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Supplementary Information

'Light Up' Protein-Protein Interaction through Bioorthogonal Incorporation of a Turn-On Fluorescent Probe into TEM-1 β-Lactamase

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1. General Information

All chemicals including the unnatural amino acid *p*-azido-L-phenylalanine (pAzF) were purchased from commercial sources. All solvents were freshly distilled and purified before use. The plasmid pEVOL–pAzF was purchased from Addgene, and primers used in this work were synthesized by Integrated DNA Technologies, Inc (Hong Kong). The concentration of protein was determined by Bradford protein assay kit using bovine serum albumin (BSA) as standard. ^{1}H-NMR spectra were recorded on a Bruker ultrashield 400 instrument. ESI-MS spectra were acquired on a Waters Micromass LCT premier mass spectrometer, and MALDI-TOF-MS spectra were measured by a Bruker BIFLEXIII spectrometer. The UV-Vis absorption and fluorescent spectra were recorded on a Cary 4000 UV-spectrophotometer and a Horiba Fluoromax-4 spectrofluorometer respectively. The CD Spectra were measured on a Jasco J-810 spectropolarimeter.

2. Synthesis and Characterization

The water soluble fluorophore EPB and its precursors were synthesized according to reported procedure in the literature with slight modification. [S1-S4]

Synthesis of 4- (trimethylsilylethynyl)benzophenone (1). A 250 ml two-necked round bottom flask was charged with $PdCl_2$ $(PPh_3)_2$ (300 mg, 0.43 mmol), CuI (150 mg, 0.79 mmol), PPh_3 (300 mg, 1.14 mmol) and 4-bromobenzophenone (2.61 g, 10 mmol). The flask was evacuated under vacuum and flushed with nitrogen for three times, followed by the injection of anhydrous mixture of THF–TEA (100 mL, 1:1 v/v). Trimethylsilylacetylene (2.0 mL, 14 mmol) was added to the suspension, and then the mixture was heated and kept at 50 °C for 24 h. After cooling to room temperature, the reaction mixture was filtered and the solid was washed with diethyl ether several times. The filtrate was concentrated under reduced pressure, and the resulting crude product was purified by silica gel column chromatography using 100:1 petroleum ether/ethyl acetate mixture as eluent to give the purified product of **1** as a white solid. Yield: 2.40 g (86%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.74–7.78

(m, 4H), 7.55–7.60 (m, 3H), 7.48 (t, J = 7.4 Hz, 2H), 0.27 (s, 9H).

Synthesis of 4, 4'-(2-bromoethoxy)benzophenone (2). To a mixture of 4, 4'-dihydroxybenzophenone (3.21 g, 15 mmol) and potassium carbonate (5.53 g, 40 mmol) in acetone (50 mL), 1, 2-dibromoethane (4.5 mL, 52 mmol) was added and the mixture was refluxed for 24 h with stirring. Any undissolved solids were removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography using 1:3 petroleum ether/chloroform as eluent to give **2** as a white powder. Yield: 4.18 g (65%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.78 (d, J = 8.8 Hz, 4H), 6.97 (d, J = 8.8 Hz, 4H), 4.38 (t, J = 6.2 Hz, 4H), 3.68 (t, J = 6.2 Hz, 4H).

Synthesis of (4-(2,2-bis(4-(2-bromoethoxy)phenyl)-1-phenylvinyl)phenyl)ethynyl)

Trimethylsilane (BPET, 3). To a 250 mL three-necked round bottom flask equipped with a reflux condenser, zinc dust (0.85g, 13 mmol) was added, then the flask was evacuated under vacuum and flushed with nitrogen for three times. After the addition of anhydrous THF (90 mL), the suspension was cooled to −5 °C, followed by the slow addition of $TiCl_4$ (0.72 ml). The resulting mixture was allowed to warm to room temperature and further stirred for 0.5 h, and was refluxed for 2.5 h afterwards. Then the mixture was cooled to −5 °C again, and charged with 0.25 mL of pyridine. After 10 minutes, a solution mixture of **1** (1.20 g, 4.3 mmol) and **2** (2.14 g, 5 mmol) in THF (25ml) was slowly added, and the mixture was refluxed overnight. The reaction was quenched with 10% K_2CO_3 aqueous solution (20 mL) and a large amount of water was added until grey solids appeared. The mixture was extracted with dichloromethane (DCM) for three times, and the combined organic layer was dried over anhydrous Na_2SO_4. The crude product was purified by silica gel column chromatography using petroleum ether/DCM as gradient eluent to give **3** for the following deprotection reaction.

Synthesis of 4, 4'-(2-(4-ethynylphenyl)-2-phenylethene-1, 1-diyl)bis((2-bromoethoxy)benzene) (PEPB, 4). To a solution of **3** (0.68 g, 1.01 mmol) in THF (15 mL), TBAF (1 M, 3 mL) was added.

The resulting mixture was stirred at room temperature for 1 h, followed by the addition of water (40 mL). The mixture was extracted with DCM for three times, and the combined organic layer was dried over anhydrous Na_2SO_4. The solvent and any volatile components were removed under reduced pressure. The crude product was purified by silica column chromatography with 1:1 petroleum ether/DCM as eluent to give **4** as a yellow solid. Yield: 0.54 g (90%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.22 (d, J = 8.0 Hz, 2H), 7.10 (m, 3H), 6.91-6.91 (m, 8H), 6.64 (t, J = 8.4 Hz, 4H), 4.22 (m, J = 6.4 Hz, 4H), 3.60 (m, J = 6.8 Hz, 4H), 3.04 (s, 1H). MALDI-MS (*m/z*): calcd for $[M]^+$ 602.03; found 602.07.

Synthesis of 2, 2'-(((2-(4-ethynylphenyl)-2-phenylethene-1, 1-diyl)bis(4, 1-phenylene))bis(oxy)) bis-(*N,N,N*-trimethylethanaminium) bromide (EPB, 5). An excess amount of trimethylamine (25% in H_2O, 10ml, 42mmol) was added to a solution of **4** (150 mg, 0.25 mmol) in THF (55 mL), and the reaction mixture was refluxed for 60 hours. During this period, aliquots of trimethyamine (25% in H_2O, 15 mL) were added at several intervals. After the reflux, the organic solvents were removed under reduced pressure. The aqueous solution was added with MeOH (10 mL), then washed with chloroform for three times. After the solvent was evaporated under reduced pressure, the residue was dried in vacuo at 50 ºC overnight to afford EPB (**5**) as a yellow powder. Yield: 124 mg (69%). ^{1}H-NMR (400 MHz, MeOH-d_4) δ 7.18 (d, J = 8.0 Hz, 2H), 7.18 (m, 3H), 6.95-7.00 (m, 8H), 6.76-6.82 (q, J = 8.8 Hz, 4H), 4.42 (m, 4H), 3.82 (m, 4H), 3.44 (s, 1H), 3.25 (m, 18H). ^{13}C-NMR (100 MHz, MeOH-d_4) δ 157.03, 156.95, 145.29, 144.11, 141.22, 140.19, 137.80, 137.77, 133.04, 133.01, 131.68, 131.62, 131.58, 128.22, 126.89, 120.88, 114.47, 114.34, 83.70, 78.03, 65.97, 62.47, 54.19, ESI-MS (*m/z*): calcd for $[M\text{-}2Br]^{2+}$ 280.17; found 280.12. Anal. Calcd. For $C_{38}H_{44}N_2O_2Br_2$: C, 63.34; H, 6.15; N, 3.89, found: C, 62.47; H, 6.30; N, 3.86.

3. Plasmid Construction

Construction of pET-24d(+)-E104TAG Bla plasmid. The wild type TEM-1 β-lactamase gene was constructed by PCR with primers TEM-F: 5'-TAGCTGATCATATGATGCACCCAGAAACGCTGGTG-3' and TEM-R: 5'-TCAGCTAAAAGCTTACCAATGCTTAATCAGTGAGGC-3'. The PCR product was digested with *Nde* I and *Hind* III and ligated to vector pET-24d (+) expression plasmid to generate the plasmid pET-24d**(+)**-TEM-1 Bla. After that, the amber mutation of E104TAG Bla gene was

introduced and amplified by site-directed mutagenesis with primer pair E104TAG-F/E104TAG-R (E104TAG-F: 5'-GAATGACTTGGTTTAGTACTCACCAGT-3'; E104TAG-R: 5'-GTGACTGGTGAGTACTAAACCAAGTCA-3') to construct the pET-24d(+)-E104TAG Bla plasmid.

Construction of β-lactamase inhibitory protein (BLIP) expression vector. The wt-BLIP gene was amplified by primer pair BLIP-F/BLIP-R (BLIP-F: 5'-AGGCTGATCATATGCACCATCACCATCACCATGCGGGGGTGATGACCGG-3'; BLIP-R: 5'-GTACAATTAAGCTTATACAAGGTCCCACTGCCG-3'). The PCR product was digested with *Nde*I and *Hind*III, and ligated to vector pRSET-K predigested to construct the pRSET-wt-BLIP plasmid. Vector pRSET-K was modified from pRSET-A (Invitrogen) by replacing the ampicillin resistance gene with kanamycin resistance gene from pET28b (Novagen). Mutant Y143A/W150A of BLIP gene was constructed by overlapping PCR. Two PCR fragments were first amplified from wild-type BLIP gene by primer pair BLIP-F/Y143A-R (BLIP-F: 5'-AGGCTGATCATATGCACCATCACCATCACCATGCGGGGGTGATGACCGG-3'; Y143A-R: 5'-CGAGGCTCGGCGCACCTCGCGTTCACGGACGGG-3') and W150A-F/BLIP-R (W150A-F: 5'-GAGGTGCGCCGAGCCTCGCGCGAACCCCGTCGAC-3'; BLIP-R: 5'-GTACAATTAAGCTTATACAAGGTCCCACTGCCG-3') separately. The final PCR product was digested with *Nde*I and *Hind*III, and ligated to vector pRSET-K to generate the pRSET-bm-BLIP expression plasmid

4. Protein Expression and Purification

Expression and purification of pAzF104-Bla. The two plasmids of pEVOL-pAzF (chloramphenicol resistance) and pET-24d(+)-E104TAG Bla (kanamycin resistance) were co-transformed into *E.coli.*BL21, and cultured on agar-solidified LB medium containing 50 μg/mL kanamycin and 25 μg/mL chloramphenicol at 37 °C overnight. A single colony was selected and inoculated in 5 mL LB medium with 50 μg/mL kanamycin and 25 μg/mL chloramphenicol at 37 °C with 250 rpm for 16h.

After that, 1 mL of the overnight culture, 1 mM pAzF, 0.2% L-arabinose, 50μg/mL kanamycin and 25μg/mL chloramphenicol were added into 200 mL fresh 2×TY medium with shaking at 250 rpm, 37 °C, till the OD_{600} reached ~0.6. 0.8 mM IPTG was then added and the protein was induced to express at 30 °C overnight. Cells were pelleted and resuspended in 10 mL Tris buffer (20 mMTris-HCl, pH 8.0, 500 mMNaCl), then lysed during three cycles of freeze–thaw. The supernatant was collected by centrifugation at 13000 rpm for 40 min at 4 °C, and purified using Ni-NTA chromatography. Approximate 200 μg of pAzF104-Bla (＞90% pure) was isolated for further usage.

Expression and purification of wt-BLIP and bm-BLIP. The plasmid of pRSET-wt-BLIP (kanamycin resistance) was transformed into *E.coli.* BL21 for expressing wt-BLIP in 100 mL LB medium with 50 μg/mL kanamycin at 37 °C, 250 rpm overnight. Cells were harvested by centrifugation and the pellets were treated with 5 mL of 6 M guanidine hydrochloride for 4 hours, and slowly diluted into 100 mL PBS buffer for protein refolding. Approximate 220 μg of pure wt-BLIP was obtained after purification by Ni-NTA chromatography. The expression and purification process of bm-BLIP was as same as the one that was described for wt-BLIP.

5. Enzyme Kinetic Data

	k_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M (s^{-1} M^{-1})
wt-Bla	1,428 ± 63	(1.83 ± 0.20) × 10^{-4}	(7.80 ± 0.51) × 10^{6}
pAzF104-Bla	2,022 ± 99	(3.98 ± 0.22) × 10^{-4}	(5.08 ± 0.53) × 10^{6}
EPB104-Bla	1,992 ± 54	(3.07 ± 0.11) × 10^{-4}	(6.49 ± 0.42) × 10^{6}

Table S1. Enzyme kinetic data of wt-Bla, pAzF104-Bla, and EPB104-Bla with ampicillin as the substrate.

6. Spectra analysis of β-lactamases

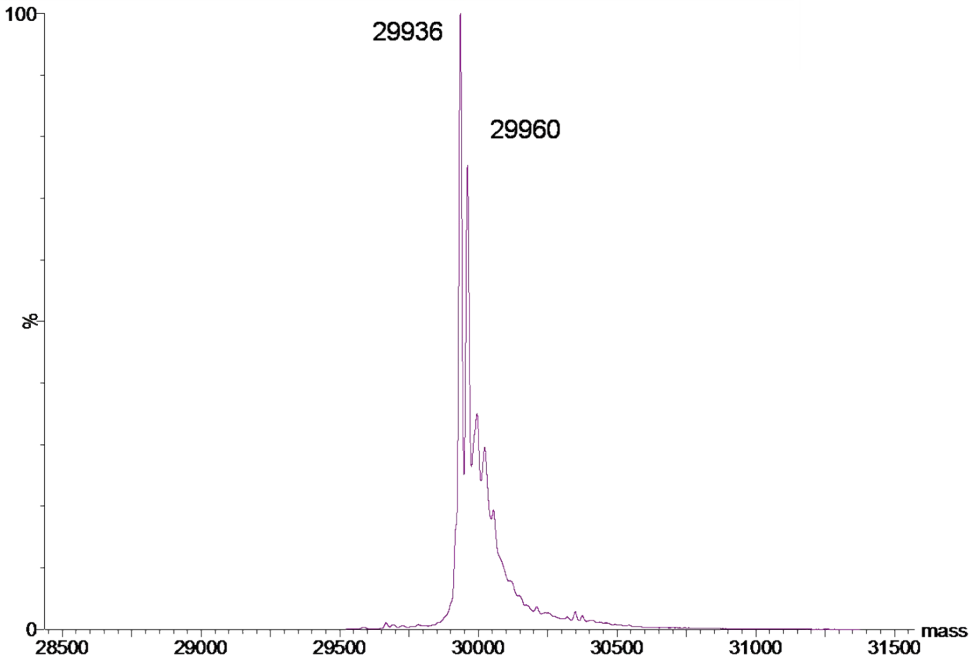

Figure S1. ESI-MS spectrum of pAzF104-Bla

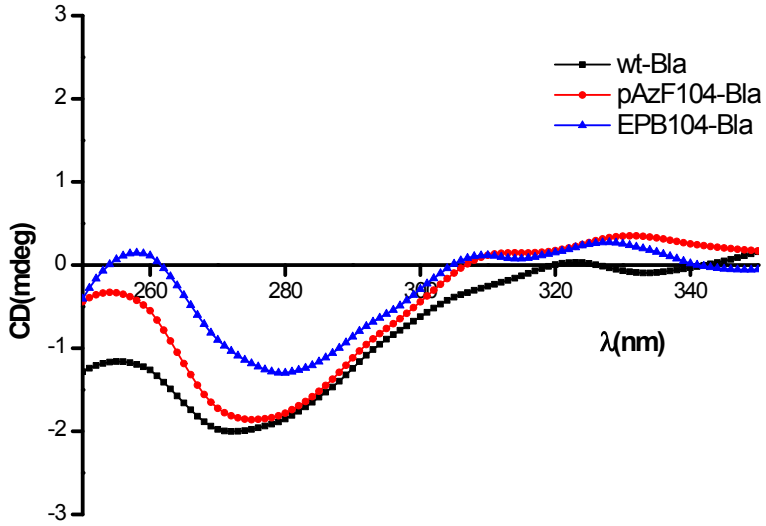

Figure S2. Near-UV CD spectra of wt-Bla, pAzF104-Bla and EPB104-Bla in PBS buffer with 1% DMSO; Bla concentration: 10 μM

7. References:

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