

Supporting Information

Assessing Changes in the Expression Levels of Cell Surface Proteins with a Turn-on Fluorescent Molecular Probe

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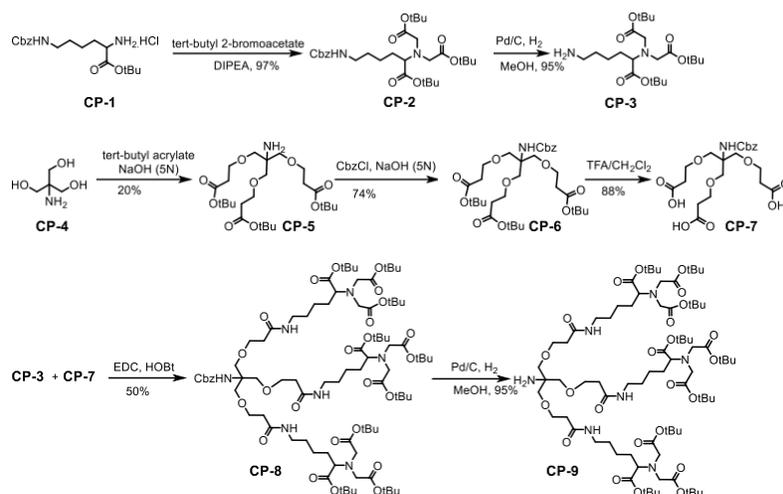
1. Materials and Methods

All solvents and reagents were obtained from commercial suppliers and used without further purification. Dry solvents were purchased from Sigma Aldrich. Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Sulfo-Cyanine5 carboxylic acid was obtained from Lumiprobe Corporation. The ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker Advance 300, 400, or 500 MHz spectrometer. The chemical shifts are represented in ppm on the δ scale down field from TMS as the internal standard. The following abbreviations were used to describe the peaks: br-broad, s-singlet, d-doublet, t-triplet, td-triplet of doublets, q-quartet, quin-quintet, and m-multiplet. Electrospray mass spectrometry was performed either with a Micromass Platform LCZ-4000 instrument at the Weizmann Institute of Science mass spectrometry facility or by using the LTQ Orbitrap Discovery hybrid FT mass spectrometer (Thermo Fisher Scientific, Inc.) equipped with an electrospray ionization ion source at the Faculty of Agriculture, Hebrew University of Jerusalem. The analytical reversed phase high-performance liquid chromatography (RP-HPLC) analysis was performed on an Agilent Technologies 1260 Infinity quaternary pump LC system, equipped with a diode-array detector using a C_{18} column. Preparative HPLC was carried out using an Agilent 218 purification system, equipped with an autosampler, a UV-Vis dual wavelength detector, and a 440-LC fraction collector operating under OpenLab ChemStation software. The elution phases consisted of 10% ACN and 0.1% TFA in H_2O (eluent A) and 90% acetonitrile and 0.1% TFA in H_2O (eluent B). Fluorescence was measured using a BioTek synergy H4 hybrid multiwell plate reader, in black flat-bottom polystyrene NBS 384-well microplates (Corning).

Abbreviations. Acetonitrile (ACN), dichloromethane (DCM), $\text{N,N}'$ -diisopropylethylamine (DIPEA), $\text{N,N}'$ -dimethylformamide (DMF), dimethyl sulfoxide (DMSO), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), fluorescein isothiocyanate (FITC), 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium3-oxid hexafluorophosphate (HATU), hydroxybenzotriazole (HOBt), methanol (MeOH), MicroScale Thermophoresis (MST), nitrilotriacetic acid (NTA), Phosphate buffer saline (PBS), reverse phase high-performance liquid chromatography (RP-HPLC), sodium dodecyl sulphate (SDS), Sulfo-Cyanine5 (sCy5), trifluoroacetic acid (TFA).

3. Synthetic Procedures:

3.1. Synthesis of tri-NTA:



The tri-NTA (CP-9) was synthesized following the earlier reported procedure. ^[1]

Synthesis of CP-2: *tert*-butyl 2-bromoacetate (2.39 mL, 16.00 mmol) and DIPEA (3.50 mL, 20 mmol) were added to a solution of N-benzyloxycarbonyl-L-lysine *tert*-butyl ester (CP-1, 1.50 g, 4.02 mmol) in 25 mL DMF. The reaction was purged with argon, heated to 55°C, and stirred overnight. The excess solvent was removed under high vacuum and then 15 mL hexane/EtOAc (3:1) was added to the solidified mixture. The mixture was filtered over sinter glass and washed with the same solvent (3 × 10 mL). Finally, the filtrate was concentrated under reduced pressure and the obtained crude was purified by column chromatography (hexane/EtOAc, 80:20) to yield the purified product (2.20 g, 97% yield). ¹H NMR (500 MHz, DMSO-*d*₆): δ 1.32-1.43 (m, 32H), 1.44-1.58 (m, 2H), 2.96 (q, *J* = 6.2 Hz, 2H), 3.23 (t, *J* = 7.4 Hz, 1H), 3.35 (d, *J* = 17.2 Hz, 2H), 3.43 (d, *J* = 17.2 Hz, 2H), 4.99 (s, 2H), 7.20 (t, *J* = 5.3 Hz, 1H), 7.24-7.42 (m, 5H). ESI-MS (*m/z*): calcd. for [M+H]⁺: 565.34; found: 565.35.

Synthesis of CP-3: CP-2 (2.20 g, 3.92 mmol) was dissolved in 50 mL MeOH and purged with argon. Next, 10% Pd/C (0.21 g, 0.196 mmol) was added and the reaction was stirred overnight under H₂ atmosphere (836 Torr). The mixture was filtered over celite, and the solvents were removed under reduced pressure. Yield: (1.60 g, 95%). ¹H NMR (300 MHz, CDCl₃): δ 1.44 (s, 18H), 1.47 (s, 9H), 1.48-1.57 (m, 4H), 1.60-1.69 (m, 2H), 2.71- 2.75 (m, 2H), 3.31 (t, *J* = 7.4 Hz, 1H), 3.43- 3.48 (m, 4H). ESI-MS (*m/z*): calcd. for [M+H]⁺: 431.31; found: 431.35.

Synthesis of CP-5: 2-amino-2-(hydroxymethyl)propane-1,3-diol (CP-4, 1.21 g, 10.00 mmol) was dissolved in DMSO (2.0 mL) and cooled to 15 °C under argon. Then, 5 N NaOH (0.20 mL) was added, followed by dropwise addition of *tert*-butyl acrylate (5.00 mL, 34.00 mmol). The reaction mixture was brought to room temperature and stirred overnight. The excess reagent and the solvent were removed under

high vacuum and the crude was purified by column chromatography (EtOAc/hexane + 0.05% v/v NH₄OH, 30:70) to yield a colorless oil (1.01 g, 20%). ¹H NMR (500 MHz, DMSO-*d*₆): δ 1.40 (s, 27 H), 2.39 (t, *J* = 6.1 Hz, 6H), 3.17 (s, 6H), 3.55 (t, *J* = 6.1 Hz, 6H). ESI-MS (*m/z*): calcd. for [M+H]⁺ 506.33; found: 506.35.

Synthesis of CP-6: Benzyl chloroformate (0.27 mL, 1.92 mmol) was added dropwise to a solution of compound CP-5 (0.75 g, 1.48 mmol) in THF (20 mL). Then, NaOH (1.85 mL, 2N, 3.70 mmol) was added. The reaction was stirred at room temperature for 4-5 hours. After complete consumption of the starting materials, as monitored by TLC, the solvent was evaporated under vacuum and the residue was re-suspended in H₂O/EtOAc (1:1). The aqueous layer was extracted with ethyl acetate (3 × 25 mL). The organic layers were combined and dried over anhydrous sodium sulphate and evaporated under reduced pressure. The resulting crude mixture was purified by flash column chromatography (EtOAc/hexane, 20:80) to yield a colorless oil (0.70 g, 74%). ¹H NMR (400 MHz, CDCl₃): δ 1.36 (s, 27H), 2.37 (t, *J* = 6.4 Hz, 6H), 3.48-3.65 (m, 12H), 4.96 (s, 2H), 7.20- 7.33 (m, 5H). ESI-MS (*m/z*): calcd. For [M+H]⁺ 640.37; found: 640.35.

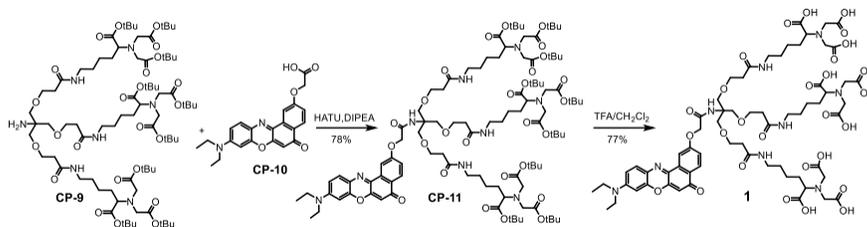
Synthesis of CP-7: TFA (5.00 mL) was added to a solution of CP6 (0.70 g, 1.09 mmol) in DCM (5.00 mL) at 0 °C. The reaction mixture was allowed to warm up to room temperature and stirring was continued for another 3 hours. After the reaction was complete, DCM and TFA were evaporated. The traces of TFA were removed by co-evaporation with DCM. To the crude compound, 40 mL ACN/H₂O (1:1) was added. The sample was frozen using liquid nitrogen and lyophilized under reduced pressure to afford a colorless powder as a product (0.45 g, 88%). ¹H NMR (300 MHz, DMSO-*d*₆): δ 2.41 (t, *J* = 6.1 Hz, 6H), 3.49 (br s, 6H), 3.56 (t, *J* = 6.1 Hz, 6H), 4.98 (s, 2H), 7.34 (br s, 4H), 7.38-7.48 (m, 1H). ESI-MS (*m/z*): calcd. for [M+H]⁺ 472.18; found: 472.18.

Synthesis of CP-8: CP-7 (0.16 g, 0.34 mmol) was dissolved in dry DCM (10 mL), and was cooled to 0 °C in an ice bath. Then Et₃N (0.17 mL, 1.20 mmol), EDC (0.19 g, 1 mmol), and HOBt (0.04 g, 0.34 mmol) were sequentially added. After 15 minutes, CP-3 (0.43 g, 1.00 mmol) was added and the reaction mixture was stirred overnight. Next, the reaction mixture was diluted with DCM (40 mL) and washed with water (3 × 10 mL). The organic layer was dried over anhydrous sodium sulphate, filtered, and concentrated under vacuum. Finally, the crude product was purified by column chromatography (DCM/MeOH, 96:4) to yield a colorless oil (0.29 g, 50%). ¹H NMR (300 MHz, CDCl₃): δ 1.44 (s, 54H), 1.45 (s, 27H), 1.51 (br s, 6H) 1.69 (br s, 12H), 2.00 (s, 2H), 2.41 (br s, 4H), 3.21 (t, *J* = 7.5 Hz, 6H), 3.29 (t, *J* = 7.5 Hz, 6H), 3.38-3.51 (m, 12H), 3.63-3.69 (m, 9H), 5.03 (br s, 2H), 5.31 (br s, 1H), 5.41 (s, 1H), 6.72 (br s, 2H), 7.34 (br s, 5H). ESI-MS (*m/z*): calcd. for [M+H]⁺ 1709.06; found: 1709.04.

Synthesis of CP-9: CP8 (0.11 g, 0.06 mmol) was dissolved in 20 mL MeOH and purged with argon. Next, 10% Pd/C (4.00 mg, 3.22 μmol) was added and the reaction was stirred overnight under H₂ atmosphere

(836 Torr). Finally, the mixture was filtered over celite and the solvent was removed under reduced pressure. Yield: (0.09 g, 95%). ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 1.23 (br s, 3H), 1.39 (s, 54H), 1.40 (s, 27H), 1.47-1.55 (m, 7H), 2.26 (t, $J = 6.2$ Hz, 6H), 3.00 (t, $J = 5.7$ Hz, 6H), 3.16 (d, $J = 9.2$ Hz, 10H), 3.22 (t, $J = 7.4$ Hz, 3H), 3.35-3.46 (m, 9H), 3.50-3.59 (m, 6H), 4.10 (br s, 2H), 7.82 (t, $J = 5.3$ Hz, 2H). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ 22.9, 27.7, 28.9, 29.7, 36.1, 38.4, 48.6, 53.2, 55.8, 64.5, 67.4, 72.3, 79.9, 80.2, 169.8, 170.0, 171.5. ESI-MS (m/z): calcd. for $[\text{M}+\text{H}]^+$ 1575.03; found: 1575.00.

3.2 : Synthesis of tri-NTA appended with Nile Red:

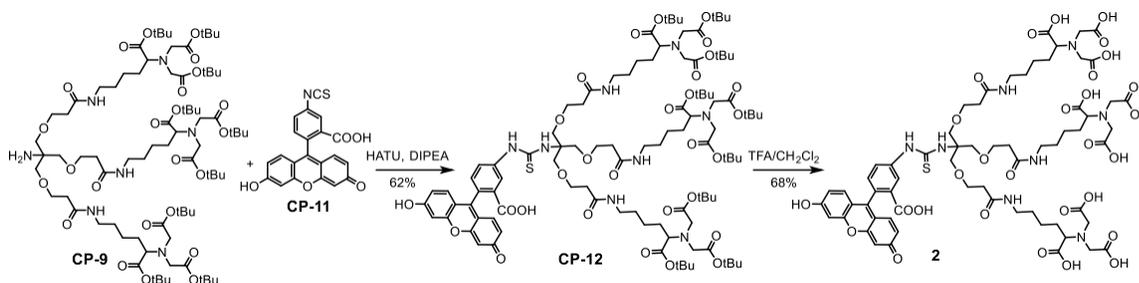


Synthesis of CP-11: To a solution of Nile Red (**CP-10**), (12.00 mg, 0.03 mmol) in CH_2Cl_2 (2 mL), synthesized following our earlier report,^[2] DIPEA (0.01 mL, 0.06 mmol) and HATU (24.00 mg, 0.06 mmol) were sequentially added and the reaction mixture was stirred for 15 minutes. Then compound **CP-9** (47.00 mg, 0.03 mmol), dissolved in CH_2Cl_2 (1.00 mL), was added dropwise and the reaction mixture was stirred at room temperature for 12 h under argon and dark conditions. After consumption of the starting materials (as monitored by TLC), CH_2Cl_2 was removed in vacuum and the residue was purified by RP-HPLC to afford **CP-11** (45.00 mg, 78%) as a dark purple solid. ^1H NMR (400 MHz, CDCl_3): δ 1.44 (s, 56H), 1.45 (s, 27H), 1.67-1.47 (m, 12H), 1.87 (s, 4H), 2.26 (t, $J = 7.0$ Hz, 6H), 2.40 (t, $J = 5.8$ Hz, 6H), 3.15-3.30 (m, 9H), 3.40-3.50 (m, 16H), 3.65-3.75 (m, 12H), 4.6 (s, 2H), 6.31 (s, 1H), 6.46 (d, $J = 2.6$ Hz, 1H), 6.71-6.65 (m, 4H), 7.03 (s, 1H), 7.24 (d, $J = 2.6$ Hz, 1H), 7.61 (d, $J = 6.4$ Hz, 1H), 8.07 (d, $J = 2.6$ Hz, 1H), 8.25 (d, $J = 8.7$ Hz, 1H). ^{13}C NMR (100 MHz, CDCl_3): δ 12.6, 23.1, 28.1, 29.7, 30.0, 36.6, 39.6, 45.1, 53.8, 59.7, 65.1, 67.5, 69.2, 80.6, 80.7, 96.3, 105.3, 107.3, 109.8, 118.1, 124.7, 126.6, 128.1, 131.2, 134.1, 139.4, 146.9, 150.9, 152.2, 159.7, 167.3, 170.7, 171.0, 172.3, 182.9. HRMS-ESI (m/z): calcd. for $[\text{M}+\text{Na}]^+$ 1971.1343; found 1971.1340

Synthesis of 1: The tert-butyl ester group was deprotected by adding TFA (1.00 mL) to a solution of **CP-11** (30.00 mg, 15.00 μmol in DCM (2 mL) at 0 $^\circ\text{C}$ and under dark conditions. The reaction mixture was warmed up to the room temperature and stirring was continued for another 3.5 h. After the reaction was completed, DCM and TFA were evaporated. The traces of TFA were removed by co-evaporation with DCM. The crude compound was washed thrice with cold diethyl ether, followed by dissolution in 3 mL $\text{ACN}/\text{H}_2\text{O}$ (1:1), freezing with liquid nitrogen, and lyophilization under high vacuum to afford a purple powder as a product (17.00 mg, 77%). ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ 1.09 (t, $J = 7.0$ Hz, 6H), 1.17 (t,

$J = 7.0$ Hz, 6H), 1.30-1.41 (m, 6H), 1.49-1.60 (m, 6H), 2.29 (t, $J = 6.2$ Hz, 6H), 2.92-3.05 (m, 6H), 3.31 (t, $J = 7.4$ Hz, 3H), 3.38 (q, $J = 7.0$ Hz, 4H), 3.41-3.54 (m, 14H), 3.54-3.59 (m, 6H), 3.61 (br s, 4H), 4.69 (s, 2H), 6.21 (s, 1H), 6.62-6.69 (m, 1H), 6.84 (dd, $J = 2.2, 9.1$ Hz, 1H), 7.30 (dd, $J = 2.2, 8.7$ Hz, 1H), 7.39 (s, 1H), 7.63 (d, $J = 9.1$ Hz, 1H), 7.79 (t, $J = 5.2$ Hz, 3H), 7.96 (d, $J = 2.2$ Hz, 1H), 8.06 (d, $J = 8.7$ Hz, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 12.4, 23.1, 28.8, 29.2, 35.9, 38.4, 44.5, 53.2, 59.7, 64.2, 66.9, 67.4, 68.3, 96.0, 104.1, 106.8, 110.2, 115.7, 118.1, 118.7, 124.0, 125.4, 127.2, 131.0, 133.6, 138.1, 146.5, 150.9, 151.8, 160.2, 166.8, 169.8, 173.1, 173.9, 181.4. HRMS-ESI (m/z): calcd. for $[\text{M}]^{2-}$ 720.7836; found: 720.7853.

3.3 : Synthesis of tri-NTA appended with Fluorescein

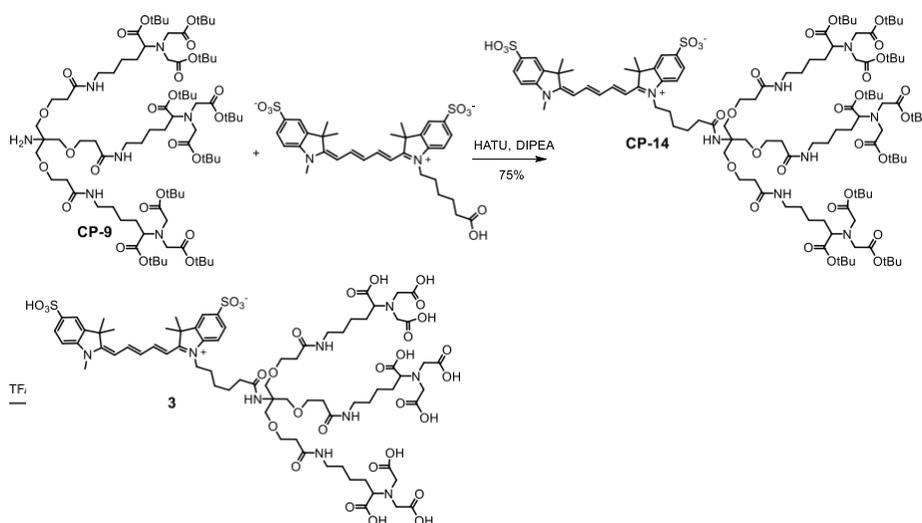


Synthesis of CP-12: Compound **CP-9** (50.00 mg, 0.032 mmol) was dissolved in CH_2Cl_2 (5 mL) and the solution was basified with DIPEA (0.012 mL, 0.064 mmol). Then fluorescein isothiocyanate (**CP-11**, 12.00 mg, 0.032 mmol) was added to the above solution and the resulting reaction mixture was stirred at room temperature for 12 h under argon and dark conditions. The solvent was removed in vacuum and the residue was dissolved in DCM (20 mL) and washed with water (2×10 mL). The organic layer was dried over anhydrous sodium sulphate, filtered, and evaporated to dryness. Finally, the residue was purified by RP-HPLC to afford **CP-12** (40.00 mg, 62%) as a bright yellow solid. ^1H NMR (400 MHz, CDCl_3): δ 1.42 (br s, 87H), 1.69-1.46 (m, 12H), 2.47 (br s, 6H), 3.10-3.30 (m, 9H), 3.35-3.50 (m, 16H), 3.60-3.90 (m, 8H), 6.55 (d, $J = 8.0$ Hz, 2H), 6.60 (br d, 2H), 6.74 (s, 2H), 6.96 (br s, 2H), 7.02 (d, $J = 8.0$ Hz, 1H), 7.12 (br s, 1H), 7.90 (br s, 1H), 8.11 (s, 1H). ^{13}C NMR (100 MHz, CDCl_3): δ 23.2, 28.1, 28.2, 28.6, 29.9, 36.4, 39.57, 54.2, 65.2, 67.9, 69.4, 81.4, 81.6, 81.8, 102.9, 111.8, 114.7, 115.3, 117.6, 119.4, 120.4, 127.0, 128.9, 130.7, 131.8, 142.1, 157.7, 160.6, 161.0, 161.4, 161.7, 167.1, 169.5, 171.2, 172.5, 180.7. HRMS-ESI (m/z): $[\text{M}+\text{Na}]^+$ 1986.0435; found: 1986.0448.

Synthesis of 2: The tert-butyl ester group was deprotected by adding TFA (1.00 mL) to a solution of **CP-12** (40.00 mg, 0.02 mmol) in DCM (2.00 mL) at 0°C and under dark conditions. The reaction mixture was warmed up to room temperature and stirring was continued for another 3.5 h. After the reaction was completed, DCM and TFA were evaporated. The traces of TFA were removed by co-evaporation with DCM. The crude compound was washed thrice with cold diethyl ether, followed by dissolution in 3.00 mL

ACN/H₂O (1:1), freezing with liquid nitrogen, and lyophilization under a high vacuum to afford a bright yellow powder as a product (20.00 mg, 68%). ¹H NMR (500 MHz, DMSO-*d*₆): δ 1.19-1.38 (m, 12H), 1.48-1.69 (m, 6H), 2.34 (br. s, 6H), 2.95-3.07 (m, 6H), 3.26-3.46 (m, 5H), 3.45-3.55 (m, 14H), 3.63 (t, *J* = 6.0 Hz, 4H), 3.69 (t, *J* = 6.0 Hz, 2H), 3.86 (br s, 2H), 6.52-6.63 (m, 4H), 6.68 (br s, 2H), 7.18 (d, *J* = 8.1 Hz, 1H), 7.27 (d, *J* = 8.1 Hz, 1H), 7.37 (br s, 1H), 7.67-7.77 (m, 1H), 7.84 (d, *J* = 4.7 Hz, 3H), 8.02-8.19 (m, 1H), 8.30 (s, 1H), 10.11 (br s, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 23.1, 28.8, 29.2, 35.9, 38.4, 40.4, 53.3, 61.9, 64.2, 67.5, 68.1, 102.2, 109.7, 112.6, 115.2, 118.1, 124.0, 126.4, 129.0, 129.8, 141.3, 151.9, 159.5, 169.8, 169.9, 173.1, 173.9, 179.7. HRMS-ESI (*m/z*): [M]⁻² 728.2381; found: 728.2396.

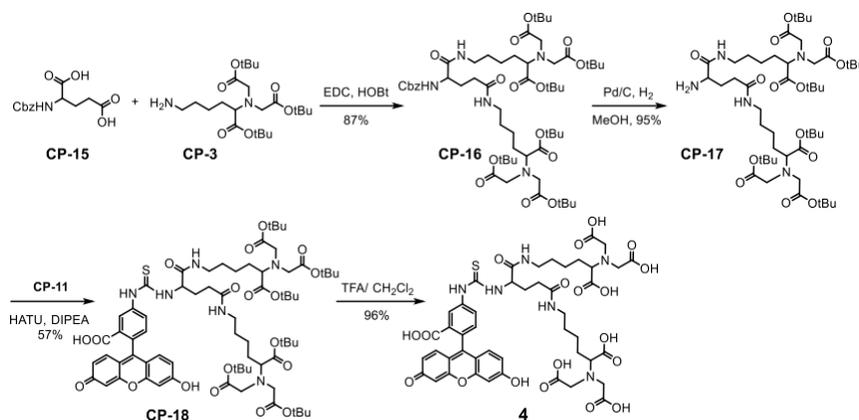
3.4 : Synthesis of tri-NTA appended with sCy5



Synthesis of CP-14: DIPEA (3.80 μL, 21.80 μmol) and HATU (9.00 mg, 21.80 μmol) were sequentially added to a solution of the scy5 carboxylic acid (CP-13, 7.00 mg, 10.90 μmol) in CH₂Cl₂ (2.00 mL). The reaction mixture was stirred for 15 minutes. Then compound CP-9 (17.00 mg, 10.90 μmol), dissolved in CH₂Cl₂ (1.00 mL), was added dropwise and the reaction mixture was stirred at room temperature for 12 h under argon and dark conditions. Finally, the solvent was removed in vacuum and the residue was purified by RP-HPLC to afford CP-14 (18.00 mg, 75%) as a blue solid. ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.23 (s, 8H), 1.37 (s, 55H), 1.44 (s, 30H), 1.49 (s, 12H), 1.68 (s, 12H), 2.06 (s, 2H), 2.25 (s, 6H), 2.98 (s, 6H), 3.21 (t, *J* = 6.9 Hz, 3H), 3.31-3.59 (m, 27H), 4.06 (s, 2H), 6.27 (t, *J* = 14.4 Hz, 2H), 6.55 (t, *J* = 11.8 Hz, 1H), 7.03 (d, *J* = 13.0 Hz, 2H), 7.16 (s, 1H), 7.32 (s, 2H), 7.63 (s, 1H), 7.80 (s, 4H), 8.35 (t, *J* = 13.0 Hz, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 13.9, 17.2, 22.0, 22.9, 24.5, 24.8, 25.7, 26.7, 26.9, 27.0, 27.5, 28.5, 28.7, 28.9, 29.0, 29.7, 30.1, 31.2, 31.3, 33.6, 35.5, 35.9, 38.4, 43.4, 48.4, 48.8, 53.2, 59.4, 64.5, 67.3, 68.2, 69.7, 79.9, 80.2, 103.2, 103.5, 110.0, 115.7, 118.7, 119.8, 119.9, 125.6, 129.9, 126.0, 140.3, 140.4, 141.9, 142.7, 145.1, 145.3, 154.2, 157.4, 157.7, 158.0, 158.3, 169.8, 170.0, 171.5, 172.1, 172.7, 173.6. HRMS-ESI (*m/z*): calcd. for [M+H]⁺ 2199.2221, found 2199.2283.

Synthesis of 3: TFA (1.00 mL) was added to a solution of **CP-14** (9.00 mg, 4.09 μmol) in DCM (2.00 mL) at 0 °C and under dark conditions. The reaction mixture was warmed up to room temperature and stirring was continued for another 3.5 h. After the reaction was completed, DCM and TFA were evaporated. Then the traces of TFA were removed by co-evaporation with DCM. Finally, the crude compound was washed thrice with cold diethyl ether, followed by dissolution in 3.00 mL ACN/H₂O (1:1), freezing with liquid nitrogen, and lyophilization under high vacuum to afford a blue powder as a product (5.00 mg, 73%). ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.18-1.36 (m, 14H), 1.44- 1.61 (m, 10H), 1.69 (s, 12H), 2.07 (t, *J* = 6.6 Hz, 2H), 2.27 (t, *J* = 5.9 Hz, 6H), 2.99 (d, *J* = 5.9 Hz, 6H), 3.33 (t, *J* = 7.3 Hz, 3H), 3.40- 3.56 (m, 23H), 3.59 (s, 3H), 4.07 (br s, 2H), 6.18-6.38 (m, 2H), 6.49-6.66 (m, 1H), 7.01 (s, 1 H), 7.25- 7.36 (m, 2H), 7.64 (d, *J* = 8.4 Hz, 2H), 7.81 (br s, 5H), 8.35 (t, *J* = 12.8 Hz, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 23.2, 27.0, 27.2, 28.9, 29.3, 34.4, 36.0, 38.4, 48.9, 48.9, 53.4, 54.9, 59.5, 64.3, 67.4, 68.3, 110.2, 112.8, 115.8, 118.8, 119.8, 119.9, 125.8, 125.8, 126.0, 126.1, 126.2, 140.5, 140.5, 142.2, 142.8, 144.9, 145.1, 154.2, 154.3, 170.0, 172.4, 172.8, 173.3, 173.8, 174.0. HRMS-ESI (*m/z*): calcd. for [M]⁻² 845.8184; found 845.8195.

3.5: Synthesis of bis-NTA appended with Fluorescein



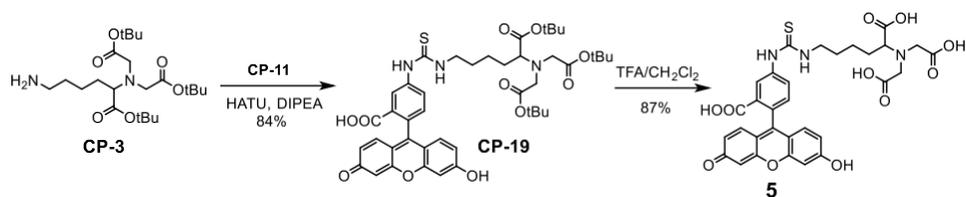
Synthesis of CP-16: Compound **CP-15** (30.00 mg, 0.11 mmol) was dissolved in dry THF (7 mL), and then EDC.HCl (60.00 mg, 0.32 mmol) and HOBt (14.00 mg, 0.11 mmol) were sequentially added. The solution was basified to pH 7-8 with Et₃N (0.04 mL, 0.32 mmol). Next, the reaction mixture was stirred for 15 minutes at room temperature under argon and thereafter, compound **CP-3** (100.00 mg, 0.23 mmol), dissolved in dry THF (3.00 mL), was added to the above solution. Stirring was continued for 24 h under argon and dark conditions. After completion of the reaction, as monitored by TLC, the solvent was removed in vacuum and the residue was purified by RP-HPLC to afford **CP-16** (106.00 mg, 87%) as a light yellow solid. ¹H NMR (400 MHz, acetonitrile-*d*₃) δ : 1.36-1.44 (m, 4H), 1.52 (s, 36H), 1.53 (m, 18H), 1.60-1.75 (m, 4H), 1.91-2.10 (m, 4H), 1.27-2.32 (m, 4H), 3.17-3.22 (m, 4H), 3.36 (td, *J* = 1.7 Hz, 7.5 Hz, 2H), 3.43-3.55 (m, 8H), 4.07 (td, *J* = 5.3, 7.5 Hz, 1H), 5.15 (s, 2H), 6.60- 6.65 (m, 2H), 6.96 (t, *J* = 5.3 Hz, 1H), 7.41-7.47 (m, 5H). ESI-MS (*m/z*): calcd. for [M+H]⁺ 1106.68; found 1106.68.

Synthesis of CP-17: Compound **CP-16** (0.60 g, 0.54 mmol) was dissolved in 30.00 mL MeOH and purged with argon. Next, 10% Pd/C (30.00 mg, 0.027 mmol) was added and the reaction was stirred overnight under H₂ (836 Torr). The mixture was filtered over Celite and the solvent was removed under reduced pressure. Yield: 0.50 g (95%). ¹H NMR (500 MHz, DMSO-*d*₆) δ: 1.17-1.27 (m, 2H), 1.39 (s, 54H), 1.45-1.69 (m, 6H), 1.69-1.81 (m, 1H), 1.84 (br s, 1H), 1.96-2.12 (m, 2H), 2.91-3.09 (m, 4H), 3.22 (t, *J* = 7.1 Hz, 2H), 3.32-3.37 (m, 5H), 3.39- 3.48 (m, 4H), 7.71 (d, *J* = 4.8 Hz, 1H), 7.77 (d, *J* = 5.2 Hz, 1H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ: 22.9, 27.8, 28.9, 29.0, 29.0, 29.2, 29.7, 29.8, 31.4, 32.1, 32.2, 34.4, 38.1, 38.2, 38.3, 53.2, 54.5, 54.9, 63.7, 64.5, 79.9, 80.3, 170.0, 171.5, 171.6, 171.7, 173.4, 174.6. ESI-MS (*m/z*): calcd. for [M+H]⁺ 972.65; found 972.63.

Synthesis of CP-18: Compound **CP-17** (100.00 mg, 102.98 μmol) was dissolved in DCM (1.00 mL) and the solution was basified to pH 7-8 with DIPEA (35.94 μL, 205.97 μmol). Then a solution of fluorescein isothiocyanate (**CP-11**, 44.00 mg, 113.27 μmol) in DCM (1.00 mL) was added to the above solution and the reaction mixture was stirred at room temperature for 7 h under argon and dark conditions. The solvent was removed in vacuum and the residue was purified by RP-HPLC to afford **CP-18** (80.00 mg, 57%) as a light yellow solid. ¹H NMR (400 MHz, acetonitrile-*d*₃): δ 1.25- 1.40 (m, 4H), 1.41- 1.46 (m, 54H), 1.46-1.56 (m, 4H), 1.57- 1.72 (m, 4H), 2.00- 2.13 (m, 2H), 2.22- 2.36 (m, 2H), 3.02- 3.14 (m, 2H), 3.19 (q, *J*=5.9 Hz, 2H), 3.33- 3.63 (m, 10H), 6.57 (dd, *J*=8.7, 2.3 Hz, 2H), 6.64 (br. s. 1H), 6.67- 6.77 (m, 3H), 6.99 (br. s., 1H), 7.14 (d, *J*=8.1 Hz, 1H), 7.75 (d, *J*=7.5 Hz, 1 H), 8.21 (br. s., 1H). ESI-MS (*m/z*): calcd. for [M+H]⁺ 1361.68, found 1361.69.

Synthesis of 4: TFA (1.00 mL) was added to a cooled solution (0 °C) of **CP-18** (11.00 mg, 8.09 μmol) in DCM (2.00 mL) under dark conditions. The reaction mixture was warmed up to room temperature and stirring was continued for another 3.5 h. After the reaction was complete, DCM and TFA were evaporated. The traces of TFA were removed by co-evaporation with DCM. The crude compound was washed thrice with cold diethyl ether, followed by dissolution in 3.00 mL ACN/H₂O (1:1), freezing with liquid nitrogen and lyophilization under high vacuum to afford a light yellow fluffy powder as a product (8.00 mg, 96%). ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.31- 1.46 (m, 12H), 1.46- 1.66 (m, 4H), 1.88- 2.22 (m, 4H), 2.73 - 2.80 (m, 2H), 2.97- 3.02 (m, 2H), 3.33 - 3.37 (m, 2H), 3.44 - 3.55 (m, 8H), 4.84- 4.89 (m, 1H), 6.54 - 6.62 (m, 3H), 6.67 - 6.70 (m, 2H), 7.19 (d, *J*=8.1 Hz, 2H), 7.64 (br. s., 2H), 7.77 (dd, *J*=8.5, 1.9 Hz, 1H), 7.86 (t, *J*=5.4 Hz, 1H), 8.34 (d, *J*=7.5 Hz, 1H), 8.38 (s, 1H), 10.17 (br. s., 1H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 22.5, 23.1, 26.7, 27.3, 28.8, 29.0, 29.2, 31.5, 38.4, 38.7, 53.2, 53.3, 56.2, 63.9, 64.2, 102.2, 109.7, 112.6, 114.3, 116.3, 117.2, 124.1, 126.5, 128.9, 129.0, 129.4, 141.3, 147.2, 151.9, 159.5, 168.5, 170.9, 173.0, 173.7, 173.8, 180.7. ESI-MS (*m/z*): calcd. for [M+H]⁺ 1025.31, found 1025.33.

3.6: Synthesis of mono-NTA appended with Fluorescein



The mono-NTA appended with fluorescein was synthesized according to a previously reported procedure.^[3]

Synthesis of CP-19: CP-3 (100.00 mg, 0.23 mmol) was dissolved in DCM (1 mL) and the solution was basified to pH 7-8 with DIPEA (0.04 mL, 0.23 mmol). Then fluorescein isothiocyanate (CP-11, 100.00 mg, 0.26 mmol) in DCM (1.00 mL) was added to the above solution and the reaction mixture was stirred at room temperature for 7 h under argon and dark conditions. The solvent was removed in vacuum and the residue was purified using RP-HPLC to afford CP-19 (0.16 g, 84%) as a dark yellow solid. ¹H NMR (500 MHz, acetonitrile-*d*₃): δ 1.46 (s, 18H), 1.48 (s, 9H), 1.50-1.58 (m, 2H), 1.67 (quin, *J* = 7.0 Hz, 2H), 1.73-1.90 (m, 2H), 3.57-3.65 (m, 2H), 3.74 (d, *J* = 17.1 Hz, 3H), 3.83 (d, *J* = 17.1 Hz, 2H), 6.60 (dd, *J* = 2.3, 8.3 Hz, 2H), 6.69-6.76 (m, 3H), 7.10 (br s, 1H), 7.14 (d, *J* = 8.3 Hz, 1H), 7.71 (d, *J* = 7.7 Hz, 1H), 8.14 (s, 1H). ESI-MS (*m/z*): calcd. for [M+H]⁺ 820.35; found: 820.35.

Synthesis of 5: TFA (1.00 mL) was added to a pre-cooled (0 °C) solution of CP-19 (0.16 g, 0.195 mmol) in DCM (2.00 mL) under dark conditions. The reaction mixture was warmed up to room temperature and stirring was continued for another 3.5 h. After completion of the reaction, DCM and TFA were evaporated. The traces of TFA were removed by co-evaporation with DCM. The crude compound was washed thrice with cold diethyl ether, followed by dissolution in 3.00 mL ACN/H₂O (1:1), freezing with liquid nitrogen and lyophilization under high vacuum to afford a light yellow fluffy powder as a product (0.11 g, 87%). ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.27-1.49 (m, 2H) 1.55 (br s, 1H), 1.57-1.74 (m, 3H), 3.36-3.43 (m, 2H), 3.45-3.52 (m, 5H), 6.54-6.59 (m, 3H), 6.59-6.63 (m, 3H), 6.67 (d, *J* = 2.2 Hz, 3H), 7.17 (d, *J* = 8.4 Hz, 1H), 7.74 (d, *J* = 7.5 Hz, 1H), 8.05 (br s, 1H), 8.23 (br s, 1H), 9.87 (br s, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 23.2, 28.1, 29.2, 43.8, 53.3, 64.3, 102.3, 102.4, 109.8, 112.6, 114.1, 114.1, 116.4, 117.0, 124.0, 126.5, 129.1, 129.5, 141.5, 147.0, 151.9, 159.5, 159.7, 159.7, 168.6, 172.9, 173.0, 173.7, 180.4. ESI-MS (*m/z*): calcd. for [M+H]⁺ 652.16; found: 652.17.

Bacterial strains and growth conditions. *E. coli* K-12 strain KRX (Promega) was used for protein expression. The details of the expression of 3 copies of hexahistidine-tag at the 7th loop of the OmpC are described in our previously published paper.^[4] Transformed bacteria with different OmpC constructs (OmpC or His-OmpC) were cultured overnight in LB medium containing ampicillin (100 μ g/ml) at 30 °C.

The next day, the bacterial cells were diluted 100-fold in fresh LB medium supplemented with ampicillin and incubated until the OD₆₀₀ reached ~0.6. Protein expression was then induced by the addition of 0.1% Rhamnose and 20 μM isopropyl-b-D-1-thiogalactopyranoside (IPTG), and cultures were incubated overnight on a shaking plate (230 rpm) set at the desired temperatures of 8 °C, 15 °C, or 37 °C. The bacterial cells were collected by centrifugation at 6000 × g for 4 min. Pellets were washed twice with PBS × 1 buffer (100 μL) and then re-suspended in 100 μL in the same buffer to an OD₆₀₀ of 0.3.

General procedure for fluorescence measurements

A sample of a probe (20 μM) and NiCl₂·6H₂O (60 μM) were mixed in Tris buffer (10 mM, pH= 7.5) and allowed to stand at room temperature for 30 minutes. Meanwhile, the bacterial cells (OmpC or His-OmpC) (OD₆₀₀ = 3.0) were transferred to Eppendorf tubes and centrifuged (6,000 g) for 4 minutes. After removing the LB medium, the pellet fraction was washed twice with Tris buffer (200 μL) and finally re-suspended in 200 μL Tris buffer. Then a probe, at a final concentration of 100 nM, was added to the bacterial suspension (60 μL), and the emission spectrum was immediately recorded. The fluorescence responses of **1-3** were measured using excitation wavelengths of 545 nm, 465nm, and 620 nm, respectively. These experiments were performed in triplicate for bacterial samples cultured at 8 °C, 15 °C, and 37 °C.

Fluorescence imaging experiments

To a 100 μL sample of the bacteria suspension, a preincubated sample of each probe, **1-5** (500 nM) and NiCl₂ (2.5 μM) was added, and the cells were incubated at room temperature for 1 h. Then each sample was washed twice with PBS, resuspended in 200 μL PBS, and placed on a glass-bottom dish (P35G-1.5-14-C; MatTek) precoated with poly-l-lysine (Sigma Aldrich) and left to adhere for 1 h. Finally, the wells were washed with PBS three times and imaged using an Olympus IX51 fluorescent microscope. The samples were imaged using 100× objective lenses.

Super-resolution imaging

Super-resolution images were obtained using a Vutara SR200 STORM (Bruker) microscope based on single-molecule localization biplane technology. His-tagged bacteria decorated with compound **3** were imaged using a 640 nm excitation laser and a 405 nm activation laser in an imaging buffer composed of cysteamine (5 mM), 7 μM glucose oxidase, 56 nM catalase, and 10% glucose in 50 mM Tris, 10 mM NaCl, at pH 8.0. Images were recorded using a ×60 NA 1.2 water immersion objective (Olympus) and an Evolve 512 EMCCD camera (Photometrics) with gain set at 50, the frame rate at 50 Hz, and a maximal power of 647, and 405 nm lasers were set at 6 and 0.05 kW/cm², respectively. The total number of frames acquired was 8000. Data were analysed by Vutara SRX software.

Microscale thermophoresis (MST)

The affinity of the tri-NTA (**2**), bis-NTA (**4**), and mono-NTA (**5**) probes to a His-tagged protein (His₆-P53) was measured using MST on a micro MST Monolith NT.115 instrument (NanoTemper Technologies, Munich, Germany). The concentrations of probes **2**, **4**, and **5** were kept constant. Each probe was separately incubated with NiCl₂ (5, 4, and 2 eq., respectively) for 30 min in PBST buffer (137 mM NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, at pH 7.4, 0.05% Tween-20). His tagged P53 was serially diluted in twofold dilutions and then the probe sample was added to each dilution series. The resulting protein-probe samples were centrifuged at 21,000 g at 4 °C for 10 minutes. After ensuring that there was no precipitation of the protein, the samples were loaded into Monolith NT.115 MST premium-coated capillaries. For probe **2** and **4**, MST measurements were conducted at 20% LED power and 20% MST power. For probe **5**, changes in the initial fluorescence were measured to determine the dissociation constant after performing SDS test successfully.^[5] The experimental data were analyzed using NT analysis and MO affinity analysis (version 2.2.7) software, and were fitted according to the following equation;

$$FB = \frac{1}{2[L]} ([L] + [P] + K_d - \sqrt{([L] + [P] + K_d)^2 - 4[L][P]})$$

Where FB is fraction bound, [L] is the probe concentration that is kept constant, [P] is the concentration of the protein, [LP] is the concentration of a bound complex of L and P, and K_d is the dissociation constant. The experiments were performed in triplicate. No binding was observed in control experiments which were performed without Ni²⁺ and with 5 mM EDTA.

Gel electrophoresis

The bacterial cells (OD₆₀₀ of 2) were collected by centrifugation at 6,000 g for 4 min. The supernatant was discarded and the pellet was washed with 1 mL of lysis buffer (10 mM Na₂HPO₄, pH=7.2), followed by centrifugation at 6,000 g for 10 min. The cell pellets were resuspended in 1 mL of lysis buffer containing a 2% protease inhibitor cocktail, and lysed by sonication for 2 min. The resulting cell lysate was centrifuged for 2 min at 12,000 rpm and the pellet was discarded. The supernatant was further centrifuged at 12,000 rpm for 30 min, and the resulting pellet was washed with the buffer and re-suspended in 170 μL of buffer. Next, 60 μL of the sample was run on Mini-PROTEAN TGX gels, 8-16% gradient gels (BioRad, Hercules, CA) at 120V for 90 min. The gels were stained with Instant Blue Coomassie Protein Stain and imaged using a BioRad ChemiDoc XRS+ imager.

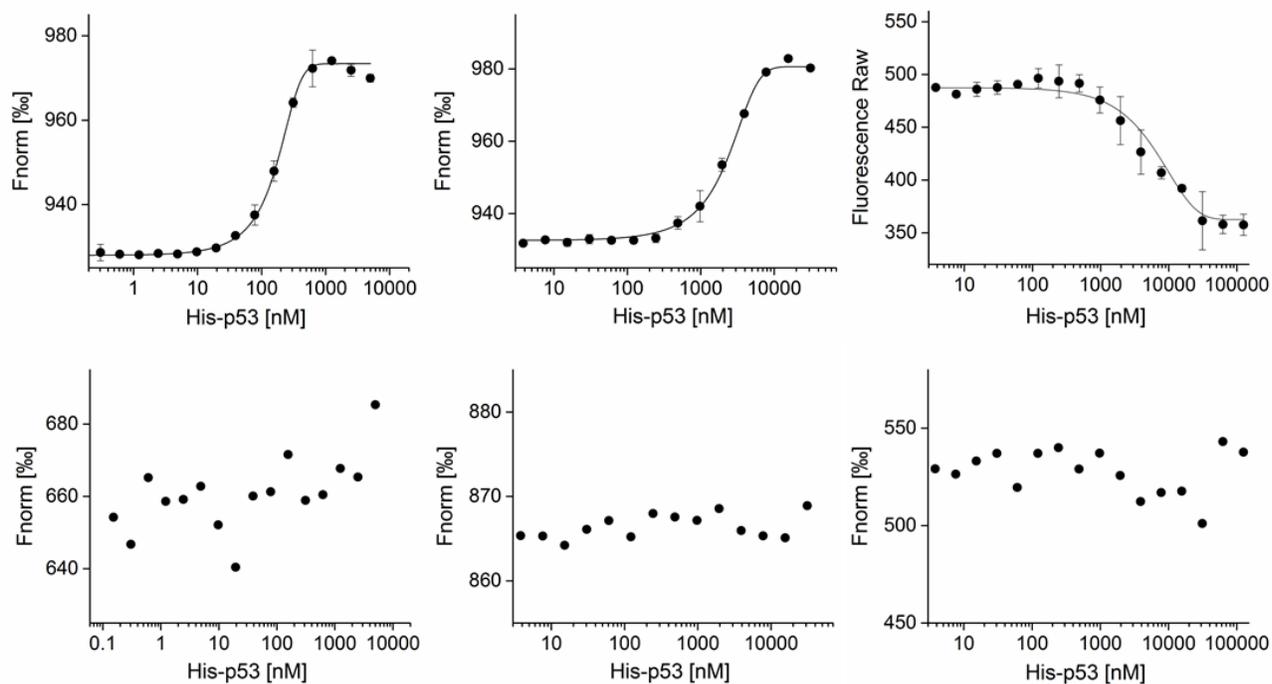


Fig. S1. MST analysis. (Top) Dose-response curves of compounds **2** (left), **4** (middle), and **5** (right) interacting with His₆-p53. (Bottom) The control experiments which were performed without Ni²⁺ and with 5 mM EDTA.

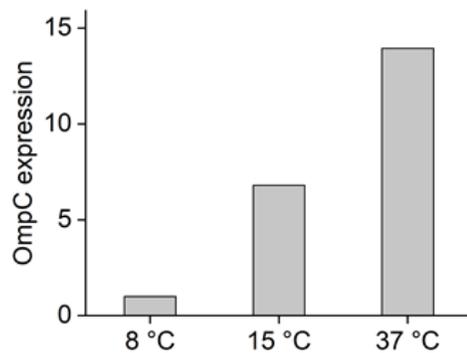


Fig. S2. Relative expression of His-OmpC in bacteria that were cultured at 8 °C, 15 °C, or 37 °C, based on quantification of the protein bands in the gel electrophoresis.

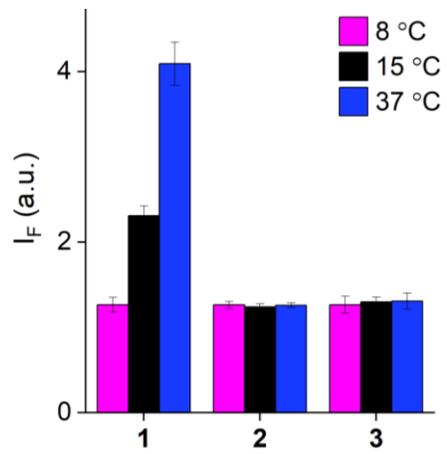
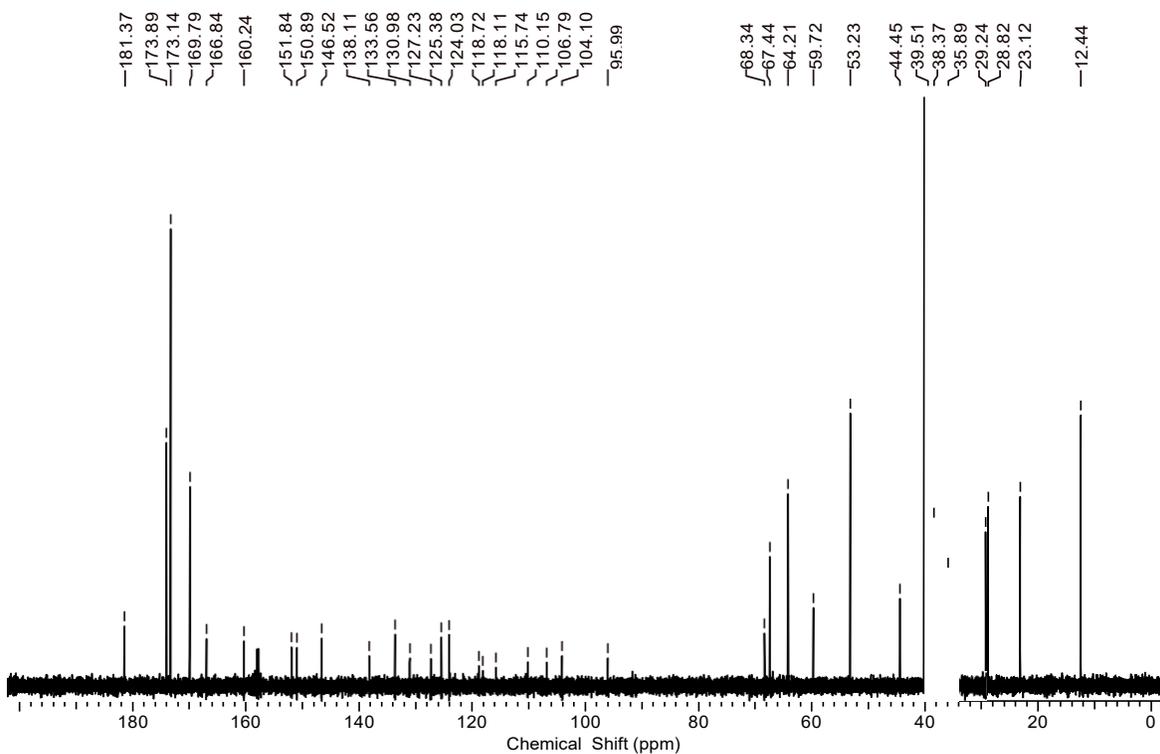
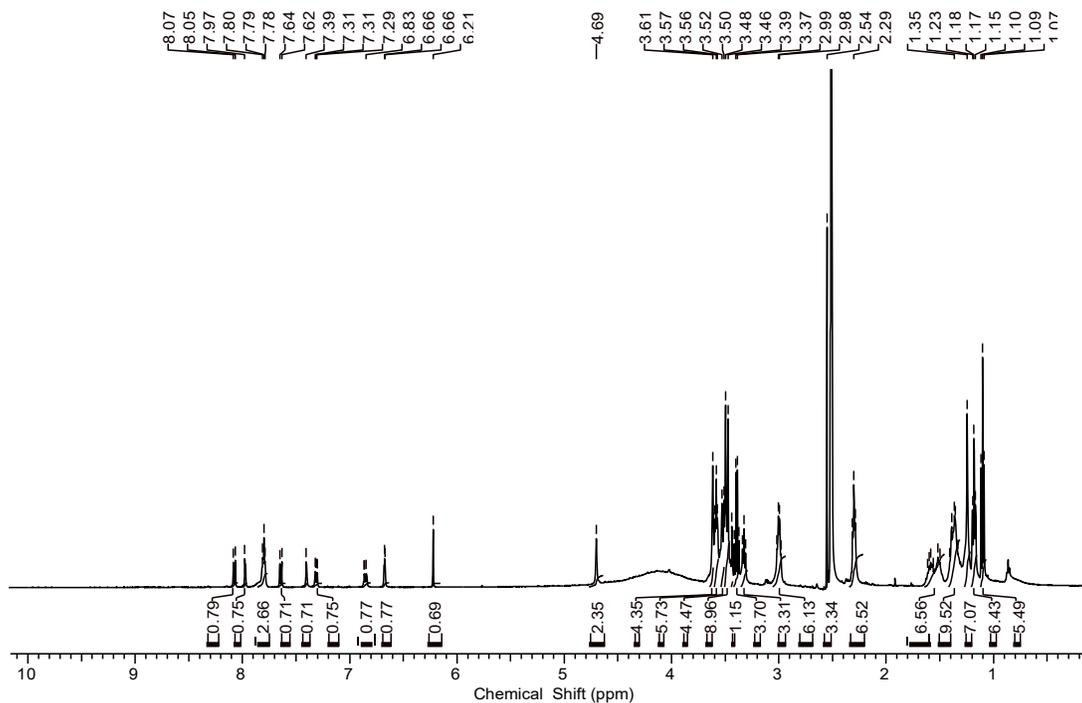


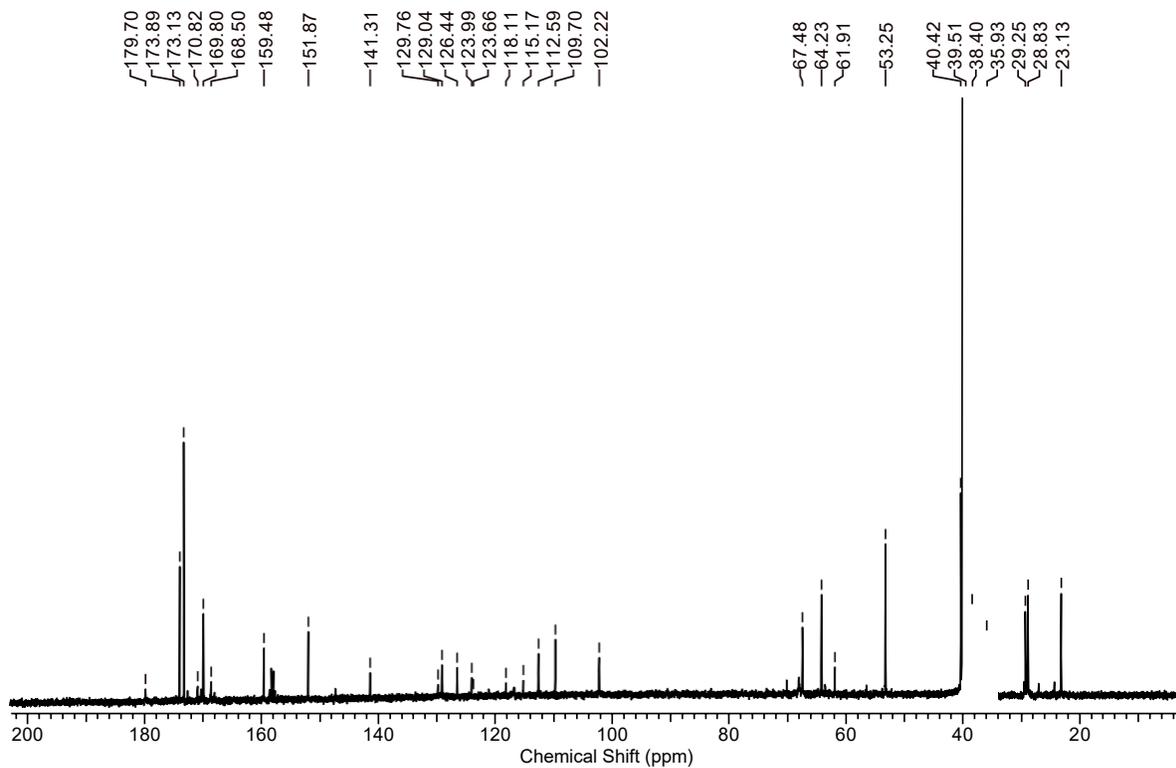
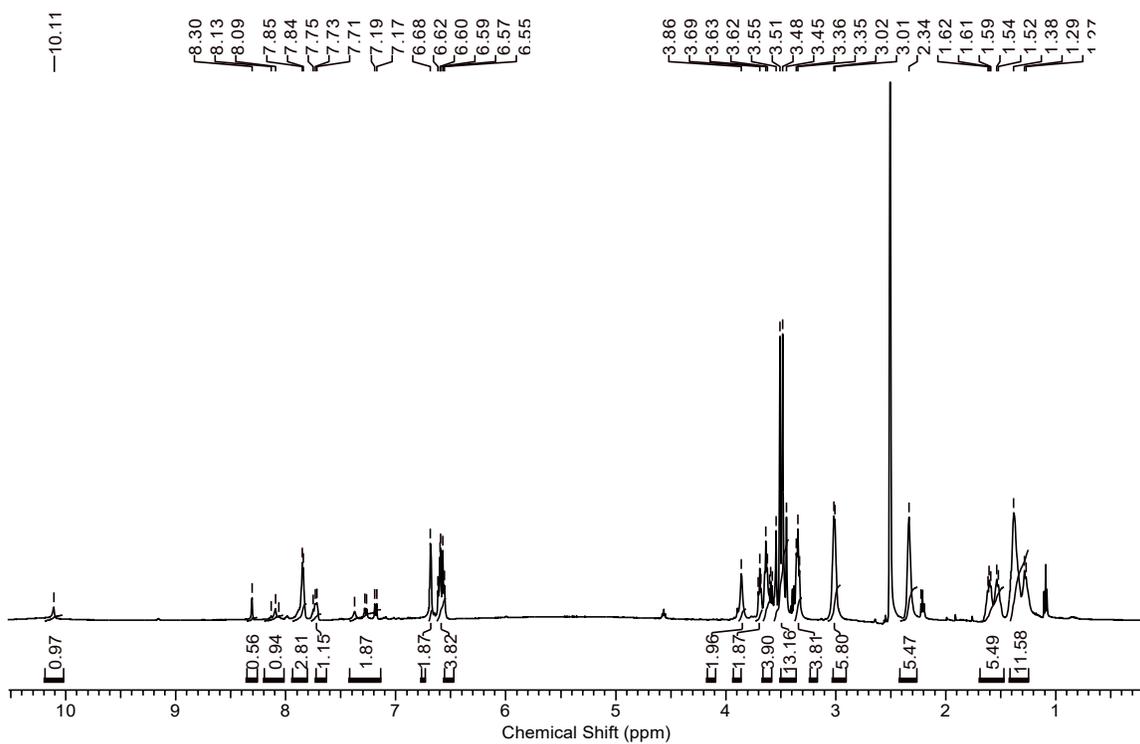
Fig. S3. Fluorescence enhancement of probes **1**, **2**, and **3** in response to bacteria expressing His-OmpC bacteria cultured at different temperatures. These experiments were performed in triplicate.

^1H and ^{13}C NMR Spectra of compounds 1-5

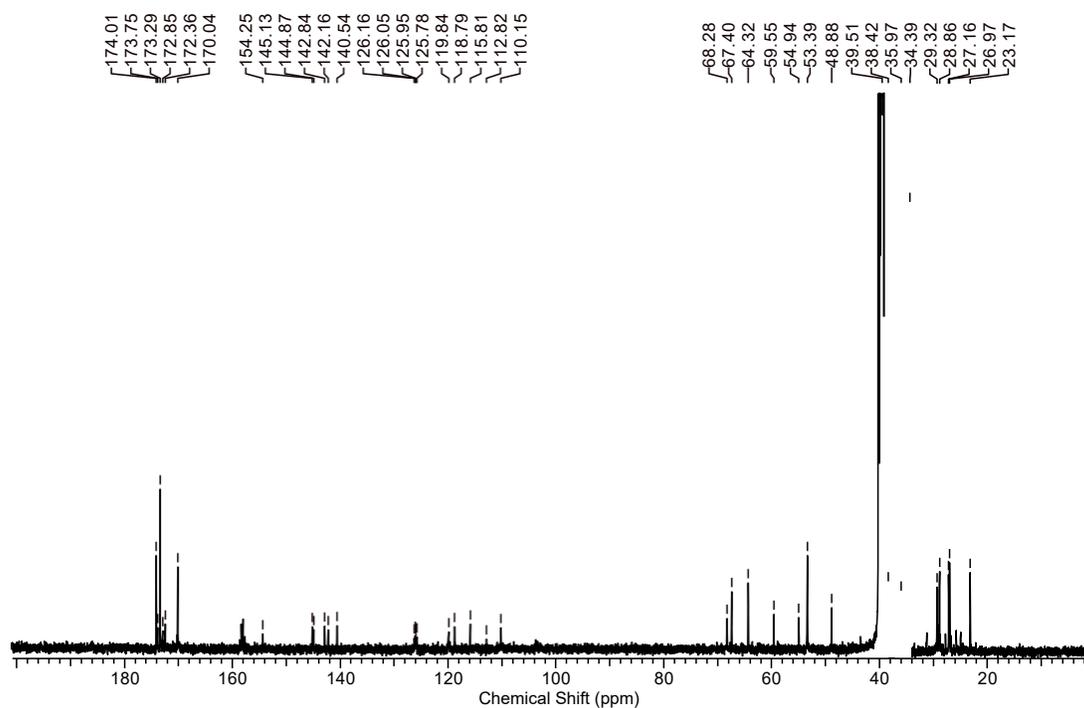
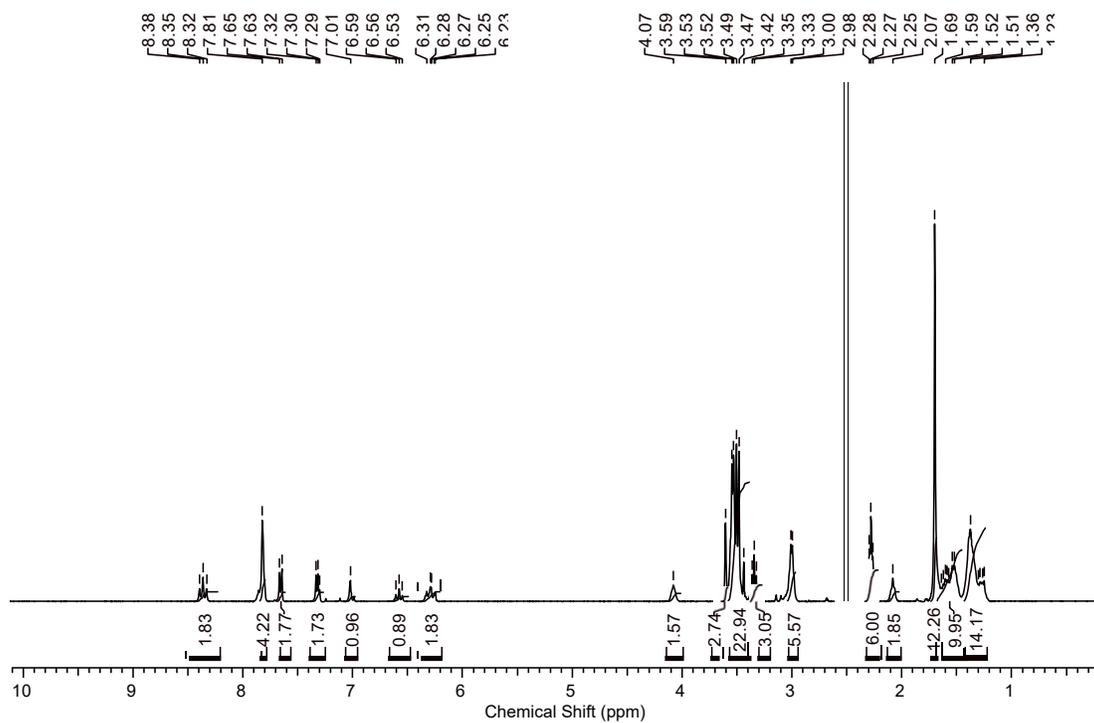
^1H and ^{13}C NMR Spectra of 1



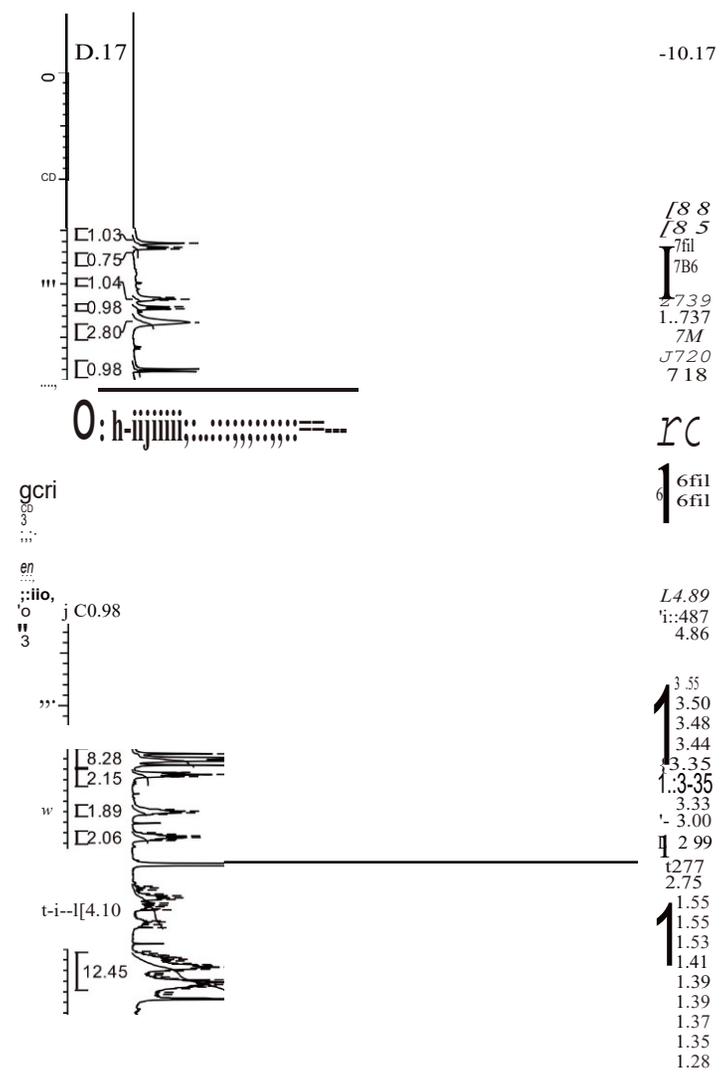
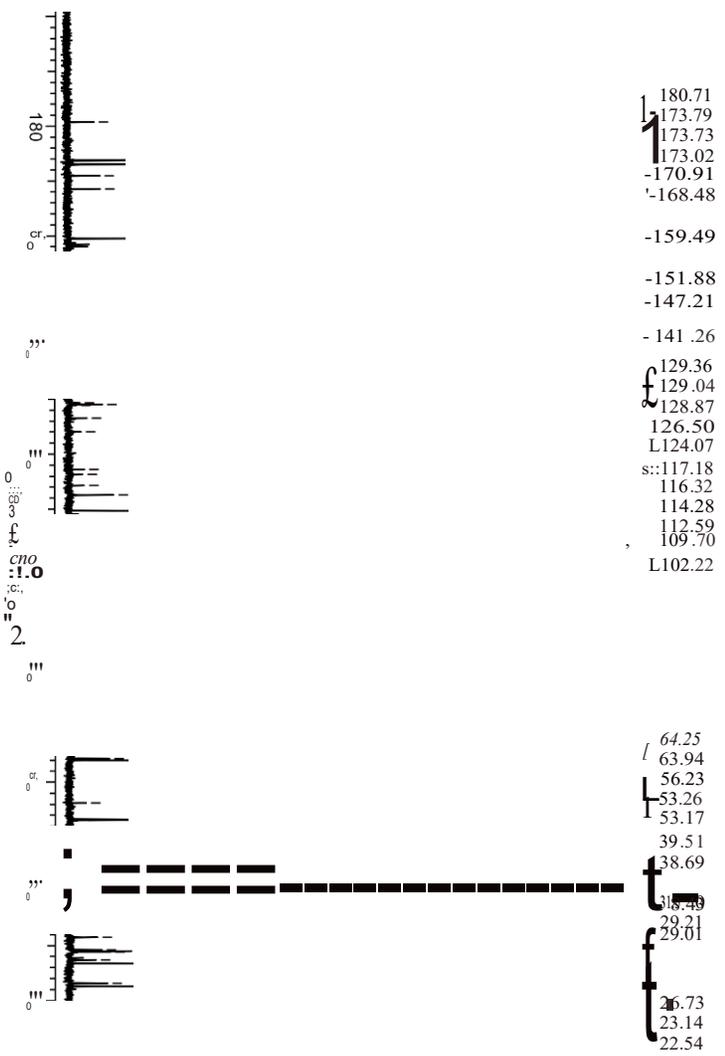
^1H and ^{13}C NMR Spectra of **2**



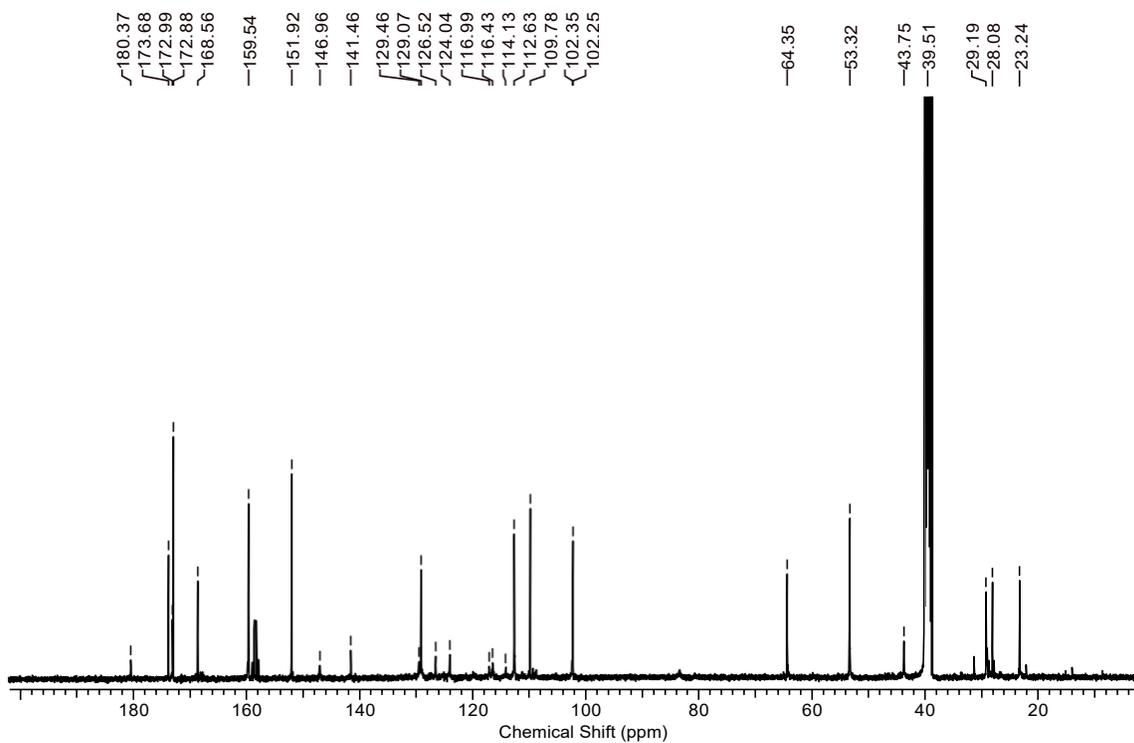
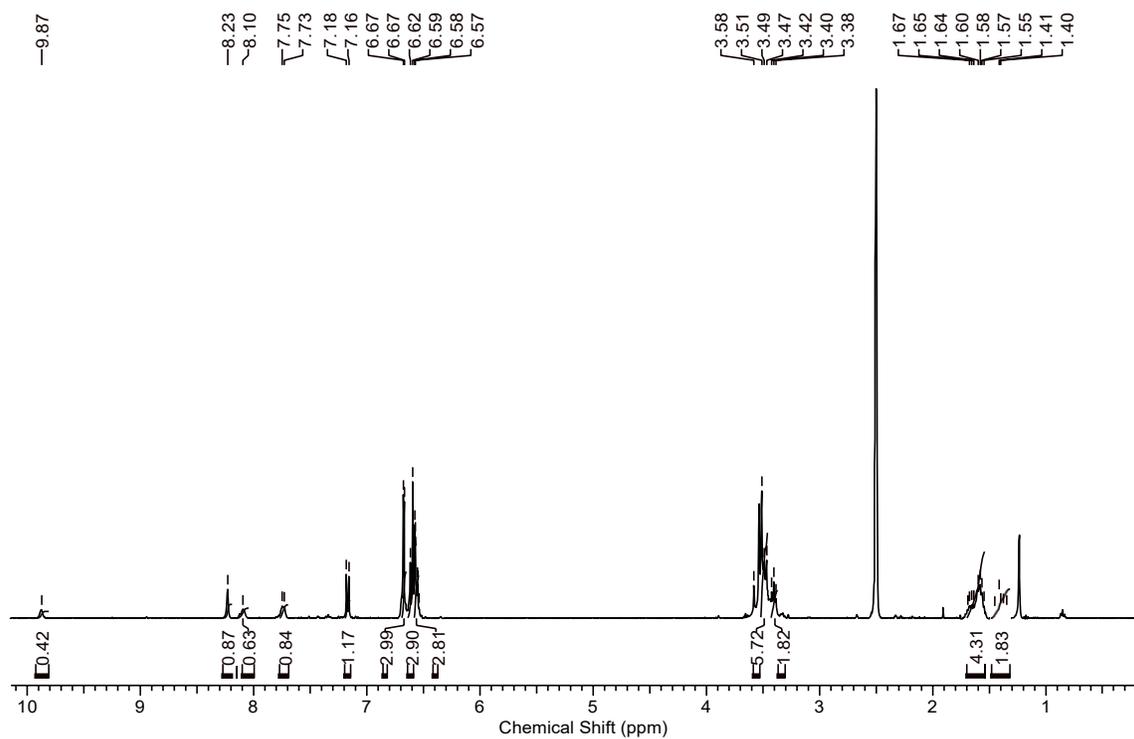
^1H and ^{13}C NMR Spectra of **3**



¹H and ¹³C NMR Spectra of 4



^1H and ^{13}C NMR Spectra of **5**



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