9-Nitrobenzo[*b*]quinolizinium as Fluorogenic Probe for the Detection of Nitroreductase *in vitro* and in *Escherichia coli*

Peter Jonas Wickhorst, Heiko Ihmels, Melanie Marianne Lammert-Baumgartner, Mareike Müller, and Holger Schönherr

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Electronic Supporting Information

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1. Equipment

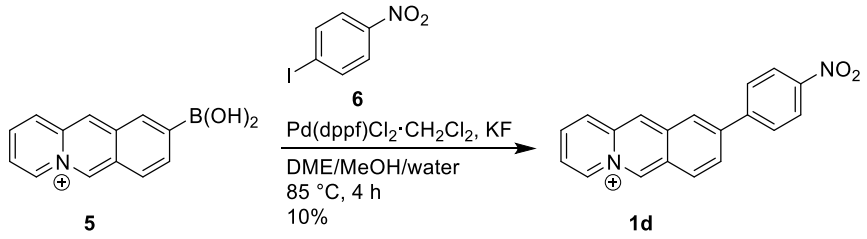
Absorption spectra: Analytik Jena Specord S600 spectrophotometer and Varian Cary 100 Bio spectrophotometer with baseline correction. Emission spectra: Varian Cary Eclipse spectrophotometer at 20 °C: Cuvettes: Quartz cells (10 mm x 4 mm). NMR spectra: Jeol ECZ 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) at 25 °C (DMSO-d_6). NMR spectra were processed with the software MestReNova and referenced to the residual solvent signal of DMSO-d_5 (^{1}H: δ = 2.50, ^{13}C: δ = 39.5). Elemental analyses data: HEKAtech EUROEA combustion analyser, by Rochus Breuer, Organische Chemie I, Universität Siegen. Melting points (uncorrected): BÜCHI 545 (BÜCHI, Flawil, CH), Tecan Reader Safire (Männedorf, Switzerland)

2. Materials

9-Nitrobenzo[*b*]quinolizinium perchlorate (**1b**),[1] 2-(4-nitrostyryl)quinolizinium tetrafluoroborate (**2**)[2] and benzo[*b*]quinolizinium-*9*-boronic acid bromide (**5**)[3] were prepared according to published procedures.[1] Nitroreductase from *Escherichia coli* (>100 units/mg), β-nicotinamide adenine dinucleotide, as disodium salt hydrate, and 4-nitroiodobenzene (**6**) were purchased from SigmaAldrich (St. Louis, USA). All solutions were prepared in PBS buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl)., Tris-HCl buffer (dilution from 1 M Tris-HCl stock buffer, pH 8.5 from jenabioscience 0.05 M and titration with HCl to pH 7.4), LB-agar (Lysogeny broth agar, Luria/Miller, Carl Roth, Karlsruhe, Germany), LB (Lysogeny broth, Luria/Miller, Carl Roth, Karlsruhe, Germany). All buffer solutions and media were prepared from purified water (resistivity 18 MΩ cm) and biochemistry-grade chemicals. The buffer solutions were filtered through a PVDF membrane filter (pore size 0.45 µm) prior to use.

3. Synthesis

The novel compound **1d** was synthesized by a Suzuki-Miyaura coupling reaction according to published standard procedure for benzo[*b*]quinolizinium derivatives.[3]



Scheme S1. Synthesis of 9-(4-nitrophenyl)benzo[*b*]quinolizinium (**1d**).

9-(4-Nitrophenyl)benzo[b]quinolizinium (**1d**).

A mixture of benzo[*b*]quinolizinium-*9*-boronic acid bromide (**5**) (152 mg, 500 µmol), 4-nitroiodobenzene (**6**) (156 mg, 625 µmol), Pd(dppf)Cl_2•CH_2Cl_2 (20 mg) and KF (116 mg, 2.00 mmol) in DME/water/MeOH (2:1:1, 6 ml) was stirred for 4 h at 85 °C under argon gas atmosphere. After cooling to r.t. the reaction mixture was diluted with MeOH (20 ml) and filtered, and aq. $NaBF_4$ solution (sat. 1.0 ml) was added to the filtrate. The precipitate was filtered off and the crude solid product was purified by column chromatography (SiO_2; CH_2Cl_2/MeOH, 9:1 v/v) to give product **1d** as red amorphous solid (19.4 mg, 50.0 µmol, 10%); mp 291–293 °C. – ^{1}H NMR (500 MHz, DMSO-d_6): 8.00 (dt, ^3J = 7 Hz, ^4J = 1 Hz, 1H, 3-H), 8.12 (s, ^3J = 7 Hz, ^4J = 1 Hz, 1H, 2-H), 8.29 (d, ^3J = 9 Hz, 2H, 3‘-H, 5‘-H), 8.43 (d, ^3J = 9 Hz, 1H, 8-H), 8.46 (d, ^3J = 9 Hz, 2H, 2‘-H, 6‘-H), 8.63 (d, ^3J = 9 Hz, 1H, 7-H), 8.66 (d, ^3J = 9 Hz, 1H, 1-H), 8.86 (s, 1H, 10-H), 9.26 (s, 1H, 11-H), 9.31 (d, ^3J = 7 Hz, 1H, 4-H), 10.46 (s, 1H, 6-H). – ^{13}C NMR (125 MHz, DMSO-d_6): δ = 122.6 (C6a), 124.4 (C2’, C6’) 125.3 (C10), 125.5 (C11), 127.0 (C1), 129.1 (C3‘, C5‘), 129.2 (C3), 129.3 (C7), 130.1 (C8), 131.5 (C2), 134.6 (C4), 135.4 (C10a), 138.0 (C11a), 140.2 (C6), 142.8 (C9), 144.1 (C4’), 147.9 (C1’). – El. Anal. for $C_{19}H_{13}BF_4N_2O_2$ x 0.5 H_2O, calc. (%): C 57.46, H 3.55, N 7.05 found (%): C 57.01, H 3.25, N 6.76.

4. Determination of fluorescence quantum yields

Solutions were prepared for each measurement as described above from stock solutions of **1b** in MeCN (c = 1.0 mM). For the detection of fluorescence spectra, the excitation and emission slits were adjusted to 5 nm, and the excitation wavelengths were fixed to 415 nm. The relative fluorescence quantum yields of **1b** were determined under identical conditions (detection wavelength, excitation wavelength, detector voltage, slit bandwidths, collection rate). The quantum yield, Φ_{fl}, was determined according to equation 1.

$$\Phi_{\mathrm{fl,\,X}} = \frac{F_X A_S}{F_S A_X} \cdot \frac{n_X^2}{n_S^2} \cdot \Phi_{\mathrm{fl,\,S}} \qquad \text{(eq. 1)}$$

The indices X and S indicate the analyte (X) and standard (S) solution.

Φ = Emission quantum yield.

F = Integral of the emission curve.

A = Absorbance at the excitation wavelength.

n = Refraction index of the solution.

Measurements were performed with coumarin 153 in ethanol as standard (Φ_{fl} = 0.544).[4]

The estimated error is ca. 10% of the given values.

5. Calculation of theoretical absorption spectra

The optimized structure, transition energies, as well as the corresponding oscillator strengths were obtained from time dependent DFT calculations [O3LYP][5] with def2-TZVP as basis set for an aqueous solution of **4** (Figure S1, Table S1). Solvent properties were simulated with the polarized continuum model (PCM).[5] The calculations were performed with ORCA Software.[6] The input files for ORCA and plots of molecular orbitals were generated with Avogadro.[7]

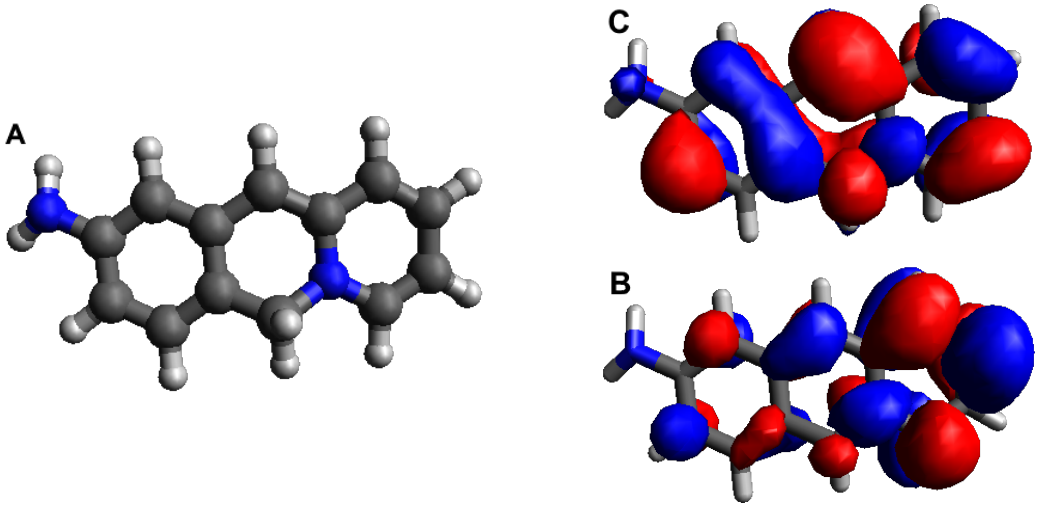


Figure S1. Optimized structure (A) and plots of the molecular orbitals of the HOMO (B) and LUMO (C) of compound **4**.

Table S1. Wavelength, λ and oscillator strength, f, of the first 10 electronic transitions of derivative **4**.

Transition	λ / nm	f
$S_0 \rightarrow S_1$	478	0.08
$S_0 \rightarrow S_2$	338	0.17
$S_0 \rightarrow S_3$	334	0.35
$S_0 \rightarrow S_4$	283	0.06
$S_0 \rightarrow S_5$	247	0.01
$S_0 \rightarrow S_6$	245	0.03
$S_0 \rightarrow S_7$	236	0.20
$S_0 \rightarrow S_8$	236	0.28
$S_0 \rightarrow S_9$	229	0.04
$S_0 \rightarrow S_{10}$	222	0.01

6. Spectrometric measurements

The absorption, emission and CD spectra were determined according to published procedures.[8,9]

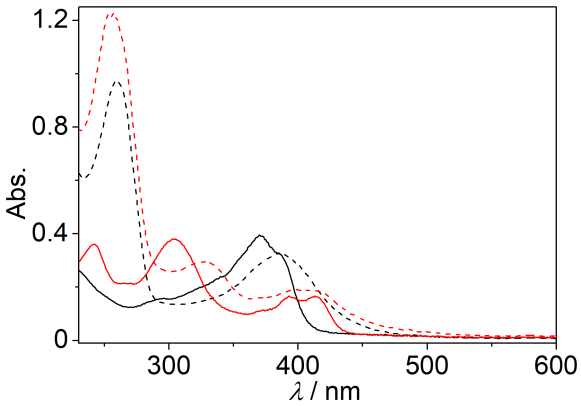


Figure S2. Absorption before (solid) and after (dashed) reaction of probe **1d** (black) and **2** (red, c = 10 µM) with nitroreductase (5 µg/ml) and NADH (50 µM) in PBS buffer (pH = 7, T = 37 °C) for 40 min; λ_{ex} = 400 nm.

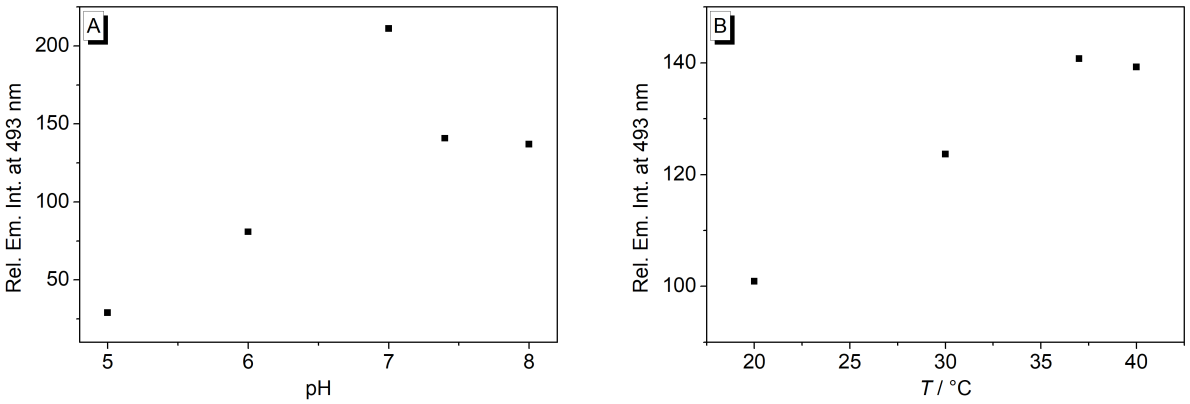


Figure S3. Relative emission intensity after incubation of **1b** (10 µM) with nitroreductase (5 µg/ml) and NADH (50 µM) for 40 min in PBS buffer at different pH (A, T = 37 °C) and different temperatures (B, pH = 7); λ_{ex} = 415 nm.

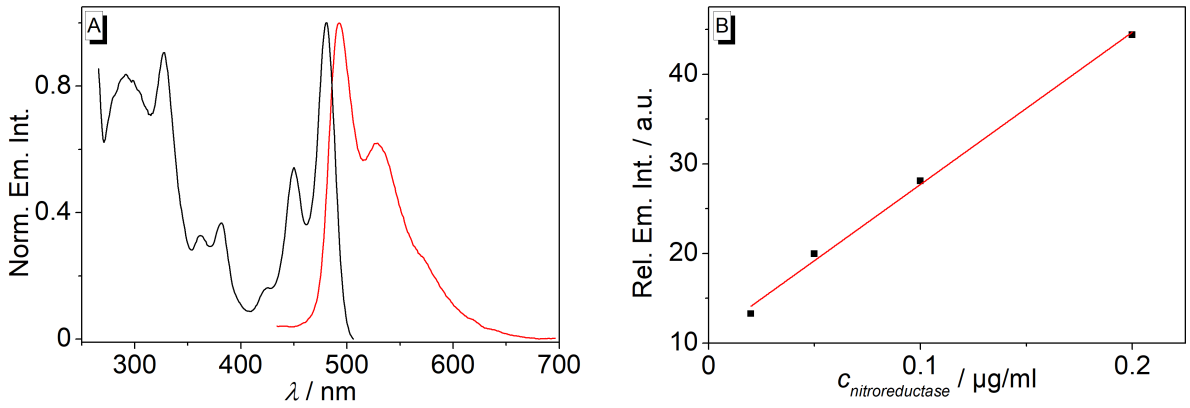


Figure S4. A: Normalized emission (red) and excitation spectrum (black) after incubation of probe **1b** (c = 10 µM) with nitroreductase (5 µg/ml) and NADH (50 µM) in PBS buffer (pH = 7, T = 37 °C) for 40 min; λ_{ex} = 415 nm, λ_{fl} = 520 nm. B: Plot of the fluorescence intensity at 490 nm versus nitroreductase concentration after incubation for 60 min. The red line represents the best fit to the theoretical model.

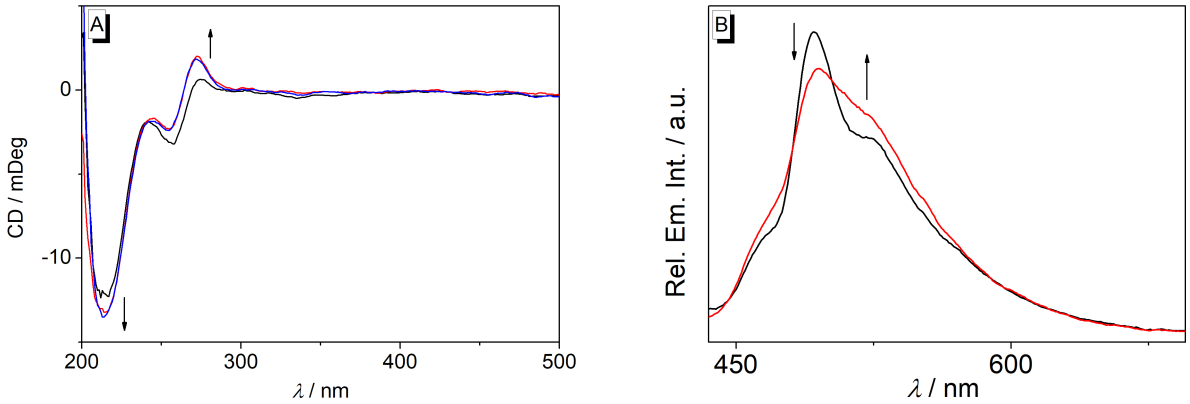


Figure S5. A: CD spectra after incubation of probe **1b** (c = 10 µM) with NADH (50 µM) and nitroreductase (5 µg/ml) for 0 min (black), 40 min (blue) and 80 min (red). B: Change of the emission after incubation of probe **1b** (c = 10 µM) with nitroreductase (5 µg/ml) and NADH (50 µM) in PBS buffer (pH = 7, T = 37 °C) for 40 min before (black) and after incubation with acetamide (100 µM) for 16 h (red); λ_{ex} = 415 nm. The arrows indicate changes of the absorption (A) and emission (B) with increasing reaction time.

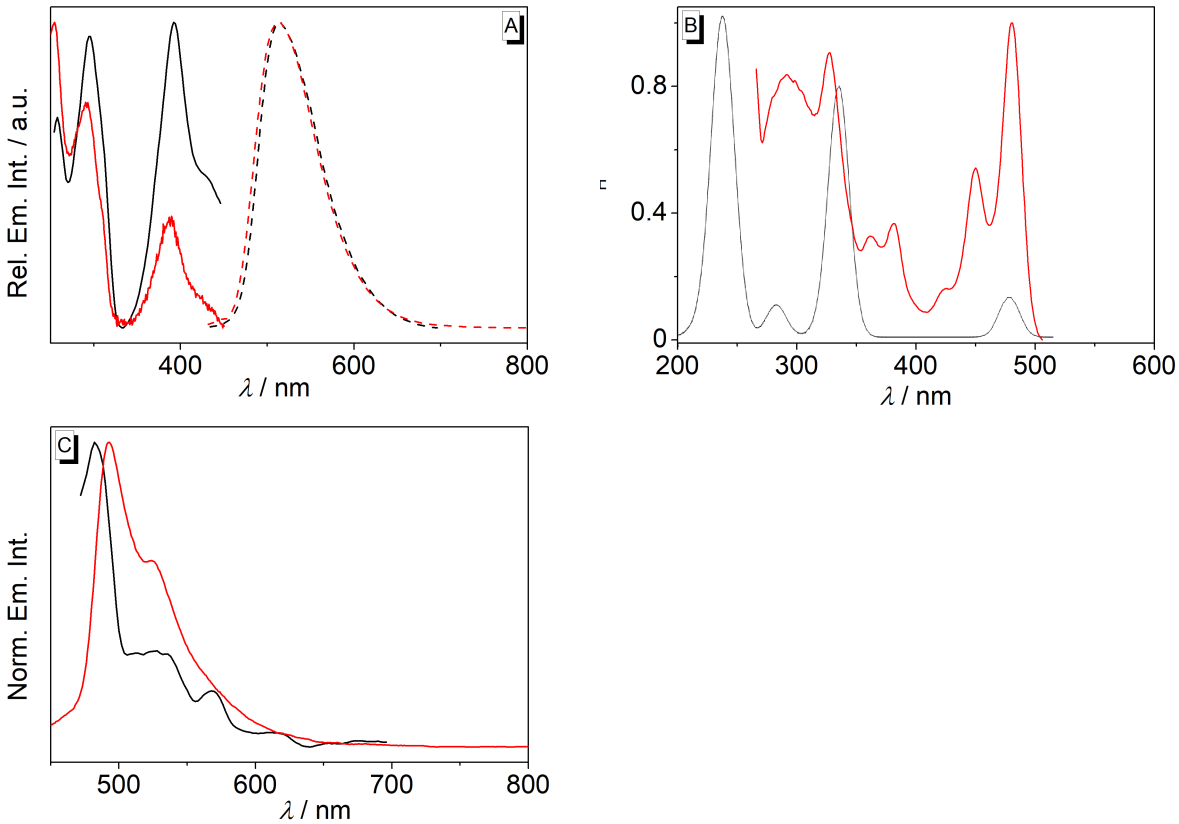


Figure S6. A: Normalized emission (dashed) and excitation spectrum (bold) of 9-aminobenzo-[*b*]quinolizinium (**1c**, black) and after incubation of probe **1b** (c = 10 µM) with nitroreductase (5 µg/ml) and NADH (50 µM) in PBS buffer (pH = 7, T = 37 °C) for 40 min and subsequent storage of the isolated product under aerobic conditions for one day; λ_{ex} = 415 nm, λ_{fl} = 515 nm. B: Excitation spectrum after reduction of probe **1b** (red, c = 10 µM) by nitroreductase and predicted absorption spectrum of the proposed product **4** (black); λ_{fl} = 520 nm. C: Emission spectrum after reduction of probe **1b** by nitroreductase (red) and $NaBH_4$ (black); λ_{ex} = 415 nm.

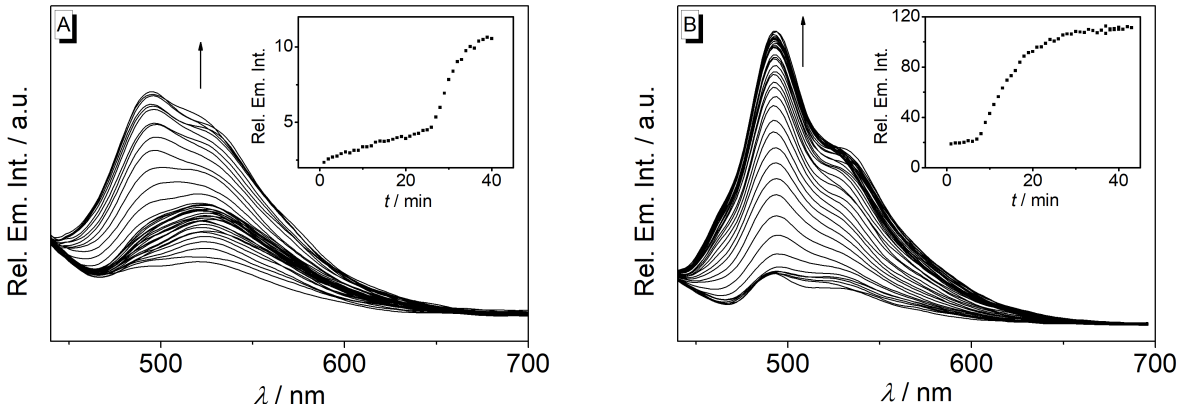


Figure S7. Change of the emission during the reaction of probe **1b** [$c_{\mathbf{1b}}$ = 2.5 µM (A), $c_{\mathbf{1b}}$ = 5 µM (B)] with nitroreductase (5 µg/ml) and NADH (50 µM) in PBS buffer (pH = 7, T = 37 °C); λ_{ex} = 415 nm. The arrows indicate the changes of emission with increasing reaction time. Inset: Plot of the probe emission at 493 nm *versus* reaction time.

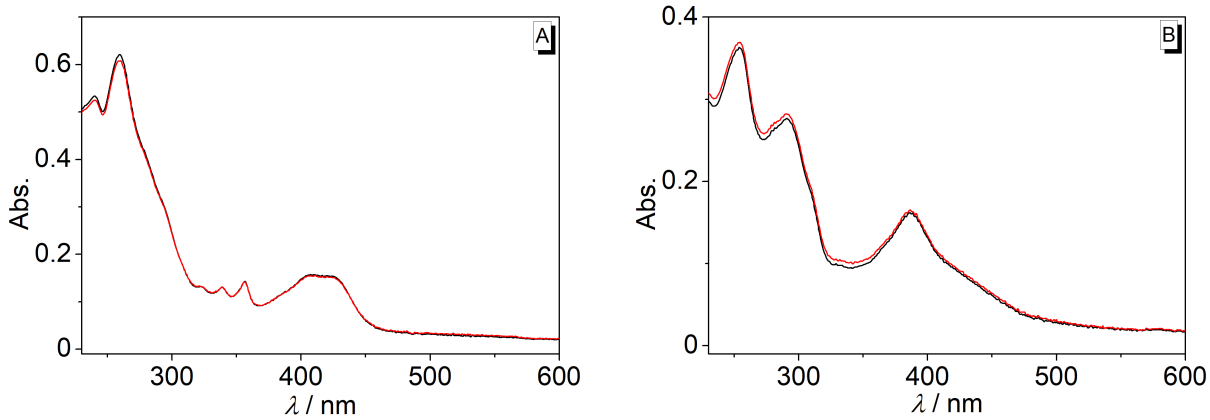


Figure S8. Absorption spectra of derivatives **1b** (A) and **1c** (B) in PBS buffer (pH = 7, *T* = 37 °C) before (black) and after (red) irradiation for 30 min with blue light (*λ* = 420 nm).

7. Fluorimetric analysis of NRT activity in live bacteria

The NTR activity was conducted according to the procedure described before by Brennecke et al.[10] As a test the nonpathogenic *Escherichia coli* W (ATCC9637) derivative Mach1™ (T1 Phage-resistant, chemical competent, purchased form Invitrogen, USA) was used.[11] *E. coli* colonies from Luria-Bertani (LB) agar plate were transferred to 5 ml LB for overnight cultures (16-20 h) at 37 °C and 200 rpm. The bacteria were harvested by centrifugation and the resulting pellet was washed by addition of 0.05 M Tris-HCl buffer (pH 7.4) with equal volume compared to the harvested culture. This washing step was repeated a second time and the bacterial pellet finally resuspended in less than half of the initial volume 0.05 M Tris-HCl buffer in order to adjust the optical density (*OD*) of the bacterial suspension to OD_{600} ≈ 2.0 (Figure S8A und C) or dilute the suspension further with Tris-HCl buffer to OD_{600} ≈ of 0.2 (Figure S8B and Fig 4). The adjusted bacterial suspension was distributed in transparent 6 well plates (Sarstedt, Nümbrecht, Germany) á 2 ml and in addition to untreated control conditions the probe **1b** (stock concentration 10 mM in DMSO) was diluted 500-fold to a final concentration of 20 µM in the bacterial culture and incubated for 4 h or 24 h at 120 rpm and 37 °C.

150 µl of these cultures have been transferred to 96 well plates (black, flat bottom, media binding, Sarstedt, Nümbrecht, Germany) for fluorimetric analysis with a multimode microplate reader (Tecan Safire, monochromator) at timepoints 0 h, 4 h, 24 h. 96well plates were sealed with transparent microplate sealing foil (AMPLISEAL, Greiner

Bio-One, Frickenhausen, Germany) and all experimental conditions were performed in technical replicates.

Pictures of fluorescence light-up of bacterial cultures incubated with 20 µM probe **1b** for 4 h in the 6 well plates (Fig 4A) have been documented via an in house built black box equipped with 365 nm LED lights.

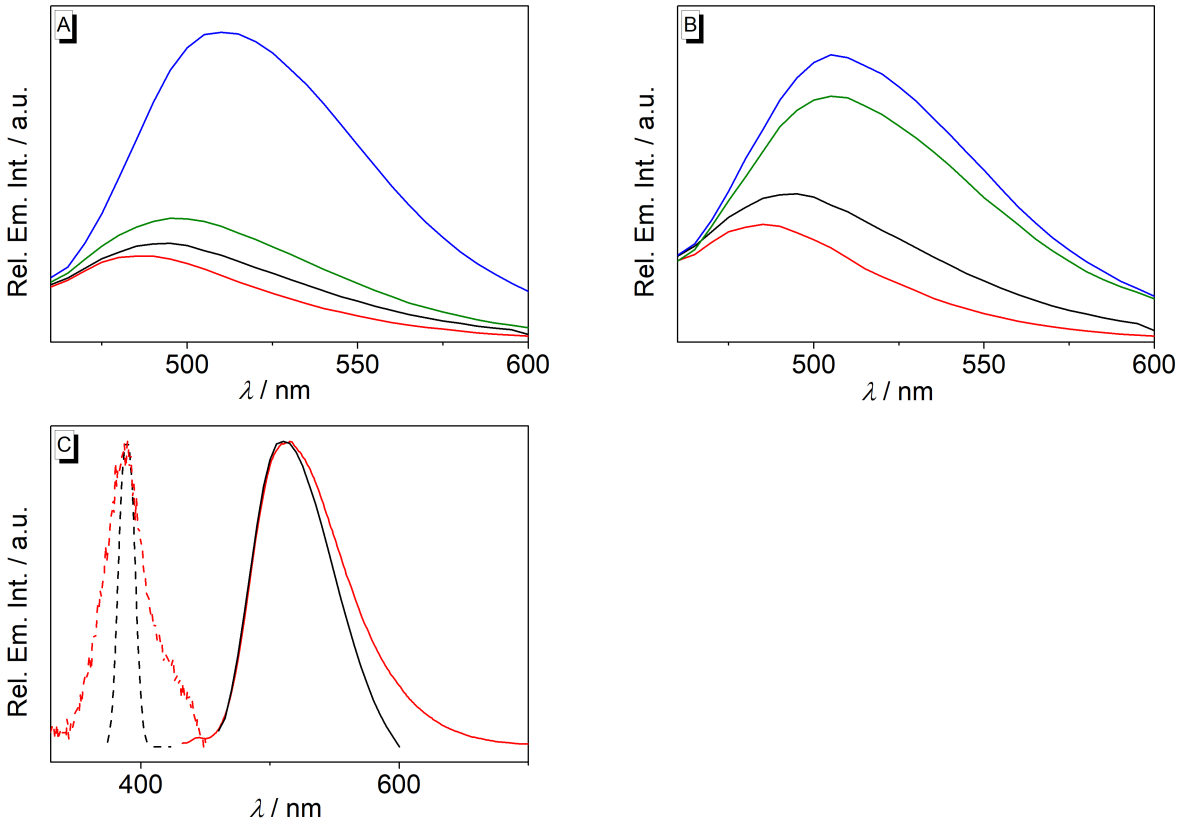


Figure S9. Relative emission intensity of *E. coli* cultures with an optical density of OD_{600} = 2 (A) and OD_{600} = 0.2 (B) before (red) and after incubation with **1b** (*c* = 20 µM) in Tris-HCl buffer for 4 h (green) and 24 h (blue) and the relative emission intensity of a pure solute of **1b** (, *c* = 20 µM) in Tris-HCl buffer (black); λ_{ex} = 399 nm. C: Normalized emission (bold) and excitation spectrum (dashed) of 9-aminobenzo[*b*]quinolizinium (**1c**, red; λ_{ex} = 415 nm, λ_{fl} = 515 nm) and after incubation of *Escherichia coli* cell cultures (OD600 = 2) with **1b** (*c* = 20 µM) in TRIS- buffer for 24 h (black; λ_{ex} = 399 nm, λ_{em} > 460 nm).

8. Toxicity testing

E. coli Mach1™ cultures were harvested as described in section 7 by centrifugation and adjusted to an OD_{600} ≈ of 0.2, re-suspended in 0.05 M Tris-HCl buffer (pH 7.4) with or without the probe **1b** (20 µM) and incubated 4 h or 24 h at 120 rpm and 37 °C. Subsequently, the bacteria suspensions were collected followed by the determination of the concentration of viable bacteria by CFU plate counting method. Suspensions were diluted serially up to 10^5 fold and 10^6 fold. Droplets of 50 µL were plated in duplicates onto LB agar plates and visible and colonies (CFUs) were counted after 24 h incubation at 37 °C.

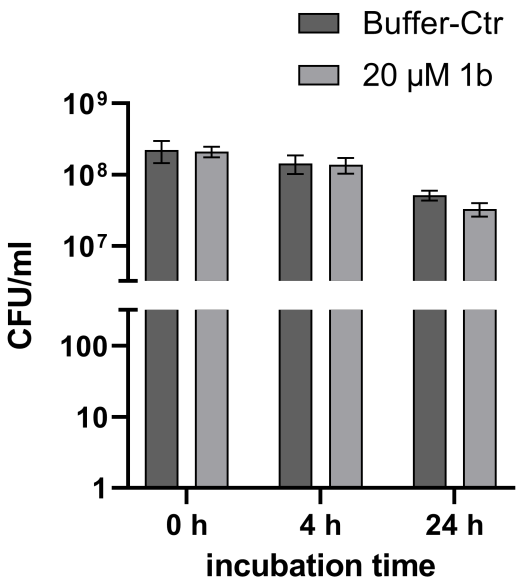


Figure S10. Determination of colony forming units (CFU) / ml as measure for the viability of *E. coli* cultures with an optical density of OD_{600} = 0.2 incubated in either Tris-HCl buffer (Buffer-Ctr) or probe **1b** (c = 20 µM) in Tris-HCl buffer at incubation starting time (0 h), 4 h or 24 h at 37 °C. Error bars indicate standard deviation (SD) of of the mean of at least 3, and max. 4 technical replicates of CFU counts.

8. NMR Spectra

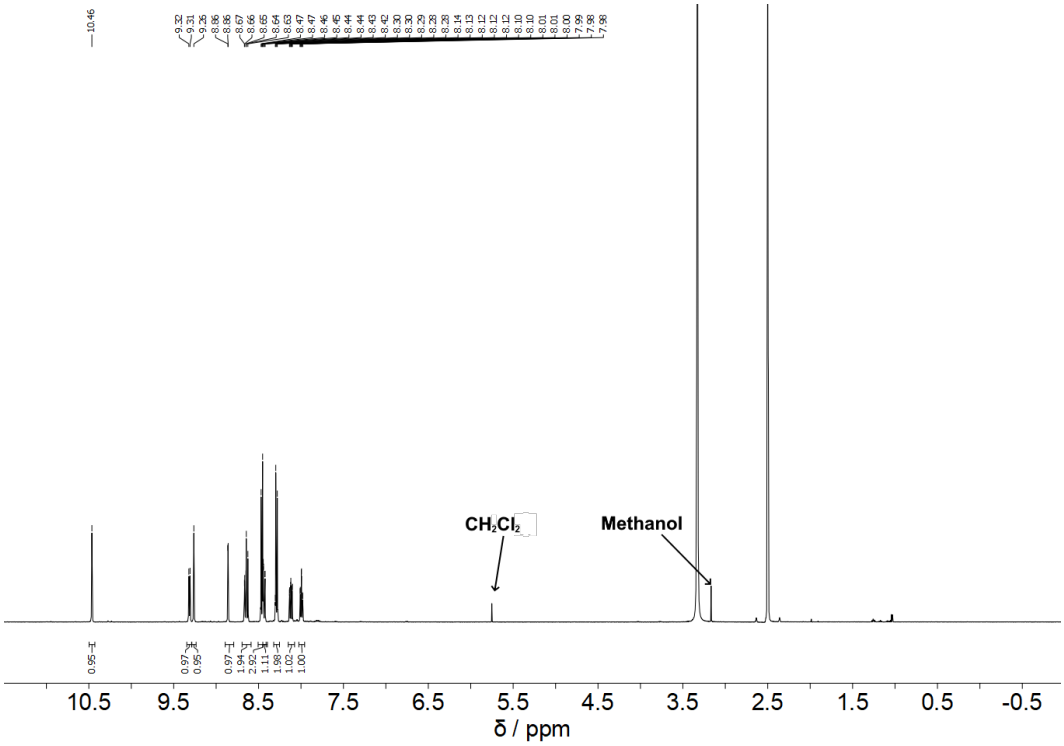


Figure S11. ^{1}H NMR spectrum (500 MHz) of derivative **1d** in DMSO-d_6.

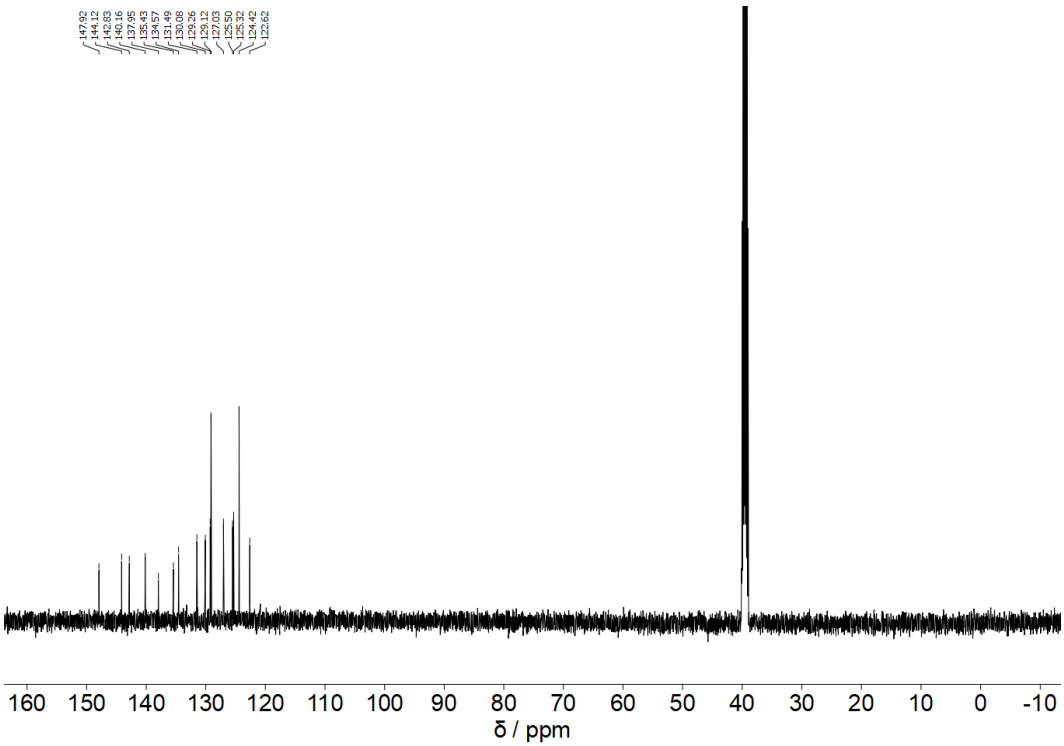


Figure S12. ^{13}C NMR spectrum (125 MHz) of derivative **1d** in DMSO-d_6.

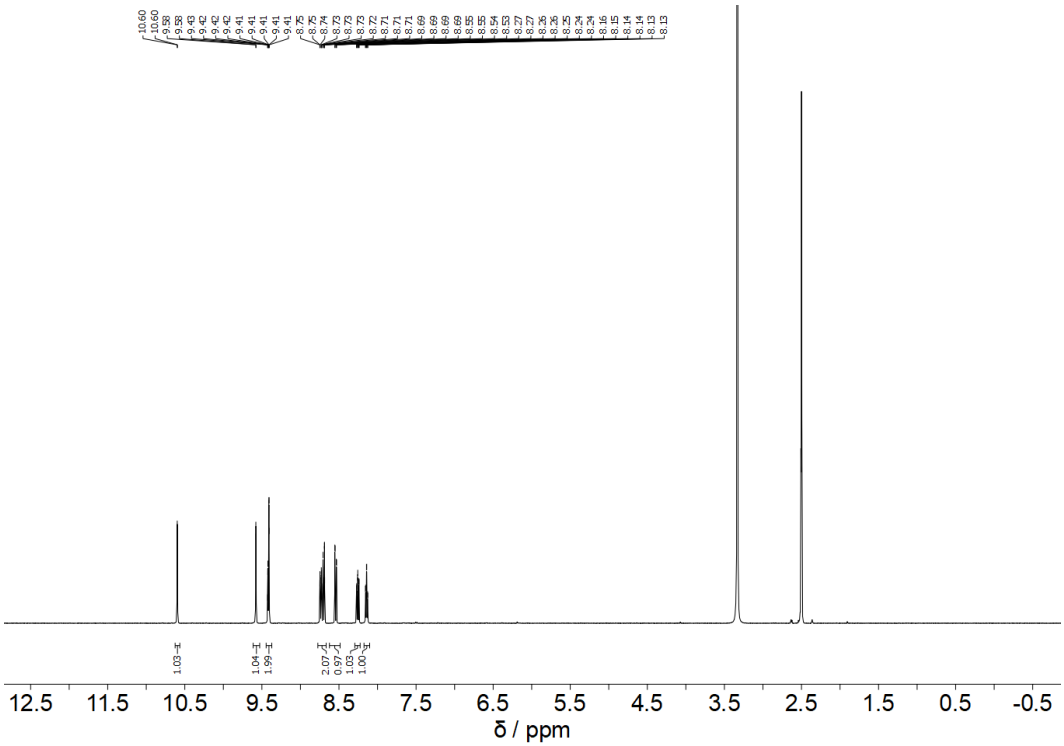


Figure S13. ^{1}H NMR spectrum (500 MHz) of derivative **1b** in DMSO-d_6.

9 References

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