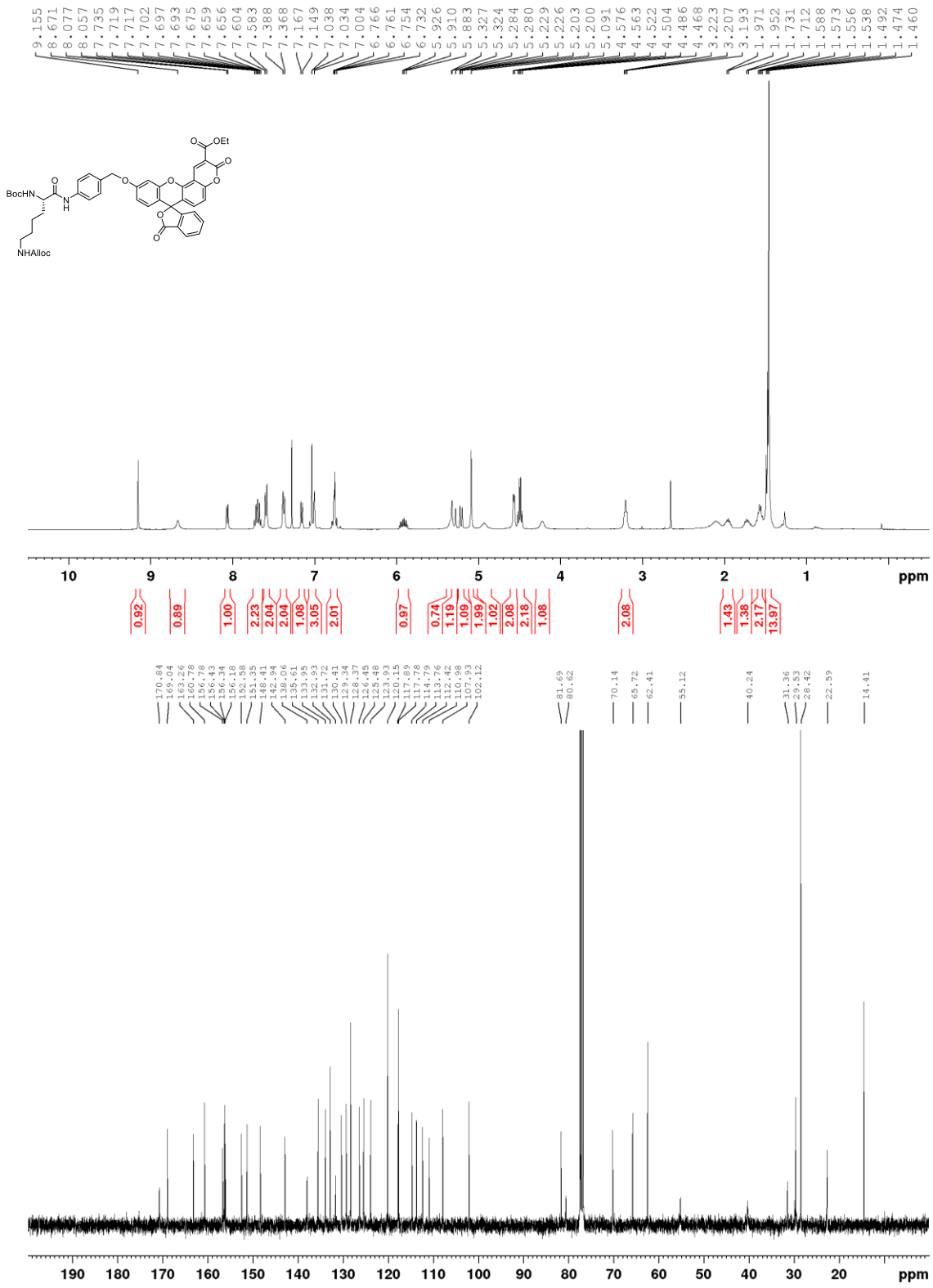
Compound 5 ($CDCl_3$, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)



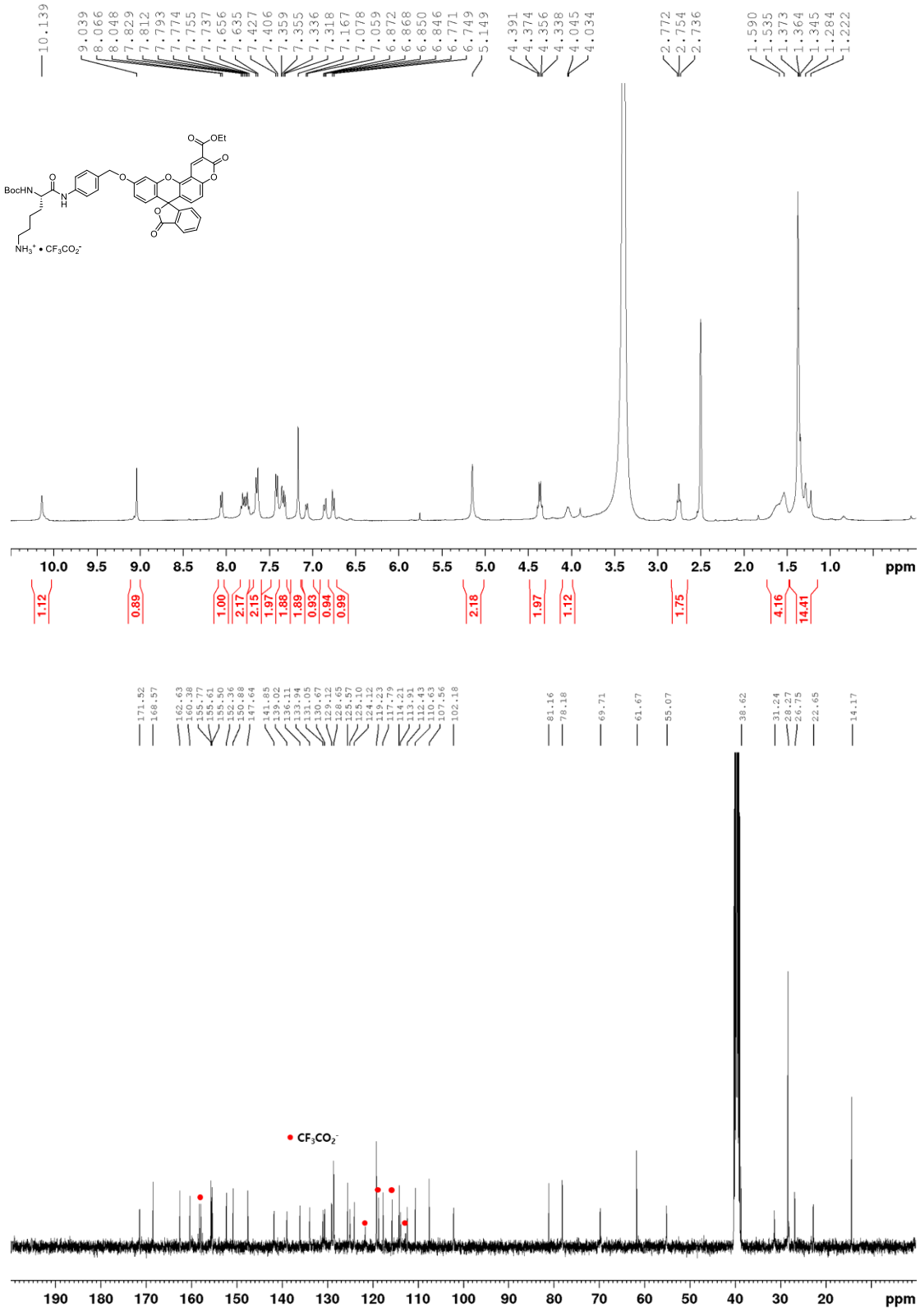
Compound 6 ($CDCl_3$, 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR)



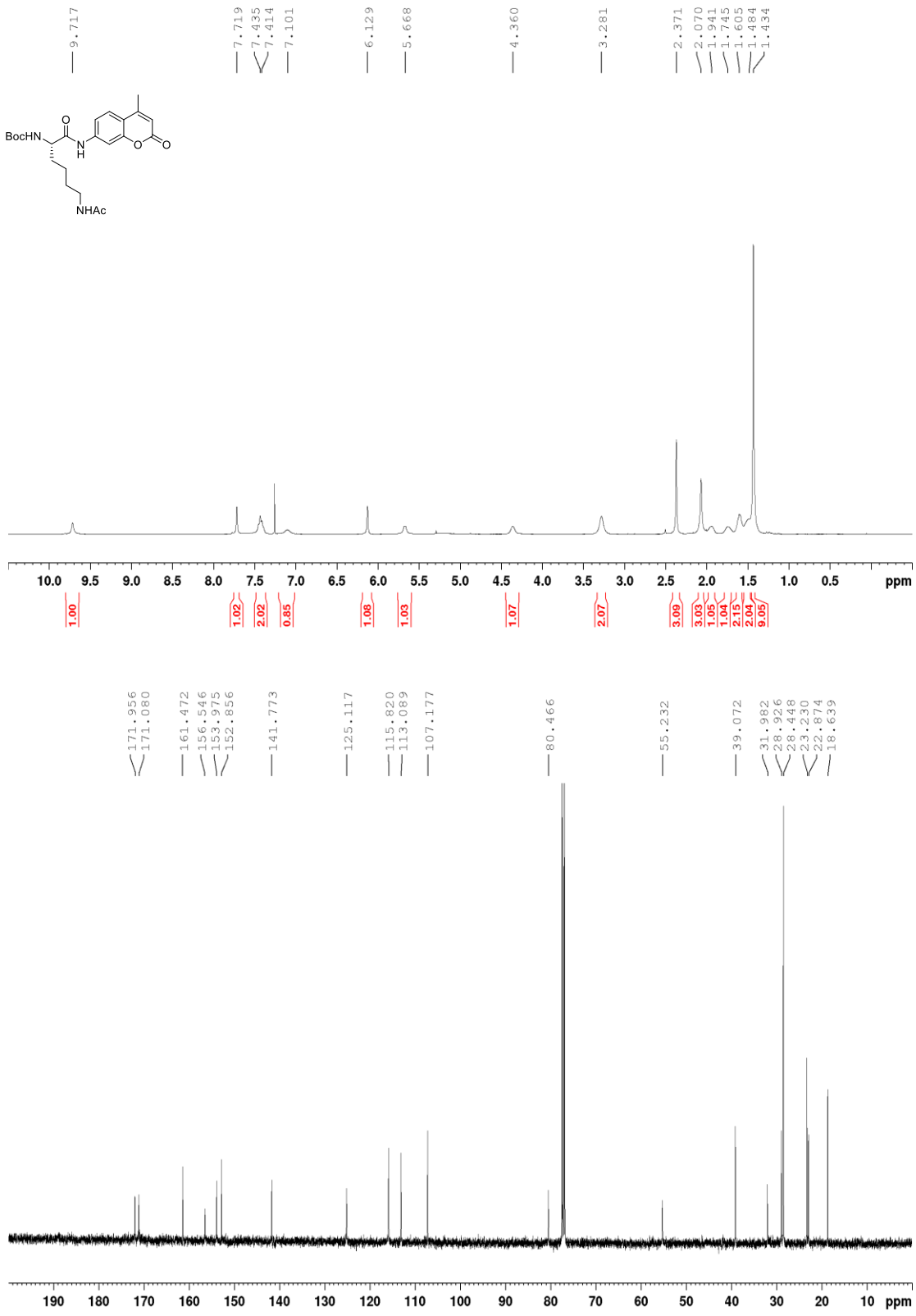
BocLys(Alloc)-AB-FC ($CDCl_3$, 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR)



BocLys-AB-FC (DMSO-d_6, 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR)



BocLys(Ac)-AMC ($CDCl_3$, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)



BocLys-AMC ($CDCl_3$, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)