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Supporting Information

for

Development of a bioorthogonal fluorescence-based assay for assessing drug uptake and delivery in bacteria

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1 General Experimental

1.1 Materials and Reagents

Cefoxitin sodium (Actavis, USA) was purchased from the Southern District Health Board Pharmacy, New Zealand. 1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 3β-[N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-Cholesterol·HCl), and 1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG (2000) amine) were obtained from Avanti Polar Lipids, USA. Propidium monoazide (PMA **4**, 20 mM in H_2O) was purchased from Biotium. L-rhamnose monohydrate, sodium azide (NaN_3), hexafluorophosphate azabenzotriazole tetramethyl uranium (HATU), ethylcarbodiimide hydrochloride, 3-azido-1-propanamine and 5-Cholesten-3β-ol were all purchased from Sigma Aldrich, USA. 4-azido-1-butanamine and 3-Azido-7-hydroxycoumarin (7N3HC **3**) were obtained from AK Scientific, Inc. Bicyclo [6.1.0] nonyne (BCN)-biotin **1** was developed and synthesised by Dr Spangler of Novartis Institutes for BioMedical Research, USA.[1] Tetrazine-BDP-FL **2** was obtained from Jena Bioscience, Germany. All other solvents and reagents were obtained from Sigma Aldrich or AK Scientific, USA.

E. coli strains, wild-type (WT, *E. coli* K-12 (BW25113, Coli Genetic Stock Center, Yale)) or isogenic tolC knock out (Δ*tolC*, *E. coli* K-12 *ΔtolC732::kan* (JW5503-1, Coli Genetic Stock Center, Yale)) modified to express streptavidin in either the periplasm or cytoplasm, were supplied by Novartis Institutes for BioMedical Research (NIBR) in Emeryville, California, United States.

Reaction solvents were purchased dry from Sigma-Aldrich, Thermo Fischer Scientific or Merck. Thin layer chromatography (TLC) was performed on 0.2 mm aluminium-backed silica gel plates 60 F_{254}, and visualised with UV light (λ = 254 nm) or basic $KMnO_4$ dip. Flash column chromatography was carried out using 40-63 µm silica gel, with AR or liquid chromatography grade solvents.

1.2 Instrumentation

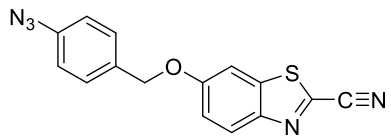
Fluorescence intensity was measured on a CLARIOstar plate reader or a POLARstar Omega Microplate reader (BMG Labtech, Germany). Reversed-phase liquid chromatography mass spectrometry was conducted on an Agilent 1290 UPLC coupled to Agilent 6550 ESI-QTOF with a Phenomenex Luna C8 column (5 µm particle, 100 Å pore size, 2 × 50 mm) with an Agilent Zorbax SB-C8, 5 µm particle, 2.1 × 12.5 mm guard column using gradient elution from 0-99% methanol in water (+ 0.1% formic acid). Expected masses of compounds were calculated using ChemBioDraw Ultra software and analysed on Skyline software.

High resolution electrospray ionisation mass spectra (HRMS-ESI) were done on a microTOFQ mass spectrometer. Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectroscopy were carried out using a 400 MHz or 500 MHz Varian NMR spectrometer. Chemical shifts are listed on the δ scale in part(s) per million (ppm) and the coupling constants (*J*), recorded in hertz (Hz). Signal multiplicities are assigned as: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; or m, multiplet. Attenuated total reflection-fourier transform infrared spectroscopy (ATR-FTIR) was carried out using a Varian 3100 FTIR (Excalibur series) instrument equipped with an attenuated total reflection accessory (GladiATR, Piketech, USA). Samples were clamped directly on to the ATR diamond crystal and the spectra were recorded over a range of 400-4000 cm^{-1} as the mean of 64 scans with a resolution of 4 cm^{-1}. The data were then analysed using Varian Resolution Pro, v4.1.0.101 software.

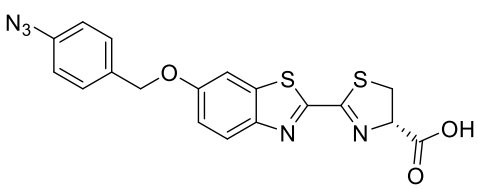
2 Synthesis

The synthesis of BCN-PEG2-Biotin **1** and it's click-generated product with benzyl azide (internal control) were synthesised and supplied by Dr Spangler as previously reported.[1] The synthesis of coumarin compounds, N3PC **5**, N3BC **6**, N34FBMeC **7**, and N34FBC **8** were conducted as previously reported.[2, 3] The synthesis of 6-[(4-azidobenzyl) oxy]-luciferin **9** and azido-cefoxitin **10** were conducted as described below.

2.1 Synthesis of 6-[(4-azidobenzyl) oxy]-luciferin (9)



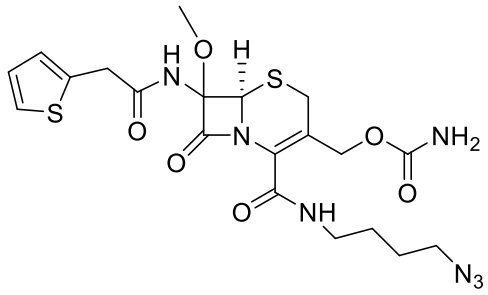
6-[(4-azidobenzyl)oxy]benzo[d]thiazole-2-carbonitrile (9a). 4-Azidobenzyl mesylate[2, 3] (0.065 g, 0.28 mmol) in 2 mL of acetonitrile was added dropwise to 2-cyano-6-hydroxy-benzothiazole (0.068 g, 0.39 mmol) and potassium carbonate (0.107 g, 0.78 mmol) in 6 mL of acetonitrile and left to stir at room temperature, in the dark, under nitrogen, for 5 days. Acetonitrile was then evaporated and the crude residue was resuspended in ethyl acetate (EtOAc; 100 mL), washed with water (3×50 mL) and brine (50 mL) and dried over $MgSO_4$ and purified by silica gel chromatography (30% EtOAc in hexane) to give the pure title compound (0.034 g, 39%), which was spectroscopically similar to that previously reported in the literature.[4] ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.10 (d, *J* = 9.0 Hz, 1H), 7.44 (d, *J* = 8.5 Hz, 2H), 7.41 (d, *J* = 2.5 Hz, 1H), 7.30 (dd, *J* = 9.0, 2.5 Hz, 1H), 7.08 (d, *J* = 8.5 Hz, 2H), 5.14 (s, 2H).



6-[(4-azidobenzyl) oxy]-luciferin (9). Prepared using a modified version of a literature procedure.[4, 5] 6-[(4-azidobenzyl)oxy]benzo[d]thiazole-2-carbonitrile (0.030 g, 0.098 mmol) and D-cysteine hydrochloride monohydrate (0.020 g, 0.114 mmol) were suspended in methanol:water 2:1 (3 mL). To this was added potassium carbonate (0.013 g, 0.094 mmol) and the reaction was allowed to stir at room temperature, under nitrogen, until the nitrile was completely consumed (observation via TLC ~3 hours). The solution was then made acidic (pH 2) with 5 M HCl and extracted with EtOAc (3×50 mL). The combined organic layers were washed with brine (50 mL) and dried over $MgSO_4$ and concentrated *in vacuo* to afford the pure title product as a white powder (0.024 g, 60%), which was spectroscopically similar to that previously reported in the literature.[4] ^{1}H NMR (400 MHz, dmso-d_6) δ 8.06 (d, *J* = 9.0 Hz, 1H), 7.85 (d, *J* = 2.6 Hz, 1H), 7.53 (d, *J* = 8.5 Hz, 2H), 7.26 (dd, *J* = 9.0, 2.6 Hz, 1H), 7.15 (d, *J* = 8.5 Hz, 2H), 5.42 (dd, *J* =

9.8, 8.3 Hz, 1H), 5.19 (s, 2H), 3.93 – 3.46 (m, 2H). ^{13}C NMR (100 MHz, dmso-d_6) δ 171.2, 164.4, 158.0, 157.8, 147.2, 139.2, 137.1, 133.4, 129.8, 124.8, 119.2, 117.5, 105.9, 78.1, 69.4, 34.7. IR: vmax/cm^{-1} 2915, 2119, 1734, 1205, 810.

2.2 Synthesis of azido-cefoxitin 10



(4-azidobutyl)-cefoxitin (10). Cefoxitin sodium (2.7 g, 6.0 mmol) and DMF (10 mL) were added into a round-bottomed flask. HATU (2.3 g, 6.0 mmol) was added and the reaction mixture was stirred at room temperature for 25 min. 4-azidobutanamine (0.53 g, 4.6 mmol) pre-dissolved in DMF (2 mL) was added dropwise over 2 min. The reaction mixture was stirred at room temperature overnight. Water was added to the mixture and the aqueous layer was extracted 3 times with EtOAc. The pooled organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified using flash column chromatography with silica gel (following column elution with 10-80% EtOAc in hexane) to give azido cefoxitin (1.8 g, 4.02 mmol, 67.5 % yield) as a pale-yellow liquid. ^{1}H NMR (400 MHz, MeOD): δ 7.28 (dd, J = 5.2, 1.6 Hz, 1H), 7.00-6.98 (m, 1H), 6.97-6.94 (m, 1H), 6.49 (m, 1H), 5.32 (s, 1H), 4.87 (s, 1H), 4.55-4.46 (m, 2H), 3.91-3.80 (m, 2H), 3.45 (s, 3H), 3.35-3.19 (m, 4H), 1.64-1.59 (m, 4H). ^{13}C NMR (100 MHz, MeOD): δ 174.0, 169.4, 162.1, 159.3, 137.0, 128.0, 127.8, 126.0, 124.3, 121.3, 97.4, 66.9, 61.5, 53.8, 52.4, 52.1, 40.2, 37.4, 27.5, 27.3. HRMS (ESI) calculated for $C_{20}H_{25}NaN_7O_6S_2^+$ [M+Na]$^+$ 546.1200 and $C_{20}H_{25}KN_7O_6S_2^+$ [M+K]$^+$ 562.0939; found *m/z* 546.1180 and 562.0916. IR: vmax/cm^{-1} 3400-3200, 2094, 1650, 1323, 1051, 800.

3 Supplementary Figures

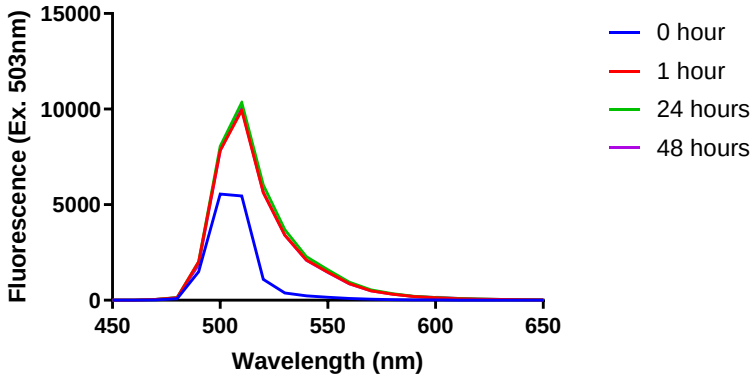


Fig. S1 Emission spectra of tetrazine-BDP-FL **2** (1 µM in PBS pH 7.4) before (0 hour) and after incubation with 20 times excess BCN-biotin **1**.

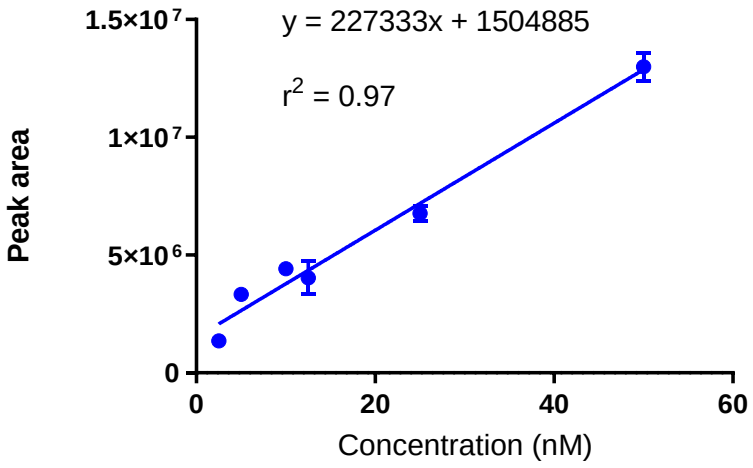


Fig. S2 *In vitro* standard curve for BCN-biotin **1** in *E. coli* lysate for the ions $[M+H]^+$ and $[M+Na]^+$ analysed via LCMS (*n*=3, mean ± SD).

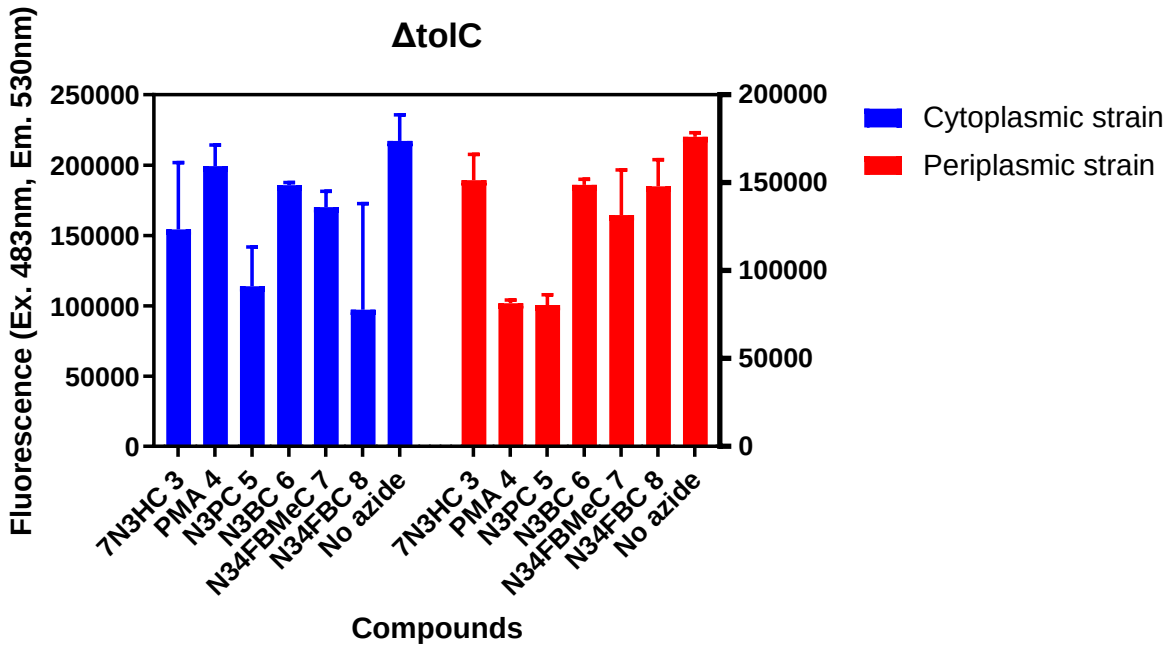


Fig. S3 Tetrazine-BODIPY-Fl **2** fluorescence measurements for azides or no azide treated cells, which have incorporated BCN-PEG$_2$-Biotin reporter **1** in in either periplasmic or cytoplasmic compartments of Δ*tolC E. coli* (*n*=3, mean ± SD).

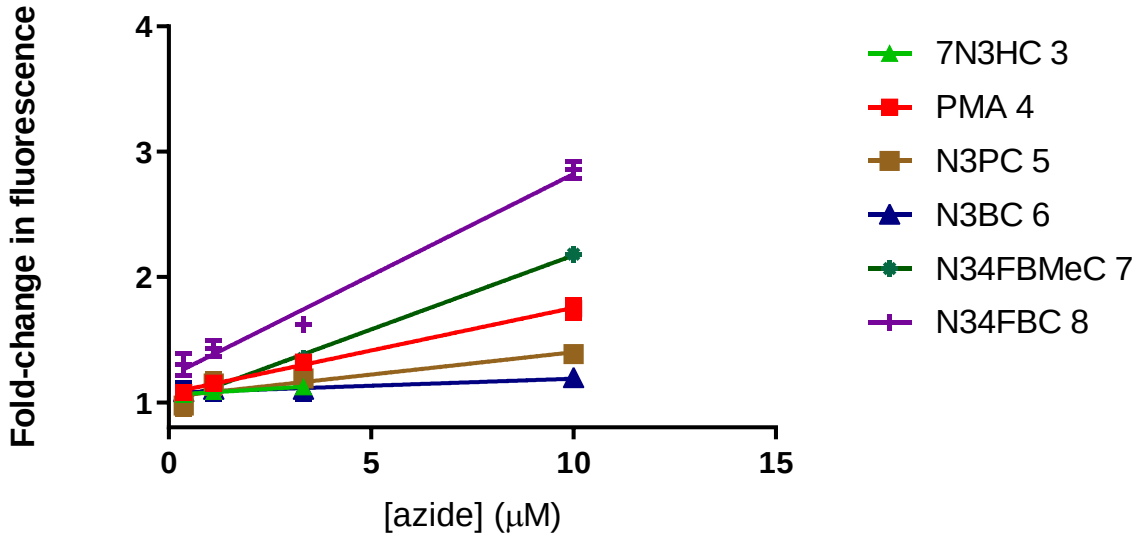


Fig. S4 *In vitro* reactivity curves for click reaction between azido compounds and BCN-biotin **1** shown as a function of fold change in fluorescence over no azide control (*n*=3, mean ± SD).

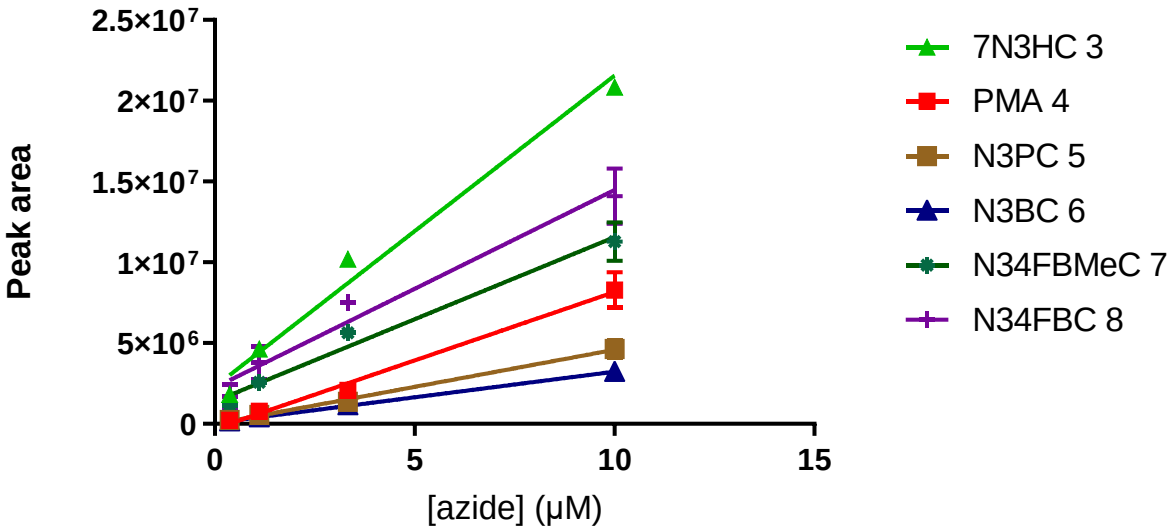


Fig. S5 *In vitro* reactivity curves for click reaction between azido compounds and BCN-biotin **1** shown as a function of MS peak area normalised to internal control (*n*=3, mean ± SD).

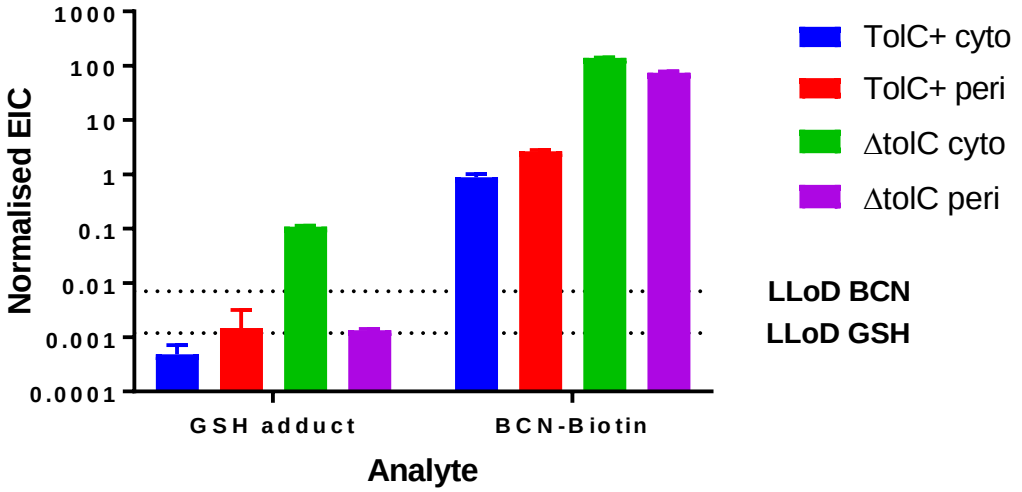


Fig. S6 Comparison of GSH-BCN adduct analyte levels against BCN-biotin **1** incorporation. The lower limits of detection (LLoD) for each species were determined from non-BCN reporter treated controls. Peak areas were normalised to OD_{600} and the internal control (*n*=3, mean ± SD).

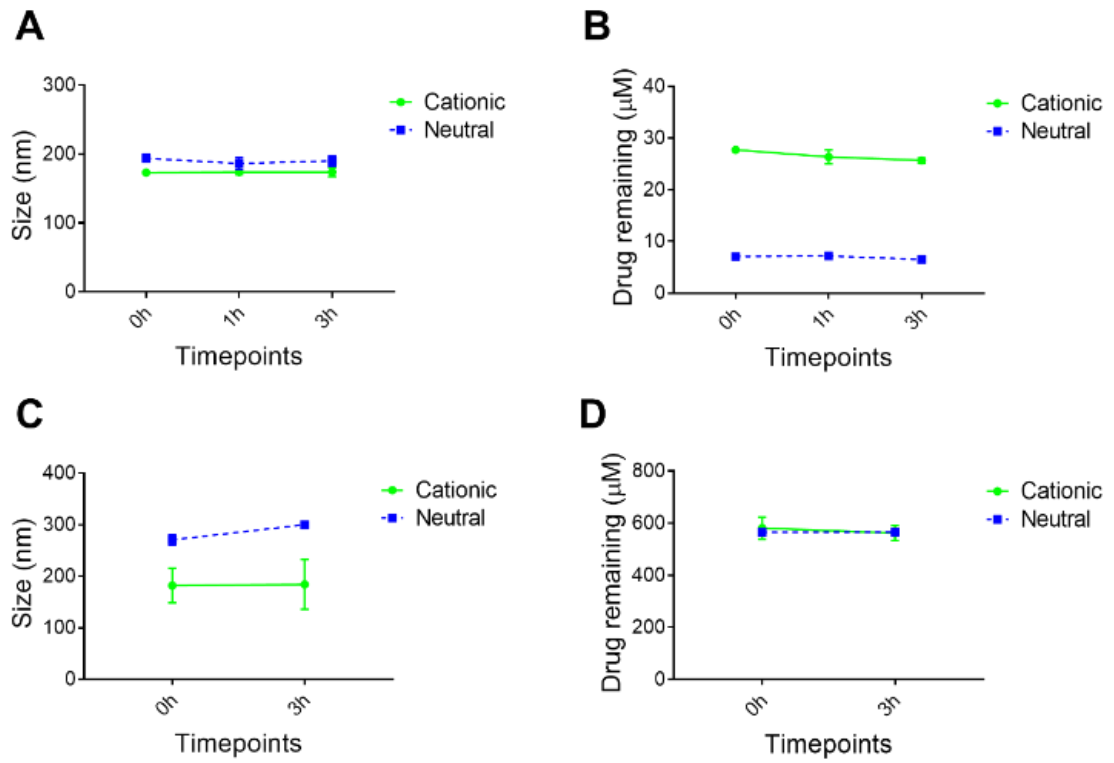


Fig. S7 *In vitro* stability of cationic and neutral azidobenzyl-luciferin **9** (A-B) and azido-cefoxitin **10** (C-D) liposomes over 3 hours of incubation at 37 °C; size and drug loading in liposomes are shown (*n*=3, mean ± SD).

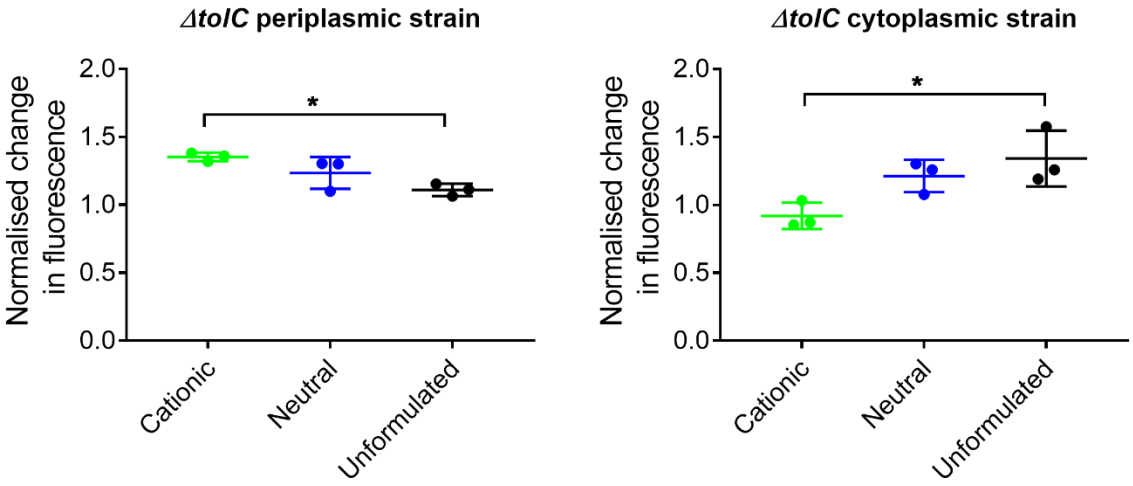


Fig. S8 Δ*tolC E. coli* strains treated with free 6-[(4-azidobenzyl) oxy]-luciferin **9** (unformulated) or with 6-[(4-azidobenzyl) oxy]-luciferin **9** formulated into cationic or neutral liposomes for 5 hours. Data has been normalised to a PBS control and analysed using one-way ANOVA followed by Dunnett's multiple comparison post hoc test (*n*=3, mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001).

4 Supplementary Tables

Table S1 Statistical significance of differences in compartment-specific compound relative uptake and accumulation compared to 7N3HC **3** utilising fluorescence assay (Figure 8).

Dunnett's multiple comparisons test	**Mean Diff.**	**95.00% CI of diff.**	**Below threshold?**	**Summary**	**Adjusted P Value**
Δ*tolC* cyto					
7N3HC **3** vs. PMA **4**	0.8189	0.4502 to 1.188	Yes	****	<0.0001
7N3HC **3** vs. N3PC **5**	0.3203	-0.04837 to 0.6889	No	ns	0.1040
7N3HC **3** vs. N3BC **6**	-0.1527	-0.5214 to 0.2159	No	ns	0.6971
7N3HC **3** vs. N34FBMeC **7**	0.8292	0.4606 to 1.198	Yes	****	<0.0001
7N3HC **3** vs. N34FBC **8**	0.7019	0.3333 to 1.071	Yes	***	0.0002
Δ*tolC* peri					
7N3HC **3** vs. PMA **4**	0.4851	0.1165 to 0.8537	Yes	**	0.0071
7N3HC **3** vs. N3PC **5**	-0.04470	-0.4133 to 0.3239	No	ns	0.9969
7N3HC **3** vs. N3BC **6**	-0.8494	-1.218 to -0.4808	Yes	****	<0.0001
7N3HC **3** vs. N34FBMeC **7**	0.7051	0.3365 to 1.074	Yes	***	0.0001
7N3HC **3** vs. N34FBC **8**	0.7965	0.4279 to 1.165	Yes	****	<0.0001

Table S2 Statistical significance of differences in compartment-specific compound relative uptake and accumulation compared to 7N3HC **3** utilising mass spectrometry assay (Figure 8).

Dunnett's multiple comparisons test	**Mean Diff.**	**95.00% CI of diff.**	**Below threshold?**	**Summary**	**Adjusted P Value**
Δ*tolC* cyto					
7N3HC **3** vs. PMA **4**	0.9878	0.3584 to 1.617	Yes	**	0.0013
7N3HC **3** vs. N3PC **5**	-0.9343	-1.564 to -0.3049	Yes	**	0.0024
7N3HC **3** vs. N3BC **6**	-0.9295	-1.559 to -0.3001	Yes	**	0.0025
7N3HC **3** vs. N34FBMeC **7**	0.6817	0.05225 to 1.311	Yes	*	0.0306
7N3HC **3** vs. N34FBC **8**	0.6831	0.05367 to 1.312	Yes	*	0.0302
Δ*tolC* peri					
7N3HC **3** vs. PMA **4**	0.6221	-0.007315 to 1.252	No	ns	0.0535
7N3HC **3** vs. N3PC **5**	-0.6909	-1.320 to -0.06146	Yes	*	0.0280
7N3HC **3** vs. N3BC **6**	-1.550	-2.180 to -0.9211	Yes	****	<0.0001
7N3HC **3** vs. N34FBMeC **7**	0.03031	-0.5991 to 0.6597	No	ns	0.9998
7N3HC **3** vs. N34FBC **8**	0.3598	-0.2696 to 0.9892	No	ns	0.4160

5 NMR Spectra

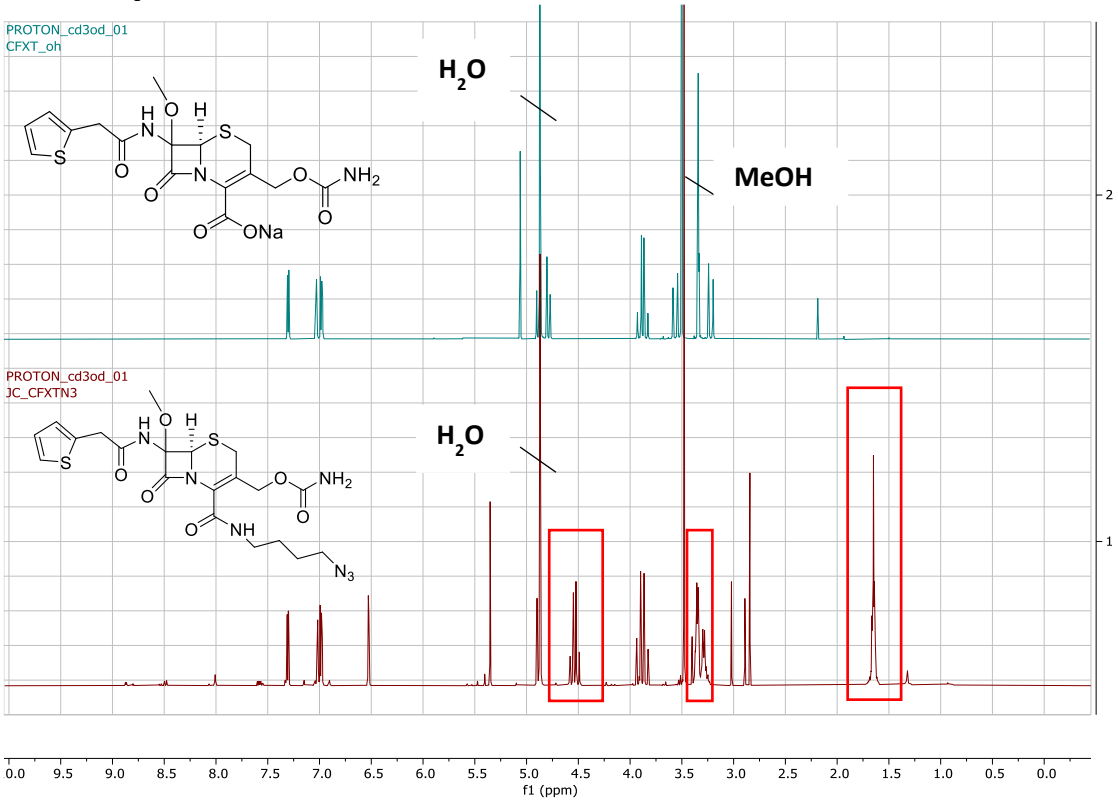


Fig. S9 400 MHz ^{1}H NMR spectra of cefoxitin (top) and azido-cefoxitin **10** (bottom) in MeOD. The red boxes indicate the proton peaks associated with 4-azido-butanamine.

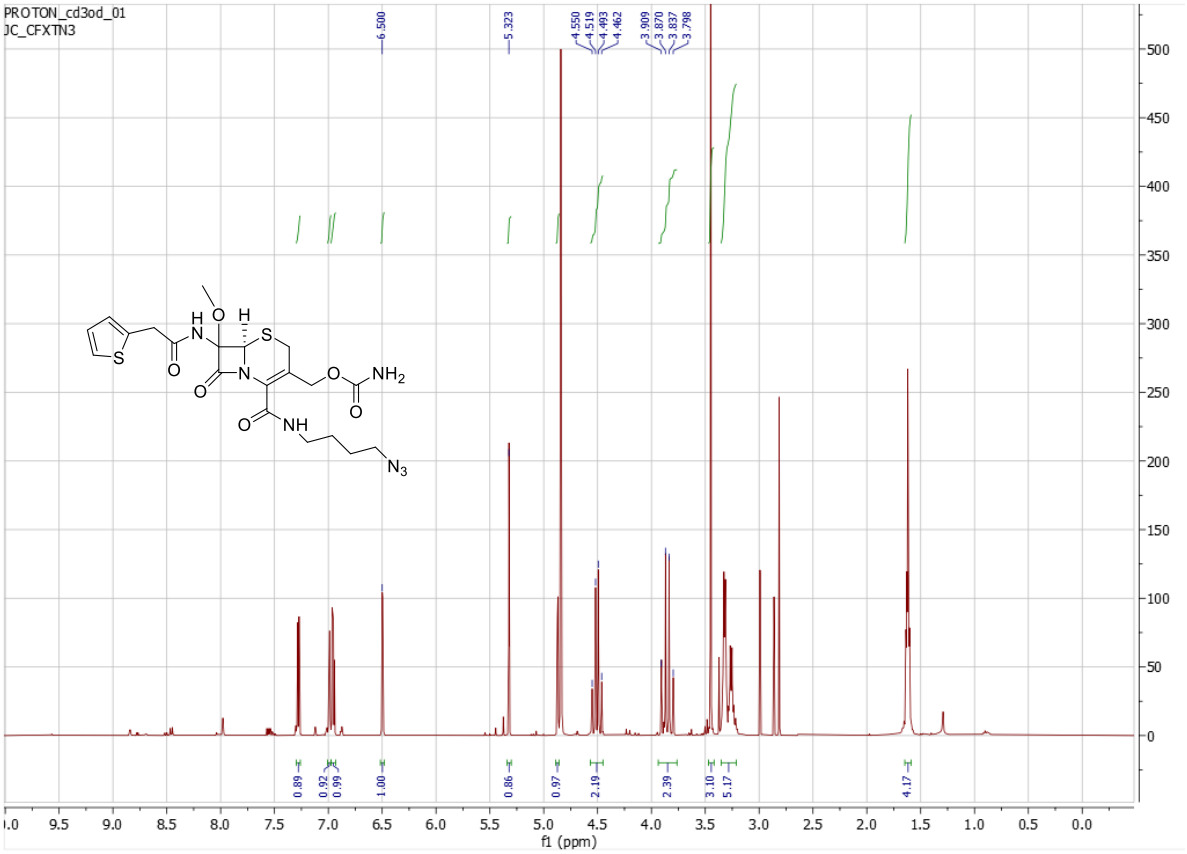


Fig. S10 400 MHz ^{1}H NMR spectra of azido-cefoxitin **10** in MeOD.

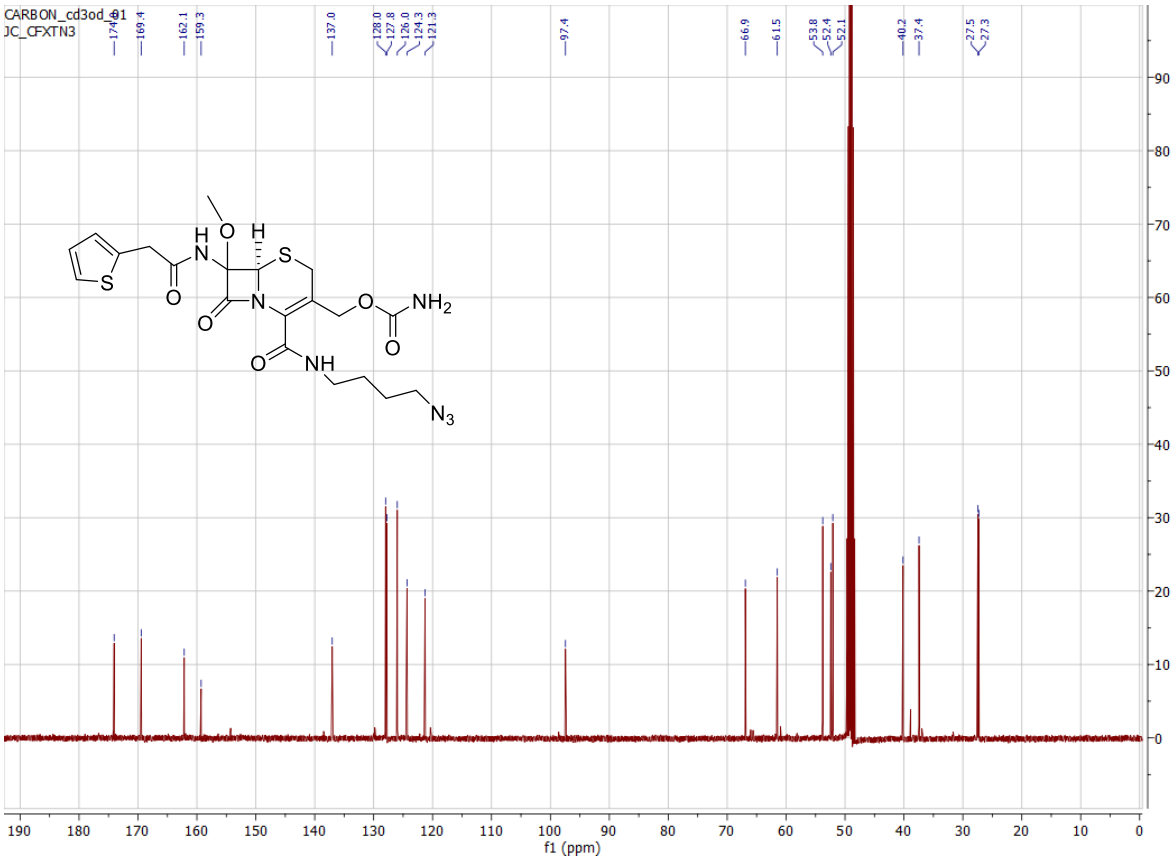


Fig. S11 100 MHz ^{13}C NMR Spectra of azido-cefoxitin **10** in MeOD.

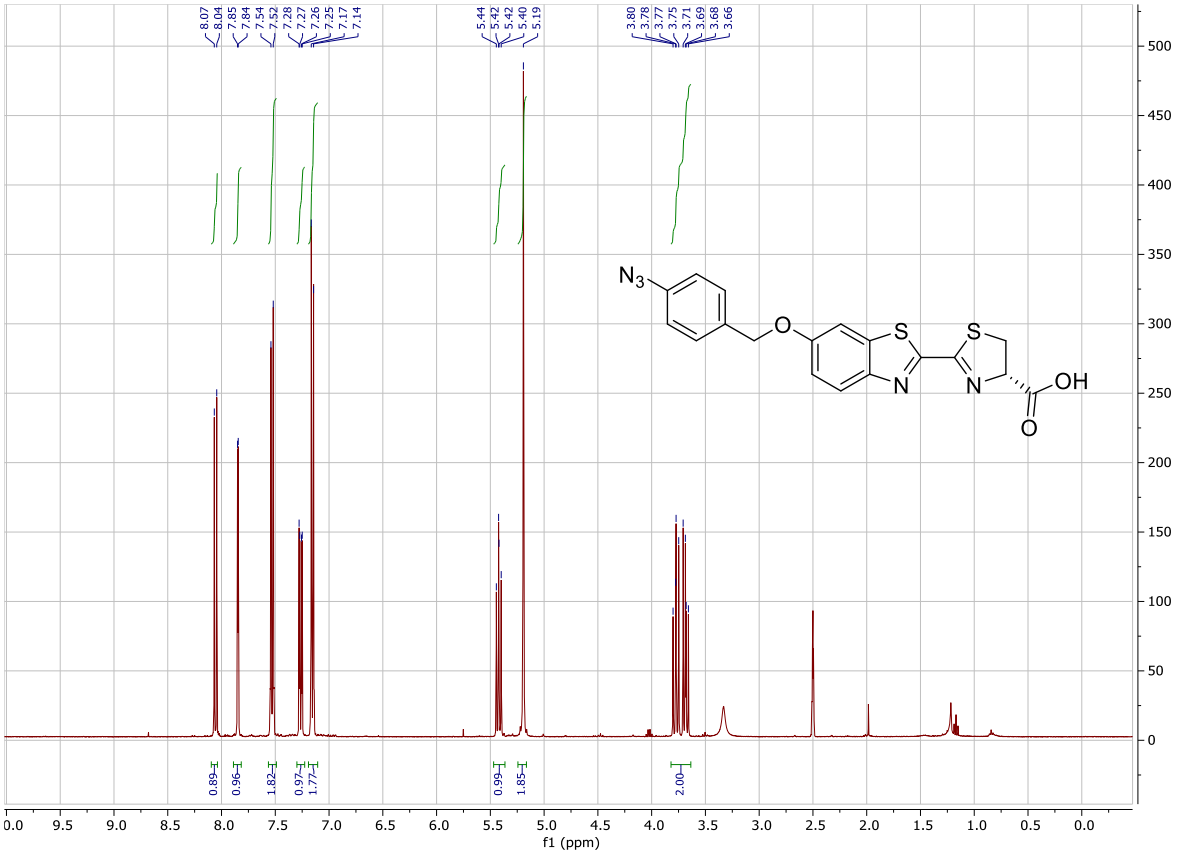


Fig. S12 400 MHz ^{1}H NMR Spectra of 6-[(4-azidobenzyl) oxy]-luciferin **9** in dmso-*d6*.

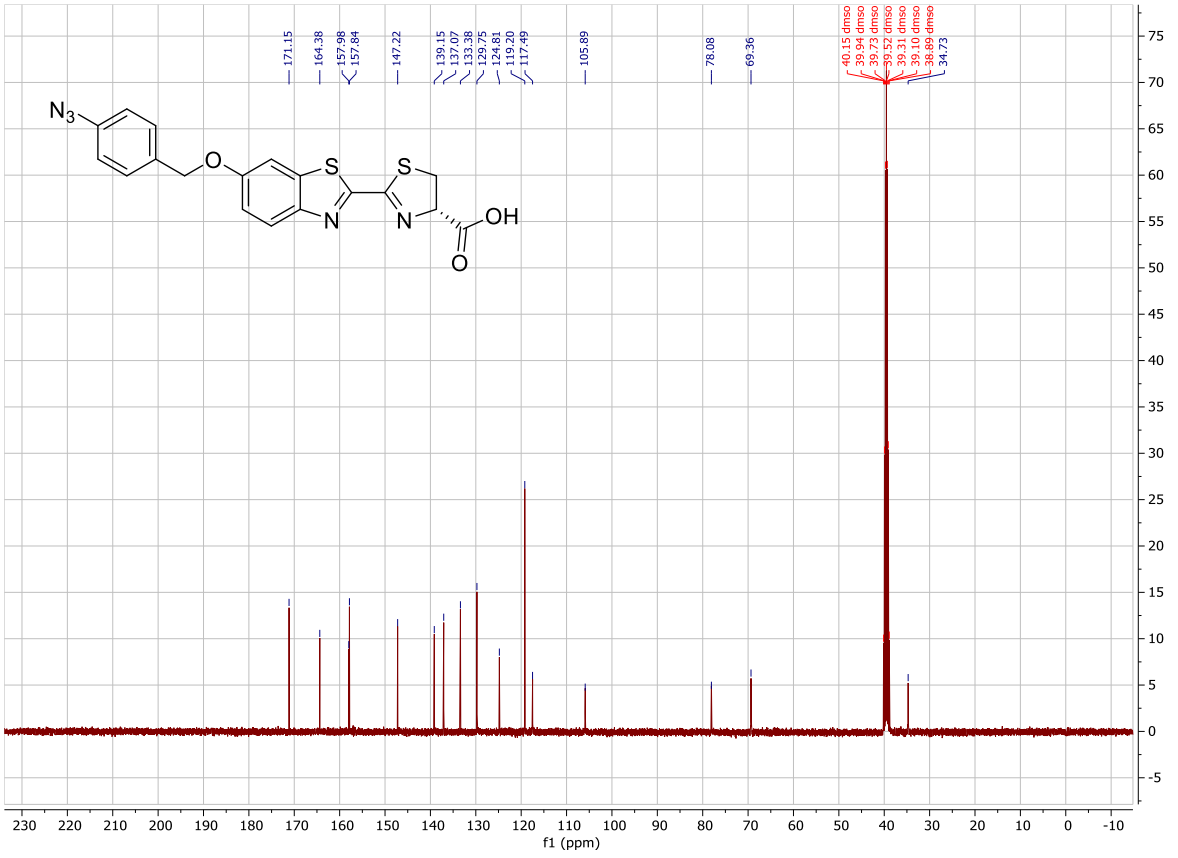


Fig. S13 100 MHz ^{13}C NMR Spectra of 6-[(4-azidobenzyl)oxy]-luciferin **9** in dmso-*d6*.

6 FTIR Spectra

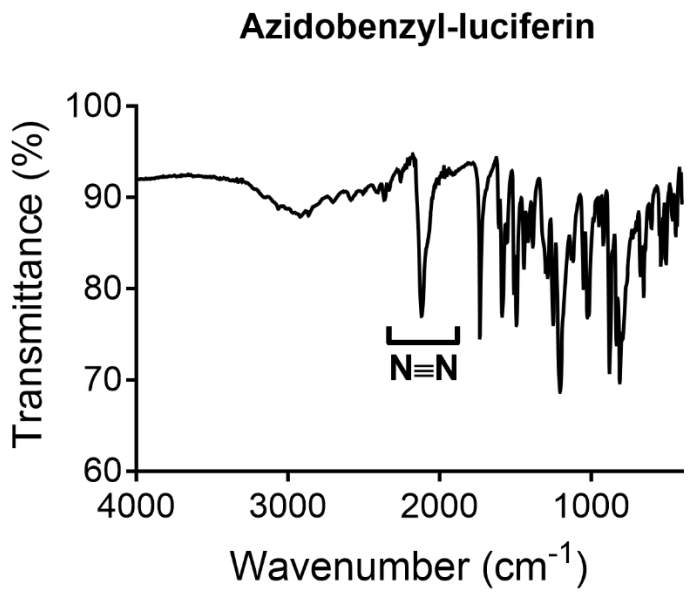


Fig. S14 Fourier-transform infrared spectroscopy (FTIR) spectra of azidobenzyl-luciferin **9**. Peak at 2100 cm^{-1} correspond to the azide group present in the compound.

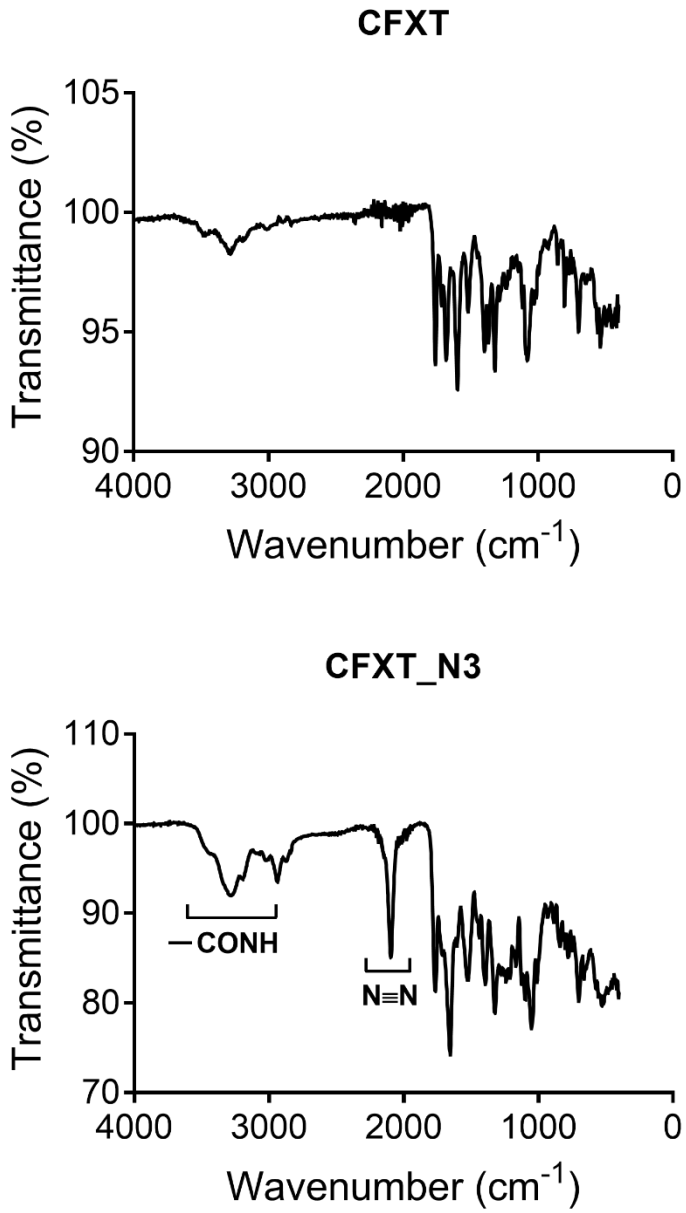


Fig. S15 Fourier-transform infrared spectroscopy (FTIR) spectra of cefoxitin (CFXT) and azido-cefoxitin **10** (CFXT_N_3). Peaks at 3200 cm^{-1} and 2100 cm^{-1} correspond to amide and azide group present in the successfully modified cefoxitin.

7 References

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