Supplementary data

Ratiometric Fluorescent Probes for Selective and Sensitive Visualization of Bacterial Microenvironment Protease Activity

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Contents

Abbreviations

ATCC = American Type Culture Collection

BHI = Brain Heart Infusion Broth

Cy = Cyanine Dyes

Cy 3-NHS = Cyanine 3-*N*-hydroxysuccinimide ester

DCM = Dichloromethane

DIPEA = Diisopropyl-ethyl amine

DMF = Dimethylformamide

DMSO = Dimethyl sulfoxide

ESI-MS = Electrospray Ionisation Mass Spectrometry

HPLC = High Performance Liquid Chromatography

HRMS = High Resolution Mass Spectrometry

LB = Luria-Bertani

LC-MS = Liquid Chromatography Mass Spectrometry

MRS = M.R.S. Broth

NB = Nutrient Broth

NMR = Nuclear Magnetic Resonance

NHS = *N*-hydroxysuccinimide

OD = Optical Density

rpm = Revolutions Per Minute

r.t. = Room Temperature

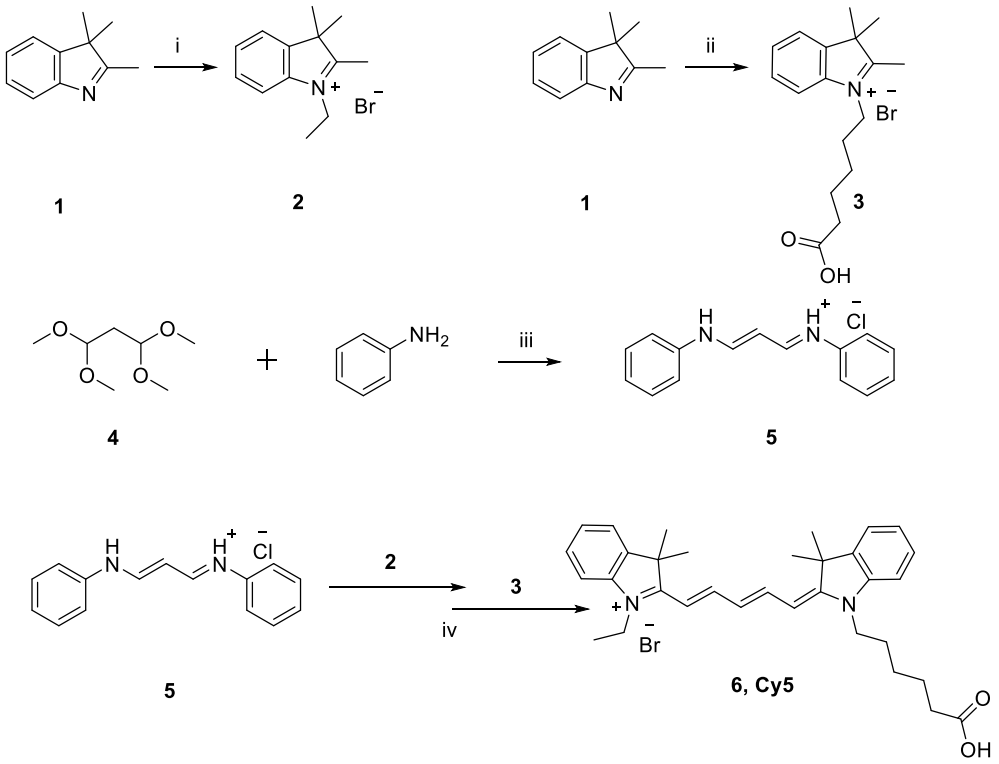
Tris = Tris(hydroxymethyl)aminomethane

TSB = Tryptone Soya Broth

General methods

All the chemicals were purchased from J&K. Commercially available reagents were used without further purification. Endoproteinase Glu-C (SspA protease) from *Staphylococcus aureus* was purchased from Aladdin. Seven bacterial strains *Staphylococcus aureus* (*S. aureus*, ATCC 25923), *Enterococcus faecium* (*E. faecium*, ATCC 29212), *Klebsiella pneumoniae* (*K. pneumoniae*, ATCC 700603), *Acinetobacter baumannii* (*A. baumannii*, ATCC 19606), *Pseudomonas aeruginosa* (*P. aeruginosa* (ATCC 27853), *Enterobacter cloacae* (*E. cloacae*, ATCC 13047)), *Micrococcus luteus* (*M. luteus*, ATCC 4698), were purchased from American Type Culture Collection (ATCC), USA. Tow yeast cells, *Pichia pastoris* GS115 and *Saccharomyces cerevisiae* INVSC1 cells, were obtained from Prof. Ping Zhu' Lab (Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China). Fluorescence emission spectra and full wavelength absorption spectra were performed on Tecan Spark™ 10M Multimode Microplate Reader. OD values were recorded in a 10 mm path quartz cell on a Metash UV-5100B spectrometer. Confocal laser scanning microscope imaging was conducted with ZEISS LSM 710 Confocal Microscope and Leica TCS SP8 X Confocal Microscope. All ^{1}H NMR spectra were recorded at 400 MHz, ^{13}C NMR spectra were recorded at 100 or 150 MHz, respectively. Mass spectra (MS) were measured with Thermo LCQ Deca XP Max mass spectrometer for electrospray ionization mass spectra (ESI).

Synthetic procedures and characterized data



Scheme S1. Synthesis of Cy 5. Reagents and conditions: (i) bromoethane, acetonitrile, reflux, 24 h, for compound **2**; (ii) 6-bromohexanoic acid, *o*-dichlorobenzene, 120 °C, 24 h, for compound **3**; (iii) hydrochloric acid, H_2O, 50 °C, 2 h, for compound **5**; (iv) compound **5** and **2**, acetic anhydride, reflux, 2 h, evaporate, then with compound **3**, anhydrous ethanol, anhydrous sodium acetate, reflux, 6 h, for **Cy 5**.

Compound **2**

Compound **1** (4.8 g, 30.15 mmol) and bromoethane (4.9 g, 44.97 mmol) were added to the acetonitrile (30 mL) and the mixture was refluxed for 24 hours. After cooling to r.t., filtered and got pale pink solid, the solid was washed with ethyl acetate (30 mL), dried and got light pink solid 6.12 g, yield 75.68 %. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.03 – 8.00 (m, 1H), 7.88 – 7.86 (m, 1H), 7.63 – 7.61 (m, 2H), 4.53 (q, J = 7.6 Hz, 2H), 2.88 (s, 3H), 1.52 (s, 6H), 1.45 (t, J = 7.2 Hz, 3H); HRMS (*m*/*z*) (M^+): calcd. for $C_{13}H_{18}N^+$ 188.1434, found 188.1434.

Compound **3**

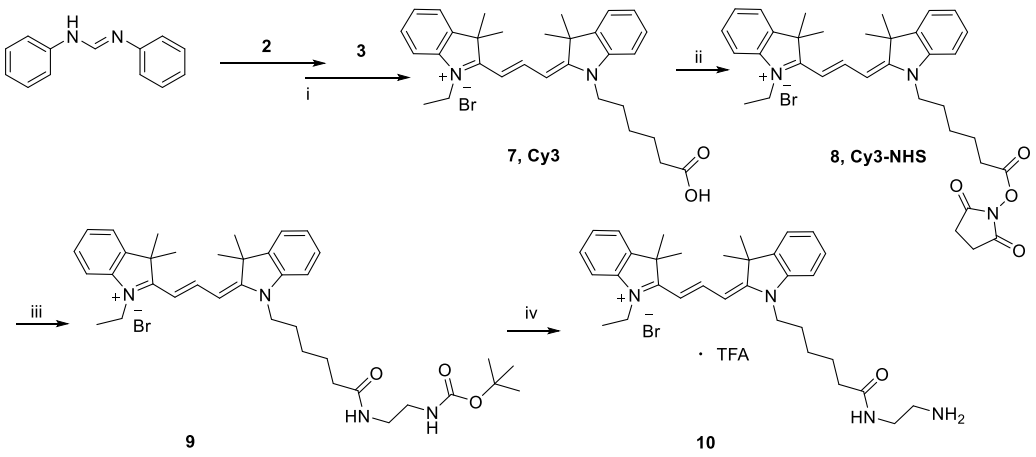
Compound **1** (3.0 g, 18.84 mmol) and 6-bromohexanoic acid (5.4 g, 27.40 mmol) were added to the *o*-dichlorobenzene (20 mL) and the mixture was heated to 120 °C for 24 h. After cooling to r.t., filtered and was washed with ethyl acetate (30 mL). Dried and got gray-white solid 4.35 g, yield 65.18 %. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.02 – 8.00 (m, 1H), 7.87 – 7.85 (m, 1H), 7.63 – 7.61 (m, 2H), 4.48 (t, J = 7.6 Hz, 2H), 2.88 (s, 3H), 1.89 – 1.81 (m, 2H), 1.88 (t, J = 7.8 Hz, 2H), 1.59 – 1.52 (m, 8H), 1.47 – 1.42 (m, 2H); HRMS (*m*/*z*) (M^+): calcd. for $C_{17}H_{24}O_2N^+$ 274.1802, found 274.1801.

Compound **5**

Compound **4** (4.9 g, 29.84 mmol) and hydrochloric acid (4.25 mL) were added to distilled water (90 mL) and stirred at 50 °C. Then the solution of aniline (4.9 g, 52.61mmol), hydrochloric acid (4.25 mL) and distilled water (70 mL) was added dropwise to the reaction and continued to stir at 50 °C for 2 h. After cooling, filtered, dried and got solid 5.68 g, yield 73.58 %; ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.68 – 8.72 (d, J = 11.6 Hz, 2H), 7. 47 (t, J = 8.4, 4H), 7.41 – 7.39 (m, 4H), 7.29 (m, 2H), 6.28 (t, J = 10.8 Hz, 1H); HRMS (*m*/*z*) (M^+): calcd. for $C_{15}H_{15}N_2^+$ 223.1230, found 223.1226.

Compound **6** (Cy 5)

The compound **5** (1.16 g, 4.48 mmol) and compound **2** (1.0 g, 3.73 mmol) were added to the acetic anhydride (20 mL) and the mixture was stirred and refluxed for 2 h. The solution was cooled down to r.t., and the solvent was evaporated under reduced pressure. Then anhydrous ethanol (20 mL), compound **3** (1.3 g, 3.67 mmol), and anhydrous sodium acetate (0.66 g, 8.05 mmol) were added to the residue, and stirred under reflux for 6 h. After the reaction was complete, the crude product was purified by silica gel column chromatography (DCM:MeOH = 30:1) to give deep blue solid 1.03 g, yield 48.58 %; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.07-7.98 (m, 2H, -Ar), 7.36 (dd, J_1 = 16 Hz, J_2 = 8.4 Hz,2H), 7.25 – 7.19 (dd, J_1 = 15.6 Hz, J_2 = 8.4 Hz,2H), 6.96 (t, J = 12.4 Hz, 2H), 6.55 (d, J = 14 Hz, 1H), 4.19 (q, J = 14.4 Hz , 2H), 4.5 (t, J = 7.6 Hz , 2H), 2.54 (t, J = 7.2 Hz, 2H), 1.87 – 1.77 (m, 4H), 1.74 (s, 6H), 1.73 (s, 6H), 1.62-1.56 (m, 2H), 1.43 (t, J = 7.2 Hz, 3H); HRMS (*m*/*z*) (M^+): calcd. for $C_{33}H_{41}O_2N_2^+$ 497.3163, found 497.3131. [1]



Scheme S2. Synthesis of Cy 3, Cy 3-NHS, compound **9** and Compound **10**. Reagents and conditions: (i) compound **5** and **2**; acetic anhydride, reflux, 2 h, then with compound **3**, acetic anhydride, pyridine, reflux, 15 min, for compound **7**; (ii) *N,N′*-disuccinimidyl carbonate, DIPEA, DCM, r.t., 2h, for compound **8**; (iii) *N*-Boc-ethylenediamine, DIPEA, DCM, r.t., 2 h, for compound **9**; (iv) 50% TFA, DCM, r.t., 1 h, for compound **10**.

Compound **7** (Cy 3)

Compound **2** (1.0 g, 6.28 mmol) and *N,N′*-diphenylformamidine (0.8 g, 4.08 mmol) were added to the acetic anhydride (10 mL) and the mixture was stirred and refluxed for 2 h. The solution was cooled down to r.t.. The compound **3** (1.5 g, 4.23 mmol) was dissolved in the mixture of acetic anhydride (8 mL) and pyridine (8 mL). The above mixture was added to the residue, and stirred and refluxed for 15 min under Ar. After the reaction was complete, the product was purified by silica gel column chromatography (DCM:MeOH = 20:1) to give dark pink solid 0.82 g, yield 36.44 %. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.57 (t, J = 12.0 Hz, 1H), 7.80 – 7.55 (m, 2H), 7.50 – 7.44 (m, 2H), 7.39 – 7.30 (m, 2H), 6.53 (t, J = 13.2 Hz, 2H), 4.32 – 4.16 (m, 4H), 2.34 (t, J = 7.2 Hz, 2H), 1.92 – 1.84 (m, 2H), 1.78 (s, 12H), 1.74 – 1.69 (m, 2H), 1.59 – 1.53 (m, 2H), 1.44 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 174.49, 174.20, 150.78, 148.55, 141.94, 141.48, 140.90, 140.74, 137.70, 128.58, 128.55, 125.32, 125.26, 124.41, 122.14, 122.08, 111.00, 110.79, 102.28, 102.12, 49.21, 49.17, 43.69, 38.91, 33.54, 26.90, 26.78, 26.75, 25.89, 24.41, 11.26; HRMS (*m*/*z*) (M^+): calcd.

for m/z, $C_{31}H_{39}O_2N_2^+$, 471.3006, found, 471.3016.

Compound **8** (Cy 3-NHS)

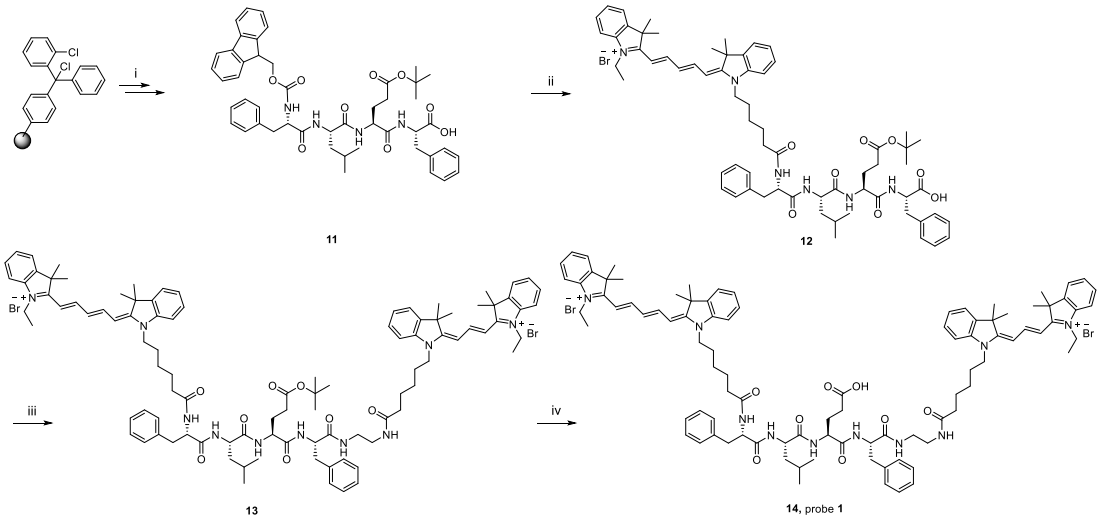
The compound **7** (500 mg, 0.9 mmol), *N*,*N*'-disuccinimidyl carbonate (279 mg, 1.09 mmol) and DIPEA (235 mg, 1.82 mmol) were added to DCM (10 mL) and the mixture was stirred at r.t. for 3 h. After the reaction was complete, the product was purified by silica gel column chromatography (DCM:MeOH = 30:1) to give dark pink solid 500 mg, yield 85.67 %. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.57 (t, J = 12.0 Hz, 1H), 7.57 (t, J = 4.4 Hz, 2H), 7.49-7.45 (m, 2H), 7.39 (m, J = 6 Hz, 2H), 6.50 (t, J = 9.4 Hz, 2H), 4.26 – 4.19 (m, 4H), 2.85 (s, 4H), 2.71 (t, J = 4.8 Hz, 2H), 1.92 – 1.86 (m, 2H), 1.80 (s, 12H), 1.77 – 1.71 (m, 2H), 1.67 – 1.61 (m, 2H), 1.45 (t, J = 6 Hz, 3H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 174.58, 174.23, 170.38, 168.71, 150.83, 150.79, 141.93, 141.91, 141.47, 140.90, 140.75, 128.59, 128.57, 125.35, 125.27, 122.14, 122.08, 111.02, 110.79, 102.22, 102.20, 102.05, 49.23, 43.63, 38.87, 29.89, 26.89, 26.77, 26.53, 25.42, 25.07, 23.93, 11.22; HRMS (*m*/*z*) (M^+): calcd. for m/z, $C_{35}H_{42}O_4N_3^+$, 568.3170, found, 568.3160.[2]

Compound **9**

The compound **8** (200 mg, 0.31 mmol), *N*-Boc-ethylenediamine (60 mg, 0.37 mmol) and DIPEA (80 mg, 0.62 mmol) were added to DCM (10 mL) and the mixture was stirred at r.t. 3 h. After the reaction was complete, the product was purified by silica gel column chromatography (DCM:MeOH = 10:1) to give dark pink solid 150 mg, yield 69.74 %. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.57 (t, J = 12.4 Hz, 1H), 7.51 (s, 2H), 7.43 (s, 2H), 7.32 – 7.28 (m, 4H), 6.68 (t, J = 12 Hz, 1H), 6.34-6.29 (m, 2H), 4.19 – 4.13 (m, 4H), 3.54-3.49 (m, 2H), 2.24 (s, 2H), 1.89-1.83 (m, 2H), 1.74 (s, 12H), 1.44 – 1.40 (m, 13H), 1.21 (t, J = 8 Hz, 3H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 174.72, 173.14, 172.90, 154.10, 153.91, 142.12, 141.63, 141.40, 141.24, 128.33, 125.38, 124.82, 124.73, 122.01, 121.96, 110.58, 110.36, 102.94, 102.70, 78.67, 65.46, 54.42, 49.14, 49.09, 43.29, 39.50, 39.09, 38.54, 35.34, 27.34, 26.76, 26.54, 26.42, 25.88, 25.06, 17.28, 15.84, 14.04, 11.73, 11.15; HRMS (*m*/*z*) (M^+): calcd. for m/z, $C_{38}H_{53}O_3N_4^+$, 613.4117, found, 613.4112.

Compound **10**

The compound **9** (100 mg, 0.14 mmol) was dissolved in the mixture of TFA (2 mL) and DCM (2 mL), and the reaction mixture was stirred at room temperature 2 h. The solvent was evaporated under reduced pressure, and the product was dark pink solid 85 mg, yield 93.61 %. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.24 (t, J = 13.2 Hz, 1H), 7.49 – 7.47 (m, 2H), 7.42 – 7.38 (m, 2H), 7.30 – 7.23 (m, 4H), 6.62 (t, J = 13.2 Hz, 1H), 6.28 (dd, J_1 = 13.6 Hz, J_2 = 8.4 Hz, 2H) 4.17 – 4.08 (m, 4H), 3.42 (t, J = 5.6 Hz, 2H), 3.03 (t, J = 5.6 Hz, 2H), 2.26 (t, J = 7.2 Hz, 2H), 1.86 – 1.80 (m, 2H), 1.71 (s, 12H), 1.68 – 1.66 (m, 2H), 1.51 – 1.45 (m, 2H), 1.38 – 1.36 (m, 3H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 177.10, 174.55, 174.44, 155.68, 155.45, 143.57, 143.04, 142.80, 142.62, 129.78, 129.72, 126.57, 126.31, 126.17, 123.44, 123.40, 111.96, 111.81, 104.20, 104.05, 66.89, 55.80, 50.60, 50.50, 44.71, 43.76, 40.77, 39.96, 38.13, 36.54, 28.20, 27.93, 27.80, 27.46, 26.28, 18.68, 17.25, 15.44, 13.13, 12.53; HRMS (m/z) (M^+): calcd. for m/z, $C_{33}H_{45}O N_4^+$, 513.3591, found, 513.3588.



Scheme S3. Synthesis of SspA protease non-lipidated probe **1**. Synthesis of SspA protease non-lipidated probe **1**. Reagents and conditions: (i) Fmoc-protected Phe amino acid, DIPEA, DMF, r.t., 4 h; then Fmoc-protected Glu amino acid and Fmoc-protected amino acid, HATU, DIPEA, DMF, r.t., 1 h; Fmoc deprotection; subsequently with 10% acetic acid/10% 2,2,2-TFE/DCM 15 min, for compound **11**; (ii) HATU, DIPEA, Cy 5, DCM, r.t., 4 h, for compound **12**; (iii) Compound **10**, HATU, DIPEA, DCM, r.t., 3 h,

for compound **13**; (iv) TFA, DCM, r.t., 2 h, for compound **14**.

Compound **11**

The compound **11** was synthesized using standard solid-phase peptide synthesis methods. The 2-chlorotrityl-chloride resin (Alfa Aesar, loading 1.0 mmol/g) was used as solid support. For loading of the resin (300 mg, 0.3 mmol), Fmoc-protected Phe amino acid (181.56 mg, 0.45 mmol) and DIPEA (116.32 mg, 0.9 mmol) were dissolved in DMF (6 mL) and the reaction mixture was added to the resin. The reaction mixture was shaken at r.t. for 4 h. The resin was washed three times with 4 ml DCM and 4 ml DMF. For Fmoc deprotection, the resin was treated two times for 15 min with 2 ml 20% piperidine/DMF. Fmoc-protected Glu amino acid (382.95 mg, 0.9 mmol), HATU (342.22 mg, 0.9 mmol) and DIPEA (232.63 mg, 1.8 mmol) were dissolved in 6 ml DMF and the reaction mixture was added to the resin containing Glu amino acid. The reaction mixture was shaken at r.t. for 1h. The resin was washed three times with 4 ml DCM and 4 ml DMF and treated two times for 15 min with 2 ml 20% piperidine/DMF for Fmoc deprotection.

A standard protocol was used for solid phase peptide synthesis: Fmoc-protected amino acid (0.9 mmol), HATU (342.22 mg, 0.9 mmol) and DIPEA (232.63 mg, 1.8 mmol) were dissolved in 6 ml DMF. The reaction mixture was added to the resin. The reaction mixture was shaken at r.t. for 1 h. The resin was washed three times with 4 ml DCM and 4 ml DMF and treated two times for 15 min with 2 ml 20 % piperidine/DMF for Fmoc deprotection the resin.

For the cleavage of the peptide from the solid-phase: the resin was treated two times for 15 min. with 2 ml 10% acetic acid/10% 2,2,2-TFE/DCM (v/v). The solvent was co-evaporated with toluene under reduced pressure, and the product was used in the next step without further purification.

HRMS (*m*/*z*) (M+H): calcd. for m/z, $C_{48}H_{57}O_9N_4^+$, 833.4120, found, 833.4106.

Compound **12**

The resin containing compound **11** (300 mg, 0.50 mmol) was treated two times for 15 min with 2 ml 20 % piperidine/DMF. HATU (380.24 mg, 1.0 mmol), DIPEA (258.48

mg, 2.0 mmol) and Cy 5 (557.61 mg, 1.0 mmol) were solved in 5 ml DCM and then added to Fmoc deprotected resin. The reaction mixture was shaken at r.t. for 4 h. For the cleavage of the compound **12** from the solid-phase: the resin was treated two times for 15 min with 10 ml 10% acetic acid/10% 2,2,2-TFA(TFA)/DCM (v/v). The solvent was co-evaporated with toluene under reduced pressure and the product was purified by silica gel column chromatography (DCM/MeOH = 10:1) to give deep blue solid 350 mg, yield 64.19 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.81 (t, J = 12.2, 3H), 7.35 (t, J = 7.2, 7H), 7.23 – 7.16 (m, 7H), 7.12 – 7.07 (m, 3H), 6.70 (t, J = 13.2 Hz, 1H), 6.29 (t, J = 15.4 Hz, 2H), 4.62 (s, 1H), 4.50 (s, 1H), 4.34 (s, 2H), 4.05 (d, J = 2.6 Hz, 2H), 3.95 (s, 2H), 3.34 – 3.10 (m, 5H), 2.42 – 2.36 (m, 1H), 2.06 – 1.90 (m, 3H), 2.06 – 1.90 (m, 3H), 1.83 – 1.74 (m, 2H), 1.69 (s,12H), 1.63 – 1.51 (m, 2H), 1.38 (s, 6H), 1.36 (s, 9H), 1.25 (s, 3H), 0.94 – 0.87 (m, 8H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ 174.21, 173.45, 172.89, 172.41, 172.17, 171.83, 153.00, 152.87, 142.05, 141.82, 141.24, 141.13, 137.58, 137.40, 129.63, 129.53, 128.98, 128.87, 128.61, 128.41, 126.76, 126.64, 126.11, 125.42, 125.23, 122.36, 122.22, 111.07, 110.48, 104.01, 103.53, 80.42, 77.36, 55.27, 49.40, 49.30, 44.40, 39.39, 37.37, 36.65, 35.50, 32.09, 29.83, 28.19, 28.13, 27.03, 26.33, 24.97, 24.71, 23.16, 21.79, 21.00, 12.47; HRMS (*m*/*z*) (M^{+}): calcd. for $C_{66}H_{85}O_8N_6^{+}$, 1089.6423, found 1089.6421.

Compound **13**

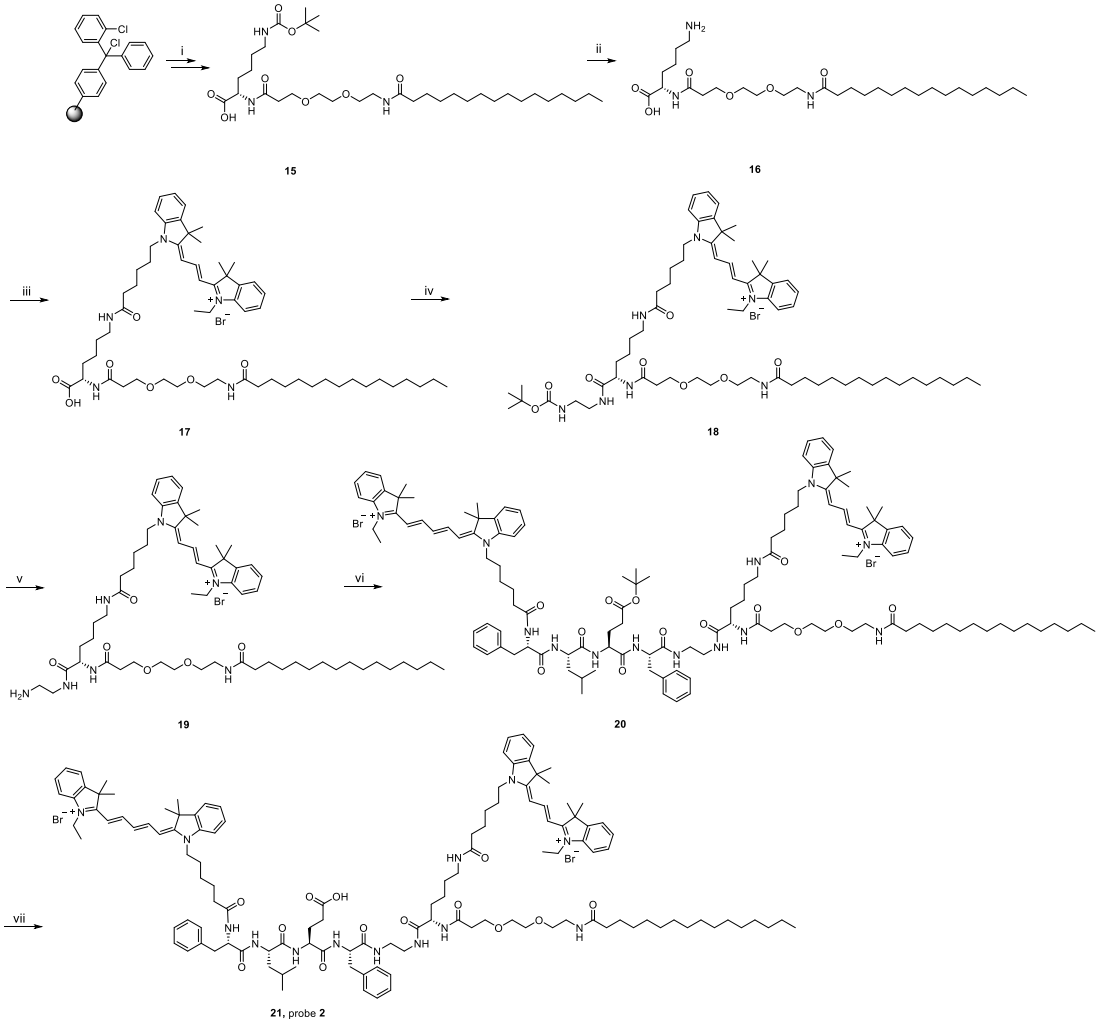
The Compound **10** (64.86 mg, 0.10 mmol), HATU (38.02 mg, 0.10 mmol) and DIPEA (25.85 mg, 0.20 mmol) were added to DCM (15 mL) and the mixture was stirred at r.t. for 15min. The compound **12** (109.04 mg, 0.1 mmol) was added to the mixture, and continued to stir at r.t. for 3 h. After the reaction was complete, the product was purified by silica gel column chromatography (DCM:MeOH = 10:1) to give deep purple solid 73 mg, yield 41.83 %. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.55 (t, J = 13.6 Hz, 1H), 8.30 – 8.22 (m, 2H), 7.75 – 7.62 (m, 1H), 7.56 (t, J = 8 Hz, 2H), 7.53 – 7.49 (m, 2H), 7.47 – 7.42 (m, 3H), 7.40 – 7.35 (m, 3H), 7.31 (t, J = 8 Hz, 4H), 7.27 – 7.24 (m, 6H), 7.24 – 7.21 (m, 4H), 7.20 – 7.15 (m, 1H), 6.61 (t, J = 12 Hz, 2H), 6.49 (dd , J_1 = 13.6 Hz, J_2 = 9.6 Hz, 2H), 6.29 (dd , J_1 = 17.6 Hz, J_2 = 14 Hz, 2H), 4.57 (dd , J_1 = 8.8 Hz,

J_2 = 5.6 Hz, 1H), 4.51 (dd , J_1 = 9.6 Hz, J_2 = 5.6 Hz, 1H), 4.31 (t, J = 6.4 Hz, 1H), 4.27 – 4.22 (m, 2H), 4.21 – 4.14 (m, 6H), 4.09 – 4.03 (m, 2H), 3.29 – 3.12 (m, 6H), 2.99 – 2.91 (m, 1H), 2.29 – 2.14 (m, 6H), 1.89 – 1.84 (m, 3H), 1.77 (s, 12H), 1.74 (s, 12H), 1.73 (s, 3H) , 1.66 – 1.61 (m, 4H), 1.55 – 1.47 (m, 3H), 1.45 – 1.37 (m, 18H), 1.31 (s, 3H), 1.0 (t, J = 7.2 Hz, 1H), 0.96 – 0.89(m, 9H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 176.31, 175.99, 175.92, 175.63, 175.23, 174.54, 174.49, 173.72, 173.54, 173.47, 155.68, 155.39, 152.19, 143.54, 143.37, 143.05, 142.90, 142.82, 142.62, 142.32, 142.18, 138.62, 138.29, 132.35, 130.33, 130.29, 130.04, 129.87, 129.81, 129.77, 129.53, 129.50, 127.90, 127.85, 126.80, 126.75, 126.60, 126.36, 126.20, 123.58, 123.53, 123.47, 123.44, 112.50, 112.24, 111.97, 111.86, 104.18, 104.15, 103.65, 103.46, 81.73, 66.65, 56.85, 56.49, 54.92, 54.10, 50.66, 50.63, 50.51, 45.11, 44.78, 44.74, 41.22, 40.32, 40.04, 40.01, 39.76, 38.60, 38.52, 36.81, 36.30, 32.55, 31.72, 30.76, 28.40, 28.34, 28.22, 28.15, 27.97, 27.82, 27.29, 26.46, 26.35, 25.81, 23.52, 21.98, 20.26, 14.40, 14.05, 12.70, 12.62; HRMS (*m*/*z*) (M^{2+}): calcd. for $C_{99}H_{128}O_8N_{10}^{2+}$ 1584.9911, found 1584.9922.

Compound **14**

The compound **13** (34.92 mg, 0.02 mmol) was dissolved in the mixture of TFA (2 mL) and DCM (2 mL). The reaction mixture was stirred at r.t. for 2 h, and the solvent was evaporated under reduced pressure. The crude product was treated with ether (15 mL), and the resulting product was filtered through a paper filter. The residue was dried under reduced pressure to afford deep purple solid 21.32 mg, yield 63.10 %.^{1}H NMR (400 MHz, Methanol-d_4) δ 8.54 (t, J = 13.2 Hz, 1H), 8.28 – 8.24 (m, 2H), 7.56 – 7.48 (m, 5H), 7.45 – 7.40 (m, 5H), 7.35 (d, J = 8.4 Hz, 2H), 7.3 (t, J = 7.6 Hz, 4H), 7.26 (s, 1H), 7.24 (s, 3H), 7.22 – 7.21 (m, 3H), 7.19 – 7.14 (m, 1H), 6.58 (t, J = 12.4 Hz, 1H), 6.46 (dd , J_1 = 13.2 Hz, J_2 = 8.8 Hz, 2H), 6.26 (dd , J_1 = 18 Hz, J_2 = 14 Hz, 2H), 4.58-4.49 (m, 2H), 4.20 – 4.12 (m, 8H), 4.04 (t, J = 7.2 Hz, 2H), 3.27 – 3.22 (m, 3H), 3.17– 3.12 (m, 3H), 2.96 – 2.91 (m, 1H), 2.27 – 2.18 (m, 6H), 1.92 – 1.83 (m, 3H), 1.76 (s, 12H), 1.72 (s, 12H), 1.65 – 1.57 (m, 6H), 1.53 – 1.50 (m, 3H), 1.43 – 1.36 (m, 10H), 1.30 (s, 3H), 0.95 – 0.89 (m, 10H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 176.33, 176.31, 176.00,

175.94, 175.64, 175.22, 174.57, 174.51, 174.48, 174.45, 173.56, 173.50, 155.66, 155.64, 155.59, 155.37, 155.32, 152.20, 143.54, 143.36, 143.05, 142.90, 142.81, 142.62, 142.32, 142.17, 138.59, 138.32, 130.30, 130.26, 130.13, 130.04, 129.81, 129.76, 129.60, 129.52, 129.48, 127.88, 126.80, 126.76, 126.51, 126.36, 126.20, 123.57, 123.53, 123.47, 123.43, 112.49, 112.23, 111.96, 111.85, 104.12, 103.62, 103.42, 56.78, 56.55, 55.01, 54.10, 50.67, 50.63, 50.59, 50.51, 50.48, 45.10, 44.77, 41.19, 40.30, 40.04, 39.99, 39.78, 38.60, 38.49, 36.80, 36.26, 31.17, 28.79, 28.33, 28.23, 28.21, 28.11, 27.95, 27.94, 27.81, 27.69, 27.29, 27.25, 26.45, 26.32, 25.81, 23.51, 21.96, 12.68, 12.59; HRMS (*m*/*z*) (M^{2+}): calcd. for $C_{95}H_{120}O_8N_{10}^{2+}$, 1528.9285, found 1528.9281.



Scheme S4. Synthesis of SspA protease lipidated probe **2**. Reagents and conditions:(i) Fmoc-protected Lys (Boc)-OH amino acid, DIPEA, DMF, r.t., 4h; 20% piperidine/DMF, 15min; Fmoc-AEEA amino acid, HATU, DIPEA, DMF, r.t., 1 h, 20% piperidine/DMF,

15 min; the palmitic acid, HATU, DIPEA, DMF, r.t., 1 h, for compound **15**; (ii) TFA, DCM, r.t., 2 h, for compound **16**; (iii) Cy 3-NHS, DCM, r.t., 3 h, for compound **17**; (iv) HATU, DIPEA, DCM, r.t., 15 min, then *N*-Boc-ethylenediamine, r.t., 3 h, for compound **18**; (v) TFA, DCM, r.t., 2 h, for compound **19**; (vi) Compound **12**, HATU, DIPEA, DCM, r.t., 15 min, then with compound **19,** r.t., 3 h, for compound **20**; (vii) TFA, DCM, r.t., 2 h, for compound **21**.

Compound **15**

The compound **15** was synthesized using standard solid-phase peptide synthesis methods. For loading of the resin (500 mg, 0.5 mmol), Fmoc-protected Lys (Boc)-OH amino acid (351.41 mg, 0.75 mmol) and DIPEA (193.86 mg, 1.50 mmol) were dissolved in 6 ml DMF and the reaction mixture was added to the resin. The reaction mixture was shaken at r.t. for 4h. The resin was washed three times with 4 ml DCM and 4 ml DMF. For Fmoc deprotection, the resin was treated two times for 15 min with 2 ml 20 % piperidine/DMF. Fmoc-AEEA (1-(9*H*-fluoren-9-yl)-3-oxo-2,7,10-trioxa-4-azatridecan-13-oic acid) amino acid (385.41 mg, 1.0 mmol), HATU (380.24 mg, 1.0 mmol) and DIPEA (258.48 mg, 2.0 mmol) were dissolved in 6 ml DMF and the reaction mixture was added to the resin containing Lys amino acid. The reaction mixture was shaken at r.t. for 1h. The resin was washed three times with 4 ml DCM and 4 ml DMF and treated two times for 15 min with 2 ml 20 % piperidine/DMF for Fmoc deprotection. The palmitic acid (256.42 mg, 1.0 mmol), HATU (380.24 mg, 1.0 mmol) and DIPEA (258.48 mg, 2.0 mmol) were dissolved in 6 ml DMF and the reaction mixture was added to the resin containing Lys amino acid and AEEA. The reaction mixture was shaken at r.t. for 1h. The resin was washed three times with 4 ml DCM and 4 ml DMF. The resin contained compound **15** was treated two times for 15 min with 10 ml 10% acetic acid/10% 2,2,2-TFE/DCM (v/v). The solvent was co-evaporated with toluene under reduced pressure and the product was treated with ether (10 mL). The resulting product was filtered through a paper filter to give white solid 232.77 mg, yield 72.30 %. ^{1}H NMR (400 MHz, Methanol-d_4) δ 4.42-4.37 (m, 1H), 3.80 – 3.72 (m, 2H), 3.64 – 3.59 (m, 4H), 3.53 (t, J = 5.6 Hz, 2H), 3.37-3.33 (m, 2H), 3.04 (t, J = 6.8 Hz, 2H), 2.56 – 2.49 (m,

2H), 2.20 (t, *J* = 7.6 Hz, 2H), 1.91-1.65 (m, 2H), 1.64 – 1.55 (m, 2H), 1.52 – 1.47 (m, 2H), 1.43 (s, 12H), 1.35 – 1.29 (m, 26H), 0.90 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (150 MHz, Methanol-d_4) δ 176.45, 175.49, 173.83, 158.53, 129.94, 129.23, 126.32, 79.84, 71.36, 71.28, 70.65, 68.23, 53.62, 41.16, 40.36, 37.38, 37.09, 33.10, 32.53, 30.82, 30.80, 30.77, 30.68, 30.55, 30.50, 30.34, 28.84, 27.07, 24.15, 23.77, 14.48; HRMS (*m*/*z*) (M+H): calcd. for $C_{34}H_{66}O_8N_3^+$, 644.4844, found 644.4833.

Compound **16**

The compound **15** (200 mg, 0.31 mmol) was dissolved in the mixture of TFA (2 mL) and DCM (2 mL) and the solution was stirred at r.t. for 2 h. The solvent was evaporated under reduced pressure to give scurf-like solid 144.41 mg, yield 85.50 %. ^{1}H NMR (400 MHz, Methanol-d_4) δ 4.47-4.44 (m, 1H), 3.81 – 3.73 (m, 2H), 3.63 – 3.60 (m, 4H), 3.54 (t, *J* = 6 Hz, 2H), 3.35 (t, *J* = 6 Hz, 2H), 2.93 (t, *J* = 6.8 Hz, 2H), 2.55 – 2.51 (m, 2H), 2.20 (t, *J* = 7.2 Hz, 2H), 1.75-1.68 (m, 2H), 1.61 (t, *J* = 6.8 Hz, 2H),1.53– 1.46 (m, 2H), 1.38 – 1.30 (m, 26H), 0.91 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 176.55, 175.07, 173.99, 71.38, 71.30, 68.21, 66.95, 53.06, 40.57, 40.35, 37.38, 37.11, 33.12, 32.36, 30.84, 30.81, 30.78, 30.70, 30.52, 30.35, 28.01, 27.09, 23.78, 15.48, 14.48; HRMS (*m*/*z*) (M^+): calcd. for $C_{29}H_{58}O_6N_3^+$, 544.4320, found 544.4326.

Compound **17**

The compound **16** (87 mg, 0.16 mmol) and Cy 3-NHS (100 mg, 0.16 mmol) were dissolved in DCM and the solution was stirred at r.t. for 3 h. After the reaction was complete, the product was purified by silica gel column chromatography (DCM:MeOH = 10:1) to give dark pink solid 108 mg, yield 63.0 %. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.52 (t, *J* = 13.6 Hz, 1H), 7.54 – 7.51 (m, 2H), 7.45 – 7.41 (m, 2H), 7.35 – 7.27 (m, 4H), 6.46 (dd, *J* = 13.2 Hz, *J* =9.2 Hz, 2H), 4.29 – 4.12 (m, 5H), 3.74 – 3.68 (m, 2H), 3.60-3.55 (m, 4H), 3.49 (t, *J* = 5.6 Hz, 2H), 3.32 – 3.30 (m, 2H), 3.11 (t, *J* = 6.4 Hz, 2H), 2.49-2.42 (m, 2H), 2.20 – 2.14 (m, 4H), 1.87 – 1.80 (m, 2H), 1.74 (s, 12H), 1.70-1.65 (m, 2H), 1.56 (t, *J* = 7.2 Hz, 2H), 1.50 – 1.34 (m, 7H), 1.29 – 1.25 (m, 26H), 0.87 (t, *J* = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 176.44, 175.98, 175.67, 173.06, 152.24, 143.41, 142.95, 142.36, 142.21, 130.08, 126.82, 123.60, 123.57, 112.54,

112.29, 103.72, 103.49, 71.38, 71.33, 70.66, 68.37, 55.64, 50.71, 50.67, 45.13, 40.40, 40.35, 40.29, 37.81, 37.10, 36.77, 33.62, 33.11, 30.83, 30.80, 30.78, 30.69, 30.52, 30.36, 30.04, 28.38, 28.26, 27.35, 27.09, 26.61, 24.09, 23.77, 14.49, 12.73; HRMS (*m*/*z*) (M^+): calcd. for $C_{60}H_{94}O_7N_5^+$, 996.7148, found 996.7132.

Compound **18**

The compound **17** (100 mg, 0.1 mmol), HATU (38 mg, 0.1 mmol) and DIPEA (26 mg, 0.2 mmol) were dissolved in DCM (10 mL) and the solution was stirred at r.t. for 15 min and *N*-Boc-ethylenediamine (24mg, 0.15 mmol) was added to the solution and continued to stir at room temperature for 3 h. After the reaction was complete, the product was purified by silica gel column chromatography (DCM:MeOH = 10:1) to give dark pink solid 96 mg, yield 78.7 %. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.57 (t, J = 13.6 Hz, 1H), 7.58 – 7.56 (m, 2H), 7.50 – 7.45 (m, 2H), 7.40 – 7.31 (m, 4H), 6.49 (d, J = 12.4 Hz, 2H), 4.29 – 4.16 (m, 5H), 3.77 (t, J = 6 Hz, 2H), 3.66-3.60 (m, 4H), 3.54 (t, J = 5.6 Hz, 2H), 3.29 – 3.24 (m, 2H), 3.18-3.14 (m, 4H), 2.53 (t, J = 6 Hz, 2H), 2.25 – 2.18 (m, 4H), 1.91 – 1.84 (m, 2H), 1.79 (s, 12H), 1.75 – 1.69 (m, 2H), 1.67 – 1.60 (m, 2H), 1.59 – 1.45 (m, 7H), 1.44 (s, 12), 1.39 – 1.25 (m, 26H), 0.91 (t, J = 6.8 Hz, 3H);^{13}C NMR (100 MHz, Methanol-d_4) δ 174.94, 174.49, 174.25, 173.09, 172.59, 157.04, 150.77, 141.94, 141.44, 140.88, 140.73, 128.61, 128.58, 126.76, 125.39, 125.32, 122.14, 122.10, 111.02, 110.81, 102.16, 102.00, 78.67, 69.87, 69.78, 69.18, 66.82, 65.45, 53.45, 49.24, 49.19, 43.63, 39.42, 39.21, 38.87, 38.85, 38.61, 35.99, 35.62, 35.30, 31.63, 31.23, 29.35, 29.32, 29.29, 29.21, 29.03, 28.87, 28.58, 27.35, 27.26, 26.90, 26.77, 25.93, 25.60, 25.16, 22.82, 22.29, 14.00, 13.01, 11.24; HRMS (m/z) (M^+): calcd. for $C_{67}H_{108}O_8N_7^+$, 1138.8254, found 1138.8247.

Compound **19**

The compound **18** (85 mg, 0.07 mmol) was dissolved in the mixture of TFA (2 mL) and DCM (2 mL), and the reaction mixture was stirred at r.t. for 2 h. The solvent was evaporated under reduced pressure, and the residue was used directly in the next step without further purification. HRMS (*m*/*z*) (M^+): calcd. for $C_{62}H_{100}O_6N_7^+$ 1038.7730, found 1038.7734.

Compound **20**

The compound **12** (30 mg, 0.05mmol), HATU (19 mg, 0.05 mmol) and DIPEA (13 mg, 0.1 mmol) were added to DCM (15 mL) and the mixture was stirred at r.t. for 15min. The compound **19** (56 mg, 0.05 mmol) was added to the mixture, and continued to stir at r.t. for 3 h. After the reaction was complete, the product was purified by silica gel column chromatography (DCM:MeOH = 10:1) to give deep purple solid 52 mg, yield 45.5 %. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.55 (t, J = 13.2 Hz, 1H), 8.29 – 8.21 (m, 2H), 7.57 – 7.54 (m, 2H), 7.50 (t, J = 6.8 Hz, 2H), 7.44 (dd, J = 13.6 Hz, J = 6.4 Hz, 4H), 7.36 (t, J = 6.8 Hz, 3H), 7.31 (t, J = 6 Hz, 3H), 7.28 – 7.23 (m, 11H), 7.20 – 7.16 (m, 1H), 6.60 (t, J = 12.4 Hz, 2H), 6.45 (t, J = 12.8 Hz, 2H), 6.28 (dd, J = 18.8 Hz, J = 13.6 Hz, 2H), 4.61 – 4.47 (m, 2H), 4.30 – 4.27 (m, 2H), 4.23 – 4.14 (m, 7H), 4.05 (t, J = 7.2 Hz, 2H), 3.74 (t, J = 5.2 Hz, 2H), 3.58 (s, 4H), 3.51 (t, J = 5.6 Hz, 2H), 3.24 – 3.13 (m, 7H), 2.97 – 2.90 (m, 2H), 2.52 (t, J = 5.6 Hz, 2H), 2.23 – 2.16 (m, 8H), 1.93 – 1.82 (m, 6H), 1.77 (s, 12H), 1.73 (s, 12H), 1.64-1.57 (m, 9H), 1.48 (t, J = 6 Hz, 4H), 1.44 – 1.36 (m, 18H), 1.27 (s, 27H), 0.95 – 0.88 (m, 12H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 176.38, 175.95, 175.71, 174.52, 174.47, 174.39, 173.96, 173.95, 173.82, 173.80, 173.57, 173.53, 155.71, 155.44, 152.24, 143.57, 143.42, 143.09, 142.93, 142.85, 142.66, 142.36, 142.21, 138.69, 138.67, 138.46, 138.43, 130.38, 130.10, 130.08, 129.85, 129.82, 129.55, 127.89, 126.88, 126.81, 126.64, 126.40, 126.25, 123.63, 123.59, 123.51, 123.48, 112.51, 112.30, 112.02, 111.90, 104.21, 104.18, 103.66, 103.50, 81.76, 71.35, 71.27, 70.67, 68.31, 54.89, 50.72, 50.66, 50.55, 45.13, 44.82, 41.37, 40.34, 40.13, 40.05, 39.92, 38.62, 37.55, 37.49, 37.12, 36.82, 36.35, 33.11, 32.82, 32.59, 30.83, 30.79, 30.70, 30.52, 30.37, 30.10, 28.46, 28.39, 28.26, 28.17, 28.01, 27.86, 27.43, 27.30, 27.09, 26.66, 26.40, 25.85, 24.32, 23.78, 23.62, 22.04, 14.51, 12.75, 12.67; HRMS (m/z) (M^+): calcd. for $C_{128}H_{183}O_{13}N_{13}^{2+}$, 1055.7040, found 1055.7042.

Compound **21**

The compound **20** (30 mg, 0.013 mmol) was dissolved in the mixture of TFA (2 mL) and DCM (2 mL), and the reaction mixture was stirred at r.t. for 2 h. The solvent was evaporated under reduced pressure, and the product was treated with ether (15 mL).

The resulting product was filtered through a paper filter. The product was dried under reduced pressure to afford deep purple solid 21 mg, yield 72.0 %. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.53 (t, J = 13.6 Hz, 1H), 8.26 – 8.22 (m, 2H), 7.55 – 7.52 (m, 2H), 7.50 – 7.46 (m, 2H), 7.44 – 7.39 (m, 4H), 7.37 – 7.28 (m, 6H), 7.26 – 7.21 (m, 11H), 7.19 – 7.11 (m, 1H), 6.58 (t, J = 12.4 Hz, 2H), 6.45 (dd, J = 13.2 Hz, J = 11.2 Hz, 2H), 6.26 (dd, J = 18.4 Hz, J = 13.6 Hz, 2H), 4.60 – 4.46 (m, 2H), 4.29 – 4.11 (m, 9H), 4.07 – 4.01 (m, 2H), 3.72 (t, J = 5.6 Hz, 2H), 3.56 (s, 4H), 3.49 (t, J = 5.6 Hz, 2H), 3.25 – 3.12 (m, 7H), 2.95 – 2.88 (m, 2H), 2.53 – 2.49 (m, 2H), 2.25 – 2.14 (m, 8H), 2.03 – 1.80 (m, 6H), 1.75 (s, 12H), 1.71 (s, 12H), 1.64-1.55 (m, 9H), 1.50-1.45 (m, 4H), 1.43-1.35 (m, 9H), 1.25 (S, 27H), 0.94 – 0.86 (m, 12H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 176.41, 176.39, 176.21, 175.96, 175.71, 175.17, 174.52, 174.49, 174.44, 173.98, 173.58, 173.55, 155.67, 155.36, 152.24, 143.57, 143.41, 143.09, 142.92, 142.84, 142.66, 142.36, 142.21, 138.67, 138.64, 138.46, 138.42, 130.36, 130.10, 130.08, 129.85, 129.81, 129.56, 127.89, 126.88, 126.81, 126.39, 126.24, 123.62, 123.59, 123.50, 123.47, 112.50, 112.29, 112.00, 111.88, 103.64, 103.45, 71.35, 71.27, 70.66, 68.30, 56.59, 54.88, 50.72, 50.67, 50.62, 50.52, 45.12, 44.81, 40.34, 40.14, 40.03, 39.93, 38.60, 37.54, 37.49, 37.12, 36.81, 36.32, 33.11, 32.81, 31.19, 30.83, 30.70, 30.52, 30.37, 30.10, 28.39, 28.26, 28.14, 28.00, 27.86, 27.42, 27.26, 27.09, 26.65, 26.37, 25.84, 24.31, 23.78, 23.61, 22.01, 14.50, 12.73, 12.65; HRMS (m/z) (M^{2+}): calcd. for $C_{124}H_{175}O_{13}N_{13}^{2+}$ 2054.3427, found 2054.3423.

Measurements of the energy transfer efficiency (*E*) and the donor-acceptor distance (*R*)

FRET involves the transfer of energy from a photoexcited donor (Cy 3) to a ground-state acceptor chromophore (Cy 5) in the proximity of the donor. The transfer of energy occurs nonradiatively, without radiation of a photon from a donor and reabsorption by an acceptor, by coupling between dipoles of a donor and an acceptor. There are at least three basic requirements for FRET to occur: (1) overlap between the emission spectrum of a donor and absorption spectrum of an acceptor, (2) coupling between donor and acceptor transition dipoles, and (3) close proximity of donor and acceptor. FRET mainly

depends on two factors, the energy transfer efficiency (*E*) and the donor-acceptor distance (*R*), which could be calculated by two equations **I** and **II**:[3]

$$E = 1 - \frac{\tau_{da}}{\tau_{d}} \qquad \textbf{(I)}$$

where τ_{da} and τ_{d} are the fluorescence lifetimes of a donor in the presence and absence of an acceptor;

$$E = \frac{1}{1+(\frac{R}{R_0})^6} \qquad \text{(II)}$$

R is the donor-acceptor distance;

R_0 is the Forster distance at which the efficiency of energy transfer is 50%. According to the literatures, the value of R_0 of Cy 3/Cy 5 (donor-acceptor system) is 60 Å.[1b, 1c]

The radiative lifetime values of a donor in the presence and absence of an acceptor, τ_{da} and τ_{d}, were obtained from fluorescence/PL measurements with Edinburgh photonics FLS980 by using compound **10** and compound **19** as the donor only moieties of probe **1** and probe **2** respectively. The energy transfer efficiency (*E*) and the donor-acceptor distance (*R*) were calculated by equations (**I** and **II**) and presented in Table S1.

Table S1. Energy transfer efficiency (*E*) and the Cy 3/Cy 5 distance (*R*) of probe **1** and probe **2**.

Compound	τ_{da}[a] (ns)	τ_{d}[b] (ns)	*E* (%)	*R* (Å)
probe **1**	1.55	3.68	58.1	56.8
probe **2**	1.56	3.47	54.9	58.1

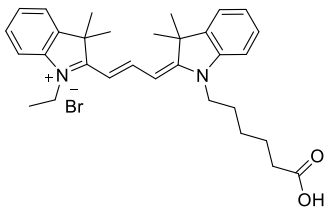
[a] the fluorescence lifetimes of probe; [b] the fluorescence lifetimes of the donor moieties of probe.

Bacteria cell culture

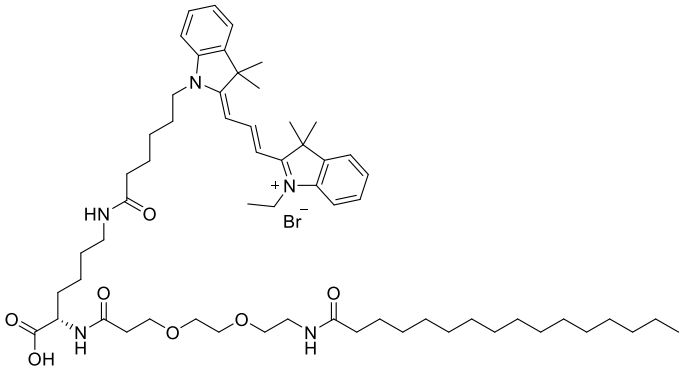
Four wild-type bacteria strains: methicillin sensitive and resistant *S. aureus*, *K. pneumoniae* and *E. cloacae* were used in this study. Tryptone Soya Broth (TSB) medium was used for culture of methicillin sensitive *S. aureus*. Nutrient broth (NB) was used for culture of methicillin resistant *S. aureus*, *K. pneumoniae* and *E. cloacae*. Single colony from the stock agar plate was added to 10 mL of liquid medium, then was grown at 37 °C on a shaker incubator (200 rpm) overnight.

Confocal imaging of bacteria treated with Cy 3 and compound 17

ESKAPE panel (comprising two Gram positive species *E. faecium* (ATCC 29212) and *S. aureus* (ATCC 25923) and four Gram negative species *K. pneumoniae* (ATCC 700603), *A. baumannii* (ATCC 19606), *P. aeruginosa* (ATCC 27853), *E. cloacae* (ATCC 13047)), and a SspA non-expression Gram positive bacteria, *M. luteus* (ATCC 4698), were cultured for 12 h in respective media at 37 °C. Bacterial strains cultured overnight in respective solution were harvested and washed twice with 20 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$ buffer. The washed cells were resuspended in 20 mM Tris-HCl (pH 7.8), 2 mM $CaCl_2$ buffer with an OD_{600} of 0.5 - 0.7. Then 200 μL aliquots were treated with 5 μM of Cy 3 and lipidated Cy 3 (compound **17**) incubation at 37 °C for 2 h respectively, and treated with 20 μg/mL of Hoechst 33258 at 37 °C for 30 min. Then a drop of the suspension was added into an 8-well chamber followed by covering with agarose pads. Fluorescence images were acquired with Leica TCS SP8 X Confocal Microscope. (HC PL Apo 63 × oil immersed optics) (λ_{ex} = 510 nm and λ_{em} = 520-610 nm, λ_{ex} = 405 nm and λ_{em} = 460 ± 25 nm).



Cy 3



Compound 17

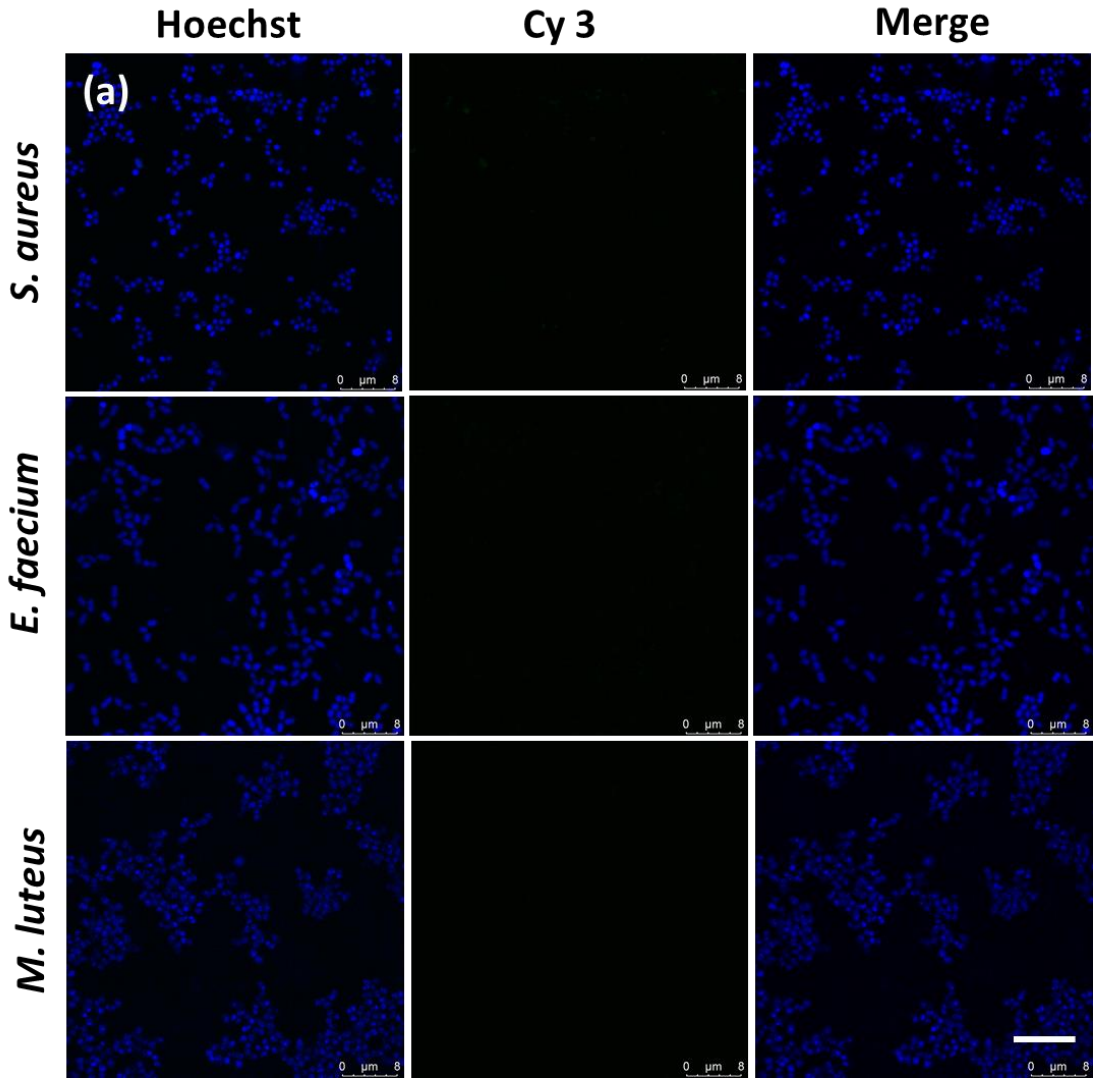


Fig. S1 (a) Fluorescence images of Gram-positive bacterial cells staining with Cy 3. All the bacterial cells were incubated with 5 μM probe at 37 °C in 20 mM Tris-HCl (pH 7.8), 2 mM $CaCl_2$ buffer for 4 h prior to fluorescence imaging. λ_{ex} = 510 nm, λ_{em} = 520-610 nm (Green, Cy 3 signal) and λ_{ex} = 405 nm and λ_{em} = 460 ± 25 nm (Hoechst signal). Scale bar = 8 μm.

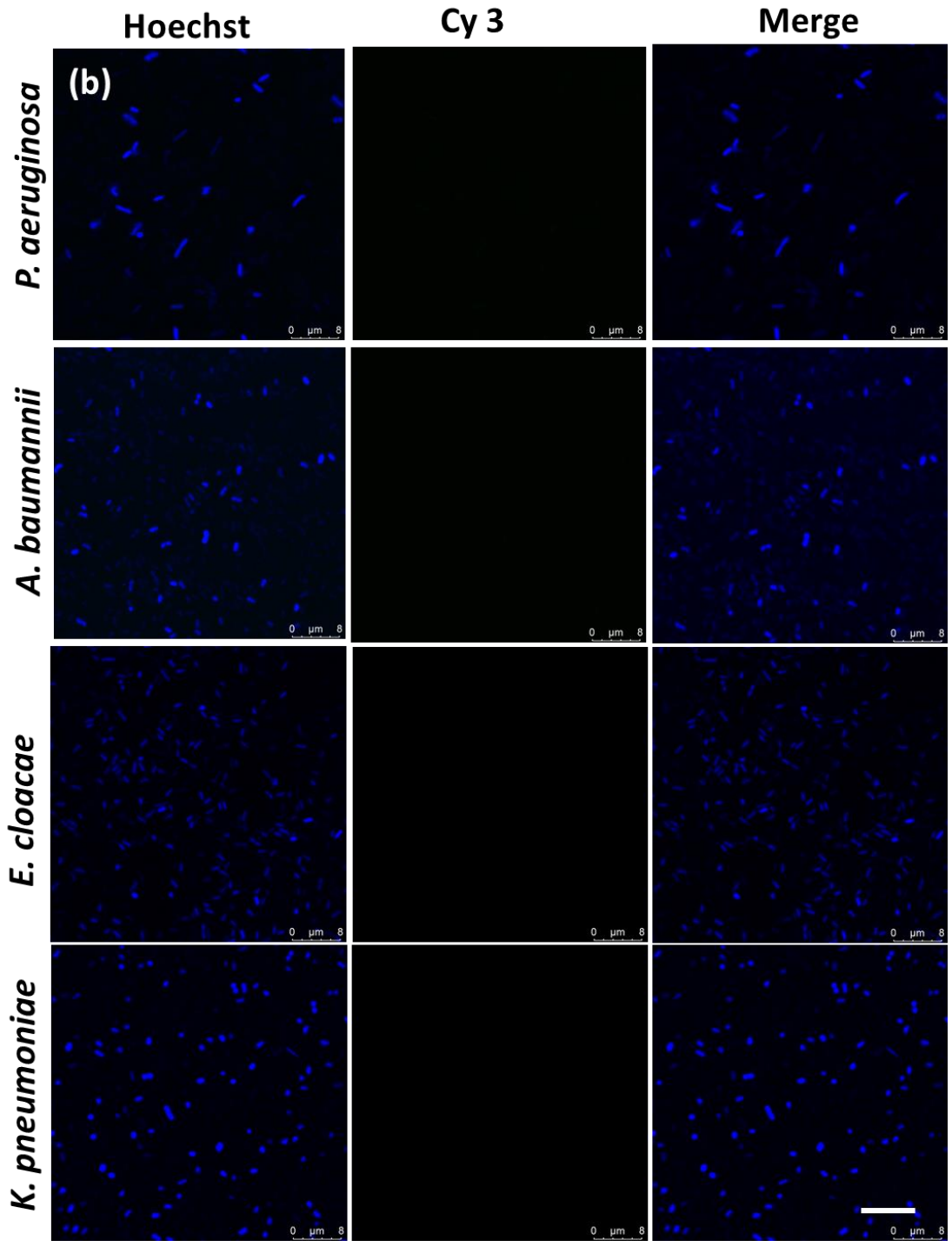


Fig. S1 (b) Fluorescence images of Gram-negative bacterial cells staining with Cy 3. All the bacterial cells were incubated with 5 μM probe at 37 °C in 20 mM Tris-HCl (pH 7.8), 2 mM $CaCl_2$ buffer for 4 h prior to fluorescence imaging. λ_{ex} = 510 nm, λ_{em} = 520-610 nm (Green, Cy 3 signal) and λ_{ex} = 405 nm and λ_{em} = 460 ± 25 nm (Hoechst signal). Scale bar = 8 μm

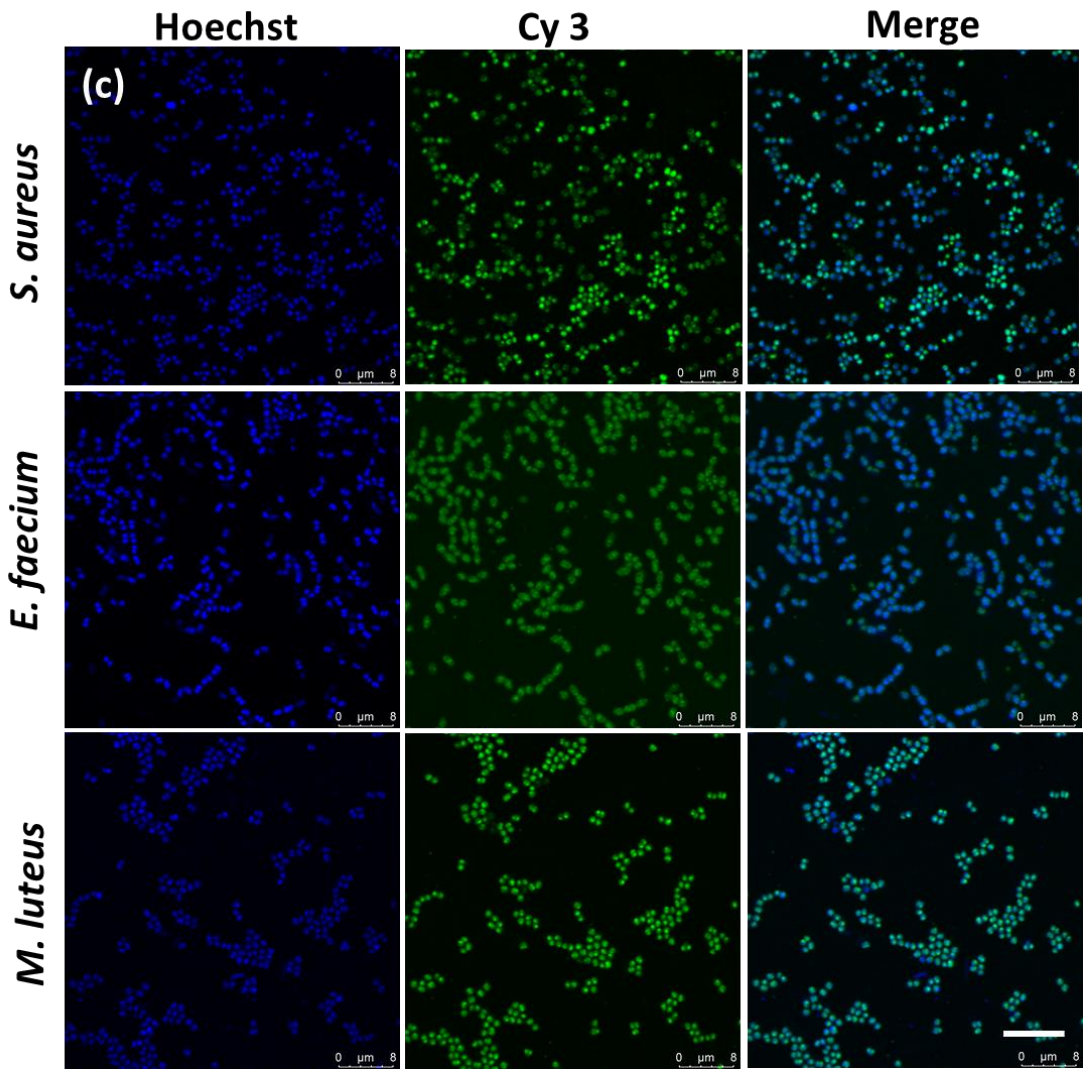


Fig. S1 (c) Fluorescence images of Gram-positive bacterial cells staining with compound **17**. All the bacterial cells were incubated with 5 μM probe at 37 °C in 20 mM Tris-HCl (pH 7.8), 2 mM $CaCl_2$ buffer for 4 h prior to fluorescence imaging. λ_{ex} = 510 nm, λ_{em} = 520-610 nm (Green, Cy 3 signal) and λ_{ex} = 405 nm and λ_{em} = 460 ± 25 nm (Hoechst signal). Scale bar = 8 μm

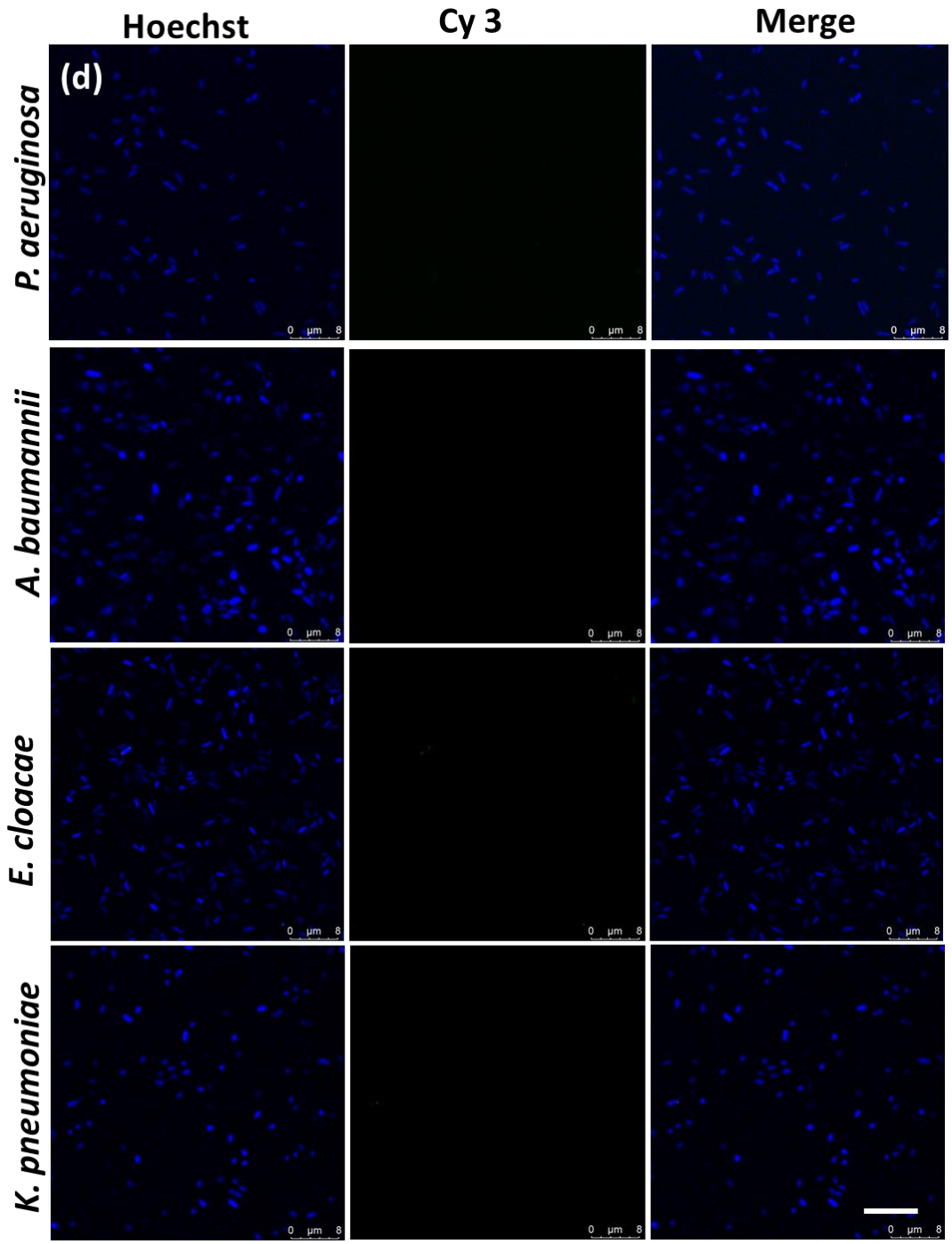


Fig. S1 (d) Fluorescence images of Gram-positive bacterial cells staining with compound **17**. All the bacterial cells were incubated with 5 µM probe at 37 °C in 20 mM Tris-HCl (pH 7.8), 2 mM $CaCl_2$ buffer for 4 h prior to fluorescence imaging. λ_{ex} = 510 nm, λ_{em} = 520-610 nm (Green, Cy 3 signal) and λ_{ex} = 405 nm and λ_{em} = 460 ± 25 nm (Hoechst signal). Scale bar = 8 µm

UV-vis absorption and fluorescence spectra

The concentration of DMSO stock solution of probe **1** and lipidated probe **2** were diluted to 0.25 μM in the 0.02 M phosphate buffer (pH = 7.4, 0.1% Caster oil, (v/v), 2 mM $CaCl_2$). The UV-Visible spectra were recorded using a Tecan Spark™ 10M Multimode Microplate Reader. Fluorescence spectroscopic studies were also performed at the excitation wavelength of 510 nm. Wavelength interval: 5.0 nm.

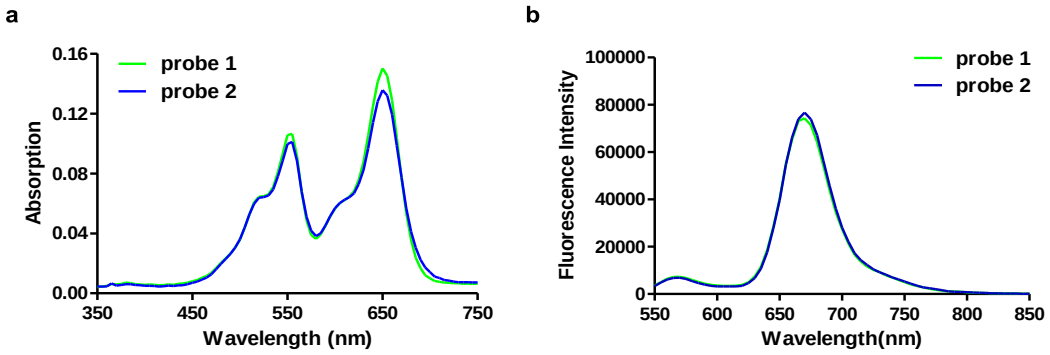


Fig. S2 (a) Absorption and (b) fluorescence emission spectra of 0.25 μM probe **1** and lipidated probe **2** in the 0.02 M phosphate buffer (pH = 7.4, 0.1% Caster oil, v/v, 2 mM $CaCl_2$). The UV-Visible and fluorescence spectra of probe **1**and lipidated probe **2** were shown with green and blue lines, respectively. λ_{ex} = 510 nm.

Inhibition studies

The concentration of DMSO stock solution of probe **1** and lipidated probe **2** were diluted to 0.25 μM in the 0.02 M phosphate buffer. Ten groups were incubated at 37 °C for 4 h, probe **1**; probe **1** /SspA (10 μg/mL); probe **1** /SspA (10 μg/mL) /3, 4-DCI (10 μg/mL); probe **1** / SspA (10 μg/mL) /3, 4-DCI (25 μg/mL); probe **1**/3, 4-DCI (25 μg/mL); probe **2**; probe **2**/SspA (10 μg/mL); probe **2**/SspA (10μg/mL)/3, 4-DCI (10 μg/mL); probe **2**/SspA (10 μg/mL)/3, 4-DCI (25 μg/mL); probe **2**/3, 4-DCI (25 μg/mL); respectively. Fluorescence spectroscopic studies were also performed at the excitation wavelength of 510 nm. Wavelength interval: 5.0 nm.

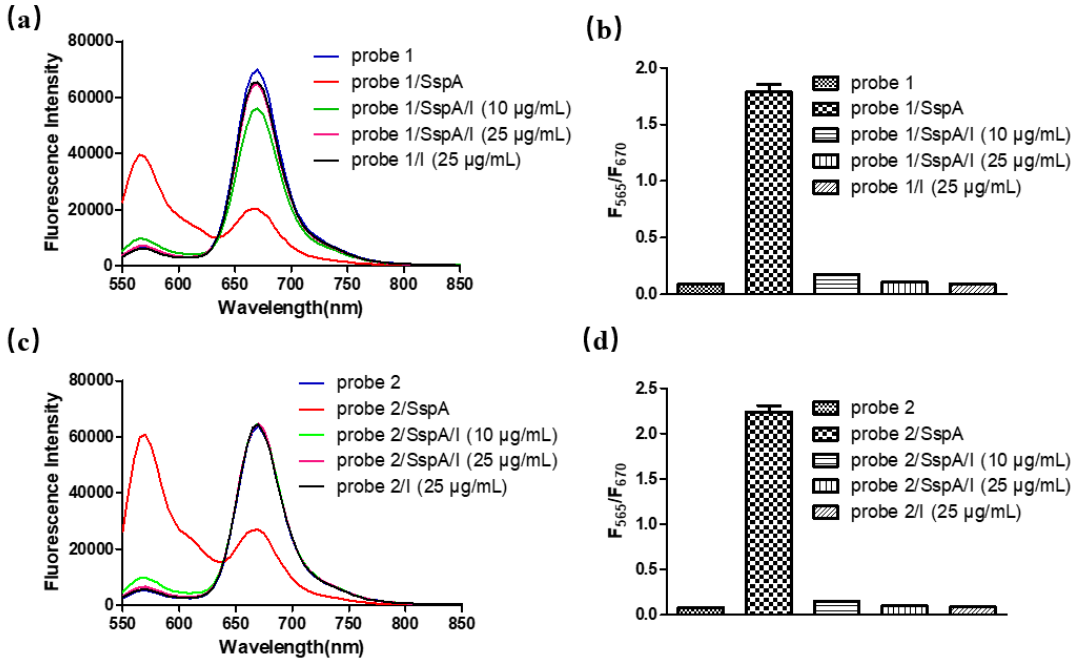


Fig. S3 Effect of inhibiter (3,4-DCI) on the fluorescence intensity of probe **1** and lipidated probe **2** reacted with SspA protease. (a, c) The UV-Visible and fluorescence spectra of probe **1**, probe **1**/SspA (10μg/mL); probe **1**/SspA /3, 4-DCI (10 μg/mL), probe **1**/SspA/3, 4-DCI (25 μg/mL), probe **1**/3, 4-DCI (25 μg/mL); probe **2**, probe **2**/SspA (10μg/mL), probe **2**/SspA /3, 4-DCI (10 μg/mL), probe **2**/SspA/3, 4-DCI (25 μg/mL), probe **2**/3, 4-DCI (25 μg/mL), respectively. (b, d) Ratiometric emission of probe **1** and probe **2** at 565 nm and 670 nm incubated with SspA protease and 3,4-DCI, respectively. λex = 510 nm.

Effects of pH and temperature

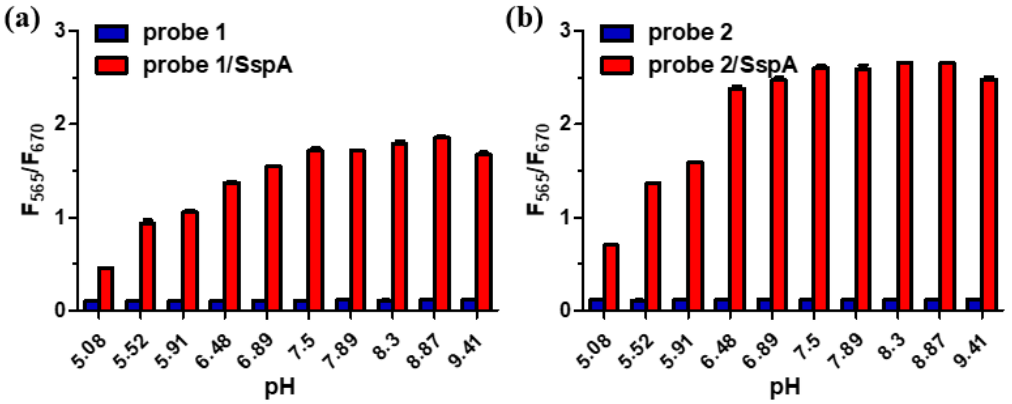


Fig. S4 Effect of pH on the fluorescence intensity of probe **1** and lipidated probe **2** (0.25 μM) reacted with SspA protease. (a) probe **1** (0.25 μM), probe **1** (0.25 μM) /SspA (10 μg/mL). (b) probe **2** (0.25 μM), probe **2** (0.25 μM)/SspA (10 μg/mL) in Na_2HPO_4-KH_2PO_4 buffer for 3h at 37 °C (λ_{ex} = 510 nm).

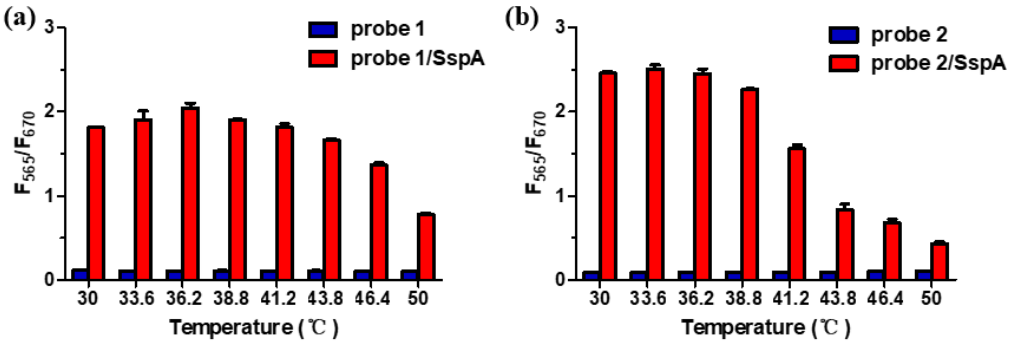


Fig. S5 Effect of temperature on the fluorescence intensity of probe **1** and lipidated probe **2** reacted with SspA. (a) probe **1** (0.25 μM), probe **1** (0.25 μM)/SspA (10 μg/mL). (b) probe **2** (0.25 μM), probe **2** (0.25 μM)/SspA(10 μg/mL) in 20 mM Tris-HCl (pH 7.8), 2 mM $CaCl_2$ buffer with 0.1% Caster oil for 3h (λex = 510 nm).

Apparent kinetic parameters for the enzymatic reaction of probe 1 and lipidated probe 2 [4]

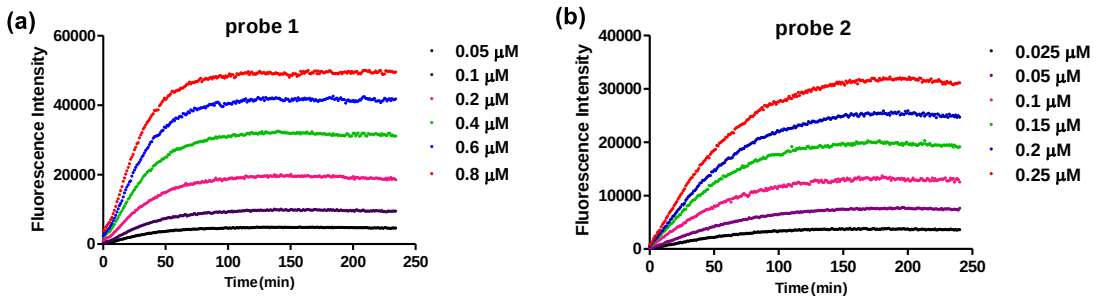


Fig. S6 Apparent kinetic parameters for the enzymatic reaction of probe **1** and lipidated probe **2**. (a) A plot of fluorescence intensity at 565 nm over an incubation time of 4h in the presence of different amounts of probe **1**: 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 μM; (b) probe **2**: 0.025, 0.05, 0.1, 0.15, 0.2, 0.25 μM. The measurements were performed in the 0.02 M phosphate buffer as co-solvent with 10 μg/mL SspA at 37 °C. λ_{ex} = 510 nm.

Lineweaver-Burk plot for the enzyme-catalyzed reaction of probe 1 and lipidated probe 2

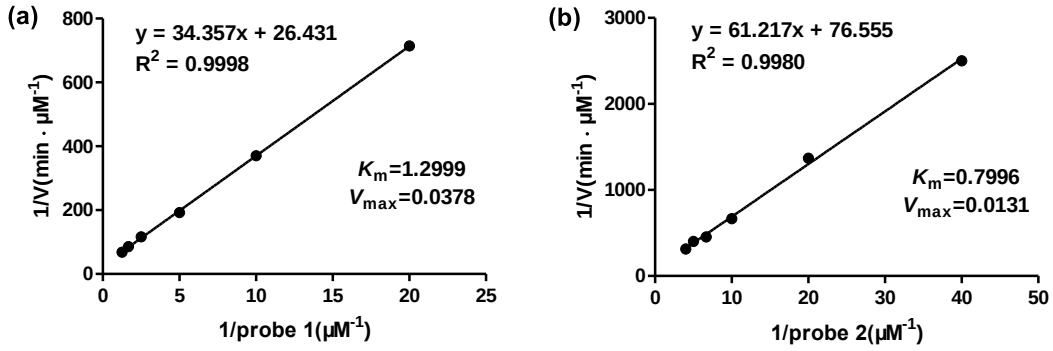


Fig. S7 Lineweaver-Burk plot for the enzyme-catalyzed reaction. The Michaelis-Menten equation was described as: $V = V_{max}$ [probe]/(K_m + [probe]), where V is the reaction rate, [probe] is the probe concentration (substrate), and K_m is the Michaelis constant. Conditions: 10 μg/mL SspA, 0.05-0.8 μM of the probe **1**, 0.025-0.25 μM of lipidated probe **2**. $\lambda_{ex}/\lambda_{em}$ = 510/565 nm. Points are fitted using a linear regression model (correlation coefficient: r^2 = 0.9998 and 0.9980, respectively).

HPLC analysis for the reaction system of probe 1

Probe **1** and the reaction product (product **A**, product **B**) of probe **1** with SspA protease were characterized by ESI-MS in Fig. S8.

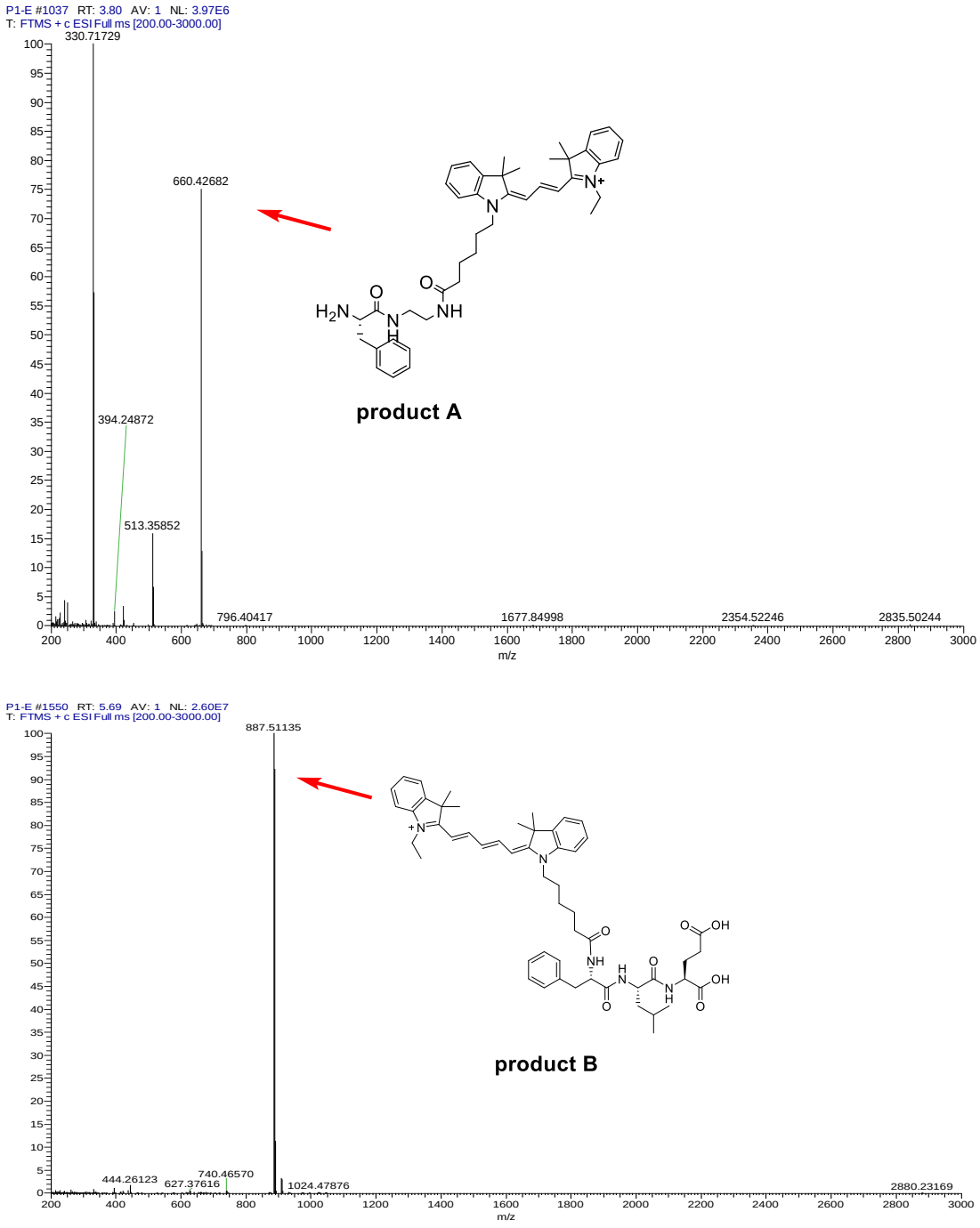


Fig. S8 ESI-MS spectrum of probe **1** reaction product **A**, product **B** and the reaction solution of probe **1** (25 μM) with SspA (10 μg/mL) after 3h, at 37 °C.

HPLC analysis for the reaction system of lipidated probe 2

Lipidated robe **2** and the reaction product (product **C**, product **D**) of lipidated probe **2** with SspA protease were characterized by ESI-MS in Fig. S9.

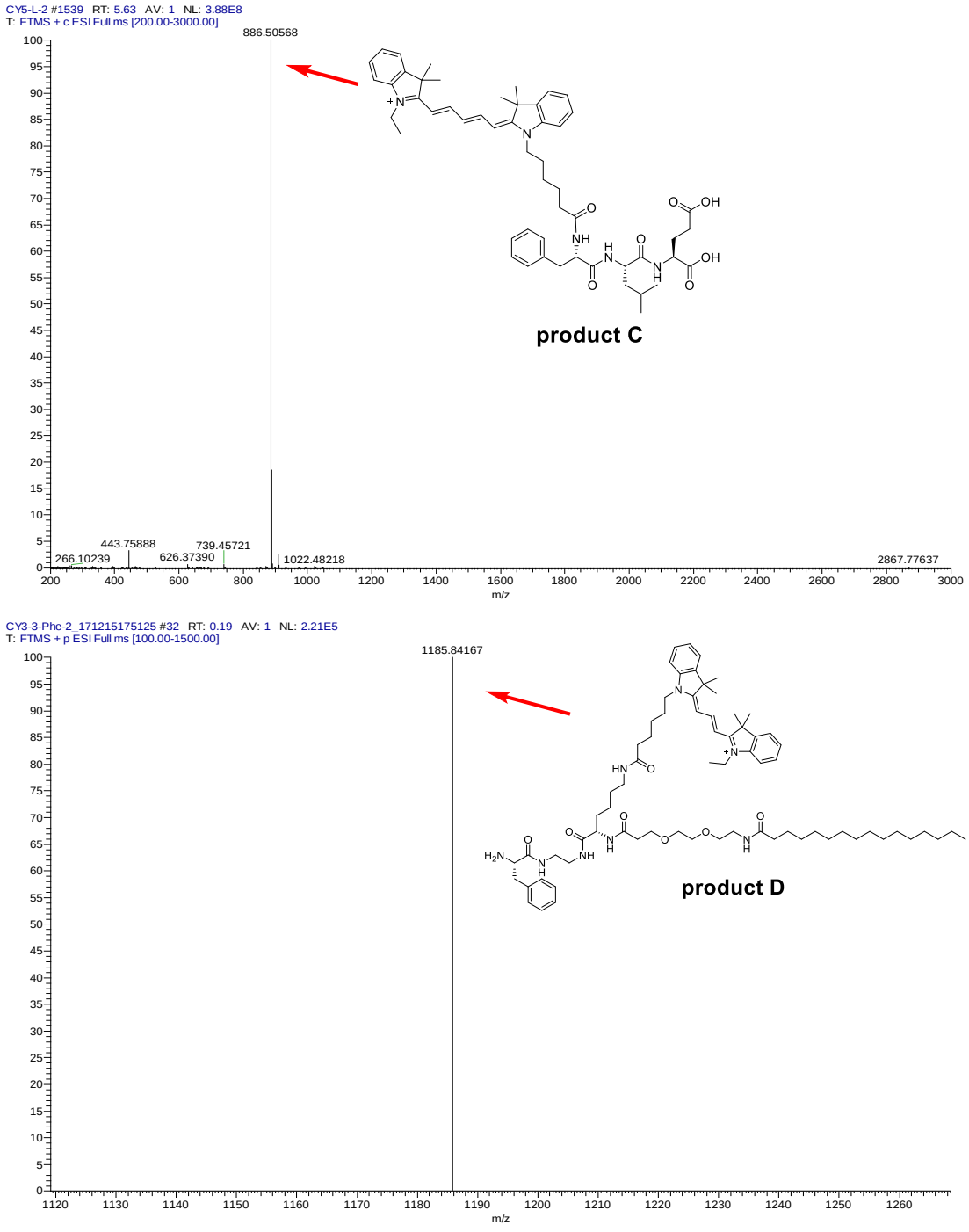


Fig. S9 ESI-MS spectrum of lipidated probe **2** reaction product **C**, product **D** and the reaction solution of lipidated probe **2** (25 μM) with SspA (10 μg/mL) after 3 h, at 37 °C.

SDS-PAGE assays [5]

To determine profiles of secreted proteins, the cell culture supernatant was condensed in a dialysis tube (MWCO10000) and solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reducing buffer. The samples were then subjected to SDS-PAGE. Following electrophoresis, the gels were shaken gently for 60 min at room temperature, in phosphate-buffered saline (PBS) containing 2.5% (vol•vol^{-1}) Triton X-100 (Sigma). Proteins were visualized by staining with Coo-massie blue.

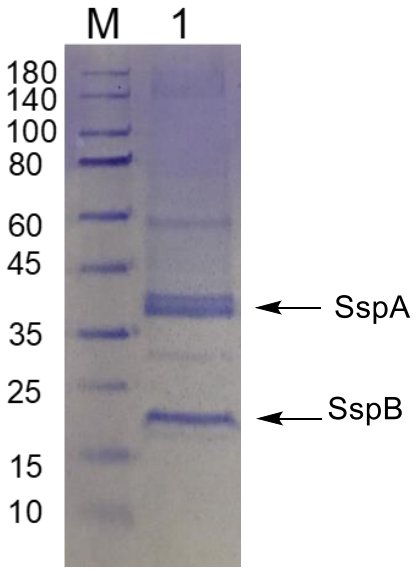


Fig. S10 SDS-PAGE of secreted proteins from *S. aureus* ATCC 25923. M: protein marker; 1: *S. aureus* ATCC 25923.

MIC assay [6]

1.0 mL aliquots of *aureus* (ATCC 25923) cells cultured in Tryptone Soya Broth (TSB) medium was resuspended in TSB at OD_{600} of 0.5, then further diluted to OD_{600} of 5×10^{-4}. Aliquots of this suspension (100 μL) were placed into a 96-well plate. The probe **1**, lipidated probe **2** and inhibitor (3,4-DCI) were diluted in DMSO and then added into the bacteria suspensions to give the desired concentration. The cultures were then added respective solutions and further incubated at 37 °C for 24 h. The wells containing the same number of cells but no compounds and the wells containing the same culture

solution but without bacterial cells were set as control groups. The plate was then read using a 96-well plate reader at 600 nm. Each concentration had triplicate values, and the whole experiment was done at three times and the MIC value was determined by taking the average of triplicate OD_{600} values for each concentration and plotting it against concentration. The data was then subjected to sigmoidal fitting. The MIC value was determined, as the point in the curve where the OD_{600} is similar to that of control having no bacteria (Table S2). Then 10 μL of supernatant from each well (3.125, 6.25, 12.5 μM) spread on the solid TSB agar plate and incubated at 37 ℃ for 8 h.

Table S2. Representative Minimal Inhibitory Concentrations (MIC) for *S.aureus* ATCC 25923

MIC (μM)	Compd.	probe **1**	probe **2**	3,4-DCI
	S.aureus	12.5	>50	>50

Fig. S11 Plate photographs for ATCC 25923 on TBS agar plates supplemented with 12.5 μM probe **1** (A1), 6.25 μM probe **1** (A2), 3.125 μM probe **1** (A3), 1.562 μM probe **1** (A4), 0.781 μM probe **1** (A5), 0.391 μM probe **1** (A6); 12.5 μM probe **2** (B1), 6.25 μM probe **2** (B2), 3.125 μM probe **2** (B3), 1.562 μM probe **2** (B4), 0.781 μM probe **2** (B5), 0.391 μM probe **2** (B6); 50 μM 3,4-DCI (C1), 25 μM 3,4-DCI (C2); positive control (only bacteria) (P); negative control (only TSB) (N).

Confocal imaging of bacteria treated with SspA inhibitor, probe 1 and probe 2

S. aureus cells (ATCC 25923) were cultured for 12 h in TSB at 37 °C. *S. aureus* cells cultured overnight in solution were harvested and washed twice with 20 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$ buffer. The washed cells were resuspended in 20 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$ buffer with an OD_{600} of 0.5 - 0.7. Then 500 μL aliquots were treated with 25 μM of SspA inhibitor, 3,4-dichloroisocoumarin (3,4-DCI) at 37 37 °C for 2 h. Then the cells with and without 3,4-DCI added with 5 μM of probe **1** and probe **2**, respectively. the cells were treated with 20 μg/mL of Hoechst 33258 at 37 °C for 30 min. Then a drop of the suspension was added into an 8-well chamber followed by covering with agarose pads. Fluorescence images were acquired with ZEISS LSM 710 Confocal Microscope (Nikon Eclipse TE2000-E, CFI Plan-Apochromat VC 63 × oil immersed optics), using a high pressure He-Ne lamp and diode laser for excitation (λ_{ex} = 510 nm and λ_{em} = 520-610 nm, λ_{em} = 610-750 nm; λ_{ex} = 405 nm and λ_{em} = 460 ± 25 nm).

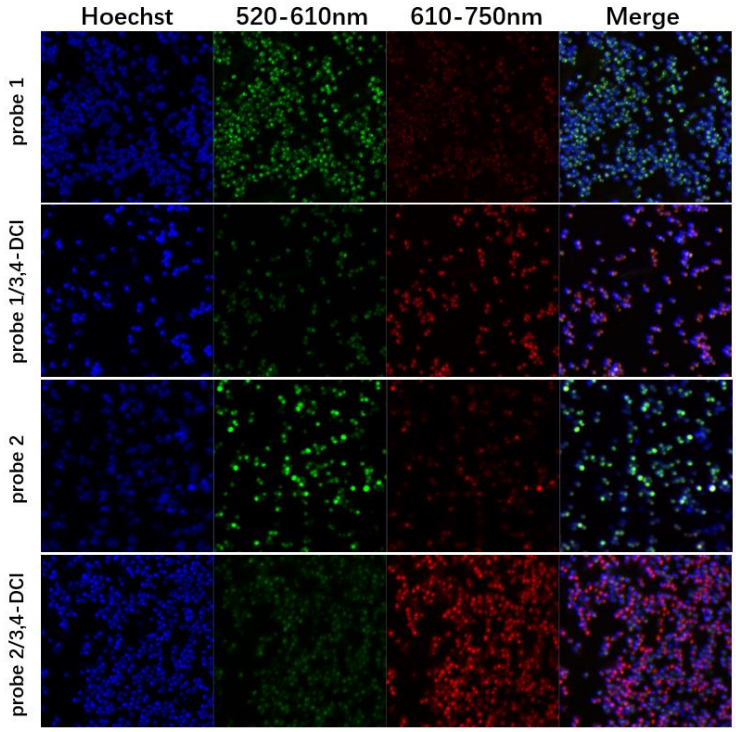
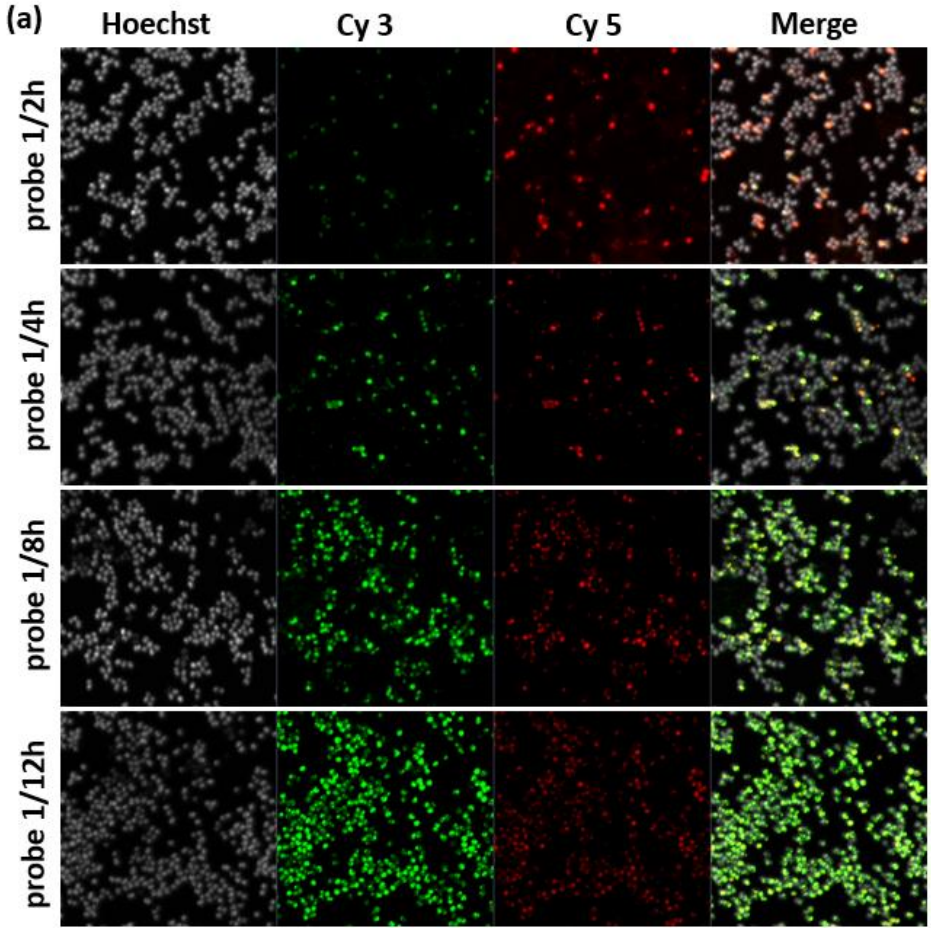


Fig. S12 Confocal microscopic images of *S. aureus* ATCC 25923 (a, b) incubated with probe **1** and (c, d) incubated with probe **2** for 12 h in the absence (a, c) or presence (b, d) of Ssp A inhibitor 3,4-DCI, respectively.

Confocal imaging of bacteria treated with probe 1 and probe 2 at different time

S. aureus cells (ATCC 25923) were cultured for 12 h in TSB at 37 °C. *S. aureus* cells cultured overnight in solution were harvested and washed twice with 20 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$ buffer. The washed cells were resuspended in 20 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$ buffer with an OD_{600} of 0.5 - 0.7. Then 500 μL aliquots were treated with 5 μM of probe **1** and probe **2**. After incubation at 37 °C for 2 h, 4h, 8h and 12h, the cells were treated with 20 μg/mL of Hoechst 33258 at 37 °C for 30 min. Then a drop of the suspension was added into an 8-well chamber followed by covering with agarose pads. Fluorescence images were acquired with ZEISS LSM 710 Confocal Microscope (Nikon Eclipse TE2000-E, CFI Plan-Apochromat VC 63 × oil immersed optics), using a high pressure He-Ne lamp and diode laser for excitation (λ_{ex} = 510 nm and λ_{em} = 520-610 nm, λ_{em} = 610-750 nm; λ_{ex} = 405 nm and λ_{em} = 460 ± 25 nm).



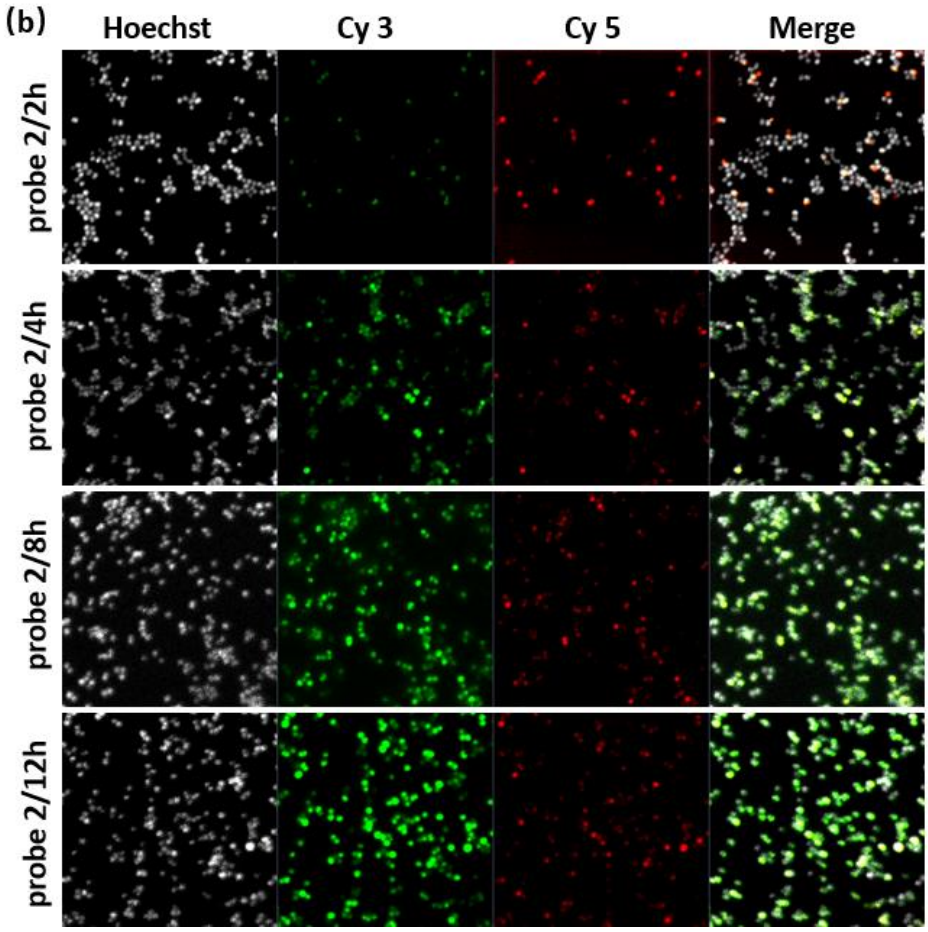


Fig. S13 Confocal microscopic images of *S. aureus* (a, b) incubated with probe **1** and probe **2** for 0.5 h, 1 h, 2 h, 12 h, respectively. green: Cy 3 520-610 nm signals; red: Cy 5 610-750 nm signals.

Confocal imaging of bacteria treated with probe 2

ESKAPE panel (comprising two Gram-positive species *E. faecium* (ATCC 29212) and *S. aureus* (ATCC 25923) and four Gram-negative species *K. pneumoniae* (ATCC 700603), *A. baumannii* (ATCC 19606), *P. aeruginosa* (ATCC 27853), *E. cloacae* (ATCC 13047)), and a SspA non-expression Gram-positive bacteria, *M. luteus* (ATCC 4698), were cultured for 12 h in respective media at 37 °C. Bacterial strains cultured overnight in respective solution were harvested and washed twice with 20 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$ buffer. The washed cells were resuspended in 20 mM Tris-HCl (pH 7.8), 2 mM $CaCl_2$ buffer with an OD_{600} of 0.5 - 0.7. Then 200 μL aliquots were treated with 5 μM of probe **2** incubation at 37 °C for 12 h, and treated with 20 μg/mL of Hoechst 33258 at 37 °C for 30 min. Then a drop of the suspension was added into an 8-well chamber followed by covering with agarose pads. Fluorescence images were acquired with Leica TCS SP8 X Confocal Microscope. (HC PL Apo 63 × oil immersed optics) (λ_{ex} = 510 nm and λ_{em} = 520-610 nm, λ_{em} = 610-750 nm; λ_{ex} = 405nm and λ_{em} = 460 ± 25 nm).

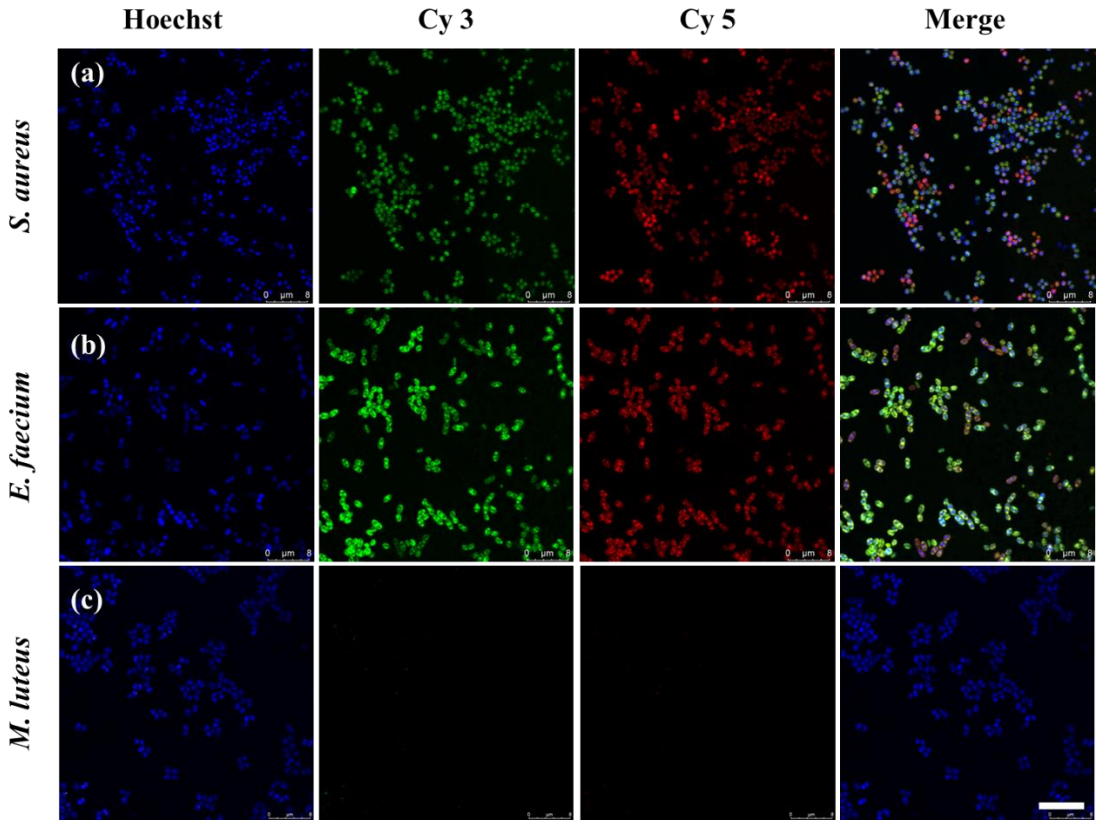


Fig. S14 (a-c) Fluorescence images of Gram-positive bacterial cells staining with probe **2**. All the bacterial cells were incubated with 5 μM probe **2** at 37 °C in 20 mM Tris-HCl (pH 7.8), 2 mM $CaCl_2$ buffer for 4 h prior to fluorescence imaging. λ_{ex} = 510 nm, λ_{em} = 520-610 nm (Cy 3 signal) and λ_{em} = 610-750 nm (Cy 5 signal); λ_{ex} = 405 nm and λ_{em} = 460 ± 25 nm (Hoechst signal). Scale bar = 8 μm.

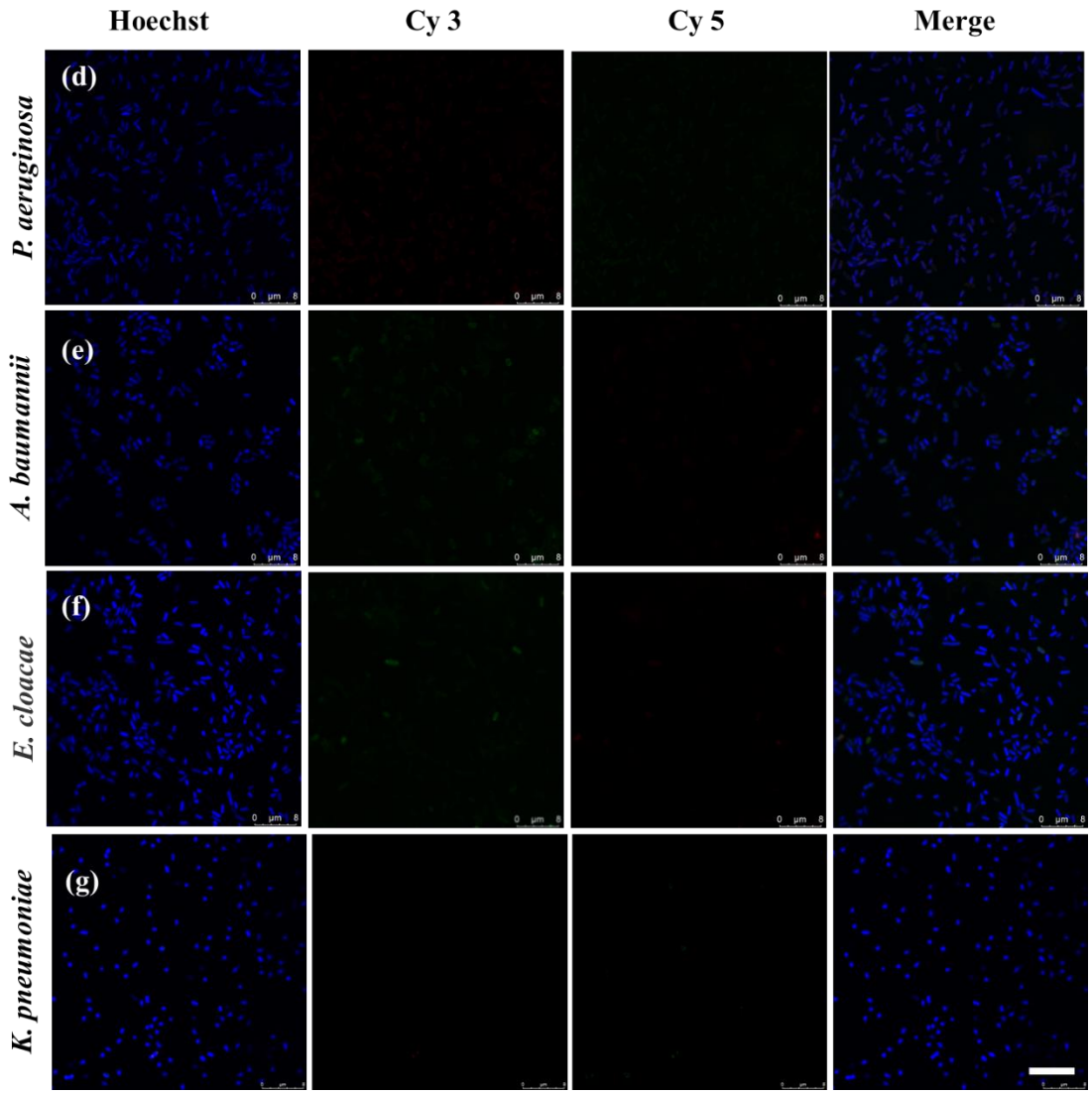


Fig. S14 (d-g) Fluorescence images of Gram-negetive bacterial cells staining with probe **2**. All the bacterial cells were incubated with 5 μM probe **2** at 37 °C in 20 mM Tris-HCl (pH 7.8), 2 mM $CaCl_2$ buffer for 4 h prior to fluorescence imaging. λ_{ex} = 510 nm, λ_{em} = 520-610 nm (Cy 3 signal) and λ_{em} = 610-750 nm (Cy 5 signal); λ_{ex} = 405 nm and λ_{em} = 460 ± 25 nm (Hoechst signal). Scale bar = 8 μm.

Confocal imaging of fungi cells treated with probe 1 and lipidated probe 2

Pichia pastoris GS115 and *Saccharomyces cerevisiae* INVSC1 cells were cultured for 12 h in respective media at 30 °C. Fungi cells cultured overnight in respective solution were harvested and washed twice with 20 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$ buffer. The washed cells were resuspended in PBS with an OD_{600} of 0.5-0.7. Then 200 µL aliquots were treated with 5 µM of probe **1** and probe **2**. After incubation at 30 °C for 4 h, and treated with 20 µg/mL of Hoechst 33258 at 37 °C for 30 min. Then a drop of the suspension was added into an 8-well chamber followed by covering with agarose pads. Fluorescence images were acquired with ZEISS LSM 710 Confocal Microscope (Nikon Eclipse TE2000-E, CFI Plan-Apochromat VC 63 × oil immersed optics), using a high pressure He-Ne lamp and diode laser for excitation (λ_{ex} = 510 nm and λ_{em} = 520-610 nm, λ_{em} = 610-750 nm; λ_{ex} = 405 nm and λ_{em} = 460 ± 25 nm).

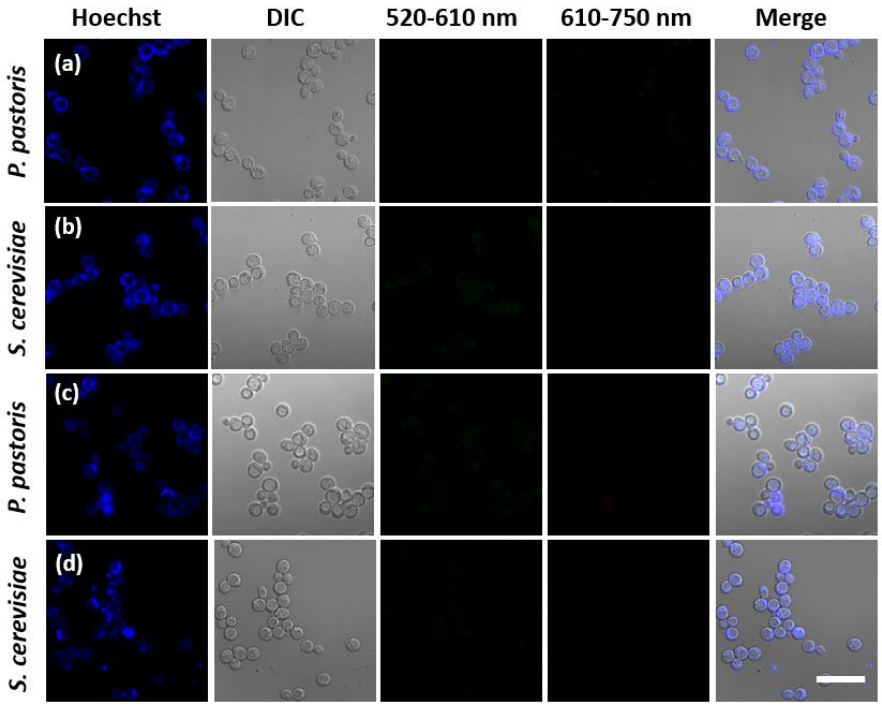


Fig. S15 (a-b) Fluorescence images of cells staining with probe **1**, (c-d) Fluorescence images of cells staining with probe **2**. All the fungi cells were incubated with 5 µM probe **1** and probe **2** at 37 °C in 20 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$ buffer for 4 h prior to fluorescence imaging. λ_{ex} = 510 nm and λ_{em} = 520-610 nm, λ_{em} = 610-750 nm; λ_{ex} = 405 nm and λ_{em} = 460 ± 25 nm. Scale bar = 20 µm.

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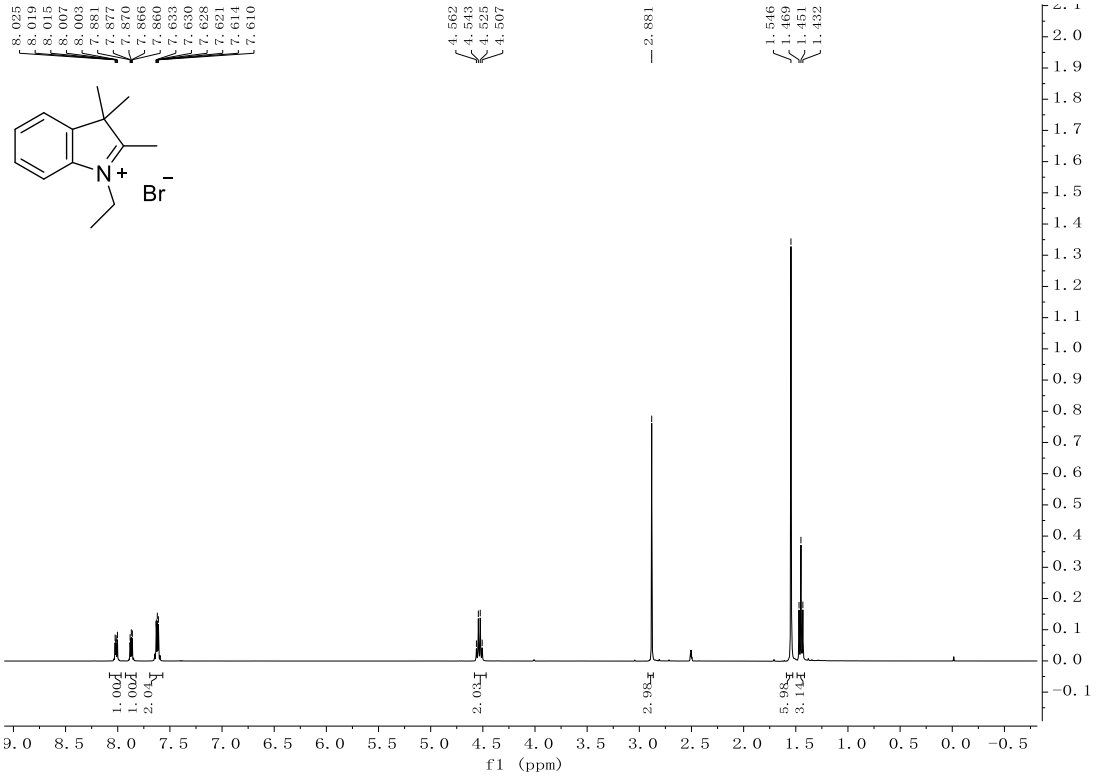
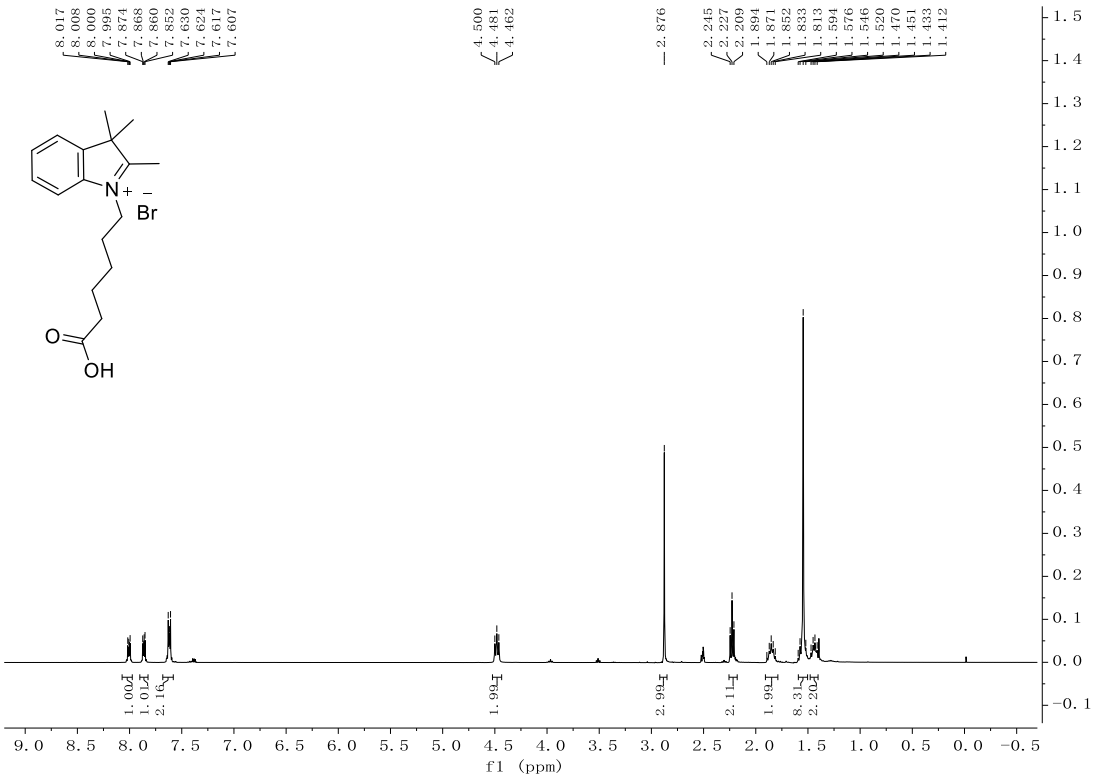
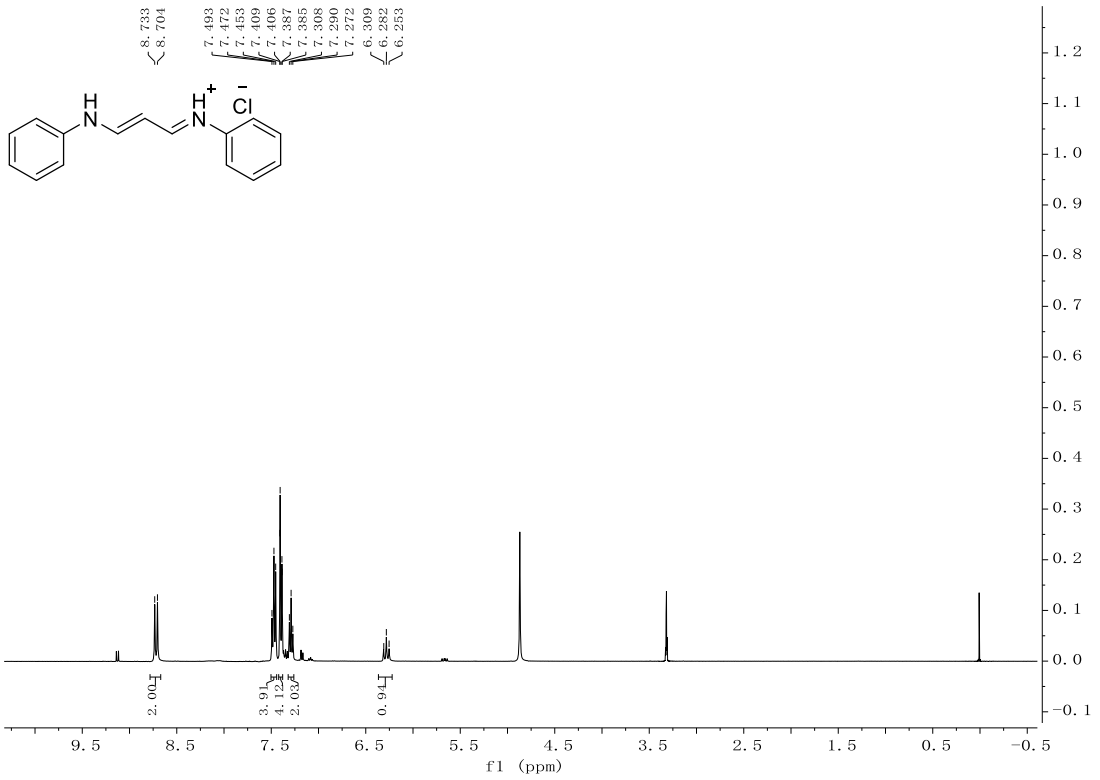
Copies of ^{1}H NMR and ^{13}C NMR spectrum of compounds



^{1}H NMR (400 MHz, DMSO-*d*6) of compound **2**



^{1}H NMR (400 MHz, DMSO-*d*6) of compound **3**



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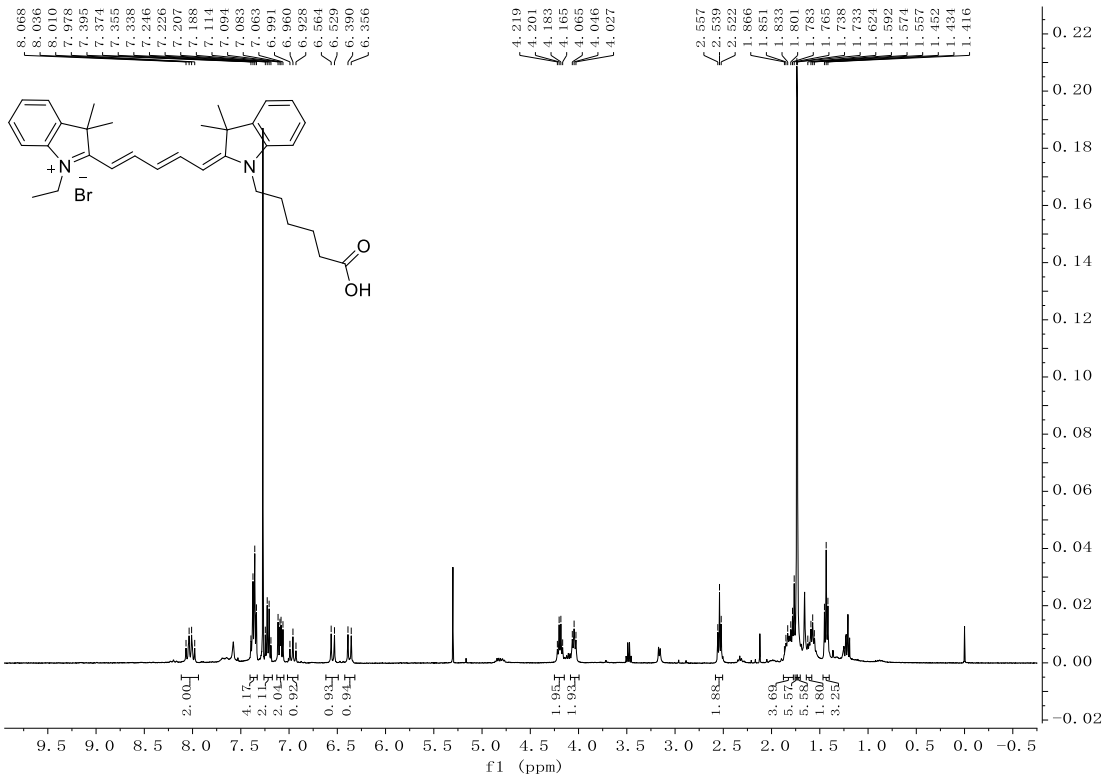
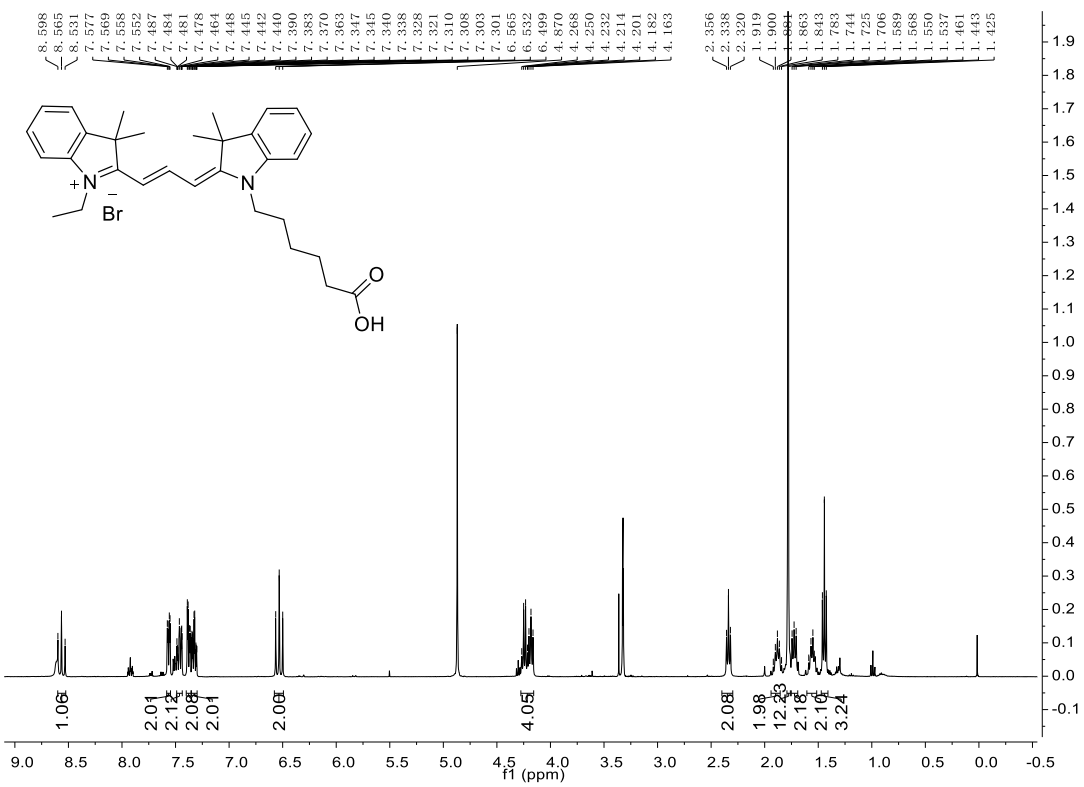
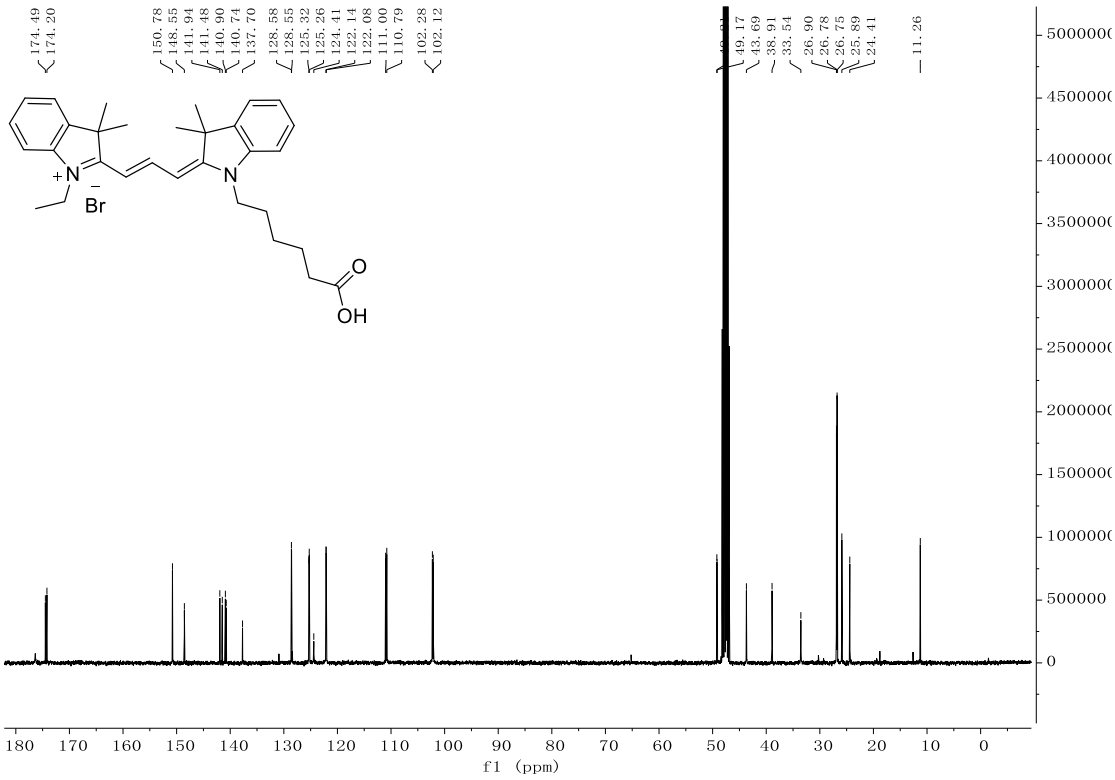


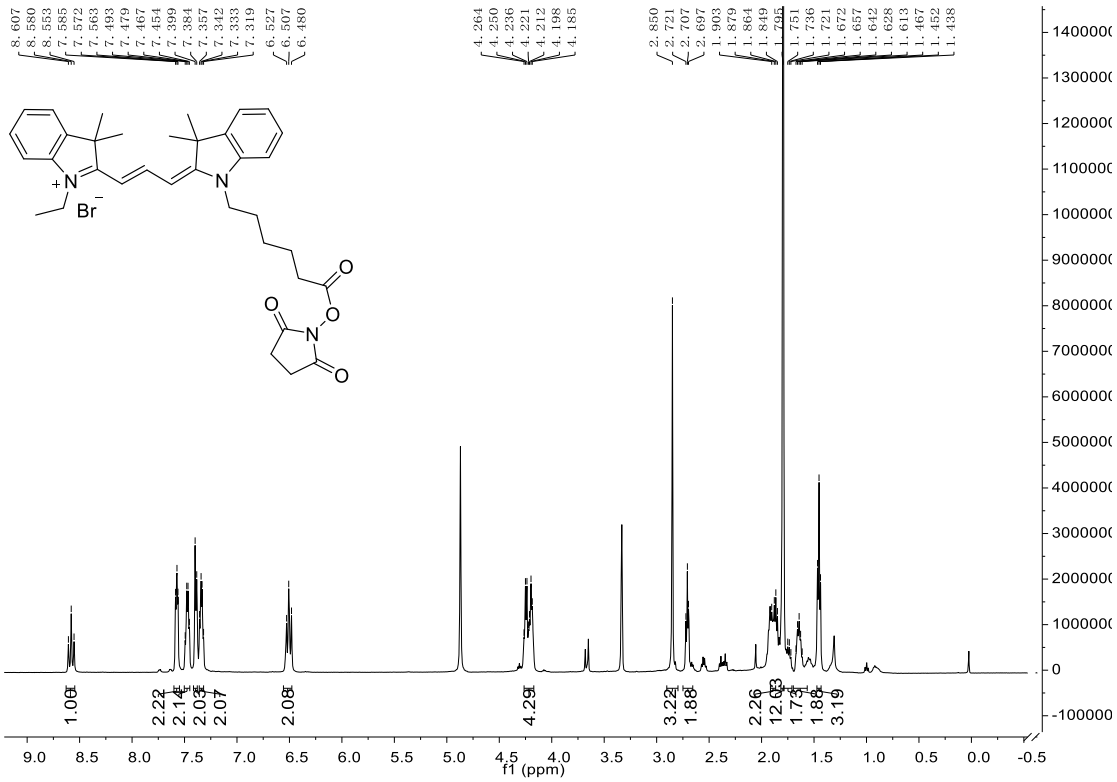
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **6**



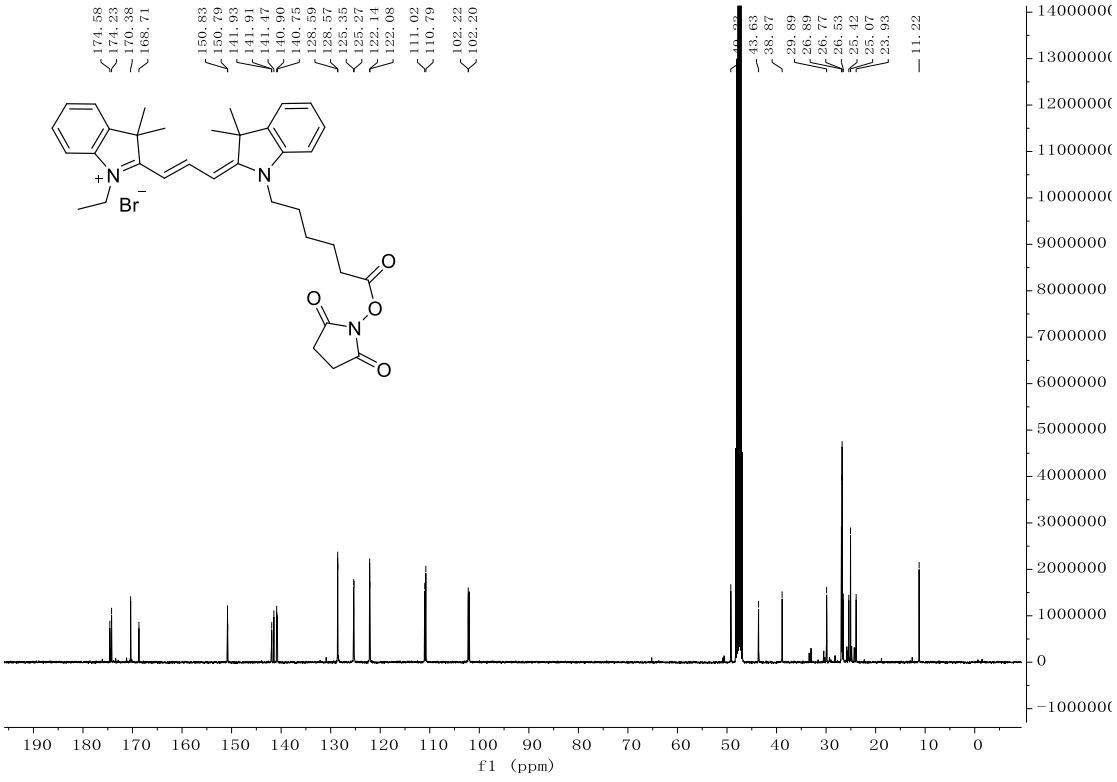
^{1}H NMR (400 MHz, Methanol-*d*4) of compound **7**



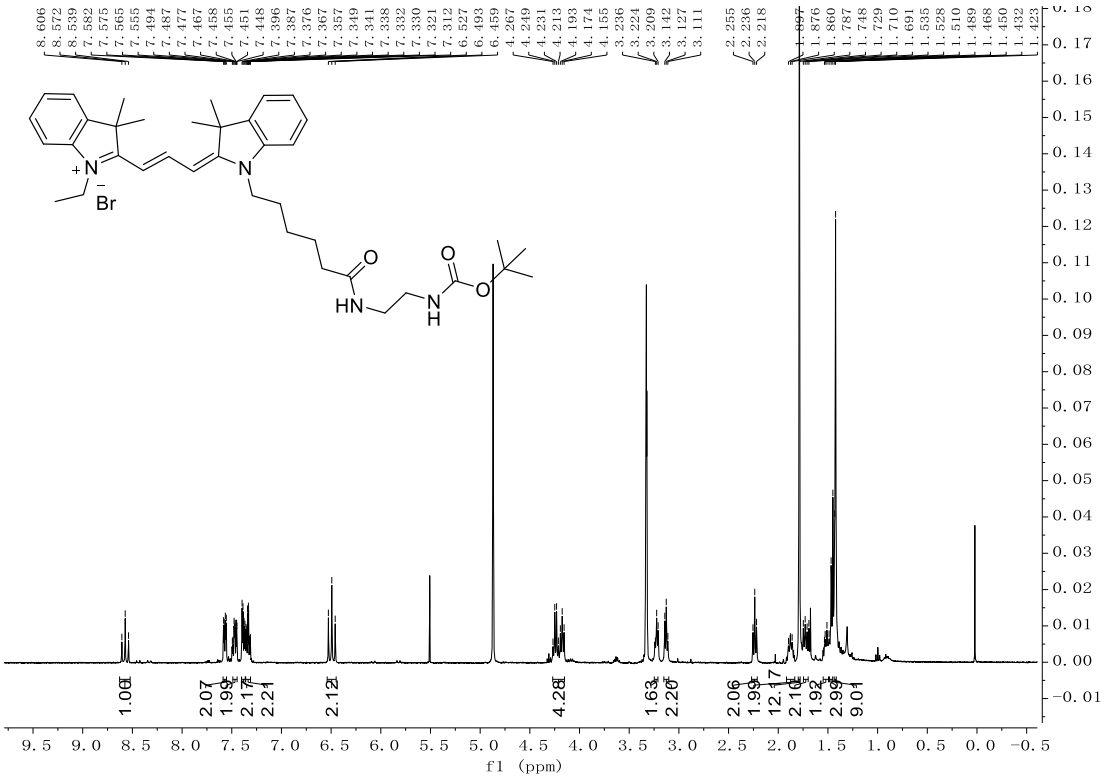
^{13}C NMR (100 MHz, Methanol-*d*4) of compound **7**



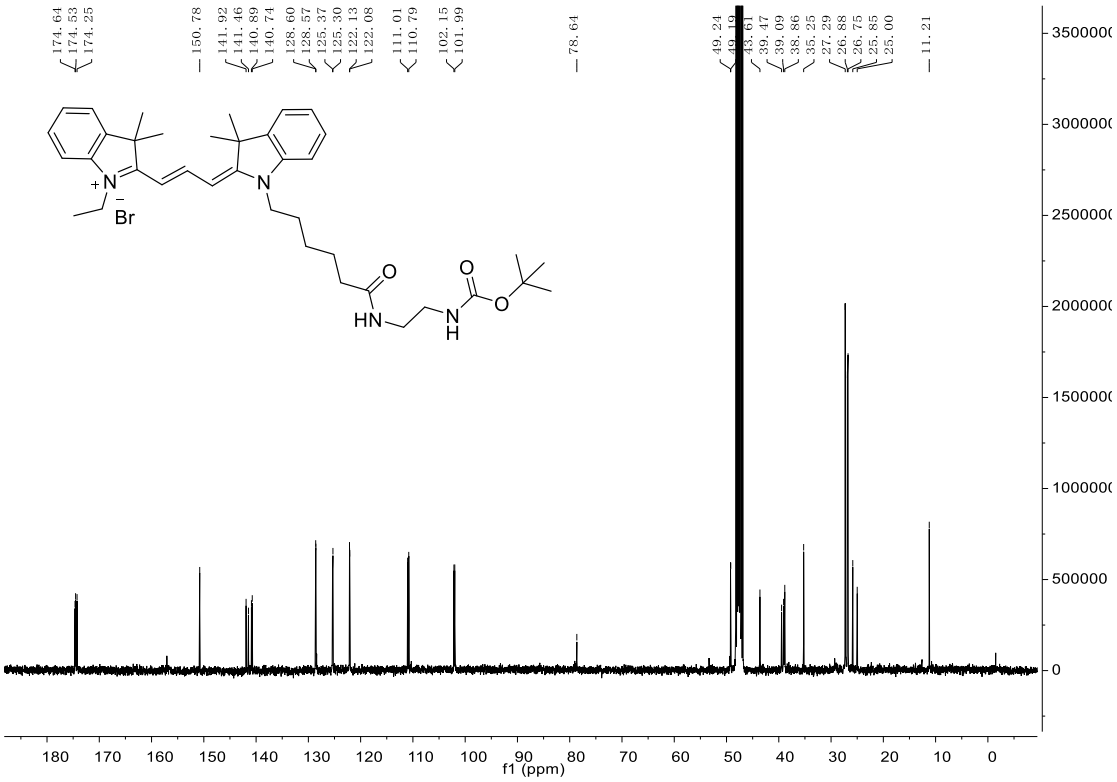
^{1}H NMR (400 MHz, Methanol-*d*4) of compound **8**



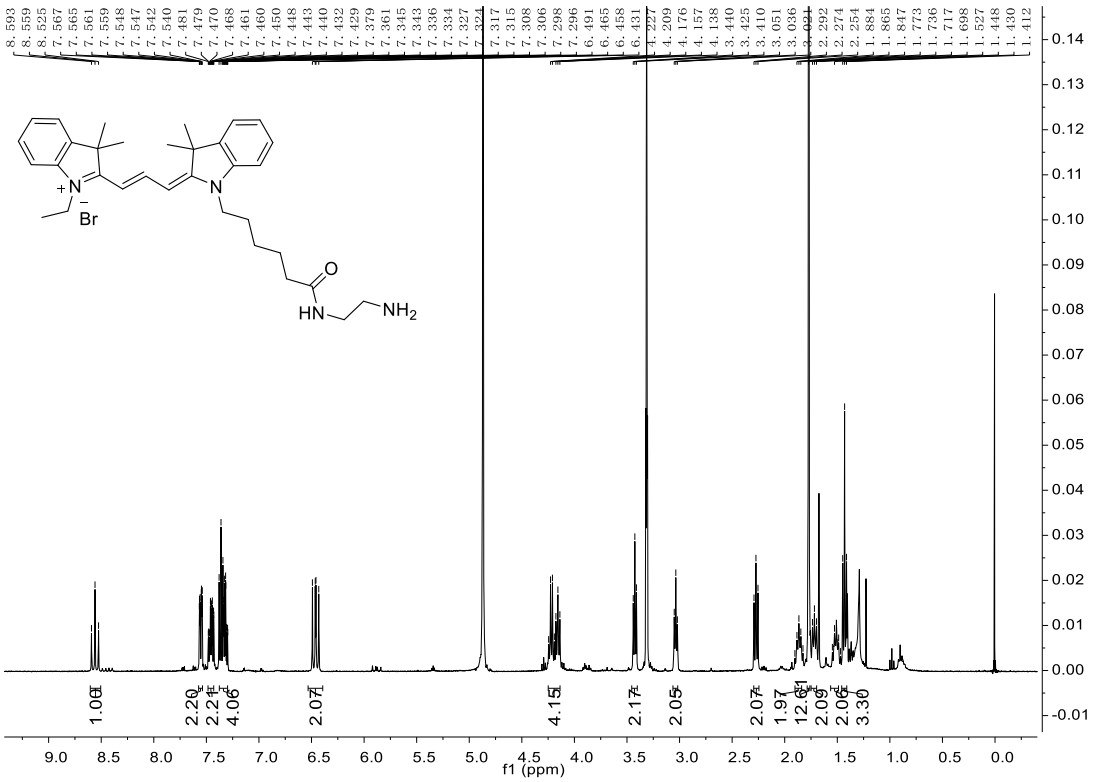
^{13}C NMR (100 MHz, Methanol-*d*4) of compound **8**



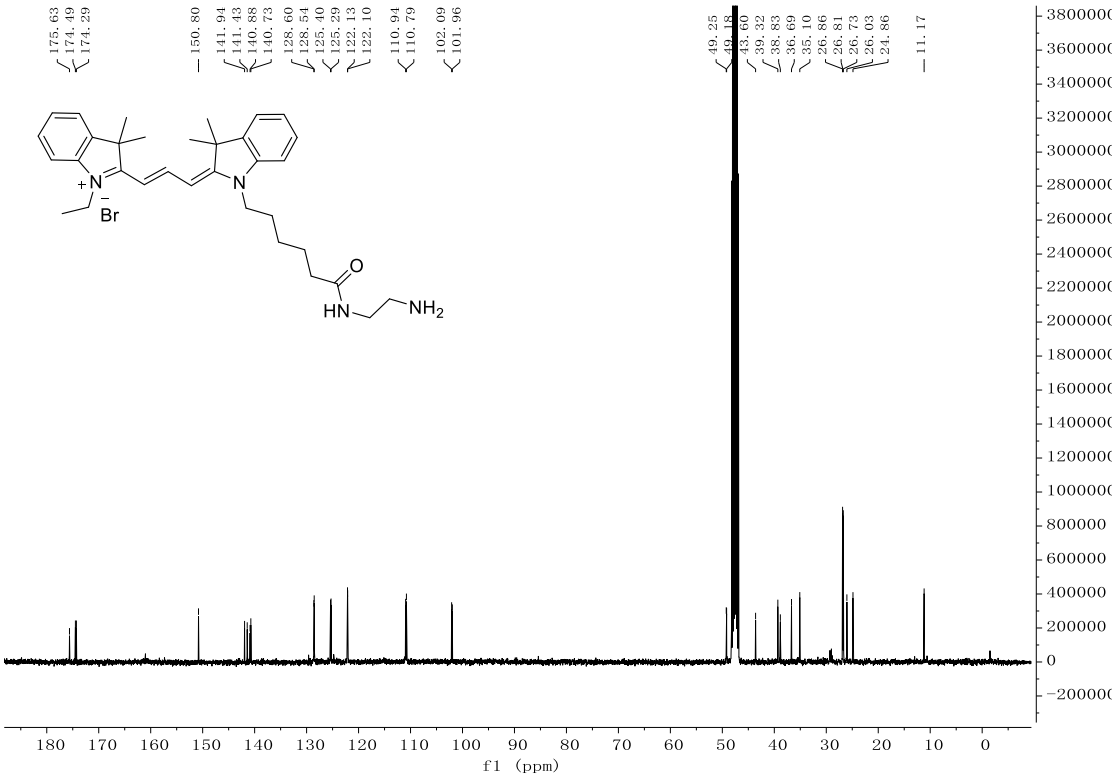
^{1}H NMR (400 MHz, Methanol-*d*4) of compound **9**



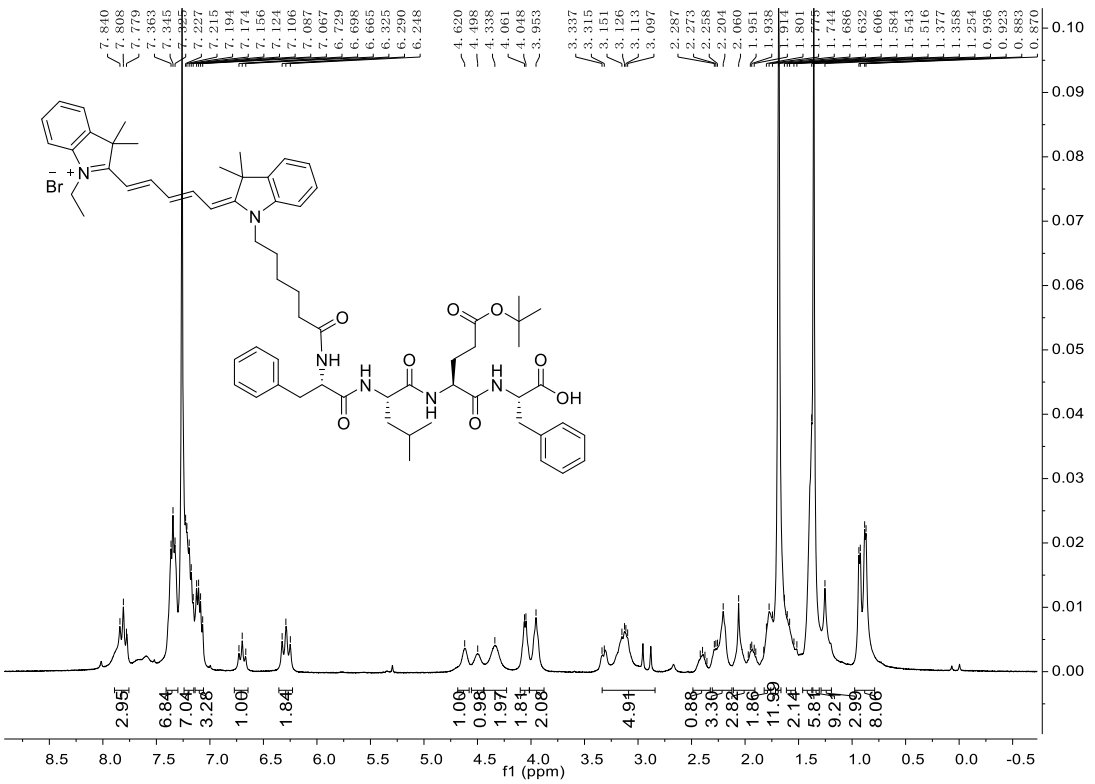
^{13}C NMR (100 MHz, Methanol-*d*4) of compound **9**



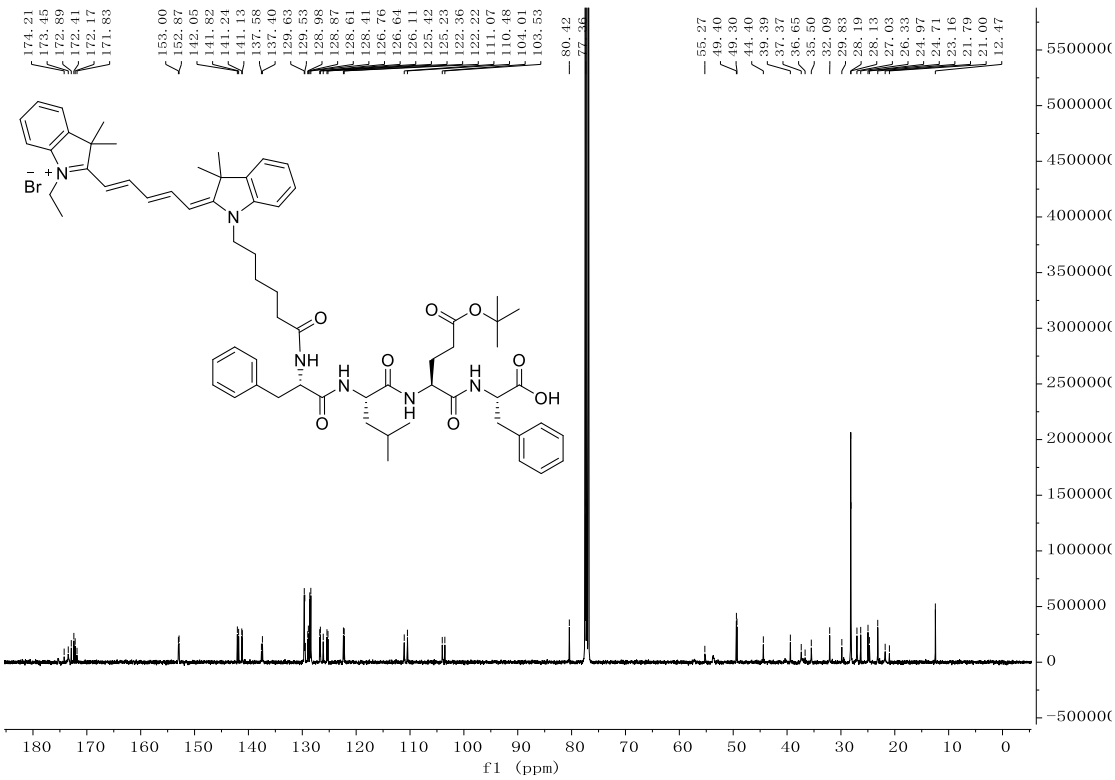
^{1}H NMR (400 MHz, Methanol-d_4) of compound **10**



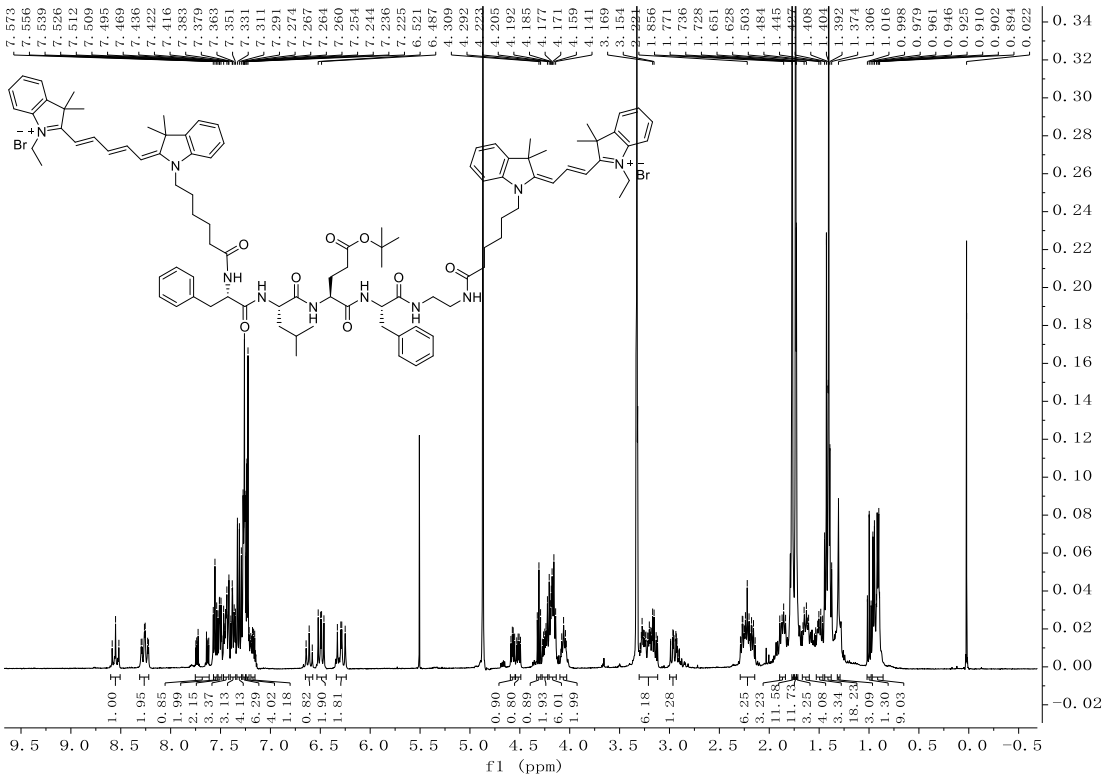
^{13}C NMR (100 MHz, Methanol-d_4) of compound **10**



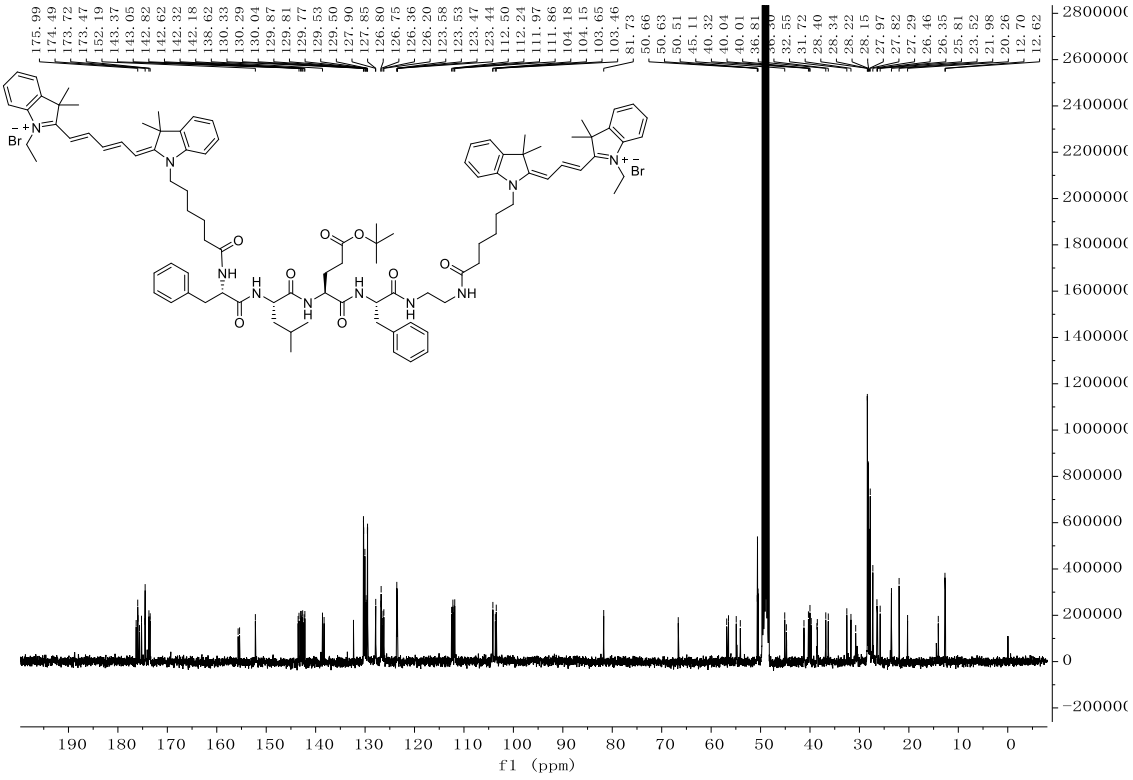
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **12**



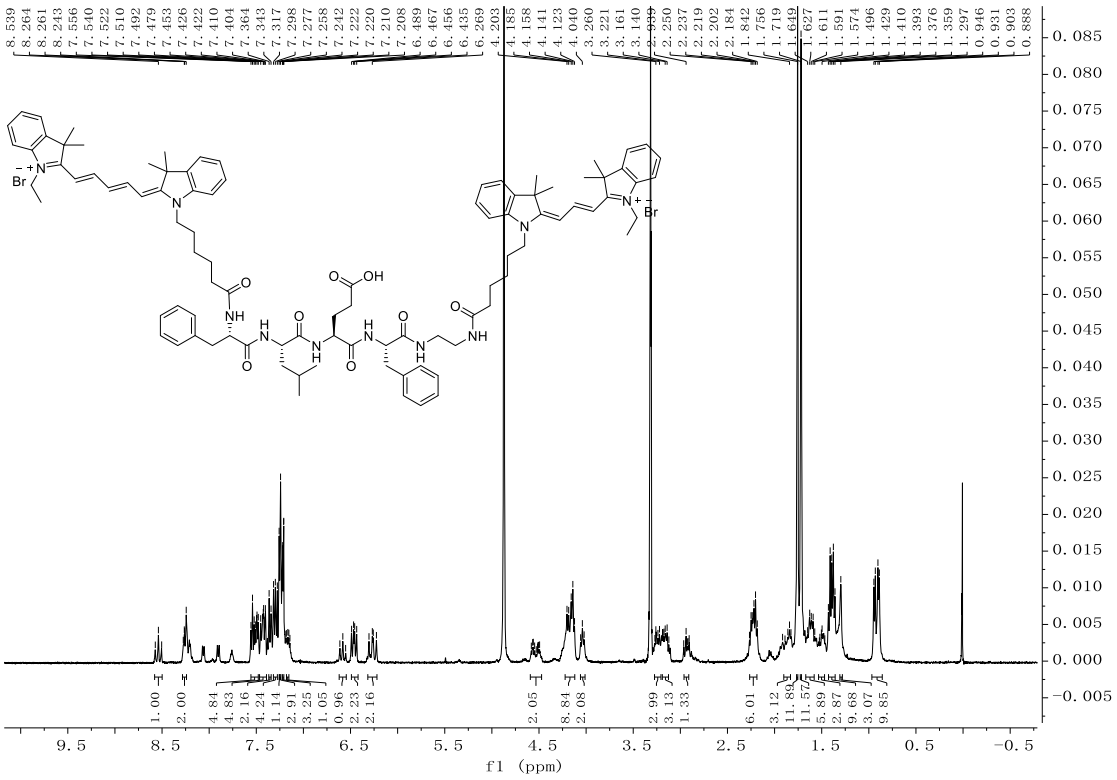
^{13}C NMR (100 MHz, Chloroform-*d*) of compound **12**



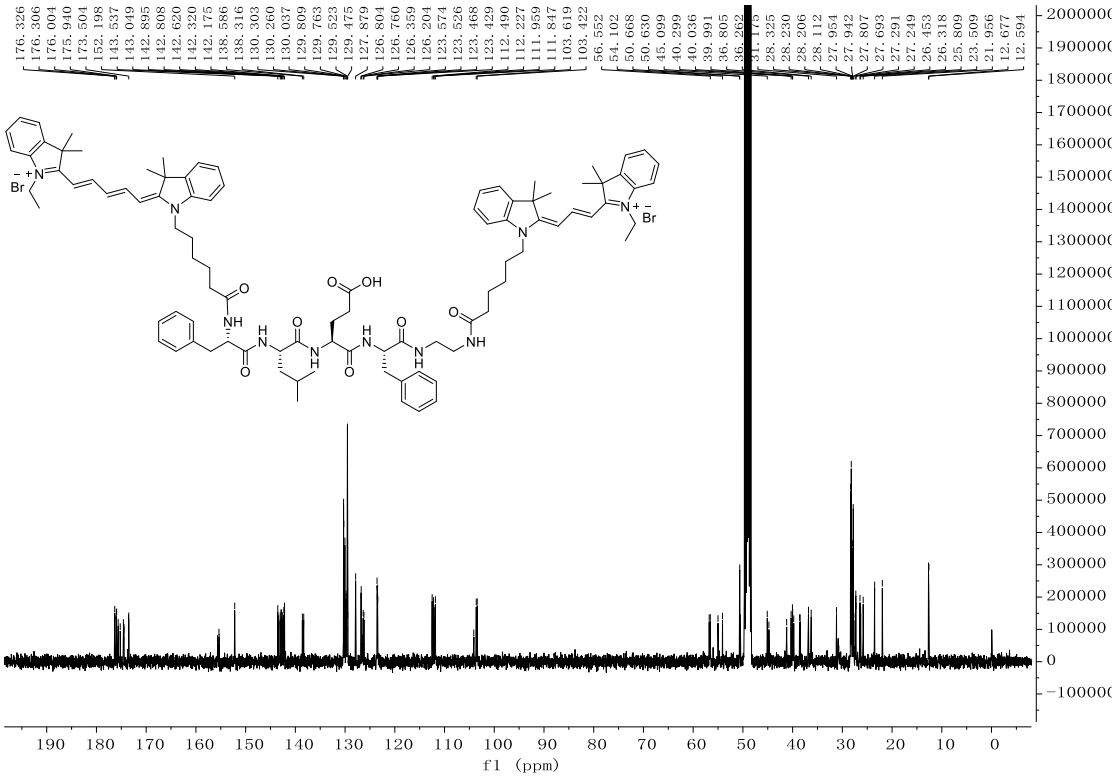
^{1}H NMR (400 MHz, Methanol-*d*4) of compound **13**



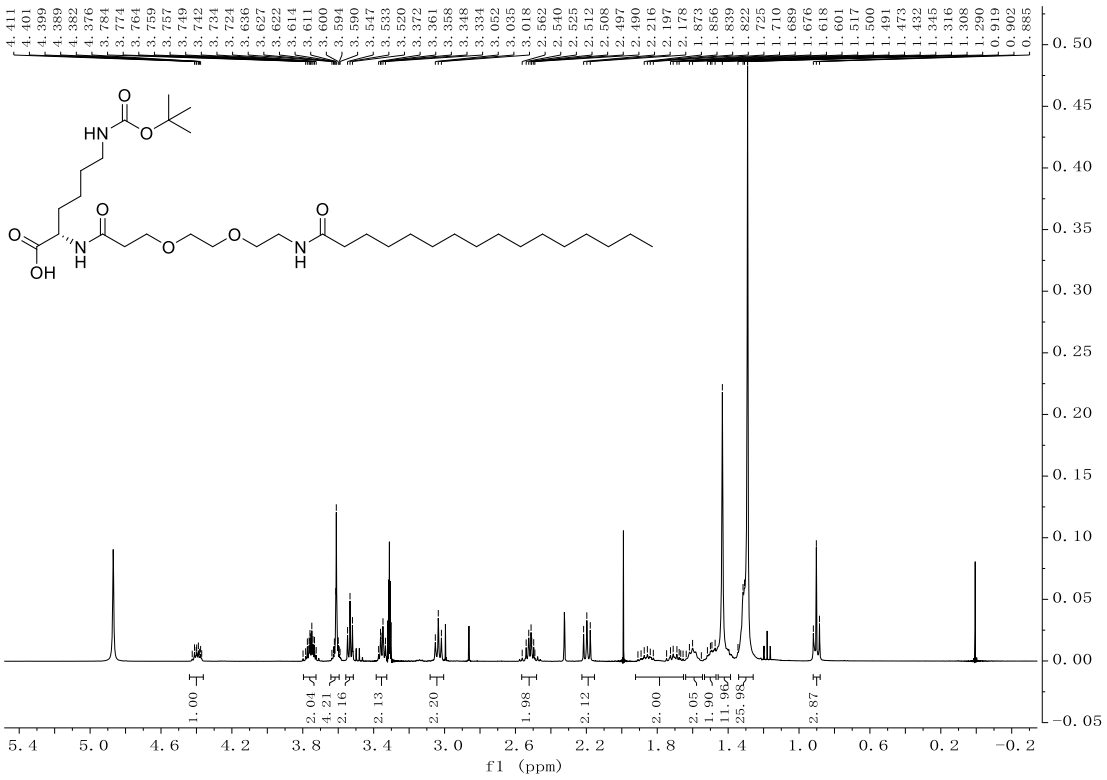
^{13}C NMR (100 MHz, Methanol-*d*4) of compound **13**



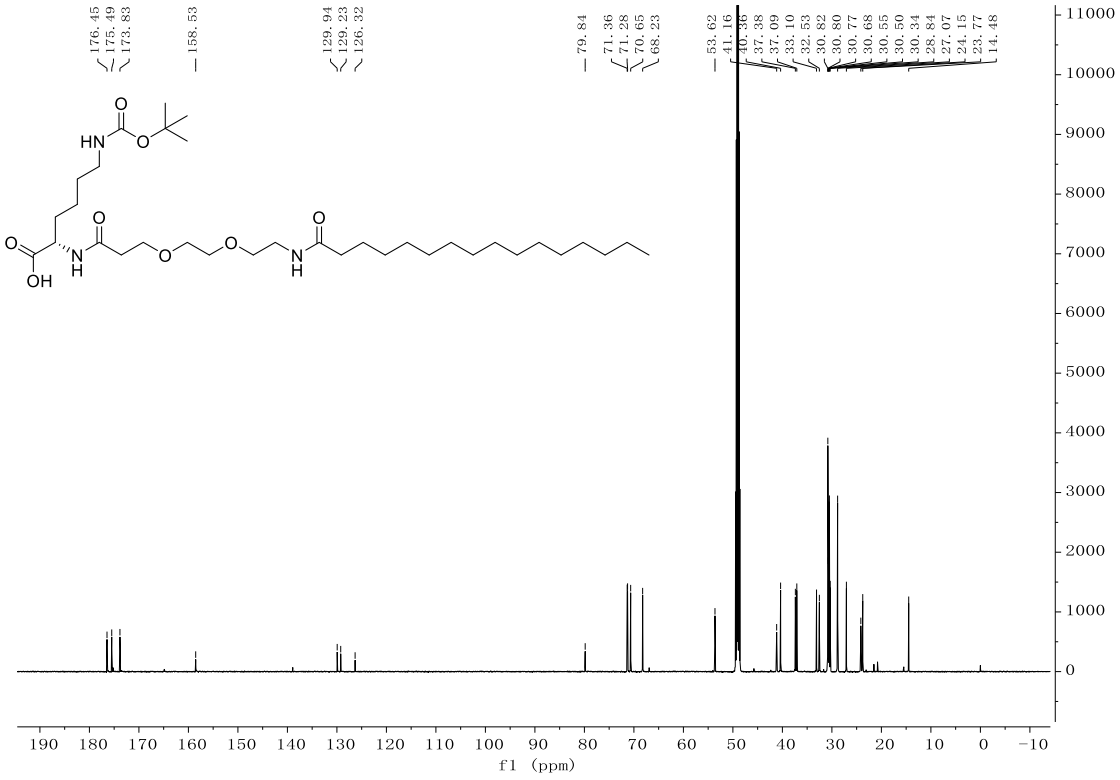
^{1}H NMR (400 MHz, Methanol-*d*4) of compound **14**



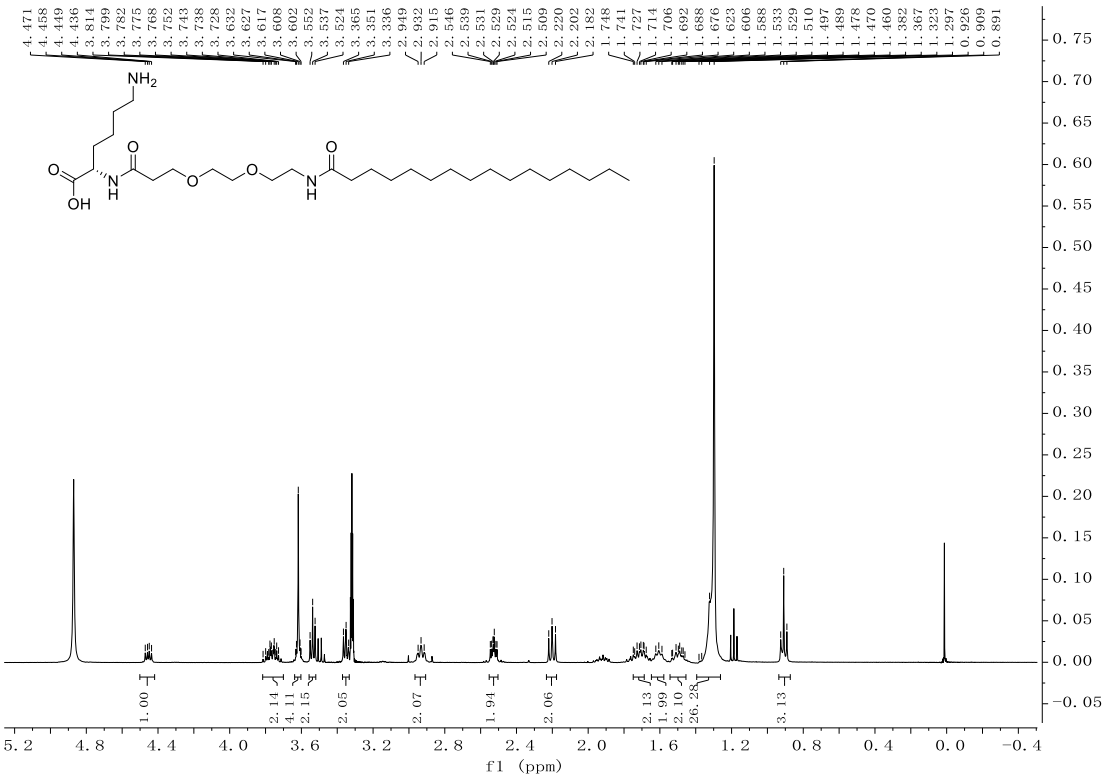
^{13}C NMR (100 MHz, Methanol-*d*4) of compound **14**



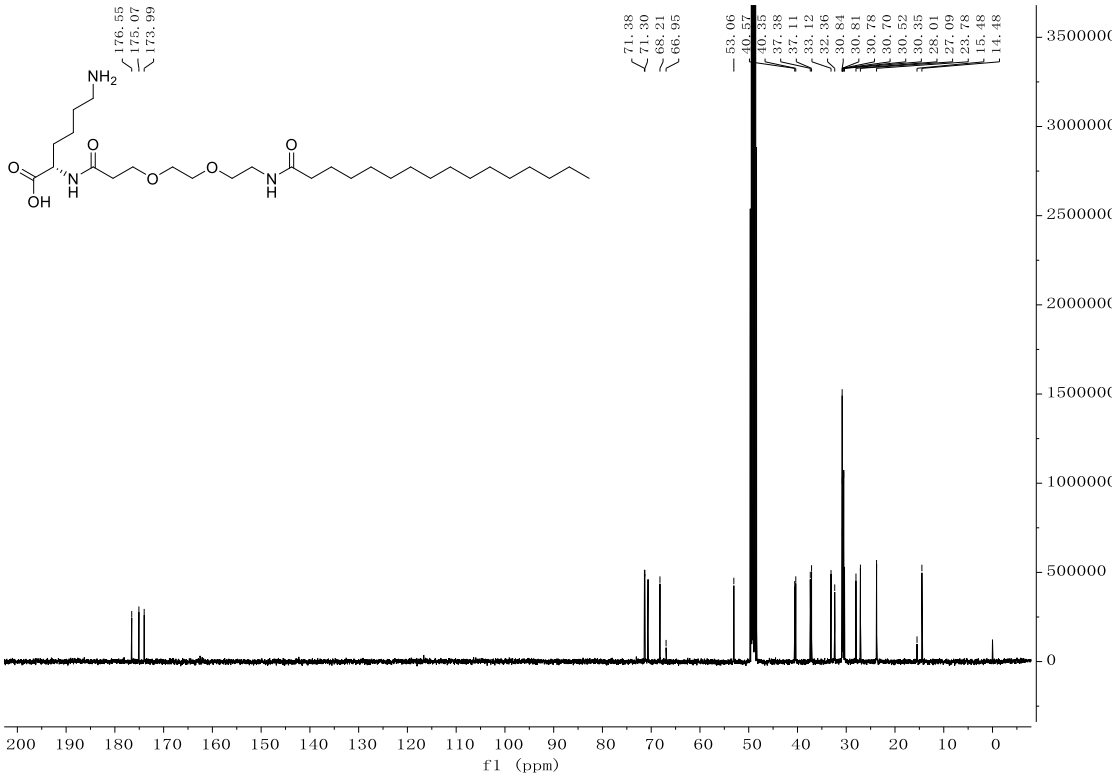
^{1}H NMR (400 MHz, Methanol-*d*4) of compound **15**



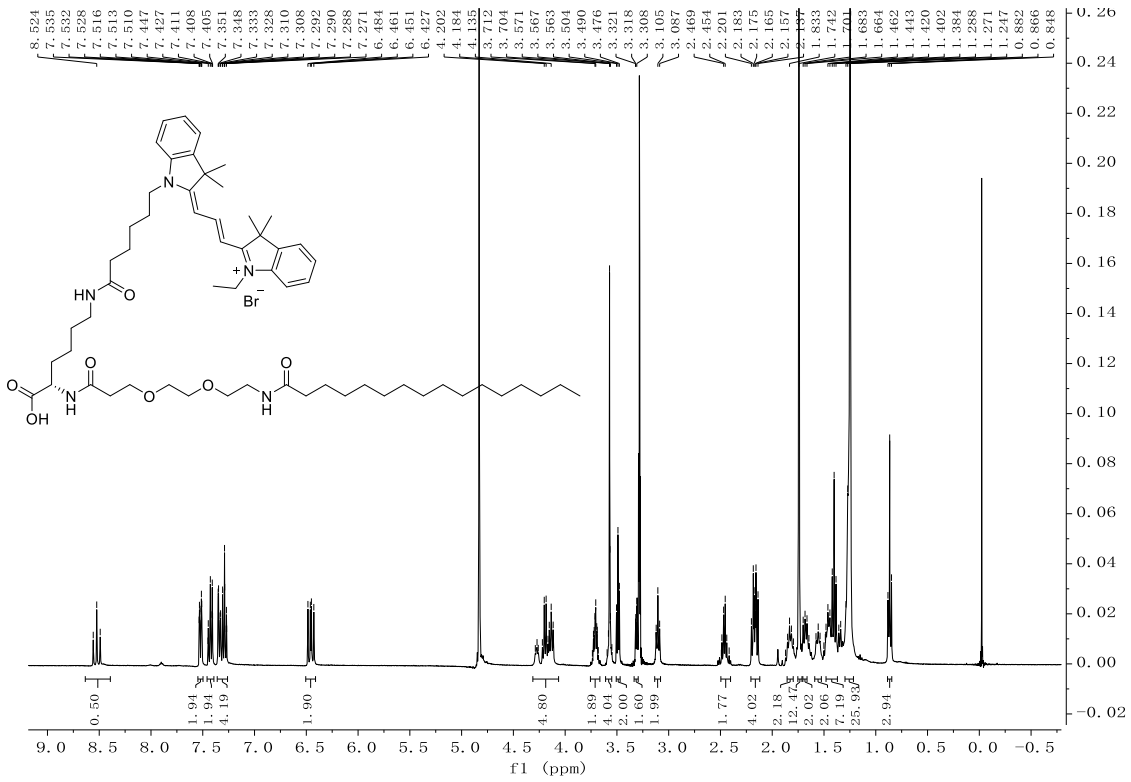
^{13}C NMR (100 MHz, Methanol-*d*4) of compound **15**



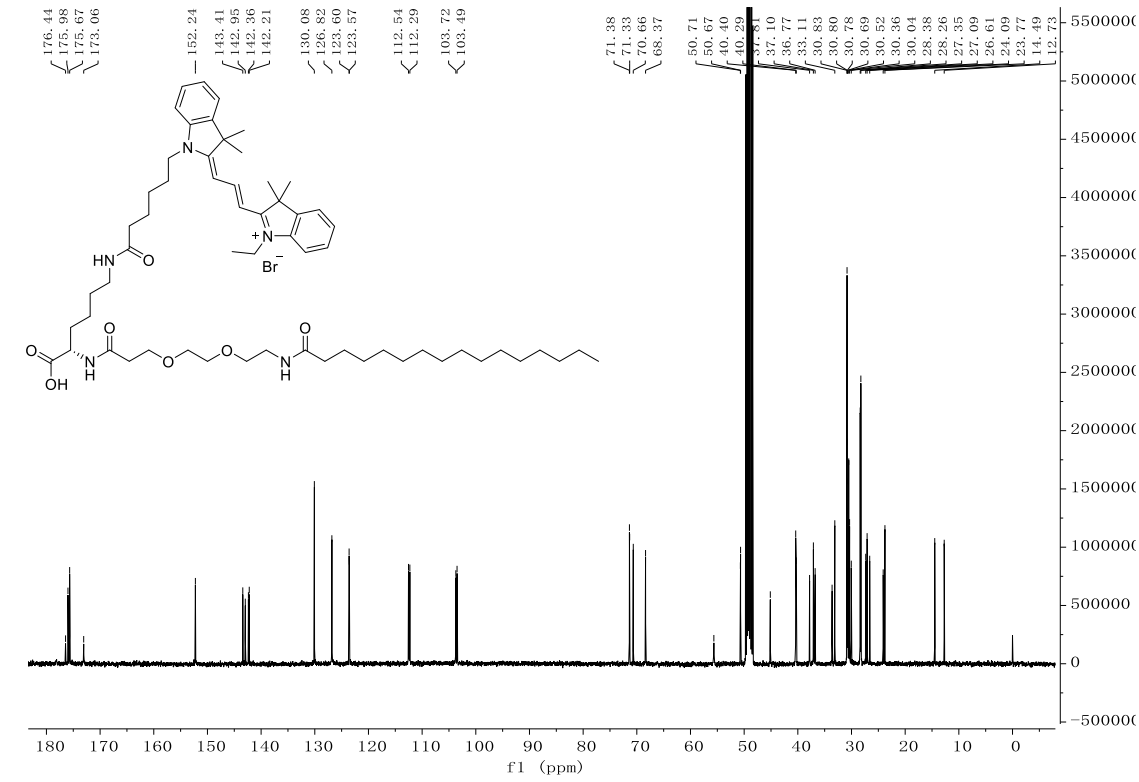
^{1}H NMR (400 MHz, Methanol-*d*4) of compound **16**



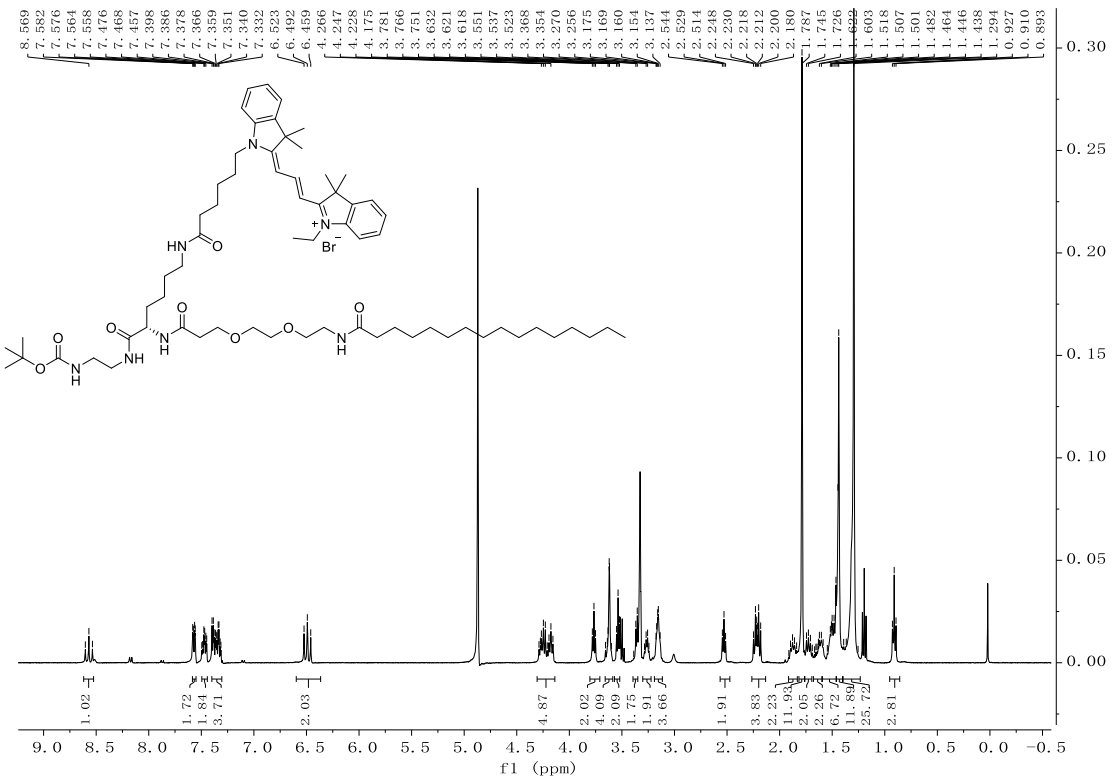
^{13}C NMR (100 MHz, Methanol-*d*4) of compound **16**



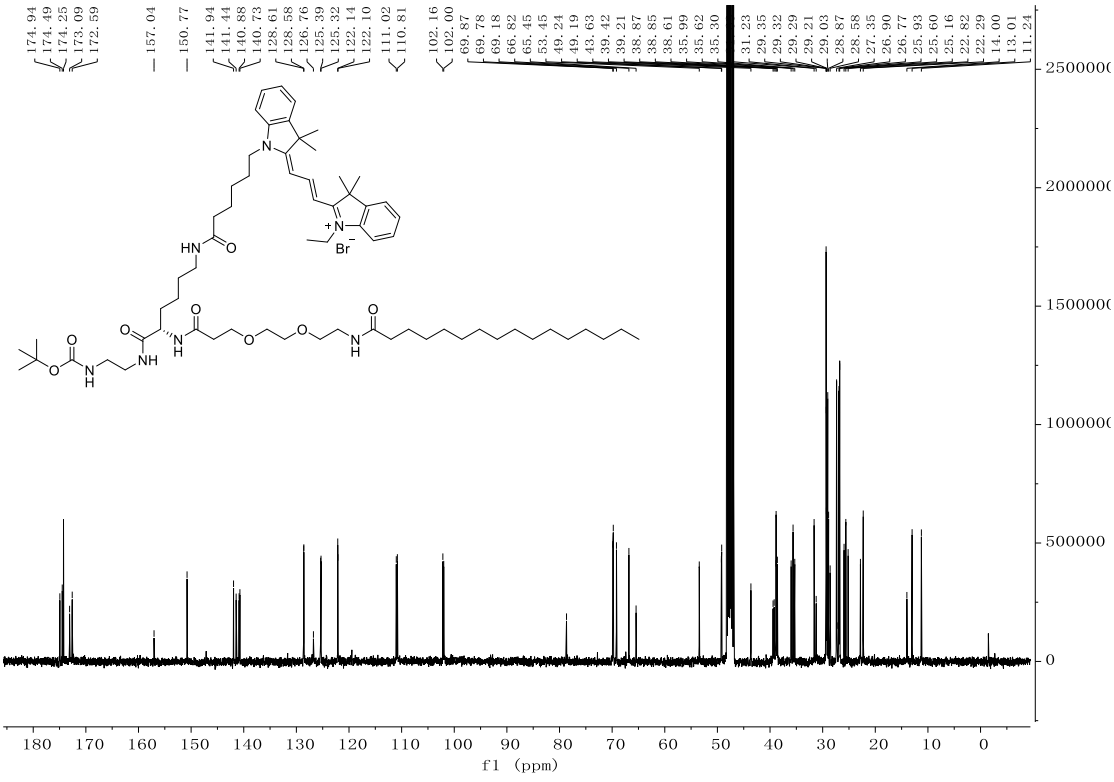
^{1}H NMR (400 MHz, Methanol-*d*4) of compound **17**



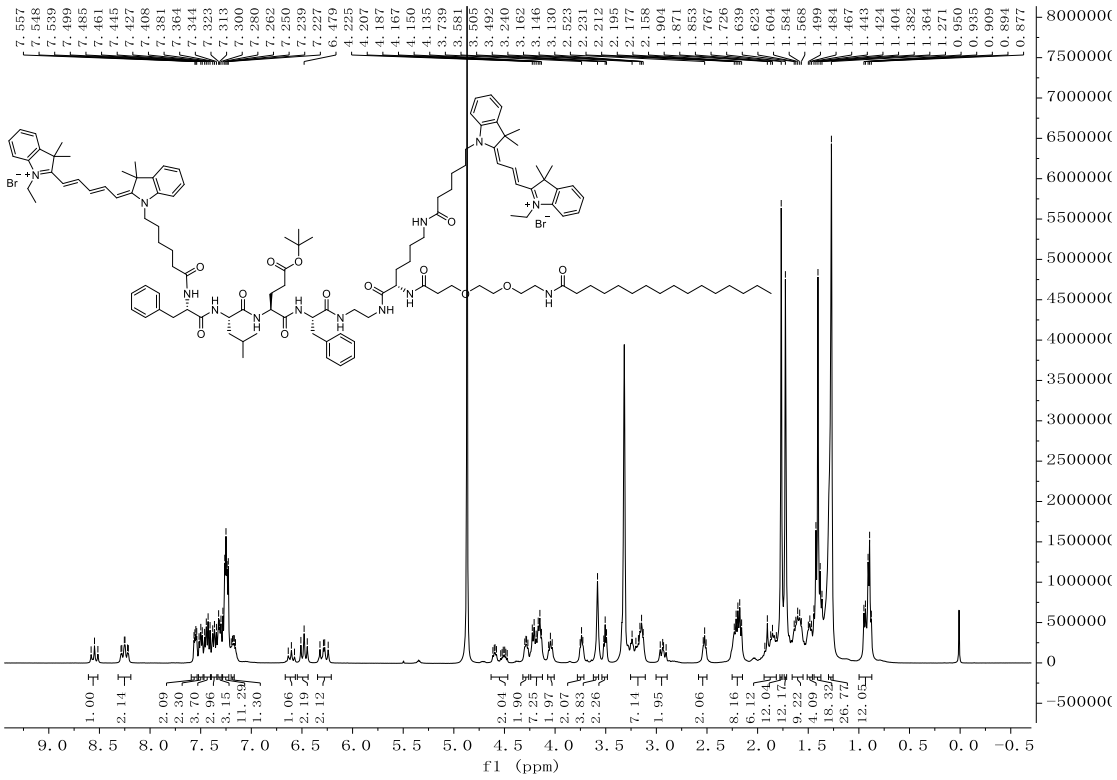
^{13}C NMR (100 MHz, Methanol-*d*4) of compound **17**



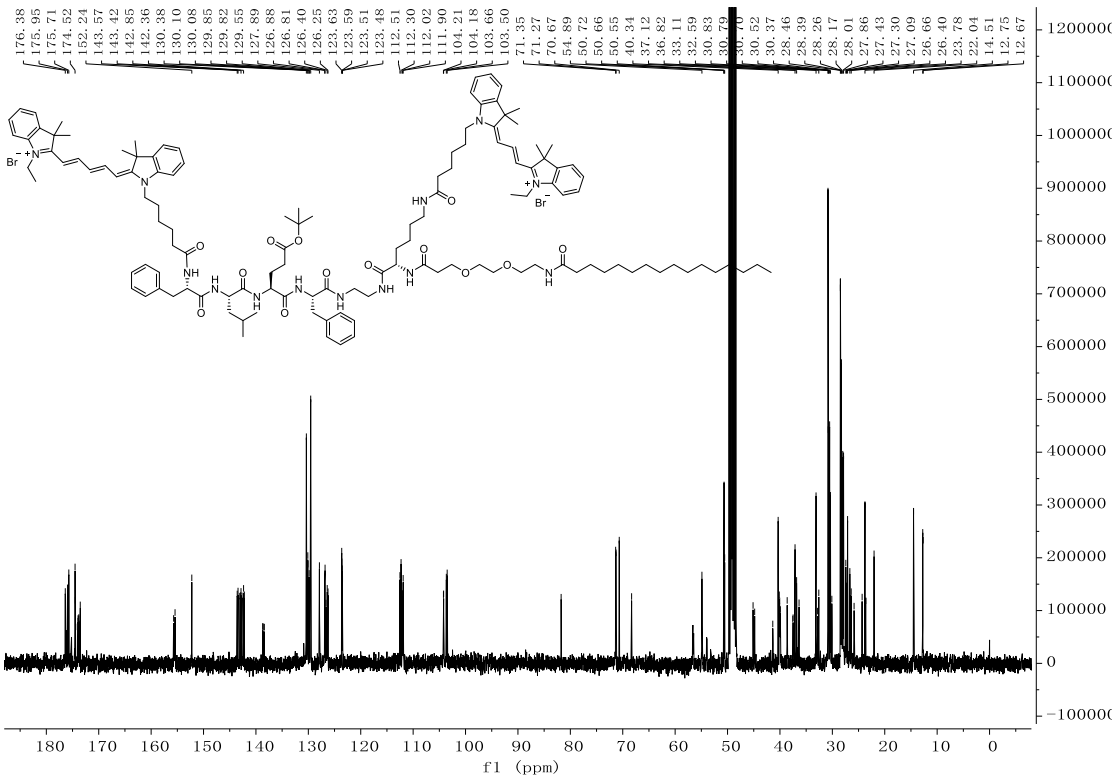
^{1}H NMR (400 MHz, Methanol-*d*4) of compound **18**



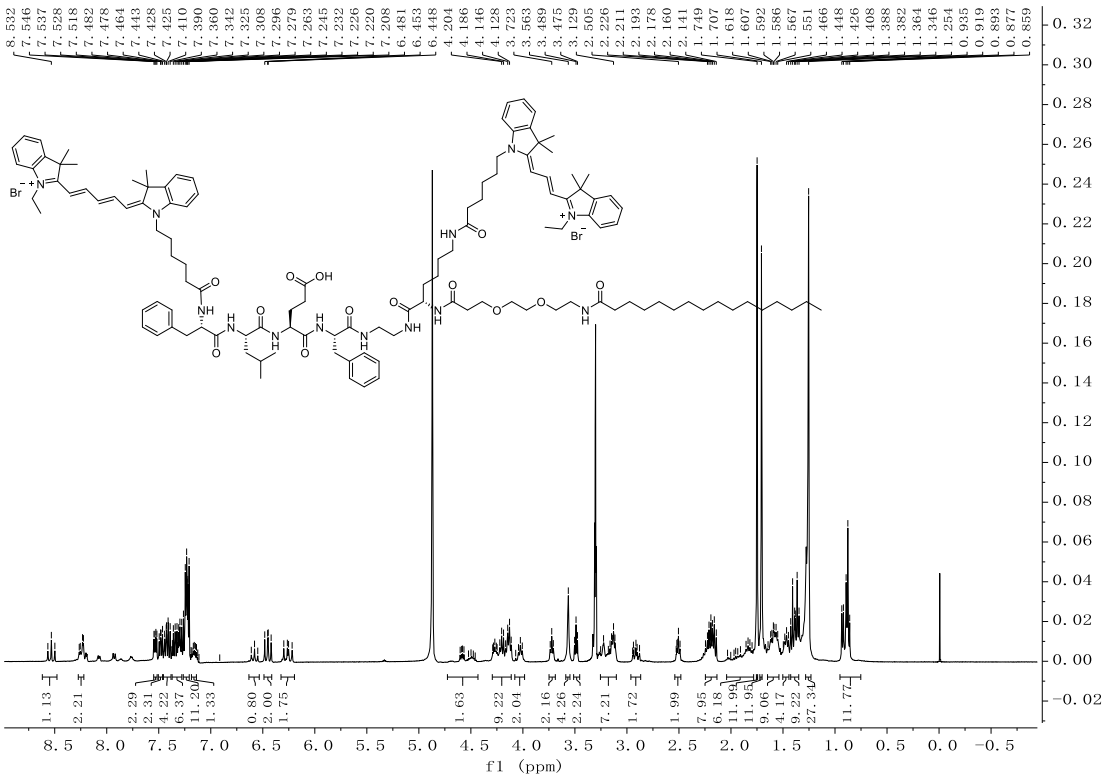
^{13}C NMR (100 MHz, Methanol-*d*4) of compound **18**



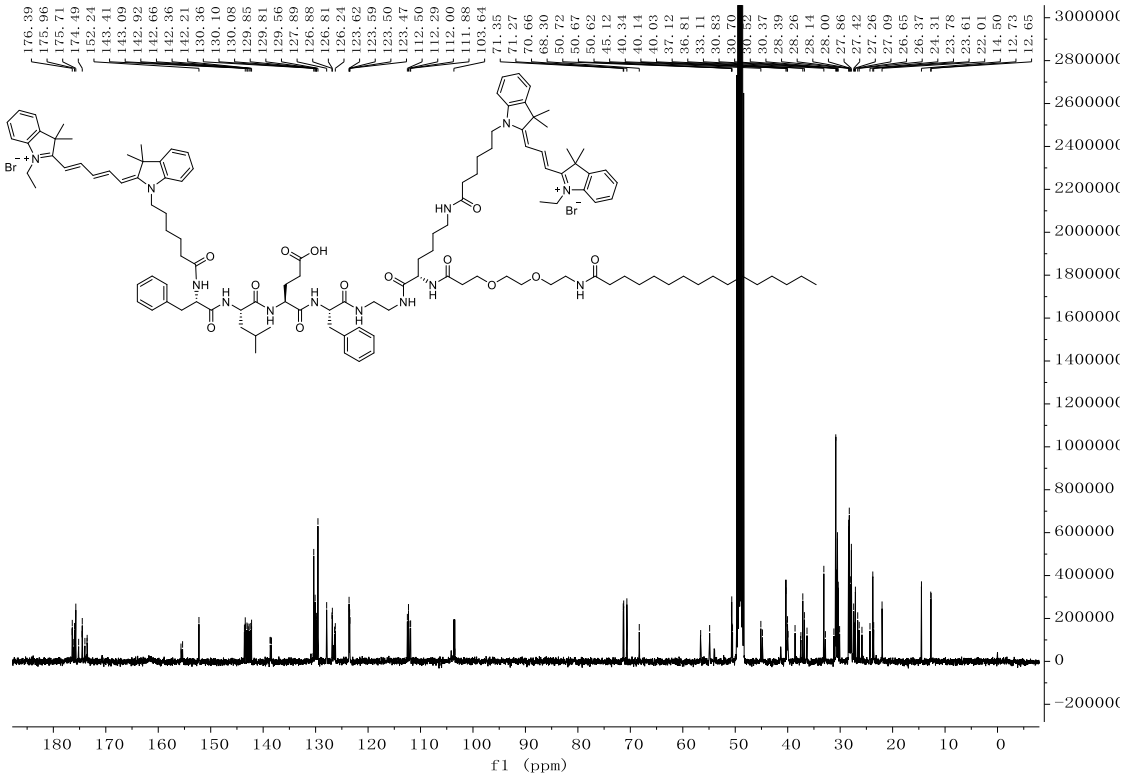
^{1}H NMR (400 MHz, Methanol-*d*4) of compound **20**



^{13}C NMR (100 MHz, Methanol-*d*4) of compound **20**



^{1}H NMR (400 MHz, Methanol-*d*4) of compound **21**



^{13}C NMR (100 MHz, Methanol-*d*4) of compound **21**