Electronic Supplementary Material (ESI) for ChemComm.
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Electronic Supporting Information

Nitroreductase-triggered activation of a novel caged fluorescent probe obtained from methylene blue

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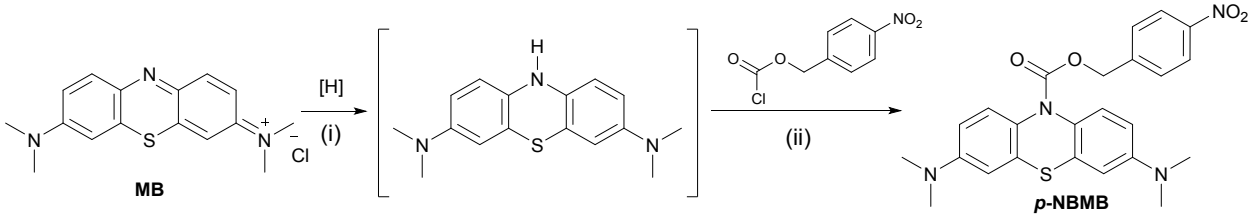
1. MATERIALS

4-Nitrobenzyl chloroformate, nitroreductase from *Escherichia coli* (*E. coli*), human DT-diaphorase, β-nicotinamide adenine dinucleotide, reduced dipotassium salt hydrate (NAD(P)H), and *p*-nitrosodimethylaniline were purchased from Sigma-Aldrich (Saint Louis, MO). Certified grade methylene blue (MB) was obtained from Acros Organics (Pittsburgh, PA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) and used as received.

2. METHODS

2-1. Synthesis of *p*-nitrobenzyl 3,7-bis(dimethylamino)-10*H*-phenothiazine-10-carboxylate (*p*-NBMB)



Scheme S1 Synthetic schemes of fluorescent probe *p*-NBMB. Reagents and conditions: (i) methylene blue, Sodium dithionate, Sodium bicarbonate, H_2O/Toluene, 50 ^oC**,** 1 h; (ii) Leuco Methylene blue, 4-nitrobenzyl chloroformate, dry toluene,(1) 0 ^oC, (2) Overnight.

Methylene blue (MB, 373.9 mg, 1 mmol) was dissolved in 10 ml of deionized water. Toluene (40 ml), sodium bicarbonate (292.4 mg, 3.48 mmol) and sodium dithionate (496.2 mg, 2.85 mmol) were added to the solution of MB, stirring at 50 °C. When the mixture turned to yellow, after 30 min, the toluene phase containing leuco-MB was transferred dropwise over 60 min into a solution of *p*-nitrobenzyl chloroformate dissolved in 40 ml of toluene, maintained in an ice bath. After overnight reaction at room temperature, the mixture was collected after washing with water three times. The obtained organic phase was evaporated under reduced pressure and then was precipitated into cold methanol. The precipitates were purified by

recrystallization from acetonitrile yielding orange solid powder: yield (52.25 mg, 11.24%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (d, *J* = 8.4 Hz, 2H), 7.47 (d, *J* = 8.3 Hz, 2H), 7.36 (d, *J* = 8.6 Hz, 2H), 6.70 (d, *J* = 2.5 Hz, 2H), 6.64 (dd, *J* = 8.9, 2.3 Hz, 2H), 5.32 (s, 2H), 2.96 (s, 12H), 1.57 (s, 2H). Found C, 61.88; H, 5.10; N, 12.06. Calcd for $C_{24}H_{24}N_4O_4S$: C, 62.05; H, 5.21; N, 12.06

2-2. Spectroscopic assessment

All spectroscopic measurements were performed in 10 mM phosphate buffered saline solution (PBS, pH 7.4, 1% DMSO). For the spectrometer measurements, a quartz cuvette with 1 cm path length was used to hold each sample. The absorbance of the solution was measured using a Genesys 6 spectrophotometer (Thermo Fisher Scientific, Inc., USA). Fluorescence spectra were acquired both using a PerkinElmer LS55 fluorescence spectrometer (Massachusetts) and an inverted microscope setup. A wavelength of 580 nm was chosen as the excitation source and fluorescence was detected in a wavelength range between 640 and 800 nm. The heterogeneity of the sample was probed using an inverted Nikon TE200U microscope with a 10x objective (NA = 1.4). The 514.5 nm laser line of an Ar^+ ion laser (~500 μW) was used to excite the sample, which was deposited on plasma cleaned glass coverslips. An Acton SP2500 spectrograph with a 150 grooves/mm grating was employed to disperse the emission and a Princeton Instruments ProEM 1024 EMCCD camera was used to record the fluorescence emission between 620 and 800 nm.

2-3. Spectral analysis of *p*-NBMB

The solutions of *p*-NBMB (1 μM) in deionized water (1% DMSO) were prepared to assess spectral analysis of *p*-NBMB. The fluorescence and absorbance of *p*-NBMB was evaluated using a UV-Visible spectrophotometer and fluorescence spectrometer, respectively, along with MB (1 μM) as a reference.

2-4. Nitroreductase activity assay

p-NBMB was dissolved in DMSO to obtain a stock solution of 10 mM and the stock solution was diluted to achieve concentrations ranging from 1 μM to 100 μM. Nitroreductase was mixed to dilute *p*-NBMB solution with an enzyme assay buffer (1 mM NAD(P)H, PBS) containing 1% DMSO to achieve final enzyme concentrations of 1 unit ml^{-1} and 2 unit ml^{-1}. All prepared samples were incubated at 37 °C. Fluorescence spectra were measured with/without nitroreductase, changing the concentration of imaging probe and enzyme.

2-5. Fluorescence imaging

Fluorescent images were obtained using IVIS Spectrum (Caliper Life Sciences, Perkin Elmer). Excitation light of 640 +/- 17 nm was used, and fluorescence emission was collected at 800 +/- 10 nm (Auto exposure, F stop 2, small binning). Mean fluorescence radiant efficiency was obtained for each well using Living Image Software (Caliper Life Sciences).

2-6. *E. coli* assay

E. coli XL1-Blue were grown in 3 mL of terrific broth without antibiotic overnight at 37 °C. At the end of the incubation, the bacteria were centrifuged at 2000xg for 10 minutes at 4 °C. The bacterial pellet was resuspended in 1 mL of PBS containing 1% DMSO. To investigate the fluorescent response of p-NBMB to intact *E. coli*, bacteria (approximately 10^9 CFU/mL) were added to each well of a black 96 well plate. Afterward, *p*-NBMB was added to a final concentration of 1 – 50 μM to each well of the 96 well plate. In addition, fluorescence response of *p*-NBMB to serial number of *E. coli* was examined. The bacterial suspension (approximately 10^9 CFU / mL) was serially diluted in PBS containing 1% DMSO in the following concentrations: undiluted, 1/10, $1/10^2$, $1/10^3$. 100 μl of PBS containing 1% DMSO, undiluted, and diluted bacterial suspension were transferred to a black 96 well plate containing 50 μM of *p*-NBMB and incubated at 37 °C for 4 hours. After the treatment, these well plates were incubated at 37 °C for 4 hours. At the end of the incubation, the fluorescence intensity was monitored in the same manner as for the measurement in section “Fluorescence imaging”. To calculate the

bacterial number, the same bacterial suspension was used. 100 µl from each dilution was spread on agar plates without antibiotics and incubated at 37 °C overnight. The colonies were counted from each plate, and colony numbers were used to calculate colony forming units (CFU) per mL in the original bacterial suspension.

2-7. Singlet oxygen ($^1O_2$) generation

Singlet oxygen generated from *p*-NBMB solution was monitored by oxidation of *p*-nitrosodimethylaniline (RNO).[1] It is known that RNO is bleached in the presence of singlet oxygen, implying that the change in the intensity of RNO absorbance indicates $^1O_2$ generation.[1] A solution of *p*-NBMB (10 µM) was prepared with the enzyme assay buffer containing 2 unit mL^{-1} of NTR and 1 mM of NAD(P)H. The buffer solution without the redox enzyme was used as a negative control. After incubation of all prepared samples for various time intervals ranging from 3 hr to 24 hr at 37 °C, RNO (13.2 µM) and histidine (21 mM) were added to the solutions. Those solutions were irradiated with a He-Ne laser (632 nm, 110 mW/cm^2, Hughes Research Laboratories, Culver City, CA). Various irradiation times ranging from 10 to 75 min were used to determine the effect of irradiation time on $^1O_2$. The optical density at 440 nm (λ_{max} of RNO) was monitored using a UV-visible spectrophotometer.

2-8. Computational Methodology

2-8.1 Protein Preparation

The crystal structure (ID: 1F5V) of *E. coli* nitroreductase NfsA (NTR) was solved by the molecular replacement method at 1.7 Å resolution.[2] We downloaded the X-ray coordinates from the RCSB Protein Data Bank (PDB).[3] The protein structure was prepared using the Protein Preparation Wizard of the Schrödinger suite[4] by adding hydrogen atoms, and adjusting bond orders, protonation states, tautomerization states of amino acids and the protein-ligand geometry. The OPLS2005 force field[5] was used for the minimization steps.

2-8.2 Receptor Grid Preparation

The receptor grid was prepared using the OpenEye suite.[6] We made sure to keep FMN as an integral part of the receptor model. The grid dimensions were 15.33 Å x 15.00 Å x 18.33 Å and it had a volume of 4325 $Å^3$. A negative image of the receptor was created with balanced extension towards solvent and protein. We adjusted the outer contour of the site shape to 600 $Å^3$ and we disabled the inner contour limit to allow small ligands to bind during the docking step. We did not specify constraints for the docking.

2-8.3 Ligand Preparation

p-NBMB was prepared using LigPrep of Schrödinger suite.[7] We adjusted the protonation and tautomerization states at pH 7.4. OMEGA version 2.4.6 of the OpenEye suite[8] was used to generate all possible conformers of the ligand using the MMF94s force field.

2-8.4 Docking

Fred v3.0.0 of the OpenEye suite[6] was used for the docking simulation. The MMF94s force field was specified for the docking step. The Chemgauss3 scoring function was used for the exhaustive search step of Fred and Chemgauss4 was used for the optimization step.

Fig. S1. ^{1}H NMR spectrum of ***p*-NBMB** in $CDCl_3$.

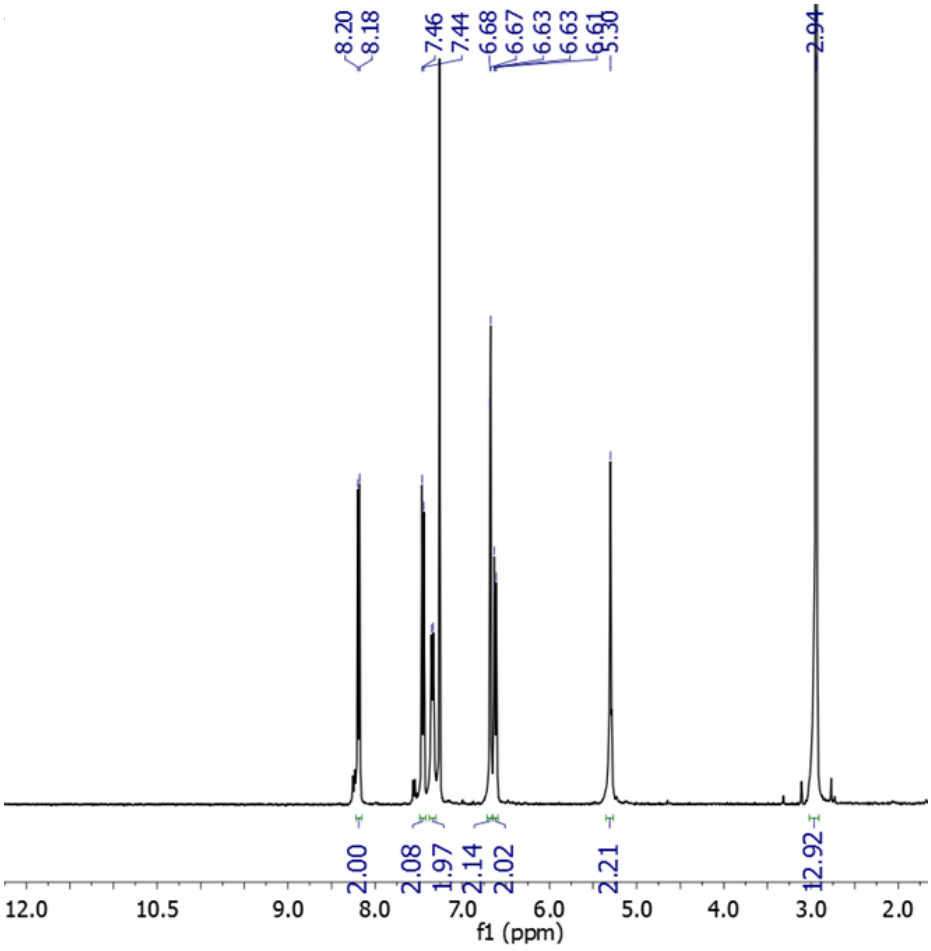


Fig. **S2.** Absorbance spectra of imaging probe *p*-NBMB (dashed line) and MB (solid line) as a reference by UV spectroscopy.

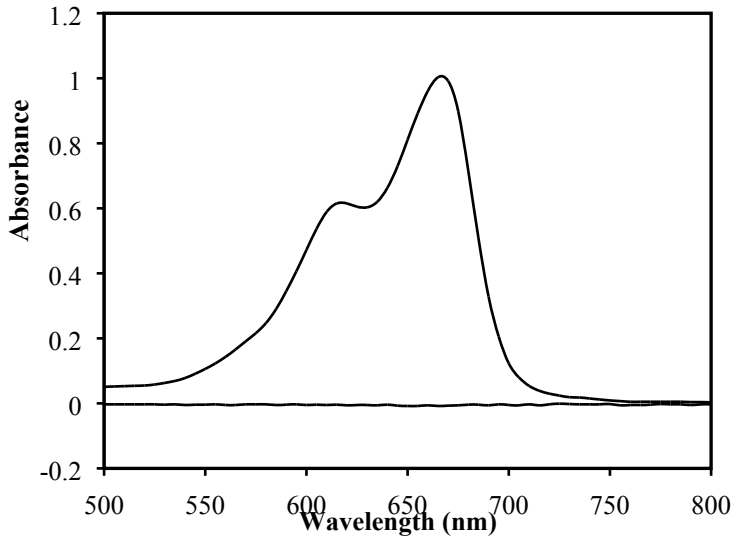


Fig. S3. Change in fluorescence intensity of *p*-NBMB (10 μM) after 3, 6, 9, 12, and 24 hours of incubation with NTR (1 unit ml^{-1})/NAD(P)H (1 mM). The negative control did not include the enzyme and NAD(P)H. Spectra were acquired in 10 mM PBS, pH 7.4 with the excitation at 580 nm**.**

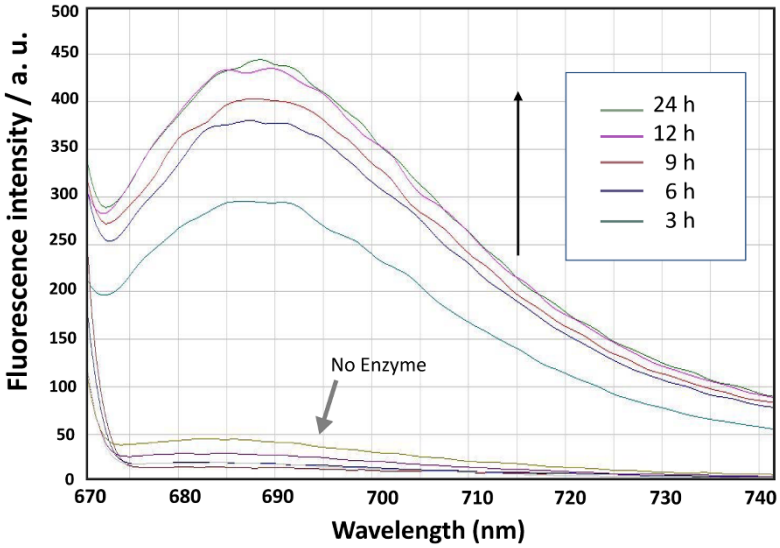


Fig. S4 Fluorescence intensity of *p*-NBMB (10 μM) treated with redox enzymes. Negative control did not include enzymes and NAD(P)H

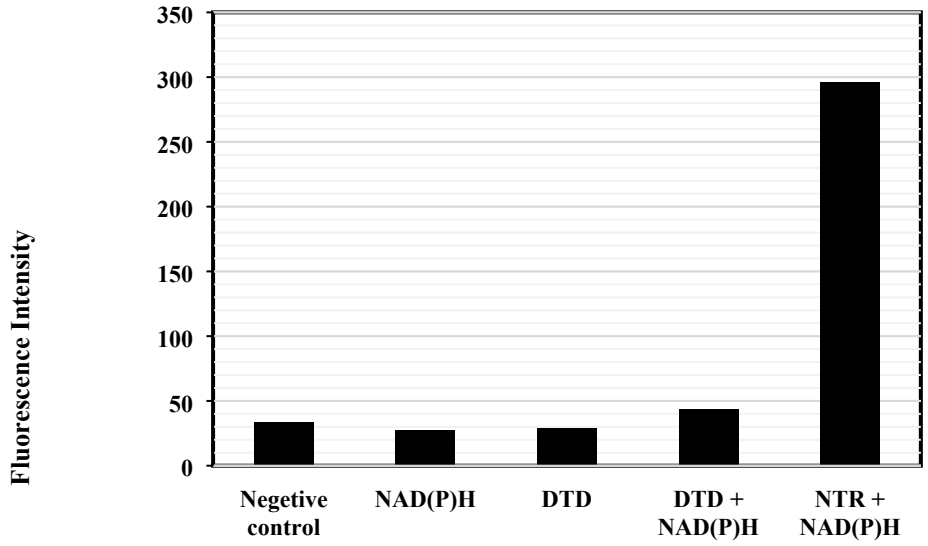


Fig. S5. Fluorescence intensity from *p*-NBMB at a concentration of 1 μM, 10 μM and 100 μM. The samples were incubated at 37 °C overnight in PBS buffer (pH 7.4) containing NTR (1 unit mL^{-1} and 2 units mL^{-1})

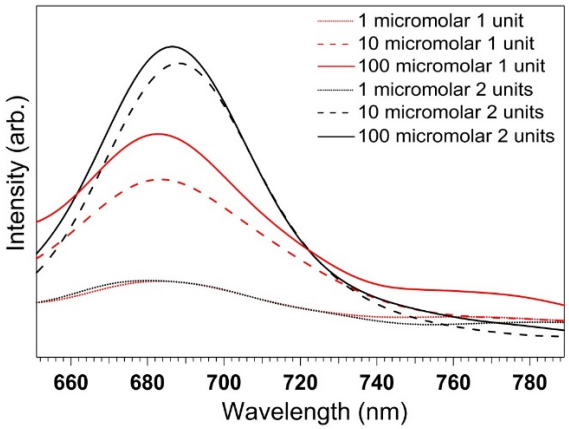


Fig. S6. Sensitivity of *p*-NBMB fluorescence to bacterial number. IVIS fluorescence images of *p*-NBMB (50 μM) incubated with a serial dilution of the intact *E. coli* K12 strain XL1-Blue suspension at 37 °C for 4 hours.

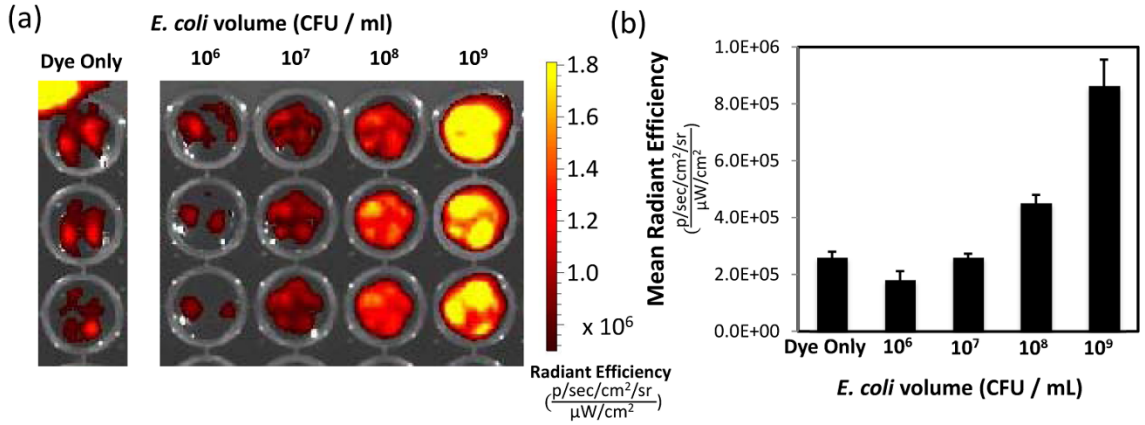


Fig. S7. Singlet oxygen ($^1O_2$) generation from *p*-NBMB solution (a) with or (b) without NTR/NAD(P)H was monitored using *p*-nitrosodimethylaniline (RNO) upon laser light irradiation at a wavelength of 632 nm. Various irradiation times ranging from 10 to 75 min were used to determine the effect of irradiation time on singlet oxygen production. The concentration of *p*-NBMB was 10 μM in the enzyme assay buffer containing NTR at a concentration of 2 unit mL^{-1} and NAD(P)H at a concentration of 1 mM. The intensity of RNO absorbance decreased in the *p*-NBMB solution containing NTR/NAD(P)H while the solution without the redox enzyme did not affect RNO absorbance.

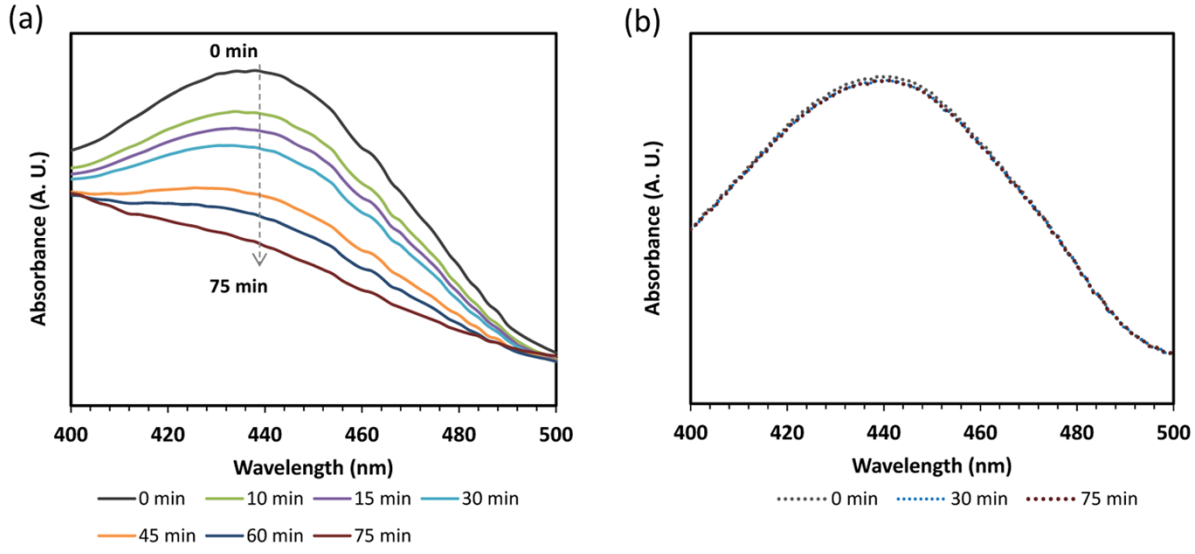
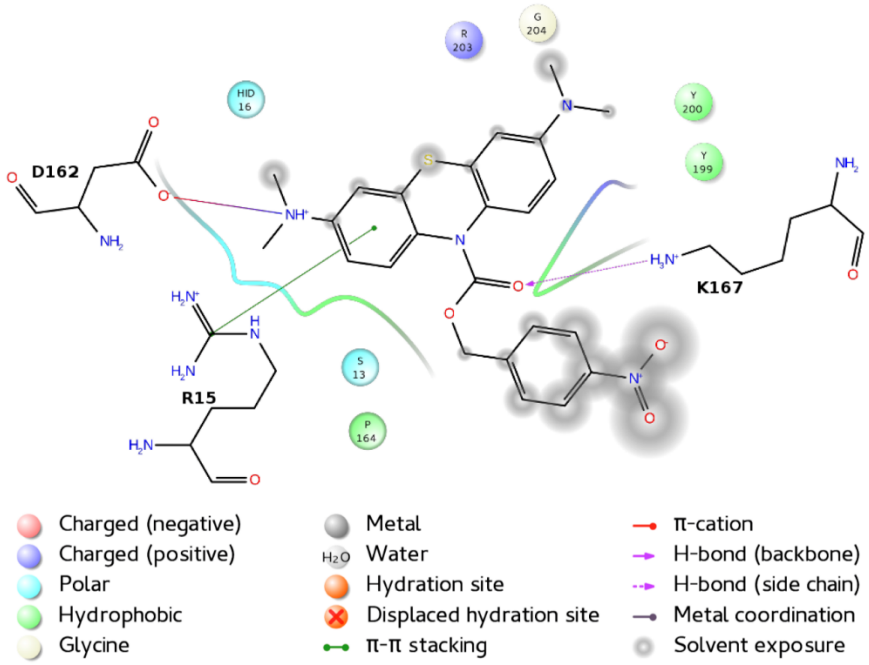


Fig. S8. The interactions between the best docking pose of *p*-NBMB and the active site amino acids of *E. coli* NTR (PDB ID: 1F5V) active site in the best docked pose. This image was prepared using Maestro.[9]



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