Supporting Information for:

Galbofloxacin: A Xenometal-Antibiotic with in vitro and in vivo Potent Efficacy Against *S. aureus*

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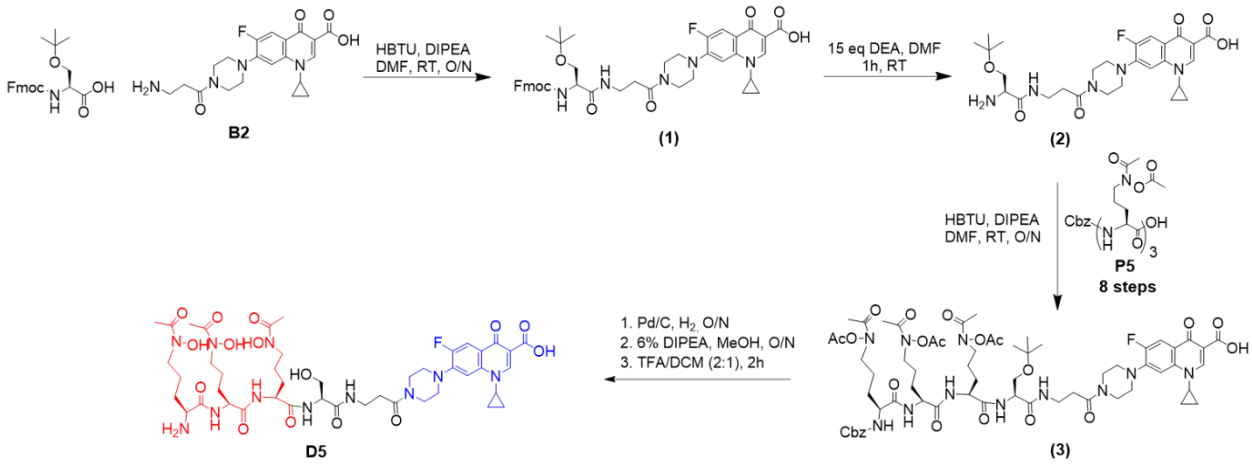
1. Chemical synthesis and characterization

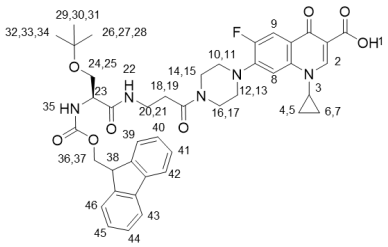
1.1 Synthesis protocols

All starting materials were purchased from Acros Organics, Alfa Aesar, Sigma Aldrich, or TCI America and used without further purification. NMR spectra (^{1}H and ^{13}C) were collected on a 700 MHz Advance III Bruker, 500 MHz or 400 MHz Bruker instrument at 25 °C and processed using TopSpin 3.5pl7. ^{19}F NMR were collected on a 400 MHz Bruker instrument at 25 °C using TFA as an internal standard (δ: -76 ppm). Chemical shifts are reported as parts per million (ppm). Mass spectrometry: low-resolution electrospray ionization (ESI) mass spectrometry and high-resolution (ESI) mass spectrometry was carried out at the Stony Brook University Institute for Chemical Biology and Drug Discovery (ICB&DD) Mass Spectrometry Facility with an Agilent LC/MSD and Agilent LC-UV-TOF spectrometers, respectively. UV-VIS spectra were collected with the NanoDrop 1C instrument (AZY1706045). Spectra were recorded from 190 to 850 nm in a quartz cuvette with 1 cm path length. HPLC: Preparative HPLC was carried out using a Shimadzu HPLC-20AR equipped with a Binary Gradient, pump, UV-Vis detector, manual injector on a Phenomenex Luna C18 column (250 mm×21.2 mm, 100 Å, AXIA packed). Method A (preparative purification method): A = 0.1% TFA in water, B = 0.1% TFA in MeCN. Gradient: 0-5 min: 95% A. 5-24 min: 5−95% B gradient. Method B (preparative purification method): A = 10 mM sodium acetate (pH = 4.5) in water, B = 100% MeCN. Gradient: 0-5 min: 95% A. 5-24 min: 5−95% B gradient. Analytical HPLC analysis was carried out using a Shimadzu HPLC-20AR equipped with a binary gradient, pump, UV-Vis detector, autoinjector and Laura radio detector on a Gemini-NX C18 column (100 mm×3 mm, 110 Å, AXIA packed). Method C: A = 0.1% TFA in water, B = 0.1% TFA in MeCN with a flow rate of 0.8 mL/min, UV detection at 254 and 280 nm. Gradient: 0-2 min: 95% A. 2-14 min: 5−95% B gradient. 14-16 min: 95% B. 16-16.6 min: 95-5% B. 16.5-20 min: 5% B. Method D (analysis of ^{67}Ga/Ga complexes): A = 10 mM sodium acetate (pH = 4.5) in water, B = 100% MeCN. Gradient: 0-2 min: 95% A. 2-14 min: 5−95% B gradient. 14-16 min: 95% B. 16-16.6 min: 95-5% B. 16.5-20 min: 5% B, UV detection at 254 and 280 nm. Purity of all intermediates and final products including radiochemical species was determined using analytical HPLC. All conjugates and complexes were ≥95% pure. ICP-OES was carried out using an Agilent 5110 ICP-OES. A 10-point standard with respect to gallium and iron was used and lines of best fit were found with R2 of 0.999. Sample concentration was determined based on this calibration curve.7-(4-(3-aminopropanoyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid, **B2 (Fragment A)** and (8S,11S,14S)-11,14-bis(3-(N-acetoxyacetamido)propyl)-4-acetyl-8-(((benzyloxy)carbonyl)amino)-2,9,12-trioxo-3-oxa-4,10,13-triazapentadecan-15-oic acid, **P5** were synthesized according to previously published procedures. [1,2]

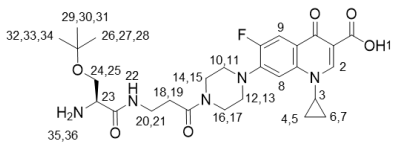


Scheme 1. Functionalization of ciprofloxacin, subsequent conjugation to protected hydroxamate fragment P5 followed by sequential deprotection steps yields the Conjugate **D5**.

(1) (S)-7-(4-(3-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(tert-butoxy)propanamido) propanoyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid. Fmoc-O-tert-butyl-L-serine (0.039 g, 0.103 mmol, 1 eq) was dissolved in DMF (2 mL) and DIPEA (54.2 µL, 0.310 mmol, 3 eq) was added. After 2 minutes, HBTU (0.058 g, 0.155 mmol, 1.5 eq) was added to the reaction mixture followed by 7-(4-β-Alanyl-1-piperazinyl)-1-cyclopropyl-6-fluoro-4-oxo-1H-quinoline-3-carboxylic acid (0.050 g, 0.124 mmol, 1.2 eq) dissolved in DMF (2 mL). The reaction mixture was stirred overnight at 40°C. Solvent was removed *in vacuo* and product was purified using preparative HPLC (Method **A**, product elutes at 100% B) to afford **1** (0.044 g, 0.058 mmol, 56%) as a yellow solid. Calculated mass for **1** ($C_{42}H_{46}FN_5O_8$): 767.3; found 768.3 $[M+H]^+$. ^1H NMR (700 MHz, DMSO-d_6): δ 1.11 (s, 9H, H-26, H-27, H-28, H-29, H-30, H-31, H-32, H-33, H-34), 1.13-1.15 (dd, 2H, H-4, H-5), 1.27-1.30 (dd, 2H, H-6, H-7), 2.49-58 (t, 2H, H-18, H-19), 3.20-3.36 (m, 6H, H-10, H-11, H-12, H-13, H-20, H-21), 3.42-3.49 (m, 2H, H-14, H-15), 3.61-3.69 (m, 4H, H-16, H-17, H-24, H-25), 3.73-3.77 (m, 1H, H-3), 4.02-4.05 (t, 1H, H-23), 4.16-4.20 (s, 2H, H-36, H-37), 4.25 (d, 1H, H-38), 7.29-7.30 (m, 2H, H-39, H-46), 7.36-7.40 (m, 4H, H-40, H-41, H-44, H-45), 7.92 (d, 1H, H-9), 7.50 (d, 1H, H-8), 7.82-7.86 (m, 3H, H-35, H-42, H-43), 8.50 (t, 1H, H-22), 8.62 (s, 1H, H-2), 15.10 (s, 1H, H-1, not included in the spectrum). ^{19}F NMR (376 MHz, DMSO-d_6,): δ -122.71. Retention time (Method C): 12.28 min (purity/peak area: > 99%).

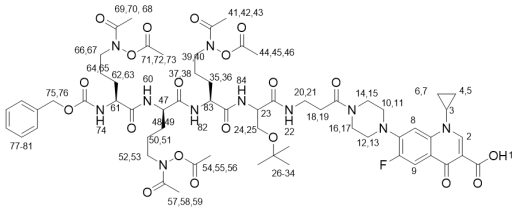


(2) (S)-7-(4-(3-(2-amino-3-(tert-butoxy)propanamido)propanoyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid. Compound **1** (0.044 g, 0.058 mmol, 1 eq) was dissolved in DMF (2 mL) and DEA (91 µL, 0.880 mmol, 15 eq) was added. The reaction was stirred at room temperature for 1 h. Solvent was removed *in vacuo* and product was purified using preparative HPLC (Method **A**, product elutes at 55% B) to afford compound **2** (0.030 g, 0.056 mmol, 96%) as a yellow solid. Calculated mass for **2** ($C_{27}H_{36}FN_5O_6$): 545.2; found 546.3 $[M+H]^+$. ^1H NMR

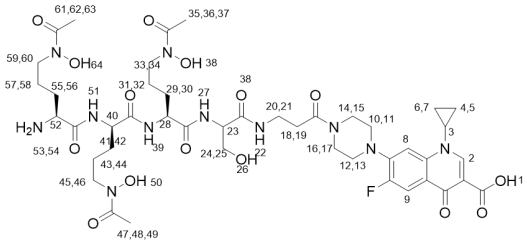


(700 MHz, DMSO-d_6):): δ 1.13 (s, 9H, H-26, H-27, H-28, H-29, H-30, H-31, H-32, H-33, H-34), 1.16-1.20 (dd, 2H, H-4, H-5), 1.29-1.33 (dd, 2H, H-6, H-7), 2.58-2.61 (t, 2H, H-18, H-19), 3.30-3.45 (m, 6H, H-10, H-11, H-12, H-13, H-20, H-21), 3.54-3.67 (m, 4H, H-14, H-15, H-16, H-17), 3.85-3.86 (m, 1H, H-3), 3.83-3.79 (t, 1H, H-23), 3.67-3.74 (dd, 2H, H-24, H-25), 7.92 (d, 1H, H-9), 7.56 (d, 1H, H-8), 8.10 (s, 2H, H-35, H-36), 8.50 (t, 1H, H-22), 8.65 (s, 1H, H-2), 15.20 (s, 1H, H-1, not included in the spectrum). ^{19}F NMR (376 MHz, DMSO-d_6,): δ -123.56. Retention time (Method C): 8.03 min (purity/peak area: > 99%).

(3) 7-(4-((8S,11S,14S,17S)-11,14-bis(3-(N-acetoxyacetamido)propyl)-4-acetyl-8-(((benzyloxy) carbonyl)amino)-17-(tert-butoxymethyl)-2,9,12,15,18-pentaoxo-3-oxa-4,10,13,16,19-pentaazadocosan-22-oyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid. **P5** (0.030 g, 0.037 mmol, 1 eq) was dissolved in DMF (2 mL) and DIPEA (20 µL, 0.113 mmol, 3 eq) was added. After 2 minutes, HBTU (0.021 g, 0.056 mmol, 1.5 eq) was added to the reaction mixture followed by compound **2** (0.024 g, 0.045 mmol, 1.2 eq) dissolved in DMF (2 mL). The reaction mixture was stirred overnight at 40ºC. Solvent was removed *in vacuo* and product was purified by preparative HPLC (Method **A**, product elutes at 76% B) to afford **3** (0.023 g, 0.018 mmol, 48%) as a yellow solid. Calculated mass for compound **3** ($C_{62}H_{84}FN_{11}O_{20}$): 1321.5; found 661.9 $[M+2H]^{2+}$. ^{1}H NMR (700 MHz, DMSO-d_6): δ 1.08 (s, 9H, H-26, H-27, H-28, H-29, H-30, H-31, H-32, H-33, H-34), 1.15-1.19 (dd, 2H, H-4, H-5), 1.22-1.33 (dd, 2H, H-6, H-7), 1.44-1.69 (m, 12H, H-35, H-36, H-37, H-38, H-48, H-49, H-50, H-51, H-62, H-63, H-64, H-65), 1.8-2.01 (broad s, 9H, H-41, H-42, H-43, H-57, H-58, H-59, H-68, H-69, H-70), 2.09-2.25 (broad s, 9H, H-44, H-45, H-46, H-54, H-55, H-56, H-71, H-72, H-73), 2.50-2.56 (t, 2H, H-18, H-19), 3.21-3.48 (m, 8H, H-3, H-24, H-25, H-39, H-40, H-52, H-53, H-66), 3.48-3.71 (m, 10H, H-10, H-11, H-12, H-13, H-14, H-15, h-16, H-17, H-20, H-21), 3.73-3.85 (m, 1H, H-47), 3.97-4.07 (m, 1H, H-83), 4.22-4.37 (m, 3H, H-61, H-23, H-67), 4.98-5.02 (m, 2H, H-75, H-76), 7.18-7.39 (m, H-77, H-78, H-79, H-80, H-81, H-8), 7.40-7.49 (s, 1H, H-84), 7.57 (s, 1H, H-22),7.90-7.96 (m, 2H, H-9, H-82), 8.09 (s, 1H, H-60), 8.66 (s, 1H, H-2), 15.01 (s, 1H, H-1, not included in the spectrum). ^{19}F NMR (376 MHz, DMSO-d_6,): δ -122.64. Retention time (Method C): 10.30 min (purity/peak area: 97.2%).



(D5) 7-(4-((7S,10S,13S,16S)-7-amino-3-hydroxy-10,13-bis(3-(N-hydroxyacetamido)propyl)-16-(hydroxymethyl)-2,8,11,14,17-pentaoxo-3,9,12,15,18-pentaazahenicosan-21-oyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid. Compound **3** (0.062 g, 0.047 mmol, 1 eq) was dissolved in MeOH (5 mL) and 10% Pd/C (0.006 g) was added to the flask. After purging the flask with H_2, the reaction mixture was stirred for 5 h under H_2-pressure (1 atm). The reaction mixture was filtered through a PVDF filter, the solvent was evaporated *in vacuo*, and the desired product was obtained as a yellow soil (0. 055 g, 0.046 mmol) and used without further purification immediately for next step. Calculated mass for $C_{54}H_{78}FN_{11}O_{18}$: 1187.5; found 1188.5 $[M+H]^+$.



Retention time (Method C): 8.72 min (purity/ peak area: 87.6%). The yellow oil was treated with 6% DIPEA in MeOH (1 mL) overnight at room temperature. Solvent was removed to afford a yellow oil (0.047 g, 0.044 mmol), the product was again used without further purification for the next step. Calculated mass for $C_{48}H_{72}FN_{11}O_{15}$: 1061.5; found 1062.5 $[M+H]^+$. Retention time (Method C): 8.07 min (purity/ peak area: 86.3%). The oil was dissolved into as solution of 2:1 TFA and DCM (1 mL). The reaction mixture was stirred overnight at room temperature. Solvent was removed *in vacuo* and product was purified using preparative HPLC (Method A, product elutes at 44% B) to afford **D5** (0.020 g, 0.020 mmol, 45%) as a yellow solid. Calculated mass for **D5** ($C_{44}H_{64}FN_{11}O_{15}$): 1005.4; found 1006.5 $[M+H]^+$. ^{1}H NMR (700 MHz, DMSO-d_6): 1.16-1.19 (dd, 2H, H-4, H-5), 1.29-1.33 (dd, 2H, H-6, H-7), 1.55-1.69 (m, 12H, H-29, H-30, H-31, H-32, H-41, H-42, H-43, H-44, H-55, H-56, H-57, H-58), 1.59 (s, 9H, H-35, H-36, H-37, H-47, H-48, H-49, H-61, H-62, H-63), 2.52-2.57 (t, 2H, H-18, H-19), 3.27-3.34 (m, 4H, H-10, H-11, H-12, H-13), 3.34-3.38 (s, 2H, H-20, H-21), 3.43-3.84 (m, 13H, H-14, H-15, H-16, H-17, H-24, H-25, H-33, H-34, H45, H-46, H-52, H-59, H-60), 4.18-4.22 (m, 1H, H-3), 4.30-4.41 (m, 2H, H-28, H-40), 5.01-5.05 (t, 1H, H-23), 7.57 (s, 1H, H-8), 7.75-7.81 (s, 2H, H-22, H-27),7.91-7.97 (m, 1H, H-9), 8.10 (m, 3H, H-53, H-54, H-51), 8.49 (s, 1H, H-39), 8.67 (s, 1H, H-2), 9.77 (broad s, 3H, H-38, H-50, H-64), 15.01 (s, 1H, H-1, not included in the spectrum). ^{19}F NMR (376 MHz, DMSO-d_6,): δ -123.07. Retention time (Method C): 7.28 min (purity/peak area: > 99%).

(Fe-D5). Compound **D5** (0.005 g, 0.004 mmol, 1 eq) was dissolved in DMF (1 mL) and $FeCl_3$ (0.002 g, 0.012 mmol, 3 eq) was added. The reaction mixture was stirred for 1h at room temperature. Solvent was removed *in vacuo* and product was purified by preparative HPLC (Method **A**, product elutes at 48% B) to afford **Fe-D5** (0.006 g, 0.003 mmol, 87%) as a red solid. Calculated mass for **Fe-D5** ($C_{44}H_{61}FFeN_{11}O_{15}$): 1058.3; found 1059.3 $[M+H]^+$. Retention time (Method D): 7.10 min (purity/peak area: > 99%).

(Ga-D5/Galbofloxacin). Compound **D5** (0.010 g, 0.009 mmol, 1 eq) was dissolved in DMF (1 mL) and $Ga(NO_3)_3$ (0.007 g, 0.029 mmol, 3 eq) was added. The pH of the solution was adjusted to 6 by adding 0.1 M NaOH. The reaction mixture was stirred for 1h at 60^{o}C and overnight at room temperature. Solvent was removed *in vacuo* and product was purified by preparative HPLC (Method **A**, product elutes at 46% B) to afford **Ga-D5** (0.006 g, 0.005 mmol, 62%) as a white solid. Calculated mass for **Ga-D5** ($C_{44}H_{61}FGaN_{11}O_{15}$): 1071.3; found 1072.4 $[M+H]^+$. Retention time (Method D): 7.13 min (purity/peak area: > 99%).

1.2 HPLC traces

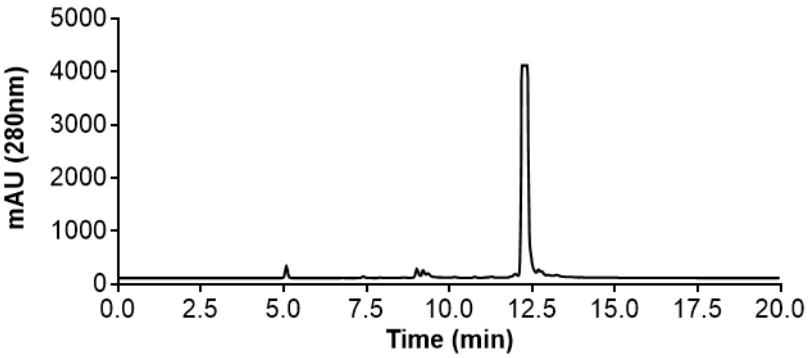


Figure S1. HPLC chromatogram of compound (**1**). Retention time (Method C): 12.28 min.

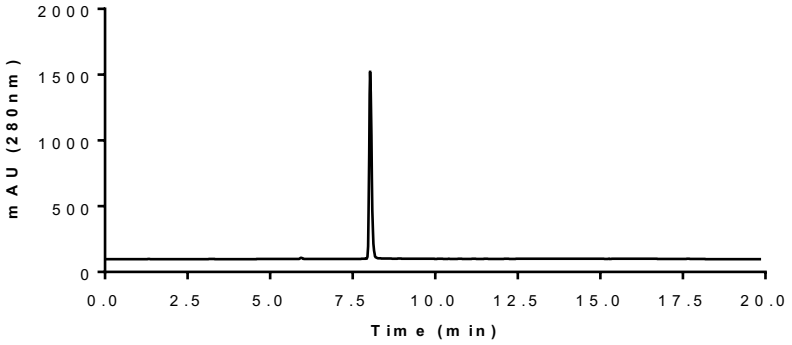


Figure S2. HPLC chromatogram of compound (**2**). Retention time (Method C): 8.03 min.

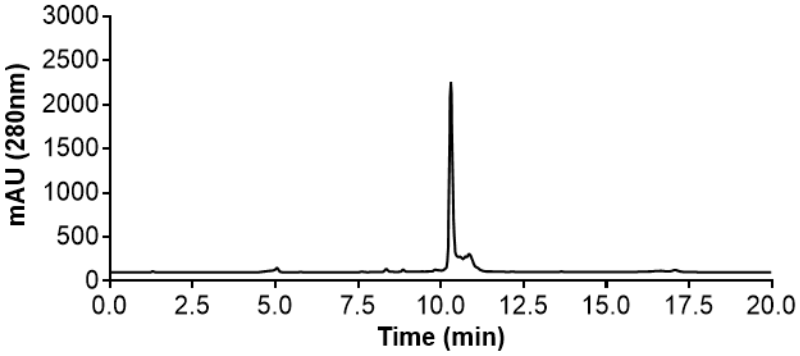


Figure S3. HPLC chromatogram of compound (**3)**. Retention time (Method C): 10.30 min.

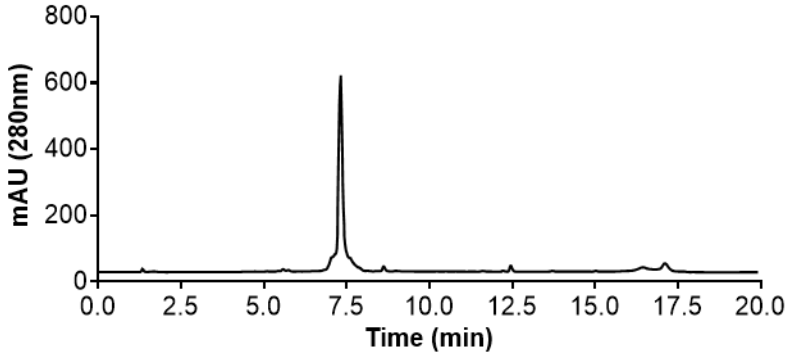


Figure S4. HPLC chromatogram of compound **D5**. Retention time (Method C): 7.28 min.

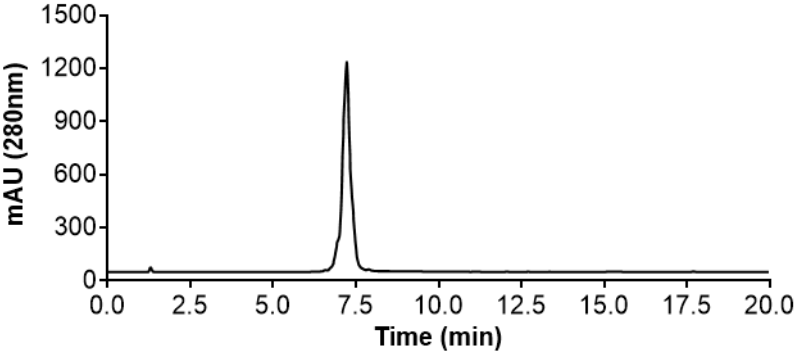


Figure S5. HPLC chromatogram for Fe-**D5**. Retention time (Method D): 7.10 min.

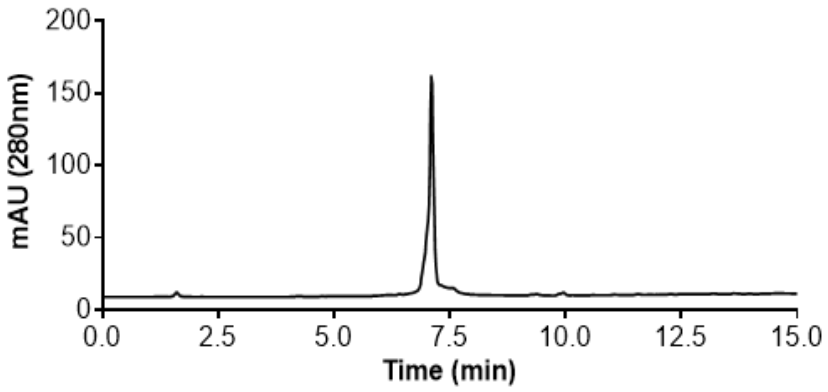


Figure S6. HPLC chromatogram of compound Ga-**D5**/Galbofloxacin. Retention time (Method D): 7.13 min.

1.3 NMR Spectra

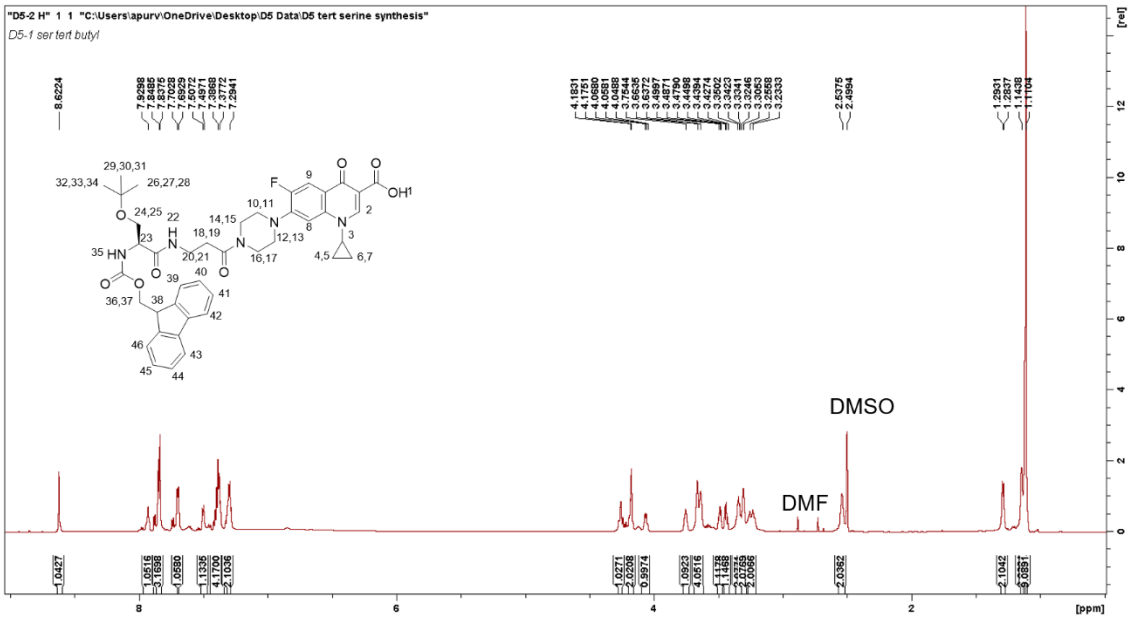


Figure S7. ^{1}H-NMR spectrum of compound **(1)**. 700 MHz, DMSO-d_6.

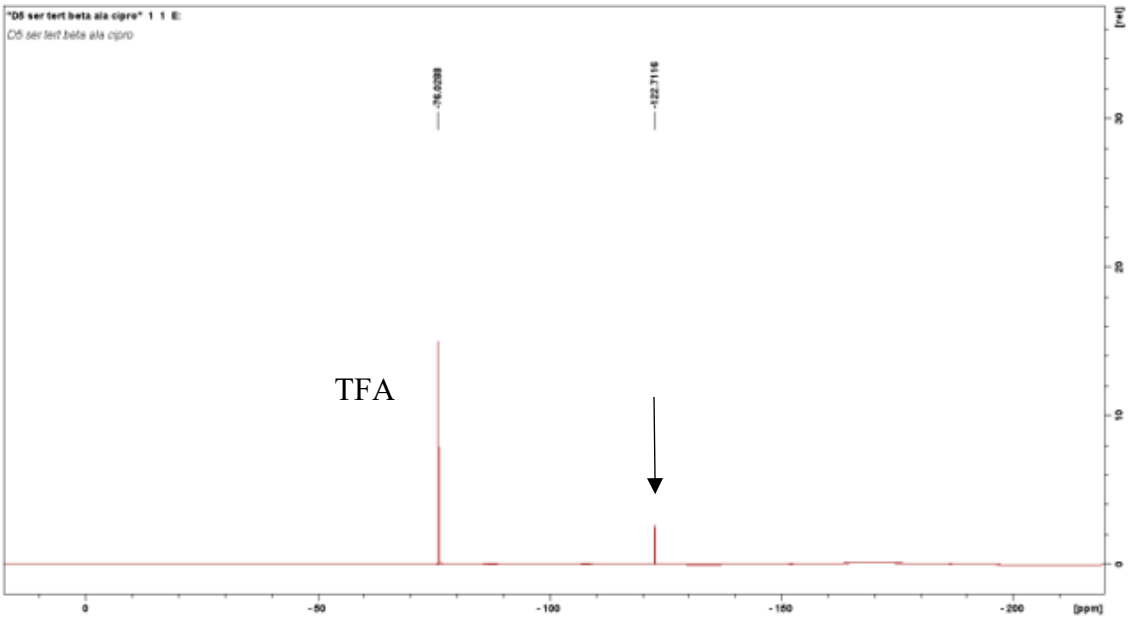


Figure S8. ^{19}F-NMR spectrum of compound **(1)**. 376 MHz, DMSO-d_6.

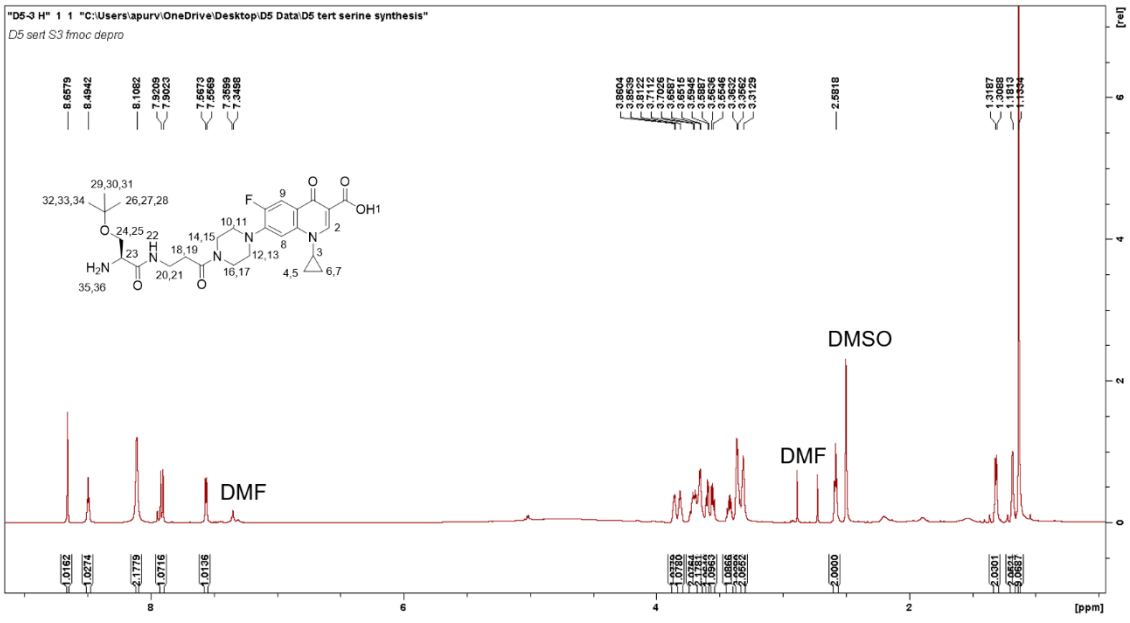


Figure S9. ^{1}H-NMR spectrum of compound **(2)**. 700 MHz, DMSO-d_6.

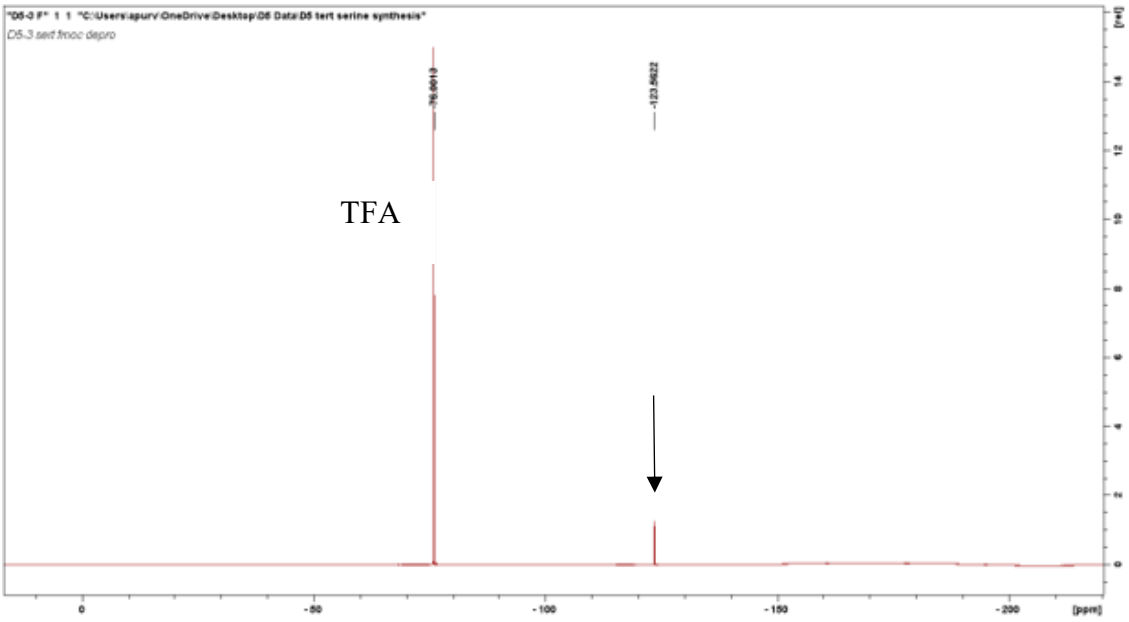


Figure S10. ^{19}F-NMR spectrum of compound **(2)**. 376 MHz, DMSO-d_6.

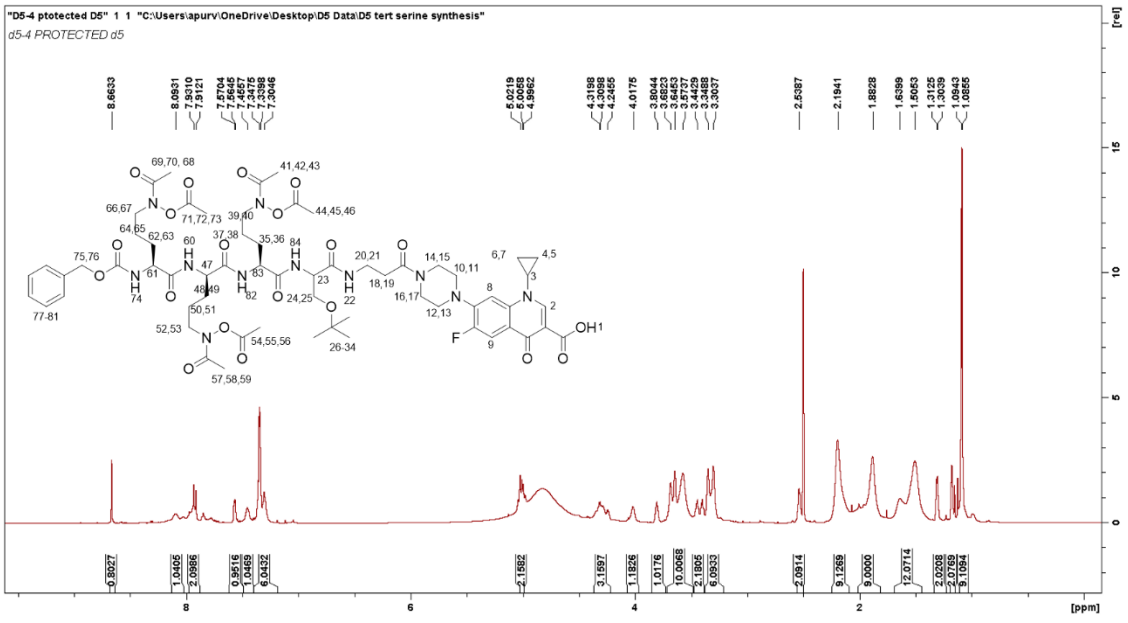


Figure S11. ^{1}H-NMR spectrum of compound **(3)**. 700 MHz, DMSO-d_6.

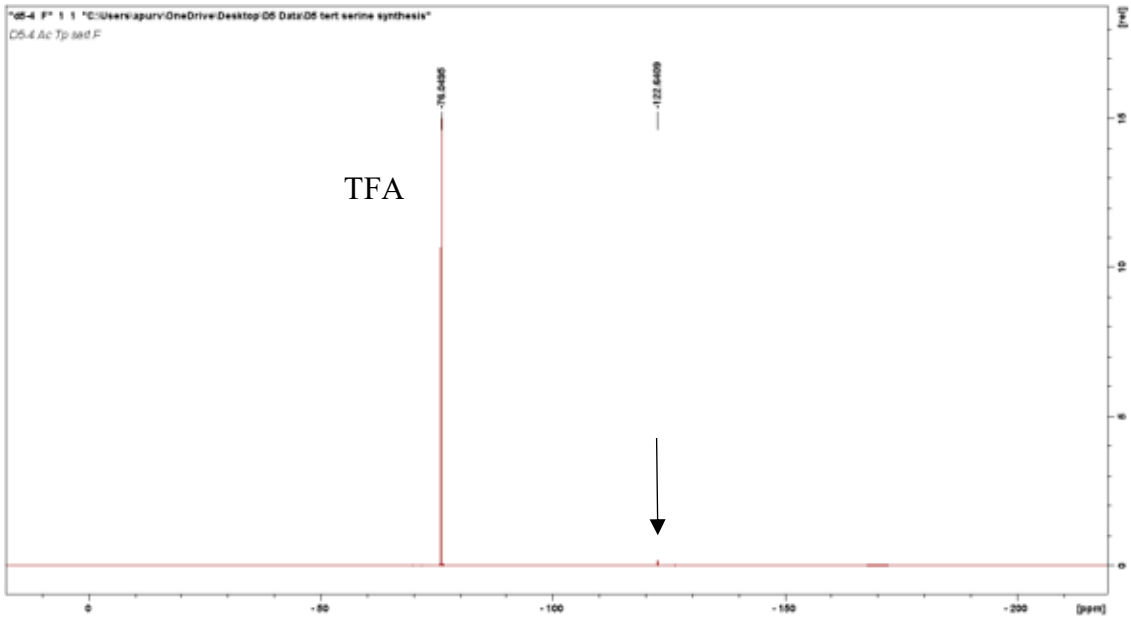


Figure S12. ^{19}F-NMR spectrum of compound **(3)**. 376 MHz, DMSO-d_6.

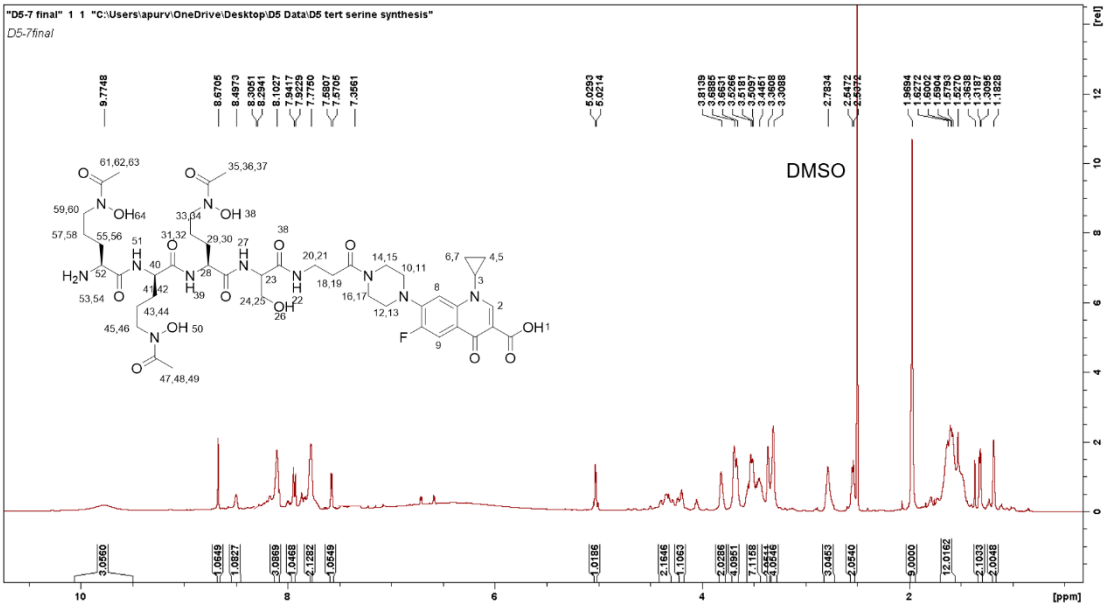


Figure S13. ^{1}H-NMR spectrum of **D5**. 700 MHz, DMSO-d_6.

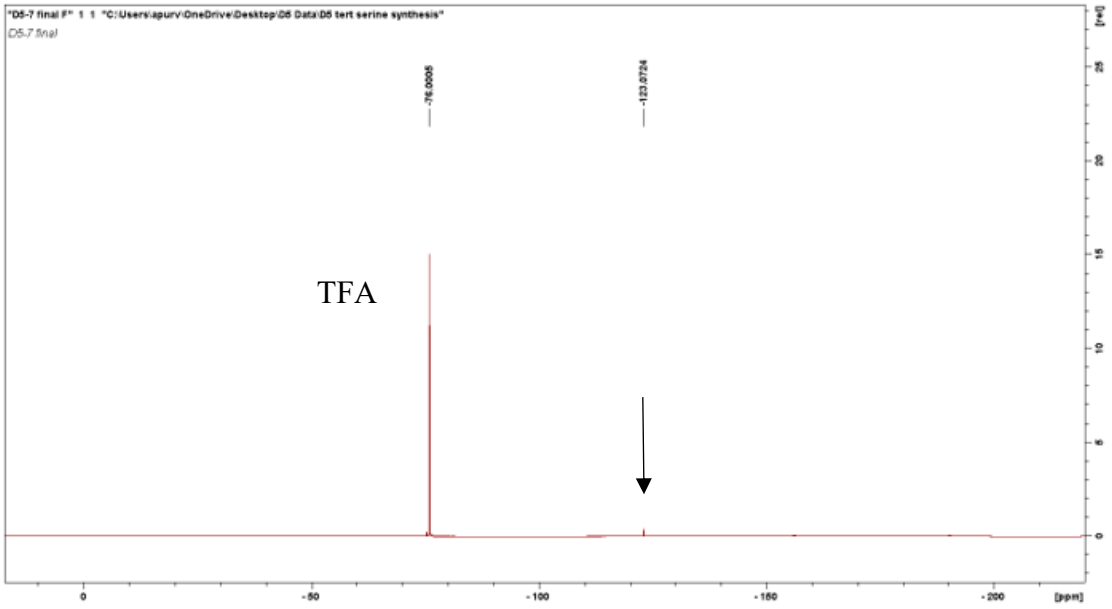


Figure S14. ^{19}F-NMR spectrum of **D5**. 376 MHz, DMSO-d_6.

1.4 HRMS

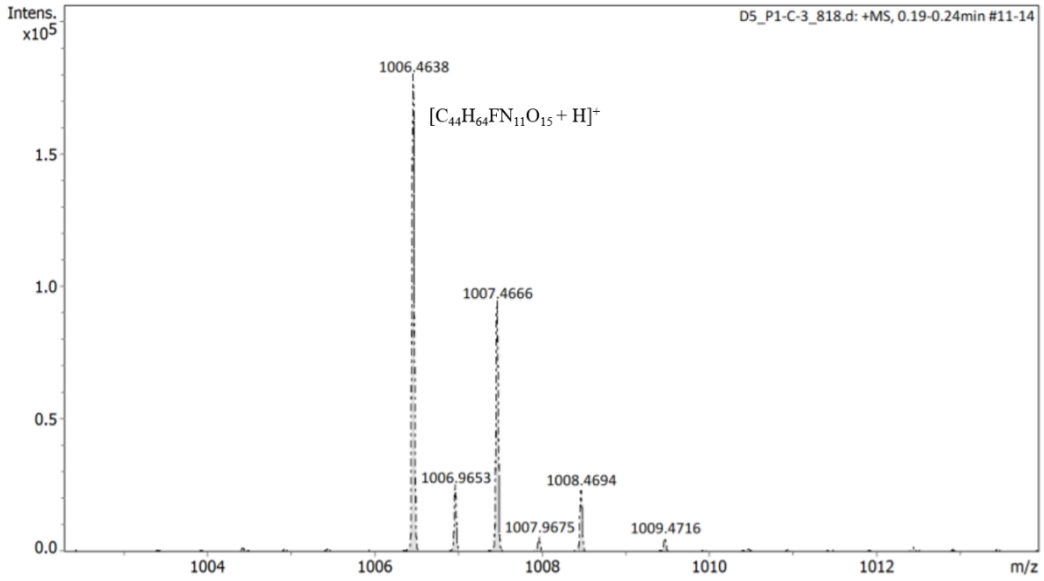


Figure S15. High resolution mass spectrum of **D5**. HRMS calculated for $C_{44}H_{64}FN_{11}O_{15}$: 1005.4567; found 1006.4638 $[M+H]^+$.

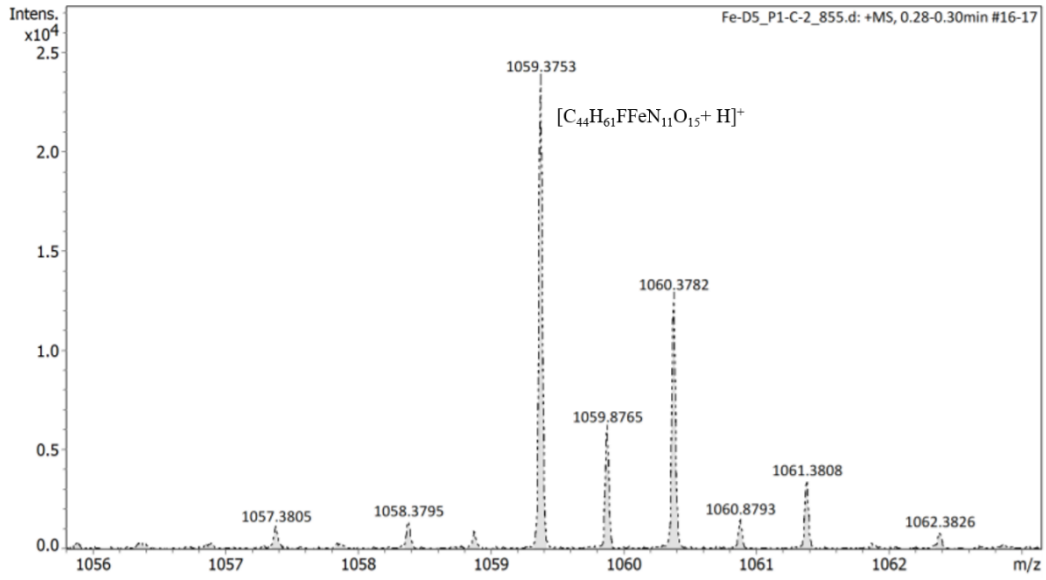


Figure S16. High resolution mass spectrum of Fe-**D5**. HRMS calculated for $C_{44}H_{61}FFeN_{11}O_{15}$: 1058.3682; found 1059.3753 $[M+H]^+$.

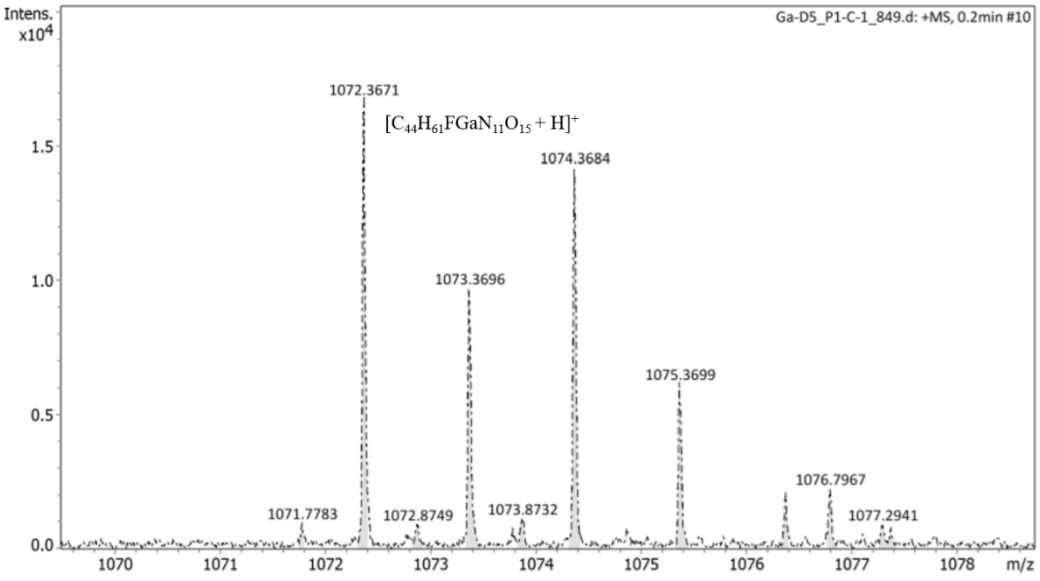


Figure S17. High resolution mass spectrum of Ga-**D5**. HRMS calculated for $C_{44}H_{61}FGaN_{11}O_{15}$: 1071.3588; found 1072.3671 $[M+H]^+$.

2. Antibacterial Assessment Assays (MIC assays)

Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. All liquids and media were sterilized by autoclaving (220 °C, 1 h) before use. All aqueous solutions and media were prepared using deionized water. Mueller-Hinton broth (MHB) commonly used for antibiotic testing was purchased from Fisher Scientific and used for all MIC assays. MHB is a non-selective and non-differential media which makes it suitable for testing different bacterial strains. It also allows for better diffusion of antibiotics and contains starch that help absorb the toxins produced by bacteria which could interfere with the antibiotics. Mueller-Hinton II broth (cation adjusted) was prepared by adding sterile aqueous solution of 0.41 mL of 1 M Ca^{2+} and 0.15 mL of 1 M Mg^{2+} to 250 mL of MHB. Iron-deficient (-Fe) MHII broth was prepared by adding 4.06 mL of a 1 mg/mL sterile aq. solution of 2,2'-bipyridine to 250 mL of MHII broth.

2.1 Sample preparation

Stock solutions of the testing compounds were prepared in sterilized deionized water. Concentration of apo-**D5** and Fragment A was analyzed using UV-Vis spectrophotometry and ciprofloxacin-based ε: 15304 $M^{-1}cm^{-1}$ was used for calculating the stock concentrations. Concentrations of Fe-**D5** and Ga-**D5** were analyzed using an ICP-OES. A 10-point standard with respect to gallium and iron was used and lines of best fit were found with R^2 of 0.999. Sample concentration was determined based on this calibration curve.

2.2 MIC assay results in *E.coli* K-12 and *P.aeruginosa* PA01

Overnight culture of *E.coli* K-12 *and P.aeruginosa* PA01 were prepared by incubating single colonies of *E.coli* K-12 *and P.aeruginosa* PA01 in 5 mL of iron deficient MHB II (cation adjusted) at 37°C on an incubator shaker for 18 h. Subsequently, the overnight culture was inoculated (1:100) into 10 mL of fresh media and incubated at 37°C until an OD_{600} ~ 0.4 was reached. The culture was further diluted to an OD_{600} of 0.001 for MIC testing. 10 µL solution of testing compound (0.3 mM) was added to the first well of the 96-well plate and serial dilutions were made down each row of the plate. 40 µL of growth media and 50 µL of diluted bacterial inoculum was also added to each well, resulting in a total volume of 100 µL and a concentration gradient of 0.3 x 10^{-4} M–1.56 x 10^{-12} M. The plates were incubated at 37°C for 18 h. Bacterial growth was determined by measuring the OD_{600} using a plate reader. The experiment was carried out in triplicates.

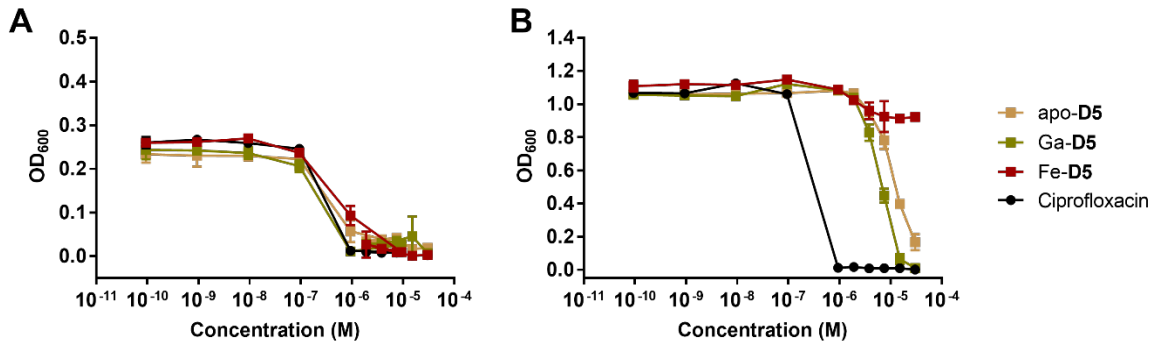


Figure S18: MIC assay of *apo*-**D5**, Fe-**D5** and Ga-**D5** in **(A)** *E.coli* K-12 and **(B)** *P.aeruginosa PA01*.

2.3 MIC assay results in *E.coli* AN 193

Overnight culture of *E.coli* AN193 was prepared by incubating single colonies of *E.coli* AN193 in 5 mL of iron deficient MHB II (cation adjusted) at 37°C on an incubator shaker for 18 h. Subsequently, the overnight culture was inoculated (1:100) into 10 mL of fresh media and incubated at 37°C until an OD_{600} ~ 0.4 was reached. The culture was further diluted to an OD_{600} of 0.001 for MIC testing. 10 µL solution of testing compound (0.3 mM) was added to the first well of the 96-well plate and serial dilutions were made down each row of the plate. 40 µL of growth media and 50 µL of diluted bacterial inoculum was also added to each well, resulting in a total volume of 100 µL and a concentration gradient of 0.3 x 10^{-4} M–1.56 x 10^{-12} M. The plates were incubated at 37°C for 18 h. Bacterial growth was determined by measuring the OD_{600} using a plate reader. The experiment was carried out in triplicates.

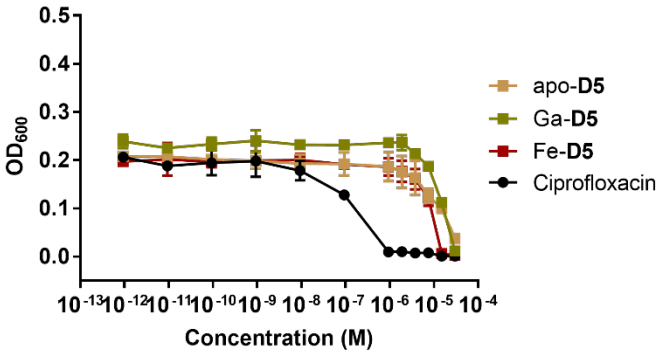


Figure S19: MIC assay of *apo*-**D5**, Fe-**D5** and Ga-**D5** in *E.coli* AN193 showing attenuation of the growth inhibitory activity.

2.4 MIC assay results for Fragment A in wt bacterial strains

Overnight culture of wt bacterial strains was prepared by incubating single colonies of *E.coli, S.aureus*, *P.aeruginosa* and *E coli* AN193 in 5 mL of iron deficient MHB II (cation adjusted) at 37°C on an incubator shaker for 18 h. Subsequently, the overnight culture was inoculated (1:100) into 10 mL of fresh media and incubated at 37°C until an OD_{600} ~ 0.4 was reached. The culture was further diluted to an OD_{600} of 0.001 for MIC testing. 10 µL solution of testing compound (0.3 mM) was added to the first well of the 96-well plate and serial dilutions were made down each row of the plate. 40 µL of growth media and 50 µL of diluted bacterial inoculum was also added to each well, resulting in a total volume of 100 µL and a concentration gradient of 0.3 x 10^{-4} M–1.56 x 10^{-12} M. The plates were incubated at 37°C for 18 h. Bacterial growth was determined by measuring the OD_{600} using a plate reader. The experiment was carried out in triplicates**.**

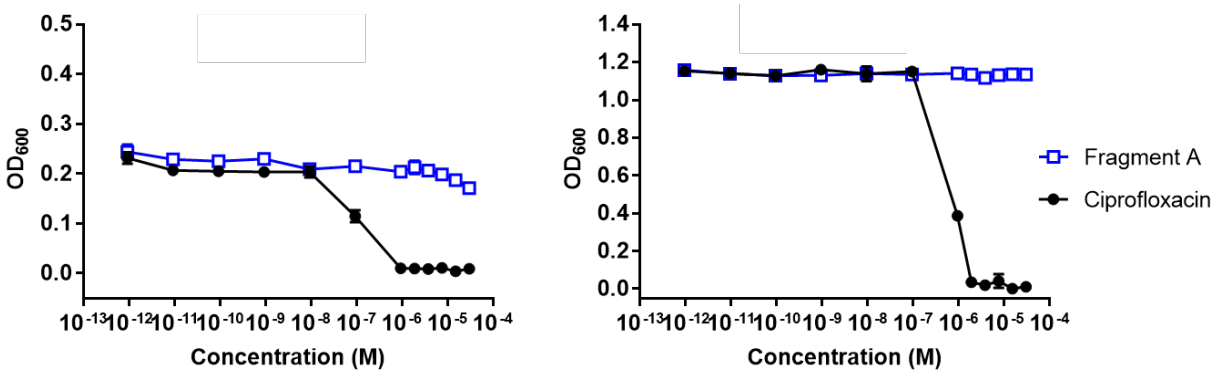


Figure S20: MIC assay of Fragment A in **(left)** *E.coli* and **(right)** *PA01* showing completely muted growth inhibitory activity.

2.6 MIC assay results for Ga-D5 in *S.aureus* Xen 29

Overnight culture of *S.aureus* Xen 29 strain was prepared by incubating single colonies of *S. aureus* Xen 29 in 5 mL of MHB II (cation adjusted) broth for 18 h. Subsequently, the overnight culture was inoculated (1:100) into 10 mL of fresh media and incubated at 37°C until an OD_{600} ~ 0.4 was reached. The culture was further diluted to an OD_{600} of 0.001 for MIC testing. 10 µL solution of testing compound (0.3 mM) was added to the first well of the 96-well plate and serial dilutions were made down each row of the plate. 40 µL of growth media and 50 µL of diluted bacterial inoculum was also added to each well, resulting in a total volume of 100 µL and a concentration gradient of 0.3 x 10^{-4} M–1.56 x 10^{-12} M. The plates were incubated at 37°C for 18 h. Bacterial growth was determined by measuring the OD_{600} using a plate reader. The plates were also imaged on IVIS Lumina Series II from Caliper Life Sciences small animal imager. Images were analyzed with Living Image software version 4.3.1. Regions of interest were determined in

quintuplicate with the ROI tool for each concentration as well as a general background ROI for background correction. The experiment was carried out in triplicates.

3. In vitro cleavage assessment (Proteinase K assay)

3.1 Sample preparation

Stock solutions of apo **D5** and Ga-**D5** were prepared in deionized water. Concentration of apo **D5** and ciprofloxacin were analyzed using UV-Vis spectrophotometry and ciprofloxacin-based ε: 15304 $M^{-1}cm^{-1}$ was used for calculating the stock concentrations. Concentrations of Ga-**D5** was analyzed using an ICP-OES. A 10-point standard with respect to gallium and iron was used and lines of best fit were found with R^2 of 0.999.

3.2 Proteinase K assay with D5 and Fe-D5

Table S1: Time dependent percentage cleavage of apo-**D5**, Ga-**D5** and Fe-**D5** on incubation with Proteinase K at 37ºC, pH = 7 (n=2).

Incubation Time (h)	apo **D5** (%)	Ga-**D5** (%)	Fe-**D5** (%)
0	0	0	0
0.25	100	9	-
1	-	28	49
2	-	40	51
3	-	45	56
4	-	55	57
5	-	45	-
6	-	68	-
7	-	98	-
8	-	100	-
9	-	-	-
10	-	-	59
24	-	-	66

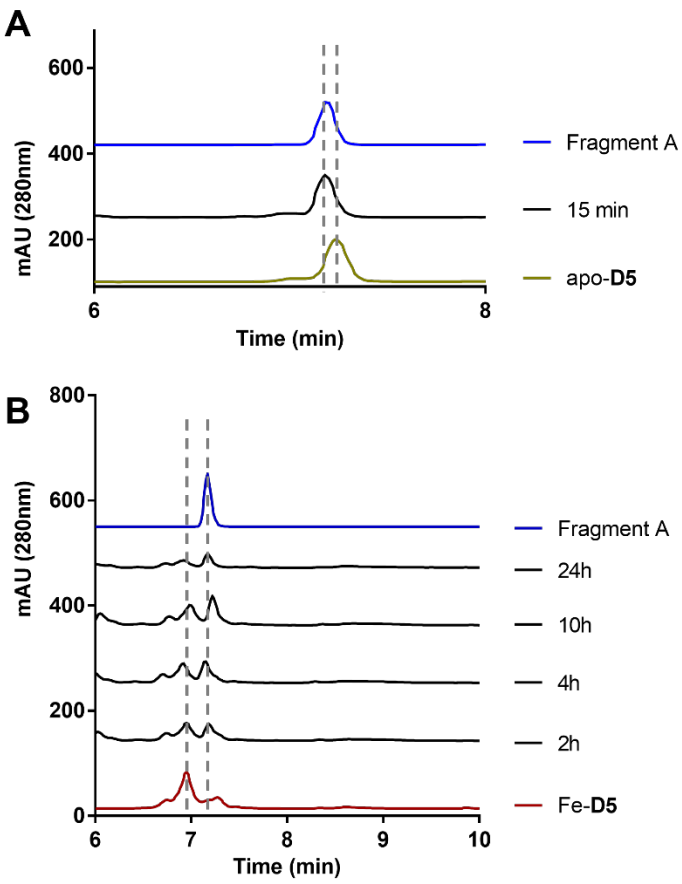


Figure S21: In-vitro cleavage assay using Proteinase K shows cleavage of apo-**D5** (t_R = 7.28 min) by appearance of a second peak corresponding to the cleaved fragment A (t_R= 7.03 min) 15 mins post incubation with Proteinase K at 37°C, pH = 7 (n=2) whereas, Fe-D5 (t_R= 6.96 min) is still 34% intact after 24h.

4. Cytotoxicity assessment (MTT assay)

4.1 Sample preparation

Stock solutions of *apo*-**D5**, Ga-**D5** and Ga-citrate were prepared in sterilized deionized water. Cisplatin was dissolved in DPBS buffer and ciprofloxacin was dissolved in DMSO and diluted with the culture medium to final concentration 0.5% of DMSO. Concentration of *apo*-**D5** and ciprofloxacin were analyzed using UV-Vis spectrophotometry and ciprofloxacin-based ε: 15304 $M^{-1}cm^{-1}$ was used for calculating the stock concentrations. Concentrations of Ga-**D5** and Ga-citrate were analyzed using an ICP-OES. A 10-point standard with respect to gallium and iron was used and lines of best fit were found with R^2 of 0.999. Sample concentration was determined based on this calibration curve. Concentration of cisplatin was calculated based on weight measurements.

4.2 MTT assay results for D5 and Ga(III)-citrate

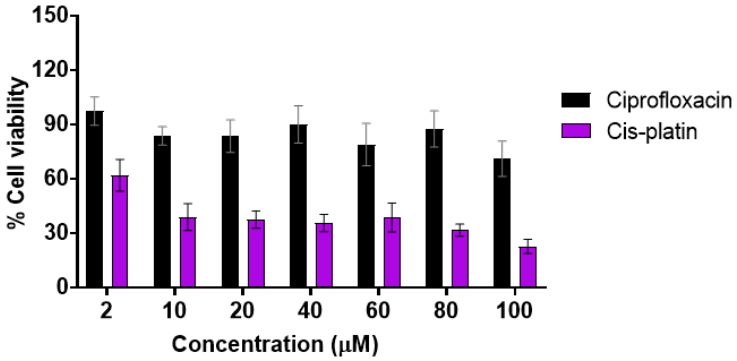


Figure S22: MTT assay of ciprofloxacin and cisplatin in HEK-293 cell line shows 80% cell viability for ciprofloxacin at 100 μM and toxic effects for cisplatin the positive control (n=3x5).

5. Radiochemical experiments

5.1 Uptake of ^{67}Ga-D5 in wt bacterial strains

Uptake was expressed as the % radioactivity found in the pellet:

$$\% \text{ uptake} = \frac{\text{cpm (pellet)}}{\text{cpm (1 mL bacterial inoculum)}}$$

Table S2: Percentage of internalized ^{67}Ga-**D5** in *E. coli* K-12 upon incubation in iron deficient bacterial culture (n=5).

Incubation Time (h)	^{67}Ga-**D5** (%)	^{67}Ga-citrate (%)
0.16	4.8±1.3	3.4±0.3
0.33	5.3±2.7	6.3±2.3
0.5	11.5±6.6	2.5±0.3
1	16.7±4.4	5.8±2.1
2	24.9±2.8	4.6±0.8

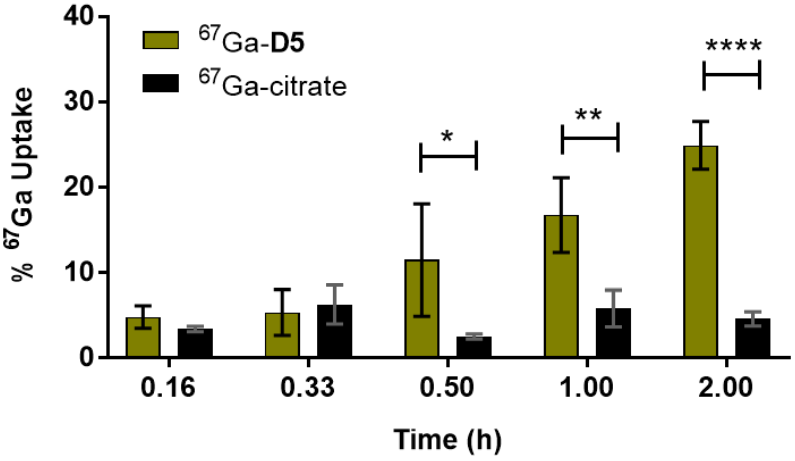


Figure S23: Time-dependent, radiochemical bacterial uptake studies in *E.coli* K12 of ^{67}Ga-**D5** iron-depleted, DP-treated media pH = 7.4 show that uptake is enhanced in comparison with ^{67}Ga-citrate.

Table S3: Percentage of internalized ^{67}Ga-**D5** in *P.aeruginosa* PA01 upon incubation in iron deficient bacterial culture (n=5).

Incubation Time (h)	^{67}Ga-**D5** (%)	^{67}Ga-Citrate (%)
0.16	6.4±0.9	3.2±1.0
0.33	7.4±4.2	3.6±1.3
0.5	14.2±4.4	2.6±0.7
1	21.5±3.8	3.6±0.9
2	27.7±5.0	3.1±1.1

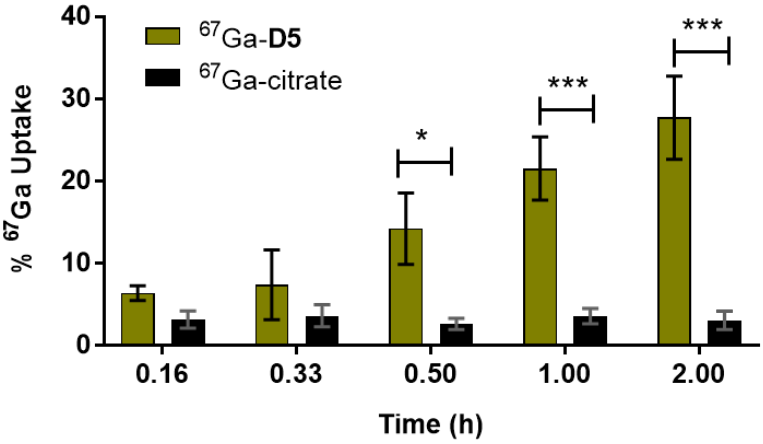


Figure S24: Time-dependent, radiochemical bacterial uptake studies in *P.aeruginosa* PA01 of ^{67}Ga-**D5** iron-depleted, DP-treated media pH = 7.4 show that uptake is enhanced in comparison with ^{67}Ga-citrate.

Table S4: Percentage of internalized ^{67}Ga-**D5** in *S.aureus* RN4220 upon incubation in iron deficient bacterial culture (n=5).

Incubation Time (h)	^{67}Ga-**D5** (%)	^{67}Ga-Citrate (%)
0.16	5.2±0.6	6.0±1.8
0.33	5.6±2.0	6.5±0.5
0.5	9.3±2.1	9.4±1.5
1	16.7±4.9	18.6±2.1
2	18.0±3.5	21.9±5.6

5.2 Challenge Uptake of ^{67}Ga-D5 in wt bacterial strains

Uptake was expressed as the % radioactivity found in the pellet:

$$\% \text{ uptake} = \frac{\text{cpm (pellet)}}{\text{cpm (1 mL bacterial inoculum)}}$$

Table S5: Percentage of internalized ^{67}Ga-**D5** complex when challenged with 200x Fe-LDFC in *E. coli* K-12 upon incubation in iron deficient bacterial culture (n=5).

Incubation Time (h)	^{67}Ga-**D5** + 200x Fe-LDFC (%)	^{67}Ga-Citrate + 200x Fe-LDFC (%)
0.16	1.2±0.5	1.3±0.2
0.33	1.1±0.4	1.1±0.2
0.5	1.1±0.4	1.3±0.2
1	2.6±3.4	1.3±0.1
2	1.4±0.2	1.6±0.5

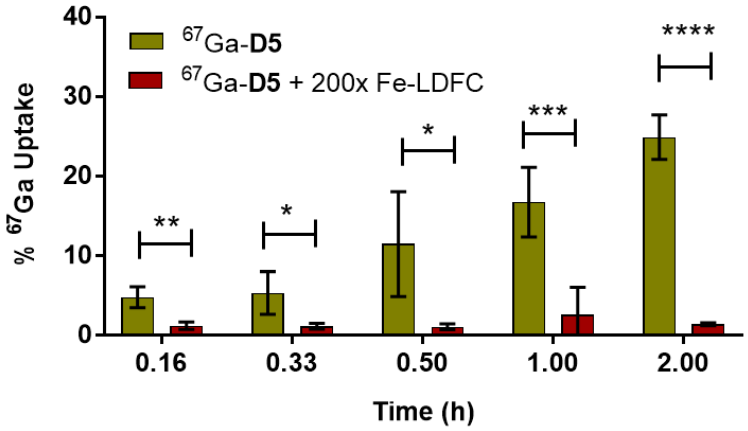


Figure S25: In the presence of 200× excess Fe−LDFC, the uptake of ^{67}Ga-**D5** in *E.coli* K-12 is attenuated significantly after 30 min ($P < 0.0001$).

Table S6: Percentage of internalized ^{67}Ga-**D5** complex when challenged with 200x Fe-LDFC in *P.aeruginosa* PA01 upon incubation in iron deficient bacterial culture (n=5).

Incubation Time (h)	^{67}Ga-**D5** + 200x Fe-LDFC (%)	^{67}Ga-Citrate + 200x Fe-LDFC (%)
0.16	1.3±0.4	2.0±0.6
0.33	1.2±0.2	1.9±0.3
0.5	1.4±0.1	1.7±0.3
1	1.3±0.2	0.8±0.3
2	1.2±0.1	1.2±0.2

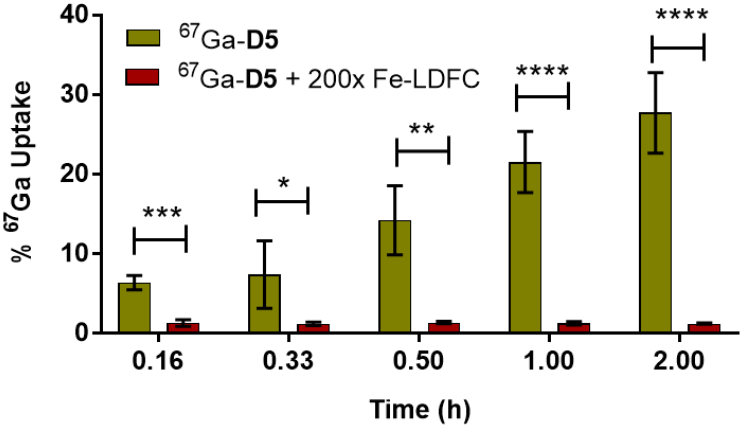


Figure S26: In the presence of 200× excess Fe−LDFC, the uptake of ^{67}Ga-**D5** in *P.aeruginosa* PA01 is attenuated significantly after 30 min ($P < 0.0001$).

Table S7: Percentage of internalized ^{67}Ga-**D5** complex when challenged with 200x Fe-LDFC in *S.aureus* RN4220 upon incubation in iron deficient bacterial culture (n=5).

Incubation Time (h)	^{67}Ga-**D5** + 200x Fe-LDFC (%)	^{67}Ga-Citrate + 200x Fe-LDFC (%)
0.16	1.3±0.2	1.9±0.3
0.33	1.2±0.2	1.8±0.3
0.5	1.2±0.1	1.5±0.5
1	1.0±0.1	1.0±0.3
2	1.1±0.1	1.3±0.2

5.3 Biodistribution

Table S8: Biodistribution of ^{67}Ga-**D5** in Balb/c mice at 1h p.i. (n=3).

Organ	^{67}Ga-**D5** (%ID/g)	^{67}Ga-citrate (%ID/g)
Blood	6.1±0.3	9.1±0.4
Heart	1.8±0.1	1.8±1.2
Lung	6.4±4.3	1.7±0.9
Liver	6.4±0.7	3.1±0.6
Spleen	5.8±2.4	1.2±1.0
Kidney	2.9±0.5	2.3±1.8
Small intestine	1.2±0.2	1.7±0.8
Muscle	0.6±0.1	0.9±0.6
Infected Muscle	1.3±0.3	2.1±1.8
Bone	3.2±2.2	1.4±1.4
Urine	172.0±55.8	54.1±0.4

5.4 Metabolite analysis

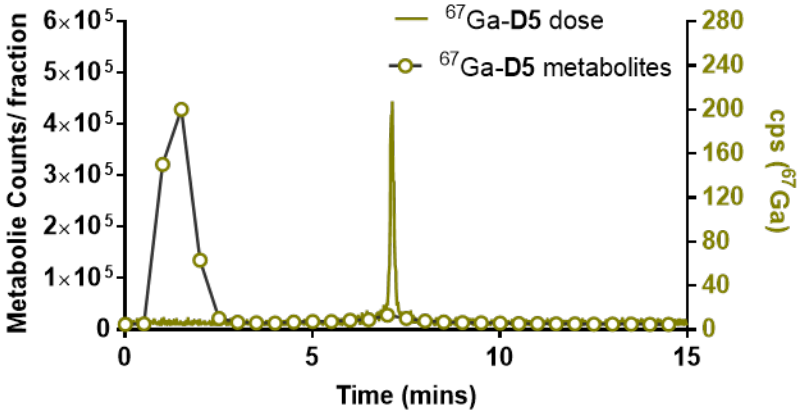


Figure S27: Metabolite analysis of ^{67}Ga-**D5** (open circles) shows detectable intact complex (5%) in the urine 1 h post injection. Radioanalytical HPLC trace of the ^{67}Ga-**D5** dose formulation prior to administration is shown as a reference (green).

6. In vivo efficacy study

6.1 Sample preparation

Stock solutions of ciprofloxacin were prepared in 0.1 M CH_3COOH and diluted using DPBS (pH = 6.5). Ga-**D5** was prepared in 10% DMSO. Concentration of ciprofloxacin was analyzed using UV-Vis spectrophotometry ε: 15304 $M^{-1}cm^{-1}$ was used for calculating the stock concentrations. Concentrations of Ga-**D5** was analyzed using an ICP-OES. A 10-point standard with respect to gallium was used and lines of best fit were found with R^2 of 0.999. Sample concentration was determined based on this calibration curve.

Table S9: Conversion table for doses of test compounds in mg/kg to µmol/kg.

Compound	Single dose (mg/kg)	Single dose (µmol/kg)
Ciprofloxacin	3.51	9.3
Ciprofloxacin	94.23	284.4
Ga-**D5**	2.5	2.3
Ga-**D5**	10	9.3

6.2 Muscle sizes

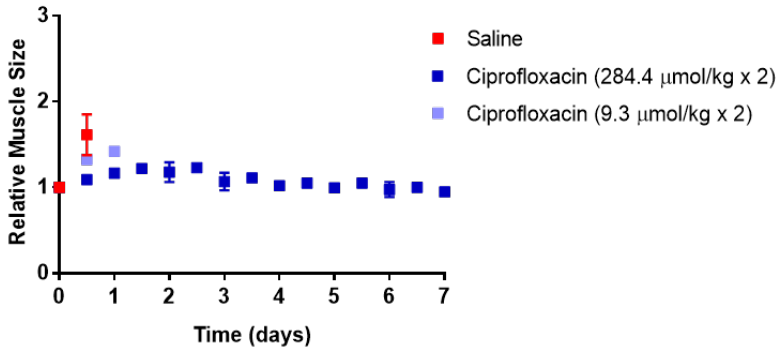


Figure S28: Relative muscle sizes of each cohort shows muscle size increasing rapidly for saline and ciprofloxacin (9.3 µmol/kg), whereas decreases over time for ciprofloxacin (284.4 µmol/kg), n=4.

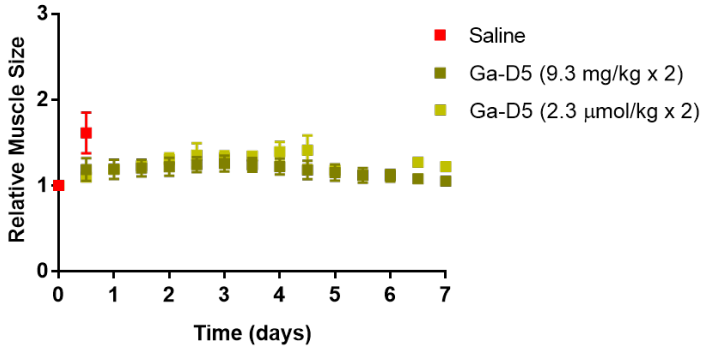


Figure S29: Relative muscle sizes of each cohort shows muscle size increasing rapidly for saline, gradually for **D5** (2.3 µmol/kg), whereas decreases over time for Ga-**D5** (9.3 µmol/kg), n=4.

6.3 Bioluminescent imaging

Mice were imaged every 12 hours using IVIS Lumina Series II from Caliper LifeSciences small animal imager. Infected muscles from mice were plated for CFU determination and the first dilution plate was also imaged on IVIS animal imager after overnight incubation at 37°C. All scans were collected with blocked excitation filter, open emission filter (from 515 nm to 840 nm) and collection time t = 5 minutes (mice) and 1 minute (bacterial plates). Images were analyzed with Living Image software version 4.3.1.

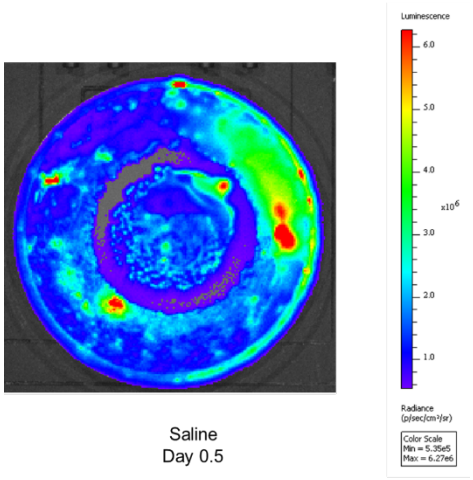


Figure S30. First dilution plate of infected muscle for saline cohort (Day 0.5).

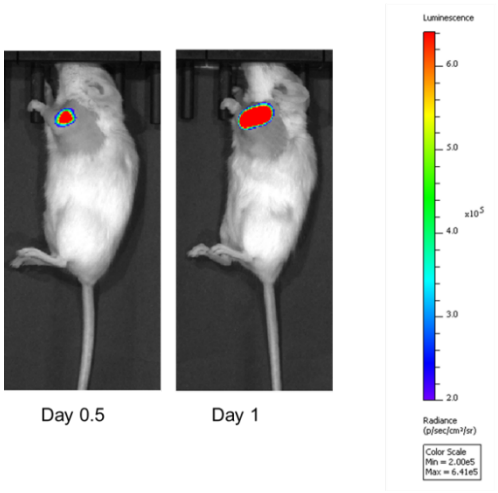


Figure S31. Ciprofloxacin (9.3 µmol/kg) treatment progression.

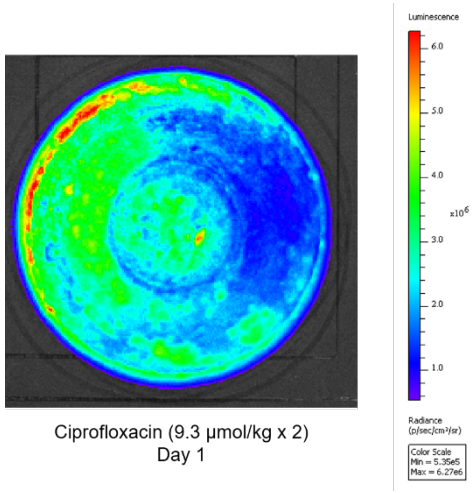


Figure S32. First dilution plate of infected muscle for ciprofloxacin (9.3 µmol/kg) cohort (Day 1).

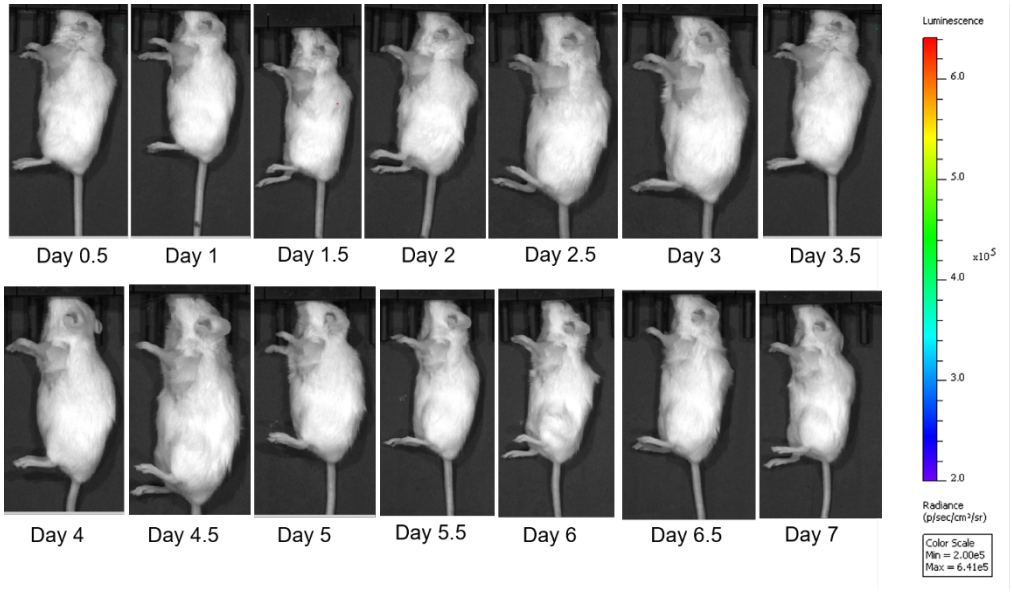


Figure S33. Ciprofloxacin (284.4 µmol/kg) 7-day treatment progression.

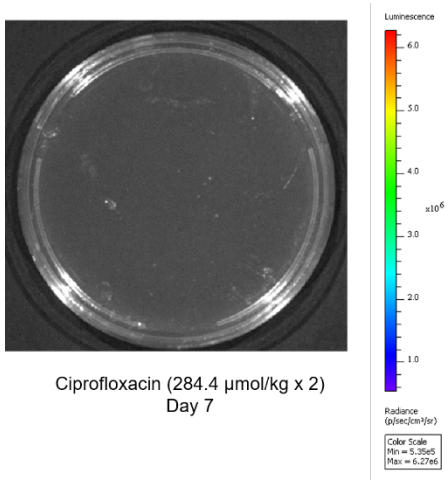


Figure S34. First dilution plate of infected muscle for ciprofloxacin (284.4 µmol/kg) cohort (Day 7).

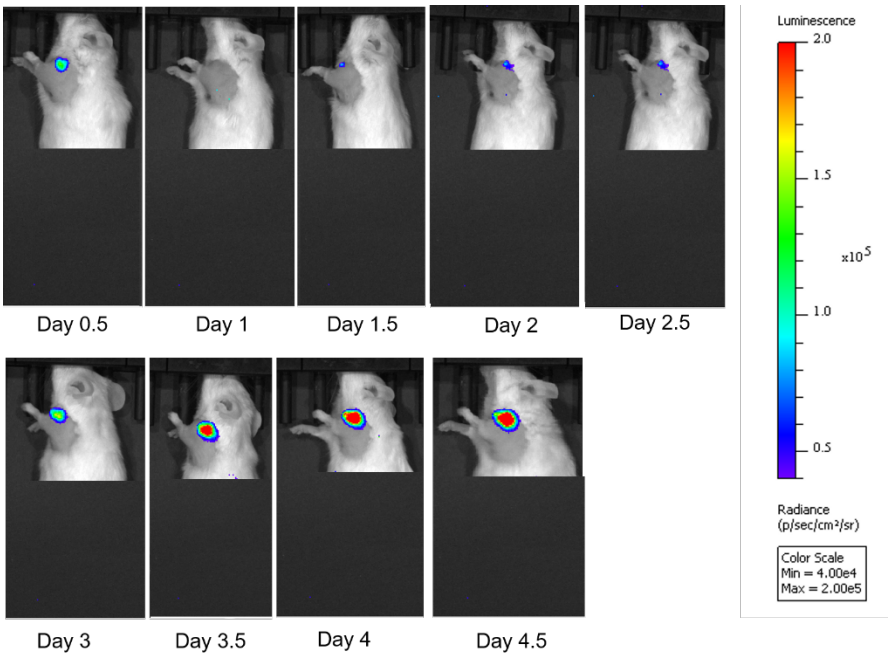


Figure S35. Ga-**D5** (2.3 µmol/kg) treatment progression.

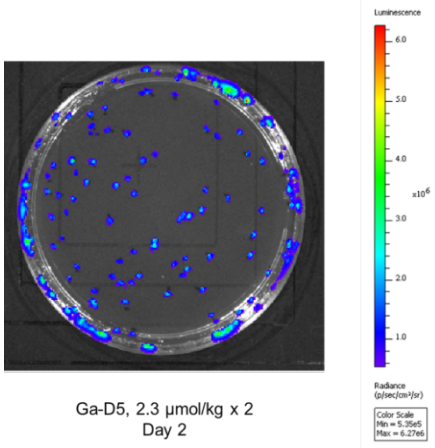


Figure S36. First dilution plate of infected muscle for Ga-**D5** (2.3 µmol/kg) cohort (Day 4.5).

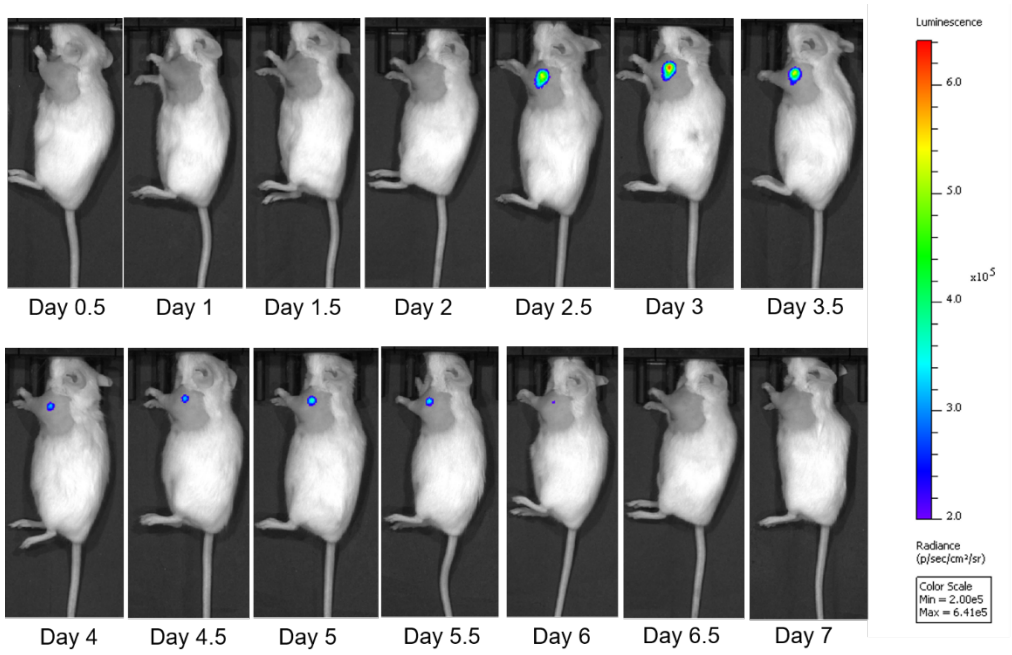


Figure S37. Ga-**D5** (9.3 µmol/kg) 7-day treatment progression.

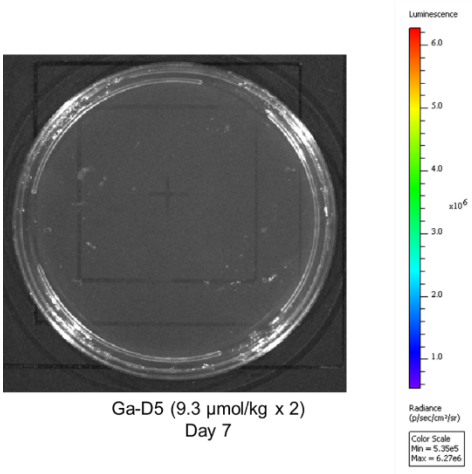


Figure S38. First dilution plate of infected muscle for Ga-**D5** (9.3 µmol/kg) cohort (Day 7).

6.4 CFU determination

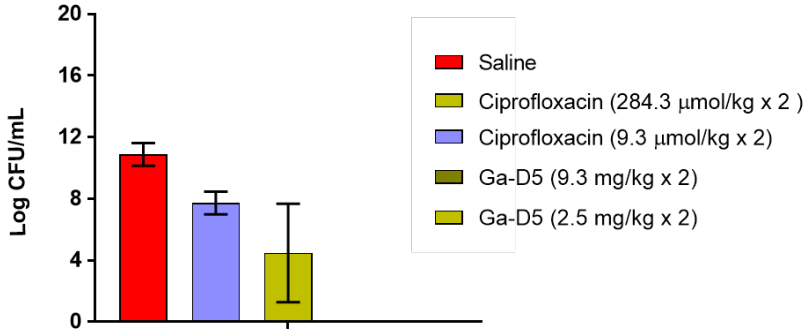


Figure S39: CFU determination for each cohort (n=4) after euthanizing the mice. No CFUs were detected for ciprofloxacin (284.4 µmol/kg) and Ga-**D5** (9.3 µmol/kg) cohort.

6.5 Bioluminescence ROI analysis

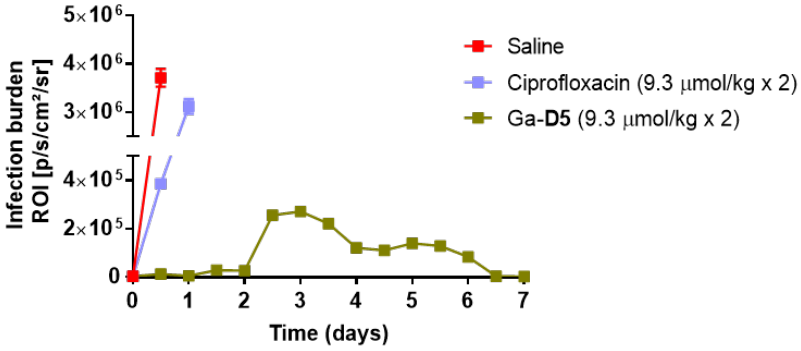


Figure S40. The infection burden increasing rapidly for the vehicle and Ciprofloxacin (9.3 µmol/kg), whereas decreases over time for Ga-**D5** (9.3 µmol/kg), n=4.

Table S10. Infection burden ROI analysis.

Compound	Single Dose (mg/kg)	Single Dose (µmol/kg)	Max survival (Day 7)	ROI (p/s/cm²/sr)
Saline	-	-	0/4	$3.7x10^6 \pm 1.9x10^5$
Ciprofloxacin	3.51	9.3	0/4	$3.1x10^6 \pm 1.6x10^5$
Ga-**D5**	10	9.3	4/4	$2.2x10^3 \pm 2.8x10^2$

7. References

1 A. Pandey, C. Savino, S. H. Ahn, Z. Yang, S. G. Van Lanen and E. Boros, *J. Med. Chem.*, 2019, **62**, 9947–9960.

2 E. K. Dolence, A. A. Minnick, C. E. Lin, M. J. Miller and S. M. Payne, *J. Med. Chem.*, 1991, **34**, 968–978.