Supporting Information

A dual-function probe for inhibition and rapid detection of *Mycobacterium tuberculosis*

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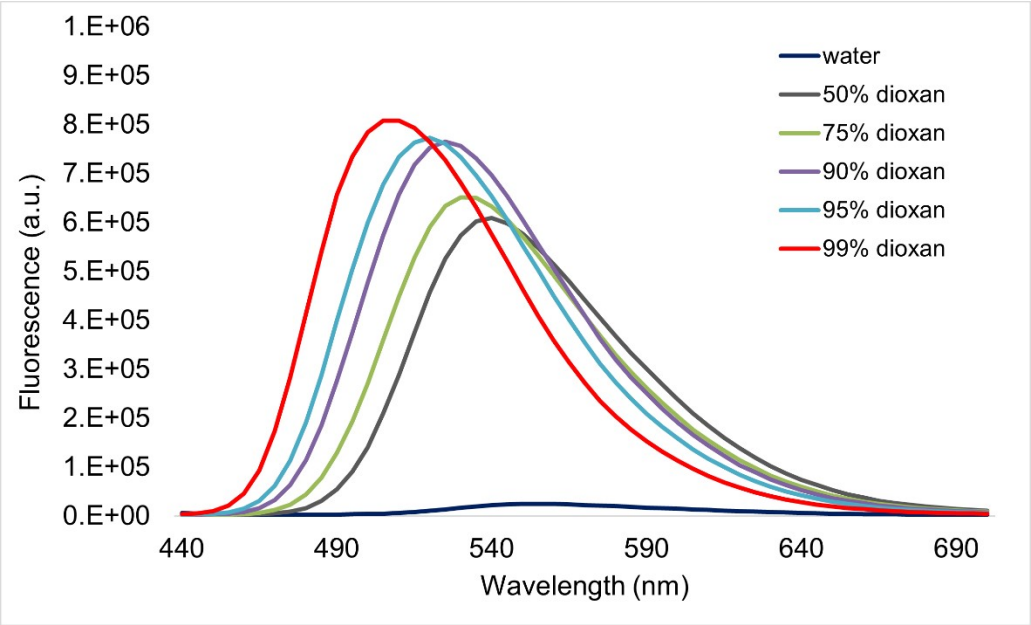


Figure S1. The fluorescence emission spectra of the environment-sensitive fluorophore dye-conjugate (ex 420 nm) in solvent systems with the specified ratio of dioxane in water.

Sample	**T_1 (ps)**	**T_2 (ps)**	**T_3 (ps)**	**B_1**	**B_2**	**B_3**	**Average Lifetime (ps)**	**χ^2**
DprE1	1270	4606	-	0.56	0.44	-	2744.7	1.39
DprE1-**NA-H**	170.2	4114.8	205.3	0.58	0.0005	-0.42	186.9	1.34

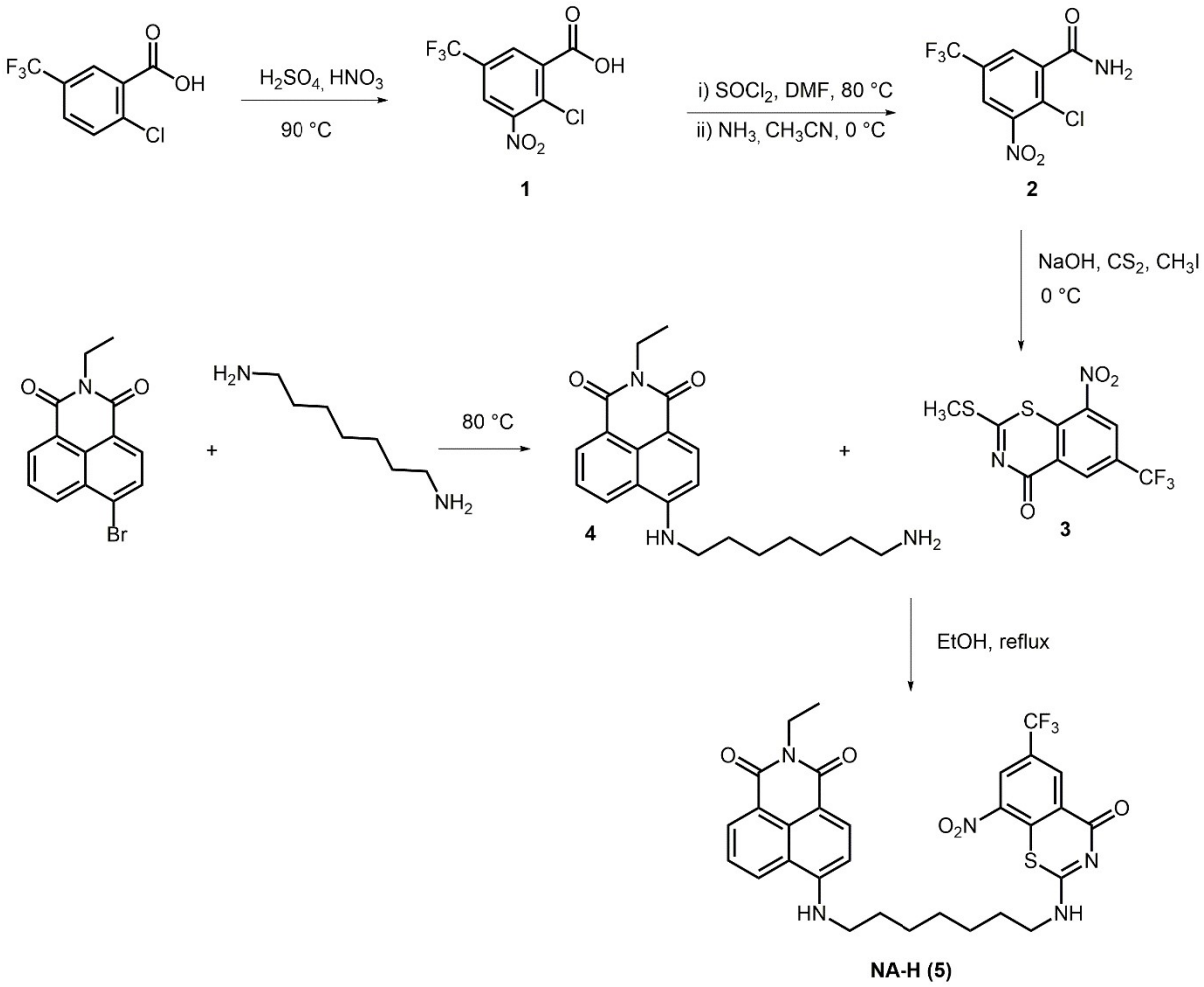
Table S1. Lifetime kinetics measurements calculated from TCSPC measurements

Supplementary Chemical Synthesis Protocols

General Information

The manipulation of all air and/or water-sensitive compounds was carried out using standard high vacuum techniques. Analytical thin layer chromatography (TLC) was carried out on Merck® aluminium backed silica gel 60 GF254 plates and visualization when required was achieved using UV light. Column chromatography was carried out on silica gel 60 GF254. NMR spectra were recorded at ambient probe temperature using AVANCE NEO 500MHz FT-NMR Spectrometer. Chemical shifts are quoted as parts per million (ppm) relative to the solvent. Coupling constants (J) are reported in hertz (Hz) with the following abbreviations: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; quin, quintet; m, multiplet. Mass was analyzed using LC-MS on an ESI source (Agilent 1290 LC/MSD) in positive and negative modes. All new compounds of the present study (denoted by an asterisk (*) were also characterized and confirmed by High-resolution mass spectra (HRMS). HRMS (ESI) were recorded on an ESI source QTOF (Agilent 1290- G6545XT) in positive and negative ion modes. M/z values are reported in Daltons.

Synthetic Scheme S1



Synthetic Procedures

Synthesis of 2-chloro-3-nitro-5-(trifluoromethyl)benzoic acid (1)

The reaction was performed as described previously (1). Briefly, to 2-chloro-5-(trifluoromethyl)benzoic acid (10g, 44.5 mmol) conc. sulphuric acid (60 ml) and conc. nitric acid (60 ml) was added and the reaction was stirred at 90 °C for 2h. The reaction mixture was cooled to r.t., and ice water (200 ml) was poured into it. The resulting white precipitate was filtered and dried under a vacuum.

^{1}H NMR (500 MHz, DMSO) δ 8.71 (s, 1H), 8.40 (s, 1H).
MS m/z (ES$^+$): $[M+H]^+$ calculated for $C_8H_4ClF_3NO_4$, 269.9; found 269.9

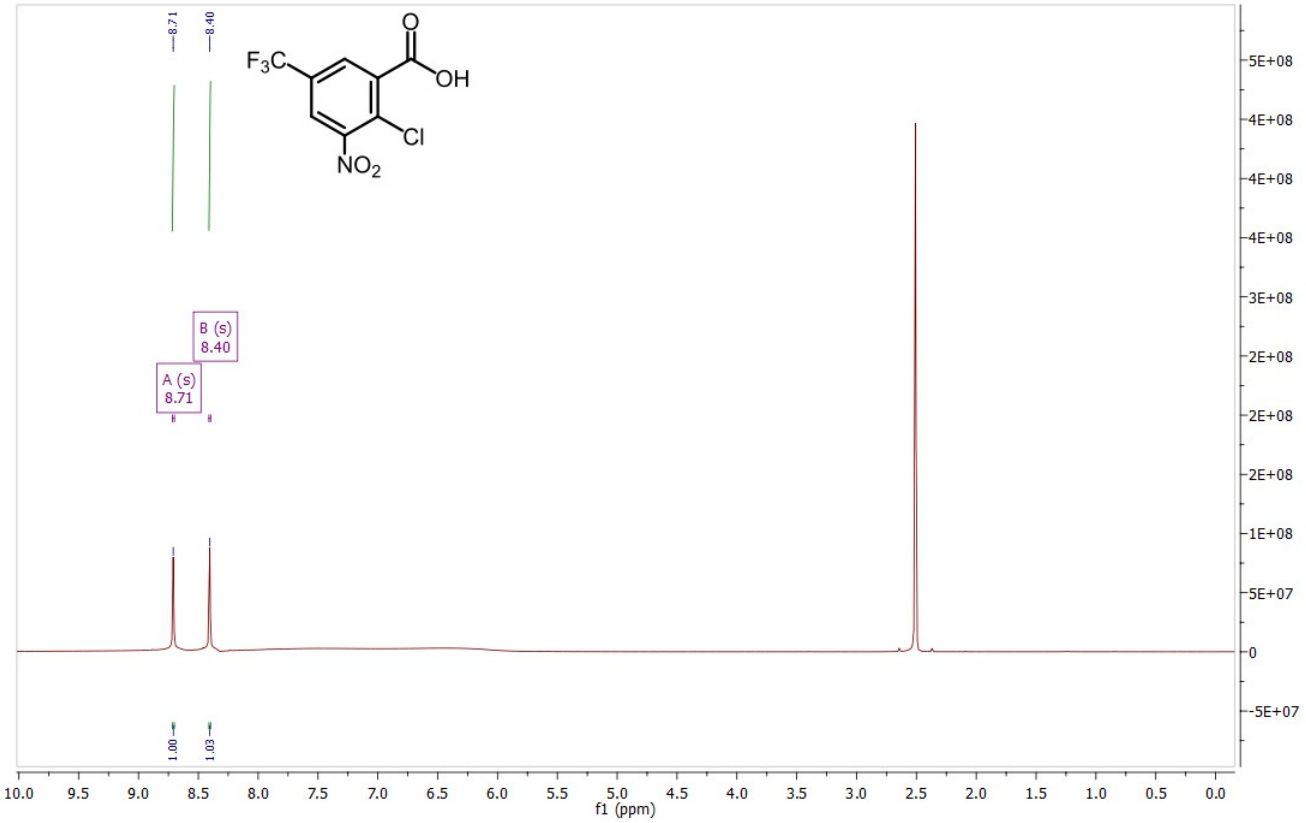


Figure S2. ^{1}H NMR (500 MHz, DMSO) for 2-chloro-3-nitro-5-(trifluoromethyl)benzoic acid (1)

Synthesis of 2-chloro-3-nitro-5-(trifluoromethyl)benzamide (2)

The reaction was adapted from a previously described method (2). Briefly, to 2-chloro-3-nitro-5-(trifluoromethyl)benzoic acid (5g, 18.5 mmol), DMF (1 ml) and excess thionyl chloride (40 ml) was added. The reaction was stirred at 80 °C for 5h, cooled to r.t. To the reaction mixture, acetonitrile (5 ml) and ice cold ammonium hydroxide solution (10 ml) were added. The reaction was stirred on ice for 1h. The resulting white precipitates were filtered and dried by desiccation.

^{1}H NMR (500 MHz, DMSO) δ 8.60 (s, 1H), 8.25 (s, 1H), 8.19 (s, 1H), 8.04 (s, 1H).

MS m/z (ES$^-$): [M-H]$^-$ calculated for $C_8H_3ClF_3N_2O_3$, 267.9; found 267.9.

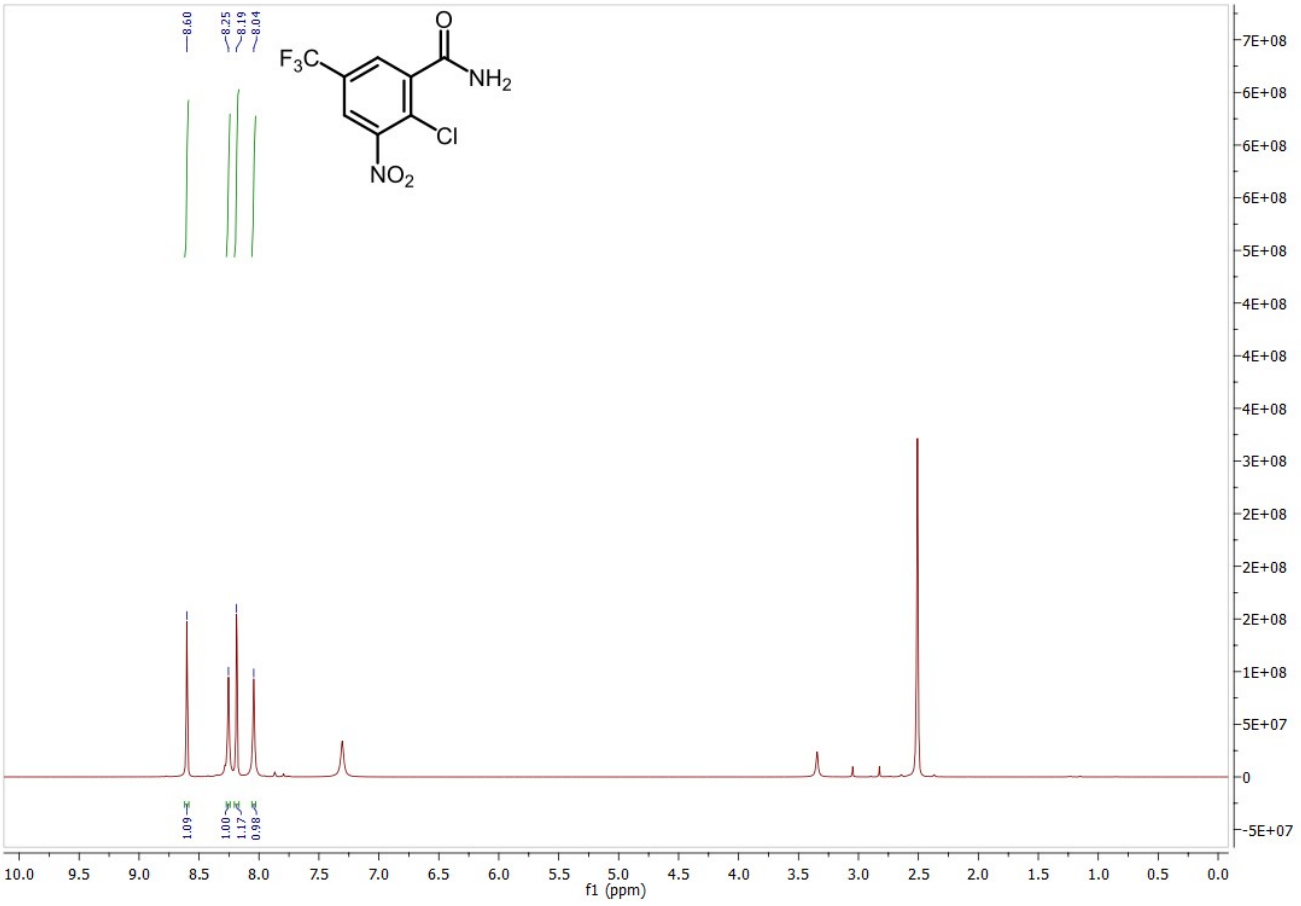


Figure S3. ^{1}H NMR (500 MHz, DMSO) for 2-chloro-3-nitro-5-(trifluoromethyl)benzamide (2)

Synthesis of 2-(methylthio)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-4-one (3)

The reaction was performed as described previously with some modifications (3). Briefly, to 1g powdered NaOH, 5 ml DMSO and 2 ml carbon disulphide were added. To the mixture, 4g of 2-chloro-3-nitro-5-(trifluoromethyl)benzamide was added and the reaction was stirred on ice for 30 min, followed by addition of 1 ml methyl iodide with continuous stirring on ice. After 30 min, ice water (100 ml) was added to the reaction mixture, the pale yellow precipitates were filtered and dried by desiccation.

^{1}H NMR (500 MHz, DMSO) δ 8.96 (s, 1H), 8.84 (s, 1H), 2.77 (s, 3H).
MS m/z (ES$^+$): $[M+H]^+$ calculated for $C_{10}H_6F_3N_2O_3S_2$, 323.0; found 323.0.

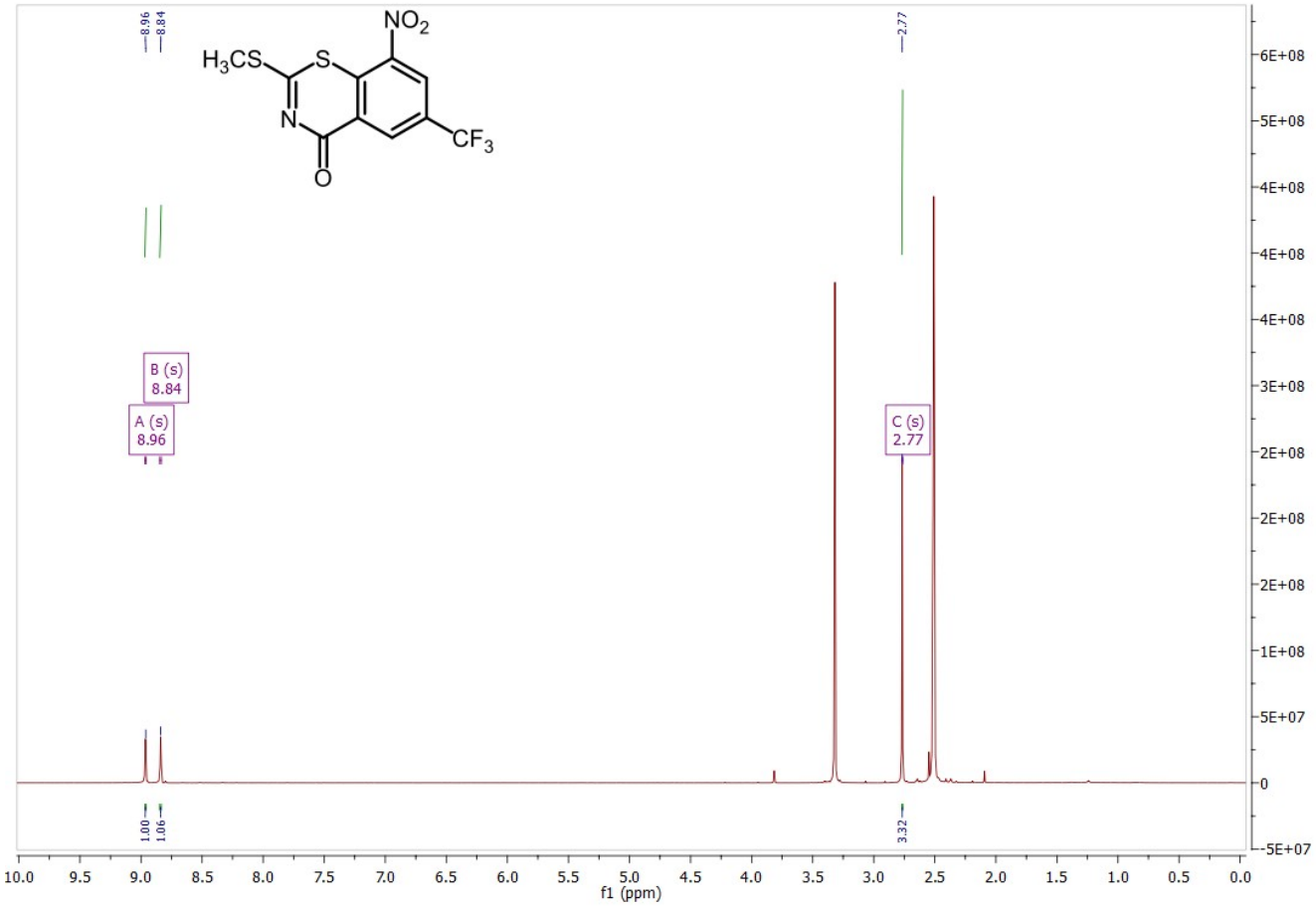


Figure S4. ^{1}H NMR (500 MHz, DMSO) for 2-(methylthio)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-4-one (3)

6-((7-aminoheptyl)amino)-2-ethyl-1H-benzo[de]isoquinoline-1,3(2H)-dione (4)*

To 6-bromo-2-ethyl-1H-benzo[de]isoquinoline-1,3(2H)-dione (0.1 g, 0.33 mmol) heptane-1,7-diamine (5 ml, 0.33 mmol, 1.0 eq.) was added dropwise and the reaction was heated at reflux overnight. The reaction mixture was cooled to r.t. and ice water (20 ml) was poured into it. The resulting yellow precipitate was separated by filtration and dried under a vacuum.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.56 (d, *J* = 7.3 Hz, 1H), 8.44 (d, *J* = 8.4 Hz, 1H), 8.11 (d, *J* = 8.1 Hz, 1H), 7.58 (d, *J* = 8.2 Hz, 1H), 6.69 (d, *J* = 8.5 Hz, 1H), 4.22 (q, *J* = 7.1 Hz, 2H), 3.39 (dd, *J* = 12.4, 7.1 Hz, 2H), 2.70 (t, *J* = 7.0 Hz, 2H), 1.80 (dt, *J* = 14.7, 7.3 Hz, 4H), 1.49 (dd, *J* = 20.3, 11.1 Hz, 4H), 1.43 – 1.36 (m, 4H), 1.32 (t, *J* = 7.1 Hz, 3H).

MS m/z (ES^-): $[M-H]^-$ calculated for $C_{21}H_{26}N_3O_2$, 352.0; found 352.0.

HRMS (ES^-) m/z: $[M - H]^-$ calculated for $C_{21}H_{26}N_3O_2$, 352.2030; found 352.2029.

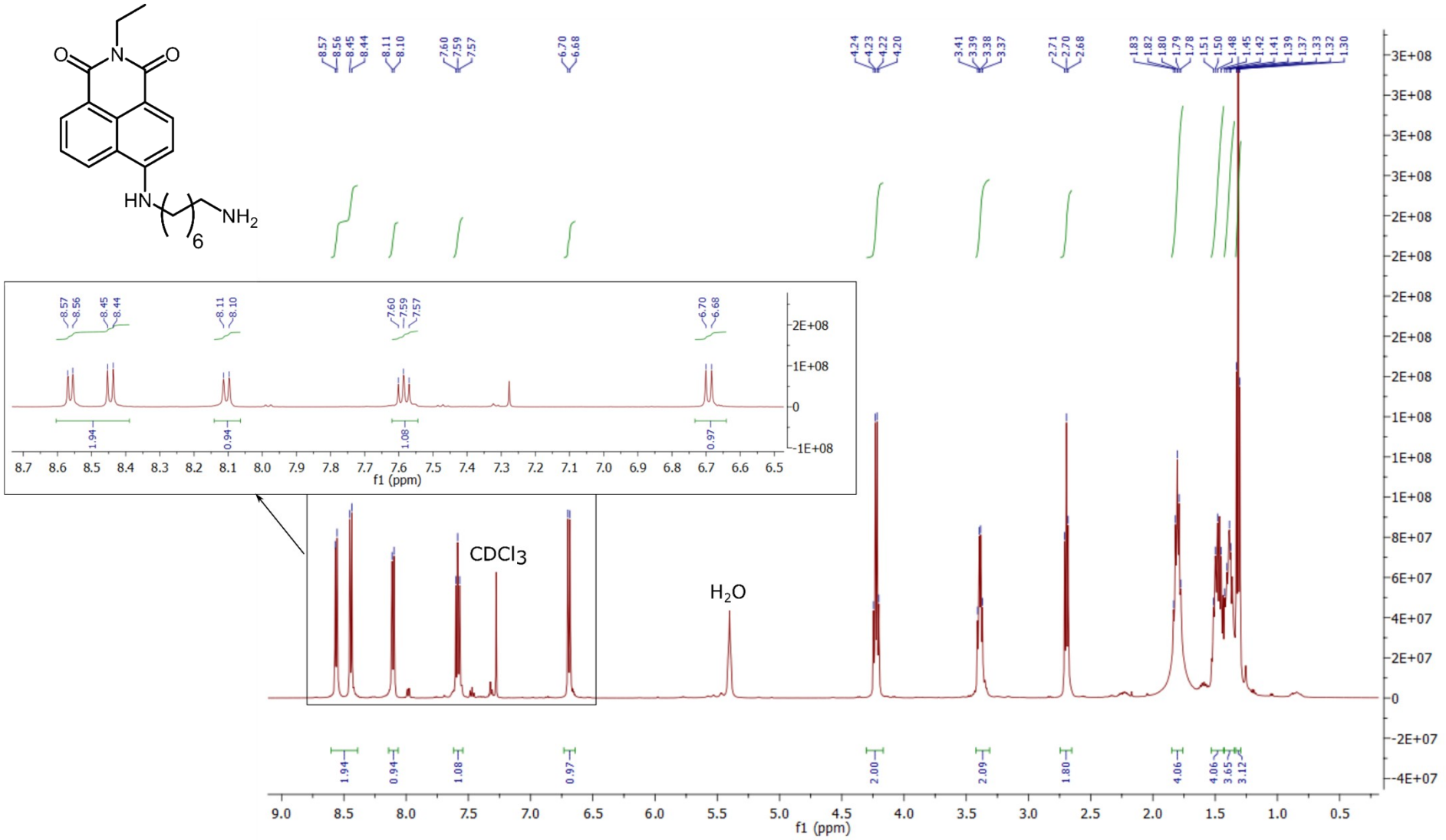


Figure S5. ^{1}H NMR (500 MHz, $CDCl_3$) for 6-((7-aminoheptyl)amino)-2-ethyl-1H-benzo[de]isoquinoline-1,3(2H)-dione **(4)**

2-ethyl-6-((7-((8-nitro-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-2-yl)amino)heptyl)amino)-1H-benzo[de]isoquinoline-1,3(2H)-dione (NA-H) (5)*

6-((7-aminoheptyl)amino)-2-ethyl-1H-benzo[de]isoquinoline-1,3(2H)-dione (0.088 g, 249 mmol) was mixed with 2-(methylthio)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-4-one (0.080 g, 249 mmol, 1 eq.). Ethanol (10 ml) was added to the reaction mixture followed by stirring at r.t. for 10 minutes. The reaction mixture was then heated at reflux overnight. After cooling, 20 ml of water was added. The resulting yellow precipitate was then filtered and dried under a vacuum.

^{1}H NMR (500 MHz, DMSO) δ 8.73 (d, *J* = 7.8 Hz, 2H), 8.66 (d, *J* = 8.4 Hz, 1H), 8.39 (d, *J* = 7.2 Hz, 1H), 8.21 (d, *J* = 8.5 Hz, 1H), 7.71 (s, 1H), 7.63 (t, *J* = 7.8 Hz, 1H), 6.72 (d, *J* = 8.6 Hz, 1H), 4.05 – 4.01 (m, 2H), 3.46 (t, *J* = 6.7 Hz, 2H), 3.35 (d, *J* = 5.7 Hz, 2H), 1.72 – 1.69 (m, 2H), 1.61 – 1.57 (m, 2H), 1.40 (m, 6H), 1.17 (t, *J* = 6.8 Hz, 3H), 1.06 (t, *J* = 7.0 Hz, 1H).

MS m/z (ES^-): $[M-H]^-$ calculated for $C_{30}H_{28}F_3N_5O_5S$, 626.0; found 626.0.

HRMS (ESI) m/z: calculated for $C_{30}H_{28}F_3N_5O_5S$, 627.1763, found 627.1706.

HRMS (ES^-) m/z: $[M - H]^-$ calculated for $C_{30}H_{28}F_3N_5O_5S$, 626.1690, found 626.1673.

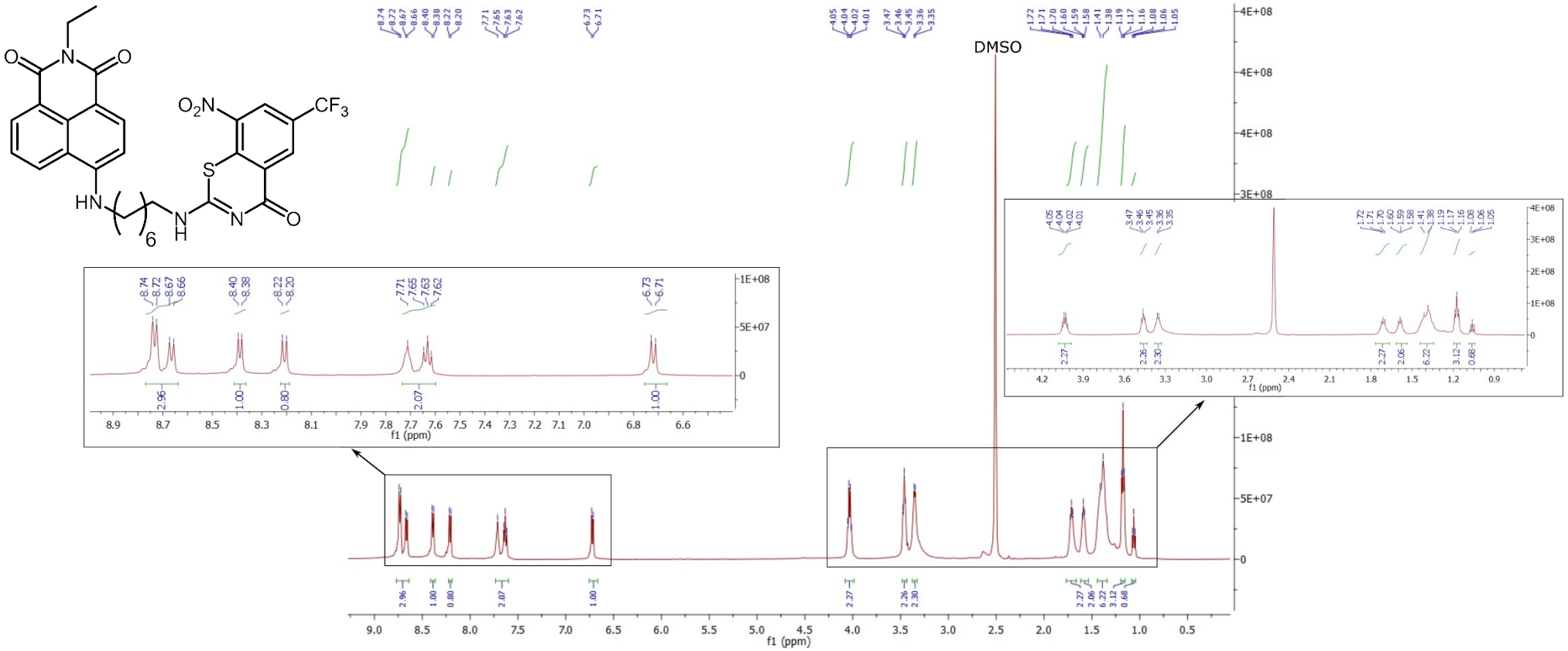


Figure S6. ^{1}H NMR (500 MHz, DMSO) for 2-ethyl-6-((7-((8-nitro-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-2-yl)amino)heptyl)amino)-1H-benzo[de]isoquinoline-1,3(2H)-dione **(NA-H) (5)**

Supplementary Biology Protocols

Fluorescence Spectroscopy

To study the fluorescence spectrum of the sensor, 10 μM of the fluorophore in water or 50%, 75%, 90%, 95%, and 99% water-1,4-dioxane (*v/v*) (pH 7.4) in 10 mm x 10 mm quartz cuvettes (hellma analytics). The data was acquired on Photon Technology International QuantaMaster™ 40. The spectra were acquired using the accompanying FelixGx v. 4.3.4 software with standard emission scan settings with lamp slit widths set at 4 nm. The compound was excited at 420 nm, and the emission scan was monitored over 440-700 nm.

Expression and Purification of Mtb DprE1 enzyme

The gene Rv3790 was cloned into the pET-16b vector between NdeI and BamHI sites. The proteins were co-expressed with Mtb and E. coli chaperones groEL (Rv0440) and groES, as described previously (4) with slight modifications. Briefly, the Mtb Rv0440 was cloned into the pGro7 (Takara, USA) vector by replacing the *E. coli* groEL with Mtb Rv0440 using the In-Fusion Snap Assembly Kit (Takara, USA). The clones were co-transformed into *E. coli* BL21(DE3) strain cells. The cells were grown at 37 °C with 0.2 mg/ml L-arabinose until OD_{600} reached 0.4 to 0.6, followed by induction with 0.5 mM IPTG at 16 °C for 16h. The following day, the cultures were centrifuged at 8000 rpm for 15 min, and the pellets were stored at -20 °C until further use. For purification, the pellet was resuspended in lysis buffer comprising 50 mM Tris pH 8.0, 200 mM NaCl, 10% glycerol, 1% sarkosyl, 5 mM imidazole, and 1 mM PMSF. The cells were lysed by sonication on ice (40% amplitude, 8s pulse on, 10s pulse off for 15 min), followed by centrifugation at 13000 rpm for 45 min. The lysate was then poured onto an immobilized metal affinity chromatography Ni-NTA column followed by stringent washing with wash buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 10% glycerol, 0.1% sarkosyl, and 5 mM imidazole) and elution with increasing concentration of imidazole (20 mM to 500 mM) in the wash buffer. The eluted fractions were analyzed on a 12% SDS-PAGE gel. Freshly purified protein (~10 mg) was loaded onto a Superdex 200 Increase 10/300 GL column equilibrated with storage buffer (25 mM Tris, pH 8.0, 100 mM NaCl, 5% glycerol, and 0.05% sarkosyl) with a flow rate of 0.2 ml/min at 4 °C. The collected fractions were stored at -80 °C.

Fluorescence Polarization

Fluorescence Polarization (FP) experiments were performed in triplicate in black 96-well plates. Dpre1 protein was serially diluted (0.078-5 μM) in the appropriate buffer (25 mM Tris, pH 8.0). The fluorophore was added at a 0.5 μM concentration. The reaction was incubated for 30 min, following which, the FP signals were recorded at 25 °C using BioTek Cytation 5 Multimode reader (Agilent, USA) equipped with fluorescence polarization filters with excitation (485 nm) and emission (528 nm) wavelengths using Gen5 software (version 3.12.08). The resulting FP signals were corrected for the baseline FP signal of the fluorophore in the absence of protein. The data were plotted as mean±SEM.

Fluorescence Lifetime Measurements

The fluorescence lifetime of tryptophan residues in DprE1 was monitored using time-correlated single photon counting (TCSPC) on a DeltaFlex™ TCSPC Lifetime Fluorometer (Horiba Scientific, Japan). The protein DprE1 (5 μM) and DprE1 mixed with the probe (100 μM) in 25 mM Tris (pH 8.0) were excited at 295 nm. The data was collected at 340 nm. The instrument response function (IRF) measured for deionized water was deconvoluted on all data, and the decay curves were fitted to the bi- and tri-exponential decay functions.

Enzyme Inhibition

The assay involves using a 2,6-dichlorophenolindophenol (DCPIP) functional assay as described by Neres et al. (5). Briefly, decaprenylphosphoryl-β-D-ribofuranose (DPR) is used as the substrate, and DCPIP as an electron acceptor. Upon reduction of DCPIP, in the absence of any inhibitor, there is a decrease in absorption at 600 nm wavelength. For inhibition studies, the enzyme (0.3 μM) was incubated with varying concentrations of compounds for 10 minutes along with substrate DPR, followed by DCPIP addition. The measurements were recorded at r.t. BioTek Cytation 5 Multimode reader (Agilent, USA) in a 96-well plate. The data were plotted as mean±SEM.

Bacterial Culture Inoculation and Metabolic Labeling

Single colonies of bacteria were inoculated in suitable media, (BD Difco Middlebrook 7H9 media supplemented with 10% v/v) OADC (Oleic, Albumin, Dextrose, Catalase), 0.5% (v/v) glycerol, and 0.5% (w/v) Tween 80 for *Mycobacterium smegmatis* mc^2155, Luria-Bertani broth for *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 and incubated at 37 °C overnight. Overnight cultures were diluted to OD_{600} of 0.5. An appropriate amount of stock fluorophore was added to the aliquots of the appropriate culture to get a final concentration of 100 μM. A suitable control with no additional probe was used, which was treated identically. Cultures were incubated for 15 min at 37 °C. The samples were then washed thrice with 1X PBS (pH 7.4) and analyzed using confocal microscopy and Flow Cytometry.

Metabolic Labeling of *Mycobacterium tuberculosis*

1 ml frozen stock of Mtb H37Rv was inoculated in 30 ml of BD Difco Middlebrook 7H9 media supplemented with 10% v/v) OADC, 0.5% (v/v) glycerol, and 0.5% (w/v) Tween 80). The cells were allowed to grow to OD_{600} of 0.5. Aliquots of 150 μl were incubated with a 100 μM probe for 15 and 60 min. After incubation, the labeled cells were harvested and washed thrice with 1X-PBS supplemented with 5% Tween 80, followed by resuspension in 1X-PBS (pH 7.4) containing 4% paraformaldehyde. The cells were then incubated at r.t. for 2 h to ensure the internal surfaces of the tube were sterilized, followed by confocal microscopy and flow cytometry measurements.

Confocal Microscopy Imaging Analysis

Samples were washed as described above. Slides were prepared by spotting a drop of sample on a 2% agarose pad, then covering it with a cover slip and sealing it with nail polish. The microscopy experiments were performed on a Nikon AIR confocal microscope 60X objective. Samples were excited by a 488 nm laser, and the images were recorded with a GFP filter (500-550 nm). Acquisition and processing of images were performed in identical settings for test and control samples.

Flow Cytometry

Samples were washed as described above. The experiments were performed on a BD FACSVerse™ Cell Analyzer using a FITC filter set (excitation, 495 nm, and emission, 519 nm). Data were collected for 10,000 cells per sample, analyzed using FlowJo, and subsequently imported into GraphPad Prism 8 for statistical analysis. The data were plotted as mean±SEM.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration of the probes against *Mycobacterium tuberculosis* was determined using the EUCAST broth microdilution reference method (6). Briefly, H37Rv culture grown in Middlebrook 7H9-10% OADC medium was diluted to 10^5 CFU/ml suspension for final inoculum. The culture was maintained in a 96-well plate incubated at 37

°C, and the growth was monitored for 7 days. A positive control (rifampicin), growth control, and sterility (negative) control were also applied.

References

1 Welch DE, Baron RR, Burton BA. α,α,α-Trifluorotoluamides as anticoccidial agents. Journal of Medicinal Chemistry. 1969 Mar;12(2):299-303.

2 Makarov V, Cole ST, Moellmann U. PCT Int. Appl. WO 2007134625, 2007.

3 Cooper M, Zuegg J, Becker B, Karoli T. PCT Int. Appl. WO 2012085654, 2012.

4 Batt SM, Jabeen T, Bhowruth V, Quill L, Lund PA, Eggeling L, Alderwick LJ, Fütterer K, Besra GS. Structural basis of inhibition of Mycobacterium tuberculosis DprE1 by benzothiazinone inhibitors. Proceedings of the National Academy of Sciences. 2012 Jul 10;109(28):11354-9.

5 Neres J, Pojer F, Molteni E, Chiarelli LR, Dhar N, Boy-Röttger S, Buroni S, Fullam E, Degiacomi G, Lucarelli AP, Read RJ. Structural basis for benzothiazinone-mediated killing of Mycobacterium tuberculosis. Science translational medicine. 2012 Sep 5;4(150):150ra121-.

6 Schön T, Werngren J, Machado D, Borroni E, Wijkander M, Lina G, Mouton J, Matuschek E, Kahlmeter G, Giske C, Santin M. Antimicrobial susceptibility testing of Mycobacterium tuberculosis complex isolates–the EUCAST broth microdilution reference method for MIC determination. Clinical Microbiology and Infection. 2020 Nov 1;26(11):1488-92.