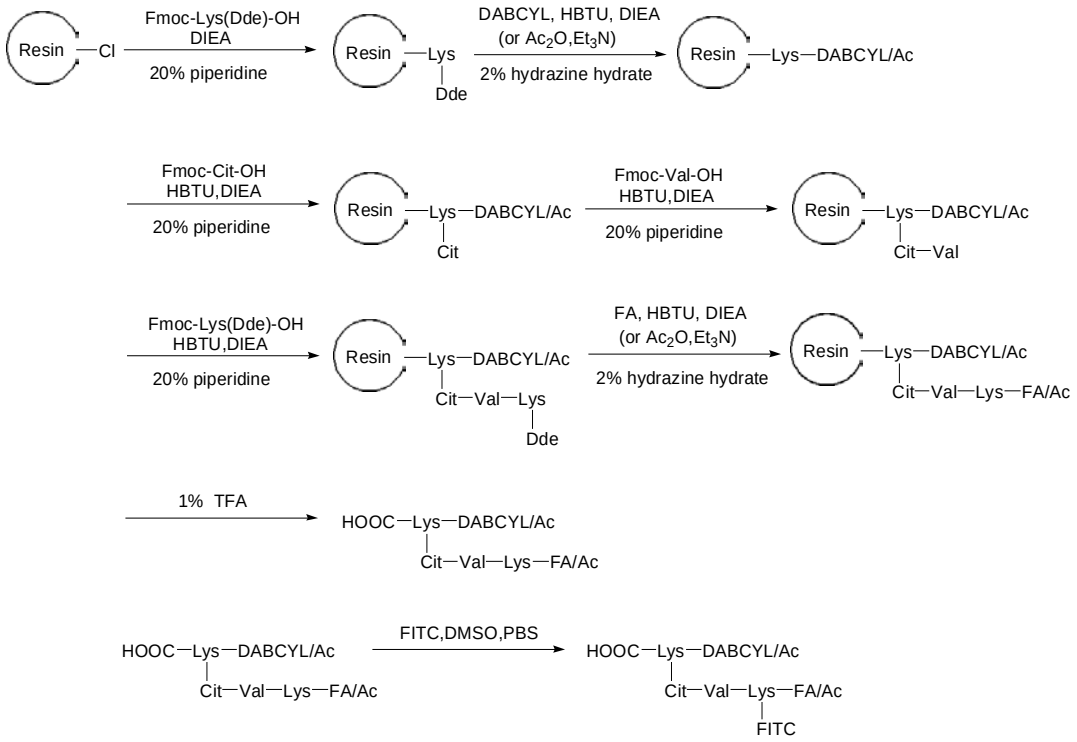
Supporting Information

The designed, cathepsin B-triggered, dual-functional fluorogenic cancer imaging probe FA-Lys(FITC)-Val-Cit-Lys(DABCYL)-OH (FA-FITC-CathepsinSubstrate-DABCYL, shortened to FFCD **1**) was synthesized according to the scheme shown in Scheme S1. At first, the peptide FA-Lys-Val-Cit-Lys(DABCYL) with DABCYL at its C-terminus and folic acid at its N-terminus was synthesized on the resin following the Fmoc solid phase peptide synthesis protocol. In this synthesis, the Dde-protected lysine (Fmoc-Lys(Dde)-OH) was used because Dde can be orthogonally de-protected using 2% hydrazine hydrate in DMF. Doing so made it convenient to selectively introduce DABCYL, FITC and folic acid to the peptide chain. After removing the Dde from the second lysine using 2% hydrazine hydrate in DMF and cleaving the peptide from the resin with 1% TFA in CH_2Cl_2, the afforded peptide was subsequently coupled to FITC at the lysine residue in DMSO/PBS to create the target probe FA-Lys(FITC)-Val-Cit-Lys(DABCYL)-OH (FFCD **1**). Following similar protocols, three controls, including the quencher-less FFC **2** (FA-Lys(FITC)-Val-Cit-Lys-OH, without DABCYL) as the "always on" control, FCB **3** (Ac-Lys(FITC)-Val-Cit-Lys(DABCYL)-OH, without folic acid) and FC **4** (Ac-Lys(FITC)-Val-Cit-Lys-OH, without DABCYL and folic acid) as the non-targeting negative controls, were synthesized by substituting the corresponding conjugate with an Ac group. The final products were purified using HPLC, and their molecular structures were characterized using UV visible and fluorescence spectroscopy (Cary 100 Bio, Varian) and ESI mass spectrometry.



Scheme S1. The synthetic procedure of FFCD **1** and the three controls (FFC **2**, FCD **3** and FC **4**).

1.1. Chemicals and materials

2-Chlorotrityl chloride resin, O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and Fmoc-N-amino acids were obtained from GL Biochem Ltd. (Shanghai, China). Other chemical agents were from Alfa Aesar (Tianjin, China).

1.2. General procedures for solid phase synthesis of FA/Ac-Lys-Val-Cit-Lys(DABCYL/Ac)-OH

Attachment of Fmoc-Lys(Dde)-OH to 2-Chlorotrityl Chloride Resin: 2-Chlorotrityl chloride resin (250 mg, 1.0 equiv, 0.25 mmol) was swelled with dry DCM (4.0 mL) for 30 min. A solution of Fmoc-Lys(Dde)-OH (159.8 mg, 1.2 equiv, 0.3 mmol), N,N-diisopropylethylamine (DIEA) (99 μL, 2.0 equiv., 0.6 mmol) in dry dichloromethane (DCM, 8.0 mL) was added to the resin, and the mixture was shaken at room temperature for 3 h. MeOH (1.0 mL) and DIEA (0.5 mL) were added, and the mixture was agitated for 30 min to cap the free sites. The resin was then filtered and washed with DCM (5 × 8.0 mL) and DMF (5 × 8.0 mL).

Fmoc deprotection: A solution of 20% piperidine in DMF (4.0 mL) was added to the resin, and the mixture was agitated for 30 min. The resin was then washed with DMF (5 × 8.0 mL).

Coupling with DABCYL: DABCYL acid (80.8 mg, 1.2 equiv, 0.3 mmol), HBTU (136.5 mg, 1.4 equiv, 0.36 mmol) and DIEA (0.6 mmol) were dissolved in 4.0 mL of DMF, and the mixed solution was added to the resin. The reaction mixture was agitated at room temperature for 3 h and then washed with DMF (5 × 8.0 mL).

Acetylating: To synthesize the controls with acetyl substitution, Ac_2O (0.6 mmol) and triethylamine (1.2 mmol) were added to the suspended resin in 4.0 mL of DMF. The reaction mixture was agitated at room temperature for 3 h and then washed with DMF (5 × 8.0 mL).

Dde deprotection: The resin was suspended in a solution of 2% hydrazine hydrate in DMF, agitated for 10 min and then washed with DMF (5 × 8.0 mL).

Coupling with HBTU/DIEA: Fmoc-Cit-OH (or Fmoc-Val-OH/Fmoc-Lys(Dde)-OH) (0.3 mmol), HBTU (0.36 mmol) and DIEA (0.6 mmol) were dissolved in DMF, and the mixed solution was added to the resin. The reaction mixture was agitated for 2 h and washed with DMF (5 × 8.0 mL).

Coupling with folic acid: Folic acid (0.6 mmol), HBTU (0.54 mmol) and DIEA (1.2 mmol) were dissolved in DMF/DMSO (1:1), and the mixed solution was added to the resin. The mixture was agitated for 8 h and then washed with DMF (5 × 8.0 mL).

Cleavage with Trifluoroacetic acid (TFA): After Dde deprotection, the resin was washed with DCM (5×8.0 mL) and suspended in a solution of 1% TFA in DCM for 8 × 1 min. The resin was removed by filtration, and the filtrate was neutralized with Et_3N. After removing the solvent in a vacuum, the residue was dissolved in CH_3OH and purified using high performance liquid chromatography (HPLC, LC-20AT, Shimadzu, Kyoto, Japan) to yield four products, respectively:

FA-Lys-Val-Cit-Lys(DABCYL)-OH (red powder, 70.2 mg, 23.3% yield, ESI-HRMS calcd for $C_{57}H_{77}N_{18}O_{12}$ $[M + H]^+$ 1205.5968, found: *m/z* 1205.5924);

FA-Lys-Val-Cit-Lys(Ac)-OH (yellow powder, 67.2 mg, 27.0% yield, ESI-HRMS calcd for $C_{44}H_{66}N_{15}O_{12}$ $[M + H]^+$ 996.5015, found: *m/z* 996.4993);

Ac-Lys-Val-Cit-Lys(DABCYL)-OH (red powder, 60.4 mg, 29.3% yield, ESI-HRMS calcd for $C_{40}H_{62}N_{11}O_8$ $[M + H]^+$ 824.4782, found: *m/z* 824.4760); and

Ac-Lys-Val-Cit-Lys(Ac)-OH (white powder, 47.0 mg, 30.6% yield, ESI-HRMS calcd for $C_{27}H_{51}N_8O_8$ $[M + H]^+$ 615.3830, found: *m/z* 615.3831).

1.3. Synthesis of FA/Ac-Lys(FITC)-Val-Cit-Lys(DABCYL/Ac)-OH by conjugation with FITC

DABCYL-Lys(Cit-Val-Lys-FA)-OH (0.04 mmol) (or other acetyl-substituted peptides) was dissolved in a mixed solvent of PBS (0.5 mL, pH = 8.0) and DMSO (2.5 mL). FITC (23.4 mg, 0.06 mmol) was added, and the mixture was stirred at room temperature overnight in the dark. The residue was purified using HPLC to yield the product FFCD **1**:

FA-Lys(FITC)-Val-Cit-Lys(DABCYL)-OH (FFCD **1**, red powder, 52.3 mg, 82.0% yield, ESI-HRMS calcd for $C_{78}H_{89}N_{19}O_{17}S$ $[M + 2H]^{2+}$ 797.8203, found: *m/z* 797.8194).

The same procedure was applied for the three controls:

FA-Lys(FITC)-Val-Cit-Lys(Ac)-OH (FFC **2**, yellow powder, 46.2 mg, 83.4% yield, ESI-HRMS calcd for $C_{65}H_{78}N_{16}O_{17}S$ $[M + 2H]^{2+}$ 693.2726, found: *m/z* 693.2717);

Ac-Lys(FITC)-Val-Cit-Lys(DABCYL)-OH (FCD **3**, red powder, 40.6 mg, 83.7% yield, ESI-HRMS calcd for $C_{61}H_{73}N_{12}O_{13}S$ $[M + H]^{+}$ 1213.5141, found: *m/z* 1213.5086); and

Ac-Lys(FITC)-Val-Cit-Lys(Ac)-OH (FC **4**, yellow powder, 33.5 mg, 83.4% yield, ESI-HRMS calcd for $C_{48}H_{62}N_{9}O_{13}S$ $[M + H]^{+}$ 1004.4188, found: *m/z* 1004.4112).

1.4. Cathepsin B Activity Assay

The enzymatic kinetic parameters of probe FFCD **1** towards Cathepsin B were determined by measuring the relative increase in fluorescence (RFU) upon the addition of cathepsin B using the plate reader. Excitation and emission wavelengths of 480 and 514 nm, respectively, were used to monitor the liberation of FITC fluorophore peptide cleavage fragment and the progress of the cleavage was calculated by comparing with the maximum RFU emitted by the fully cleaved FFCD **1**. First, FFCD **1** (2, 4, 6, 8, 10, 20, 30 and 40 μmol) was dissolved in 20 μL of DMSO and 180 μL of H_2O. It was then diluted with 0.8 mL of buffer solution (25 mM acetate and 1 mM EDTA, pH 5.0) and incubated with cathepsin B (50 nM) at 37 °C. After 20 min, the reaction was terminated with PBS (10×). Assays were done in triplicate using the same concentration of enzyme.

Kinetic parameters including maximum enzymatic rate (V_{max}), and Michaelis constant (K_m) were calculated from the standard Michaelis-Menten plots (V versus [S]), which were found to be 32.7 nM min^{-1} and 2.43 μM, respectively. The catalytic constant (k_{cat}), defined as the number of catalytic events per minute per enzyme molecule, was determined to be 0.65 min^{-1}.

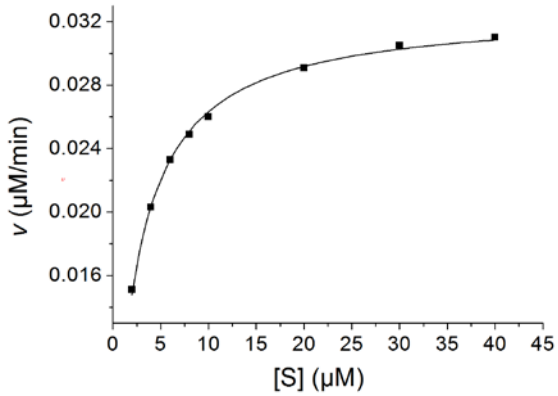


Fig. S1. Determination of the enzymatic parameters by variation of the initial cleavage rate of FFCD **1** by cathepsin B (50 nM) with increasing concentration of FFCD **1**.

1.5. Living cell Confocal Laser Scanning Microscopy (CLSM) detection

The fluorescent probes might be washed away in the process of cell fixation and washing. To avoid this possibility, we also detected the living cell CLSM imaging. The procedure for cell

culture was the same as described in the fixing cell CLSM detection. KB cells that were continuously cultured in folic acid-free RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 ℃ and 5% CO_2 were seeded in a glass-bottomed Petri dish and then placed in a 24-well plate at a concentration of 5×10^4 cells/well 24 h before initiating experiments. Two hours before the experiments, the medium was removed and replaced with 1.0 mL of fresh folic acid-deficient RPMI-1640. After incubation with a series of the prepared probes at 37 ℃ for 4 h, the cells were washed three times with PBS and detected with CSLM directly. Confocal images were acquired using a Laser Scanning Confocal Microscope (TCS SP8, Leica, Wetzlar, Germany). The results are shown in Figure S2.

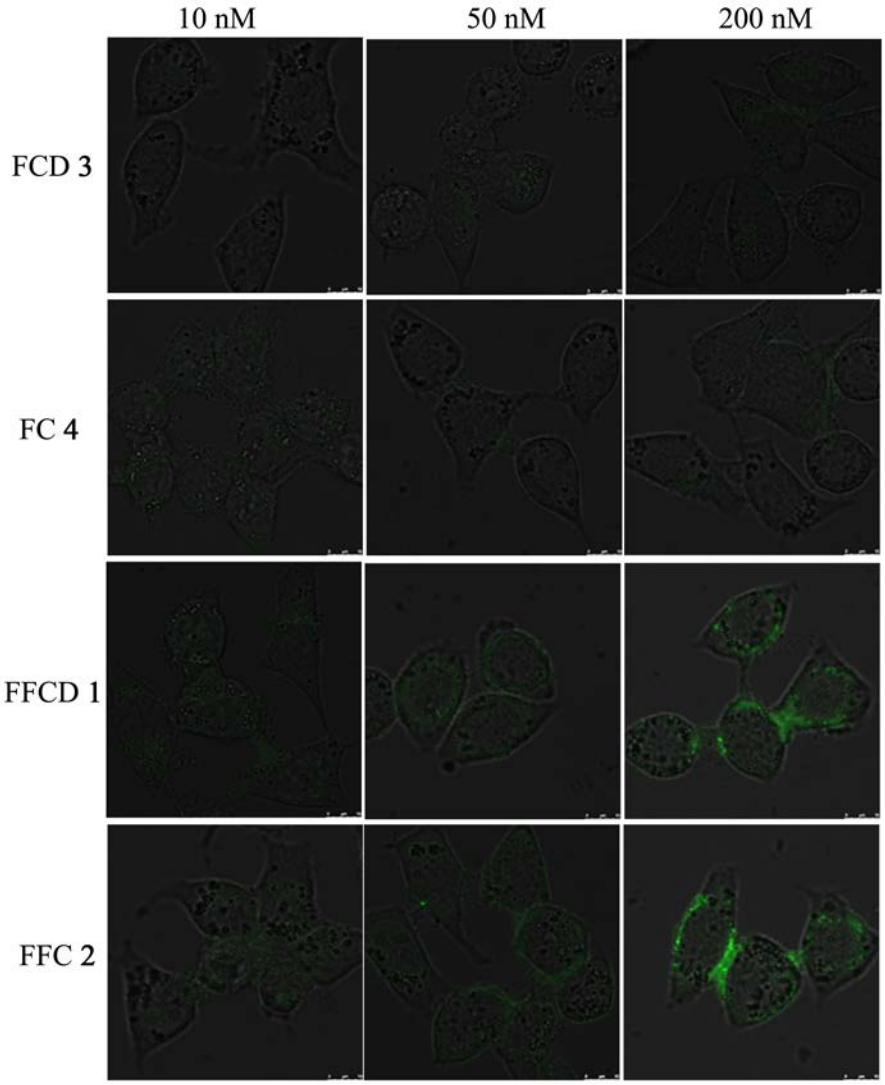


Fig. S2. Confocal microscopic images of living KB cells after incubation with FFCD **1**, FFC **2**, FCD **3** or FC **4** (10 nM, 50 nM or 200 nM) at 37 °C for 4 h. The cells were detected for FITC (green) fluorescence using a confocal microscope directly after washing with PBS.

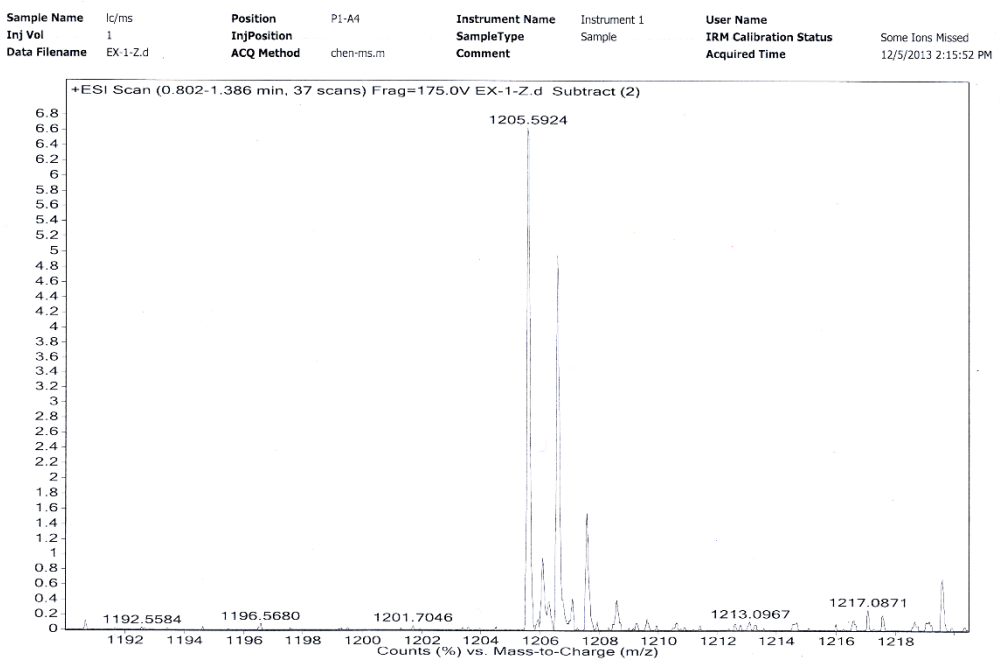


Fig. S3. FA-Lys-Val-Cit-Lys(DABCYL)-OH (ESI-HRMS calcd for $C_{57}H_{77}N_{18}O_{12}$ $[M + H]^+$ 1205.5968, found: *m/z* 1205.5924).

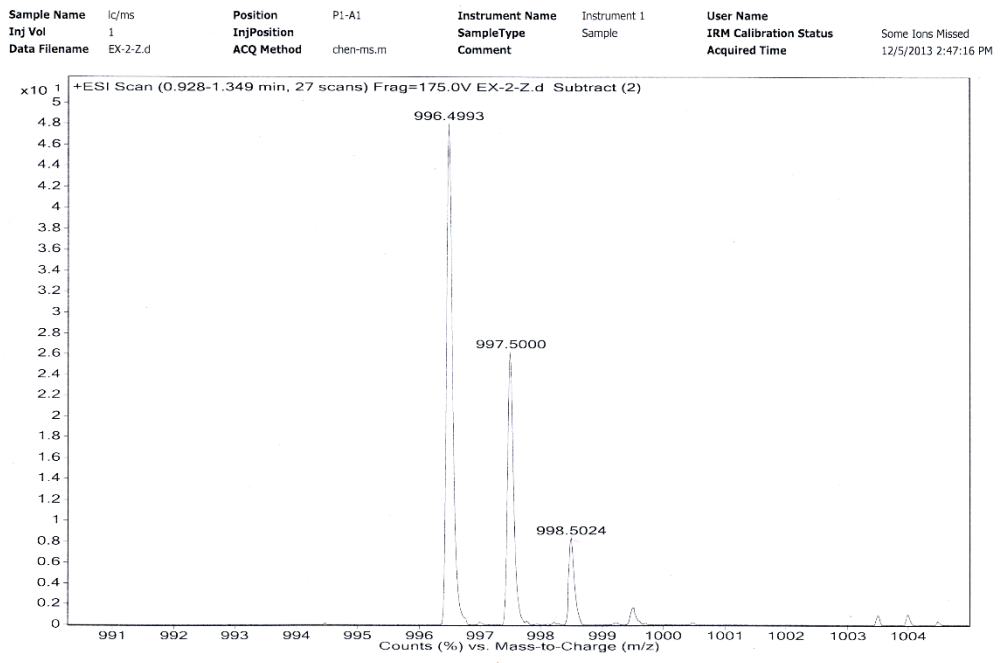


Fig. S4. FA-Lys-Val-Cit-Lys(Ac)-OH (ESI-HRMS calcd for $C_{44}H_{66}N_{15}O_{12}$ $[M + H]^+$ 996.5015, found: *m/z* 996.4993).

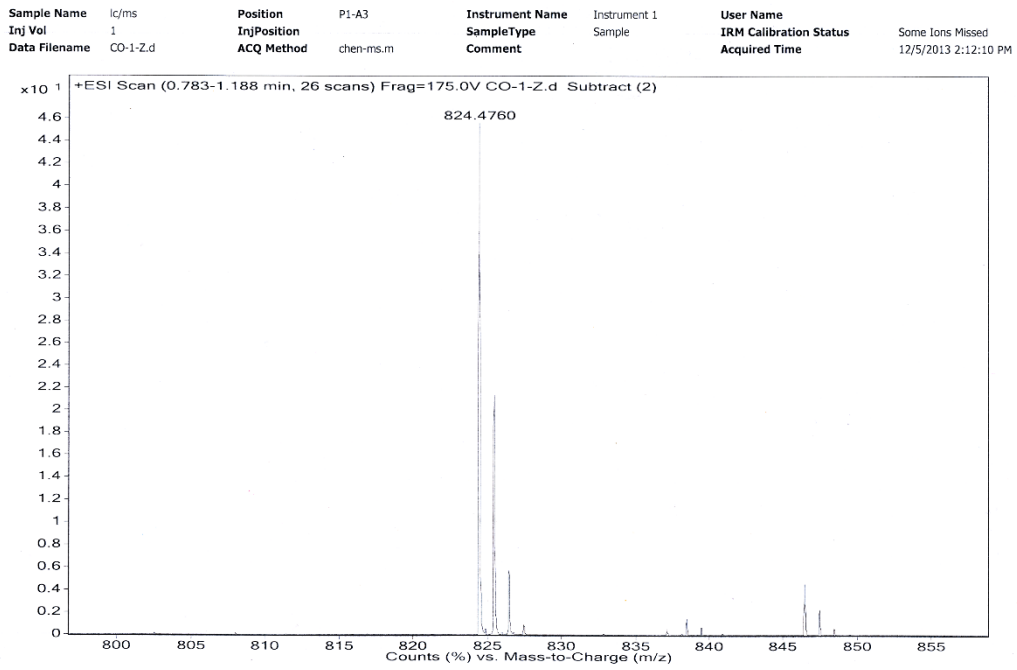


Fig. S5. Ac-Lys-Val-Cit-Lys(DABCYL)-OH (ESI-HRMS calcd for $C_{40}H_{62}N_{11}O_8$ $[M + H]^+$ 824.4782, found: *m/z* 824.4760).

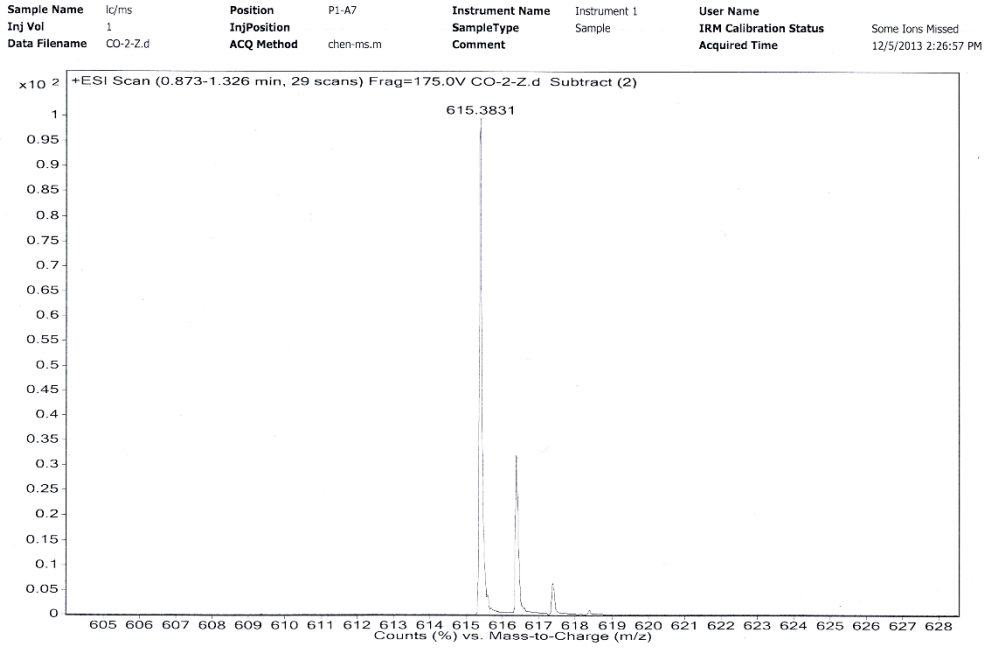


Fig. S6. Ac-Lys-Val-Cit-Lys(Ac)-OH (ESI-HRMS calcd for $C_{27}H_{51}N_8O_8$ $[M + H]^+$ 615.3830, found: *m/z* 615.3831).

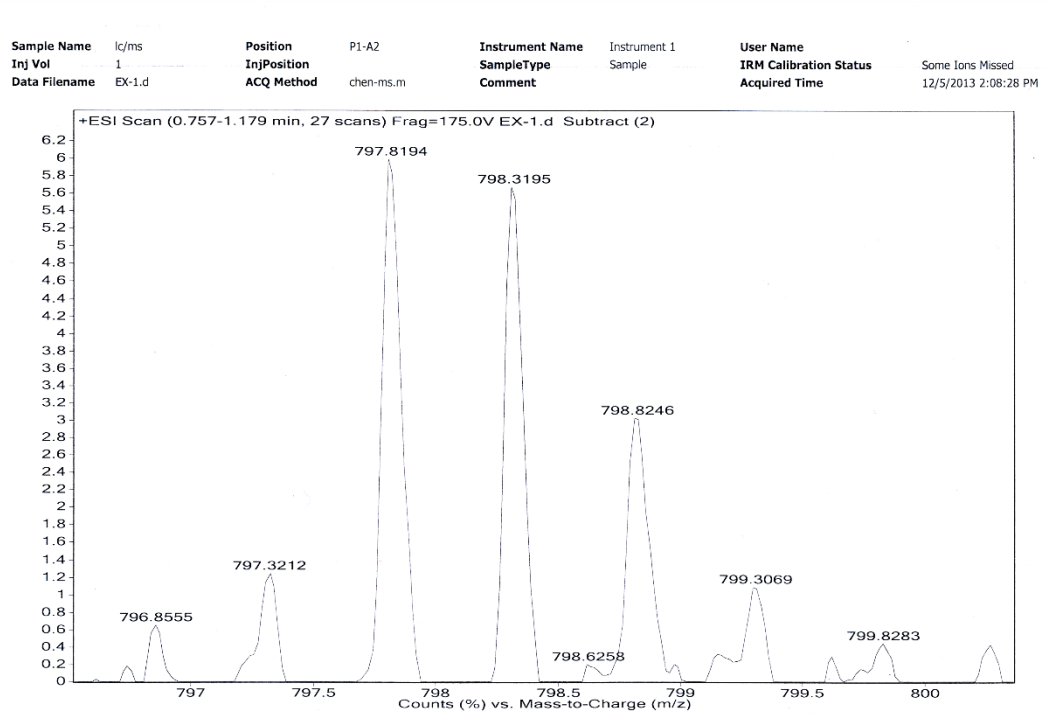


Fig. S7. FA-Lys(FITC)-Val-Cit-Lys(DABCYL)-OH (FFCD **1**, ESI-HRMS calcd for $C_{78}H_{89}N_{19}O_{17}S$ $[M + 2H]^{2+}$ 797.8203, found: *m/z* 797.8194).

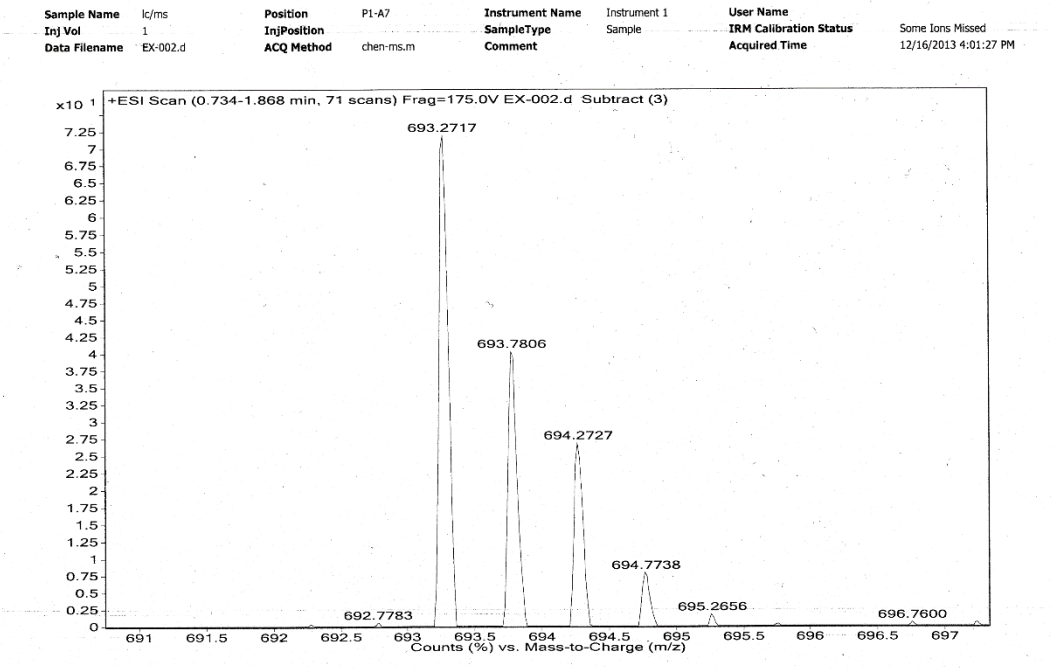


Fig. S8. FA-Lys(FITC)-Val-Cit-Lys(Ac)-OH (FFC **2**, ESI-HRMS calcd for $C_{65}H_{78}N_{16}O_{17}S$ $[M + 2H]^{2+}$ 693.2726, found: *m/z* 693.2717).

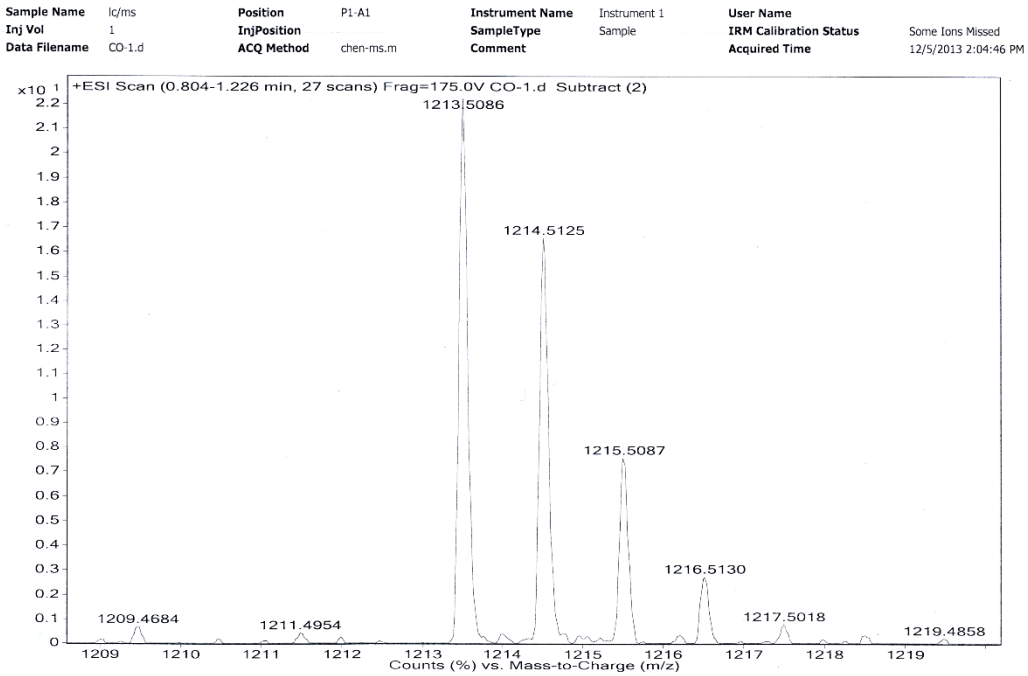


Fig. S9. Ac-Lys(FITC)-Val-Cit-Lys(DABCYL)-OH (FCD **3**, ESI-HRMS calcd for $C_{61}H_{73}N_{12}O_{13}S$ $[M + H]^+$ 1213.5141, found: *m/z* 1213.5086).

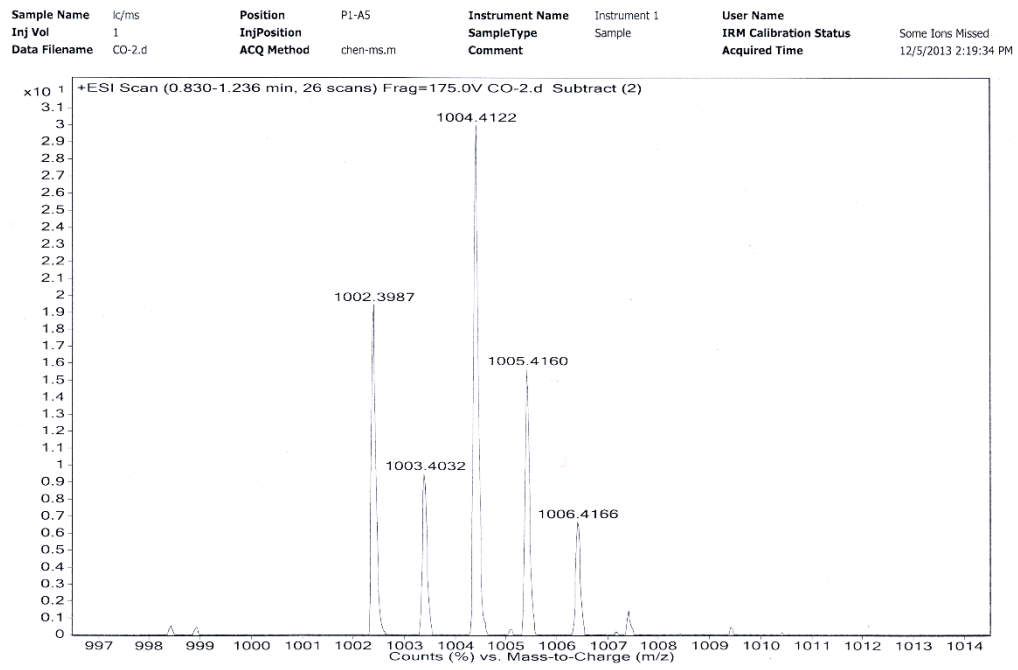
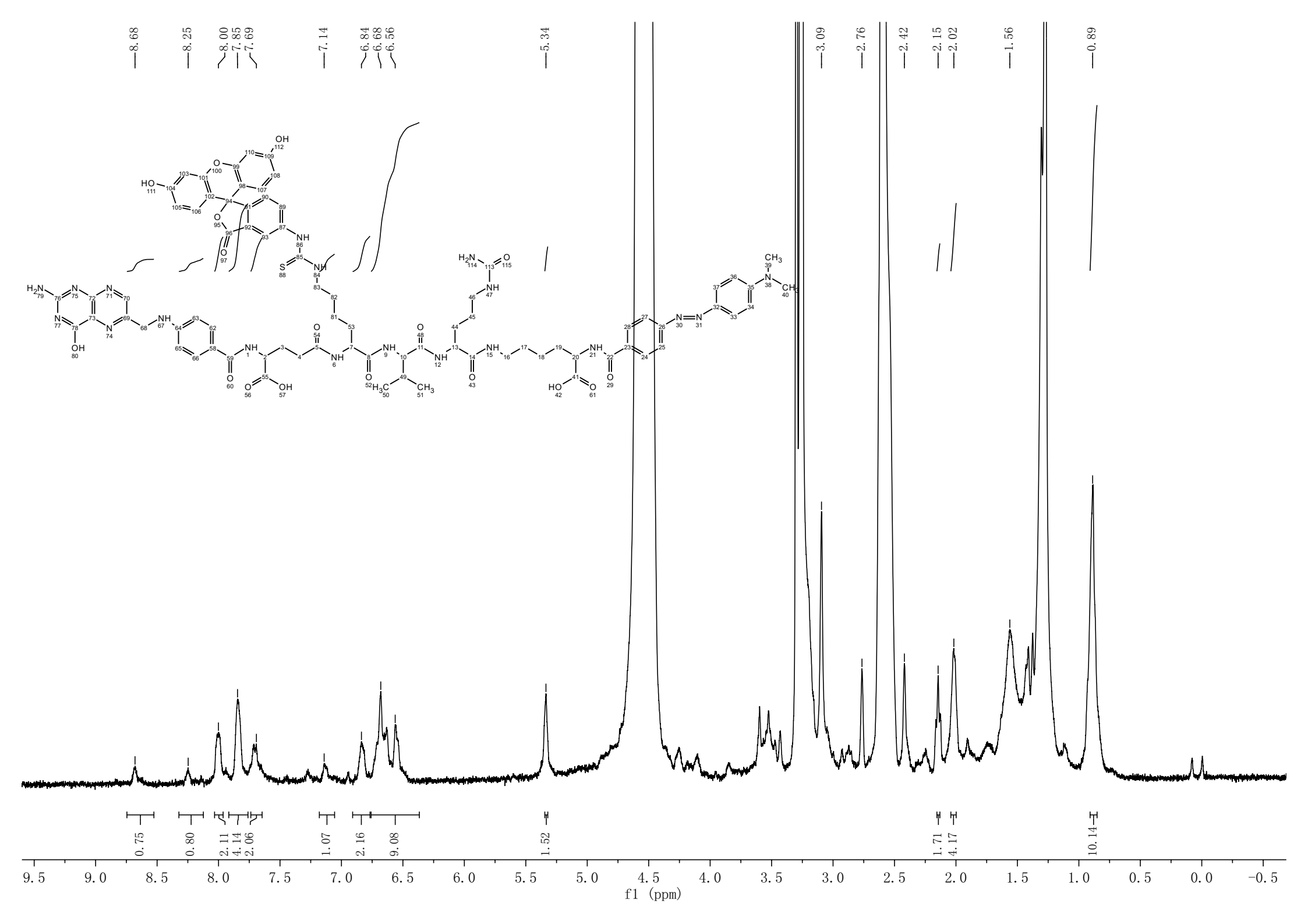


Fig. S10. Ac-Lys(FITC)-Val-Cit-Lys(Ac)-OH (FC **4**, ESI-HRMS calcd for $C_{48}H_{62}N_9O_{13}S$ $[M + H]^+$ 1004.4188, found: *m/z* 1004.4112).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2.02
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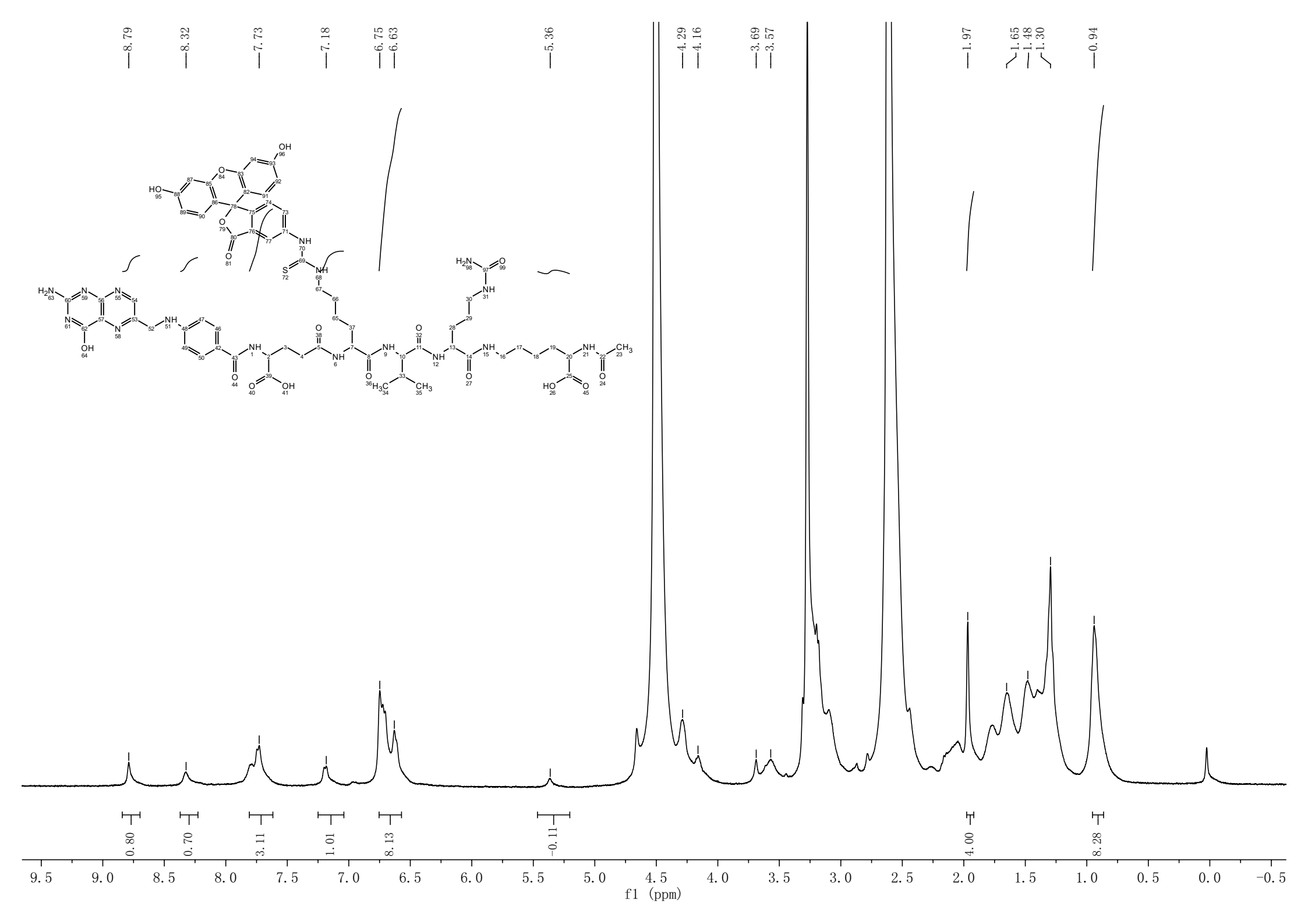
f1 (ppm)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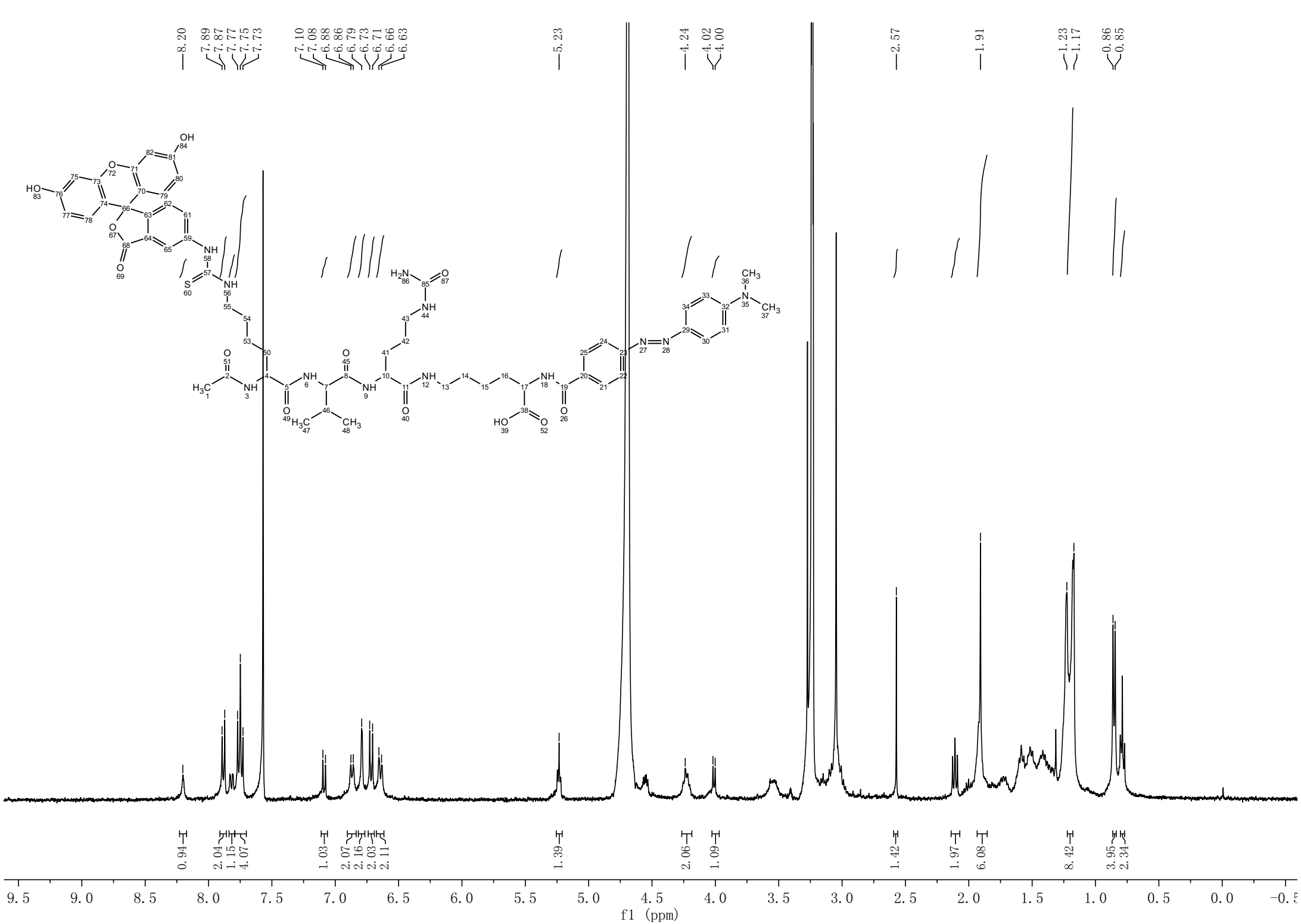
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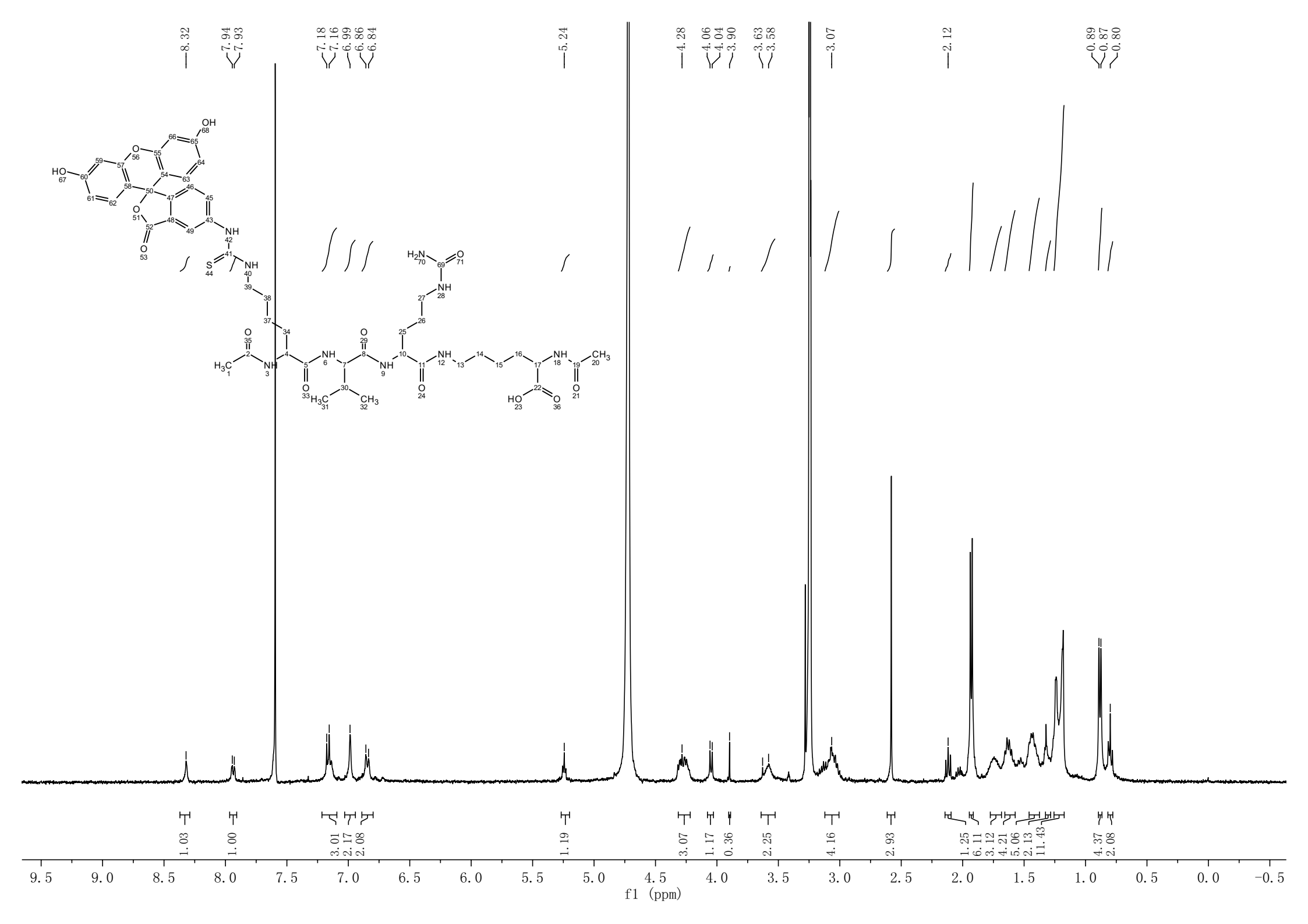
f1 (ppm)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