Chemiluminescence Detection of Hydrogen Sulfide Release by β-Lactamase catalyzed β-Lactam Biodegradation: Unprecedented Pathway for Monitoring β-Lactam Antibiotic Bacterial Resistance

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1. General Information

All reactions requiring anhydrous conditions were performed under an Argon atmosphere. All reactions were carried out at room temperature unless stated otherwise. Chemicals and solvents were either A.R. grade or purified by standard techniques. Thin-layer chromatography (TLC): silica gel plates Merck 60 F254: compounds were visualized by irradiation with UV light. Column chromatography (FC): silica gel Merck 60 (particle size 0.040-0.063 mm), eluent given in parentheses. Reverse-phase high-pressure liquid chromatography (RP-HPLC): C18 5u, 250x4.6mm, eluent given in parentheses. Preparative RP-HPLC: C18 5u, 250x21mm, eluent given in parentheses. ¹H-NMR spectra were measured using Bruker Avance operated at 300, 400 and 600 MHz. ¹³C-NMR spectra were measured using Bruker Avance operated at 101 and 151 MHz. Chemical shifts were reported in ppm on the δ scale relative to a residual solvent (CDCl₃: δ = 7.26 for ¹H-NMR and 77.16 for ¹³C-NMR, DMSO-d₆: δ = 2.50 for ¹H-NMR and 39.52 for ¹³C-NMR). Mass spectra were measured on Waters Xevo TQD. Chemiluminescence was recorded on Molecular Devices Spectramax i3x and SpectraMax M5 plate reader. Fluorescence was recorded on Tecan infinite 200 Pro. All general reagents, including salts and solvents, were purchased from Sigma-Aldrich. Light irradiation for photochemical reactions: LED PAR38 lamp (19W, 3000K). β-Lactamase, Bacillus cereus 569/H9 (βLBC) was purchased from Merck Millipore; all other β-lactamases were obtained from Biosynth-Carbosynth.

Abbreviations

ACN - Acetonitrile, CDI - 1,1'-Carbonyldiimidazole, DCC - N,N'-Dicyclohexylcarbodiimide, DCM - dichloromethane, DIPEA - N,N'-Diisopropylethylamine, DMF - N,N'-Dimethylformamide, DMBA - Dimethylbarbituric acid, DMAP - 4-(Dimethylamino)-pyridine, DMSO - Dimethyl sulfoxide, EDC - 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide, EEDQ - N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, EtOAc - Ethylacetate, HBTU - 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyloxonium hexafluorophosphate, Hex- Hexane, TFA - Trifluoroacetic acid, TEA - Triethylamine, THF - Tetrahydrofuran, TMSCl - Trimethylsilyl chloride, PBS - Phosphate-buffered saline.
2. Synthetic Schemes and Experimental Procedures

Scheme 1. Synthesis of probe 1.

Compound 3:

Compound 1\(^1\) (50 mg, 0.13 mmol, 1 eq), compound 2\(^2\) (70 mg, 0.26 mmol, 2 eq) and 4-DMAP (33 mg, 0.26 mmol, 2 eq) were dissolved in DCM (4 mL). Then EDC (21 mg, 0.13 mmol, 1 eq) was added and the reaction mixture was stirred for 30 minutes at room temperature. After consumption of compound 1 (as indicated by TLC) the reaction was diluted with EtOAc (30 mL), washed with 1M HCl (10 mL), dried over Na\(_2\)SO\(_4\) and concentrated under reduced pressure. The crude mixture was purified using silica column chromatography to elute pure 3 as a white solid (39 mg, 47%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.50 (d, \(J = 4.2\) Hz, 1H), 8.42 (dd, \(J = 7.8, 1.3\) Hz, 1H), 7.99 (d, \(J = 8.1\) Hz, 1H), 7.82 (d, \(J = 16.1\) Hz, 1H), 7.57-7.67 (m, 4H), 7.38 (t, \(J = 7.3\) Hz, 1H), 7.29 (t, \(J = 8.1\) Hz, 1H), 7.14-7.11 (m, 1H), 6.52 (d, \(J = 15.9\) Hz, 1H), 3.36 (s, 3H), 3.29 (s, 1H), 2.16 (s, 1H), 2.00-1.72 (m, 12H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 170.4, 163.6, 158.9, 149.0, 146.4, 141.8, 139.3, 139.1, 138.6, 138.4, 134.5, 132.6, 130.0, 129.3, 129.0, 126.5, 126.4, 125.5, 125.3, 121.5, 121.0, 120.5, 57.6, 39.2, 38.8, 37.2, 33.1, 29.9, 28.3. MS (ES+): m/z calc. for C\(_{33}\)H\(_{30}\)ClNO\(_5\)S\(_2\): 619.1; found: 620.5 [M + 1]\(^+\).
Probe 1:

Enol-ether 3 (30 mg, 0.048 mmol, 1 eq) and catalytic amount (~2 mg) of methylene blue were dissolved in 10 mL of DCM. Oxygen was bubbled through the solution while irradiating with yellow light for 5 minutes. The reaction progress was monitored by RP-HPLC. Upon completion, solvent was removed and the crude mixture was then purified by preparative RP-HPLC [70-100% ACN in water (0.1 % TFA), 20 min] to afford probe 1 as white solid (26.8 mg, 85%). 1H NMR (400 MHz, DMSO-d6) δ 8.44-8.50 (m, 2H), 8.14 (d, J = 8.6 Hz, 1H), 8.00 (dd, J = 8.3, 1 Hz, 1H), 7.74-7.83 (m, 2H), 7.50-7.56 (m, 3H), 7.25-7.30 (m, 1H), 6.75 (d, J = 16 Hz, 1H), 3.15 (s, 3H), 2.88 (s, 1H), 2.16 (m, 1H), 1.95 (s, 1H), 1.30-1.78 (m, 12H). 13C NMR (101 MHz, DMSO-d6) δ 167.3, 163.7, 157.4, 150.5, 146.5, 141.4, 138.8, 135.9, 135.5, 134.7, 133.0, 131.5, 131.20, 130.23, 127.6, 126.9, 126.7, 126.4, 125.4, 124.9, 122.5, 120.4, 111.6, 96.1, 50.1, 36.4, 33.9, 33.7, 32.3, 31.7, 31.5, 26.1, 25.8. MS (ES+): m/z calc. for C33H30ClNO7S2: 651.1; found: 652.5 [M + 1]+.

Scheme 2. Synthesis of probe 2.
Compound 6:

![Chemical structure of Compound 6]

Aldehyde 4 (0.700 g, 2.1 mmol, 1 eq) and phosphonium salt 5 (1.160 g, 2.3 mmol, 1.1 eq) were dissolved in dry toluene (14 mL) under argon atmosphere. TEA (0.35 mL, 2.5 mmol, 1.2 eq) was added and the reaction mixture was stirred for 2 h at room temperature. Upon completion, the reaction mixture was diluted with Et₂O (25 mL) and the organic layer was washed with sat. NH₄Cl (20 mL). The separated aqueous layer was extracted one more time with Et₂O (25 mL). The combined organic layers were dried with MgSO₄, filtered and concentrated in vacuo. The crude product was purified using silica column chromatography to elute compound 6 as a white solid (0.700 g, 70%). ¹H NMR (300 MHz, CDCl₃) δ 7.93 (d, J = 16.2 Hz, 1H), 7.38 (d, J = 8.0 Hz, 1H), 6.86 (d, J = 8.0 Hz, 1H), 6.59 (d, J = 16.2 Hz, 1H), 6.27 (s, 1H), 4.32 (t, J = 8.4 Hz, 2H), 3.31 (s, J = 7.2 Hz, 3H), 3.27 (s, 1H), 2.12 (s, 1H), 1.96-1.58 (m, 12H), 1.08 (t, J = 8.4 Hz, 2H), 0.08 (s, 9H). ¹³C NMR (151 MHz, CDCl₃) δ 167.4, 150.7, 139.6, 138.8, 136.5, 132.9, 126.8, 123.7, 122.2, 121.6, 120.5, 62.9, 57.4, 39.2, 38.9, 37.2, 33.0, 29.9, 28.5, 17.5, -1.3. MS (ES+): m/z calc. for C₂₆H₃₅ClNO₄Si: 474.2; found: 475.2 [M + 1].

Compound 8:

![Chemical structure of Compound 8]

Compound 7 (340 mg, 1.1 mmol, 1.3 eq) and CDI (191 mg, 1.18 mmol, 1.4 eq) were dissolved in dry THF (8.4 mL) under argon atmosphere and stirred for 1 h at room temperature. Then, compound 6 (400 mg, 0.84 mmol, 1 eq) followed by DMAP (10 mg, 0.08 mmol, 0.1 eq) were added and the stirring was continued for an additional 21 h at room temperature. After completion, the solid was filtered off and washed with Et₂O (20 mL) and filtrate was concentrated under reduced pressure. The crude product was purified using silica column chromatography to elute compound 8 as white solid (0.264 g, 41%). ¹H NMR (300 MHz, CDCl₃) δ 8.50-8.40 (m, 2H), 8.12 (dd, J = 8.1, 0.7 Hz, 1H), 7.72 (d, J = 16.1 Hz, 1H), 7.63-7.53 (m, 4H), 7.43 (td, J = 7.8, 1.0 Hz, 1H), 7.28 (d, J = 8.3 Hz, 1H), 7.05 (ddd, J = 6.7, 4.9, 1.7 Hz, 1H), 6.50 (d, J = 16.0 Hz, 1H), 4.26 (t, J = 8.4 Hz, 2H), 3.36 (s, 3H), 3.29 (s, 1H), 2.17 (s, 1H), 1.97-1.58 (m, 12H), 1.00 (t, J = 8.4 Hz, 2H), 0.02 (s, J = 3.2 Hz, 9H). ¹³C NMR (151 MHz, CDCl₃) δ 166.5,
Compound 9:

![Diagram of Compound 9]

Compound 8 (80 mg, 0.1 mmol, 1 eq) was dissolved in DCM (10 mL). Methylene blue (3.3 mg, 0.01 mmol, 0.1 eq) was added to reaction mixture and the final blue solution was stirred under O₂ atmosphere (balloon) while irradiating with yellow light for 3 h. Upon completion, the crude mixture was eluted with Et₂O on a short silica gel column and the desired product 9 (82 mg, 98%) was obtained as a pale pink foam. ¹H NMR (600 MHz, CDCl₃) δ 8.49-8.41 (m, 2H), 8.14-8.09 (m, 2H), 7.77-7.69 (m, 2H), 7.61-7.55 (m, 3H), 7.43 (t, J = 7.3 Hz, 1H), 7.06 (ddd, J = 6.6, 4.9, 1.4 Hz, 1H), 6.56 (d, J = 16.0 Hz, 1H), 4.27 (t, J = 8.4 Hz, 2H), 3.26 (s, 3H), 3.02 (s, 1H), 2.24 (d, J = 2.2 Hz, 1H), 2.0 (s, 1H), 1.89-1.32 (m, 11H), 1.00 (t, J = 8.4 Hz, 2H), 0.01 (s, 9H). ¹³C NMR (151 MHz, CDCl₃) δ 166.3, 165.0, 158.4, 149.7, 139.6, 137.4, 136.3, 135.3, 134.8, 132.4, 131.2, 128.3, 126.7, 125.6, 125.3, 123.5, 121.9, 120.9, 111.7, 63.3, 50.0, 39.4, 36.7, 34.1, 33.8, 32.4, 31.8, 31.7, 29.8, 26.3, 25.9, 17.4, -1.3. MS (ES+): m/z calc. for C₃₈H₄₂ClNO₇SeSi: 799.1; found: 800.1 [M + 1]⁺.

Probe 2:

![Diagram of Probe 2]

Compound 9 (70 mg, 0.09 mmol, 1 eq) was dissolved in dry DCM (4 mL) and cooled to 0 °C. TFA (0.5 mL, 6.53 mmol) was added and the final solution was allowed to stay in fridge (4 °C) for 28 h (HPLC conversion-probe 2:9 = 70:30). The crude reaction mixture was concentrated and purified by preparative RP-HPLC (isocrat CH₃CN/30 mM NH₄HCO₃ = 6:4) to afford probe 2 as an off-white solid (28 mg, 46%). ¹H NMR (600 MHz, CD₃OD) δ 8.50 (d, J = 7.6 Hz, 1H), 8.36 (d, J = 4.0 Hz, 1H), 8.07 (t, J = 8.9
Hz, 2H), 7.91 (d, \( J = 8.4 \) Hz, 1H), 7.71-7.60 (m, 3H), 7.55-7.47 (m, 2H), 7.16 (dd, \( J = 6.7, 5.1 \) Hz, 1H), 6.68 (d, \( J = 16.0 \) Hz, 1H), 3.22 (s, 3H), 2.95 (s, 1H), 2.29 (d, \( J = 12.1 \) Hz, 1H), 2.03 (s, 1H), 1.89-1.37 (m, 12H). \(^{13}\)C NMR (151 MHz, CD\(_3\)OD) \( \delta \) 171.5, 165.1, 157.7, 148.9, 138.3, 138.0, 134.5, 133.8, 132.4, 132.3, 131.9, 130.6, 127.3, 126.6, 126.2, 125.0, 122.0, 121.1, 111.3, 48.6, 33.6, 33.6, 31.8, 31.5, 31.3, 26.3, 25.9. MS (ES+): m/z calc. for C\(_{33}\)H\(_{30}\)ClNO\(_7\)SSe: 699.1; found: 700.1 [M + 1]+.

Scheme 3. Synthesis of probe 3.

Compound 12:

Compound 10\(^3\) (200 mg, 0.48 mmol, 1 eq) was dissolved in DMF (2 mL) under argon. K\(_2\)CO\(_3\) (0.100 mg, 0.724 mmol, 1.5) was added followed by compound 11\(^6\) (228 mg, 0.531 mmol, 1.1 eq) and the final solution was heated to 50 °C for 5 h. After starting material (compound 10) was consumed as indicated by TLC, the reaction was diluted with EtOAc (30 mL), washed with water (20 mL), dried over Na\(_2\)SO\(_4\) and concentrated under reduced pressure. The crude product was then purified using silica column chromatography to elute 12 as a white solid (0.324 g, 88%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.45 (d, \( J = 2.2 \) Hz, 1H), 8.36 (dd, \( J = 8.7, 2.2 \) Hz, 1H), 7.94 (d, \( J = 16.2 \) Hz,
1H), 7.74 (d, J = 8.7 Hz, 1H), 7.50 (dd, J = 14.3, 8.2 Hz, 3H), 7.31-7.23 (m, 2H), 7.11 (d, J = 8.0 Hz, 1H), 6.50 (d, J = 16.1 Hz, 1H), 6.07-5.89 (m, 1H), 5.46-5.23 (m, 2H), 5.00 (s, 2H), 4.71 (dt, J = 5.7, 1.4 Hz, 2H), 3.45 (s, 3H), 3.33 (s, 3H), 3.28 (s, 1H), 2.07 (d, J = 17.6 Hz, 1H), 2.05-1.57 (m, 12H). 13C NMR (101 MHz, CDCl3) δ 166.4, 153.8, 149.9, 148.5, 139.8, 139.5, 138.9, 138.6, 137.0, 136.8, 133.8, 133.0, 132.3, 132.9, 129.7, 129.5, 128.2, 127.9, 125.8, 125.3, 120.3, 119.5, 118.5, 75.2, 65.5, 57.5, 40.0, 39.3, 39.2, 38.8, 37.2, 33.1, 29.9, 28.5, 28.3. MS (ES+): m/z calc. for C38H38ClN3O10S: 763.2; found: 764.6 [M + 1]+.

**Compound 13:**

A mixture of compound **12** (50 mg, 0.066 mmol, 1 eq), DMBA (31 mg, 0.196 mmol, 3 eq) and Pd(PPh3)4 (8 mg, 0.006 mmol, 0.1 eq) in DCM (2 mL) was stirred at 50 °C for 3 hours. Upon completion, the reaction mixture was concentrated under reduced pressure and crude mixture was purified by preparative RP-HPLC (50-100% ACN in water, 20 min) to afford compound **13** (38 mg, 79%) as a white solid. 1H NMR (400 MHz, CDCl3) δ 8.44 (d, J = 2.2 Hz, 1H), 8.33 (dd, J = 8.7, 2.2 Hz, 1H), 7.98 (d, J = 16.1 Hz, 1H), 7.74 (d, J = 8.7 Hz, 1H), 7.51 (dd, J = 8.2, 3.7 Hz, 3H), 7.27 (d, J = 8.4 Hz, 2H), 7.14 (d, J = 8.0 Hz, 1H), 6.47 (d, J = 16.1 Hz, 1H), 5.43 (s, 1H), 5.02 (s, 2H), 3.44 (s, 3H), 3.35 (s, 3H), 3.29 (s, 1H), 2.09 (s, 1H), 2.01-1.57 (m, 12H). 13C NMR (101 MHz, CDCl3) δ 171.6, 154.0, 149.9, 148.5, 141.1, 139.8, 139.4, 139.1, 136.9, 136.8, 133.7, 133.3, 129.8, 129.2, 128.3, 127.8, 125.7, 125.6, 119.5, 119.5, 75.3, 57.5, 40.0, 39.2, 38.8, 37.1, 33.1, 29.9, 28.3. MS (ES+): m/z calc. for C38H34ClN3O10S: 723.2; found: 724.5 [M+H]+.
**Probe 3:**

Enol-ether 13 (30 mg, 0.041 mmol, 1 eq) and catalytic amount of methylene blue (~3 mg) were dissolved in 8 mL of DCM. Oxygen was bubbled through the solution while irradiating with yellow light for 5 minutes. The reaction was monitored by RP-HPLC. After completion, the reaction mixture was concentrated by evaporation under reduced pressure. The crude product was purified by preparative RP-HPLC [70-100% ACN in water (0.1 % TFA), 20 min] to afford probe 3 as a white solid (26.3 mg, 85%).<ref>

**Probe 4:**

Compound 14 (50 mg, 0.308 mmol, 1 eq), compound 7 (70 mg, 0.617 mmol, 2 eq) and 4-DMAP (75 mg, 0.0.617 mmol, 2 eq) were dissolved in DCM (4 mL). Then EDC (48 mg, 0.308 mmol, 1 eq) was added and the reaction mixture was stirred for 60 minutes at room temperature. After consumption of compound 14 (as indicated by TLC) the reaction was diluted with EtOAc (30 mL), washed with 1M HCl (10 mL), dried over Na₂SO₄ and concentrated under reduced pressure. The crude mixture was purified using silica column chromatography to elute pure probe 4 as a white solid (108 mg, 77%).

**1H NMR (400 MHz, CDCl₃) δ 8.48 – 8.43 (m, 1H), 8.34 (dd, J = 7.8, 1.4 Hz, 1H), 8.14 – 8.02 (m, 1H), 7.46 (dd, J = 7.8, 1.4 Hz, 1H), 7.39 (dd, J = 7.8, 1.4 Hz, 1H), 7.17 (dd, J = 7.8, 1.4 Hz, 1H), 6.95 (dd, J = 7.8, 1.4 Hz, 1H), 6.70 (dd, J = 7.8, 1.4 Hz, 1H), 5.80 (dd, J = 7.8, 1.4 Hz, 1H).**

**13C NMR (101 MHz, CDCl₃) δ 161.8, 156.1, 151.3, 148.5, 146.0, 137.1, 136.8, 134.8, 133.0, 132.0, 130.3, 127.8, 127.3, 126.7, 123.6, 120.7, 111.8, 96.0, 75.5, 50.0, 36.5, 33.9, 33.7, 32.4, 32.2, 31.7, 26.1, 25.8.** MS (ES+): m/z calc. for C₃₅H₃₄ClN₃O₁₂S: 755.2; found: 756.5 [M+H]+.
(d, J = 8.2 Hz, 1H), 7.74 (d, J = 9.6 Hz, 1H), 7.62 – 7.52 (m, 4H), 7.45 – 7.37 (m, 1H),
7.30 – 7.21 (m, 2H), 7.07 (ddd, J = 6.7, 4.9, 1.8 Hz, 1H), 6.44 (d, J = 9.6 Hz, 1H). 13C
NMR (101 MHz, CDCl3) δ 165.7, 160.3, 158.3, 154.9, 153.1, 149.6, 142.9, 139.4,
137.5, 134.7, 132.2, 128.9, 128.2, 126.6, 126.1, 122.1, 121.0, 118.6, 117.2, 116.6,
110.7. MS (ES+): m/z calc. for C21H13N3O4SSe: 455.0; found: 456.3 [M+H]+.
3. Spectroscopic Data:

3a. Evaluation of the selectivity of probes 1-3 towards H₂S and RSH.

In this assay, we studied the detection selectivity of probes 1-3 towards H₂S and RSH. Both probe 1 and 2 presented high selectivity for detection H₂S in comparison to RSH, while probe 3 presented much lower selectivity (Figure S1).

**Figure S1:** Chemiluminescence kinetic profiles (Up, over 60 min), total light emission (down left, over 4 min) and signal-to-noise (down right, over 4 min) of probes 1-3 [10 μM] in PBS (pH 7.4), in the presence and absence of L-cysteine-hydrochloride and NaSH [100 μM] (The probes were pre-incubated for 30 minutes at 27 °C in PBS (pH 7.4) prior to use).
3b. Determination of the Limit of Detection (LOD) of probe 1 for detection of H$_2$S:

In this measurement, the LOD of probe 1 was determined using various concentrations of NaSH. (LOD = 2.3 × 10$^{-7}$ M or 0.0128 µg/mL).

**Figure S2:** Signal/noise ratio values for **probe 1** [10 µM], 10% DMSO in PBS (pH 7.4) plotted against different NaSH concentrations.
3c. Detection of H\textsubscript{2}S-released from antibiotics degradation by βLBC.

**General assay protocol:**

All antibiotics stocks solutions were prepared using DMSO or water according to their solubility. All enzyme stocks solutions were prepared according to their manufacturer solubility protocol. Two-step protocol was used to detect H\textsubscript{2}S released from antibiotics [1 mM] after incubation with enzyme [βLBC = 10 U/mL and all others 2 U/mL] using a chemiluminescent probes 1-3 [25 µM].

**Two-step protocol:**

**For 25 µM assay (final volume of assay 0.250 mL):**

1. 0.175 mL PBS (pH 7.4) + 5 µL antibiotic stock 50 mM in DMSO (final conc. 1 mM) + 20 µL βLBC [100 U/mL] stock solution in PBS (final conc. 10 U/mL). [For other enzymes; 0.193 mL PBS + 5 µL antibiotic stock 50 mM in DMSO (final conc. 1 mM), 2 µL enzyme [200 U/mL] stock solution in PBS (final conc. 2 U/mL)].

Negative controls (background): 0.195 mL PBS + 5 µL antibiotic stock of 50 mM in DMSO (final conc. 1 mM).

Incubation of plate for 30 min at room temperature.

2. Addition of 50 µL dioxetane probe 1 working solution [Pre-incubated for 45 min, 0.750 mL PBS + 50 µL of 2 mM probe (final conc. 25 µM)]. The luminescence was measured every minute for 2 h with SpectraMax i3x plate reader at 27 °C.

Results of the antibiotics with probe 1, producing a high signal-to-noise ratio shown in the manuscript (Figure 4). Whereas antibiotics, which are producing a low signal-to-noise ratio are presented below (Figure S3):
Figure S3: Chemiluminescence kinetic profiles [Left] and total light emission [Right] (over 2 h) produced by probe 1 [25 μM] in PBS (pH 7.4) with four different antibiotics [1 mM] in the presence and absence of βLBC [10 U/mL]. Probe 1 was pre-incubated for 45 min in PBS (pH 7.4) prior to use; antibiotics and βLBC were incubated for 30 min prior to measurement.
3d. Formation of byproduct A upon biodegradation of probe 1 in the presence of ceftizoxime or cefalexin with βLBC

In this experiment, RP-HPLC chromatograms showing gradual formation of byproduct A upon decomposing of probe 1 [100 µM] in the presence of Cefalexin [3 mM] and βLBC [20 U/mL] (Figure S4). The formation of byproduct A and Benzoate were confirmed by LCMS and their authentic samples by cross injection in HPLC. The same byproduct A detection experiment with ceftizoxime explained in manuscript (Figure 6).

Figure S4: RP-HPLC chromatograms showing gradual formation of byproduct A upon decomposing of probe 1 [100 µM] in the presence of Cefalexin [3 mM] and βLBC [20 U/mL]. HPLC elution gradient ACN and water with 0.1 TFA (30-100%).
3e. General procedure for the data given in Figure 8 (Detection of antibiotic resistant bacteria using probe 1).

Eight antibiotic resistant bacterial strains were inoculated from agar plates into culture tubes with nutrient broth (5 g/L peptone, 5 g/L NaCl; 2 g/L yeast extract, 1 g/L meat extract, pH 7.4) and incubated for 19 h at 37 °C and 150 rpm. Broth cultures were diluted to an optical density (600 nm) of 0.1 with sterile saline (0.9% NaCl). In wells of a white 96-well plate, 0.1 mL culture samples were mixed with 90 µL PBS and 10 µL Ceftizoxime or sulopenem stock solution (25 mM, final concentration 1 mM) and incubated for 30 min at room temperature. Then, 50 µL PBS with 0.125 mM of probe 1 (5x working solution, pre-incubated 1 h at room temperature) was added and luminescence (relative light units, RLU) was measured for 1 h at room temperature with a plate reader (500 ms integration time). Results of assay explained in the manuscript (Figure 8).
3f. Detection of carbapenem-resistant bacteria with Faropenem and probe 3.

Two imipenem (a carbapenem) resistant bacterial strains, *Pseudomonas aeruginosa* RKI 48/09 (VIM-15) and *Klebsiella pneumonia* RKI 92/08 (KPC-2), and a cephalosporinase-positive strain, *Escherichia coli* RKI 66/09 (CMY-2, AmpC), were inoculated from agar plates into culture tubes with nutrient broth and incubated for 22 h at 37°C and 150 rpm. In parallel, the two carbapenemase-positive strains were cultivated in nutrient broth supplemented with 8 and 4 mg/L imipenem (Imp), respectively. In addition, antibiotic sensitive strains *Pseudomonas aeruginosa* ATCC10145, *Klebsiella pneumoniae* RKI 2867/81 and *Escherichia coli* ATCC 25922 were similarly cultivated in nutrient broth. Broth cultures were diluted to an optical density (600 nm) of 0.5 with sterile saline (0.9% NaCl). In wells of a white 96-well plate, 0.19 ml cell suspension samples were mixed with 10 µL Faropenem stock solution (20 mM in dimethyl sulfoxide, final concentration 0.8 mM) and 50 µL 5x working solution of probe 3 (0.125 mM in PBS, final concentration 25 µM). Luminescence was measured for 1 h at room temperature in a plate reader (500 ms integration time). Results are shown in Figure S5.

![Figure S5](image.png)

**Figure S5:** Integrated luminescence signals and signal-to-background (s/b) for antibiotic resistant bacteria and sensitive reference strains after addition of Faropenem [0.8 µM] and probe 3 [25 µM]. Background: antibiotic sensitive reference strain of the same species. Orange color bars: Assay 1, blue color bars: Assay 2.
3g. General procedure for the data given in Figure 9 (one-step assay for antibiotic resistant bacteria with probe 1 and inhibition effect-obtained by 3-Aminophenylboronic acid).

Two strains expressing serine-type beta-lactamases, *Klebsiella pneumonia* RKI 92/08 (KPC-2) and *Escherichia coli* RKI 66/09 (CMY-2, AmpC) and two antibiotic sensitive reference strains, *Klebsiella pneumonia* RKI 2867/81 and *Escherichia coli* ATCC 25922 were inoculated from agar plates into culture tubes with nutrient broth and incubated for 19 h at 37°C and 150 rpm. Broth cultures were diluted to an optical density (600 nm) of 1.0 with sterile saline (0.9% NaCl). In wells of a white 96-well plate 90 µL PBS with 50 µM of 0.125 mM probe 1 (pre-incubated in PBS for 1 h at room temperature, final concentration 25 µM) was mixed with 100 µL cell suspension OD600 1.0 (final OD600 0.4) or sterile medium and 10 µL of 25 mM Cefazolin (final concentration 1 mM). In addition, 7 mM (final concentration) of the known serine beta-lactamase inhibitor 3-aminophenylboronic acid was added to a subset of wells. Immediately after all reagents had been added, luminescence (relative light units, RLU) was measured at room temperature for 15 min every min with a plate reader (500 ms integration time). Results are shown in the manuscript (Figure 9).
3h. Determination of the Limit of Detection (LOD) of probe 1 for the detection of antibiotic resistant bacteria using Ceftizoxime:

Strains cultivated in nutrient broth overnight at 37°C, cell density set to 10⁹ CFU/mL by optical density, dilution series prepared in sterile saline. Assay in white 96-well plate: 0.1 mL cell suspension mixed with 0.1 mL PBS with 2.5 mM Ceftizoxime and 2.5% DMF (Final Ceftizoxime concentration: 1.25 mM) background control wells: 0.1 mL cell suspension mixed with 0.1 mL PBS with 2.5% DMF. 96-well plate incubated for 30 min at room temperature. 0.05 mL PBS with 0.125 mM AquaSpark S-S Probe added per well (final probe concentration 25 µM) Luminescence measured for 30 min. Limit of Detection (LOD) = 3.1⁶ cells of antibiotic resistant bacteria.

![Figure S6: Integrated luminescence signals and signal-to-background (s/b) for antibiotic resistant bacteria and sensitive reference strain [1.0⁹ to 3.1⁶ CFU/mL] after addition of Ceftizoxime [1.23 mM] and probe 3 [25 µM]. Background: antibiotic sensitive reference strain of the same species.](image)
3i. Determination of the relative rate constant of the reaction of hydrogen sulfide release by β-lactamase-catalyzed β-lactam biodegradation of various antibiotics.

The order of reaction is pseudo zero. Because the concentration of antibiotics [1 mM] used in the experiment is considerably high in compare with the concentration of probe 4 [25 µM], the order of the reaction could be considered pseudo zero order. The constants below were calculated from the fluorescent probe activation assay after the conversion of RLU measured to concentration of H₂S emitted. In pseudo zero order reaction the slope of such graph would represent the rate constant of the reaction.

Equation

\[ R = K \text{ [antibiotics concentration]}^0 \]

**Figure S7:** Correlation between H₂S release vs time upon incubation with probe 4 [25 µM] in PBS, pH 7.4, and antibiotics [1 mM] in the presence and absence of enzyme (Beta-lactamase from *Bacillus cereus*) [10 U/mL]. Antibiotics and βLBC were incubated for 30 min prior to measurement.
| Sr. No. | Antibiotics    | Rate constant (K) |
|---------|----------------|-------------------|
| 1       | Ceftizoxime    | $1.1 \times 10^{-3}$ µM/s |
| 2       | Cefalexin      | $6.0 \times 10^{-4}$ µM/s |
| 3       | Faropenem      | $4.0 \times 10^{-4}$ µM/s |

**Table S1**: Rate constant of the reaction of hydrogen sulfide release by β-lactamase-catalyzed β-lactam biodegradation of various antibiotics.
4. NMR and MS Spectra:

$^1$H-NMR and $^{13}$C-NMR spectra of compound 3:
MS of compound 3
$^1$H-NMR and $^{13}$C-NMR spectra of probe 1:
MS of probe 1:
$^{1}$H-NMR and $^{13}$C-NMR spectra of compound 6:
MS of compound 6
$^1$H-NMR and $^{13}$C-NMR spectra of compound 8:
MS of compound 8:

![Mass Spectrogram](image_url)

Exact Mass: 767.1

| m/z       | Charge | Relative Abundance |
|-----------|--------|--------------------|
| 768.14962 | z=1    |                    |
| 144.98242 | z=1    |                    |
| 124.08696 | z=1    |                    |
| 221.03269 | z=1    |                    |
| 163.09708 | z=1    |                    |
| 325.97526 | z=2    |                    |
| 391.28462 | z=1    |                    |
| 519.13957 | z=1    |                    |
| 536.16629 | z=1    |                    |
| 519.13957 | z=1    |                    |
| 684.20409 | z=1    |                    |
| 730.07101 | z=1    |                    |
| 740.11813 | z=2    |                    |
| 768.14962 | z=1    |                    |
| 830.07101 | z=1    |                    |
| 934.26470 | z=1    |                    |
| 850.25277 | z=1    |                    |
$^1$H-NMR and $^{13}$C-NMR spectra of compound 9:
MS of compound 9:

![MS spectrum of compound 9](image-url)

- Formula: AuTM-445
- RT: 0.24-0.28
- AV: 3
- NL: 1.61E7
- T: FTMS + p ESI Full ms [80.00-950.00]

Exact Mass: 799.1
$^1$H-NMR and $^{13}$C-NMR spectra of **probe 2:**
MS of probe 2:
\(^1\)H-NMR and \(^{13}\)C-NMR spectra of compound 12:
MS of compound 12:
$^{1}$H-NMR and $^{13}$C-NMR spectra of compound 13:
MS of compound 13:
\(^1\)H-NMR and \(^{13}\)C-NMR spectra of **probe 3:**
MS of probe 3:
$^1\text{H}-\text{NMR}$ and $^{13}\text{C}-\text{NMR}$ spectra of probe 4:
MS of probe 4:
5. References:

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6. APPENDIX

Explanation why 1st group triggers the probe
Figure 1 Established position numbering for cephems
Explanation why 2\textsuperscript{nd} group does not trigger the probe

Known to be stable at pH 7
valid for all cepham with good leaving group at methylene in 3-position

Easy to detect

Poor messenger
Thiocarboxylic acid do not trigger RSH probes

Very stable structure free of acetal-like functionality
LCMS method description

All samples were measured on Agilent 1260 system with single quadrupole MSD featured with multimode (ESI+APCI) ionization chamber. The ionization chamber parameters were as follows:

a) positive mode scan signal: ionization mode MM-ES+APCI; fragmentor ramp enabled; percent cycle time 100.00 %; mass range 40-900 m/z; fragmentor variable with ramp: mass 121.05 value 30, mass 922.01 value 150; sheath gas temperature 200°C; vaporizer temperature 180°C, gas flow 7L/min; nebulizer pressure 3105 Torr; quadrupole temperature 100°C, capillary voltage 3000V; charge voltage 1500V; corona current 3 µA.

b) negative mode scan signal: ionization mode MM-ES+APCI; fragmentor ramp enabled; percent cycle time 100.00 %; mass range 40-900 m/z; fragmentor variable with ramp: mass 121.05 value 100, mass 922.01 value 300; sheath gas temperature 200°C; vaporizer temperature 180°C, gas flow 7L/min; nebulizer pressure 3105 Torr; quadrupole temperature 100°C, capillary voltage 4000V; charge voltage 2000V; corona current 40 µA.

The HPLC part of the device comprised a quadruple gradient pump, an headspace autosampler, Phenomenex Luna C8 5µm, 4.6x150 mm column and a DAD detector at 254 nm. The acidic elution method was a combination of 7 min gradient from 5% to 100% MeOH in 0.1% formic acid and subsequent 8min elution with pure MeOH. The acidic elution was used for both polarities as the beta-lactams open spontaneously under basic conditions.

Sample preparation for the LCMS assay

All antibiotics were purchased from commercial suppliers (sulopenem, cetazidime, imipenem, ceftizoxime from Biosynth-Carbosynth EN, the rest from Merck-Aldrich). A 10 mM stock solutions of inspected antibiotics in PBS (prepared by dissolving Merck-Aldrich P3813-1PAK in RODI water and set to 1L total volume) were made. Beta lactamase enzyme (Bacillus cereus 569/H9 from Aldrich, 426205-500U) stock solution was prepared by dissolving 1mg of dry enzyme in PBS and set by PBS to total volume of 1mL. Every LCMS sample was made by mixing 0.25 mL of antibiotic stock solution with 0.25mL of lactamase stock solution in a 2mL head space vial and incubation at RT (45min on linear shaker, 60 shakes per minute). 50 µL of the resulting solution was directly injected to LCMS after a period specified later. Stock solutions of ATB were directly injected in 10 µL loads to acquire data of starting material standards.

Faropenem decay promoted by B. Cereus metallo-lactamase, assayed by LCMS

The analysis with negative ionization was captured directly after the incubation period (injected after 45 min of incubation), positive ionization experiment started after 90 min of incubation and negative ionization experiment was repeated with identical result after 110 min of incubation.
Faropenem had hydrolysed completely during 45 min, furnishing 2 major peaks eluting at 4.08 and 4.47 min, 2 minor UV active peaks at 3.45 and 5.25 min (having only positive MS signal) and one large positive peak at 2.54 min with no UV absorption, which belongs to 2-amino-2-(hydroxymethyl)-1,3-propanediol (m/z 122.1 in positive mode), the key part of TRIS buffer in which was the enzyme lyophilised by manufacturer (this peak is notable in all presented LCMS data and won’t be commented again). The two major peaks had almost identical MS spectra in both polarities. Here we list the spectra for described peaks:
After inspecting all major ions detected, we have constructed major decomposition pathway for the faropenem. Major changes after opening the beta-lactam ring are a) decarboxylation, which is likely happening in the MS detector b) retro-aldol reaction.¹ Those major products are almost identical in its fragmentation and share almost the same molar weight. The proposed mechanism contains several

¹ Drawz, S. M.; Babic, M.; Bethel, C. R.; Taracila, M.; Distler, A. M.; Ori, C., ... Bonomo, R. A. *Biochemistry* **2010**, *49*(2), 329–340, doi:10.1021/bi9015988
intermediates with SH-group, capable to trigger a RSH sensing probe, and allows several ways to generate H₂S from outlined thioacetal and thiohemiaminal structures.

Scheme 1: Metalo-lactamase mediated decomposition pattern for faropenem.

Sulopenem decay promoted by B. Cereus metallo-lactamase, assayed by LCMS

The analysis with negative ionization was captured directly after the incubation period (injected after 45 min of incubation), positive ionization experiment started after 90 min of incubation and negative ionization experiment was repeated with identical result after 110 min of incubation. Sulopenem decomposed only partially during this period and had to incubate overnight (10h overall) to reach complete conversion. Following chromatogram shows the completed reaction mixture after 10 h.

Two major UV-absorbing peaks were detected in the hydrolysed mixture at 3.68 an 4.05 min. Negative ionization signal provided only these two peaks. Positive signal was also collected at 2.54 min, originating from the TRIS amine (m/z 122.1).
Proposed decomposition pathway is roughly like the one for faropenem. The greatest difference opens the explanation of high detection rate of this antibiotic by RSH sensing probes: all the key intermediates can eliminate 2,3-dihydrothiophene 1-oxide via retro-Michael pathway and products are thiols, capable of reaction with the RSH sensing probe. 2,3-Dihydrothiophene-1-oxide signal was the strongest ion in the positive ionization MS spectrum for both major peaks, which strongly supports our explanation.
Penicillin V decay promoted by B. Cereus metallo-lactamase, assayed by LCMS

The analysis with negative ionization was captured directly after the incubation period (injected after 45 min of incubation at RT), positive ionization experiment started after 90 min of incubation. Penicillin V decomposed completely during this period. Only one major peak with higher polarity (6.51 min) was formed and remained untouched during the incubation. One minor peak was detected at 6.72 min.

Fragment analysis is rather simple here: the hydrolysed beta lactam produces very stable compound, which then resists any further chemical changes. This is explaining very low luminescence of all tested beta-lactamase penicillin degradation mixtures with RSH sensing probe. The possible transformation through penillic acid, known from human metabolism is not likely in used reaction system and the key intermediate – the appropriate m/z for penillic acid - was not practically detected (the ion was present in very low concentration only). On the other hand, the ion with m/z 246 was developed in decent concentration, which speaks for good chance to produce H2S through decarboxylation/elimination pathway at elevated temperatures. This process can occur in limited scale at RT and furnish small, but detectable H2S levels, which then generated observed weak luminescent signal with H2S sensitive probe.
Figure 2 EIC signal for m/z 349-351 in the chromatogram with negative ionization, corresponding to the penillic intermediate.
Meropenem decay promoted by B. Cereus metallo-lactamase, assayed by LCMS

The analysis with positive ionization was captured directly after the incubation period (injected after 45 min of incubation at RT), negative ionization experiment started 60 min after the incubation was started. Meropenem decomposed completely during this period. Only one major peak with slightly higher polarity was formed. Observed ions were corresponding to the expected hydrolytic pathway for all carbapenems: beta-lactam ring opening followed by retro-aldol elimination of acetaldehyde. The product then can undergo series of decarboxylations during the ionization process. Weak signal with RSH sensing probe can be explained by formation of the thiol intermediate with m/z 175.1 in the positive mode.
Cefotaxime decay promoted by B. Cereus metallo-lactamase, assayed by LCMS

The analysis with positive ionization was captured directly after the incubation period (injected after 50 min of incubation at RT), negative ionization experiment started 70 min after the incubation was started. Meropenem decomposed completely during this period. Only one broad major peak with slightly lower polarity was formed (5.22 min). MS signals of both polarities were very weak, which is given by zwitterion character of most molecules. Observed ions are corresponding with expected hydrolytic pathway: the acetoxy group eliminates along with the hydrolysis of the beta lactam ring. The gamma-lactone intermediate is easy detectable by its decarboxylated fragment ion (m/z 368 in acquired negative MS spectra). Under the positive ionization, m/z 201 and 101 are most remarkable, corresponding to the acylamide abstracted from the 7-position and its 2-aminothiazolyl fragment. Clearly, no formation of RSH or even H2S is allowed during this hydrolytic decomposition, which agrees with poor signal measured by thiol sensing luminescent probes.
Imipenem decay promoted by B. Cereus metallo-lactamase, assayed by LCMS

The analysis with positive ionization was captured directly after the incubation period (injected after 70 min of incubation at RT), negative ionization experiment started after 90 min after the incubation was started. Imipenem decomposed completely during this period. Only one major peak with slightly higher polarity was formed (2.94 min). According to MS signals, imipenem undergoes lactamase mediated transformation quite in accord with other penems: first the lactamase opens the lactam ring and then catalyses the retro-aldol reaction. Formed product is then fragmented in MS ionization chamber through NH3 eliminating steps forming isocyanide fragments, which in parallel decarboxylate. No product nor fragment possessing SH group was detected, which explains low signal after treatment with the RSH sensing probes.
imipenem

Exact Mass: 273.08

CO₂

NH₃

Exact Mass: 229.09

Exact Mass: 256.05

NH₃

CO₂

Exact Mass: 169.08

Exact Mass: 212.06
Ceftizoxime decay promoted by B. Cereus metallo-lactamase, assayed by LCMS

The analysis with positive ionization was captured directly after the incubation period (injected after 80 min of incubation at RT), negative ionization experiment started 100 min after the incubation was started. Ceftizoxime decomposed completely during this period. One major peak with lower polarity was formed (5.25 min) and one minor peak with slightly higher polarity (4.93 min). As the tested sample was the sodium salt of ceftizoxime, M+Na adducts were observed in higher intensity. Major decomposition pathway follows the cefotaxime scheme with one important exception: detected ions with m/z 243 and m/z 134 in the positive mode signal can be explained by the opening of the dihydrothiazine ring, when an aldehyde structure and corresponding enamine-thiol is formed. We believe this process can occur at lower temperatures as well and explains high signal strength with RSH sensing probes.
Ceftazidime decay promoted by B. Cereus metallo-lactamase, assayed by LCMS

The analysis with positive ionization was captured directly after the incubation period (injected after 80 min of incubation at RT), negative ionization experiment started 100 min after the incubation was started. Ceftazidime decomposed completely during this period. One major peak with higher polarity was formed (3.92 min) and one minor peak with even higher polarity (3.18 min). MS signal of both polarities was weak, which was caused by zwitterion character of relevant structures, and heavily fragmented, which is resulting from fragile structure. Pyridinium ring eliminates readily after the lactam ring was opened and the oxime side chain is also steady for fast decarboxylation in the ionization chamber. One tentative thiol structure with m/z 146 was identified, which could eventually explain weak luminescent signal observed in this case.
Cefazoline decay promoted by B. Cereus metallo-lactamase, assayed by LCMS

50 min of incubation at RT), negative ionization experiment started 70 min after the incubation was started. Cefazoline decomposed completely during this period. Two small peaks were formed at The analysis with positive ionization was captured directly after the incubation period (injected after 50 min of incubation at RT), negative ionization experiment started 70 min after the incubation was started. Cefazoline decomposed completely during this period. Two small peaks were formed at 5.14 and 5.51 min. In this case the captured ions clearly display the mechanism of H₂S release through hydrolytic opening of the dihydrothiazine ring, producing positive ions with m/z 263, 279 and 295. 5-Methyl-1,3,4-thiadiazole-2-thiol is another RSH component generated quickly after the lactam ring was

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**Diagram:**

- Cefazidine
  - Hydrolysis
  - Formation of intermediates with exact masses: 169.02, 272.06, 145.02, 100.01, 397.09

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50 min of incubation at RT; negative ionization experiment started 70 min after the incubation was started. Cefazoline decomposed completely during this period. Two small peaks were formed at The analysis with positive ionization was captured directly after the incubation period (injected after 50 min of incubation at RT), negative ionization experiment started 70 min after the incubation was started. Cefazoline decomposed completely during this period. Two small peaks were formed at 5.14 and 5.51 min. In this case the captured ions clearly display the mechanism of H₂S release through hydrolytic opening of the dihydrothiazine ring, producing positive ions with m/z 263, 279 and 295. 5-Methyl-1,3,4-thiadiazole-2-thiol is another RSH component generated quickly after the lactam ring was
hydrolysed, nevertheless this electron-poor thiol has only minimal triggering effect on used hydrogen sulphide sensing probes.
