Electronic Supplementary Information

β-lactamase triggered visual detection of bacteria using cephalosporin functionalized biomaterials

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Contents

1 Materials

7-amino-3-chloromethyl-3-cephem-4-carboxylic acid p-methoxybenzyl ester hydrochloride (ACLE) was purchased from AK Scientific (Union City, CA). 4-nitrobenzenethiol (NBT) and 3-maleimidopropionic acid were purchased from TCI Chemicals (Tokyo, Japan). Nitrocefin was purchased from P212121, LLC (Boston, MA). Deuterated dimethyl sulfoxide (DMSO-d_6) was obtained from Cambridge Isotope Laboratories (Andover, MA). Triethylamine (TEA), 4-methylmorpholine (NMM), anhydrous dichloromethane (DCM), anhydrous dimethylformamide (DMF), hexanes, diethyl ether, ethyl acetate, thin layer chromatography (TLC) silica gel 60 on glass plates, disodium hydrogen phosphate anyhydrous, sodium dihydrogen phosphate anhydrous, CENTA, dimethyl sulfoxide (DMSO), trifluoroacetic acid (TFA), anisole, thiol functionalized 4-arm-poly(ethylene glycol) (4-arm-PEG-SH; 20 kDa), βL from *Bacillus cereus* (βL-BC; cat.# P0389, 28 kDa, 2817.8 U/mg protein, 4.72% protein), βL from *Pseudomonas aeruginosa* (βL-PA; cat.# L6170, 30 kDa, 1080 U/mg protein, 1% protein), βL from *Enterobacter cloacae* (βL-EC; cat.# P4524, 20-26 kDa, 0.37 U/mg protein, 56.45% protein), collagenase from *Clostridium histolyticum*, phosphate buffered saline (PBS), sodium nitrate, cation-adjusted Müller-Hinton broth (CMHB), α-cyano-4-hydroxycinnamic acid, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), N,N-disopropylethylamine (DIPEA), and hydrochloric acid (HCl) were acquired from Millipore Sigma (St. Louis, MO). Methanol, silica gel, tryptic soy broth (TSB), and SYLGARD 184 silicone elastomer kit were purchased from Thermo Fisher Scientific (Waltham, MA). Methoxy-poly(ethylene glycol)-thiol (mPEG-thiol; 1.7 kDa) was purchased from Laysan Bio, Inc. (Arab, AL). *Staphylococcus aureus* strains 25923 and 29213, methicillin-resistant *S. aureus* (MRSA) MW2, *B. cereus* 13061, *Escherichia coli* 25922, and *E. cloacae* 13047 were purchased from ATCC (Manassas, VA). *P. aeruginosa* PA01 was generously donated by Walter Reed Army Institute of Research (Silver Spring, MD). *E. coli* DH5-α was purchase from Life Technologies (Carlsbad, CA). Bis-maleimide-PEG_3 (mal-PEG-mal, 494.5 Da) was purchased from BroadPharm (San Diego, CA). Repligen Biotech cellulose ester 500-1000 Da molecular weight cut-off (MWCO) dialysis tubing was obtained from Spectrum Labs Inc. (Rancho Dominguez, CA). Ultra-high-purity nitrogen gas (99.999%) was obtained from Airgas (Warwick, RI). Ultrapure deionized water (18.2 MΩ·cm, Millipore Sigma, Billerica, MA) was utilized in all experiments. Room temperature (RT) referred to in this work is approximately 23°C.

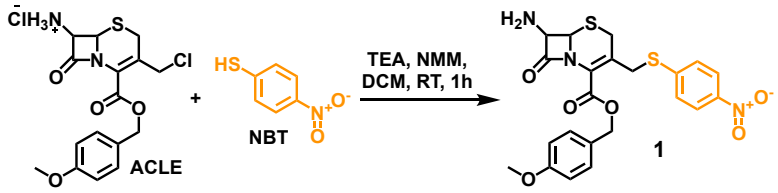
2 Instrumentation

^{1}H-NMR, ^{13}C-NMR, and 2D-NMR were recorded on a Bruker DRX Avance 400 MHz spectrometer or a Bruker Ascend 600 MHz spectrometer. Data for ^{1}H and ^{13}C NMR are reported with chemical shifts stated in δ in units of parts per million (ppm) relative to DMSO-d_6 (^{1}H-NMR: δ = 2.50 ppm, ^{13}C-NMR: δ = 39.52 ppm). High resolution mass spectrometry (HRMS) electrospray ionization (ESI) was conducted on an Agilent 6530 liquid chromatography (LC) mass spectrometer. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS was conducted using an AXIMA Performance equipped with a 50 Hz nitrogen laser (Shimadzu Scientific Instruments, Columbia, MD). The matrix utilized was α-cyano-4-hydroxycinnamic acid. Size exclusion chromatography (SEC) was performed on a 1260 Infinity II LC system equipped with multiple

wavelength ultraviolet-visible (UV-vis) and refractive index detectors (Agilent Technologies, Santa Clara, CA). An Agilent AdvanceBio SEC 130 Å pore size column (7.8 × 300 mm; 2.7 μm particle size) was used under isocratic flow conditions with 100 mM sodium nitrate with 0.1% (v/v) trifluoroacetic acid (pH 6.5) as the mobile phase and at a flow rate of 0.35 mL/min. UV-vis spectral scans and kinetic experiments were performed using a Cytation 3 microplate reader (BioTek®, Winooski, VT) using 96-well UV-capable plates (Corning Inc., Corning, NY), unless otherwise specified.

3 Synthesis and purification of 1

Compound **1** was synthesized adapting previously reported methods.[1, 2] ACLE (600 mg, 1.48 mmol) was dissolved in anhydrous DCM (20 mL) and stirred at RT under N_2. TEA (390 μL, 2.8 mmol) was slowly added in three portions over 20 minutes to the ACLE mixture. NMM (200 μL, 1.8 mmol) and NBT (370 mg, 2.4 mmol) were added sequentially thereafter. The reaction was stirred at RT and monitored by TLC (30% hexanes/70% ethyl acetate). After 1 hour, DCM was evaporated using a Buchï rotary evaporator. The conjugate was purified using flash column chromatography (silica gel, 70% to 100% gradient ethyl acetate in hexanes as eluent) to yield compound **1** as a yellow solid (586 mg, 1.20 mmol, 81% yield). HRMS-ESI m/z: Calculated for $C_{22}H_{21}N_3O_6S_2^+$ $[M+H]^+$: 488.09; Found: 488.0948. ^{1}H-NMR (600 MHz, DMSO-d$_6$) δ (ppm): 8.07 (d, J = 8.9 Hz, 2H), 7.46 (d, J = 8.9 Hz, 2H), 7.31 (d, J = 8.6 Hz, 2H), 6.87 (d, J = 8.6 Hz, 2H), 5.18 (d, J = 11.9 Hz, 1H), 5.11 (d, J = 11.9 Hz, 1H), 4.96 (d, J = 5.0 Hz, 1H), 4.77 (d, J = 5.0 Hz, 1H), 4.26 (d, J = 12.9 Hz, 1H), 4.07 (d, J = 12.9 Hz, 1H), 3.73 (s, 3H), 3.70 (d, J= 18.0, 1H), 3.50 (d, J= 18.0, 1H), 2.33 (br, s, 2H). Note, Δ_2 isomer is not assigned in this spectrum but marked with an asterisk.



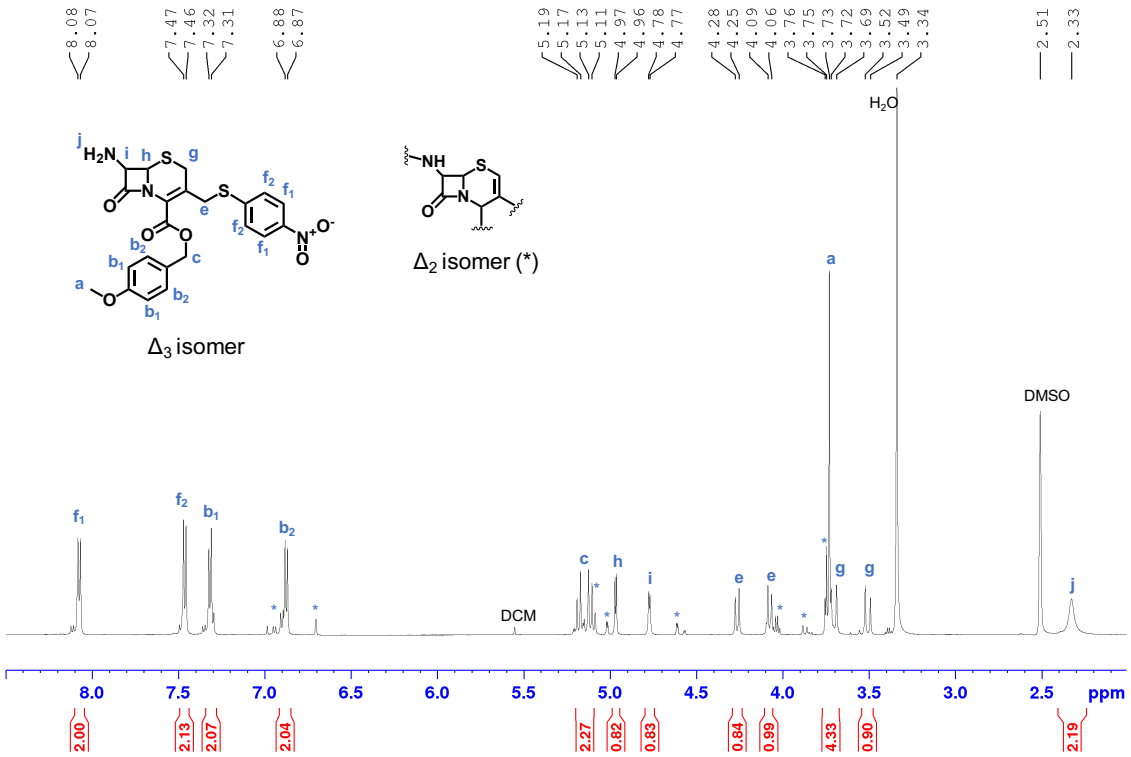


Figure S1: ^{1}H-NMR spectrum of compound **1** (DMSO-d_6, 600 MHz).

4 Synthesis and purification of 2

Compound **1** (70 mg, 0.14 mmol) was deprotected in a solution of TFA:anisole:DCM (49 mL total) at a 1:1:5 volumetric ratio under N_2 on ice for 4 hours. The solvents were then evaporated using a Buchï rotary evaporator. The deprotected compound was dissolved in DMSO at a concentration of ∼8 mg/mL and diluted 1:10 in methanol. The suspension was left at 4°C overnight to precipitate, then centrifuged at 5,000 rpm for 30 minutes at 4°C. The supernatant was removed and the pellet redissolved in DMSO to repeat the rinsing process. The final substrate **2** pellet was frozen and lyophilized yielding a yellow solid (17 mg, 0.046 mmol, 32% yield). HRMS-ESI m/z: Calculated for $C_{14}H_{13}N_3O_5S_2^+$ $[M+H]^+$: 368.03; Found: 368.0366. ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 8.13 (d, J = 8.9 Hz, 2H), 7.56 (d, J = 8.9 Hz, 2H), 4.96 (d, J = 5.0 Hz, 1H), 4.75 (d, J = 5.0 Hz, 1H), 4.29 (d, J = 12.6 Hz, 1H), 4.14 (d, J = 12.6 Hz, 1H), 3.68 (d, J = 18.0 Hz, 1H), 3.47 (d, J = 18.0 Hz, 1H), 3.33 (s, 2H).

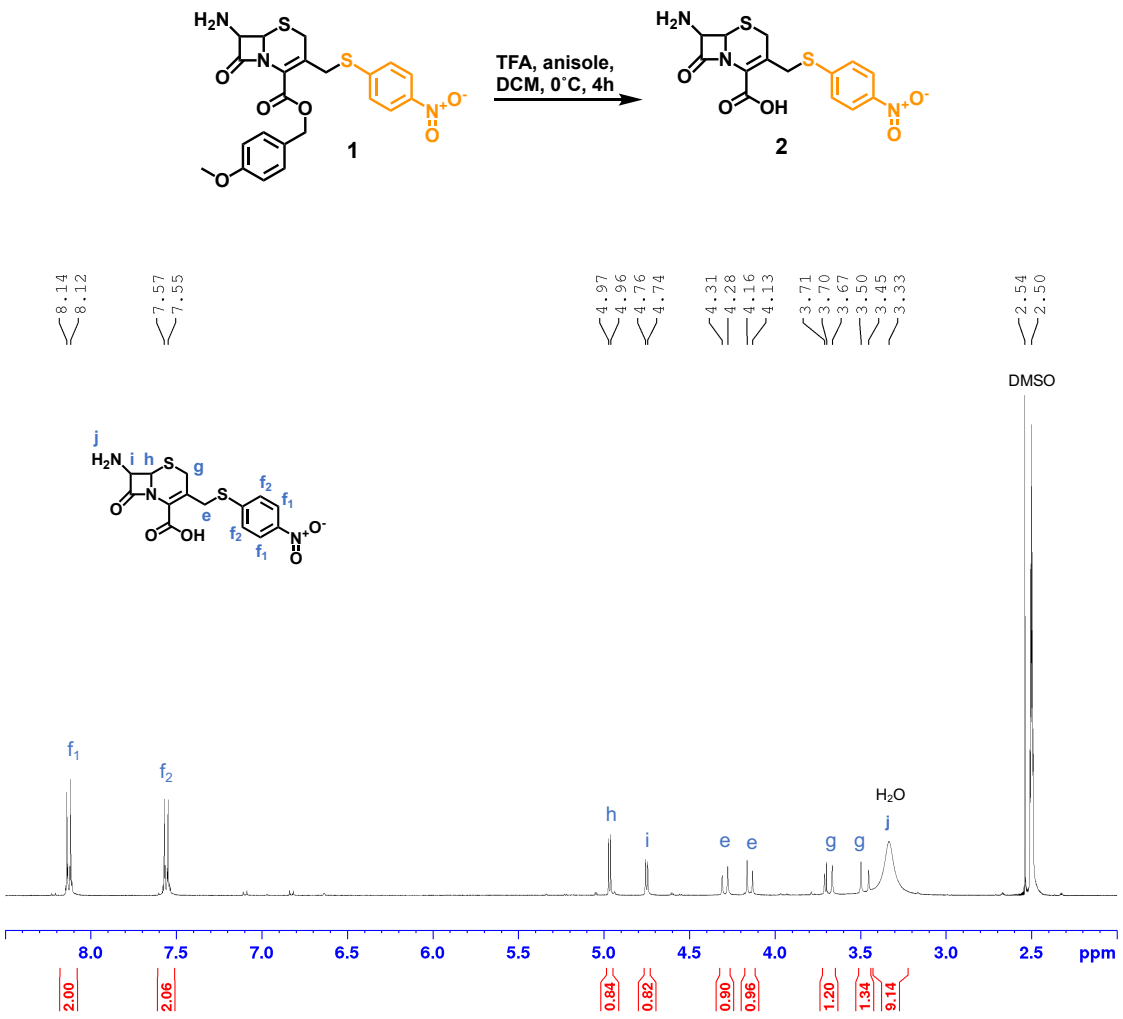


Figure S2: ^{1}H-NMR spectrum of compound **2** (DMSO-d$_6$, 400 MHz).

5 Substrate 2 incubation with βLs

In order to test the response of substrate **2** to different βLs, 135 μL of each βL dissolved in 0.15 M PB (pH 7) or 1× PBS (pH 7.4) was added to 15 μL of substrate **2** dissolved in DMSO, achieving final concentrations of 544 μM of **2** and 10% (v/v) DMSO in buffer. Enzyme concentration and time for UV-vis measurement were selected to ensure successful measurement of substrate hydrolysis. Substrate **2** was incubated with 1 unit (U)/mL βL-EC for 3 hours (**Figure S3a**), 50 U/mL βL-PA for 90 minutes (**Figure S3b**), or 200 U/mL βL-BC for 30 minutes (**Figure S3c**) at 37°C before UV-vis spectra were measured from 230 to 600 nm. Note, 1 U of enzyme was defined as hydrolyzing 1.0 μmole of benzylpenicillin per min at pH 7.0 at 25°C.

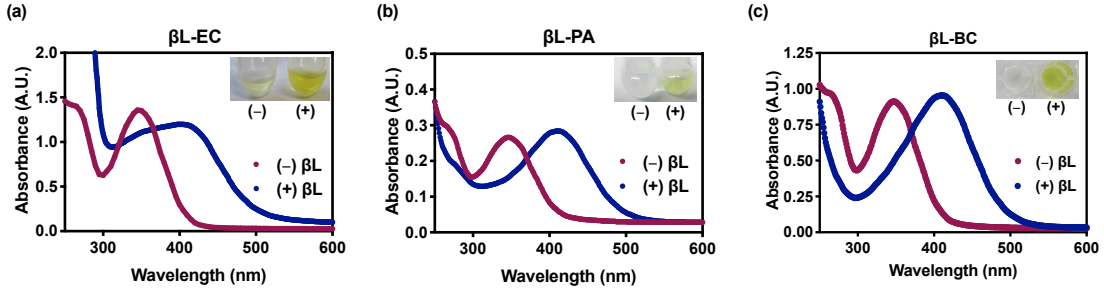


Figure S3: Absorbance spectra of **2** (544 μM) incubated in PB (with 10% (v/v) DMSO) with (a) 1 U/mL of βL-EC for 3 hours, (b) 50 U/mL of βL-PA for 90 minutes, or (c) 200 U/mL βL-BC for 30 minutes at 37°C. Insets: Images of **2** incubated with (+) or without (-) βLs in 1× PB.

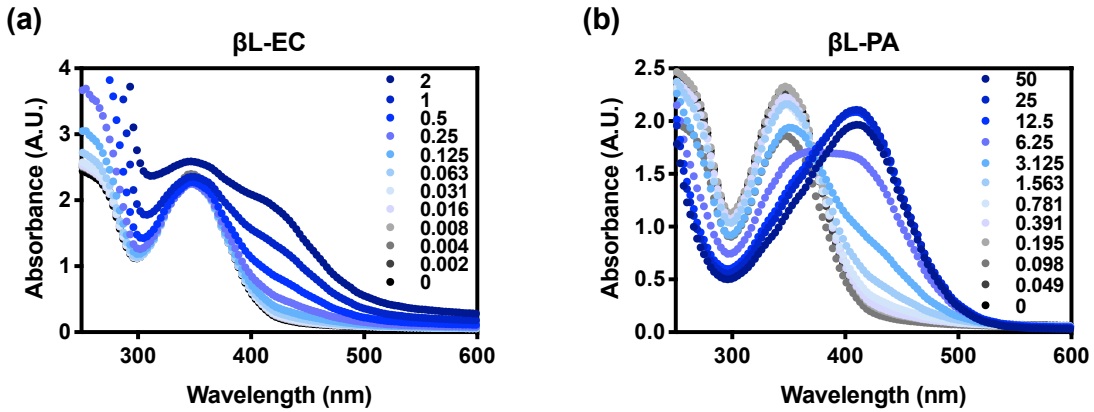


Figure S4: Spectral scan of **2** (544 μM) incubated in 1× PBS (with 10% (v/v) DMSO) with different concentrations (U/mL) of (a) βL-EC or (b) βL-PA for 45 minutes at 37°C.

To study enzyme concentration dependent effects on substrate **2** hydrolysis, **2** was incubated with different concentrations of βL-EC, βL-PA, or βL-BC in 1× PBS (pH 7.4). Substrates in DMSO and enzymes in 1× PBS were mixed as described above. After 45 minutes of incubation at 37°C, digital images of the wells from the bottom of the 96-well plate were taken (**Figure 1a**) and absorbance spectra were measured (**Figures 1b** and **S4**). Absorbance at 410 nm was plotted with respect to enzyme concentration (**Figure 1c**), and the linear region was fit using linear regression (**Figure S5**). The limit of enzyme detection (LOD) was calculated using **Equation 1**.

$$LOD = 3.3 \times \frac{standard\ deviation\ of\ blank}{slope\ of\ regression\ line} \tag{1}$$

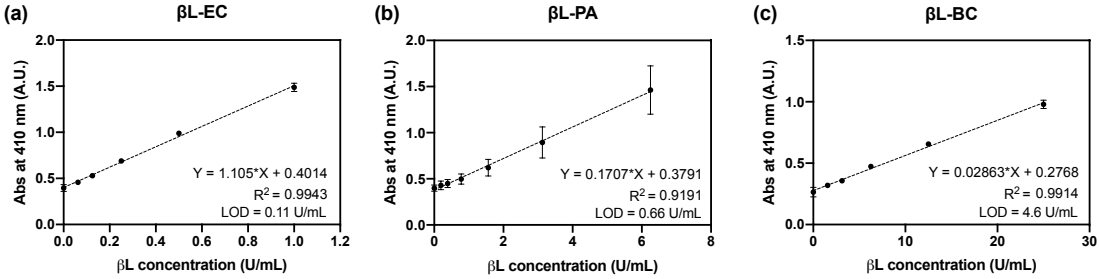


Figure S5: Linear regions of plots of absorbance at 410 nm as a function of βL concentration (**Figure 1c**) showing linear regression fits used to measure the LOD for substrate **2** (544 μM) when incubated with (a) βL-EC, (b) βL-PA, or (c) βL-BC in 1× PBS (with 10% (v/v) DMSO) for 45 minutes at 37°C. Data are shown as mean ± standard deviation (n = 3).

6 Enzyme-substrate kinetics

The kinetics of substrate **2**, CENTA, and nitrocefin hydrolysis in the presence of βL-BC, βL-PA, βL-EC were investigated using the Michaelis-Menten model. The three substrates were dissolved in DMSO due to the low aqueous solubility of **2** and nitrocefin at concentrations ranging from 0.68 to 5.4 mM. Enzyme stocks were prepared in 0.15 M PB (pH 7) at different concentrations (U/mL) for each substrate-βL combination and were warmed to 37°C. 15 μL of **2**, CENTA, or nitrocefin followed by 135 μL of βL was added to 96-well plates to achieve final concentrations of 68 to 544 μM for substrates in 10% (v/v) DMSO in PB. Absorbance at 410, 405, and 485 nm, for **2**, CENTA, and nitrocefin, respectively, were immediately monitored over time at 37°C. The absorbance was plotted against time for each substrate-βL combination, and the initial slope of each curve was determined and used with the molar extinction coefficient of the respective substrate to calculate the initial velocity of the reaction (**Figure S6**). The K_M, k_{cat}, and k_{cat}/K_M values were calculated by fitting this data with the Michaelis-Menten equation [3] using GraphPad Prism^{TM} (summarized in **Table 1**).

To measure the molar extinction coefficients of **2**, CENTA, and nitrocefin, used in the calculation of initial velocities, 15 μL of each substrate (in DMSO) and 135 μL of βL-BC (in 0.15 M PB, pH 7) was added to a 96-well plate to achieve final concentrations of 68 to 544 μM for substrates and 25 U/mL for βL-BC in 10% DMSO (v/v) in PB. The absorbance at 410 nm for **2**, 405 nm for CENTA, and 485 nm for nitrocefin was monitored for 30 minutes. The maximum absorbance achieved for each substrate at each concentration was recorded and absorbance versus substrate concentration plots were generated for each substrate. The slope of each plot was determined and recorded as the extinction coefficient for each substrate. The coefficients were calculated to be 1331, 6221, and 15068 $M^{-1}cm^{-1}$ for **2**, CENTA, and nitrocefin, respectively.

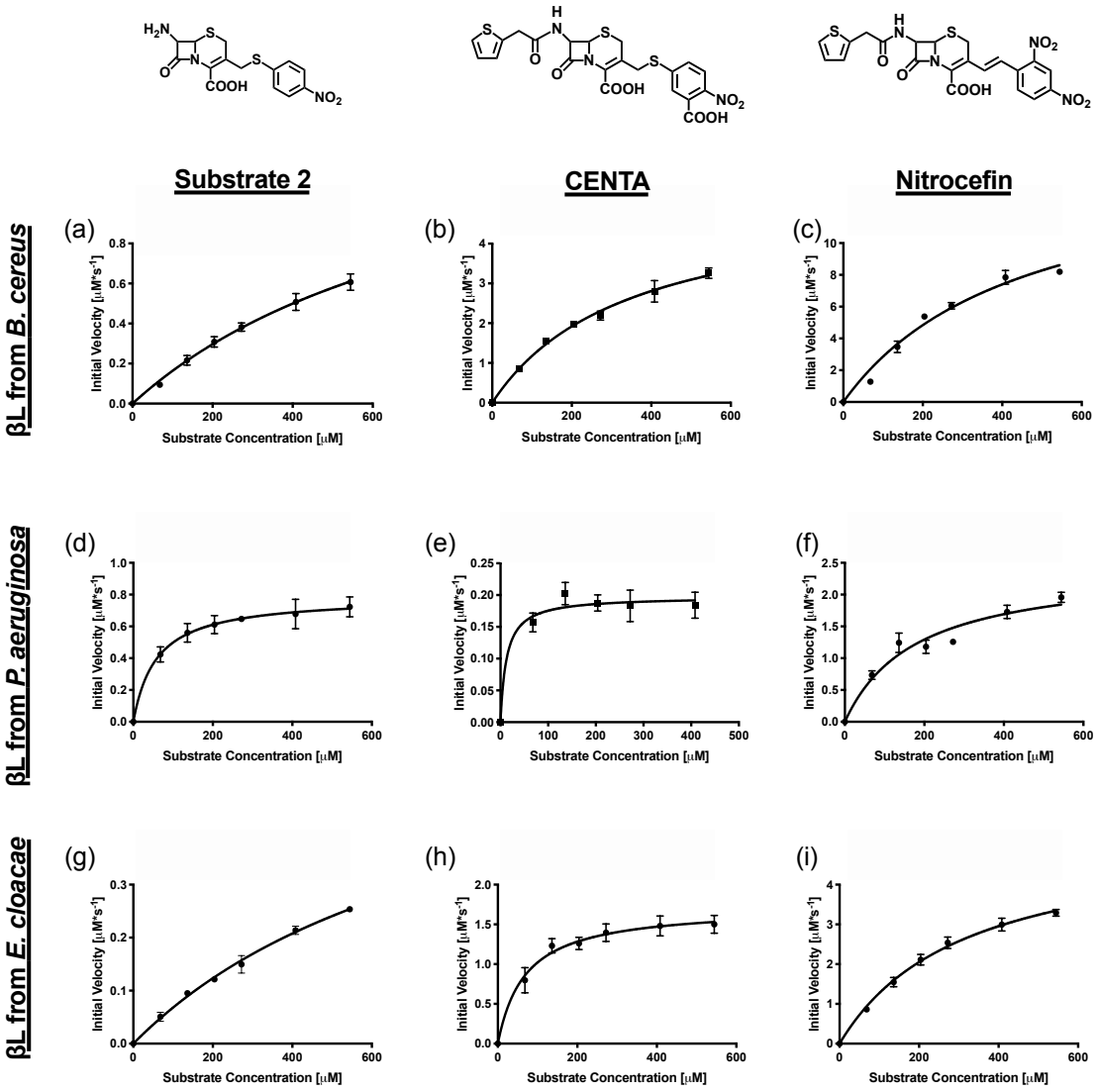


Figure S6: Initial velocity versus substrate concentration for (a) **2**, (b) CENTA, and (c) nitrocefin with 25 U/mL of βL-BC, (d) **2** with 25 U/mL of βL-PA, (e) CENTA with 10 U/mL of βL-PA, and (f) nitrocefin with 10 U/mL of βL-PA, (g) **2** with 1 U/mL of βL-EC, (h) CENTA with 0.01 U/mL of βL-EC, and (i) nitrocefin with 0.005 U/mL of βL-EC. Data are shown as mean $\pm$ standard deviation (n = 3). Each data set has been fit to the Michaelis-Menten equation to calculate kinetic parameters, K_M, k_{cat}, and k_{cat}/K_M, using GraphPad Prism^{TM} (**Table 1**).

7 Investigating potential substrate 2 hydrolysis in collagenases

To test potential non-specific color change induced by collagenases, substrate **2**, nitrocefin, and CENTA (544 μM) were incubated with 1 mg/mL of collagenase from *Clostridium histolyticum* in 1× PBS supplemented with 1 mM of calcium chloride (needed for collagenase activity), blank buffer, or 30 U/mL of βL-PA in 1× PBS at 37°C. Substrates in DMSO and enzymes in aqueous buffers were mixed as described in **Section 5**. The solutions were incubated shaking (120 rpm) at 37°C and digital images of the wells were taken from the bottom of the 96-well plate after 0.5, 2, and 5 hours (**Figure 1d**).

8 Investigating antibacterial activity of substrate 2

Potential antibacterial effects of **2** were tested against different gram-positive and gram-negative bacteria (**Table S1**) in a microdilution assay. **2** was dissolved in DMSO at 2.56 mg/mL and then diluted to a concentration of 256 μg/mL in sterile 1× PBS. Substrate **2** and a control of 10% (v/v) DMSO in CMHB or TSB were serially diluted 1:2 with CMHB or TSB in 96-well plates. Bacteria were grown overnight in CMHB (*S. aureus* 25923, *S. aureus* 29213, MRSA MW2) or TSB (*B. cereus* 13061, *P. aeruginosa* PA01, *E. cloacae* 13047), then diluted 1:1000, and then added in their logarithmic growth phase to the wells at a final concentration of 1×10^5 colony forming units (CFU)/mL. Positive controls of bacteria cultured in CMHB or TSB only and negative controls of CMHB or TSB without bacteria were included. After 16-18 hours of shaking (100 rpm) at 37°C, the optical density at 600 nm (OD_{600}) was measured using a plate reader. The normalized bacteria density was calculated using **Equation 2**:

$$Normalized\ bacteria\ density = \frac{sample\ OD_{600} - negative\ control\ OD_{600}}{positive\ control\ OD_{600} - negative\ control\ OD_{600}} \quad (2)$$

The minimum inhibitory concentration (MIC) of **2** was determined as the lowest concentration of **2** at which the normalized bacteria density transitioned from zero to greater than zero and is summarized in **Table S1**.

Table S1: MIC of **2** against different strains of bacteria

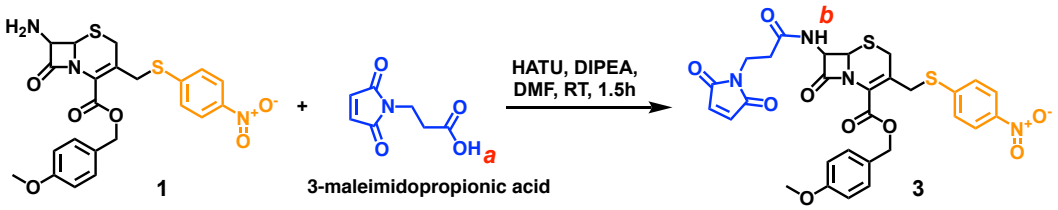
Bacteria	Gram (+/-)	MIC (μg/mL)	MIC (μM)
S. aureus 25923	+	8	21.8
S. aureus 29213	+	8	21.8
MRSA MW2	+	>128	>348.4
B. cereus 13061	+	>128	>348.4
P. aeruginosa PA01	-	>128	>348.4
E. cloacae 13047	-	>128	>348.4

9 Synthesis and purification of 3

Compound **1** (151 mg, 0.31 mmol), 3-maleimidopropionic acid (344 mg, 1.24 mmol), and HATU (567 mg, 1.49 mmol) were dissolved in anhydrous DMF (4 mL). The mixture was stirred for 15 minutes under N_2 at RT before DIPEA was added (370 μL, 2.18 mmol). The reaction was allowed to proceed for another 75 minutes and was monitored by TLC (20% ethyl acetate/80% hexanes). The crude reaction was transferred to a separatory funnel and partitioned between DCM and 0.1 M HCl to separate the layers. DCM was rinsed again with HCl and then rinsed twice with water. The organic layer was washed with brine and dried over sodium sulfate, filtered, and concentrated *in vacuo*. For further purification, the product was dissolved in DMSO at a concentration of ~50 mg/mL and diluted 1:3 in water. The suspension was left at 4°C overnight and then centrifuged at 5,000 rpm for 20 minutes at 4°C. The supernatant was removed and the rinsing process was repeated once more. The final pellet of compound **3** was frozen and lyophilized yielding a yellow solid (118 mg, 0.19 mmol, 60% yield). HRMS-ESI m/z: calculated for $C_{29}H_{26}N_4O_9S_2^+$ $[M+H]^+$: 639.11; Found: 639.1218. Note, 1D and 2D NMR experiments below (**Figures S7 - S13**) suggest the presence of two isomers. Δ_3 and Δ_2 isomers have been reported in literature for similar compounds.[4–6] The Δ_2 isomer resonances are marked with an asterisk.

^{1}H-NMR (600 MHz, DMSO-d_6) δ (ppm): 9.05 (d, J = 7.5 Hz, 0.5H, j*), 8.98 (d, J = 8.3 Hz, 0.5H, j), 8.08 (d, J = 9 Hz, 1H, f_1), 8.07 (d, J = 9 Hz, 1H, f_1), 7.47 (d, J = 9 Hz, 1H, f_2), 7.46 (d, J = 9 Hz, 1H, f_2), 7.32 (d, J = 8.7 Hz, 1H, b_1), 7.31 (d, J = 8.7 Hz, 1H, b_1), 6.99 (s, 2H, l), 6.91 (d, J = 8.7 Hz, 1H, b_2), 6.88 (d, J = 8.7 Hz, 1H, b_2), 6.70 (s, 0.5H, g*), 5.63 - 5.59 (dd, J_1 = 4.8 Hz, J_2 = 8.3 Hz, 0.5H, i), 5.37 - 5.34 (dd, J_1 = 3.9 Hz, J_2 = 7.5 Hz, 0.5H, i*), 5.22 - 5.05 (m, 3.5H, overlapping c/g'*/h), 4.25 (d, J = 12.9, 0.5H, e), 4.13 (d, J = 12.9, 0.5H, e), 4.11 (d, J = 14.4 Hz, 0.5H, e*), 3.89 (d, J = 14.4 Hz, 0.5H, e*), 3.74 (s, 1.5H, a*), 3.73 (d, J = 17.7 Hz, 0.5H, g), 3.72 (s, 1.5H, a), 3.64 - 3.55 (m, 2H, k_2), 3.54 (d, J = 17.7 Hz, 0.5H, g), 2.51 - 2.45 (m, 2H, k_1).

^{13}C-NMR (150 MHz, DMSO-d_6) δ (ppm): 170.66 (2C; 3,4), 170.27 (7*), 170.26 (7), 166.89 (14*), 164.70 (10*), 163.54 (10*), 161.43 (14*), 159.37/159.35 (21,21*), 146.04 (24*), 145.71 (24), 145.14 (29), 144.84 (29*), 134.56 (2C; 1,2), 130.39 (17, 18) 130.19 (17*, 18*), 128.10 (25, 26), 127.22 (25*, 26*), 126.95/126.88 (16,16*), 126.37 (12), 124.77 (13), 123.85 (2C; 27,28), 120.53 (11*), 118.00 (12*), 113.84/113.75 (19, 20), 67.29/67.15 (15,15*), 60.59 (8*), 59.01 (8), 57.84 (9), 55.06/55.04 (22,22*), 52.57 (9*), 49.98 (13*), 36.06 (23), 34.48 (23*), 33.71/33.69 (5,5*), 33.21/33.17 (6,6*), 27.28 (11).



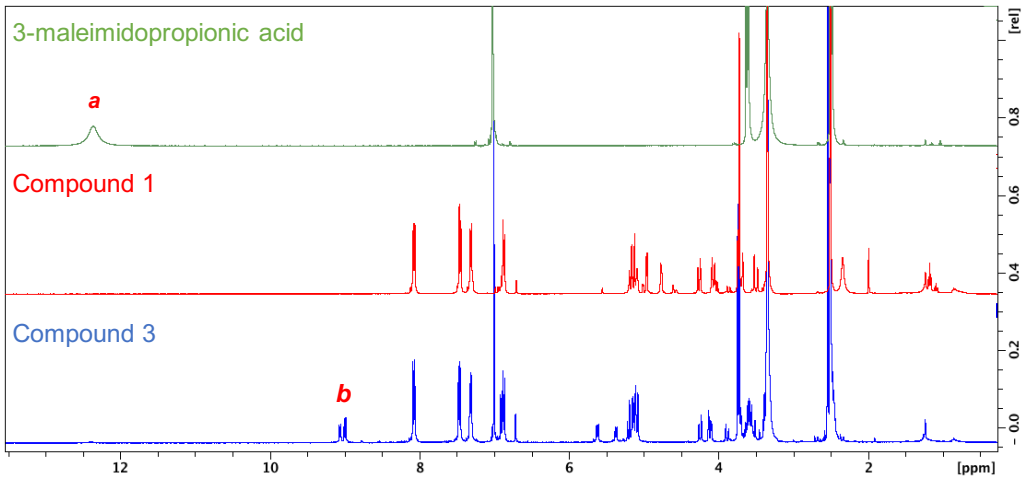


Figure S7: ^{1}H-NMR spectra of 3-maleimidopropionic acid, compound **1**, and compound **3** (DMSO-d_6, 400 MHz). Compound **3** spectrum includes the protons of compound **1** and of 3-maleimidopropionic acid minus the proton of the carboxylic acid (12.36 ppm; marked *a*), and the appearance of the newly formed amide (9.07 - 8.95 ppm; marked *b*).

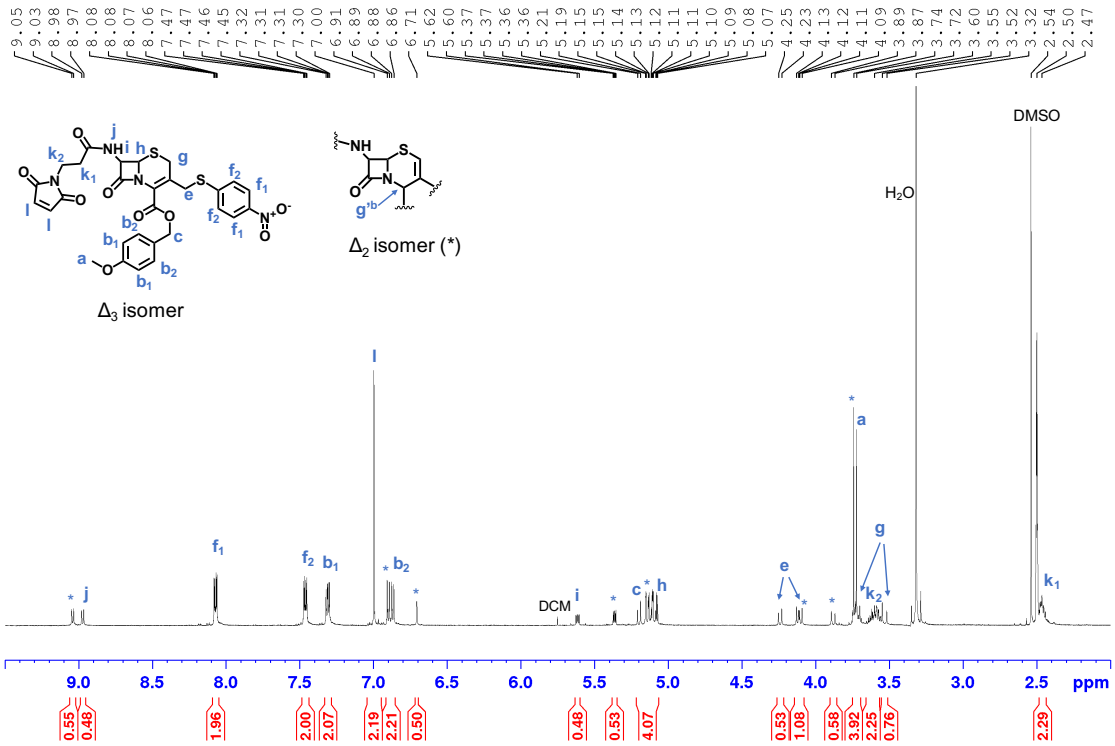


Figure S8: ^{1}H-NMR spectrum for compound **3** (DMSO-d_6, 600 MHz).

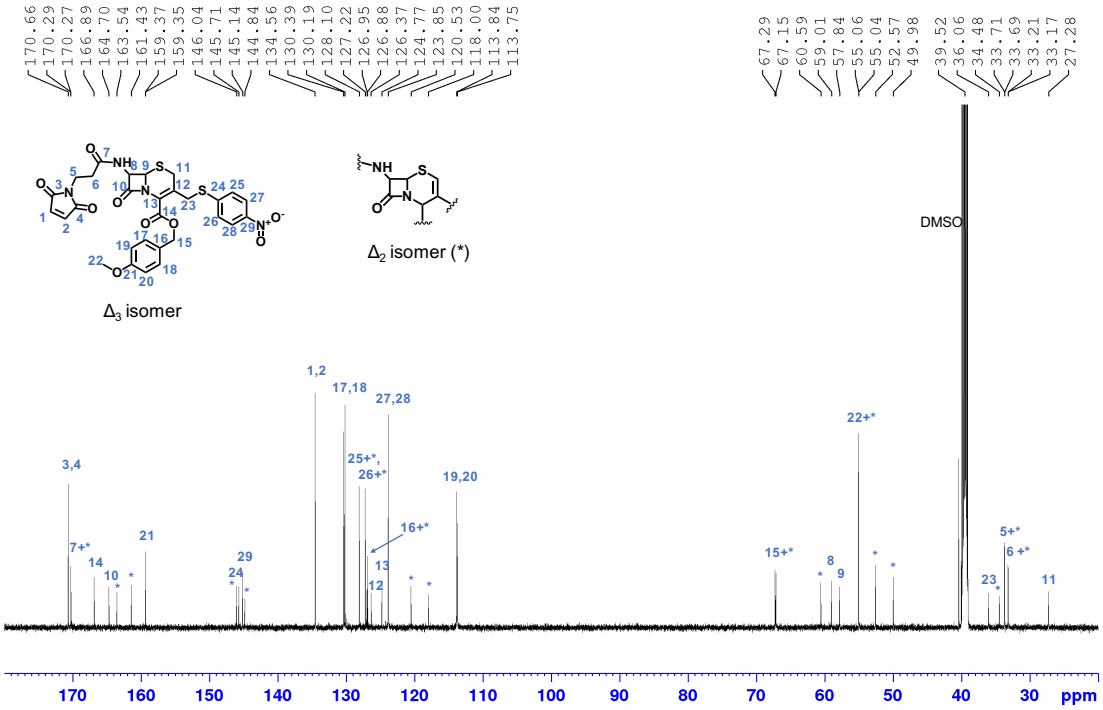


Figure S9: ^{13}C-NMR spectrum for compound **3** (DMSO-d_6, 150 MHz).

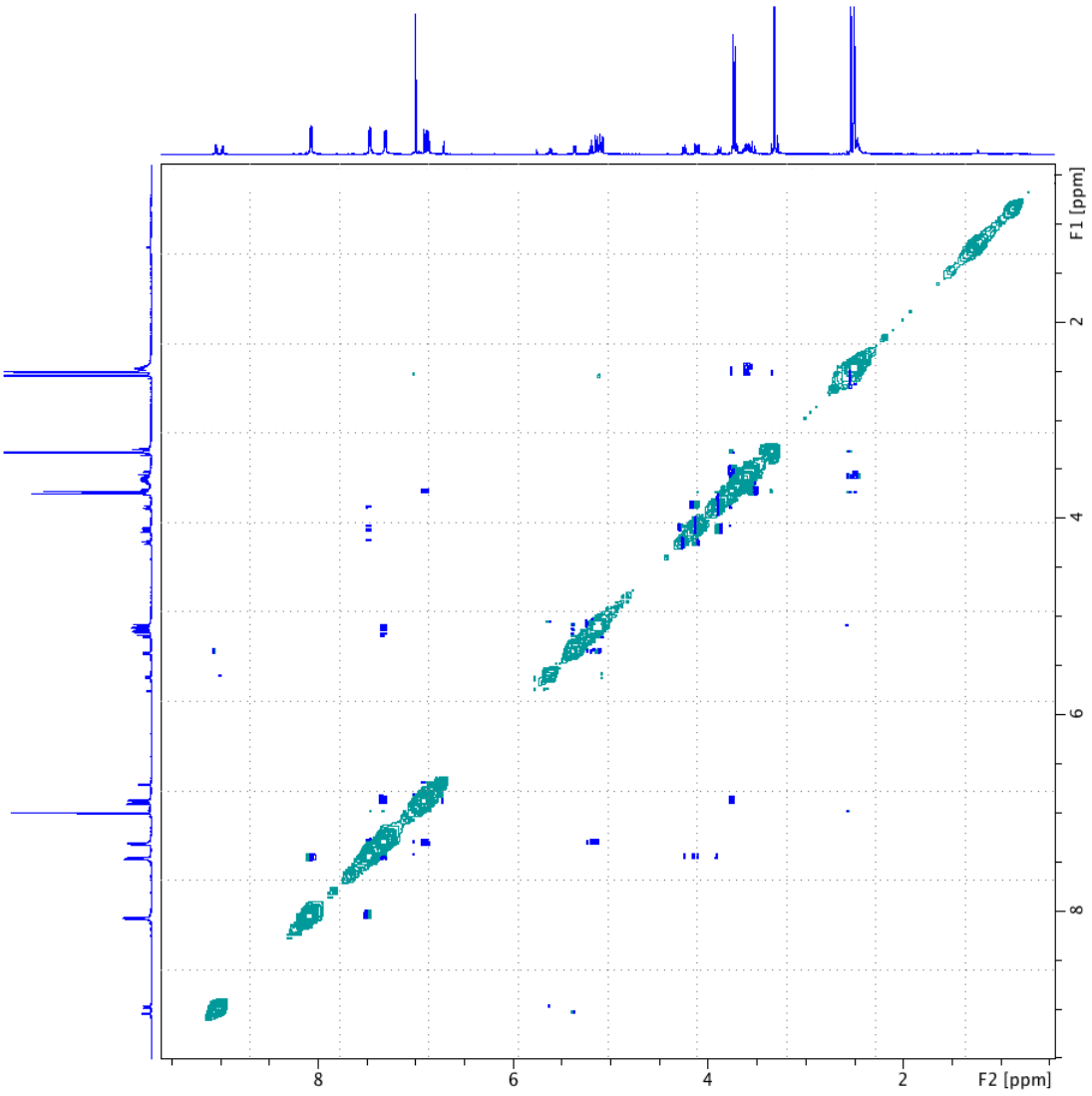


Figure S10: Nuclear Overhauser effect spectroscopy (NOESY) spectrum for compound **3** (DMSO-d_6, 400 MHz).

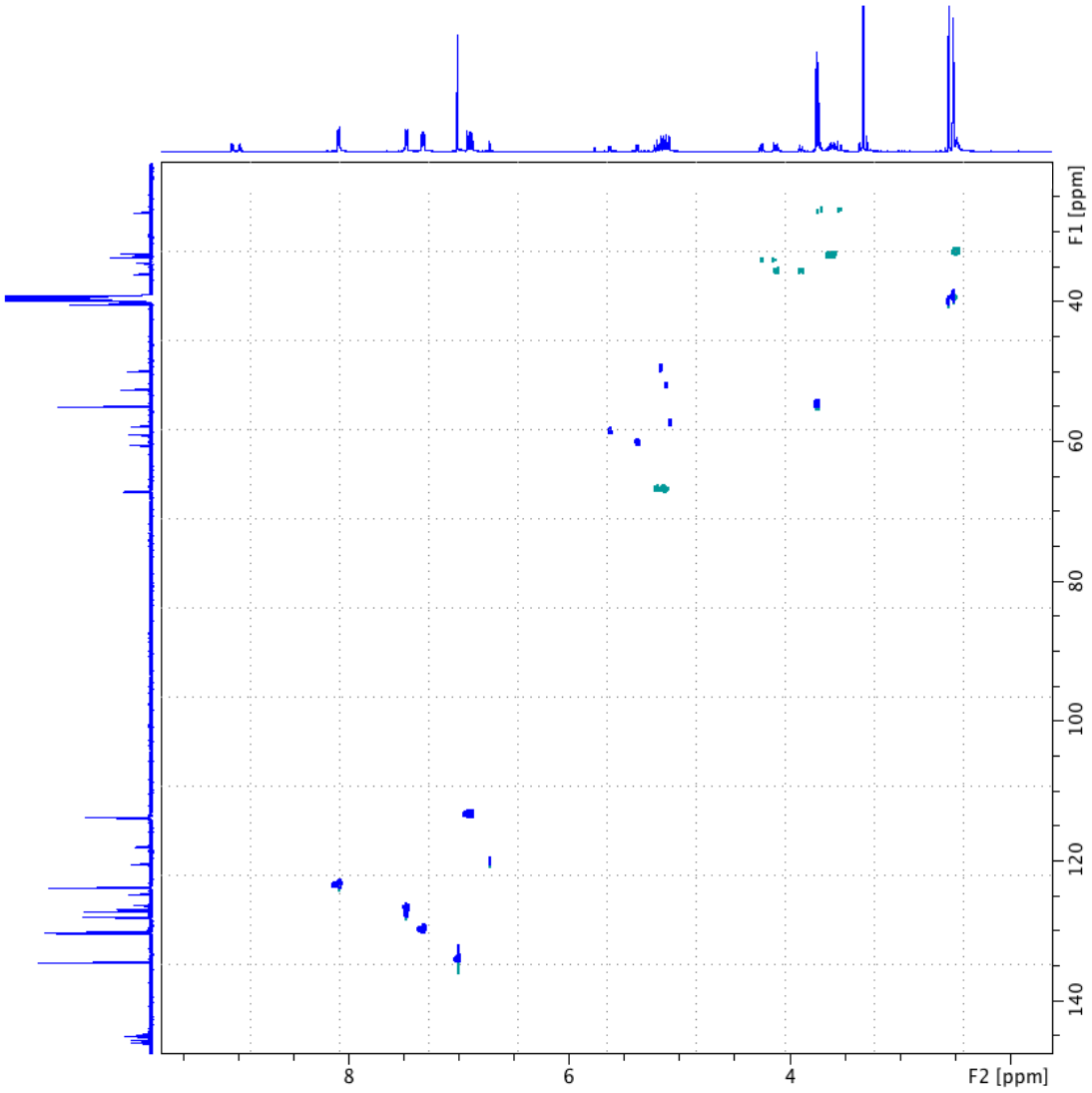


Figure S11: ^{1}H - ^{13}C heteronuclear single quantum correlation (HSQC) spectrum for compound **3** (DMSO-d_6, 600 MHz).

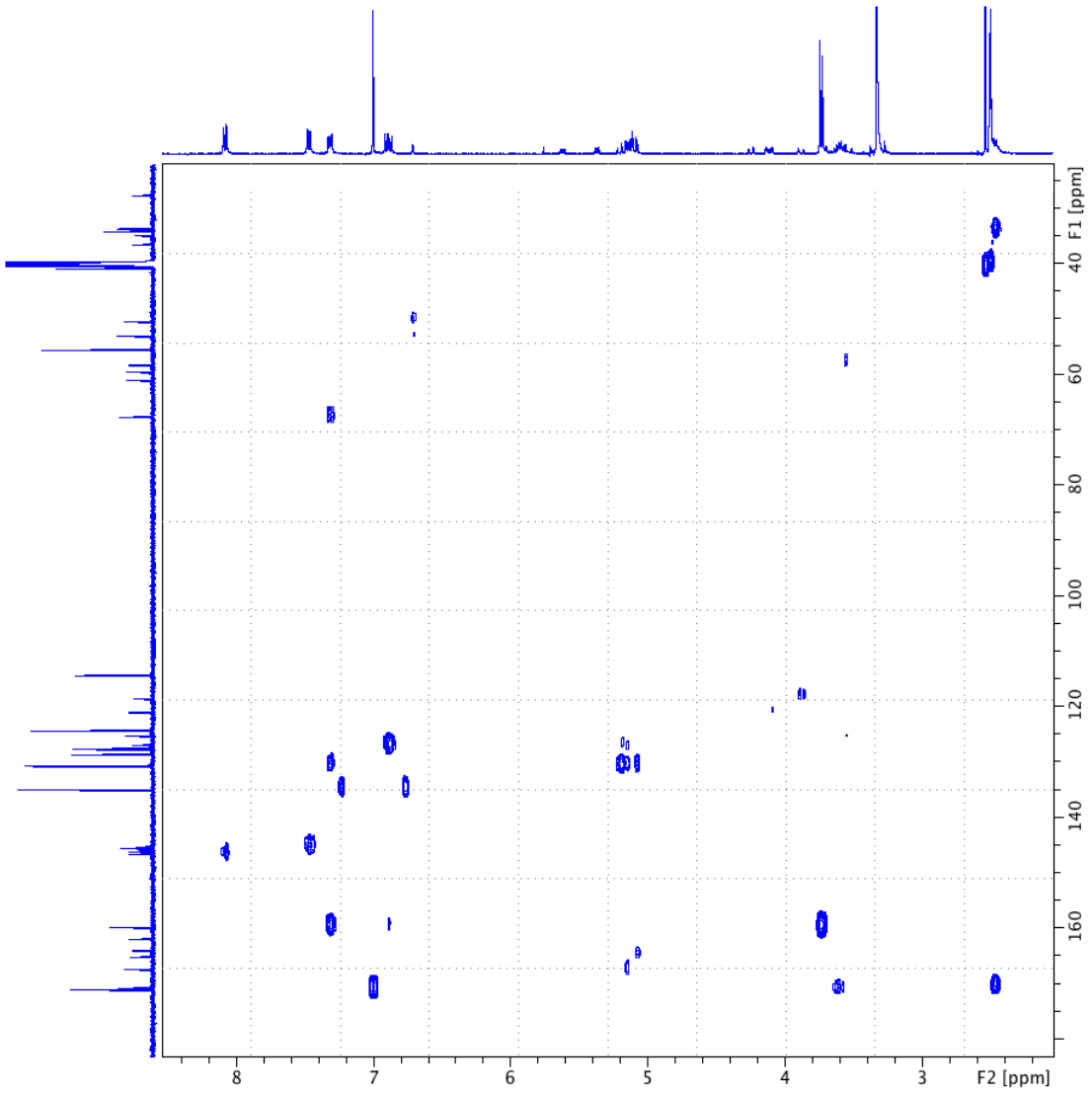


Figure S12: ^{1}H - ^{13}C heteronuclear multiple-bond correlation (HMBC) spectrum for compound **3** (DMSO-d_6, 600 MHz).

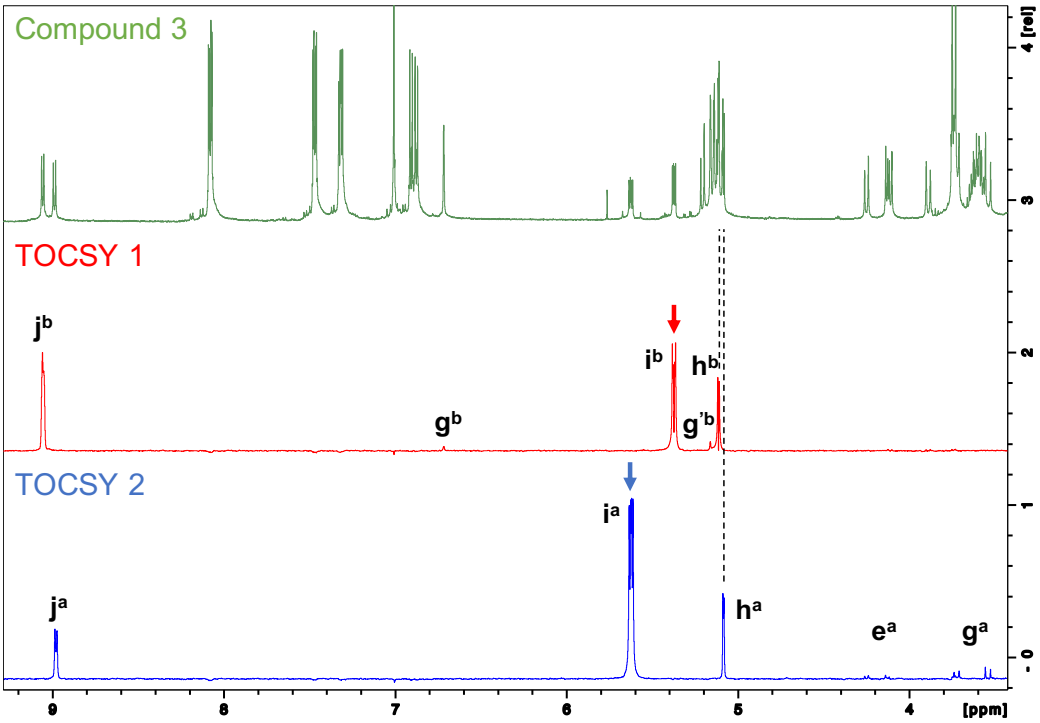
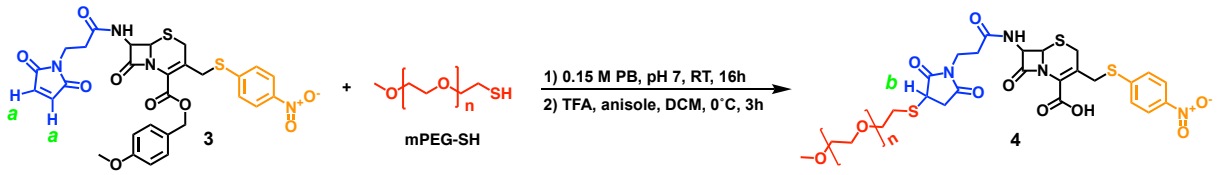


Figure S13: ^{1}H-NMR spectrum of compound **3** and two 1D selective total correlation spectroscopy (TOCSY) experiments (DMSO-d_6, 600 MHz). The arrows indicate the irradiated resonances.

10 Synthesis and purification of 4

Compound **3** (30 mg, 0.047 mmol) dissolved in DMSO (175 μL) was added to mPEG-thiol (40 mg, 0.023 mmol) dissolved in 0.15 M sodium phosphate buffer (PB; 1 mL; pH 7) and the solution was left spinning at RT for 16 hours. The product was then dialyzed in 500-1000 Da MWCO cellulose ester dialysis tubing in water for 6 hours, frozen, and lyophilized. The dried product was deprotected in a solution of TFA:anisole:DCM mixed at a 1:1:5 volumetric ratio (10.5 mL total) on ice for 3 hours. The DCM and TFA were evaporated using a Buchï rotary evaporator, then the product was precipitated and rinsed twice in cold diethyl ether (pelleted by centrifuging at 4,000 $\times$g for 5 minutes at -10°C). Lastly, conjugate **4** was dialyzed in water for 24 hours, frozen, and lyophilized.

Conjugation was confirmed using ^{1}H-NMR (**Figure S14**), MALDI-TOF MS (**Figure S15**), and SEC (**Figure S16**). Both SEC and NMR also indicated that any free, non-conjugated **3** had been successfully removed. Response to βL-BC was characterized using SEC (**Figure S16**) and UV-vis spectroscopy (**Figure S17**).



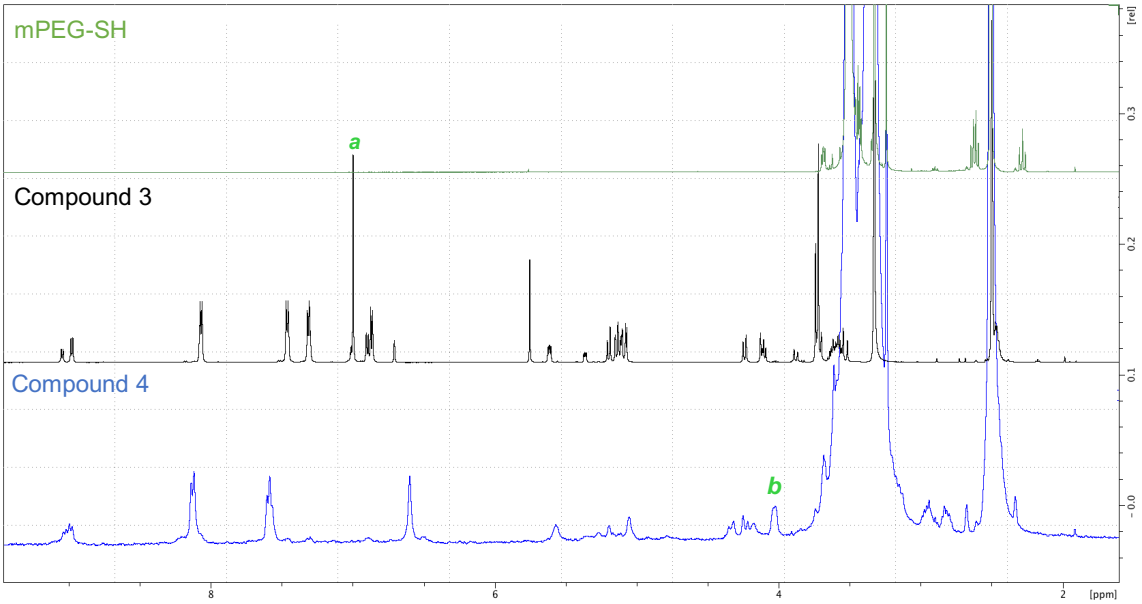


Figure S14: ^{1}H-NMR spectra of mPEG-SH, compound **3**, and compound **4** (DMSO-d_6, 400 MHz). Compound **4** spectrum indicates presence of PEG repeat units, compound **3** protons minus those of the *p*-methoxybenzyl protecting group and the maleimide protons (7.0 ppm; marked *a*). It also shows the appearance of what is expected to be one of the maleimide-thiol adduct protons (4.02 ppm; marked *b*) in compound **4**, suggesting successful conjugation. The other two protons on the adduct would likely appear in the region of 2.4 - 3.2 ppm,[7] obscured by the protons of the PEG repeat units.

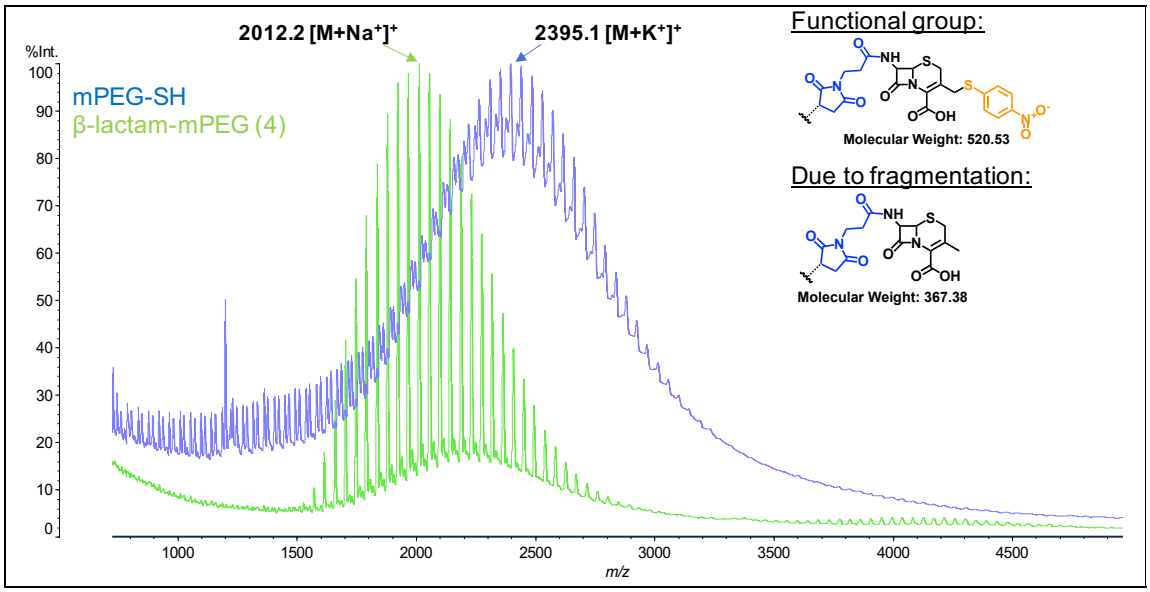


Figure S15: MALDI-TOF mass spectra of unmodified mPEG-SH (green) and β-lactam-mPEG conjugate **4** (blue) demonstrating an increase in polymer molecular weight (MW) (+ 367 Da) upon conjugation of compound **3** to mPEG-SH. The difference between the peaks is lower than the expected 520 Da (MW of deprotected compound **3**) likely due to fragmentation during ionization and the loss of the 4-nitrobenzenethiol group. Similar fragmentation has been reported in MALDI-TOF analysis of proteins and polymers with sulfur containing terminal groups and terminal groups with maximal absorbance wavelengths near the laser wavelength (here, 345 nm for 4-nitrobenzenethiol and 337 nm laser).[8–10]

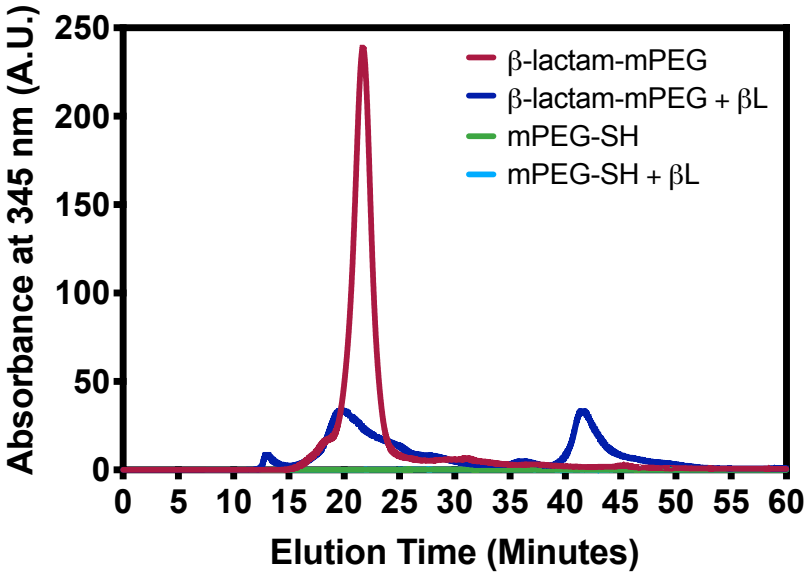


Figure S16: Size exclusion chromatograms of 1 mg/mL mPEG-SH or β-lactam-mPEG conjugate **4** indicating decreased absorbance at 345 nm for **4** incubated in 200 U/mL of βL-BC compared with incubation in 1× PBS for 3 hours at 37°C.

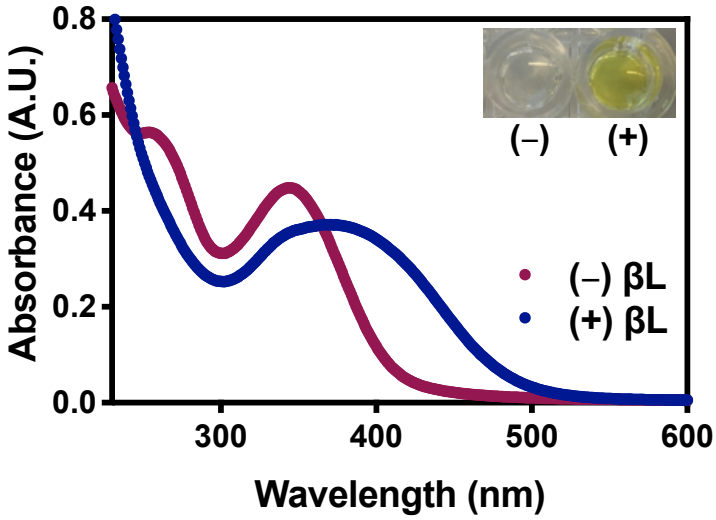


Figure S17: Spectral scan of β-lactam-mPEG **4** after incubation in 1× PBS with or without βL-BC. 450 μM of **4** was incubated with 200 U/mL of βL-BC for 3 hours at 37°C and then diluted to 0.3 mg/mL before absorbance was measured using a PerkinElmer Lambda 950 spectrophotometer. Inset: Images of **4** incubated with (+) or without (-) βL in 1× PBS.

11 Product 4 *in vitro* bacteria-responsive color change

βL-producing bacteria, *B. cereus* 13061, *E. cloacae* 13047, and *P. aeruginosa* PA01, and non-βL-producing bacteria, *E. coli* DH5-α, were grown overnight in 1× TSB, then diluted 1:1000 and grown to mid-logarithmic growth phase. **4** was dissolved in 1× PBS at 4056 μM and then serially diluted 1:2 in 50 μL of 1× PBS in a 96-well plate. 50 μL of TSB or bacteria in TSB was added to each well to a final concentration of 1×10^7 CFU/mL. The final solutions were 50% (v/v) TSB in 1× PBS and were incubated shaking (100 rpm) at 37°C for 18-24 hours before digital images of the wells were taken (**Figure 2a**).

12 β-lactam-PEG hydrogel formation and βL-responsive color change

20% (w/v) PEG hydrogels incorporating tethered substrate **3** were formed following similar previously reported procedures.[11] As depicted in **Scheme 1**, compound **3** (in DMSO) was incubated with 4-arm-PEG-SH (20 kDa) (in 0.1× PBS, pH 5) at a 4:1 thiol to maleimide molar ratio with shaking (100 rpm) at 37°C for 15 minutes. Subsequently, mal-PEG-mal (2 kDa) (in DMSO) was added to the solution at a 1:1 thiol to maleimide molar ratio to form hydrogels; the mixture (50 μL) was briefly vortexed then quickly transferred to a 5 mm circular polydimethylsiloxane (PDMS) mold, and incubated at 37°C for 45 minutes. Control non-responsive hydrogels were formulated similarly but without the addition of compound **3**. The hydrogels were rinsed in methanol and then DCM (with shaking at RT), before TFA and anisole were added (1:1:5 TFA:anisole:DCM; 3.5 mL total) to deprotect the carboxylic acid on the β-lactam substrate; hydrogels were left shaking on ice for 4 hours. After deprotection, the hydrogels were thoroughly rinsed in DCM followed by methanol. Before the hydrogels were incubated with βLs, they were rinsed and equilibrated in 1× PBS overnight at 4°C. The hydrogels were cut in half before 100 μL of either 1× PBS or 400 U/mL of βL-BC in 1× PBS was added onto the hydrogels; a color change was observed within <10 minutes (**Figure 2b**).

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