

Supporting Information

Site-specific immobilization of biomolecules by a biocompatible reaction between cysteine and 2-cyanobenzothiazole

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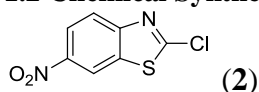
1. Material and Methods

1.1 Reagents and instruments

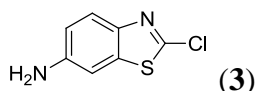
All chemicals and solvents were purchased from commercial suppliers and applied directly in the experiment without further purification. The progress of the reaction was monitored by TLC on pre-coated silica plates (Merck 60 F254, 250 μm thickness), and spots were visualized by KMnO_4 , UV light or iodine. Merck silica gel 60 (70-200 mesh) was used for column chromatography purification. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker model DPX-400 MHz NMR spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($\text{Si}(\text{CH}_3)_4 = 0.00$ ppm) or residual solvent peaks ($\text{CHCl}_3 = 7.26$ ppm). ^1H NMR coupling constants (J) are reported in Hertz (Hz), and multiplicity is indicated as the following: s (singlet), d (doublet), t (triplet), m (multiplet), dd (doublet of

doublet). Mass spectra were obtained on PC Sciex API 150 EX ESI-MS system. High resolution mass spectrum was carried out on ABI Qstar Elite Q-TOF. Fluorescence signal was recorded with a FluoroMax-4 fluorescence photometer. Fluorescence images were acquired using a Leica TCS SPE Confocal Scanning Microscope.

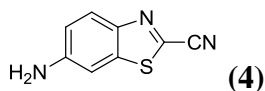
1.2 Chemical Synthesis



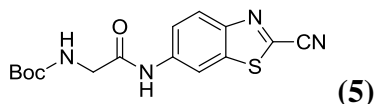
2-Chlorobenzothiazole (2.6 g, 11.8 mmol) was added dropwise to H₂SO₄ (concentrated, 15 mL) in ice-water bath. Potassium nitrate (1.31 g, 12.9 mmol) was then added. The resulting mixture was stirred at ice-water bath for 30 minutes and then at room temperature for 1 hour. The solution was subsequently poured onto ice. The precipitate was obtained by filtration and further purified with chromatography (hexane/ethyl acetate = 10:1-3:1) to give compound 1 (2.6 g, 78.8%). ¹H NMR (300 MHz, CDCl₃) δ 8.75 (d, *J* = 2.1 Hz, 1H), 8.40 (dd, *J*₁ = 2.1 Hz, *J*₂ = 9.0 Hz, 1H), 8.08 (d, *J* = 9.0 Hz, 1H); LC-MS (ESI) calcd for [M+H]⁺:214.96, found: 214.95.



Tin(II) chloride dehydrate (4.0 g, 17.7 mmol) was added to the solution of compound 2 (500 mg, 2.3 mmol) in the mixture of ethanol (38 mL), water (25 mL) and hydrochloric acid (4.8 M in water, 5 mL). The resulting mixture was stirred at reflux for 4 hours. The reaction mixture was subsequently cooled down and basified with aqueous sodium hydroxide solution. The precipitate was removed by filtration. The desired filtrate was concentrated under reduced pressure, and the residue was extracted with ethyl acetate (30 mL × 2). The combined organic phase was washed with brine (30 mL × 1), dried over sodium sulfite, concentrated and purified with chromatography (hexane/ethyl acetate = 5:1-2:1) to give compound 2 (249 mg, 57.9%). ¹H NMR (500 MHz, CDCl₃) δ 7.70 (d, *J* = 8.5 Hz, 1H), 6.98 (d, *J* = 2.5 Hz, 1H), 6.81 (dd, *J*₁ = 2.1 Hz, *J*₂ = 9.0 Hz, 1H), 3.84 (s, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 148.4, 144.9, 144.1, 137.8, 123.3, 115.6, 105.1; LC-MS (ESI) calcd for [M+H]⁺:184.99, found: 184.95.

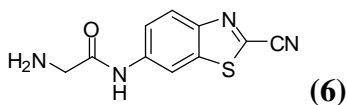


Potassium cyanide (1.0 g, 15.3 mmol) was added to DMSO (80 mL), and the solution was heated at 135 °C under an Ar atmosphere overnight. The temperature was then lowered to 120 °C, and a solution of compound 3 (840 mg, 4.32 mmol) in DMSO was added. When TLC showed compound 3 completely used up, the reaction mixture was cooled down and poured into a solution of potassium dihydrogen phosphate in water (1.0 M, 200 mL). The aqueous layer was then extracted with ethyl acetate (50 mL × 2). The combined organic layer was washed with water (50 mL × 2) and brine (50 mL × 1), dried over sodium sulfite, concentrated, and purified with chromatography (hexane/ethyl acetate = 5:1-2:1) to give compound 3 (200 mg, 24.9%). ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.70 (d, *J* = 9.0 Hz, 1H), 7.16 (d, *J* = 2.1 Hz, 1H), 6.99 (dd, *J*₁ = 2.1 Hz, *J*₂ = 9.0 Hz, 1H), 6.11 (brs, 2H); ¹H NMR (75 MHz, DMSO-*d*₆) δ 151.0, 143.7, 138.8, 128.2, 125.5, 117.8, 114.7, 102.7; LC-MS (ESI) calcd for [M+H]⁺:176.02, found: 176.25.



N-methyl morpholine (NMP) (450 mg, 4.44 mmol) and isobutyl chlorformate (473 mg, 3.46 mmol) was added to the solution of Boc-glycine (680 mg, 3.88 mmol) in THF (10 mL) in ice-water bath. The resulting mixture was stirred for 20 minutes in ice-water bath. Compound 4 (170 mg, 0.96 mmol) was then added. The mixture was stirred at ice-water bath for 2 hours and then at room temperature for 2 hours. It was

subsequently diluted with ethyl acetate (100 mL) and washed with saturated NaHCO₃ aqueous solution (30 mL × 1) and brine (30 mL × 1), dried with sodium sulfite, concentrated, and purified with chromatography (hexane/ethyl acetate = 10:1-3:1) to give compound **5** (192 mg, 57.8%). ¹H NMR (300 MHz, CDCl₃) δ 8.91 (brs, 1H), 8.62 (d, *J* = 2.1 Hz, 1H), 8.10 (d, *J* = 9.0 Hz, 1H), 7.42 (dd, *J*₁ = 2.1 Hz, *J*₂ = 9.0 Hz, 1H), 5.34 (m, 1H), 3.99 (d, *J* = 6.0 Hz, 1H), 1.49 (s, 9H); ¹³C NMR (75 MHz, CDCl₃) δ 168.4, 148.7, 138.1, 136.8, 135.3, 125.3, 120.5, 112.9, 111.3, 81.3, 77.2, 46.0, 28.2; LC-MS (IT-TOF) calcd for [M+H]⁺:333.10, found: 333.09.



TFA (4 mL) was added to the solution of compound **5** (180 mg, 0.54 mmol) in DCM (10 mL). The resulting solution was stirred at room temperature for 8 hours. All the solvent was then removed under reduced pressure, and the desired residue was further purified by prep-HPLC to give the final compound (40 mg, 22.6%). ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.03 (s, 1H), 8.71 (d, *J* = 2.1 Hz, 1H), 8.25 (m, 4H), 7.79 (dd, *J*₁ = 2.1 Hz, *J*₂ = 9.0 Hz, 1H), 3.88 (s, 2H); ¹H NMR (75 MHz, DMSO-*d*₆) δ 166.0, 148.4, 139.1, 137.2, 136.1, 125.5, 121.0, 114.0, 112.1, 41.7; LC-MS (IT-TOF) calcd for [M+H]⁺:233.04, found: 233.04.

1.3 Protein Expression and Purification

1.3.1 Preparation of N-Cys EGFP

pTwin1-intein-EGFP plasmid was transformed into an expression host ER2566. The transformed bacterial cells were grown in LB medium supplemented with ampicillin (100 µg/mL) at 37 °C in an orbital shaker. When the cell density reaches 0.6, a solution of IPTG (0.5 mM) was added to the cell culture to induce protein expression. The cells were left shaking overnight at room temperature. On the following day, the cells were harvested by centrifugation at 4000 rpm for 30 min. The resulting cell pellets were resuspended in lysis buffer (20 mM Tris buffer, pH = 8.5, 500 mM NaCl, 1mM EDTA) and lysed by sonication method on ice. The cell debris was then pelleted down by centrifugation at 13,000 rpm and 4 °C for 30 min.

The column was packed with 1 ml chitin beads and washed with column buffer (20 mM Tris buffer, pH = 8.5, 500 mM NaCl, 1mM EDTA) for three times. The crude cellular lysates were loaded onto the column and incubated at 4 °C for 30 min. The beads were then washed with column buffer for five times. 1 ml of cleavage buffer (20 mM Tris buffer, pH = 7.0, 500 mM NaCl, 1mM EDTA) was subsequently added to the column and incubated with agitation at room temperature for 20 hours. Finally the proteins were eluted out and dialyzed into PBS buffer.

1.3.2 Preparation of N-His EGFP

Pdest17-EGFP was transformed into an expression host BL21-Ai. The transformed bacterial cells were grown in LB medium supplemented with ampicillin (100 µg/mL) at 37 °C in an orbital shaker. When the cell density reaches 0.6, a solution of L-arabinose (final concentration 0.2%) was added to the cell culture to induce protein expression. The cells were then left shaking at room temperature overnight. On the following day, the cells were harvested by centrifugation at 4000 rpm for 30 min. The resulting cell pellets were resuspended in lysis buffer (20 mM Tris buffer, pH = 8.0, 10 mM imidazole) and lysed by sonication method on ice. The cell debris was subsequently pelleted down by centrifugation at 13,000 rpm and 4 °C for 30 min.

The column was packed with 200 µl of Ni-NTA beads and washed with lysis buffer for three times. The crude cellular lysates were loaded onto column and incubated at 4 °C for 1 hour. The beads were washed with washing buffer (20 mM Tris buffer, pH = 8.0, 20 mM imidazole) for three times. Finally the proteins were eluted out with elution buffer (20 mM Tris buffer, pH = 8.0, 200 mM imidazole) and dialyzed into PBS buffer.

1.4 Preparation of CBT-functionalized Slide

Glass slides (Sigma-Aldrich) were immersed in a piranha solution (sulfuric acid/hydrogen peroxide, 7:3) overnight before slide functionalization. An amine handle was first introduced to the slides by silanization with (aminopropyl) triethoxysilane in ethanol. The amine slides were then reacted with succinic anhydride to yield carboxylic acid-functionalized slides. Following that, a solution of N-hydroxysuccinimide, HBTU and DIEA were applied onto glass surface to generate NHS-derivatized slides. Finally, a solution of glycine-CBT in 10 mM of NaHCO₃ (pH 9) was incubated with the slides for 40 min. The slides were then washed with ethanol, water and air dried. The unreacted NHS groups were quenched with a solution of 2 mM aspartic acid in NaHCO₃ buffer (500 mM, pH = 9.0). These slides were then washed with deionized water, dried and set for printing.

1.5 Microarray Preparation

For the small molecule immobilization experiment, various concentrations of Cys-TER in the spotting buffer (PBS: DMSO = 1:1) were prepared in 384 well before spotting. For the protein immobilization experiment, different concentrations of proteins were prepared in PBS buffer supplemented with 0.1 mM of TCEP. The spotting process was performed using an ESI SMA arrayer (Ontario, Canada) followed by incubation in a humidity chamber. After the incubation, the slides were washed with PBST buffer (PBS with 0.5% Tween 20), rinsed with deionized water and dried with nitrogen. The slides were then scanned with a microarray scanner (Tecan Trading AG).

1.6 Peptide Synthesis

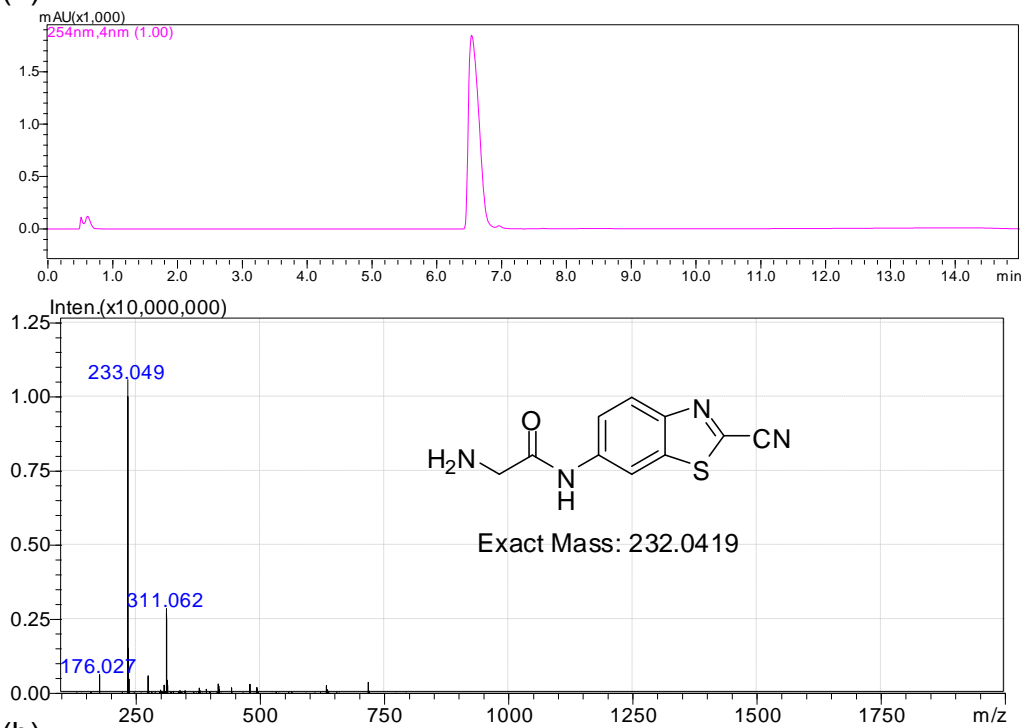
Peptides were synthesized using standard Fmoc chemistry with a microwave peptide synthesizer (Liberty 1). Rink amide resin was used as solid support. Amino acid residues were coupled to the resin through HOBT/HBTU/DIEA method. The Fmoc group was deprotected with 20% piperidine. At the end of the peptide coupling, the peptides were cleaved from the resin using a TFA cleavage cocktail (92.5% TFA, 2.5% EDT, 2.5% H₂O and 1% TIS). The peptides were then purified by HPLC before slide coating process.

1.7 Cell Adhesion Study

NIH-3T3 fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and an appropriate amount of antibiotics (penicillin and streptomycin). Glass slides were coated with 200 μM of peptides in a humid chamber for 3 hours. Excess peptide substrates were washed away using PBS buffer and ethanol. Subsequently the cells were seeded onto glass slides coated with different peptide substrates and incubated in an incubator for 48 hours. The cells were then imaged with a confocal microscope (Leica TCS SPE).

2. Supplementary Figures

(a)



(b)

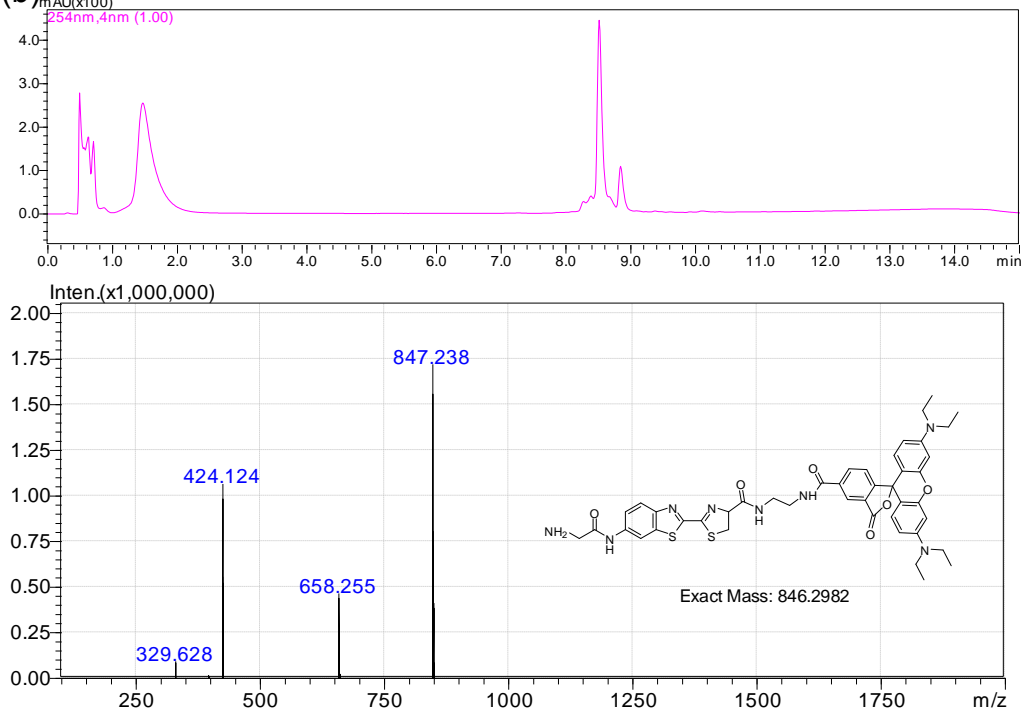


Fig. S1. LC-MS analysis of the reaction between CBT and cysteine-TER in PBS buffer (10 mM, pH =7.4). (a) CBT only. (b) The reaction mixture after 1h. Both peaks (8.0-9.0 min) show the target molecular weight.

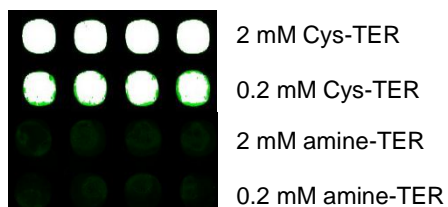


Fig. S2. Small molecule immobilization experiment. The negative control (amine-TER) did not show noticeable fluorescence signal under the same experimental conditions.

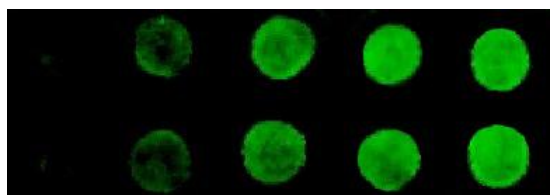


Fig. S3. Different concentrations of N-Cys-EGFP were immobilized onto CBT-functionalized slides (From left to right: 0.2, 1, 5, 25, 50 μ M).

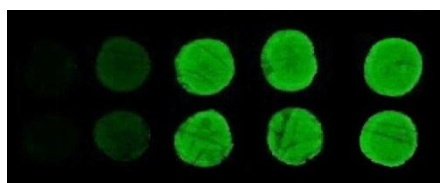


Fig. S4. Time course experiment of N-Cys-EGFP immobilization onto microarray (From left to right : 15, 30, 60, 120, 180 min).

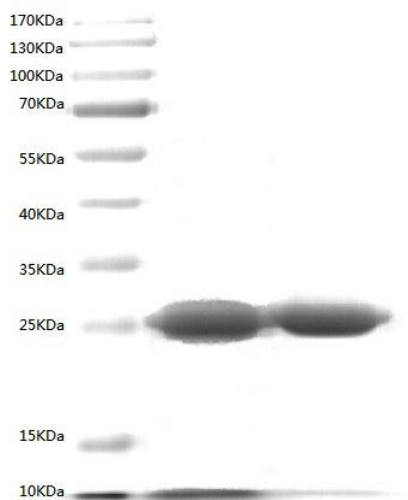
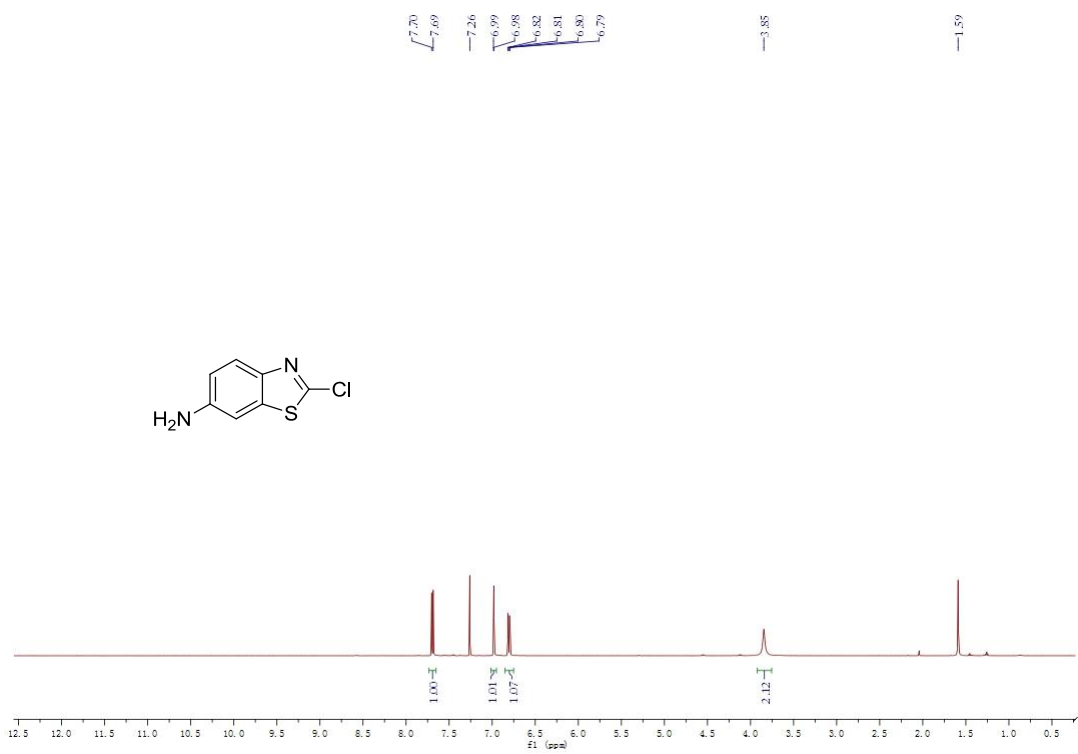
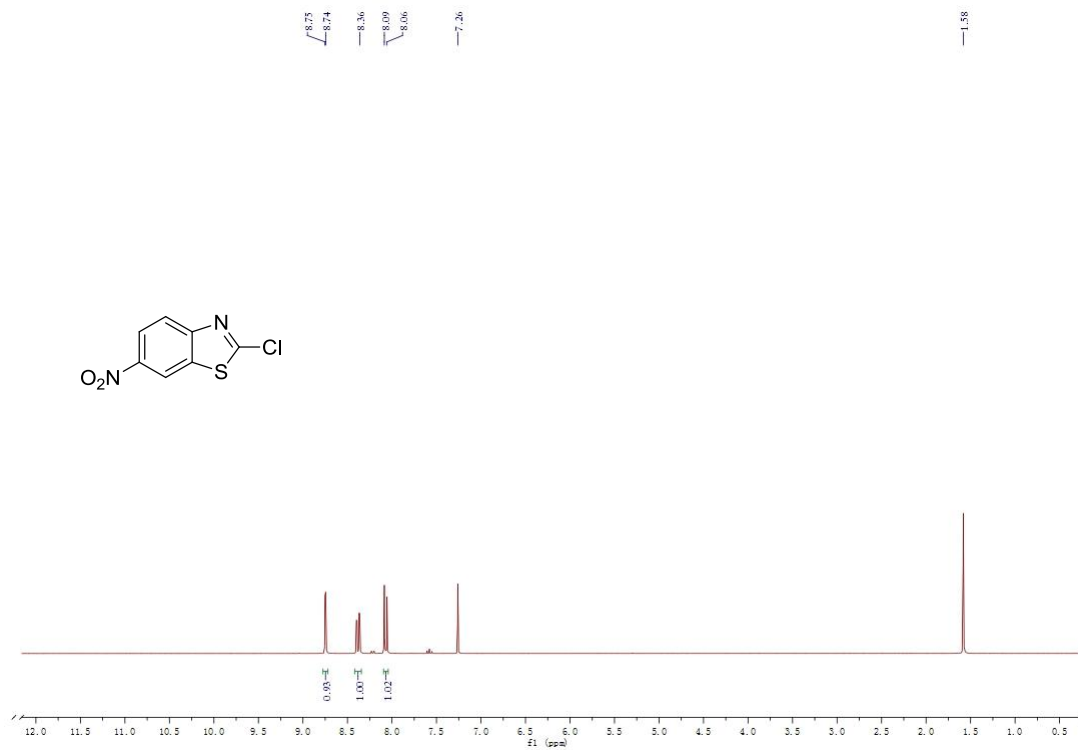
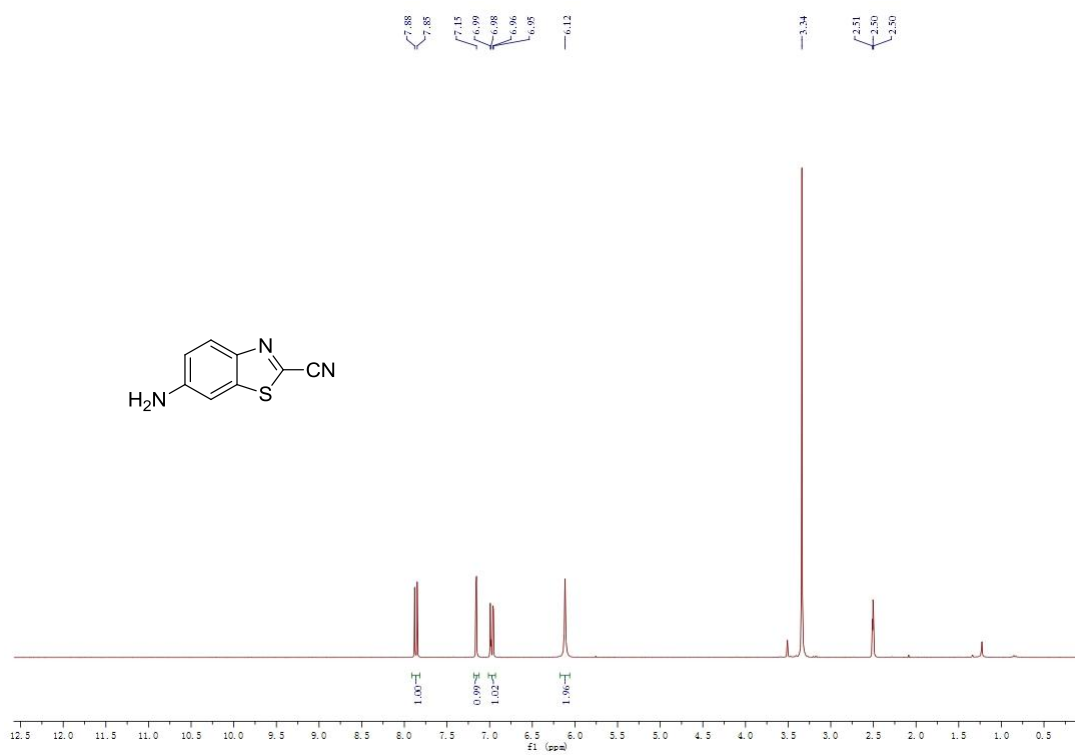
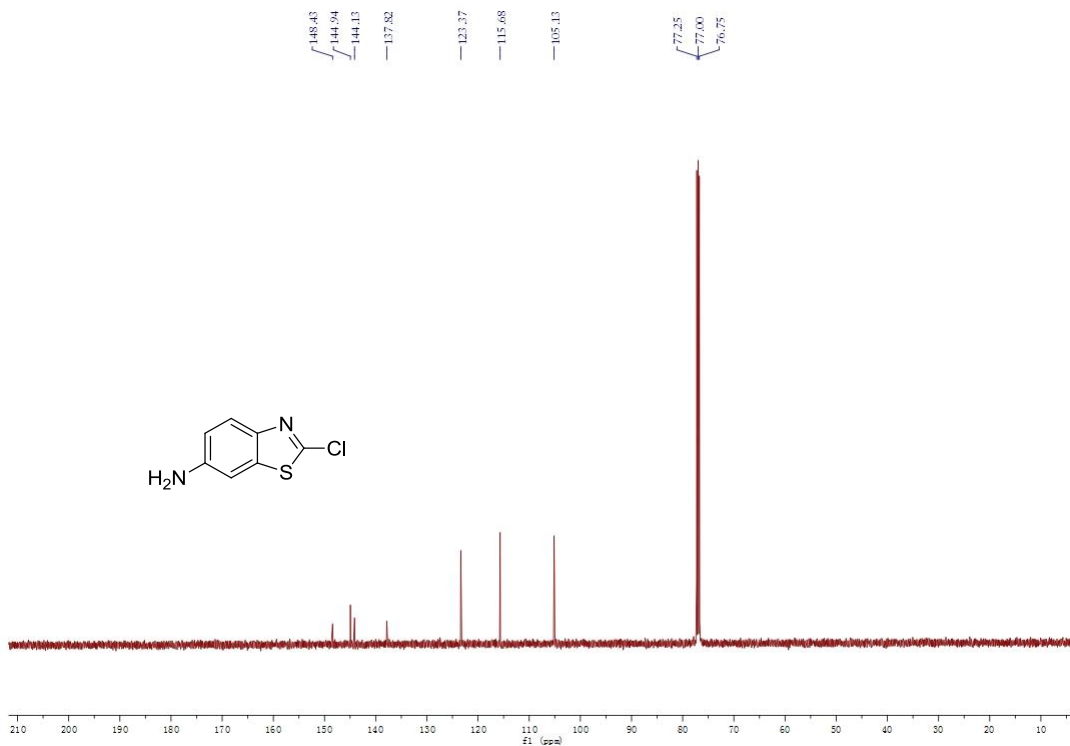
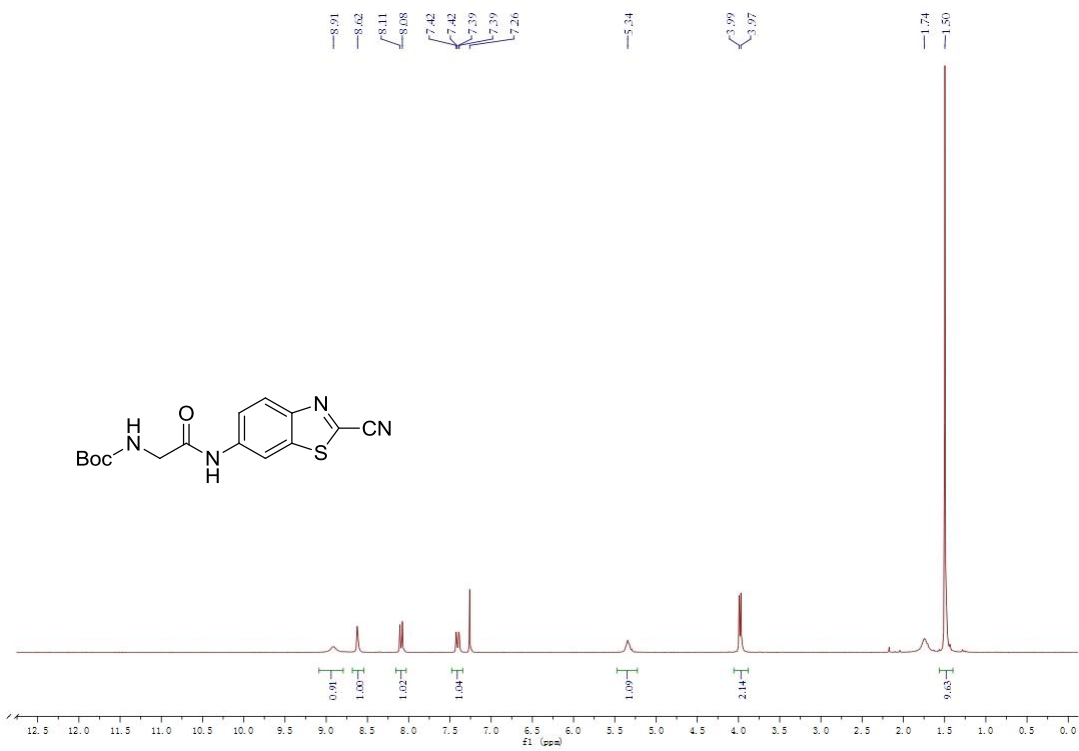
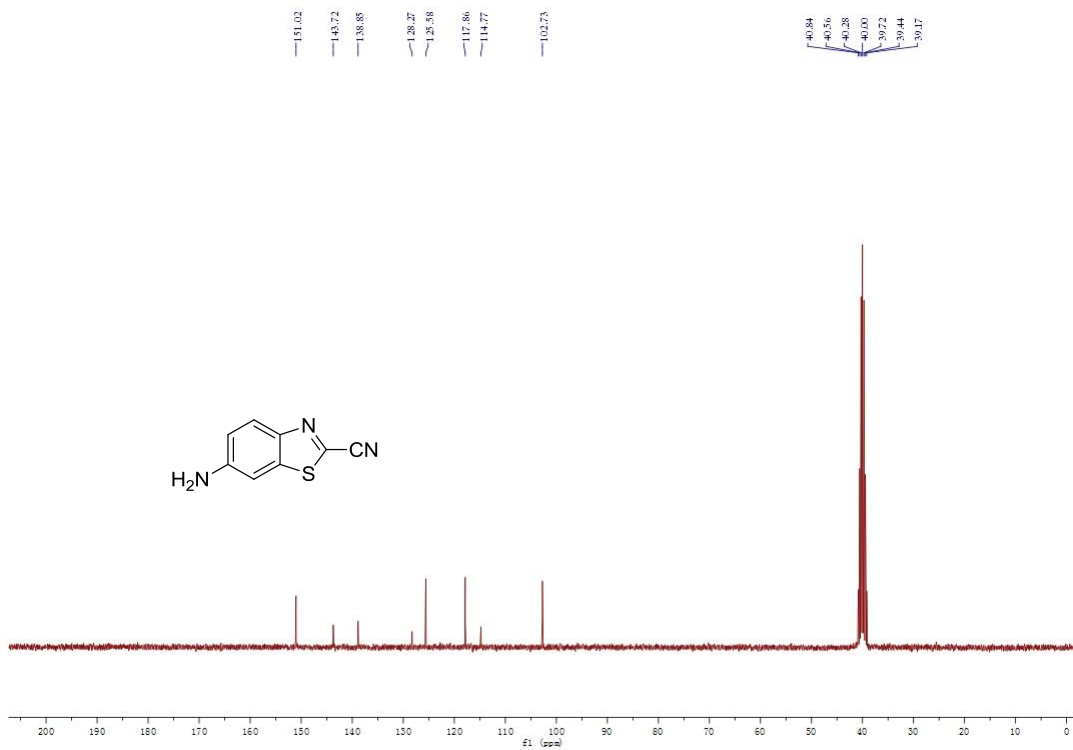
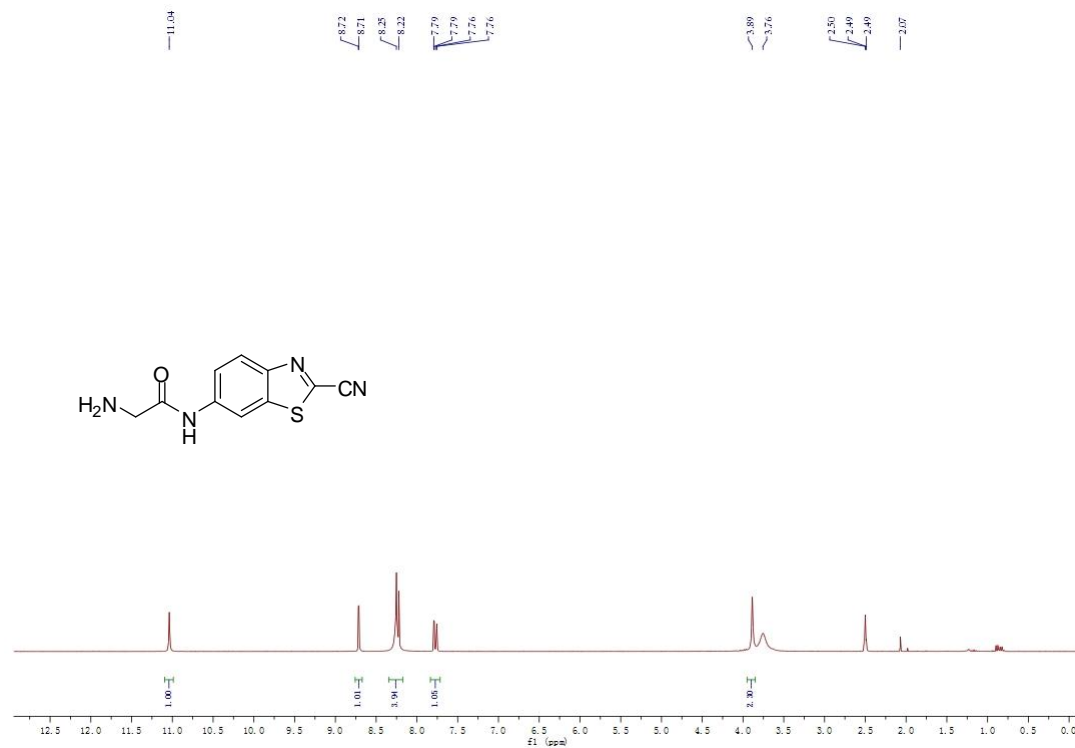
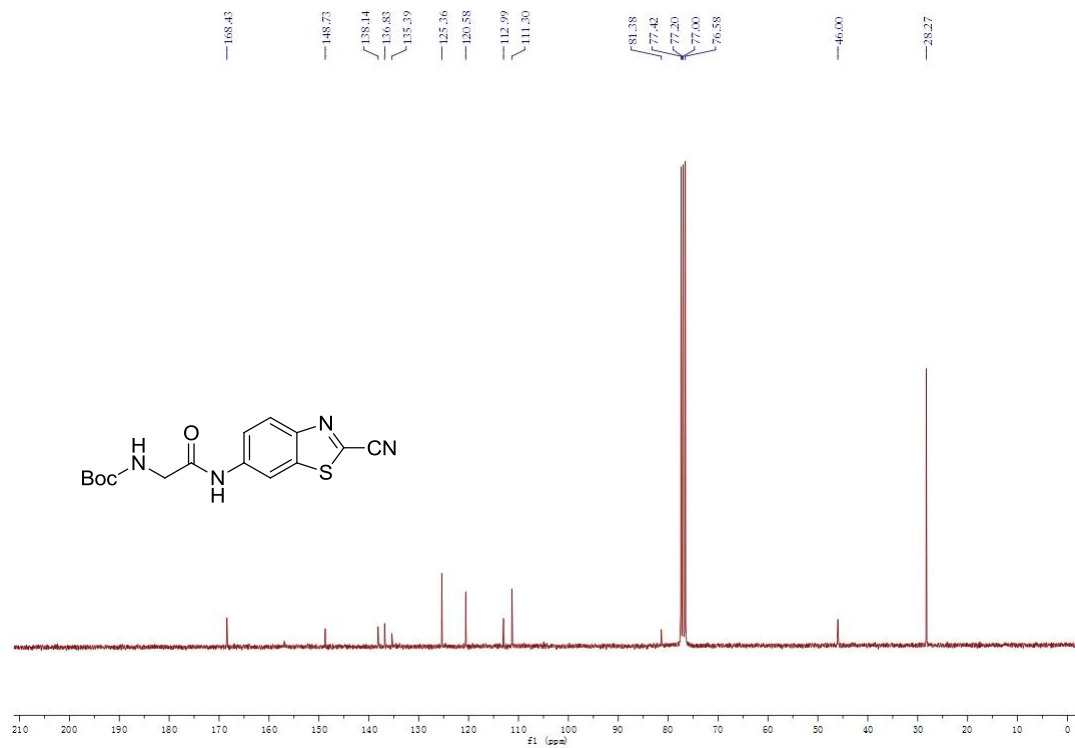


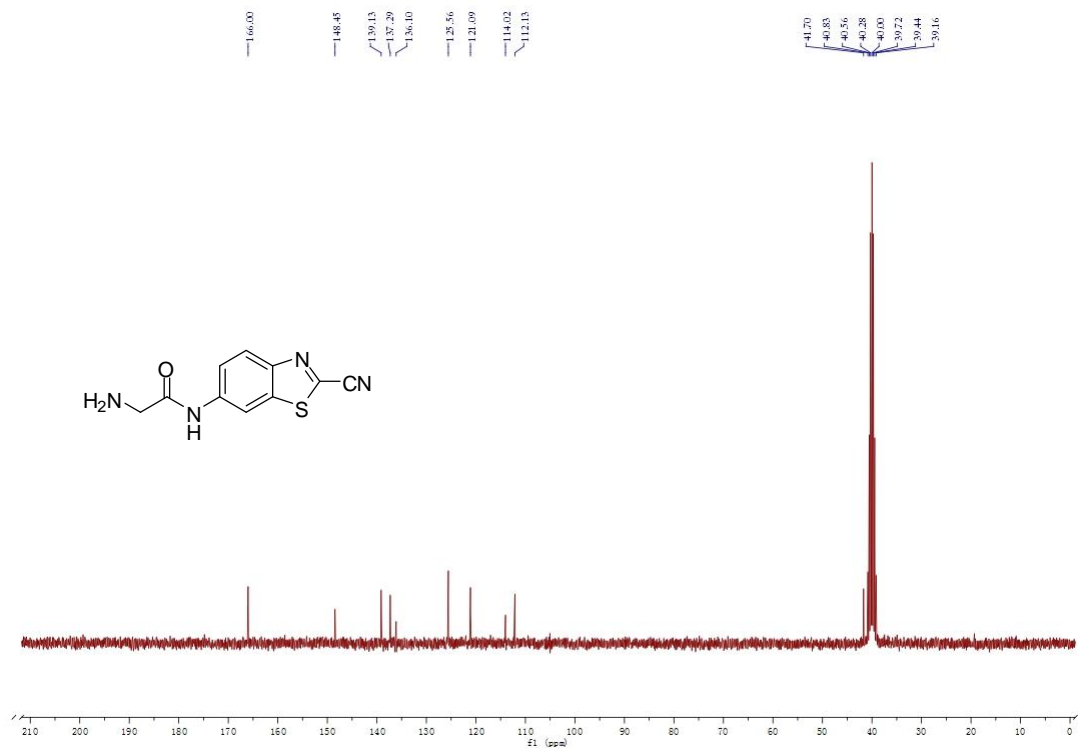
Fig. S5. SDS PAGE analysis of protein (from left to right: protein marker, N-His-EGFP, N-Cys-EGFP).











Reference:

[1]. Hideo Takakura, Ryosuke Kojima, Yasuteru Urano, Takuya Terai, Kenjiro Hanaoka and Tetsuo Nagano, *Chem. Asian J.* **2011**, 6, 1800 – 1810.

[2]. Hongjun Ren, Fei Xiao, Ke Zhan, Young-Pil Kim, Hexin Xie, Zuyong Xia, and Jianghong Rao, *Angew. Chem. Int. Ed.* **2009**, 48, 9658 –9662.