Supporting Information for

Expanding the Functionality of Proteins with Genetically Encoded Dibenzo[*b*,*f*][1,4,5]thiadiazepine: a Photo-Transducer for Photo-Click Decoration

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General Information

Unless otherwise indicated, all solvents and starting materials were purchased from commercial sources and used directly without further purification. Anhydrous solvents, purchased from Acros Organics (DMF and THF), were used as received. Commercially available chemicals were obtained from Adamas, Acros Organics, Aldrich Chemical Co., Alfa Aesar and TCI and used as received unless otherwise stated. The ^{1}H, ^{13}C spectra were recorded on a Brüker Avance spectrometer (^{1}H: 400 MHz, ^{13}C: 101 or 150 MHz). Chemical shifts (δ) for ^{1}H and ^{13}C NMR spectra are given in ppm relative to TMS. The residual solvent signals were used as references for ^{1}H and ^{13}C NMR spectra and the chemical shifts converted to the TMS scale ($CDCl_3$, 7.26 ppm for ^{1}H NMR and 77.16 ppm for ^{13}C NMR; DMSO-*d*$_6$, 2.50 ppm for ^{1}H-NMR and 39.5 ppm for ^{13}C-NMR). Shifts multiplicity was reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, brs. = broad. Exact ESI mass spectra were recorded on a SHIMADZU LCMS-IT-TOF or a Thermo Q Exactive™ mass spectrometry instrument. LC/ESI-MS data were obtained on a Thermo LTQ-XL mass spectrometer. The photo-irradiation power density of various light sources in photo-chemical transformation experiments were measured by an optical power meter produced by Thorlabs: a 311 nm UV lamp (10.8 mW/cm^2), a 405 nm LED array (13.4 mW/cm^2), a 373 nm LED array (38.5 mW/cm^2).

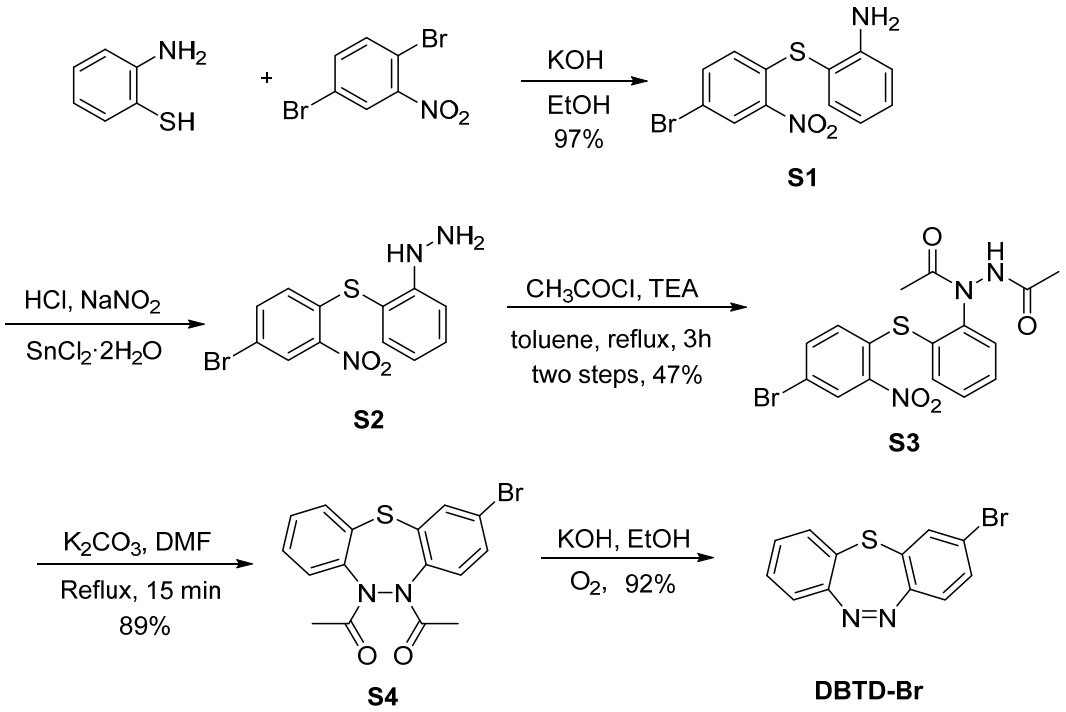
AccuPrime™ *pfx* DNA Polymerase was obtained from Invitrogen; T4 DNA ligase and restriction enzymes were obtained from New England Biolabs; oligonucleotide primers and gene fragments were synthesized by Sangon Biotech, and plasmids were sequenced by Tsingke Biotechnology; Plasmid DNA isolation was carried out with E.Z.N.A.® Plasmid Mini Kit I (from Omega Bio-tek®); Ni-NTA resin was obtained from Thermo Fisher. UV-Vis absorption spectra and Optical density (OD) were measured by using Thermo NANODROP 2000C Spectrophotometer. Fluorescence and western blot images were captured on a ChampChemi™ 610 Fluorescence/Chemiluminscence Gel Imager. Fluorescence cell imaging experiments were carried out on an Olympus IX83 fluorescence microscope, and confocal fluorescence cell imaging was performed with a Zeiss 780 laser scanning confocal fluorescence microscope. Fluorescence intensities were analyzed with ImageJ software. The three-dimensional reconstruction imaging derived from Z-stacked image slices was collected on the Olympus IX83 fluorescence microscope and analyzed by Imaris Viewer software after deconvolution calculation to increase the resolution in each slice.

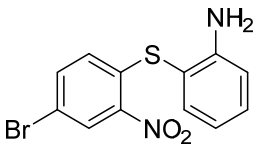
Ultraviolet and visible spectrophotometric (UV-Vis) absorption spectra were recorded on a HORIBA Fluoromax-4 Spectrofluorometer Detector in a 0.1×1.0 cm optical path quartz cuvette (0.1 cm optical path face was used for photo-stimulation), and a deuterium arc & halide lamp was used as the light source (Purchased from Shanghai Wenyi Photoelectric Technology Co., Ltd. China). The dynamic absorbance data of photo-switching performance were recorded on an in-house assembled fiber optic spectroscopic instrument based on a fast-response modular spectrometer connected with a Qpod 2e thermostatic cuvette holder (Quantum Northwest), and the

temperature was stabilized to 298 K. A power-controllable 405 nm diode laser was used for implementing the programable and controlled photo-stimulation connecting to the cuvette holder through 1000 μm quartz optical fibers (power density at the stimulation face of the cuvette = 250 $mW \cdot cm^{-2}$).

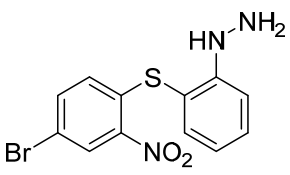
Synthetic routes for DBTDA and DASyd probes

Scheme S1: Synthesis of **DBTD-Br** according to the methods in a literature procedure[1]



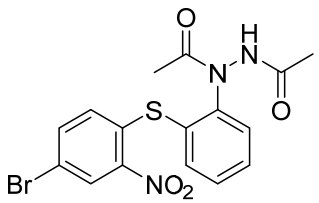


2-[(4-Bromo-2-nitrophenyl)thio]aniline (S1): To a stirring solution of 1,4-dibromo-2-nitrobenzene (20.0 g, 71.72 mmol) in ethanol (150 mL), 2-aminobenzenethiol (9.2 mL, 86.06 mmol) and potassium hydroxide (4.83 g, 86.06 mmol) was added react until the raw material was converted completely at room temperature. Then, the precipitated solid was filtered, washed with *n*-hexanes and dried under vacuum to give the title compound as yellow solid (22.5 g, yield 97%). ^{1}H-NMR (400 MHz, Chloroform-*d*) δ 8.40 (d, J = 2.2 Hz, 1H), 7.44 (m, 2H), 7.34 (m, 1H), 6.88-6.80 (m, 2H), 6.72 (d, J = 8.8 Hz, 1H), 4.28 (s, 2H). ^{13}C-NMR (101 MHz, Chloroform-*d*) δ 149.4, 145.6, 137.8, 136.7, 136.6, 132.7, 128.8, 128.8, 119.4, 118.0, 115.8, 111.6. HRMS (ESI) calcd. For $C_{12}H_{10}BrN_2O_2S^+$ 324.9641 [M+H$^+$], found 324.9637.

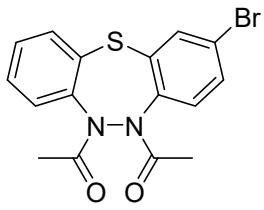


{2-[(4-Bromo-2-nitrophenyl)thio]phenyl}hydrazine (S2): To a solution of **S1** (5.0 g, 14.8 mmol) in a mixture of H_2O (20.0 mL) and concentrated HCl (12 M, 15.0 mL), a solution of $NaNO_2$ (1.07 g, 15.5 mmol) in H_2O (4 mL) was added dropwise at 0 °C and the reaction mixture was stirred and kept at this temperature for 1 h. Then, a solution of $SnCl_2·2H_2O$ (5.59 g, 29.5 mmol)

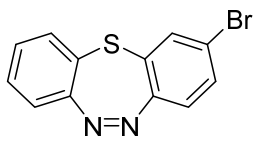
in concentrated HCl (12 M, 15.0 mL) was added dropwise and the mixture was stirred at room temperature for 2 h. Next, a solution of NaOH was added into mixture, precipitate was crashed out and filtered, washed with brine, Et_2O and dried in vacuum at 40 °C overnight. A yellow brown solid was obtained corresponding to the desired product. The product was used without further purification for the subsequent reactions. ^{1}H-NMR (400 MHz, Chloroform-*d*) δ 8.40 (d, J = 2.2 Hz, 1H), 7.50 (m, 1H), 7.44 (dd, J = 2.6, 1.8 Hz, 1H), 7.42 (dd, J = 3.8, 1.8 Hz, 1H), 7.25 (d, J = 1.4 Hz, 1H), 6.86 (td, J = 7.6, 1.4 Hz, 1H), 6.65 (d, J = 8.8 Hz, 1H), 6.15 (s, 1H), 3.54 (s, 2H). ^{13}C-NMR (101 MHz, Chloroform-*d*) δ 152.3, 145.6, 137.7, 136.6, 136.4, 132.8, 128.8, 128.7, 119.3, 118.1, 111.9, 110.4. HRMS (ESI) calcd. For $C_{12}H_{11}BrN_3O_2S^+$ 339.9750 [M+H$^+$], found 339.9751.



N-[2-(4-bromo-2-nitrothiophenyl)phenyl]-N,N′-diacetyl hydrazine (S3): Acetyl chloride (1.42 mL, 20.0 mmol) was added on an ice-cooled solution of triethylamine (2.78 mL, 20.0 mmol) and **S2** (3.39 g, 10.0 mmol) in anhydrous toluene (50.0 mL). Then, the solution was stirred at 120 °C for 4 h and traced with TLC till the conversion was completed. The mixture was then evaporated to dryness in vacuum and the residue was purified through flash chromatography (Hexanes/EtOAc = 1/1) to give the desired product as a yellow solid (2.81 g, two steps yield 47%). ^{1}H-NMR (400 MHz, DMSO-*d*$_6$) δ 10.80 (s, 3H), 10.22 (s, 1H), 8.32 (d, J = 2.4 Hz, 1H), 8.26 (d, J = 2.4 Hz, 3H), 7.81 (d, J = 7.8 Hz, 1H), 7.71-7.63 (m, 6H), 7.60-7.53 (m, 10H), 7.46-7.39 (m, 3H), 6.91 (d, J = 8.8 Hz, 3H), 6.72 (d, J = 8.8 Hz, 1H), 2.04 (s, 9H), 1.85 (s, 9H), 1.84 (s, 3H), 1.59 (s, 3H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ 171.92, 169.08, 146.50, 144.90, 137.18, 136.68, 136.66, 131.84, 131.46, 130.13, 129.74, 129.16, 127.71, 117.90, 20.66, 20.56. HRMS (ESI) calcd. for $C_{16}H_{15}BrN_3O_4S^+$ 423.9961 [M+H$^+$], found 423.9958.

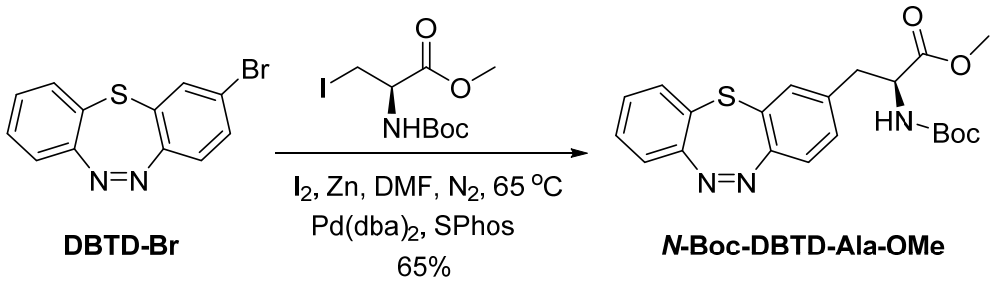


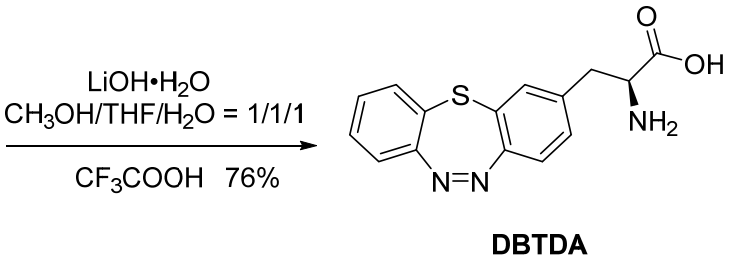
1,1'-{2-bromodibenzo[b,f][1,4,5]thiadiazepine-5,6-diyl}bis (ethan-1-one) (S4): Compound **S3** (2.00 g, 4.73 mmol) and an equimolecular amount of potassium carbonate (654 mg, 4.73 mmol) were dissolved in DMF. Then, the solution was stirred at 120 °C for 30 min and traced with TLC till the conversion was completed. The reaction mixture was added water (50.0 mL) and extracted with EtOAc (50.0 mL × 3). The organic layer was washed with brine, dried over Na_2SO_4 and then evaporated to dryness in vacuum. The residue was purified through flash chromatography (Hexanes/EtOAc = 2/1) to give the desired product as a colorless solid (1.70 g, yield 89%). ^{1}H-NMR (400 MHz, DMSO-*d*$_6$) δ 7.83-7.23 (m, 7H), 2.12 (s, 3H), 2.03 (s, 3H). HRMS (ESI) calcd. for $C_{16}H_{14}BrN_2O_2S^+$ 376.9954 [M+H$^+$], found 376.9961.

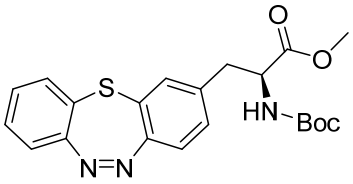


2-Bromodibenzo[b,f][1,4,5]thiadiazepine (DBTD-Br): The corresponding compound **S4** (1.40 g, 3.72 mmol) was dissolved in 20.0 mL of ethanol containing 11.17 mmol of potassium hydroxide. The solution was stirred at 80 °C for 4 h. After the mixture cooled to room temperature, air flow was bubbled into the solution and the crystalline yellow solid was formed, and filtered and washed with ethanol. This product was obtained as a yellow crystalline solid (0.90 g, yield 92%). ^{1}H-NMR (400 MHz, Chloroform-*d*) δ 7.61 (dd, J = 8.0, 1.4 Hz, 1H), 7.56 (dd, J = 8.4, 2.2 Hz, 1H), 7.51 (d, J = 2.0 Hz, 1H), 7.50- 7.41 (m, 2H), 7.36-7.27 (m, 2H). ^{13}C-NMR (101 MHz, Chloroform-*d*) δ 151.7, 150.4, 134.2, 132.71, 132.4, 131.9, 130.3, 130.0, 129.6, 128.9, 127.7, 123.7. HRMS (ESI) calcd. for $C_{12}H_8BrN_2S^+$ 290.9586 [M+H$^+$], found 290.9585.

Scheme S2: Synthesis of **DBTDA**[2]

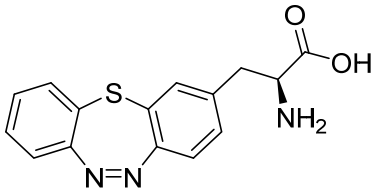






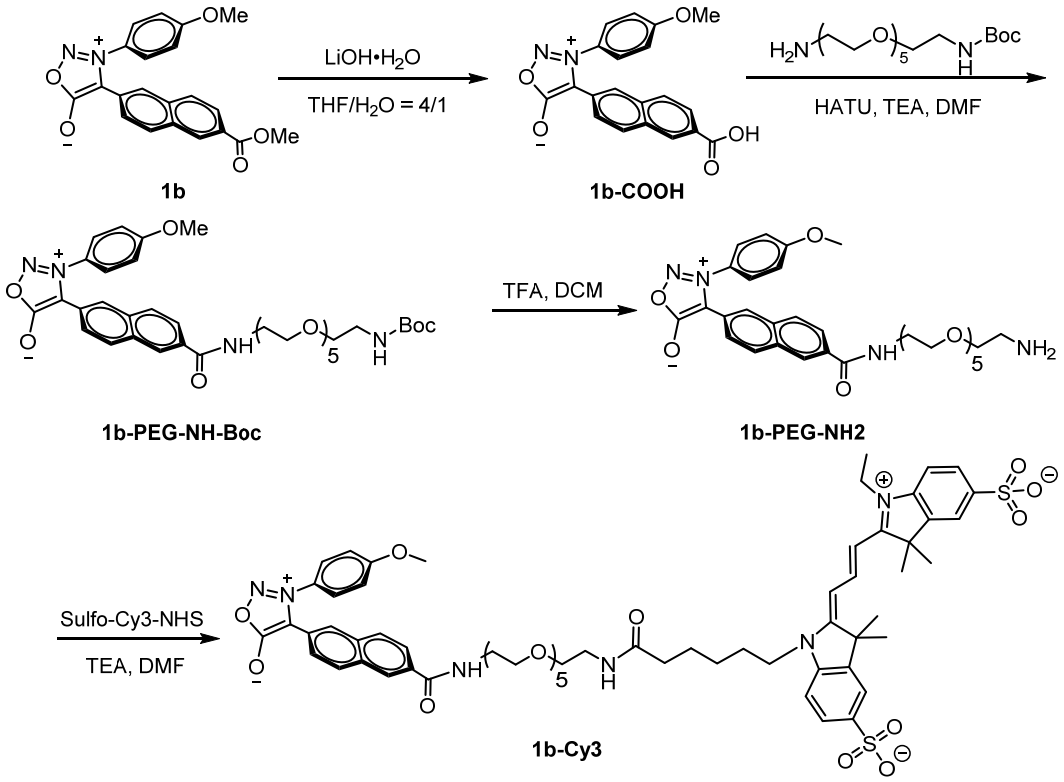
Methyl-(S)-2-[(tert-butoxycarbonyl)amino]-3-{dibenzo[b,f][1,4,5]thiadiazepin-2-yl}propanoate (*N*-Boc-DBTD-Ala-OMe): In nitrogen atmosphere, zinc dust (1.58 g, 25.0 mmol) was added to a flame-dried, nitrogen-purged side arm round-bottomed flask. Anhydrous DMF (5.0 mL) was added via syringe, followed by a catalytic amount of iodine (333 mg, 1.25 mmol). A color change of the DMF solution was observed from colorless to yellow and back to colorless again. Protected methyl (*R*)-2-((*tert*-butoxycarbonyl)-amino)-3-iodopropanoate (*N*-Boc-β-iodo-l-Ala-OMe, 2.74 g, 8.32 mmol) was added immediately, followed by an additional and catalytic amount of iodine (333 mg, 1.25 mmol). The solution was stirred at room temperature for 5 min; Pd(dba)$_2$ (98.0 mg, 0.170 mmol), SPhos (68.2

mg, 0.170 mmol) and **DBTD-Br** (965 mg, 3.33 mmol) were added to the solution of organozinc reagent and the mixture was heated at 70℃ for 12 h, under a positive pressure of nitrogen gas. The reaction mixture was added with water (50.0 mL) and extracted with EtOAc (50.0 mL × 3). The organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuum. The residue was purified through flash chromatography (Hexanes/EtOAc = 4/1) to give the desired product as a yellow solid (921 mg, yield 67%).^{1}H-NMR (400 MHz, DMSO-*d*$_6$) δ 7.62 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.59-7.52 (m, 2H), 7.46-7.36 (m, 3H), 7.33-7.27 (m, 2H), 4.30-4.18 (m, 1H), 3.60 (s, 3H), 3.05 (dd, *J* = 13.8, 4.8 Hz, 1H), 2.85 (dd, *J* = 13.8, 10.6 Hz, 1H), 1.25 (s, 9H). ^{13}C-NMR (101 MHz, DMSO-*d*$_6$) δ 172.11, 155.24, 151.23, 149.83, 140.46, 132.24, 131.80, 130.61, 130.12, 129.82, 129.68, 127.07, 78.27, 54.26, 51.86, 35.54, 27.99. HRMS (ESI) calcd. for $C_{21}H_{24}N_3O_4S^+$ 414.1482 [M+H$^+$], found 414.1481.

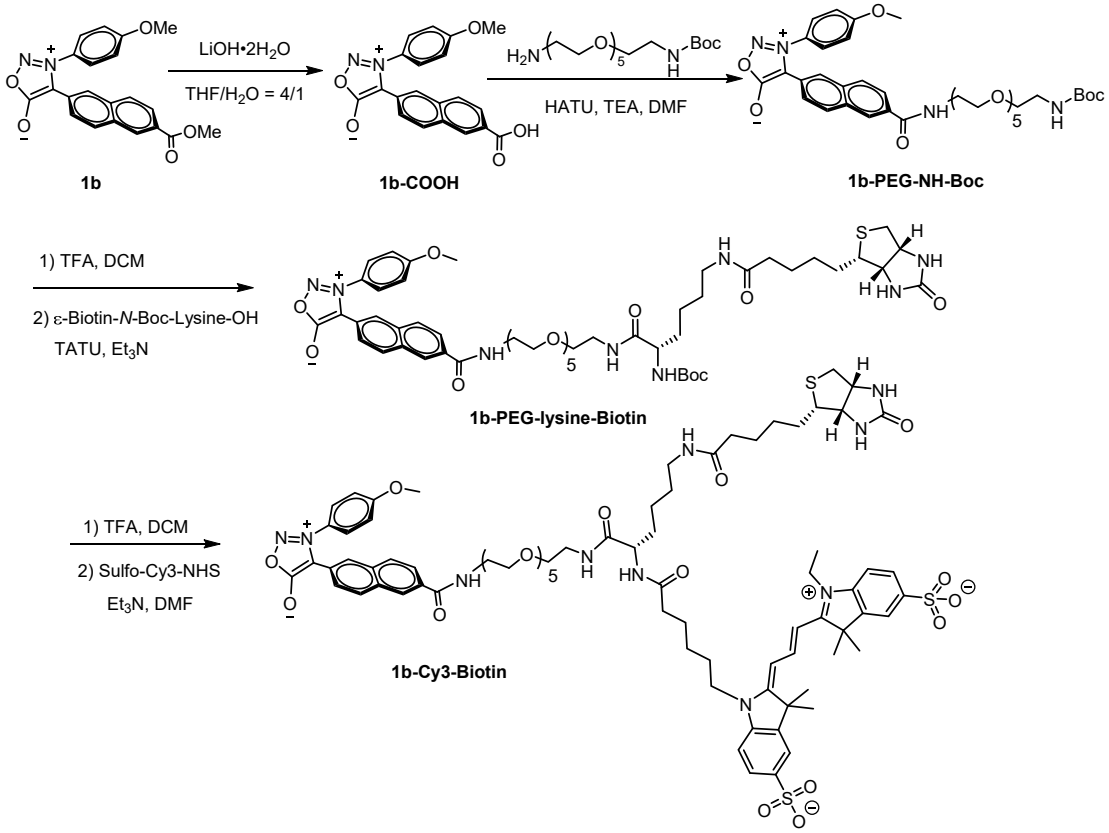


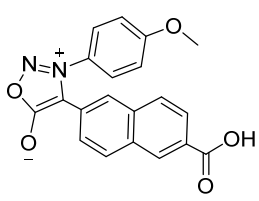
(S)-2-amino-3-{dibenzo[b,f][1,4,5]thiadiazepin-2-yl}propanoic acid (DBTDA): To a stirring suspension of ***N*-Boc-DBTD-Ala-OMe** (250 mg, 0.60 mmol) in CH_3OH/THF/H_2O = 1/1/1, $LiOH·H_2O$ (33.6 mg, 0.80 mmol) was added to react until the raw material was converted completely. The reaction mixture was acidified with dilute HCl (1 M) solution to pH = 4-5. Then the mixture was extracted with EtOAc (50.0 mL × 3). The organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuum to give a crude product of carboxylic acid. Then the crude product was added with 2 mL TFA was stirred at room temperature for 15 min. Then, the solvent was evaporated and the residue was purified through a C18 reverse-phase chromatography to give the desired ncAA as a yellow solid (150 mg, yield 76%). ^{1}H-NMR (400 MHz, DMSO-*d*$_6$) δ 7.66-7.53 (m, 3H), 7.50-7.38 (m, 3H), 7.37-7.32 (m, 1H), 3.50 (dt, *J* = 8.0, 3.8 Hz, 1H), 3.14 (dt, *J* = 13.9, 4.0 Hz, 1H), 2.92 (m, 1H). ^{13}C-NMR (101 MHz, DMSO-*d*$_6$) δ 169.6, 151.6, 150.3, 140.3, 132.9, 132.3, 131.3, 130.6, 130.5, 130.3, 130.2, 127.7, 127.4, 55.1, 36.0. HRMS (ESI) calcd. for $C_{15}H_{12}N_3O_2S^-$ 298.0656 [M-H$^+$], found 298.0654.

Scheme S3: Synthesis of **1b-Cy3**[3]

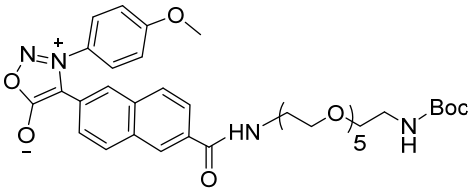


Scheme S4: Synthesis of **1b-Cy3-Biotin**

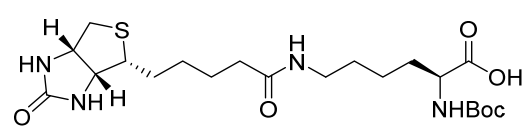




4-(6-carboxynaphthalen-2-yl)-3-(4-methoxyphenyl)-1,2,3-oxadiazol-3-ium-5-olate (1b-COOH): To a stirring suspension of **1b** (120 mg, 0.32 mmol) in THF/H_2O = 4/1 (5 mL), LiOH·H_2O (23 mg, 0.96 mmol) was added. The reaction mixture was stirred at room temperature overnight. When the hydrolysis is completed, the reaction mixture was acidified with dilute HCl (1 M) solution to pH = 4-5. Then the mixture was extracted with EtOAc (3 mL × 3) and concentrated under vacuum to give the crude product. The crude product was purified by flash chromatography on silica gel (eluting with 10% MeOH in DCM) to yield a white powder (118 mg, yield 95%). ^{1}H-NMR (400 MHz, DMSO-*d*$_6$) δ 8.48 (s, 1H), 8.06 – 7.98 (m, 2H), 7.94 (d, J = 8.7 Hz, 1H), 7.83 (d, J = 8.6 Hz, 1H), 7.70 – 7.63 (m, 2H), 7.18 (dd, J = 9.5, 2.8 Hz, 3H), 3.84 (s, 3H).

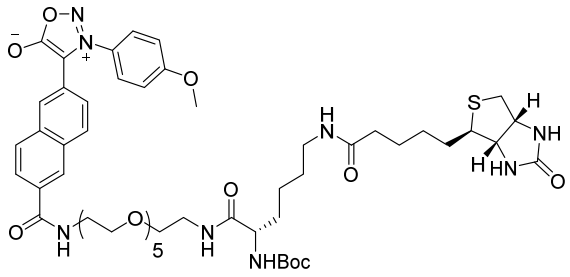


4-{6-[(2,2-dimethyl-4-oxo-3,8,11,14,17,20-hexaoxa-5-azadocosan-22-yl)carbamoyl]naphthalen-2-yl}-3-(4-methoxyphenyl)-1,2,3-oxadiazol-3-ium-5-olate (1b-PEG-NH-Boc): To a stirring solution of **1b-COOH** (15.0 mg, 0.0410 mmol) in anhydrous DMF (2 mL), HATU (18.9 mg, 0.050 mmol), Et_3N (17.1 μL, 0.123 mmol) and *tert*-butyl-(17-amino-3,6,9,12,15-pentaoxah-eptadecyl)-carbamate (Boc-NH-PEG$_5$-$CH_2CH_2NH_2$, 18.9 mg, 0.050 mmol) and was added to react until the raw material was converted completely. Get rid of the DMF by air flow drying and the reaction was quenched by adding 1 mL water. Then the mixture was extracted by EtOAc (2.0 mL × 3) and the crude product was purified by flash chromatography on silica gel (eluting with 10% EtOH in DCM) to yield a white solid (29.4 mg, yield 98%). ^{1}H-NMR (600 MHz, DMSO-*d*$_6$) δ 8.73 – 8.66 (m, 1H), 8.39 (d, J = 1.7 Hz, 1H), 8.03 (d, J = 1.7 Hz, 1H), 7.97 – 7.89 (m, 3H), 7.71 – 7.66 (m, 2H), 7.24 (dd, J = 8.7, 1.8 Hz, 1H), 7.21 – 7.17 (m, 2H), 6.78 – 6.70 (m, 1H), 3.86 (s, 3H), 3.60 – 3.37 (m, 22H), 3.08 – 3.01 (m, 2H), 1.36 (s, 9H).

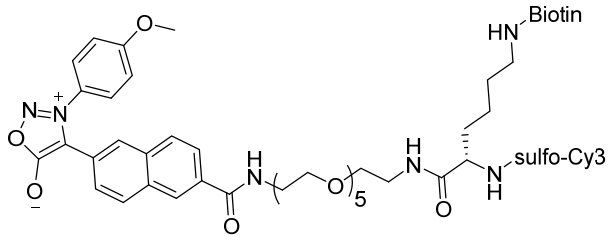


***N*^{2}-(*tert*-butoxycarbonyl)-*N*^{6}-(5-((3aR,4R,6aS)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoyl)-L-lysine (*N*-Boc-Biotin-Lysine):** To a stirring solution of *N*^{6}-(*tert*-butoxycarbonyl)-L-lysine (60 mg, 0.24 mmol) in anhydrous DMF (2 mL), Biotin-NHS (100 mg, 0.293 mmol) and Et_3N (104 μL, 0.75 mmol) was added to react until the raw material was converted completely. Get rid of the DMF by air flow drying and the reaction was quenched by adding 1 mL water. Then the mixture was acidified with formic acid and redissolved in water/acetonitrile = 2/1 (3 mL) and the crude product was purified by reverse phase flash chromatography on a Ginimi NX-C18 column (eluting with linear gradient from 10% CH_3CN in water to 90% CH_3CN in water with 0.1% HCOOH) to yield a white solid (105 mg, yield 91%). ^{1}H-NMR (400 MHz, DMSO-*d*$_6$) δ 12.40 (s, 1H), 7.73 (t, J = 5.6 Hz, 1H), 7.00 (d, J =

8.0 Hz, 1H), 6.41 (s, 1H), 6.35 (s, 1H), 4.30 (dd, *J* = 7.7, 5.1 Hz, 1H), 4.12 (ddd, *J* = 7.7, 4.4, 1.9 Hz, 1H), 3.88 – 3.72 (m, 1H), 3.09 (ddd, *J* = 8.5, 6.1, 4.3 Hz, 1H), 2.99 (q, *J* = 6.4 Hz, 2H), 2.82 (dd, *J* = 12.4, 5.1 Hz, 1H), 2.57 (d, *J* = 12.4 Hz, 1H), 2.04 (t, *J* = 7.4 Hz, 2H), 1.71 – 1.40 (m, 6H), 1.38 (s, 9H), 1.38-1.19 (m, 6H). ^{13}C-NMR (101 MHz, DMSO-*d*$_6$) δ 174.7, 172.3, 163.2, 156.1, 78.4, 61.5, 59.7, 56.5, 55.9, 53.9, 40.3, 38.6, 35.7, 30.9, 29.3, 28.7, 28.5, 25.8, 23.6. HRMS (ESI) calcd. For $C_{21}H_{37}N_4O_6S^+$ 473.2428 [M+H$^+$], found 473.2427.



4-(6-(((S)-2,2-dimethyl-4,7-dioxo-6-(4-(5-((3aS,4R,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)butyl)-3,11,14,17,20,23-hexaoxa-5,8-diazapentacosan-25-yl)carbamoyl)naphthalen-2-yl)-3-(4-methoxyphenyl)-1,2,3-oxadiazol-3-ium-5-olate (1b-PEG-lysine-Biotin): Compound **1b-PEG-NH-Boc** (6.8 mg, 9.4 × 10^{-3} mmol) was dissolved in 1 mL TFA/DCM (1/1) at 0 °C, then stirred at room temperature for 20 min. The reaction mixture was concentrated. Without any purification, the residue was redissolved in DMF. TEA (6.5 μL, 0.050 mmol) and ***N*-Boc-Biotin-Lysine** (5 mg, 0.010 mmol) was added. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was purified by reverse phase HPLC preparation with a C18 column (A: HCOOH/H_2O 0.1%; B: HCOOH/ACN 0.1%) affording **1b-PEG-lysine-Biotin** as a white foam (7.3 mg, yield 76%). HRMS (ESI) calcd. For $C_{53}H_{75}N_8O_{14}S^+$ 1079.5118 [M-H$^+$], found 1079.5121.



1b-Cy3-Biotin: Compound **1b-PEG-lysine-Biotin** (7.0 mg, 6.8 × 10^{-3} mmol) was dissolved in 0.50 mL TFA/DCM (1/1) at 0 °C, then stirred at room temperature for 20 min. After getting rid of the TFA by air flow, the residual TFA and TFA salt of desired intermediate was neutralized by adding Et_3N (4.1 μL, 0.029 mmol) in anhydrous 200 μL DMF to get the crude solution of the primary amine product. After the crude product was redissolved, then, Sulfo-Cy3-NHS (6.0 mg, 8.2 × 10^{-3} mmol) in 0.2 mL anhydrous DMF were added under N_2 atmosphere, after the reaction was reacted for 12 h at room temperature, the mixture was concentrated and purified by reverse phase HPLC preparation with a Gimini NX-C18 column (A: HCOOH/H_2O 0.1%; B: HCOOH/ACN 0.1%, linear gradient from 95% B with 5% A to 5% B with 95% A) affording 1b-Cy3-Biotin as a red solid (9.5 mg, yield 88%). HRMS (ESI) calcd. For $C_{79}H_{102}N_{10}O_{19}S_3^-$ 1590.6485 [M-H$^+$], found 1590.6458.

Materials and methods

Plasmid construction

All amino acid sequence of the plasmids used in this work are presented in **Supplementary Note** and all primer sequences used in this work are listed in **Table S2**. The sfGFP gene was synthesized and then ligated into pETDuet-1 vector by Sangon biotech to construct plasmid pET-sfGFP-His6. For site-directed mutagenesis, primers of sfGFP-Q204TAG-f and sfGFP-Q204TAG-r were used to perform PCR using plasmid pET-sfGFP-His6 as template to generate plasmid pET-sfGFP-Q204TAG-His6. Using the corresponding primers, we were able to construct the plasmid pET-sfGFP-K3TAG-His6 and pET-sfGFP-N149TAG-His6 according to the above procedure. The sfGFP gene carrying Q204TAG mutation was amplified by PCR and was ligated into pBAD-Myc-His A vector to construct plasmid pBAD-sfGFP-Q204TAG.

The OmpC gene was synthesized by Sangon biotech, and then subcloned into pET-11a, using NdeI-BamHI cloning sites, to construct the plasmid pET-OmpC-His6. For site-directed mutagenesis, primers of OmpC-Y232TAG-f and OmpC-Y232TAG-r were used to perform PCR using plasmid pET-OmpC-His6 as template to generate plasmid pET-OmpC-Y232TAG-His6[4].

The *Mm*PylRS plasmid bearing a chloramphenicol gene with an amber codon at Q98 and a sfGFP gene with an amber codon at Q204 was constructed from pACYC177 and pEvol-PylT-*Mm*PylRS for positive selection. The pYSneghc plasmid harboring barnase gene with two TAG amber codons at both Q3 and G66 mutations was constructed by members of our laboratory previously from pBAD-43 for purpose of negative selection.

Plasmid library construction for the directed evolution

Using plasmid pEvol-*Mm*PylRS as a template, Tyr384 was mutated to phenylalanine (Phe) by site-directed mutagenesis with AccuPrime™ *p*fx DNA Polymerase (Invitrogen). Then the PCR product was sequentially digested with DpnI (Thermo Fisher); residues N346 and C348 were allowed to be mutated to Gly, Ala and Val randomly via mixed primers; residue A302 was allowed to be mutated to Ala and Thr randomly via mixed primers; residue W417 was fully randomized with the NNK codon via site saturation mutagenesis (**Table S1**). These mutations were performed by iterative overlap extension PCR with AccuPrime™ *p*fx DNA Polymerase (Invitrogen). Then the PCR products were sequentially digested with SalI and BcuI (Thermo Fisher) to create sticky ends, and ligated into the precut pEvol-*Mm*PylRS plasmid with the same restriction enzymes using T4 DNA ligase (New England Biolabs) to afford the *Mm*DBTDRS library. The quality of the *Mm*DBTDRS library constructions were confirmed with randomized nucleotide at designated positions in the open reading frame (ORF) of structural gene by DNA sequencing.

Screening of the *Mm*DBTDRS library for encoding the DBTDA

After the construction of *Mm*PylRS library, the interactive positive and negative selection cycles were implemented to allow the *Mm*PylRS variants that can synthesize the aminoacyl-tRNA$_{CUA}$ for survival in the presence of certain level of antibiotic pressure as positive selection and selectively synthesize DBTDA aminoacyl-tRNA$_{CUA}$ to be picked out by eliminated others through expressing the toxic RNase gene as negative selection. For positive selection, the plasmids harboring the *Mm*DBTDRS library was transformed into Top10 Competent cells containing the plasmid pBAD-sfGFP-Q204TAG. Colonies were grown on GMML agar in Petri dishes supplemented with tetracycline (34 μg·mL^{-1}), ampicillin (100 μg·mL^{-1}), chloramphenicol (40 μg·mL^{-1}) and L-arabinose (0.2%). In the presence of 2 mM DBTDA, suppression of nonsense codon allowed the bacteria to propagate under the antibiotic pressure to form colonies with sfGFP co-expressed, and harvested for plasmids extraction to ensure the quality of the screened library by sequencing. The collected plasmids extracts were used then for the negative selection. For negative selection, the purified plasmid library was then transformed into Top10 competent cells harboring pYSneghc (barnase-Q3TAG-G66TAG) and grown on LB agar growth medium in Petri dishes supplemented with tetracycline (34 μg·mL^{-1}), ampicillin (100 μg·mL^{-1}), L-arabinose (0.2%). In the absence of the 2 mM DBTDA, suppression of nonsense codon by undesired amino acid substrate allows the expressing of the toxic barnase RNase gene, leading to bacterial suicide of undesired monoclone. The recovered plasmids extracts were used then for the next positive selection. The positive and negative selection process was cycled and iteratively repeated until the *E. coli* titers began to recover with sfGFP fluorescence signal observed and a few of consensuses DNA sequence from the *Mm*PylRS plasmid library obtained. These single colonies were picked and corresponding *Mm*PylRS mutants were cloned into pEvol plasmid and subjected to protein expression in BL21(DE3) cell with pET-sfGFP-TAG to identify a unique DBTDA synthetase. The BL21(DE3) monoclone that could express full-length of sfGFP was picked and sequenced, and mutations at the indicated codon positions were confirmed.

Molecular docking

The crystal structure of *Mm*OmeRS (PDB: 3QTC) with co-crystalized *O*-methyl-L-tyrosine as ligand was obtained from the Protein Data Bank. To predict the binding-conformation of DBTDA to the appropriate binding site, the 3D structure and geometry of the *Mm*OmeRS were simplified by removing water molecules, solvent molecules and the original ncAA ligand from the crystal structures. We next constructed the parent structure model of *Mm*DBTDRS by replacing the mutated amino acid substitutions (A302T, N346A, C348G, Y384F, W417T) in the corresponding residues of the *Mm*OmeRS template. Before docking, in the model of *Mm*DBTDRS, all the hydrogen atoms and the side chain of residues were displayed. On the other hand, the crystal structure of DBTD (CCDC: 1905279) was obtained free

of charge from the Cambridge Crystallographic Data Centre. Therefore, the docking ligand *cis*-DBTDA was built based on the crystal structure of DBTD and optimized to a minimized ligand energy by applying a CHARMM force field. Following the minimization, DBTDA was positioned roughly in the active cavity of the *Mm*DBTDRS receptor to furnish a *Mm*DBTDRS-DBTDA complex in which the binding sites were defined within a 5Å spherical space covering the active residues of the *Mm*DBTDRS enzyme Afterwards, molecular docking study was performed by applying a molecular dynamics (MD) simulated-annealing-based algorithm[5],; The top hit value was set at 1 to acquire the best docking pose; The pose cluster radius was set to 0.5 Å to ensure that the docked poses were sufficiently diverse; Other parameters were set as default. CDOCKER was employed to search a series of molecular docking results with interaction scoring and energy ranking. All computational experiments and conformational analysis of the complexes were carried out on the Discovery Studio software; The results of molecular docking were evaluated depending on CDOCKER score and CDOCKER interaction energy of each complex against the related targets; All docking images were generated and visualized by Pymol software. The docking results were presented in Table S3. Two representative docking postures include: an optimal pose with azo moiety facing inward and one with azo moiety facing outward were highlighted in blue color with the scores and docking energy presented.

Protein expression and purification

For protein expression and purification, we co-transformed the pET-sfGFP-XTAG-His_6 plasmid (with a C-terminal hexahistidine tagged sfGFP gene with an amber codon designated at position K3, N149 or Q204, respectively; X represent K3, N149, or Q204) and pEvol-*Mm*DBTDRS plasmid (which genetically encodes DBTDA) into *E. coli* strain BL21 (DE3) competent cells, Colonies were grown on LB growth medium in Petri dishes supplemented with tetracycline (34 $\mu g \cdot mL^{-1}$) and ampicillin (100 $\mu g \cdot mL^{-1}$). A single colony from LB agar plate was picked and inoculated in 5 mL LB growth medium supplemented with tetracycline (34 $\mu g \cdot mL^{-1}$) and ampicillin (100 $\mu g \cdot mL^{-1}$) overnight (37 ºC, 250 rpm), and then inoculated at 37 ºC, 250 rpm in a 1:100 dilution supplemented with tetracycline (34 $\mu g \cdot mL^{-1}$) and ampicillin (100 $\mu g \cdot mL^{-1}$) of 50 mL fresh LB growth medium. When the OD600 reached 0.4 to 0.5, 2 mM DBTDA was added to LB growth medium, and the protein expression was induced by adding 1 mM IPTG and 0.2% arabinose when OD600 reached 0.6. After 8 h induction at 37 ºC, 250 rpm. Cells were harvested by centrifugation (30 min, 10,000 g, 4 ºC) and bathed on ice and resuspended in 3 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH = 8.0). Next, cells were lysed by sonication in an ice/water bath for 30 times (2s each with 6s interval). The cell lysates were centrifuged (30 min, 12,000 g, 4 ºC) to get rid of insoluble debris. The supernatant was incubated with 0.2 mL Ni-NTA resin (Thermo HisPur) (2 h, 4 ºC). The slurry was then loaded to a separation column and the protein-bound resin was washed three times with 3 mL lysis buffer and washed twice

with 3 mL washing buffer (300 mM NaCl, 50 mM Na_2HPO_4, 50 mM imidazole, pH = 8.0). The protein was finally eluted from the resin by using elution buffer (300 mM NaCl, 50 mM Na_2HPO_4, 250 mM imidazole, pH = 8.0). The eluted fractions were collected, concentrated and subjected to buffer-exchange to PBS buffer (pH = 7.4) using an Amicon Ultra-15 Centrifugal Filter (10k MWCO, Millipore). The protein purity was analyzed by SDS-PAGE and LC/ESI-MS. Equal protein loading was assessed by staining the SDS-PAGE gel with Coomassie Brilliant Blue according to the manual of a commercial staining solution and imaged under white light source. The concentration of the protein was determined by measuring the absorbance at 485 nm and derived by the extinction coefficient was 8.33×10^4 $M^{-1}cm^{-1}$ for sfGFP which was reported in the literature[6].

Optimization of conditions for incorporation of DBTDA into protein in *E. coli*

The *E. coli* strain BL21 (DE3) competent cells harboring plasmids pET-sfGFP-Q204TAG-His6 and pEVOL-*Mm*DBTDRS were inoculated in 5 mL LB growth medium supplemented with tetracycline (34 μg·mL^{-1}) and ampicillin (100 μg·mL^{-1}) overnight (37 ºC, 250 rpm). For optimization of concentration of DBTDA, cells were inoculated in a 1:100 dilution to fresh LB growth medium supplemented with tetracycline (34 μg/mL) and ampicillin (100 μg/mL) (37 ºC, 250 rpm). When the OD600 reached 0.4 to 0.5, DBTDA (from 0 to 4 mM) was added into the LB growth medium, and the protein expression was induced by adding 1 mM IPTG, 0.2% arabinose when OD600 reached 0.6; For optimization the pH of the LB growth medium: cells were inoculated in a 1:100 dilution to 25 mL fresh LB growth medium at different pH (The pH of growth medium was adjusted in the range from 6.5 to 9.0 using 0.1M NaOH or 0.1M HCl) supplemented with tetracycline (34 μg/mL) and ampicillin (100 μg/mL) (37 ºC, 250 rpm). When the OD600 reached 0.4 to 0.5, DBTDA (2 mM) was added into the LB growth medium, and the protein expression was induced by adding 1 mM IPTG, 0.2% arabinose when OD600 reached 0.6. After 8 h induction at 37 ºC, 250 rpm, 100 μL of bacterial cultures were centrifuged at 10,000 g for 10 min, resuspended with 900 μL PBS (pH = 7.4), and cells were seeded in 35-mm glass-bottom tissue culture dish, 1% agarose (w/v) was introduced onto the culture dish which was then sealed by using a autoclaved coverslip. The *E. coli* cells were then imagined under using Olympus IX83 fluorescence microscope with corresponding filters for sfGFP. Fluorescence intensity was analyzed using ImageJ software to compare the expression level. Then, 25 mL of bacterial cultures were harvested by centrifugation (30 min, 10,000 g, 4 ºC) and bathed on ice and resuspended in 3 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH = 8.0). Next, cells were sonicated in an ice/water bath for 30 times (2s each with 6s interval). The cell lysate was centrifuged (30 min, 12,000 g, 4 ºC). The supernatant was incubated with 0.2 mL Ni-NTA resin (Thermo HisPur) (2 h, 4 ºC). The slurry was then loaded to a separation column and the protein-bound resin was washed three times with 3 mL lysis buffer and washed twice with 3 mL washing buffer (300 mM NaCl, 50 mM Na_2HPO_4, 50 mM imidazole, pH = 8.0). The protein

was finally eluted from the resin by using the elution buffer (300 mM NaCl, 50 mM Na_2HPO_4, 250 mM imidazole, pH = 8.0). The eluted fractions were collected, concentrated and subjected to buffer-exchange to PBS buffer (pH = 7.4) using an Amicon Ultra-15 Centrifugal Filter (10k MWCO, Millipore). The protein purity was analyzed by SDS-PAGE and LC-ESI/MS. Equal protein loading was assessed by staining the SDS-PAGE gel with Coomassie Brilliant Blue according to the manual of a commercial staining solution and imaged under white light source. The concentration of the protein was determined by measuring the absorbance at 485 nm, the extinction coefficient was 8.33×10^4 $M^{-1}cm^{-1}$ for sfGFP which was reported in the literature.[6] The protein samples were desalted on a ZIPTIP (C18 resin, Millipore) and analyzed by Thermo LTQ-XL mass spectrometer. Data about the peptide charge-ladder were deconvoluted using the ProMass for Xcalibur software.

The LC-MS/MS analysis of sfGFP-Q204DBTD for studying the residue specificity of the genetic incorporation process

The SDS loading buffer (5X) and DTT (1 M) were added to the PCR tubes containing 10 μL purified sfGFP-Q204DBTD protein (10 mM), the mixture was heated to 85 ºC for 8 min, further resolved by gel electrophoresis by using a 12% polyacrylamide gels. Protein loading was assessed by staining the SDS-PAGE gel with Coomassie Brilliant Blue according to the manual of a commercial staining solution and imaged under white light source. The target protein band on the gel was collected and then subjected to in-gel digestion using trypsin followed by LC−MS/MS analysis (Q Exactive plus, Thermo). The mass spectroscopic raw data of the peptide fragments were analyzed and spliced by searching the fragments containing the desired residues by using the Thermo proteome discoverer.

Photo-click reaction between sfGFP-DBTD and DASyd 1a

We incubated the three purified sfGFP-DBTD mutant proteins (10 μM) with DASyd **1a** (50 μM, from 500 μM stock solution in DMSO) in quartz test tube to derive the final concentration of DASyd **1a** as 25 μM and the final concentration of sfGFP-DBTD as 5 μM in 20 μL total volume. The reaction mixtures were photo-irradiated with the 311 nm (a hand-held UV lamp) and the 405 nm (LED array) combination for 2 min. Then, aliquots of 10 μL reaction mixture were analyzed by LC-ESI/MS with an Aeris WIDEPORE 3.6 u C4 column (50 x 2.1 mm) and mass spectroscopic data were collected in the positive mode. The intact protein mass was derived by deconvoluting the charge ladders by using the ProMass software.

Photo-click reaction between sfGFP-DBTD and DASyd 1b-Cy3

For in-gel fluorescence imaging purpose, we incubated the purified sfGFP-N149DBTD (10 μM) with **1b-Cy3** (100 μM) in PCR tubes to derive the final concentration of **1b-Cy3** as 50 μM and the final concentration of sfGFP-N149DBTD

as 5 μM in 20 μL total volume. The reaction mixtures were photo-irradiated with the 405 nm LED array (0-120 s). After irradiation, SDS loading buffer (5X) and DTT (1 M) were added to the PCR tubes containing the 20 μL reaction mixture which was heated to 85 ºC for 8 min, and subjected to further analysis by gel electrophoresis using a 12% polyacrylamide gels and imaged with a CHAMPCHEMI multicolor fluorescence imaging system using 590 nm (for Cy3 emission) optical filters. Equal protein loading was assessed by staining the SDS-PAGE gel with Coomassie Brilliant Blue according to the manual of a commercial staining solution and imaged under white light source.

For LC-ESI/MS analysis, we incubated the three purified sfGFP-DBTD mutant protein (10 μM) with **1b-Cy3** (250 μM) in PCR tubes to obtain the final concentration of **1b-Cy3** as 125 μM and the final concentration of sfGFP-DBTD as 5 μM in 20 μL total volume. The reaction mixtures were photo-irradiated with the 405 nm LED array for 3 min. Then, aliquots of 10 μL reaction mixture were analyzed by LC-MS by using an Aeris WIDEPORE 3.6 u C4 column (50 x 2.1 mm), and mass spectroscopic data were collected in the positive mode. The intact protein mass was derived by deconvoluting the charge ladders by using the ProMass software.

Determination of the yield of the photo-click reaction between sfGFP-DBTD and 1b-Cy3 via UV-Vis spectral analysis

For quantitative analysis of the photo-labelling yield, we incubated the purified sfGFP-DBTD (10 μM) with **1b-Cy3** (250 μM) in 0.2 mL PCR tubes to obtain the final concentration of **1b-Cy3** as 125 μM and the final concentration of sfGFP-DBTD as 5 μM. The reaction mixture was photo-irradiated with the 405 nm LED array for 3 min. Next, the reaction mixture was purified to get rid of the excessive amount of DASyd **1b-Cy3** probe and other low-molecular-weight compounds via a Sephadex G-50 column in sodium phosphate buffer, by collecting the colored high-molecular-weight fractions. The eluted fraction was then concentrated using an Amicon Ultra-15 Centrifugal Filter (10k MWCO, Millipore). The reaction yield was then determined via a spectrophotometric analysis in which the absorbance at 485 nm and 552 nm were used as characteristic absorption signals. The molar extinction coefficient of Cy3 dye: ε_{552} = 150 000 M^{-1} cm^{-1} at 552 nm and the molar extinction coefficient of the sfGFP: ε_{485} = 83 300 M^{-1} cm^{-1} at 485 nm are used herein to calculate their molar concentrations, respectively. The calculation also has to be adjusted because the absorbance of the Cy3 dye has partially overlapped with the absorbance of the sfGFP at 485 nm, which is approximately 15.1% of the absorbance of Cy3 dye at 552 nm. Also, if the desired photo-conjugation was one molecule of the protein tagged with one molecule of Cy3 dye, the labelling yield could be calculated based on the following equations:

$$[\text{Cy3}] = [\text{sfGFP-}\mathbf{2b\text{-}Cy3}] = (\text{Abs}_{552})/(\varepsilon_{552} \times l)$$

$$[\text{total sfGFP}] = [(Abs_{485})-0.151\times(Abs_{552})]/(\varepsilon_{485}\times l)$$

$$\text{Yield (\%)} = [\text{sfGFP-}\mathbf{2b\text{-}Cy3}]/[\text{total sfGFP}] \times 100\% = [(Abs_{552})/\varepsilon_{552}]/[\ [(Abs_{485})-0.151\times(Abs_{552})]/\varepsilon_{485}] \times 100\%$$

The LC-MS/MS analysis of sfGFP-Q204DBTD after photo-reacted with DASyd 1b to identify the specificity of the photo-click process

20 μL purified sfGFP-Q204DBTD protein (20 μM) were added with 2 μL DASyd **1b** (10 mM stock solution in DMSO, final concentration = 200 μM) and 78 μL PBS (pH = 7.4). The reaction mixture was photo-irradiated with the 405 nm LED array for 2 min. After irradiation, aliquots of 20 μL reaction products, SDS loading buffer (5X) and DTT (1 M) were mixed in the PCR tubes. Then, the mixture was heated to 85 ºC for 8 min, further analyzed by gel electrophoresis using a 12% polyacrylamide gels, protein loading was assessed by staining the SDS-PAGE gel with Coomassie Brilliant Blue according to the manual of a commercial staining solution and imaged under white light source. The target protein band on the gel was collected and then subjected to in-gel digestion using trypsin followed by LC−MS/MS analysis (Q Exactive plus, Thermo). The mass spectroscopic raw data of the peptide fragments were analyzed and spliced by searching the fragments containing the desired residues by using the Thermo proteome discoverer.

Analysis of sfGFP-N149-2b-Cy3 stability in bio-mimicking environment via SDS-PAGE in-gel fluorescence

The photo-conjugated product, sfGFP-N149-**2b-Cy3**, was purified via a Sephadex G-50 gel GPC column to remove undesired small molecule dyes. Then 9.2 μM purified sfGFP-N149-**2b-Cy3** was incubated with the PBS buffer (pH = 7.4) or the PBS buffer containing final 5 mM concentration GSH (cytoplasm mimicking) for 24 hours at 37 ºC. In addition, the purified sfGFP-N149-**2b-Cy3** was also incubated with either the PBS buffer (pH=7.4) or the PBS buffer containing final 5 mM concentration GSH, and then irradiated with the 405 nm LED array for 5 min (photo-stability). Then, all of the tested mixture were heated to 85 ºC for 8 min, revolved by gel electrophoresis using a 12% SDS-polyacrylamide gels, respectively. Further, the SDS-PAGE was imaged with a CHAMPCHEMI multicolor fluorescence imaging system using 590 nm (for Cy3 emission) optical filters. Equal protein loading was assessed by staining the same SDS-PAGE gel with Coomassie Brilliant Blue according to the manual of a commercial staining solution and imaged under white light source. The protein degradation efficient and the quenching off the Cy3 fluorophore for sfGFP-N149-**2b-Cy3** were calculated by quantitative analysis of the stained protein bands via ImageJ software. The images of the SDS-PAGE were displayed in Fig. S21.

Photo-labelling of sfGFP-N149DBTD in cell lysate with 1b-Cy3 analyzed via SDS-PAGE in-gel fluorescence imaging

The *E. Coli* cells expressing sfGFP-N149DBTD at 37 °C, 250 rpm for 10 h. The bacterial cultures were harvested by centrifugation (30 min, 10,000 g, 4 °C) and bathed on ice and resuspended in 3 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH = 8.0). Then, cells were sonicated in an ice/water bath for 30 times (2 s each with 6 s interval) to release the overexpressed sfGFP-N149DBTD protein. To 10 μL supernatant of the cell lysates were added 5 μL of **1b-Cy3** (250 μM) in PBS (pH = 7.4) and then irradiated with the 405 nm LED array for 3 min. After the photo-ligation procedure, SDS loading buffer (5X) and DTT (1 M) were added to the PCR tubes containing 15 μL reaction mixture, heated to 85 ºC for 8 min which was further revolved by gel electrophoresis using a 12% polyacrylamide gels and imaged with a CHAMPCHEMI multicolor fluorescence imaging system using 590 nm (for Cy3 emission) optical filters. Equal protein loading was assessed by staining the SDS-PAGE gel with Coomassie Brilliant Blue according to the manual of a commercial staining solution and imaged under the white light source.

Photo-labelling of intracellular sfGFP-N149DBTD in living cells with DASyd-Fl, analyzed by SDS-PAGE in-gel fluorescence imaging

The transformed *E. coli* cells were incubated at 37 °C, 250 rpm for 10 h with 2 mM DBTDA to over-express sfGFP-N149DBTD inside the cells. The bacterial cultures were harvested by centrifugation (30 min, 10,000 g, 4 °C) and resuspended with 10 mL of PBS buffer, and centrifuged at 10,000 g for 15 min. This process was repeated for three times in order to completely exchange the LB growth medium and also to remove residual DBTDA. The pellets were resuspended in 1.0 mL PBS buffer to give an OD600 = 1. **DASyd-Fl** was added to the cell suspension to derive the final concentration of 25 μM in 100 μL of the diluted culture, then the mixtures were incubated at 37 ºC for 10 minutes in a shaker and further irradiated with the 373 nm + 405 nm LED array at room temperature for 5 min to label the sfGFP-N149DBTD inside the intact *E. coli* cells. Then, samples were centrifuged at 10,000 g for 3 min and washed with PBS buffer, this process was repeated for 3-5 times to ensure complete removal of unreacted **DASyd-Fl**. For in-gel fluorescence imaging, after the completion of the photo-click ligation with **DASyd-Fl**, the cells were collected and resuspended in 60 μL of SDS loading buffer (2X), heated for 10 min at 90 °C and centrifuged at 10,000 g for 15 min. 15 μL of the supernatant was resolved by gel electrophoresis using a 12% polyacrylamide gels and imaged with a CHAMPCHEMI multicolor fluorescence imaging system by using blue optical filters. Equal protein loading was assessed by staining the same SDS-PAGE gel with Coomassie Brilliant Blue according to the manual of a commercial staining solution and imaged under white light source. The images of the SDS-PAGE were displayed in Fig. S22.

Dynamic absorbance spectroscopic studies for photo-control of the switching of *cis*-DBTD to PSS either as small molecule or on proteins

The absorbance spectra were recorded in PBS (pH = 7.4) at 298K by using a HORIBA Fluoromax-4 Spectrofluorometer Detector. The DBTDA (50 μM, 0.1% MeOH) and the purified protein samples (19.1 μM) were transferred into a 0.1×1.0 cm optical path quartz optical cuvette (405 nm laser irradiation optical path was 0.1 cm), the absorbance signal of samples before/after irradiation with the 405 nm laser were then monitored. Spectral range of the samples were recorded from 250 to 600 nm, interval = 0.2 nm per data point over the recorded wavelength range. The 405 nm laser power density was set at 250 mW/cm^2.

Photo-switching kinetic studies of the sfGFP-DBTD variants under irradiation of the 405 nm laser

The rate constant of the photo-switching process of the sfGFP-DBTD variants was measured via dynamic spectral tracking upon a programmable 405 nm laser irradiation intermittently (laser on for 4s and off for 6s per cycle) with 19.1 μM protein samples in PBS (pH = 7.4), 298K. The laser irradiation was sat in front of a quartz lens to adjust the spot size of the laser beam from the laser emitting fiber head of the diode laser to the cuvette, while the irradiation face of the cuvette was exposed to the laser irradiation spot as evenly as possible. The purified protein samples (19.1 μM) were transferred into a 0.1×1.0 cm optical path quartz cuvette (405 nm laser irradiation optical path was 0.1 cm), and the cuvette was placed in the cuvette holder with a thermostat for 5 min to equilibrate to designated temperature. The dynamic absorbance signals were read out by monitoring several specific absorbance wavelengths simultaneously. Kinetic runs were recorded using the following instrumental parameters: monitoring wavelength, $\lambda_{moni.}$ = 285 nm, 386 nm and 457 nm; Interval 35 millisecond per data point over the recorded time range (2 min). All data processing was then performed by using the Origin pro software.

Photo-labelling of OmpC-Y232DBTD on living *E. coli* with either 1b-Cy3 and 1b-Cy3-Biotin

We co-transformed the pET-OmpC-Y232TAG plasmid and pEvol-*Mm*DBTDRS plasmid into *E. coli* strain BL21 (DE3) competent cells. Colonies were formed on LB agar in Petri dishes supplemented with tetracycline (34 $\mu g \cdot mL^{-1}$) and ampicillin (100 $\mu g \cdot mL^{-1}$). A single colony from LB agar plate was picked and inoculated in 5 mL LB growth medium supplemented with tetracycline (34 $\mu g \cdot mL^{-1}$) and ampicillin (100 $\mu g \cdot mL^{-1}$) overnight (37 ºC, 250 rpm), and then inoculated in a 1:100 dilution supplemented with tetracycline (34 $\mu g \cdot mL^{-1}$) and ampicillin (100 $\mu g \cdot mL^{-1}$) of 10 mL fresh LB growth medium, and shaken at 37 ºC, 250 rpm. When the OD600 reached 0.4 to 0.5, 2 mM DBTDA was added to the LB growth medium, and the protein expression was induced by adding 1 mM IPTG and 0.2% arabinose when OD600

reached 0.6. After 8 h induction at 30 ºC, 250 rpm, the *E. coli* cells harboring pET-OmpC-Y232TAG either in the presence or absence of DBTDA were collected by centrifugation (15 min, 10,000 g) and resuspended and washed with 10 mL of PBS buffer, and centrifuged at 10,000 g for 15 min. This process was repeated for three times in order to completely exchange the LB growth medium and to remove residual DBTDA. The pellets were resuspended in 1.0 mL PBS to give an OD600 = 1 for imaging purpose. Either **1b-Cy3** or **1b-Cy3-Biotin** was added to the cell suspension to derive the final concentration of 25 μM in 100 μL of the diluted culture, then the mixtures were irradiated with the 405 nm LED array at room temperature for 5 min to label the OmpC-Y232DBTD on living *E. coli* cells. Then, samples were centrifuged at 10,000 g for 3 min and washed with PBS buffer, this process was repeated for 3-5 times to ensure complete removal of unreacted **1b-Cy3** or **1b-Cy3-Biotin**.

For confocal microscopic imaging, *E. coli* cells were seeded in 35-mm glass-bottom tissue culture dishes, 1% agarose (w/v) was melted and introduced into the petri dish which was sealed afterward by using an autoclaved coverslip, and solidified by cooling to room temperature slowly. The immobilized living *E. coli* cells were then imaged immediately under the confocal fluorescence microscope with Cy3 dye acquisition setting.

For In-gel fluorescence imaging, after the completion of the photo-click ligation with **1b-Cy3**, the cells were collected and resuspended in 60 μL of SDS loading buffer (2x), heated for 10 min at 90 °C and centrifuged at 10,000 g for 15 min. 15 μL of the supernatant was resolved by gel electrophoresis using a 12% polyacrylamide gels and imaged with a CHAMPCHEMI multicolor fluorescence imaging system by using 590 nm (for Cy3 emission) optical filters. Equal protein loading was assessed by staining the same SDS-PAGE gel with Coomassie Brilliant Blue according to the manual of a commercial staining solution and imaged under white light source.

For western blot to study the selectivity in the complex of cell lysate, after the photo-click ligation with **1b-Cy3-Biotin**, the cells were collected and resuspended in 60 μL of SDS loading buffer (2X), heated for 10 min at 90 °C and centrifuged at 10,000 g for 15 min. 15 μL of supernatant was first separated by gel electrophoresis using a 12% polyacrylamide gels and then transferred to PVDF membrane (GE, Hybond P, 0.45 μm) via semi-dry electroblotting (25 V, 30 min). Then the electroblotted PVDF membrane was blocked by a commercial blocking buffer containing BSA and Triton X-100 in TBS buffer. After blocking, the transferred PVDF membrane was stained with the NeutrAvidin™, Oregon Green™ 488 conjugate for recognition of the biotin target under gentle agitation for 1 h. After thoroughly washed by the blocking buffer, the PVDF membrane was imaged with a CHAMPCHEMI multicolor fluorescence imaging system by using 590 nm (for Cy3 emission) and 535 nm (for NeutrAvidin OG488 conjugate emission) optical filters to detect the fluorescent bands of the desired protein with two colors sequentially. Equal protein loading was also assessed by staining the SDS-PAGE gel with Coomassie

Brilliant Blue according to the manual of a commercial staining solution in an identical SDS-PAGE gel without electro-transferring and imaged under white light source.

Spatiotemporally resolved fluorescence imaging of the *E. coli* cells

The *E. coli* cells overexpressing OmpC-Y232DBTD were collected by centrifugation (15 min, 10,000 g) and washed with 10 mL of PBS buffer, resuspended and then centrifuged at 10,000 g for 15 min. This process was repeated for three times in order to remove the LB growth medium and residual DBTDA in the culture buffer to avoid interference from small molecules. Then, the pellets were then resuspended in 1 mL PBS to give a suspension with OD600 = 1. The *E. coli* cells were seeded in 35-mm glass-bottom tissue culture dishes, and fixed with 4% PFA for 1 h, and washed with PBS to remove PFA. **1b-Cy3-Biotin** (5 μM) was diluted in PBS and added into *E. coli* cells culture dishes. A cluster of *E. coli* cells of interest were irradiated by projecting a grid-patterned 405 nm LED photo-stimulation via a digital micromirror device (DMD) embedded in an epifluorescence microscope with a designated time sequence, and the cells were further washed with PBS for three times to wash away unreacted **1b-Cy3-Biotin** and quenched reagents. After the photo-ligation procedure, the region of interest covering the irradiated cells were then imagined immediately under the fluorescence microscope with corresponding filter set for Cy3 fluorophore.

Assessment of bacterial viability after the photo-click labelling on OmpC

The live *E. coli* cells over-expressing OmpC-Y232DBTD were collected, washed and resuspended in PBS buffer (pH = 7.4) to give a cell suspension with OD600 = 1. Then, **1b-Cy3** was added to the cell suspension to derive the final concentration of 25 μM in 100 μL of the diluted culture, then the mixtures were irradiated with the 405 nm LED array at room temperature for 5 min to label the OmpC-Y232DBTD on outer membrane. After photo-labelling, samples were centrifuged at 10,000 g for 3 min and washed with PBS buffer, this process was repeated for 3-5 times to ensure complete removal of unreacted **1b-Cy3.** After this experimental procedure, the treated cells (10 μL) were diluted with LB growth medium, and then plated on a LB agar plate supplemented with tetracycline (34 $\mu g \cdot mL^{-1}$) and ampicillin (100 $\mu g \cdot mL^{-1}$). Next, the smeared agar petri dish was placed in incubators at 37 °C for 12 hours to allow colonies formation. After imaging under white light to identify the colonies on the agar plate, we can manually count the colonies on the plate in order to assess the viability of the cells after the photo-labelling procedure. As for the three control experiments, the *E. coli* cells treated with **1b-Cy3** without photo-stimulation or only subjected to photo-stimulation without compound treatment or washed without photo-labelling procedure were also smeared on agar plate to evaluate the viability for comparison. The images of the control groups (including the untreated *E. coli* cells) and the experimental group were displayed in Fig. S27.

Supplemental Notes

Note S1: The amino acid sequence of *Mm*DBTDRS

MDKKPLNTLISATGLWMSRTGTIHKIKHHEVSRSKIYIEMACGDHLVVN
NSRSSRTARALRHHKYRKTCKRCRVSDEDLNKFLTKANEDQTSVKVKVVSA
PTRTKKAMPKSVARAPKPLENTEAAQAQPSGSKFSPAIPVSTQESVSVPASVS
TSISSISTGATASALVKGNTNPITSMSAPVQASAPALTKSQTDRLEVLLNPKDEI
SLNSGKPFRELESELLSRRKKDLQQIYAEERENYLGKLEREITRFFVDRGFLEI
KSPILIPLEYIERMGIDNDTELSKQIFRVDKNFCLRPMLTPNLYNYLRKLDRAL
PDPIKIFEIGPCYRKESDGKEHLEEFTMLAFGQMGSGCTRENLESIITDFLNHLG
IDFKIVGDSCMVFGDTLDVMHGDLELSSAVVGPIPLDREWGIDKPTIGAGFGL
ERLLKVKHDFKNIKRAARSESYYNGISTNL*

Note S2: The amino acid sequence of sfGFP-K3TAG-His6

MS*GEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTT
GKLPVPWPTLVTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKD
DGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYITA
DKQKNGIKANFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSA
LSKDPNEKRDHMVLLEFVTAAGITHGMDELYKELHHHHHH*

Note S3: The amino acid sequence of sfGFP-N149TAG-His6

MSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICT
TGKLPVPWPTLVTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFK
DDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSH*VYITA
DKQKNGIKANFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSA
LSKDPNEKRDHMVLLEFVTAAGITHGMDELYKELHHHHHH*

Note S4: The amino acid sequence of sfGFP-Q204TAG-His6

MSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICT
TGKLPVPWPTLVTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFK
DDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIT

ADKQKNGIKANFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLST*SA
LSKDPNEKRDHMVLLEFVTAAGITHGMDELYKELHHHHHH*

Note S5: The amino acid sequence of OmpC-Y232TAG-His6

MKVKVLSLLVPALLVAGAANAAEVYNKDGNKLDLYGKVDGLHYFSDN
KDVDGDQTYMRLGFKGETQVTDQLTGYGQWEYQIQGNSAENENNSWTRVA
FAGLKFQDVGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYGSDNFMQQRGN
GFATYRNTDFFGLVDGLNFAVQYQGKNGNPSGEGFTSGVTNNGRDALRQNG
DGVGGSITYDYEGFGIGGAISSSKRTDAQNTAA*IGNGDRAETYTGGLKYDA
NNIYLAAQYTQTYNATRVGSLGWANKAQNFEAVAQYQFDFGLRPSLAYLQS
KGKNLGRGYDDEDILKYVDVGATYYFNKNMSTYVDYKINLLDDNQFTRDA
GINTDNIVALGLVYQFHHHHHH*

where * is the stop codon, TAG.

Supplemental Tables

Table S1: the *Mm*DBTDRS mutation library constructed in this research work for the directed evolution.

Synthetase	Mutation sites				
*Mm*PylRS	A302	N346	C348	Y384	W417
*Mm*DBTDRS library	A/T	A/G/V	A/G/V	F	NNK

Table S2: The primer sequences used for PCR in this research work either to introduction the TAG amber stop codon or to create the mutation library for the directed evolution of the synthetase.

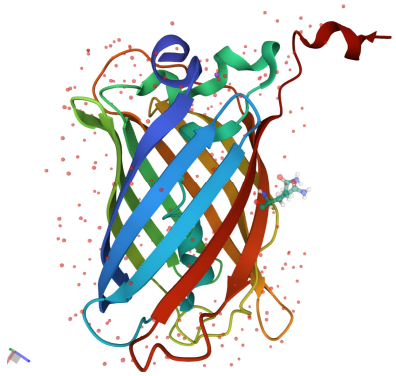
Plasmids	Oligo-nucleotide	Primer sequence (5'-3')
sfGFP-Q204TAG	forward	tgtcgacatagtctgccctttcgaaagatcc
	reverse	caaccattacctgtcgacatagtctgccct
sfGFP-N149TAG	forward	taactcacacTAGgtatacatcacggcag
	reverse	GatgtatacCTAgtgtgagttatagttgtactcg
sfGFP-K3TAG	forward	CATatgagttagggagaagaacttttcactggag
	reverse	tcttctccctaactcatATGTATATCTCCTTC
OmpC-Y232TAG	forward	GCTGCTTAGATCGGTAACGGCGACCGTGC TGAAAC
	reverse	CGTTACCGATcTAAGCAGCGGTGTTCTGA GCATcagtac
*Mm*PylRS-Y384F	forward	tgcatggtctttggggataccttgatgtaatgc
	reverse	tatccccaaagaccatgcaggaatcgcctac
*Mm*PylRS-A302 A/T	forward	gacccatgcttaccccaaacctttacgacccatgcttgctccaaaccttac
	reverse	aggtttggggtaagcatgggtctcaggcagaagaggtttggagcaa gcatgggtctcaggcagaag
*Mm*PylRS-N346 G/A/V-C348G/A/V	forward	CatgctgGBSttcGBScagatgggatcgggat B=C/G/T; S=G/C
	reverse	AtcccatctgCVWgaaCVWcagcatggtaaactcttcgagg V=G/C/A; W=A/T
*Mm*PylRS-W417 NNK	forward	aatggggtattgataaacccNNKataggggcaggtttcg N=A/T/G/C; K=G/T
	reverse	AcctgcccctatMNNgggtttatcaataccccat N=A/T/G/C; M=C/A

Table S3: The docking results with scoring and interaction energies of DBTDA to *Mm*DBTDRS

Pose number	CDOCKER energy (kcal·mol^{-1})	CDOCKER interaction energy (kcal·mol^{-1})	remarks
1	-34.5	-44.2	The most favorable pose and docking site, showing the azo-moiety is facing inward of the synthetase
2	-33.8	-42.3	
3	-32.9	-42.5	
4	-32.3	-39.0	
5	-31.5	-39.0	
6	-30.8	-41.4	
7	-30.7	-39.3	
8	-29.5	-37.6	
9	-27.7	-33.3	The favorable pose and docking site, showing the azo-moiety is facing outward of the synthetase (Figure S3)
10	-27.5	-37.9	

Supplemental Figures

(a)



MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGK
LPVPWPTLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDG
NYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADK
QKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVL**LPDNHYLSTUSALS**
KDPNEKRDHMVLLEFVTAAGITHGMDELYK

(b)

#1	b+	b2+	b3+	b4+	Seq.	y+	y2+	y3+	y4+	#2
15	1707.77214	854.38971	569.92890	427.69849	Q	3046.47273	1523.74000	1016.16243	762.37364	27
16	1835.83071	918.41900	612.61509	459.71314	Q	2918.41415	1459.71071	973.47624	730.35900	26
17	1949.87364	975.44046	650.62940	488.22387	N	2790.35558	1395.68143	930.79004	698.34435	25
18	2050.92132	1025.96430	684.31196	513.48579	T	2676.31265	1338.65996	892.77573	669.83362	24
19	2147.97408	1074.49068	716.66288	537.74898	P	2575.26497	1288.13612	859.09317	644.57170	23
20	2261.05815	1131.03271	754.35757	566.01999	I	2478.21221	1239.60974	826.74225	620.30851	22
21	2318.07961	1159.54344	773.36472	580.27536	G	2365.12814	1183.06771	789.04757	592.03749	21
22	2433.10656	1217.05692	811.70704	609.03210	D	2308.10668	1154.55698	770.04041	577.78213	20
23	2490.12802	1245.56765	830.71419	623.28746	G	2193.07974	1097.04351	731.69810	549.02539	19
24	2587.18078	1294.09403	863.06511	647.55065	P	2136.05827	1068.53277	712.69094	534.77003	18
25	2686.24920	1343.62824	896.08792	672.31776	V	2039.00551	1020.00639	680.34002	510.50683	17
26	2799.33326	1400.17027	933.78260	700.58877	L	1939.93709	970.47219	647.31722	485.73973	16
27	2912.41732	1456.71230	971.47729	728.85979	L	1826.85303	913.93015	609.62253	457.46872	15
28	3009.47009	1505.23868	1003.82821	753.12298	P	1713.76897	857.38812	571.92784	429.19770	14
29	3124.49703	1562.75215	1042.17053	781.87972	D	1616.71620	808.86174	539.57692	404.93451	13
30	3238.53996	1619.77362	1080.18484	810.39045	N	1501.68926	751.34827	501.23460	376.17777	12
31	3375.59887	1688.30307	1125.87114	844.65518	H	1387.64633	694.32680	463.22030	347.66704	11
32	3538.66220	1769.83474	1180.22558	885.42101	Y	1250.58742	625.79735	417.53399	313.40231	10
33	3651.74626	1826.37677	1217.92027	913.69202	L	1087.52409	544.26568	363.17955	272.63648	9
34	3738.77829	1869.89278	1246.93095	935.45003	S	974.44003	487.72365	325.48486	244.36546	8
35	3839.82597	1920.41662	1280.61351	960.71195	T	887.40800	444.20764	296.47418	222.60746	7
36	4120.88825	2060.94776	1374.30094	1030.97752	-C10H3NO(-1)	786.36032	393.68380	262.79162	197.34554	6
37	4207.92028	2104.46378	1403.31161	1052.73553	S	505.29804	253.15266	169.10420	127.07997	5
38	4278.95739	2139.98234	1426.99065	1070.49481	A	418.26601	209.63664	140.09352	105.32196	4
39	4392.04146	2196.52437	1464.68534	1098.76582	L	347.22890	174.11809	116.41448	87.56268	3
40	4479.07349	2240.04038	1493.69601	1120.52383	S	234.14483	117.57605	78.71980	59.29167	2
41					K	147.11280	74.06004	49.70912	37.53366	1

Figure S1. The LC-MS/MS analysis of sfGFP-Q204DBTD. (a) The amino acid sequences of sfGFP and the portions of spliced sequence detected and covered in the LC-MS/MS analysis which were marked in green color, showing the coverage up to 85% of the full length. (b) The table showed the raw m/z data and searching results in the LC-FTMS mass spectrometry. And the de novo sequenced peptide region covering the key 204TAG residue was displayed, and related peptide fragments identified with b (red) and y (blue) cation signals were marked with mass-to-charge

ratio (m/z). The genetic encoded DBTDA at 204 position was confirmed by detecting the prcesence of Q-$C_{10}H_3NO(_{-1})S$ (molecular formula where the original glutamine was replaced with DBTDA) to specify the incorporation of DBTDA into sfGFPQ204 residue.

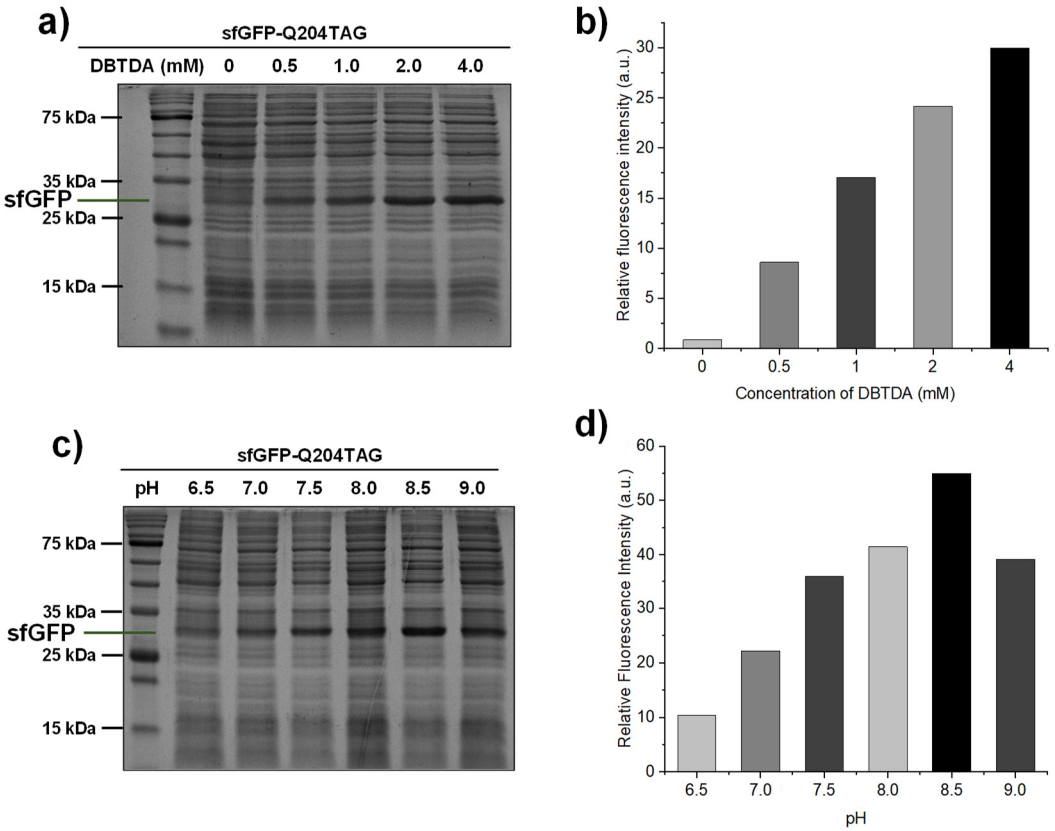
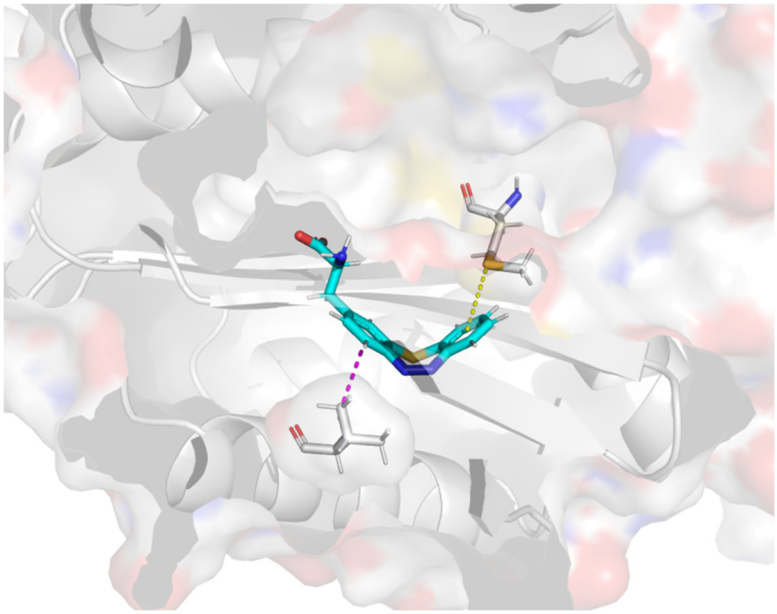


Figure S2. Optimization of the incorporation efficiency of DBTDA to express sfGFP-Q204DBTD in *E. coli* BL21(DE3). (a) SDS-PAGE analysis of sfGFP-Q204DBTD expression in the presence of various concentrations of DBTDA (from 0 to 4 mM) supplied in the culture medium. (b) Fluorescence intensity analysis of sfGFP-Q204DBTD expressed in the presence of various concentrations of DBTDA (from 0 to 4 mM) measured via qualification of microscopic imaging. (c) SDS-PAGE analysis of sfGFP-Q204DBTD expression under various pH (from 6.5 to 9.0) of the LB medium. (d) Fluorescence intensity analysis of sfGFP-Q204DBTD expression under various pH (from 6.5 to 9.0) measured via qualification of microscopic imaging.

a)



b)

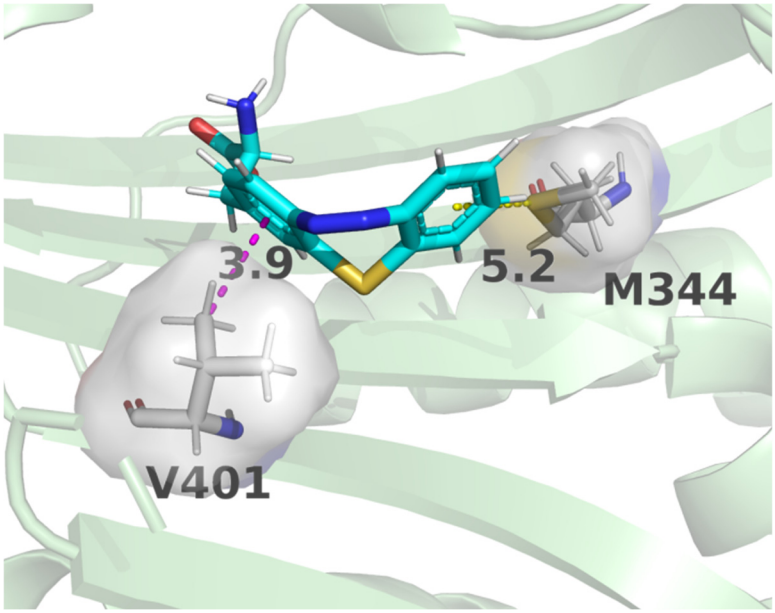


Figure S3. A molecular docking results of DBTDA to the active sites of *Mm*DBTDRS in which azo-moiety of DBTDA was facing outward from the binding pocket of the synthetase. (a) The primary interactions showing in the docking model of DBTDA (Cyan) in *Mm*DBTDRS (Gray) complex with the binding free energy (-33.3 kcal·mol^{-1}) (front view). (b) The amino acid residues (Gray) in the active pocket of *Mm*DBTDRS that interacted with DBTDA (Cyan) are shown in stick and labelled: M344 (sulfur-π interaction), V401 (alkyl-π interaction).

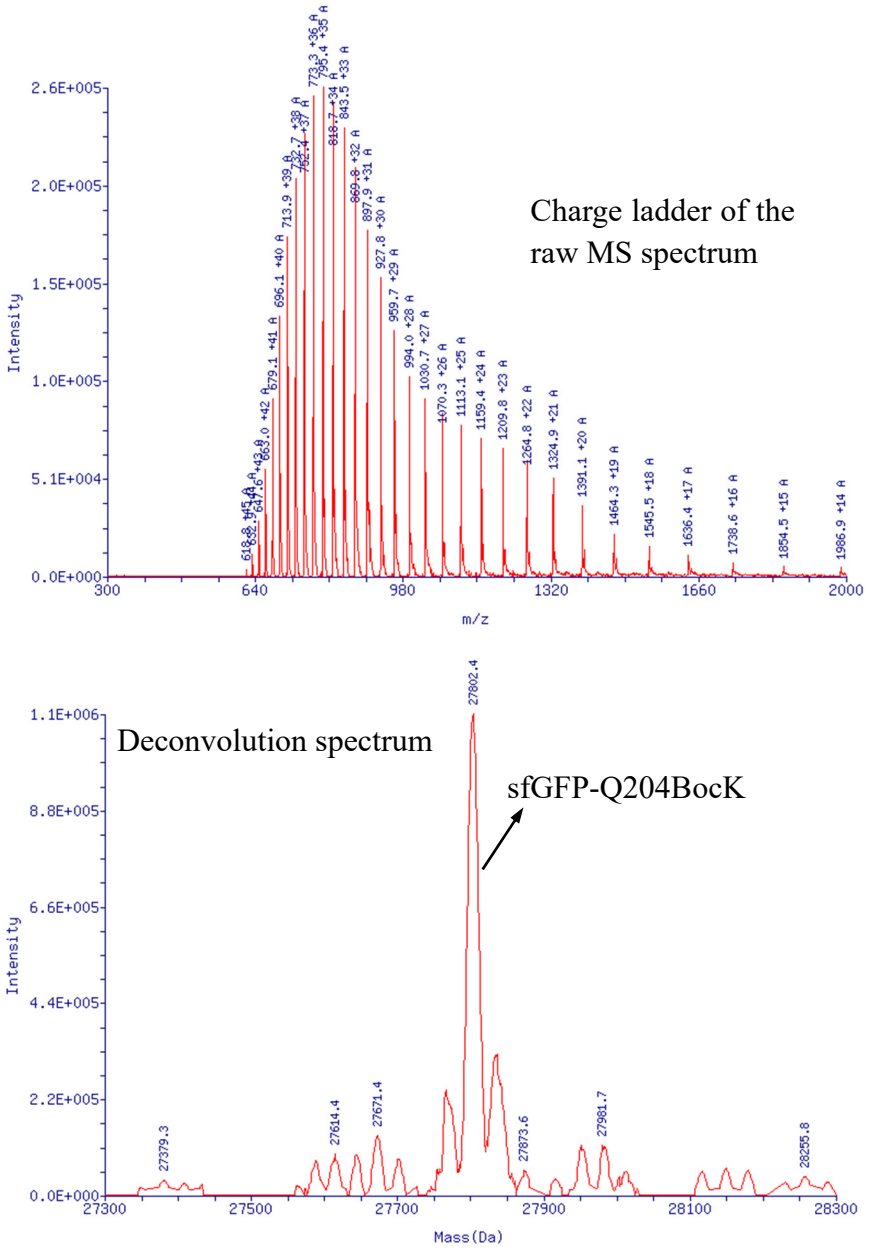


Figure S4. Deconvoluted LC-MS analysis of sfGFP-Q204BocK with the charge ladder and the deconvoluted mass spectrum. Calcd. mass 27830.2 [M-Met+H^+], found 27802.4 Da. Yield = 11.8 mg·L^{-1}.

Sequence is M S K G E E L F T G V V P I L V E L D G D V N G H K F S V R G E G E G D A T N G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T # S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K E L H H H H H H, where # is the BocK.

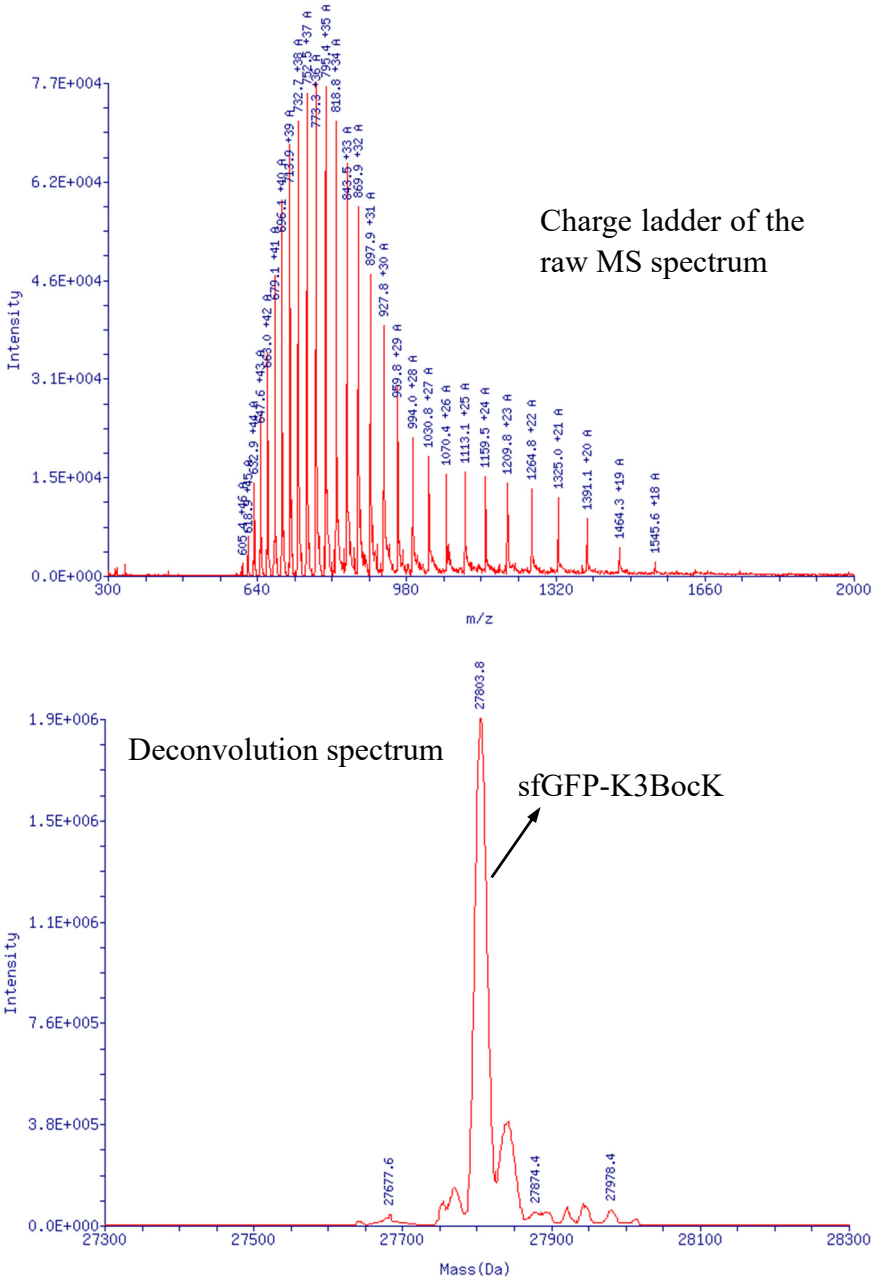


Figure S5. Deconvoluted LC-MS analysis of sfGFP-K3BocK with the charge ladder and the deconvoluted mass spectrum. Calcd. mass 27830.2 [M-Met+H^+], found 27803.8 Da. Yield = 10.7 mg·L^{-1}.

Sequence is M S # G E E L F T G V V P I L V E L D G D V N G H K F S V R G E G E G D A T N G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K E L H H H H H H, where # is the BocK.

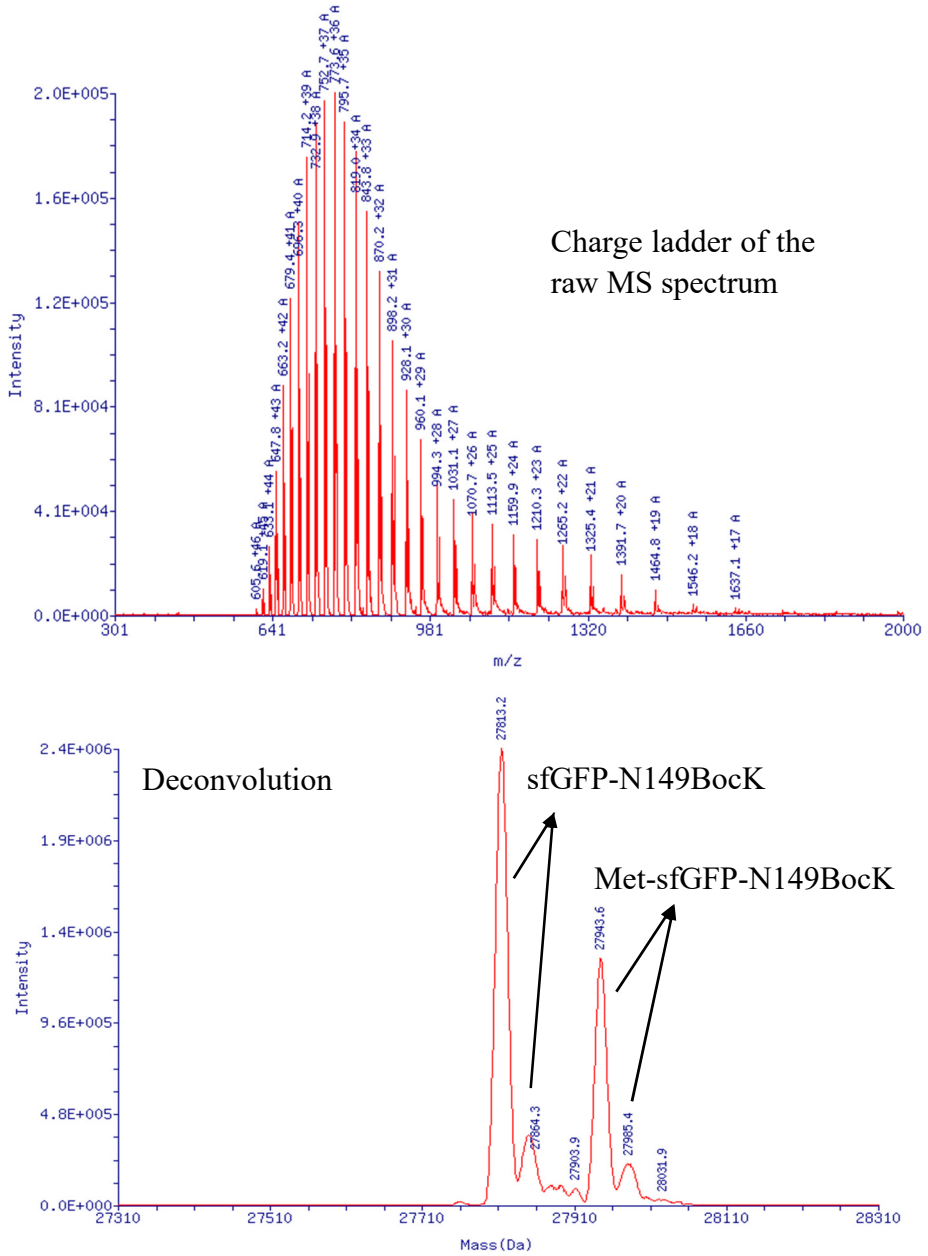


Figure S6. Deconvoluted LC-MS analysis of sfGFP-N149BocK with the charge ladder and the deconvoluted mass spectrum. Calcd. mass 27844.2 [M-Met+H^+], found 27813.2 Da. Yield = 12.4 mg·L^{-1}. The peak of Met-sfGFP-N149BocK represents the intact sfGFP protein without losing the first methionine (Met) in its protein sequence during the native PTM in live cells.

Sequence is M S K G E E L F T G V V P I L V E L D G D V N G H K F S V R G E G E G D A T N G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H # V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K E L H H H H H H, where # is the BocK.

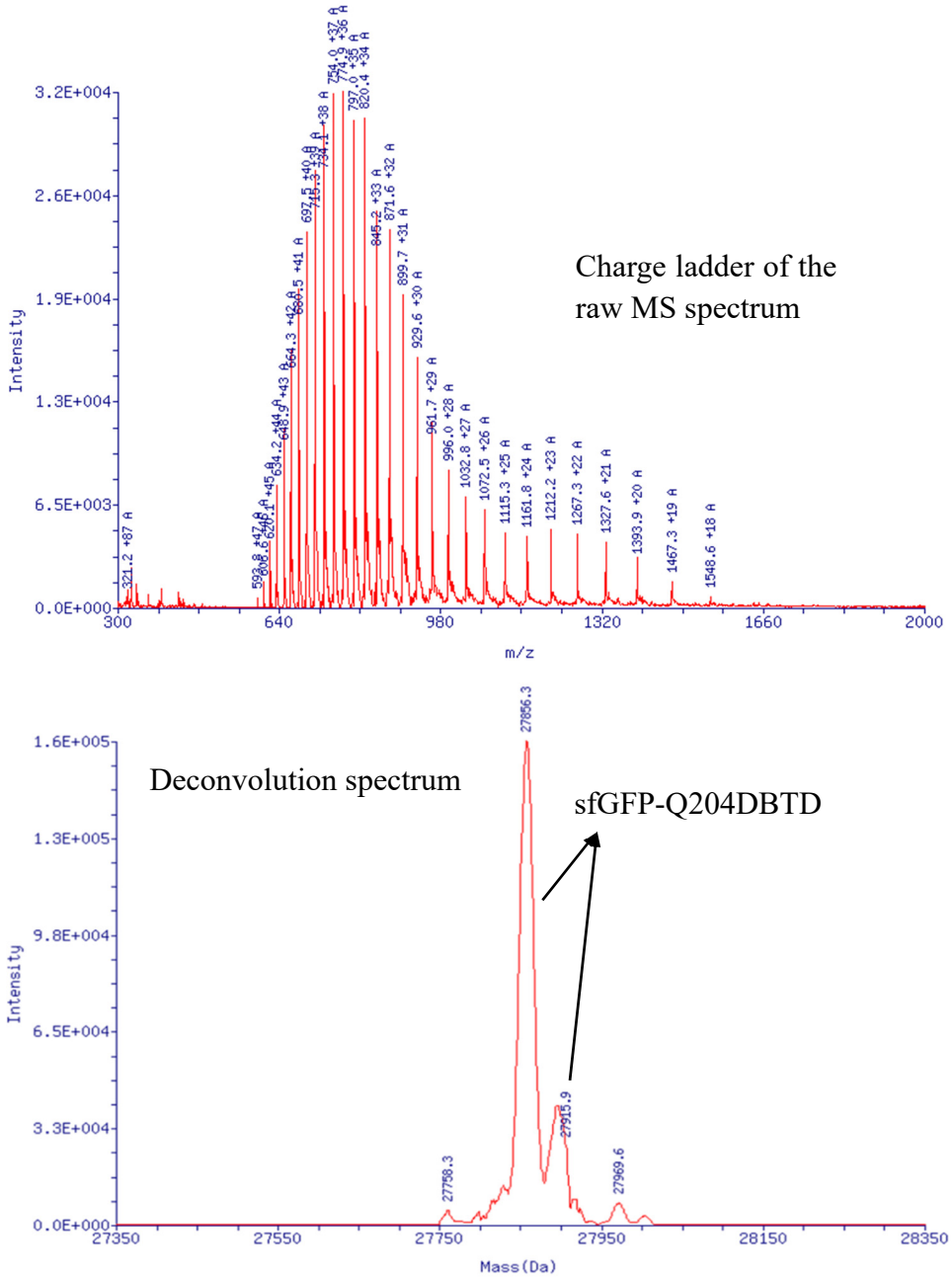


Figure S7. Deconvoluted LC-MS analysis of sfGFP-Q204DBTD with the charge ladder and the deconvoluted mass spectrum. Calcd. mass 27883.3 [M-Met+H^+], found 27856.3 Da. Yield = 4.8 mg·L^{-1}

Sequence is M S K G E E L F T G V V P I L V E L D G D V N G H K F S V R G E G E G D A T N G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T # S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K E L H H H H H H, where # is the DBTDA.

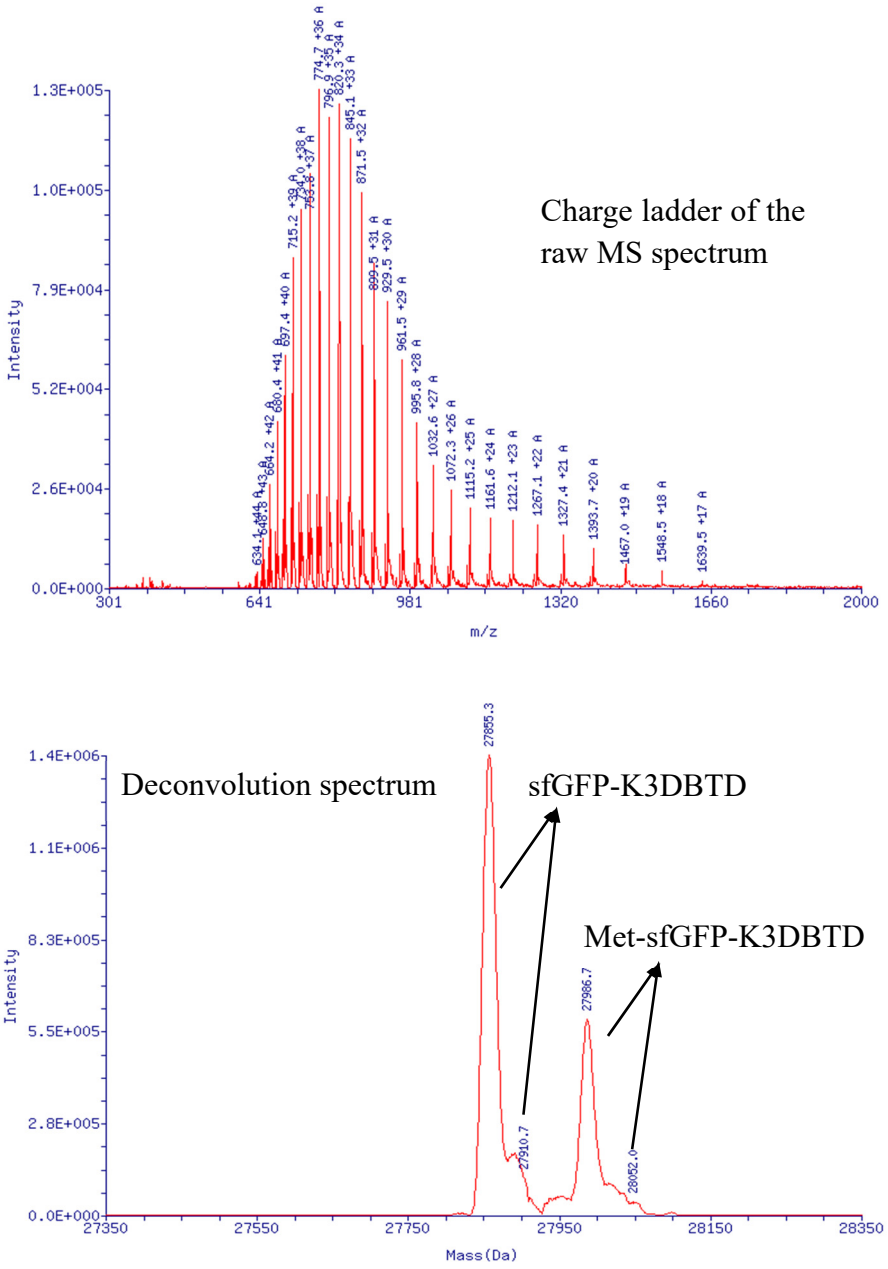


Figure S8. Deconvoluted LC-MS analysis of sfGFP-K3DBTD with the charge ladder and the deconvoluted mass spectrum. Calcd. mass 27883.2 [M-Met+H^+], found 27855.3 Da. Yield = 5.1 mg·L^{-1}. The peak of Met-sfGFP-K3DBTD represents the intact sfGFP protein without losing the first methionine (Met) in its protein sequence during the native PTM in live cells.

Sequence is M S # G E E L F T G V V P I L V E L D G D V N G H K F S V R G E G E G D A T N G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K E L H H H H H H, where # is the DBTDA.

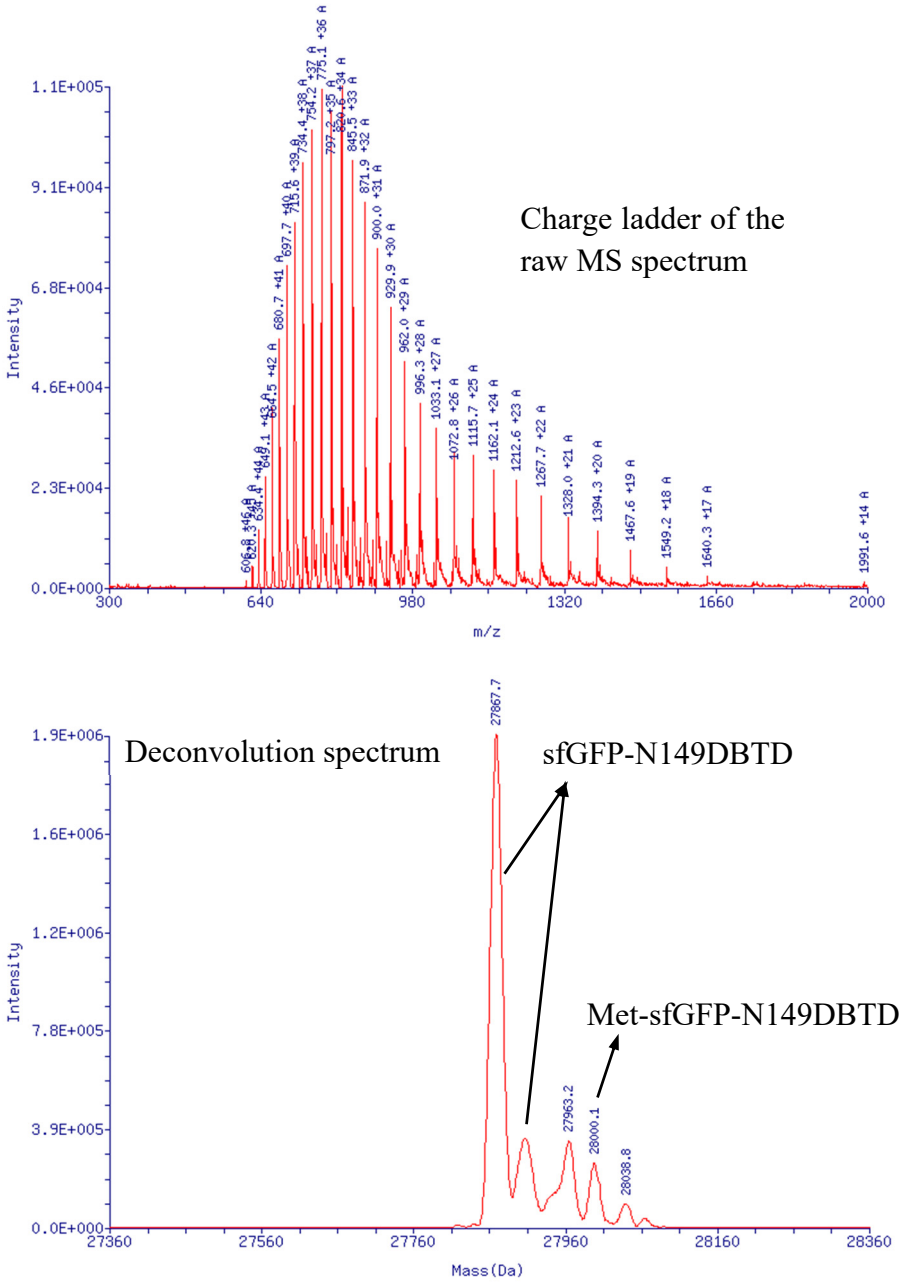


Figure S9. Deconvoluted LC-MS analysis of sfGFP-N149DBTD with the charge ladder and the deconvoluted mass spectrum. Calcd. mass 27897.1 [M-Met+H^+], found 27867.7 Da. Yield = 5.3 mg/L. The peak of Met-sfGFP-N149DBTD represents the intact sfGFP protein without losing the first methionine (Met) in its protein sequence during the native PTM in live cells.

Sequence is M S K G E E L F T G V V P I L V E L D G D V N G H K F S V R G E G E G D A T N G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H # V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K E L H H H H H H, where # is the DBTDA.

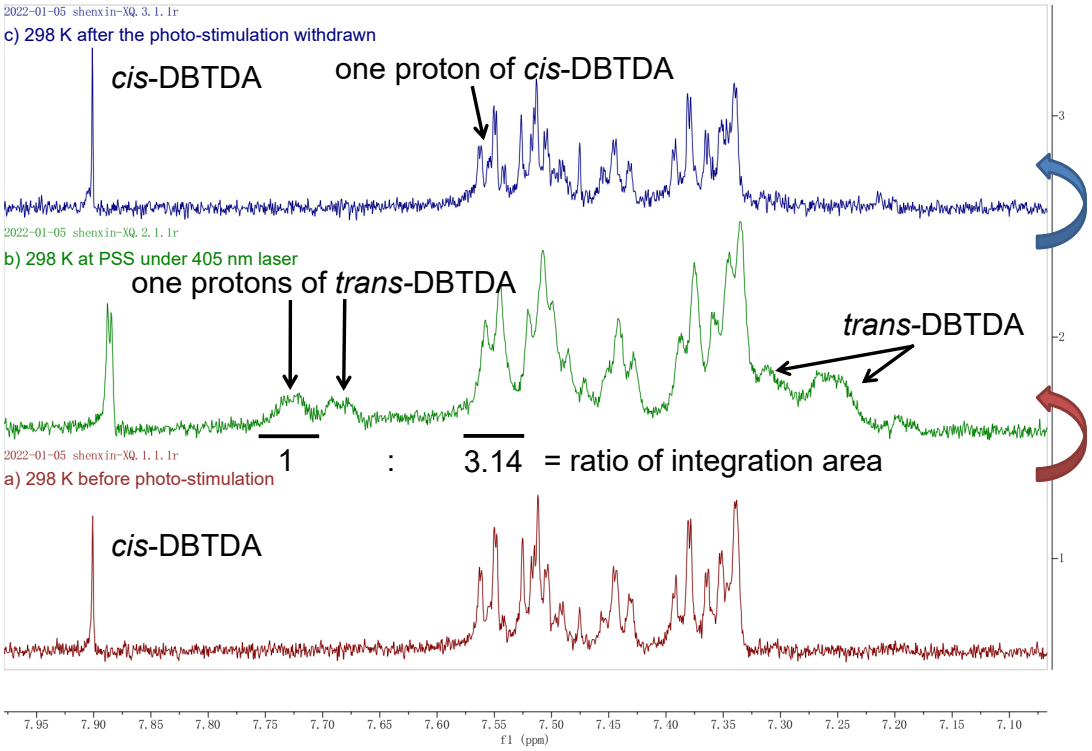


Figure S10. ^{1}H NMR spectra to study the photostationary state (PSS) of DBTDA under exposure of 405 nm laser continuously in methanol-d_4 solvent at 298 K. (a) The initial spectrum of *cis*-DBTDA before photo-stimulation. (b) The spectrum recorded under 405 nm laser irradiation continuously, (*trans*)/(*cis*) = 1/3.14. (c) The spectrum recorded after the irradiation of the 405 nm laser withdrawn. 1.0 mM DBTDA in methanol-d_4 as solvent was transfered into a coaxial quartz NMR tube embedded with a quartz optical-fiber (core diameter: 1000 μm), aligning at the axial direction, ^{1}H NMR, 600 MHz. The "metastable" configuration (ring-strain energy loaded) of *trans*-DBTDA was determined to 24.2% in total amount of DBTDA based on the ratio of the integration area of one proton of the molecule. The optical fiber (1 mm diameter, quartz) guided 405 nm laser source for in-situ NMR study of the PSS was set to 250 mW at the fiber output port.

The same 405 nm laser source with the same power setting was applied to dynamic absorbance tracking experiments shown in Fig. 3a-b in the manuscript, in which the DBTDA concentration was 50 μM in the 0.1 cm (irradiation path) × 1.0 cm (absorbance path) cuvette. Therefore, the "metastable" configuration (ring-strain energy loaded) of *trans*-DBTDA was also estimated to be 24.2% in the diluted conditions, which displayed a Δabsorbance of 0.096 at 285 nm after reaching the PSS. On the sfGFP residues, however, the Δabsorbance at 285 nm was only 0.0153 for sfGFP-N149DBTD and 0.0187 for sfGFP-Q204DBTD, despite the total protein concentration were only 19.1 μM. Considering the potential effect of protein microenvironment on the photoisomerization of the DBTD residues, we could infer that the proportion of DBTD isomerized to the energy-loaded state (*trans*-DBTD

residue) at the sfGFP residue is about 10% *trans*-DBTD for sfGFP-N149DBTD and 12% *trans*-DBTD for sfGFP-Q204DBTD by comparing the Δabsorbance value at 285 nm.

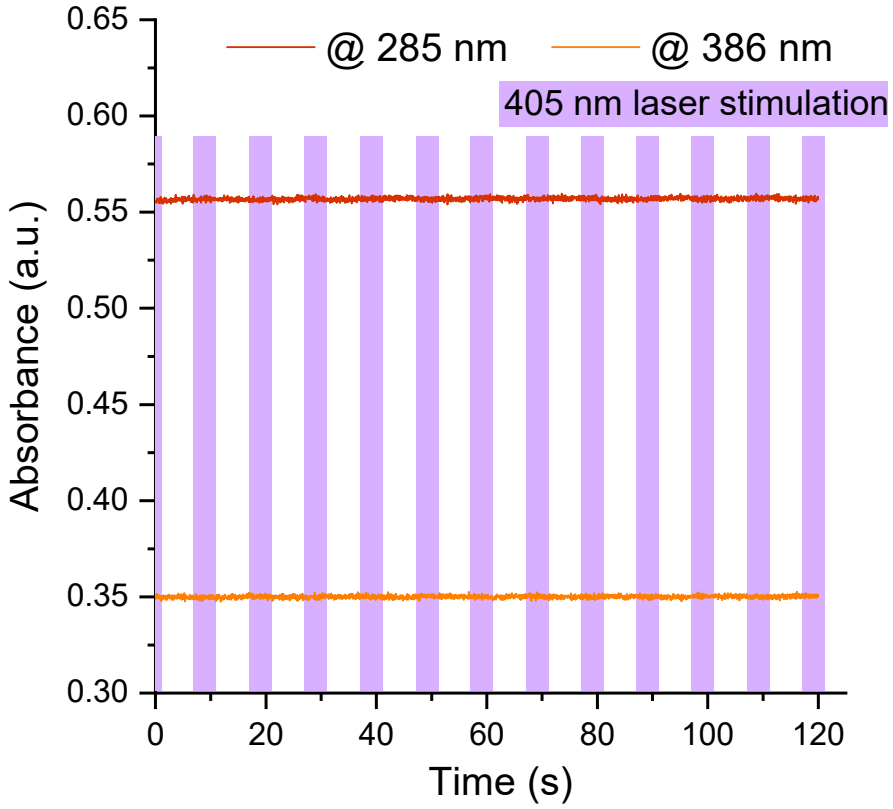


Figure S11. Photo-switching of the wild-type sfGFP under the programmble irradiation sequence of the 405 nm laser. Under an alternating on/off of 405 nm laser photo-stimulation, the absorbance singal of the wild-type sfGFP did not show any significant changes over the time. The absorbance of the sfGFP was measured via the dynamic spectrum tracing upon a 405 nm laser irradiation intermittently (laser on for 4s and off for 6s per cycle) at 19.1 μM in PBS (pH = 7.4). Monitoring wavelength, $\lambda_{moni.}$ = 285 nm and 386 nm.

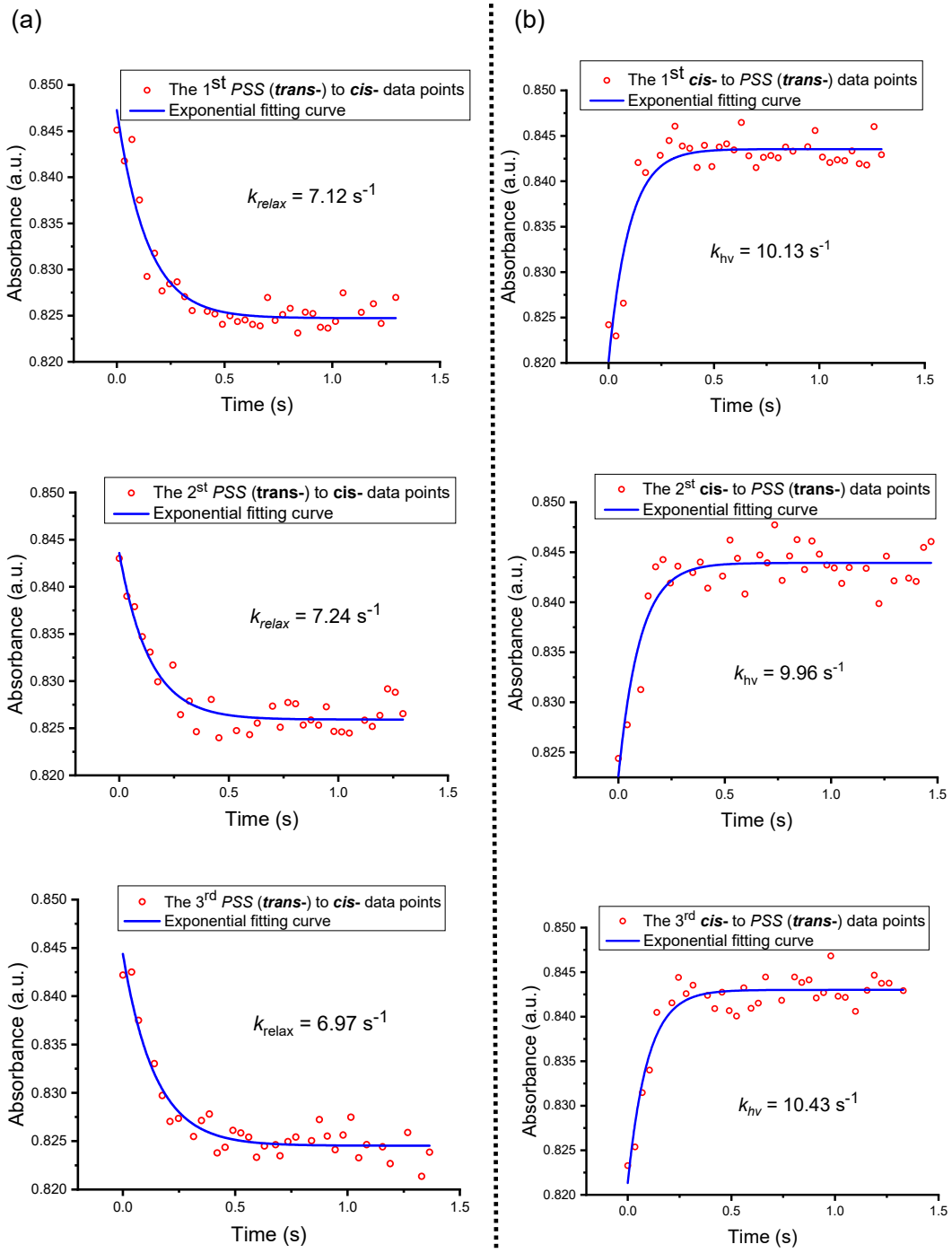


Figure S12. Kinetic studies for the photo-chemical energy conversion process of the DBTD residue on sfGFP-Q204DBTD between its *cis*-state and the PSS_{405nm} by monitoring the absorbance intensity evolution at 285 nm with the photo-stimulation of the 405 nm laser on/off (laser on for 4s and off for 6s per cycle) in PBS (pH = 7.4). (a) Detailed data points with exponential fitting curves of the absorbance intensity evolution at 285 nm. And the averaged thermodynamic switching rate in dark (k_{relax}, the *trans*- to the *cis*-) was determined to be 7.1 ± 0.14 s^{-1}, 298 K. (b) Detailed data points with exponential fitting curves of the absorbance intensity evolution at 285 nm.

And the averaged photo-switching rate (k_{PSS}, the *cis*- to the PSS_{405nm}) value was determined to be $10 \pm 0.54\ s^{-1}$, 298 K.

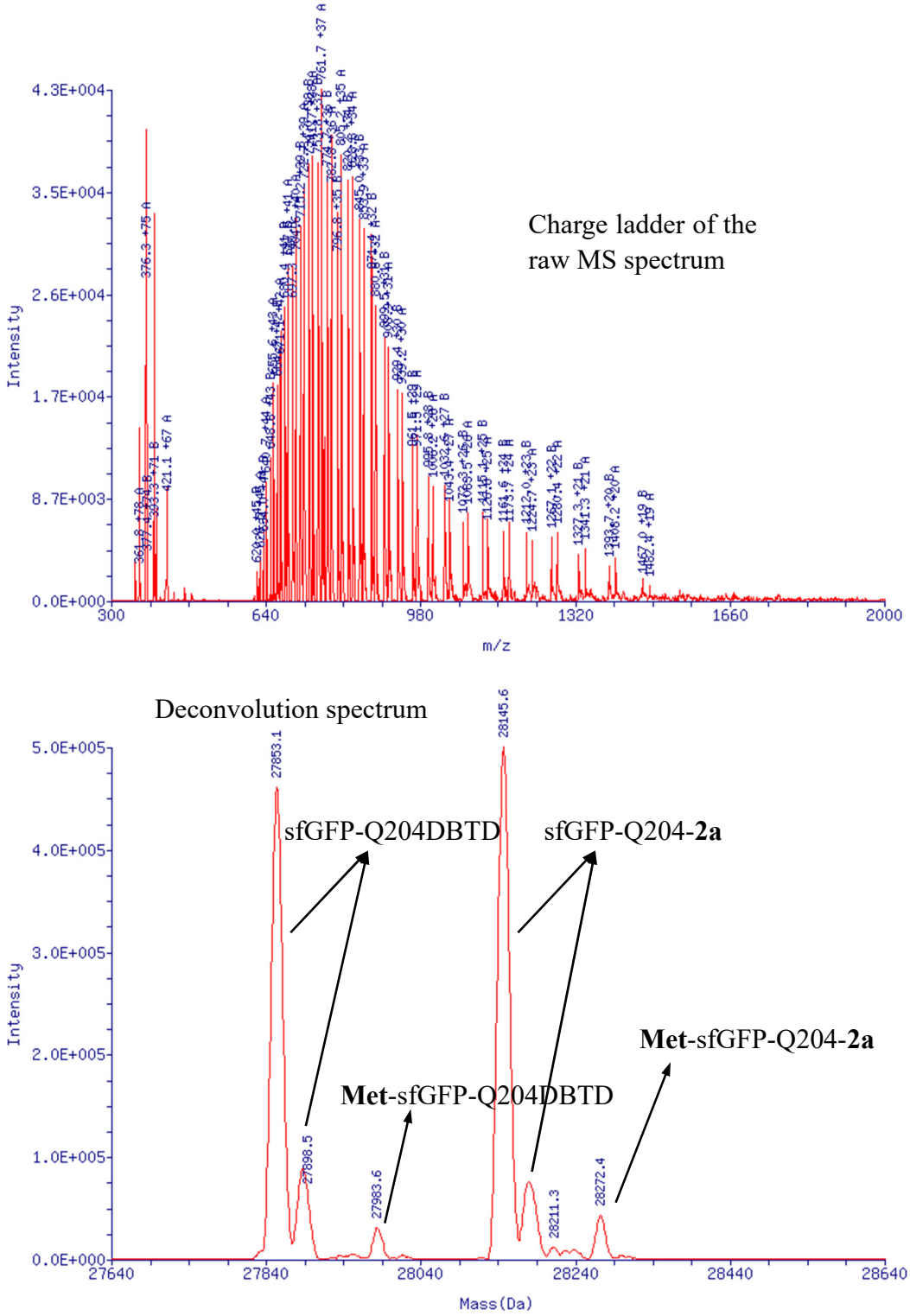


Figure S13. LC-MS analysis of sfGFP-Q204DBTD (10 μM) after the photo-click reaction with DASyd **1a** (100 μM) under irradiation of 311 nm + 405 nm for 2 min. Calcd. mass 28147.4 Da (starting material sfGFP-Q204DBTD adding the nitrile imine derived from DASyd **1a**), found 28145.6 Da. Yield = 52.07% (calculated by comparing the percentage of the product to that of the starting material plus product). The peak of Met-sfGFP-Q204DBTD and Met-sfGFP-Q204-**2a** represents the intact

sfGFP protein without losing the first methionine (Met) in its protein sequence during the native PTM in live cells.
Sequence is M S K G E E L F T G V V P I L V E L D G D V N G H K F S V R G E G E G D A T N G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T # S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K E L H H H H H H, where # is the position to incorporate ncAA.

Mass (Da)	Intensity	Score	Delta Mass	%Total
28145.6	4.98E+005	42.38	0.0	44.64
27853.1	4.59E+005	43.02	-292.5	41.11

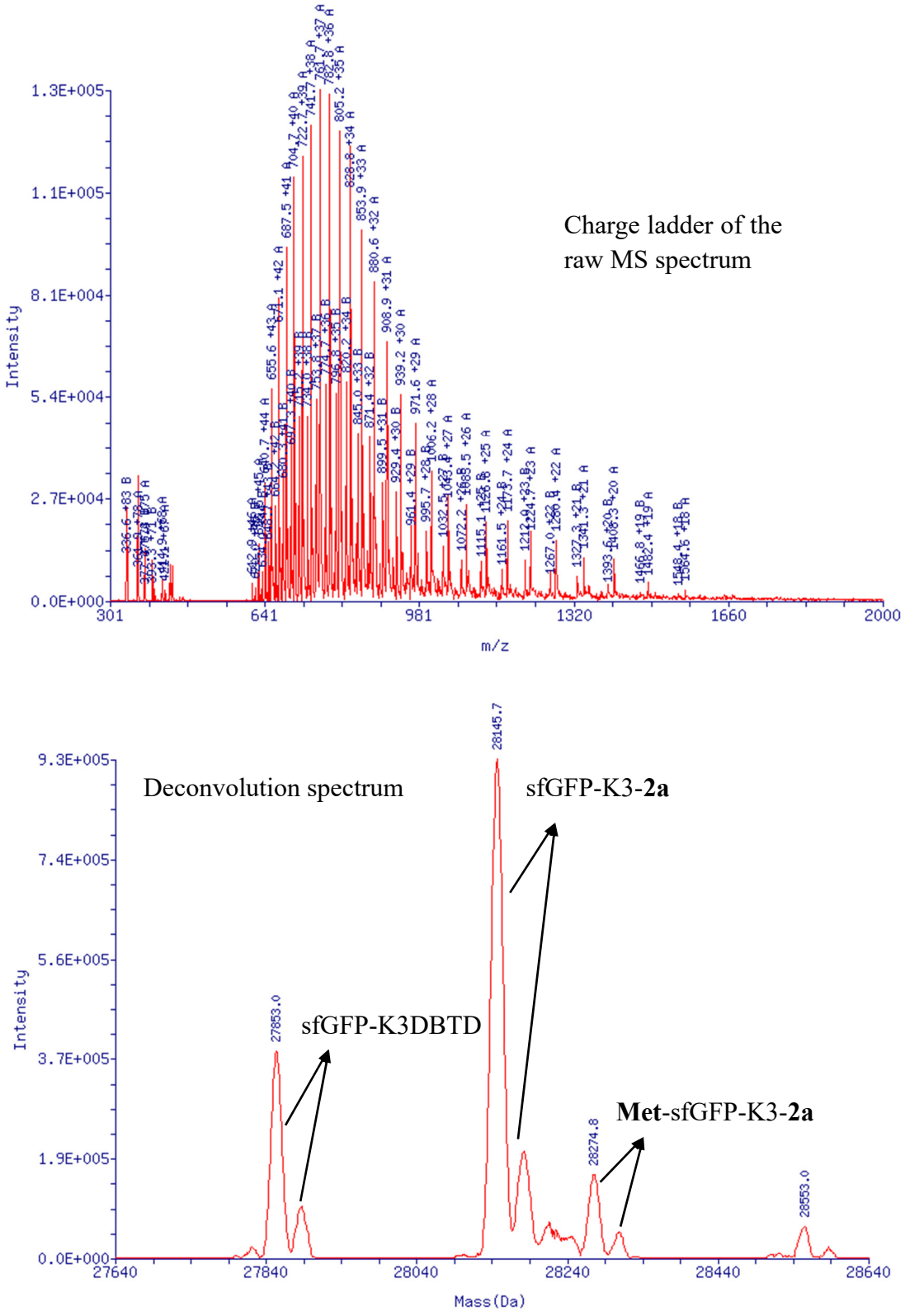


Figure S14. Deconvoluted LC-MS analysis of sfGFP-K3DBTD (10 µM) after the photo-click reaction with DASyd **1a** (100 µM) under irradiation of 311 nm + 405 nm for 2 min. Calcd. mass 28145.0 Da (starting material sfGFP-K3DBTD adding the

nitrile imine derived from DASyd **1a**), found 28145.7 Da. Yield = 70.68% (calculated by comparing the percentage of the product to that of the starting material plus product). The peak of Met-sfGFP-K3-**2a** represents the intact sfGFP protein without losing the first methionine (Met) in its protein sequence during the native PTM in live cells.

Sequence is M S # G E E L F T G V V P I L V E L D G D V N G H K F S V R G E G E G D A T N G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K E L H H H H H H, where # is the position to incorporate ncAA.

Mass (Da)	Intensity	Score	Delta Mass	%Total
28145.7	**9.27E+005**	**36.27**	**0.0**	**60.15**
27853.0	**3.85E+005**	**25.11**	**-292.7**	**24.95**

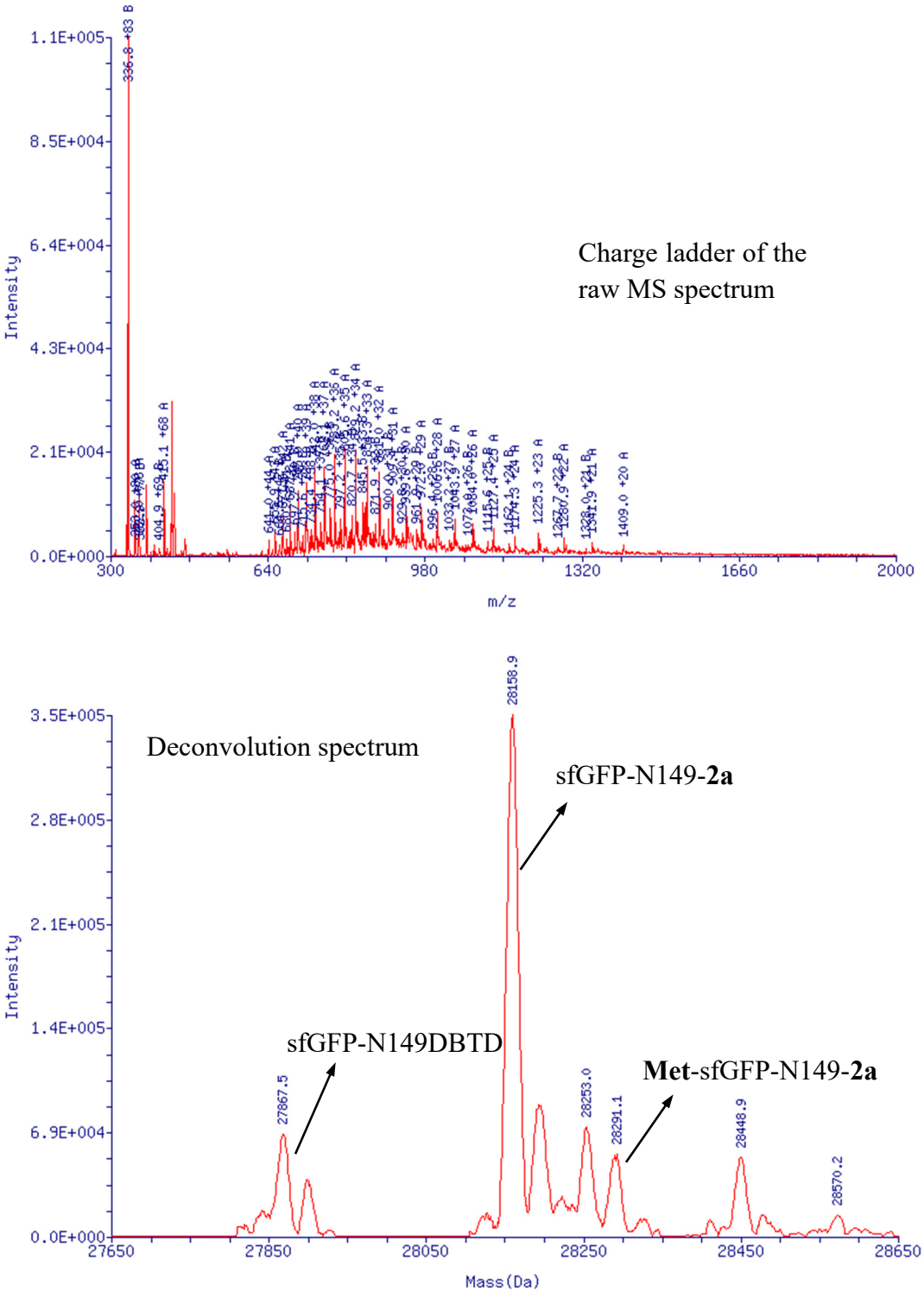


Figure S15. Deconvoluted LC-MS analysis of sfGFP-N149DBTD (10 µM) after the photo-click reaction with DASyd **1a** (100 µM) under irradiation of 311 nm + 405 nm for 2 min. Calcd. mass 28159.5 Da (starting material sfGFP-N149DBTD adding the nitrile imine derived from DASyd **1a**), found 28158.9 Da. Yield = 85.50% (calculated

by comparing the ion count of the product to that of the starting material plus product). The peak of Met-sfGFP-N149-**2a** represents the intact sfGFP protein without losing the first methionine (Met) in its protein sequence during the native PTM in live cells. Sequence is M S K G E E L F T G V V P I L V E L D G D V N G H K F S V R G E G E G D A T N G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H # V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K E L H H H H H H, where # is the position to incorporate ncAA.

Mass (Da)	Intensity	Score	Delta Mass	%Total
28158.9	**3.47E+005**	**28.77**	**0.0**	**53.17**
27867.5	**6.78E+004**	**6.52**	**-291.4**	**10.38**

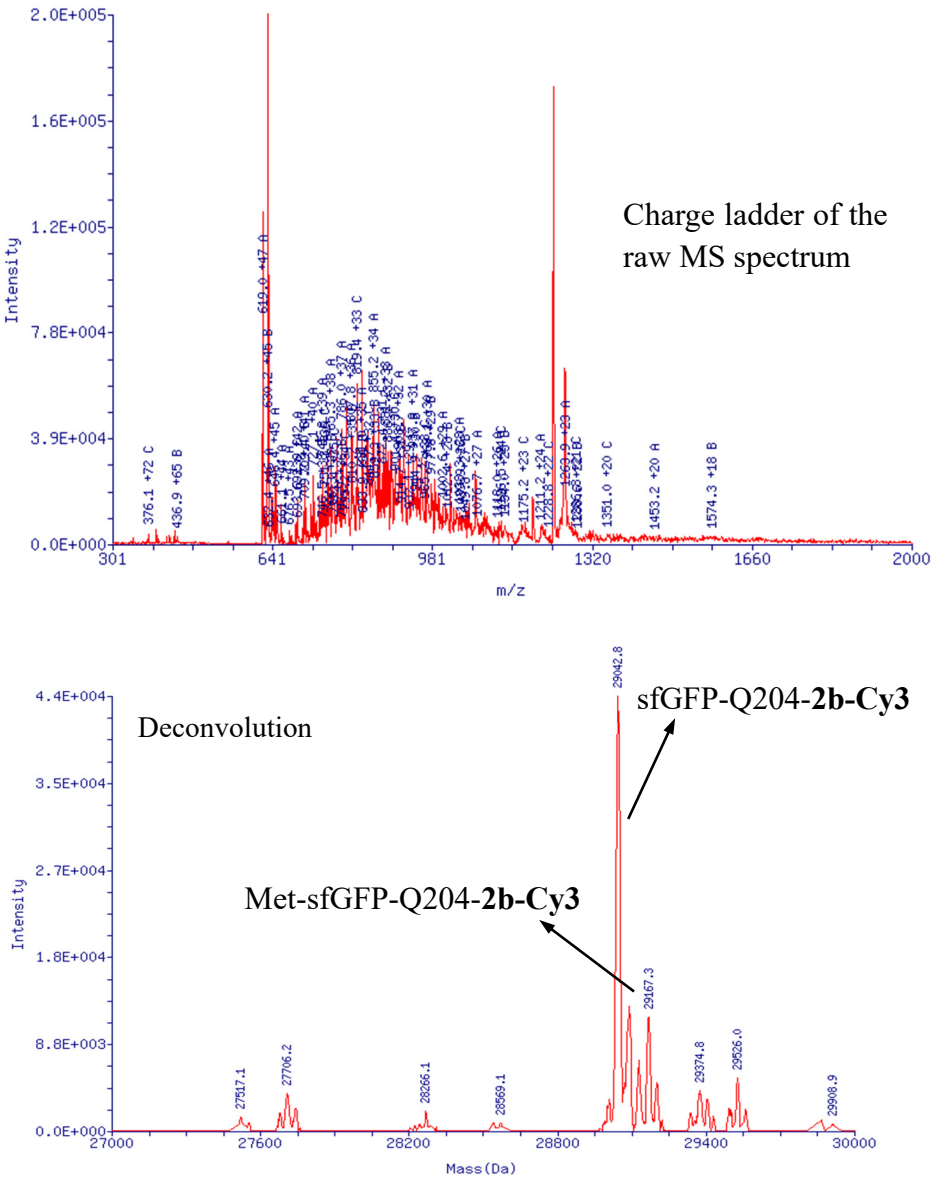


Figure S16. Deconvoluted LC-MS analysis of sfGFP-Q204DBTD (10 μM) after the photo-click reaction with **1b-Cy3** (250 μM) under irradiation of 405 nm for 3 min. Calcd. mass 29045.0 Da (starting material sfGFP-Q204DBTD adding the nitrile imine derived from **1b-Cy3**), found 29042.8 Da. (calculated by comparing the ion count of the product to that of the starting material plus product). The peak of Met-sfGFP-Q204-**2b-Cy3** represents the intact sfGFP protein without losing the first methionine (Met) in its protein sequence during the native PTM in live cells.
Sequence is M S K G E E L F T G V V P I L V E L D G D V N G H K F S V R G E G E G D A T N G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T # S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K E L H H H H H H, where # is the position to incorporate ncAA

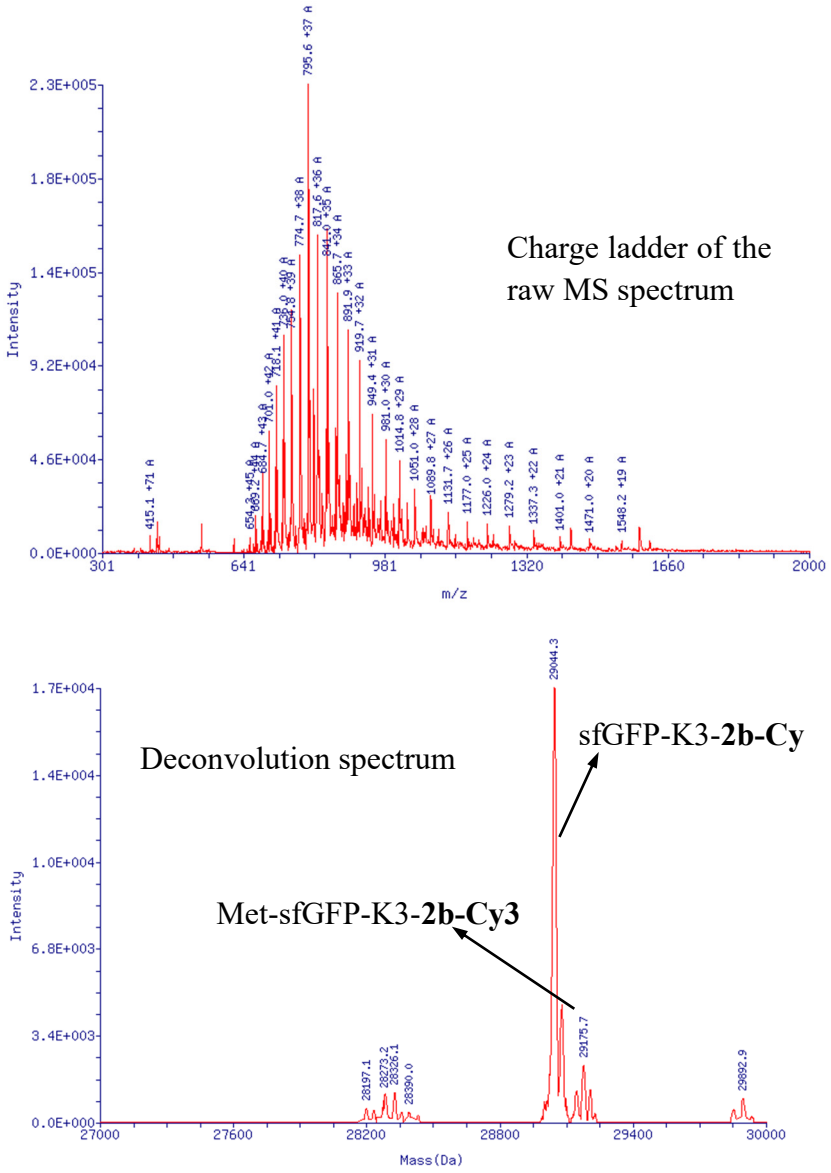


Figure S17. Deconvoluted LC-MS analysis of sfGFP-K3DBTD (10 μM) after the photo-click reaction with **1b-Cy3** (250 μM) under irradiation of 405 nm for 3 min. Calcd. mass 29045.4 Da (starting material sfGFP-K3DBTD adding the nitrile imine derived from **1b-Cy3**), found 29044.3 Da. (calculated by comparing the ion count of the product to that of the starting material plus product). The peak of Met-sfGFP-K3-**2b-Cy3** represents the intact sfGFP protein without losing the first methionine (Met) in its protein sequence during the native PTM in live cells.
Sequence is M S # G E E L F T G V V P I L V E L D G D V N G H K F S V R G E G E G D A T N G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K E L H H H H H H, where # is the position to incorporate ncAA.

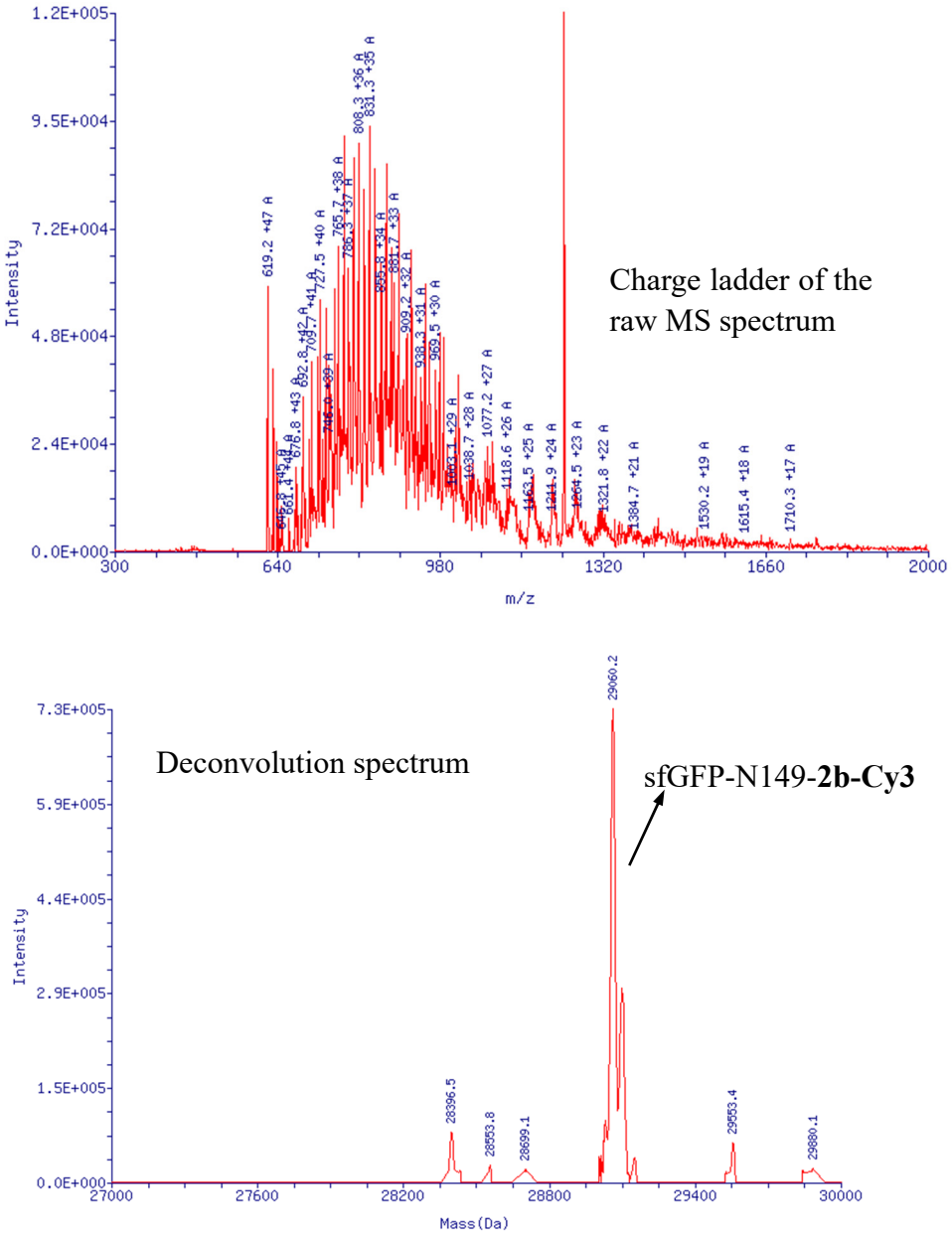
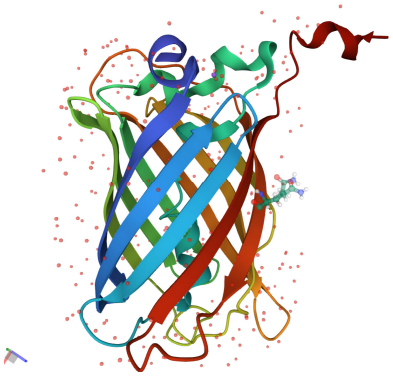


Figure S18. Deconvoluted LC-MS analysis of sfGFP-N149DBTD (10 μM) after the photo-click reaction with **1b-Cy3** (250 μM) under irradiation of 405 nm for 3 min. Calcd. mass 29059.5 Da (starting material sfGFP-N149DBTD adding the nitrile imine derived from **1b-Cy3**), found 29060.2 Da. (calculated by comparing the ion count of the product to that of the starting material plus product).
Sequence is M S K G E E L F T G V V P I L V E L D G D V N G H K F S V R G E G E G D A T N G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K R H D F F K S A M P E G Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H # V Y I T A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G I T H G M D E L Y K E L H H H H H H, where # is the position to incorporate ncAA.

(a)



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KDPNEKRDHMVLLEFVTAAGITHGMDELYK

(b)

#1	b^{-}	b^{2-}	b^{3-}	Seq.	y^{-}	y^{2-}	y^{3-}	#2
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2	257.12443	129.06585	86.37966	Q	3037.41890	1519.21309	1013.14449	24
3	371.16736	186.08732	124.39397	N	2909.36033	1455.18380	970.45829	23
4	472.21504	236.61116	158.07653	T	2795.31740	1398.16234	932.44398	22
5	569.26780	285.13754	190.42745	P	2694.26972	1347.63850	898.76142	21
6	682.35187	341.67957	228.12214	I	2597.21696	1299.11212	866.41050	20
7	739.37333	370.19030	247.12929	G	2484.13289	1242.57009	828.71582	19
8	854.40027	427.70377	285.47161	D	2427.11143	1214.05935	809.70866	18
9	911.42174	456.21451	304.47876	G	2312.08449	1156.54588	771.36635	17
10	1008.47450	504.74089	336.82968	P	2255.06302	1128.03515	752.35919	16
11	1107.54291	554.27509	369.85249	V	2158.01026	1079.50877	720.00827	15
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21	2261.11969	1131.06348	754.37808	T	1006.41275	503.71001	336.14243	5
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23	2963.34574	1482.17651	988.45343	S	290.17105	145.58916	97.39520	3
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(c)

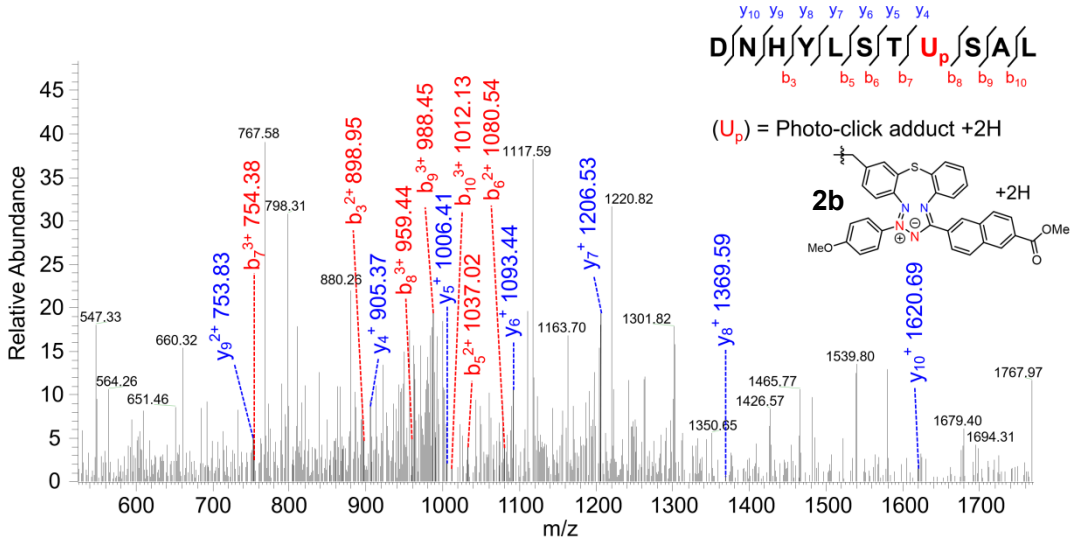

Figure S19. The LC-MS/MS analysis of sfGFP-Q204DBTD after photo-reaction with DASyd **1b**. (a) The amino acid sequences of sfGFP and the portions of spliced sequence detected and covered in the LC-MS/MS analysis which were marked in green color, showing the coverage up to 97% of the full length. (b) The table showed the raw m/z data and searching results in the LC-FTMS mass spectrometry. The *de novo* sequenced peptide region covering the key 204 residue was displayed. And related peptide fragments identified with b (red) and y (blue) cation signals were marked in mass-to-charge ratio (m/z). The genetic encoded and photo-conjugated **2b** at 204 position was confirmed by detecting the prcesence of Q-$C_{30}H_{21}N_3O_2S$ (the molecular formula where the original glutamine, Q, was replaced by DBTDA + **1b** - CO_2), indicating that the photo-click bioconjugation with DASyd 1b was specific on the DBTD residue at site 204 of the encoded sfGFP-Q204DBTD protein. (c) LC-MS/MS tandem mass spectra containing the peptide fragments from digested QQNTPIGDGPVLLPDNHYLST**U$_p$**SAL in the sfGFP-Q204-**2b**. The peptide fragments with corresponding mass-to-charge ratio (m/z) were indicated above the peaks, U_p = **2b**.

Molar concentrations of Cy3 dye and sfGFP protein (the core fluorophore) could be calculated based on their characteristic absorbance peaks at different wavelength, respectively. Therefore, the ratio values of the Molar concentration could reflect the photo-labelling yield if the protein mixture is free of Cy3 small molecule which would interfere with the protein absorbance. Therefore, the photo-labelled protein mixture was firstly purified through a Sephadex G-50 column to get rid of the free dyes, and was subsequently concentrated with a spin-column (10 kDa cutoff). The Molar extinction coefficient of Cy3 dye: ε_{552} = 150 000 M^{-1} cm^{-1} at 552 nm and the Molar extinction coefficient of the sfGFP: ε_{485} = 83 300 M^{-1} cm^{-1} at 485 nm are used in this study to calculated their Molar concentration, respectively. The calculation also has to be adjusted because the absorbance of the Cy3 dye has partially overlapped with the absorbance of the sfGFP at 485 nm, which is approximately 15.1% of the absorbance of Cy3 dye at 552 nm. As a result, the molar concentrations of Cy3 dye and sfGFP protein were calculated based on the following:

Also, if the desired photo-conjugation was one molecule of the protein tagged with one molecule of Cy3 dye, the labelling yield could be calculated based on the following equations:

$$[\text{Cy3}] = [\text{sfGFP-}\mathbf{2b\text{-}Cy3}] = (\text{Abs}_{552})/(\varepsilon_{552} \times l)$$

$$[\text{total sfGFP}] = [(\text{Abs}_{485}) - 0.151 \times (\text{Abs}_{552})]/(\varepsilon_{485} \times l)$$

$$\text{Yield (\%)} = [\text{sfGFP-}\mathbf{2b\text{-}Cy3}]/[\text{total sfGFP}] \times 100\% = [(\text{Abs}_{552})/\varepsilon_{552}]/[\,[(\text{Abs}_{485}) - 0.151 \times (\text{Abs}_{552})]/\varepsilon_{485}] \times 100\%$$

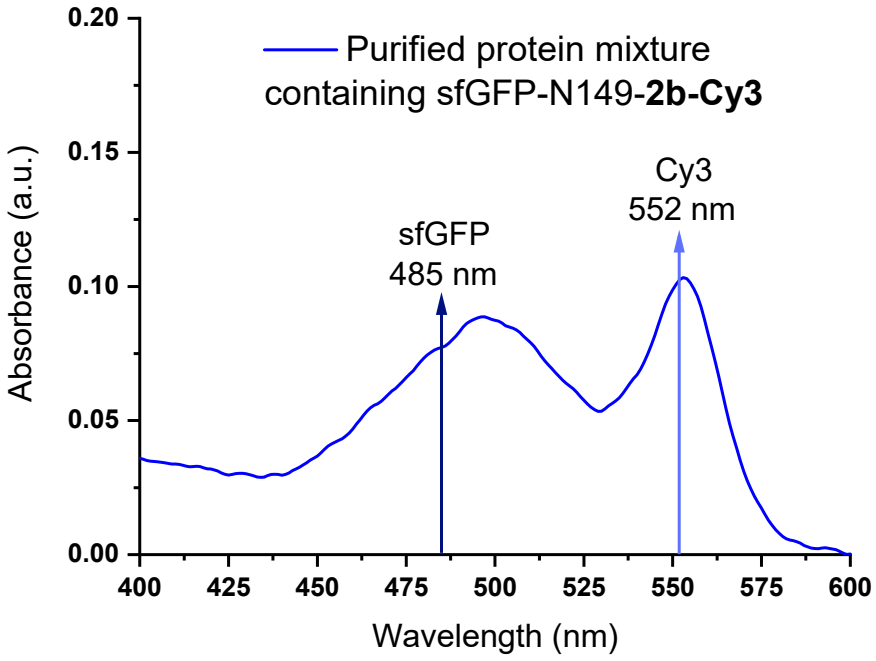

Figure S20. UV-vis spectrum of the purified protein mixture after the photo-click reaction with **1b-Cy3** for determining protein photo-labelling yield. The protein mixture mainly contains sfGFP-N149DBTD and sfGFP-N149-**2b-Cy3**. The absorbance values at 485 nm and 552 nm were measured to be 0.077 and 0.102 on the pedestal of a NanoDrop 2000 spectrophotometer, respectively, whereas the l was 0.1 cm.

Stability of sfGFP-N149-**2b-Cy3**					
	1	**2**	**3**	**4**	**5**
37 °C (24h)	-	+	+	-	-
GSH (5 mM)	-	-	+	-	+
405 nm (5 min)	-	-	-	+	+
Gray value CBB (%)	100	97.5	75.4	88.6	80.5
Cy3 intensity value (%)	100	101	53.2	86.3	81.9

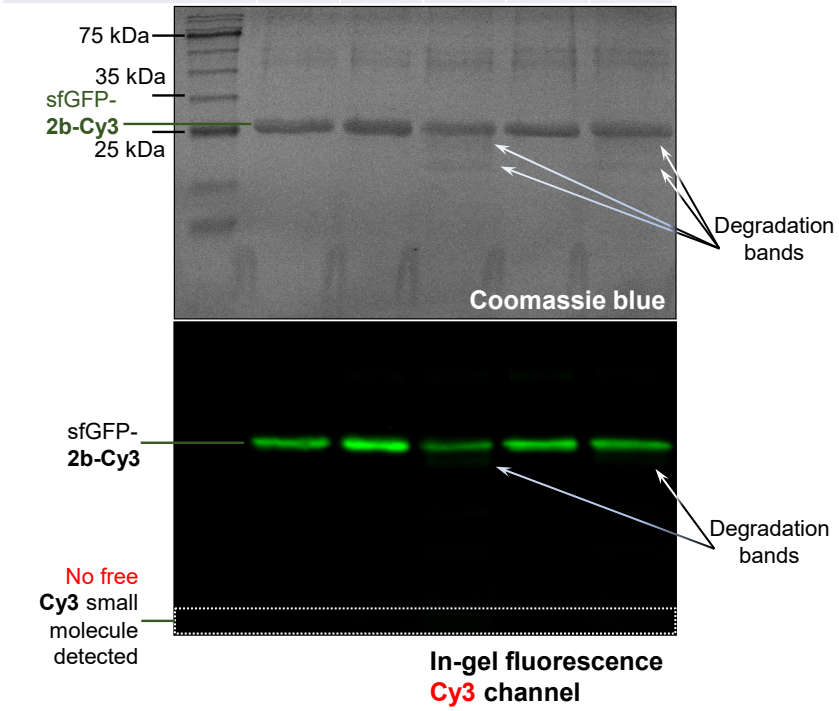

Figure S21. The in-gel fluorescence analysis of the stability of purified sfGFP-N149-**2b-Cy3**. Protein degradation was calculated based on the relative gray value of the sfGFP bands in the Coomassie brilliant blue (CBB) stained gel image by using ImageJ software. The in-gel fluorescence image for the Cy3 fluorescence channel was analyzed quantitatively to evaluate whether the Cy3 fluorophore was released due to the breaking of the covalent linkage formed by photo-click reaction, and also to investigate whether the Cy3 fluorophore was quenched by GSH addition by using ImageJ software.

Lane 1: The purified sfGFP-N149-**2b-Cy3**.

Lanes 2 and 3: The sfGFP-N149-**2b-Cy3** was incubated with the PBS buffer (pH = 7.4) or the PBS buffer containing 5 mM GSH at 37°C for 24 hours, respectively.

Lanes 4 and 5: The sfGFP-N149-**2b-Cy3** was irradiated with or without the 405 nm LED array for 5 minutes at room temperature, respectively.

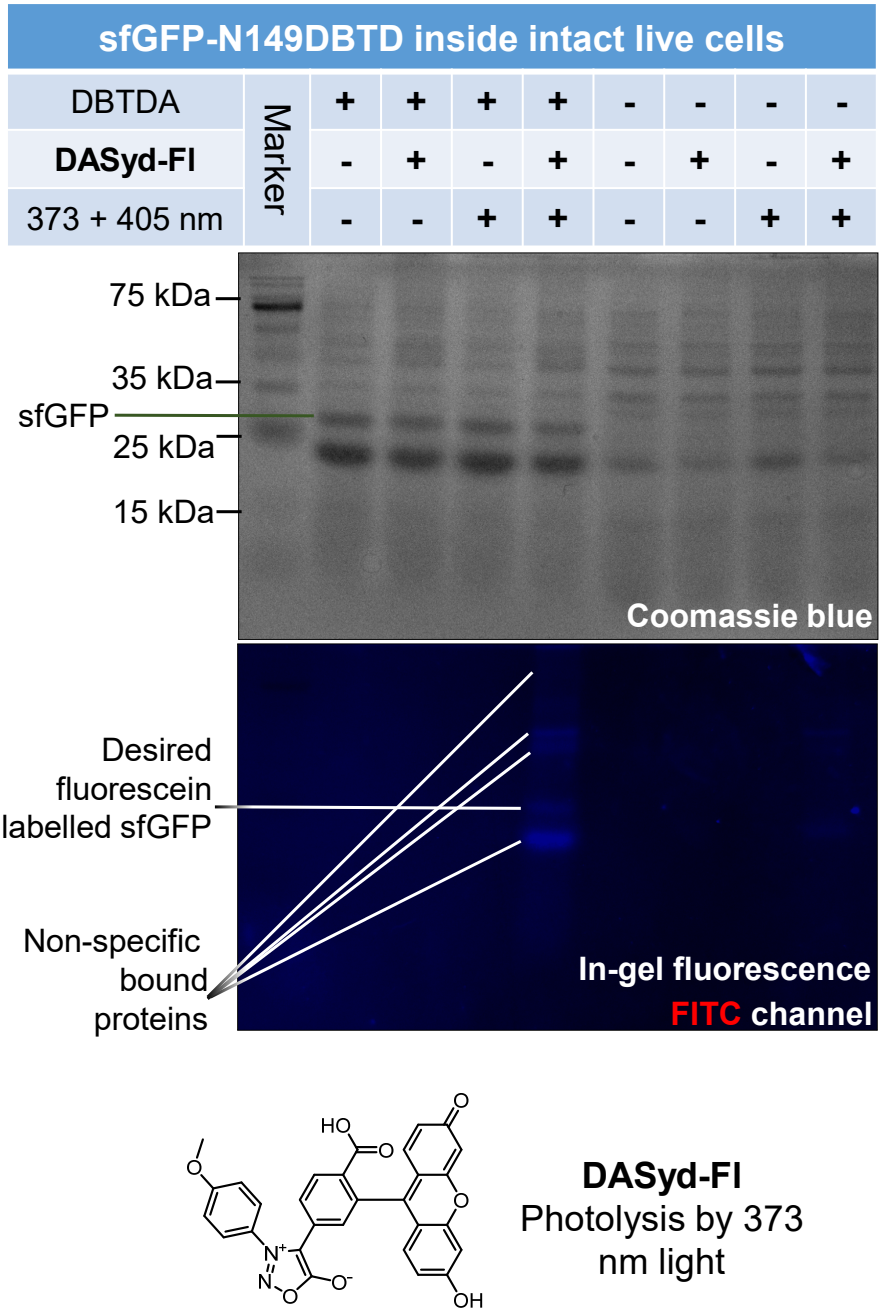

Figure S22. In-gel fluorescence analysis for photo-labelling of the intracellular sfGFP-N149DBTD in *E. coli* cells after photo-irradiation with **DASyd-Fl**, and control experiments to clarify the selectivity of the photo-conjugation inside living cells.

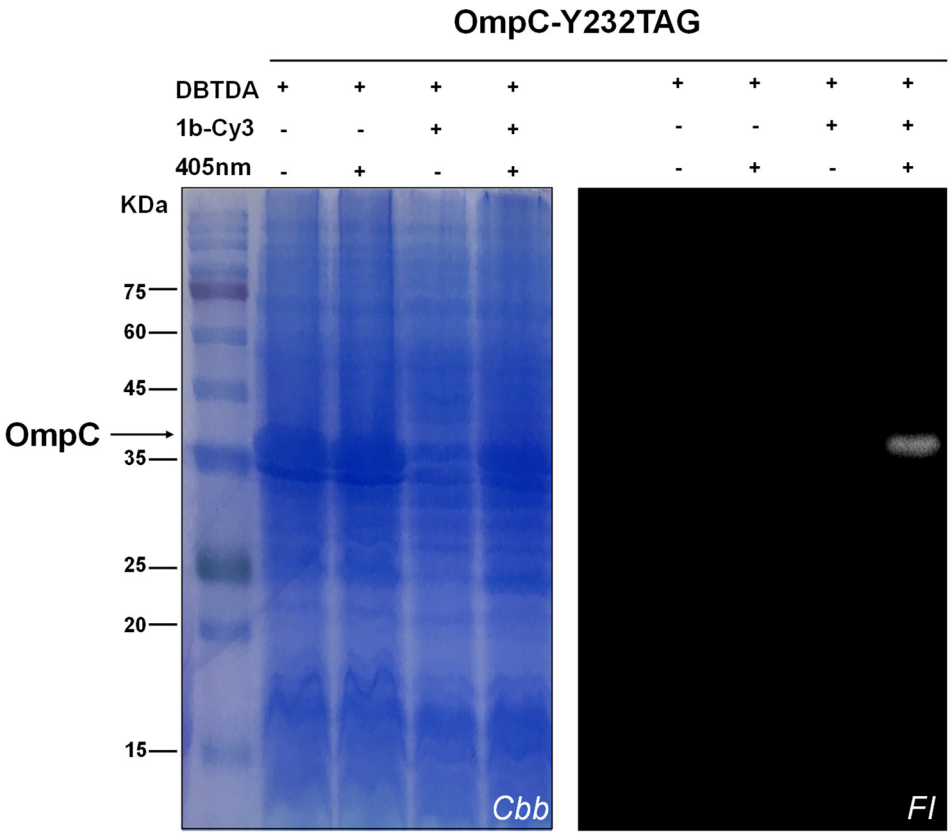

Figure S23. In-gel fluorescence analysis for photo-labelling of OmpC-Y232DBTD on living *E. coli* cells after photo-irradiation with **1b-Cy3** and control experiments to clarify selectivity on living cells.

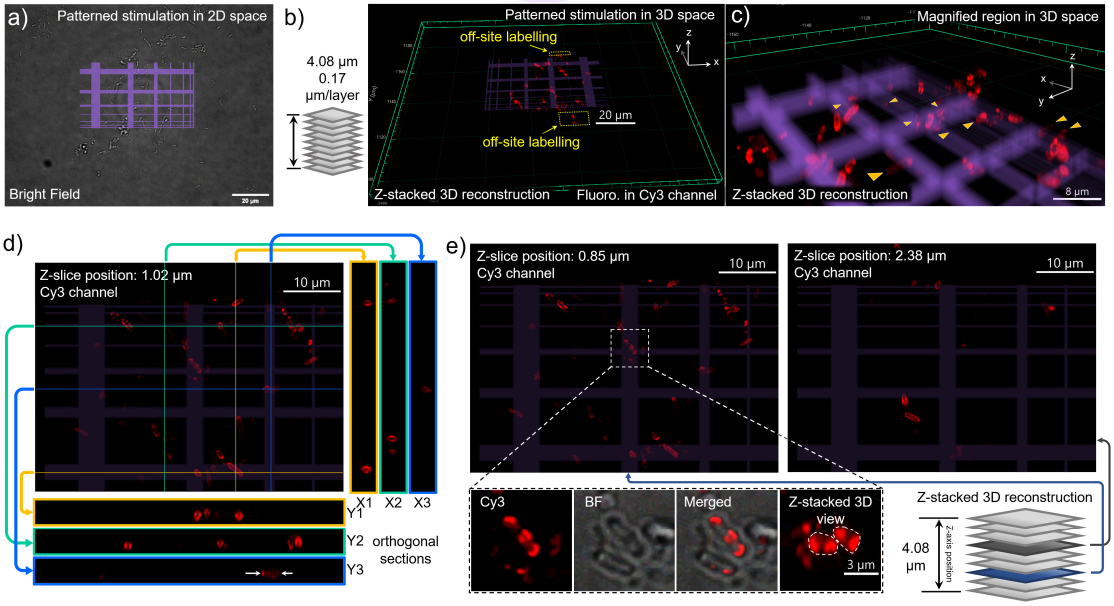

Figure S24. Photo-click labelling of OmpC protein on *E. coli* cells with enhanced spatiotemporal control, and the Cy3 fluorescence distribution analysis for the labelled OmpC in 3D space. (a) Differential Interference Contrast (DIC) image of *E. coli* cells with a grid pattern of 405 nm photo-stimulation shown in 2D space. (b) 3D reconstruction rendering to display the Cy3-labelled *E. coli* cells overlapping within the photo-stimulation pattern. Z-stacking of 25-slice tomograms. Interval of each layer = 0.17 µm, full thickness in Z-axis = 4.08 µm. Experimental conditions: 5 µM **1b-Cy3-Biotin**, 405 nm, 30 s, then washed with PBS for three times before imaging. There were some off-site labelled *E. coli* cells observed outside of the grid photo-stimulation pattern marked with yellow boxes because of the scattering of light or diffusion of the activated nitrile imine intermediate. (c) Magnified region of interest in the rendered 3D space to show the fluorescence labelling at subcellular level. Yellow arrows were pinpointing to the featured cells with partial fluorescence labelling on the outer membrane. (d) Diagram to show cross-section images at three different X-Z and Y-Z coordinate planes to display the labelled *E. coli* cells on different Z-axis cross-section of the grid photo-stimulation pattern. Z-slice position: 1.02 µm. (e) Other Z-slice images with magnified region focused on a pair of featured E. coli cells with fluorescence labelling on both poles. Z-slice positions: 0.85 and 2.38 µm.

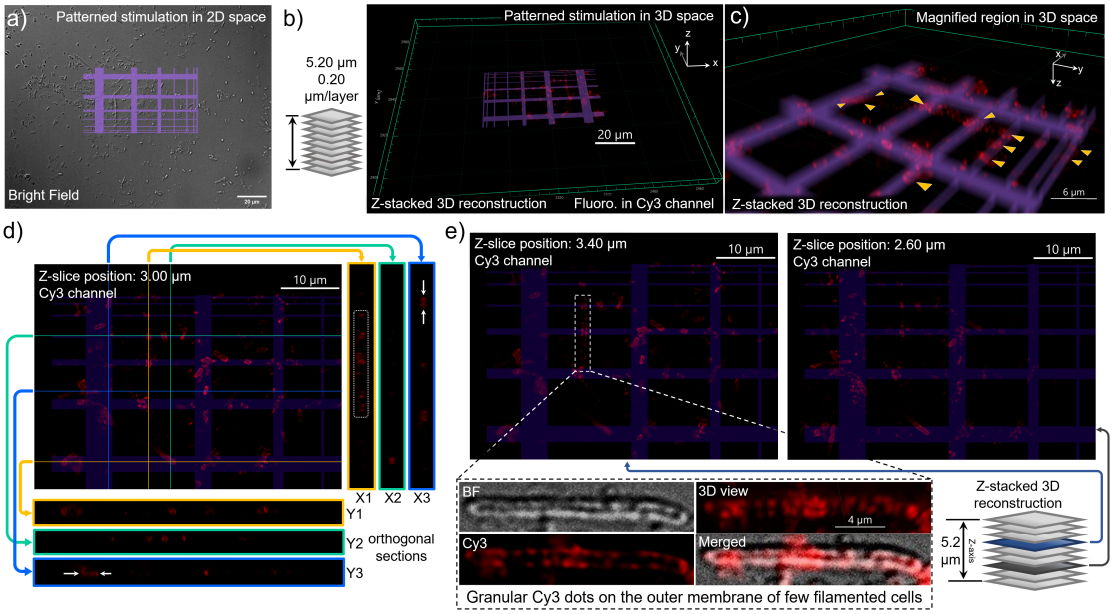

Figure S25. Photo-click labelling of OmpC protein on *E. coli* cells with enhanced spatiotemporal control, and the Cy3 fluorescence distribution analysis for the labelled OmpC in 3D space. (a) Differential Interference Contrast (DIC) image of *E. coli* cells with a grid pattern of 405 nm photo-stimulation shown in 2D space. (b) 3D reconstruction rendering to display the Cy3-labelled *E. coli* cells overlapping within the photo-stimulation pattern. Z-stacking of 27-slice tomograms. Interval of each layer = 0.20 µm, full thickness in Z-axis = 5.20 µm. Experimental conditions: 5 µM **1b-Cy3-Biotin**, 405 nm, 30 s, then washed with PBS for three times before imaging. There were some off-site labelled *E. coli* cells observed outside of the grid photo-stimulation pattern marked with yellow boxes because of the scattering of light or diffusion of the activated nitrile imine intermediate. (c) Magnified region of interest in the rendered 3D space to show the fluorescence labelling at subcellular level. Yellow arrows were pinpointing to the featured cells with partial fluorescence labelling on the outer membrane. (d) Diagram to show cross-section images at three different X-Z and Y-Z coordinate planes to display the labelled *E. coli* cells on different Z-axis cross-section of the grid photo-stimulation pattern. Z-slice position: 3.0 µm. (e) Other Z-slice images with magnified region focused on filamentation of a group of *E. coli* cells with fluorescence labelled OmpC aggregated into uniformly scattered spots (zoom-in images). Z-slice positions: 3.40 and 2.60 µm.

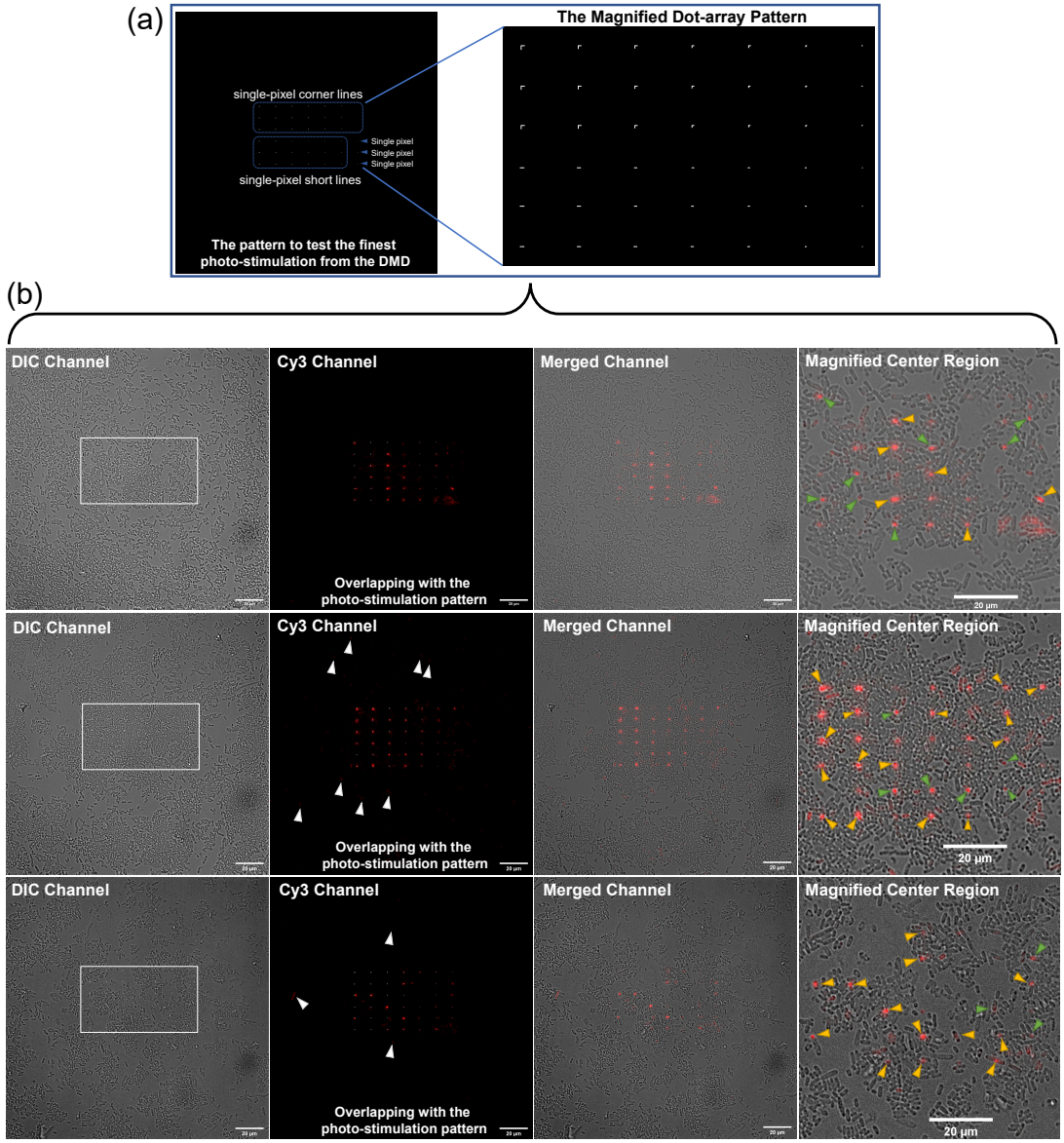

Figure S26. Photo-click labelling of OmpC-DBTD protein on fixed *E. coli* cells with maximum spatiotemporal control of the DMD system in the microscope via projecting a dot-array photo-stimulation pattern, and the Cy3 fluorescence analysis for single bacterial cell labelling performance, targeting the OmpC on outer membrane. Experimental conditions: 5 μM **1b-Cy3-Biotin**, 405 nm, 20 s, then washed with PBS for three times before imaging. (a) The dot-array photo-stimulation pattern in the entire microscopic imaging field and the enlarged stimulus pattern to demonstrate the detailed punctate shapes. The dot-array was programmed to contain many single-pixel excitations, single-pixel line excitations and single-pixel corner line excitations to possibly demonstrate the finest resolution of the current DMD photo-stimulation system. (b) The imaging results after the photo-click labelling in various channels, including DIC, Cy3 channel, merged images, as well as the magnified photo-stimulation center regions. The fluorescence labeled single bacterial cells overlapping with the dotty photo-stimulation in the resulting images were pinpointed

by yellow arrows, which showed significantly stronger Cy3 fluorescence signals in comparison with other cells close but not right on the dot-array spots. The single bacterial cells showing a subcellular spot or partial membrane fluorescence signal were pinpointed with green arrows, colocalizing well with single photo-stimulation dots. The background labelling of cells outside of the illumination regions were also marked by white arrows. The dot-array photo-stimulation experiments were independently replicated for at least three times.

A single-pixel 405 nm excitation projected via the DMD on the specimen (via the 100× oil immersion objective) was a spot only 90 nm in diameter (78 μm × 44 μm stimulation field, with 861 × 483 pixels resolution for the DMD) theoretically, which actually would be larger than 135 nm (0.135 μm) because the Abbe diffraction limit for the microscope is roughly $d = \lambda/2NA = 135$ nm, where $\lambda = 405$ nm, numerical aperture (NA. of the 100× oil immersion objective) = 1.5. The single-pixel photo-stimulation in the microscopic system is finer than the size of a single *E. coli* cell (size: length < 3 μm, width ≈ 1 μm).

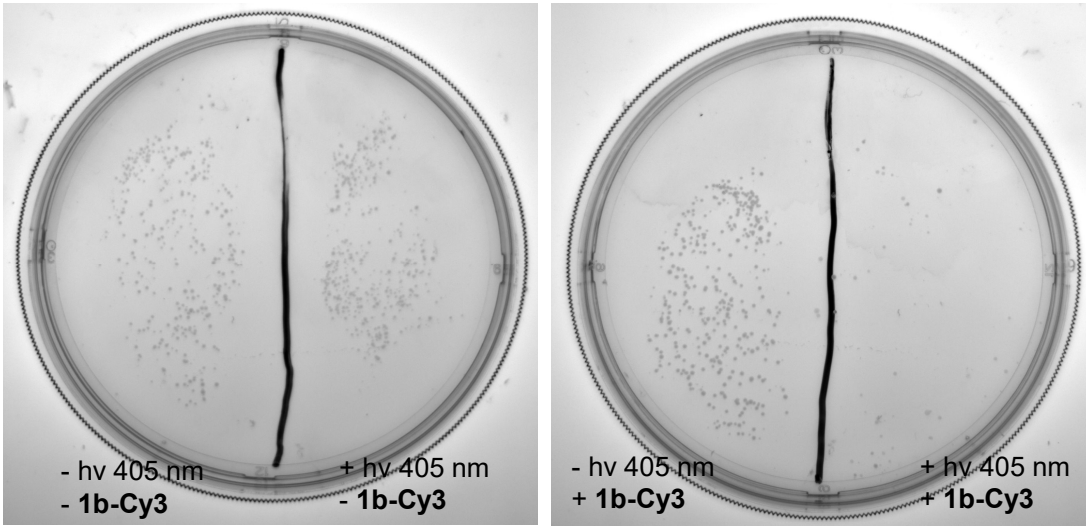

Figure S27. Assessment of bacterial viability after the photo-click labelling toward the DBTD residue on OmpC. By imaging the colonies on the LB plate, we were able to perform the manually counting of the number of the colonies on agar plates illuminated by transmitted light to evaluate the viability of the bacteria after photo-induced conjugation reaction.

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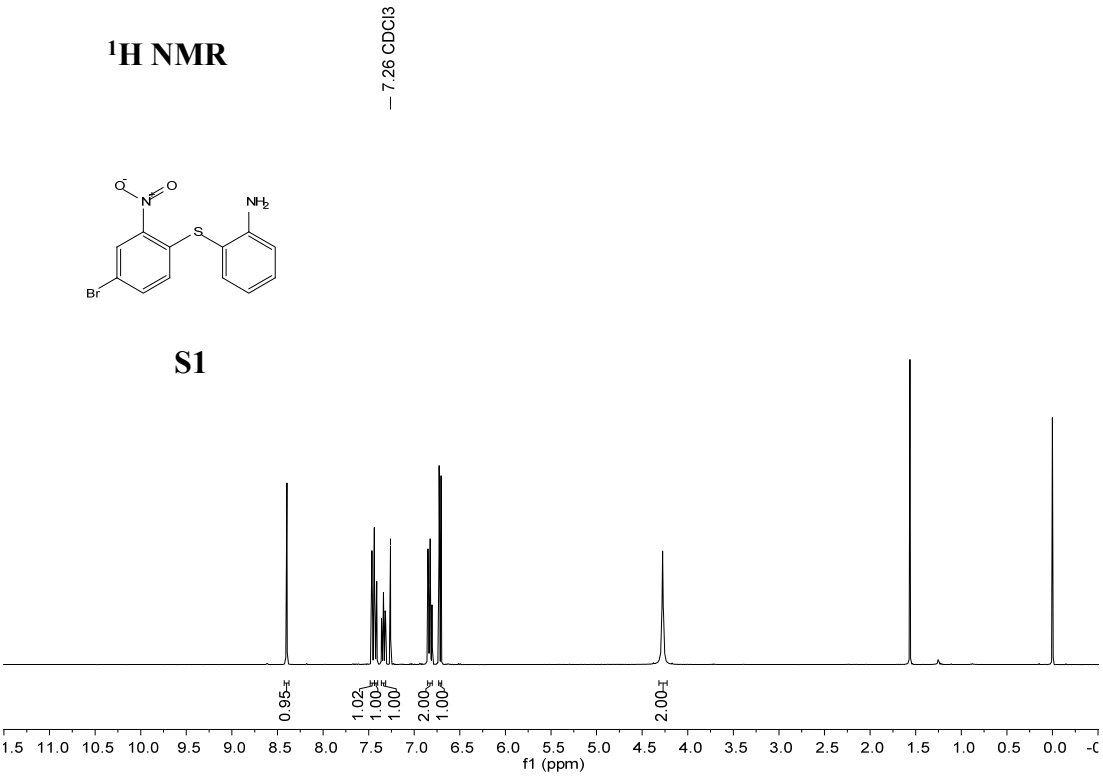
^{1}H and ^{13}C NMR Spectra

^{1}H NMR



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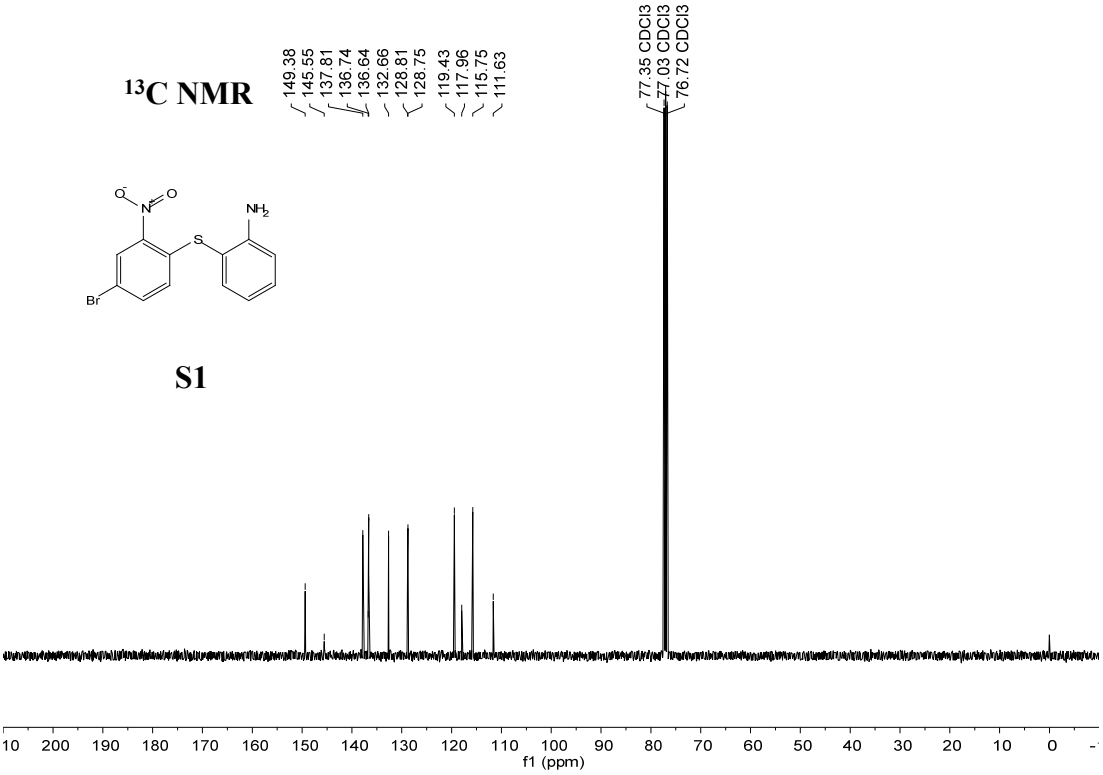

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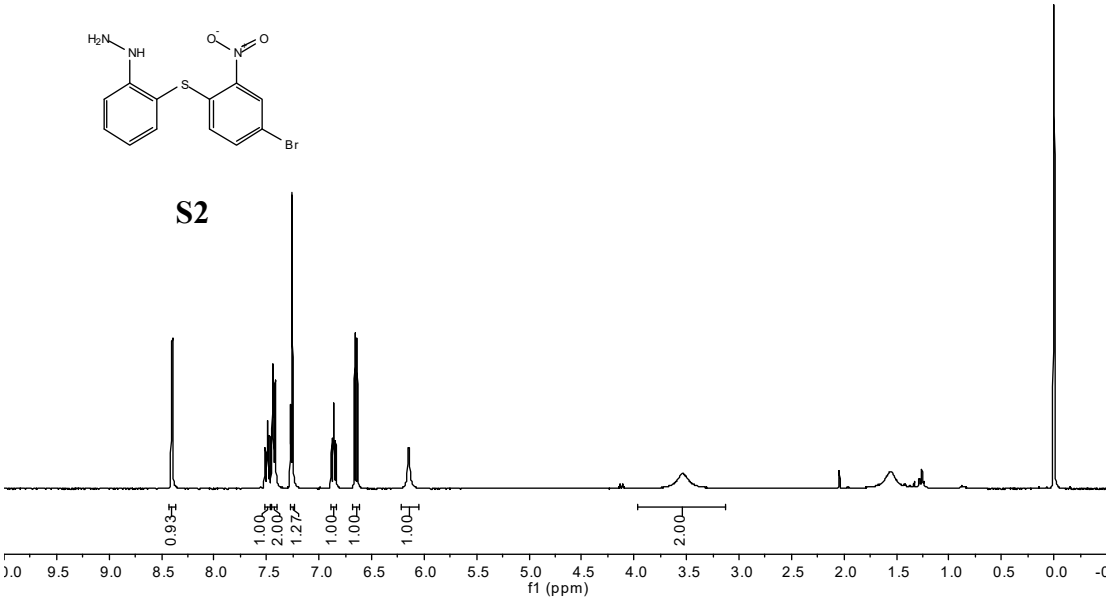

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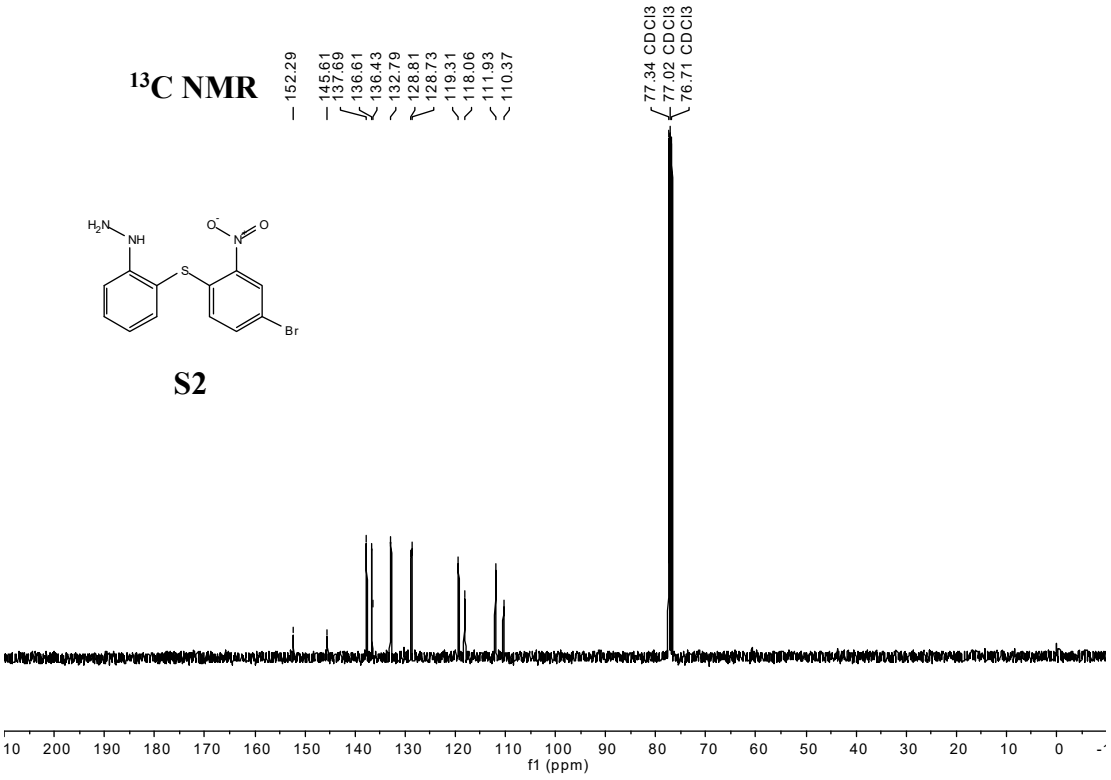

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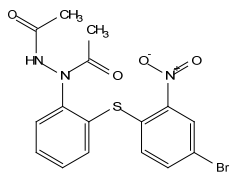

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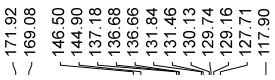

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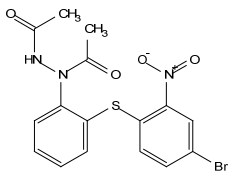

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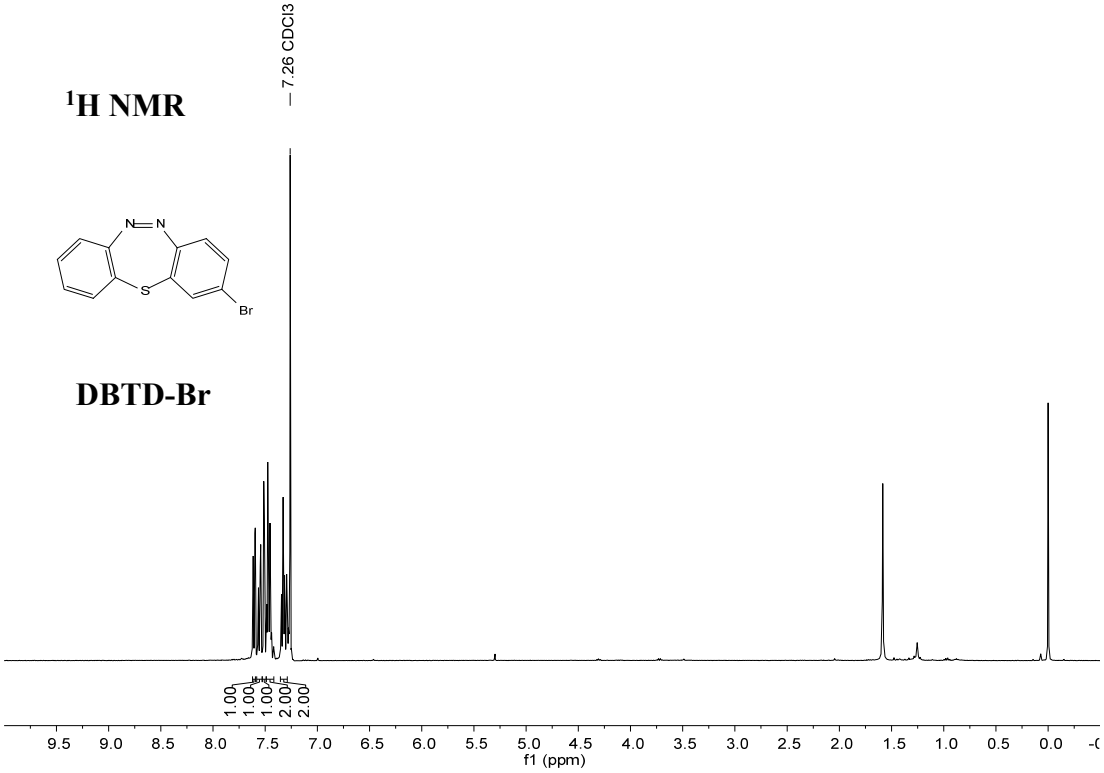

DBTD-Br

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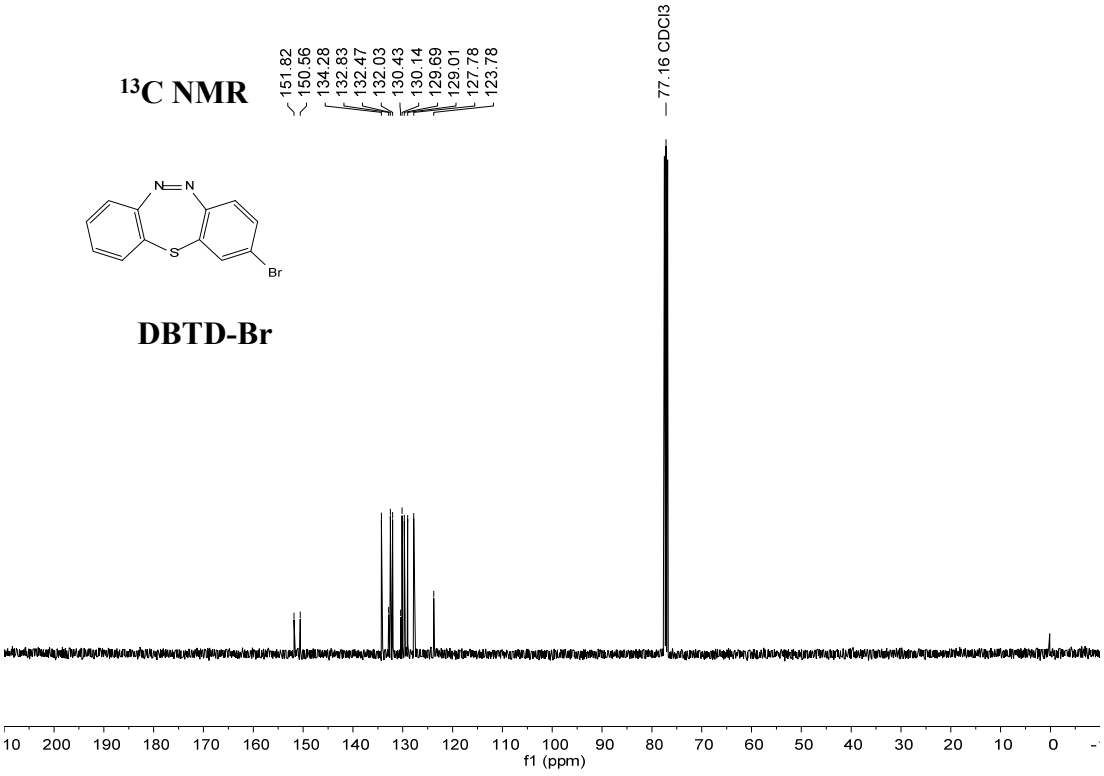

DBTD-Br

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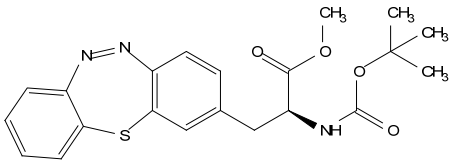

***N*-Boc-DBTD-Ala-OMe**

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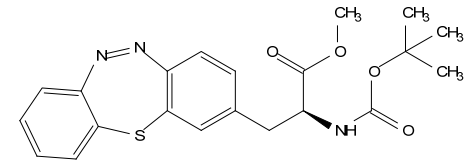

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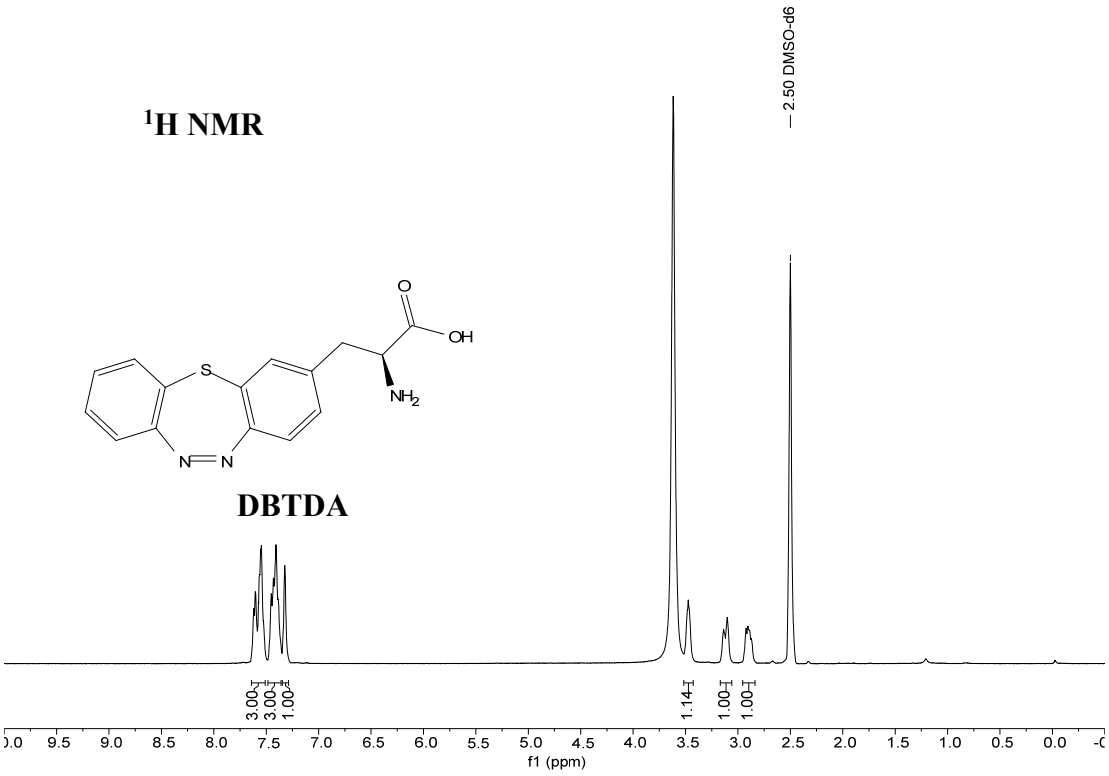
^{1}H NMR



DBTDA

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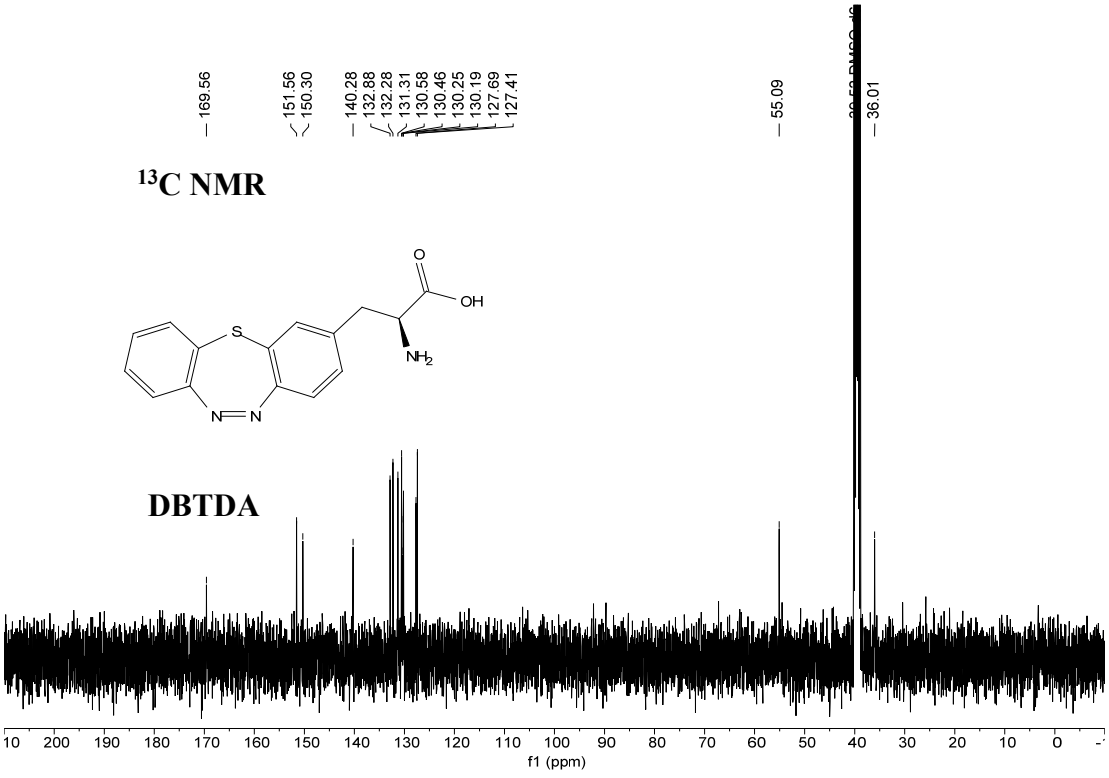

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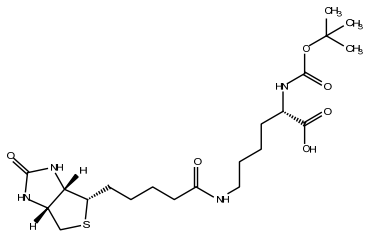

***N*-Boc-Biotin-Lysine**

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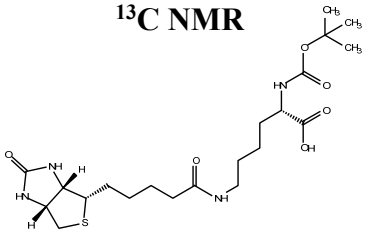

***N*-Boc-Biotin-Lysine**