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Exploitation of antibiotic resistance as a novel drug target: development of a β-lactamase-activated antibacterial prodrug

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ABSTRACT

Expression of β-lactamase is the single most prevalent determinant of antibiotic resistance, rendering bacteria resistant to β-lactam antibiotics. In this article, we describe the development of

an antibiotic pro-drug that combines ciprofloxacin with a β-lactamase-cleavable motif. The pro-drug is only bactericidal after activation by β-lactamase. Bactericidal activity comparable to ciprofloxacin is demonstrated against clinically-relevant *E. coli* isolates expressing diverse β-lactamases; bactericidal activity was not observed in strains without β-lactamase**.** These findings demonstrate that it is possible to exploit antibiotic resistance to selectively target β-lactamase-producing bacteria using our pro-drug approach, without adversely affecting bacteria that do not produce β-lactamase. This paves the way for selective targeting of drug-resistant pathogens without disrupting or selecting for resistance within the microbiota, reducing the rate of secondary infections and subsequent antibiotic use.

INTRODUCTION

Antimicrobial drug resistance is a global health emergency, threatening advances in many areas of medicine including surgery, cancer chemotherapy, organ transplantation and survival of pre-term infants.[1,2] The most prevalent and important resistance determinant is the β-lactamase enzyme, which hydrolyses members of the β-lactam class of antibiotic (e.g. penicillin, cephalosporins and carbapenems) and thereby prevents engagement with their therapeutic targets the penicillin-binding proteins (PBPs).[3,4] Of particular concern are the extended-spectrum β-lactamases (ESBLs) such as the CTX-M class, which are able to cleave a wide range of clinically-relevant β-lactam antibiotics.[5–7]

Urinary tract infections (UTIs) are the most prevalent type of bacterial infection globally. These infections have a high rate of recurrence and can also lead to serious invasive infections such as sepsis, particularly in the elderly.[8,9] *E. coli* is the most common causative organism (~75% cases), of which ~50% are resistant to β-lactam antibiotics due to β-lactamase expression.[8,10] As

a consequence of the high rate of β-lactam resistance in UTI pathogens, second-line, broad-spectrum antibiotics such as ciprofloxacin are increasingly used therapeutically.[11,12] Unfortunately, these broad-spectrum antibiotics are associated with disruption to the beneficial bacteria that colonize the gastro-intestinal tract and other surfaces, known as the microbiota.[13–17] This disruption can lead to serious secondary infections by antibiotic-resistant bacteria such as *Clostridium difficle* or fungi such as *Candida albicans*, leading to colitis and thrush respectively.[13,18] This is because antibiotics target conserved processes in bacteria such as cell wall, protein, DNA or RNA biosynthesis, which not only occur in the pathogens that cause infection but also in the members of the microbiota.[19,20]

An additional complication associated with some second-line therapeutics such as ciprofloxacin is host toxicity. Ciprofloxacin holds two black box warnings, one for increased risk of tendonitis and tendon rupture and one for exacerbation of muscle weakness in myasthenia gravis sufferers.[21] Additionally, in 2015, the FDA officially recognized fluoroquinolone-associated disability (FQAD) as a syndrome. FQAD describes a range of disabling and potentially permanent side effects including disturbances of tendons, joints, muscles, nerves, the nervous system and induction of type 2 diabetes.[22,23] As a result, strategies with the potential to mitigate host toxicity by reducing exposure to ciprofloxacin are needed.

Given the drawbacks associated with broad-spectrum antibiotics, efforts have been made to limit their use.[11,23–25] However, these efforts have had limited success with usage rates increasing globally, particularly in low- and middle-income countries.[26] In part, this is due to a lack of access to fast and efficient diagnostic techniques and the need to respond quickly to serious bacterial infections with effective and cost-efficient treatment regimens that target a wide range

of different bacterial pathogens.[27] There is, therefore, a pressing need to develop new therapeutics that kill a broad range of different pathogens without damaging the host microbiota.

Since β-lactamase enzymes are not found in mammalian cells, we hypothesized that we could exploit this enzyme as a novel anti-bacterial target. Furthermore, β-lactamase expression is prevalent amongst UTI pathogens, which can both colonize the gut and cause infection of the GU tract.[8,10] Consequently, this represents an opportunity to selectively target disease-causing bacteria without causing significant disruption to the microbiota or select for drug resistance as has been reported for broad-spectrum antibiotics such as ciprofloxacin.[28–30] Therefore, the aim of this work was to develop a small molecule antibacterial agent that is selectively active against bacteria that express β-lactamase. To do this, we employed a pro-drug strategy that utilized a β-lactam cleavable motif linked to the broad-spectrum antibiotic ciprofloxacin.

In support of our approach, the use of β-lactams as prodrug modifiers in antibody-directed enzyme prodrug therapy approaches has been explored in disease areas such as cancer (**1** – **3**, Fig 1).[31–35] Additionally, β-lactam-fluoroquinolone conjugates have been proposed as a co-drug strategy to treat bacterial infections (**4** and **5**, Fig 1).[36–39] However, our approach is different in that it is designed to selectively deliver a broad-spectrum, bactericidal antibiotic to only bacteria that express β-lactamase, whilst having minimal effect on bacteria which do not express the resistance determinant. By contrast, previous dual activity co-drug approaches were designed to have broad-spectrum activity against both drug-sensitive bacteria and those that express β-lactamase.

Herein we describe the design and development of the pro-drug, including optimization of the β-lactam motif to reduce the antibacterial activity of the intact molecule and increase the efficiency

of β-lactamase mediated ciprofloxacin release. This is, to our knowledge, the first example of a β-lactam-fluoroquinolone pro-drug with selective activity against drug-resistant bacteria.

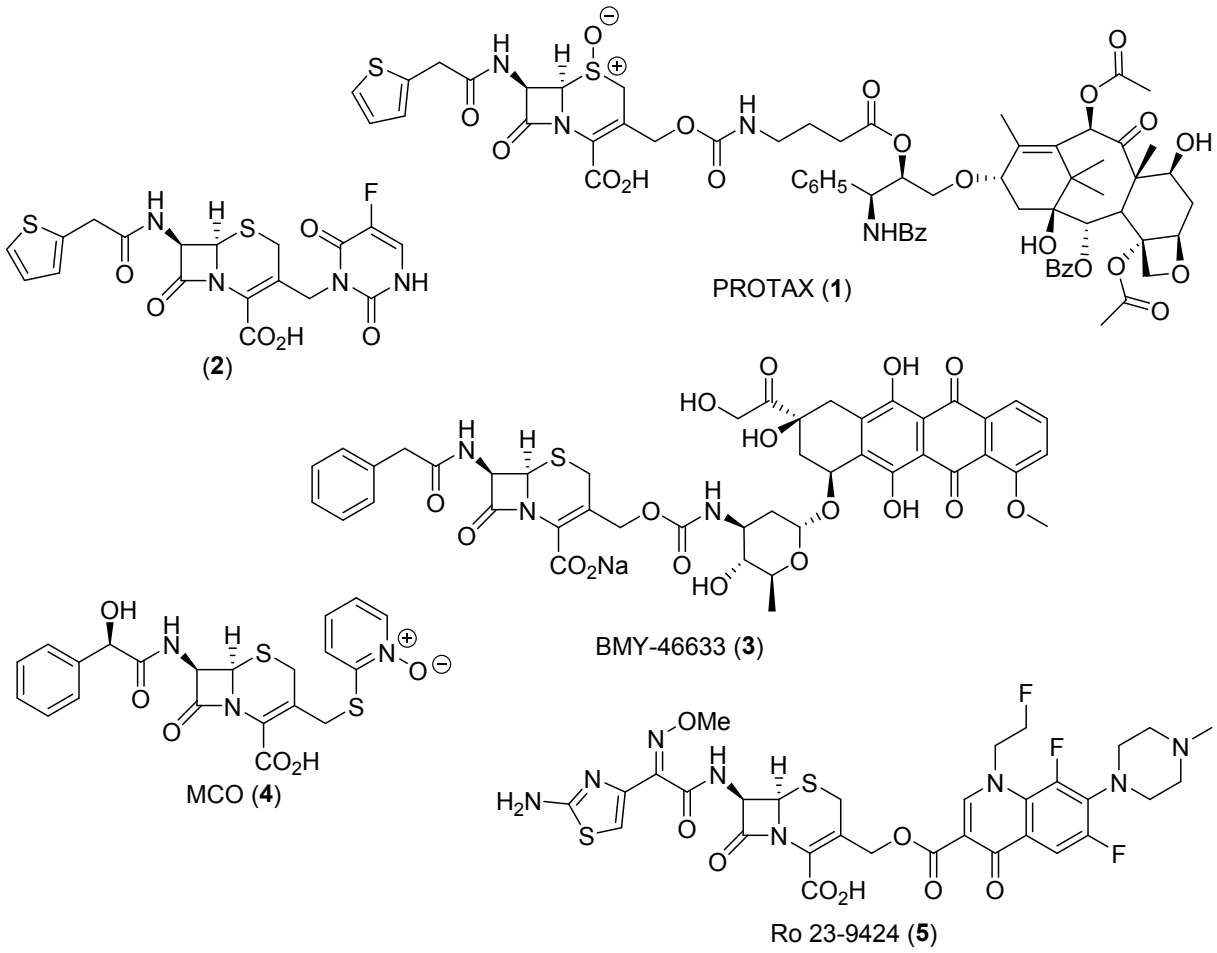


Figure 1. Selected representative examples of cephalosporin pro-drugs (PROTAX **1**,[33] **2**,[34] and BMY-46633 **3**[35]) and codrugs (MCO **4**[38] and Ro 23-9424 **5**[39]).

RESULTS AND DISCUSSION

Prodrug design

In order to create a pro-drug molecule that is selectively activated in β-lactamase producing bacteria it was important to select a β-lactamase cleavable motif, linkage strategy and active

antibiotic that gave a stable non-bactericidal intact molecule and enabled the rapid and efficient release of the antibiotic upon activation by β-lactamase. The success of this strategy required a pro-drug motif that would enable efficient substrate turnover rather than inhibition of the β-lactamase enzyme. The cephalosporin class of β-lactams are efficiently hydrolysed by β-lactamases and have been previously employed as a prodrug motif as cleavage of the β-lactam ring is associated with the loss of the functional group at the 3'-position (Fig 2a).[40] In addition, the chemistry associated with changing the 3'-substituent of cephalosporins is well-established and a wide variety of substituents at the C-3' position are tolerated by β-lactamases.[37,41,42] Consequently, a cephalosporin core was selected as the β-lactam component. To achieve the desired selectivity profile, ciprofloxacin was attached via the carboxylic acid to give the 3'-cephem ester **6** (Fig 2b). Derivatization of the carboxylic acid group of fluoroquinolone antibiotics is associated with a significant decrease in antibacterial activity due to a decreased ability to bind to bacterial DNA-enzyme complexes.[43] Whilst this choice of attachment site was selected to remove the ciprofloxacin activity from the intact prodrug, it remained likely that the pro-drug molecule would retain antibacterial activity as a result of the ability of the cephem portion of the molecule to interact with PBPs. Therefore, to further increase selectivity it was essential to undertake a program of optimization of the β-lactam motif to reduce PBP activity and increase or maintain β-lactamase activity. Initial optimization was performed on the cephalosporin portion of the prodrug to enable the rapid generation of analogues and evaluation of biological activity. The cephalosporin analogues with the most desirable activity profile were then selected for preparation as the full pro-drug.

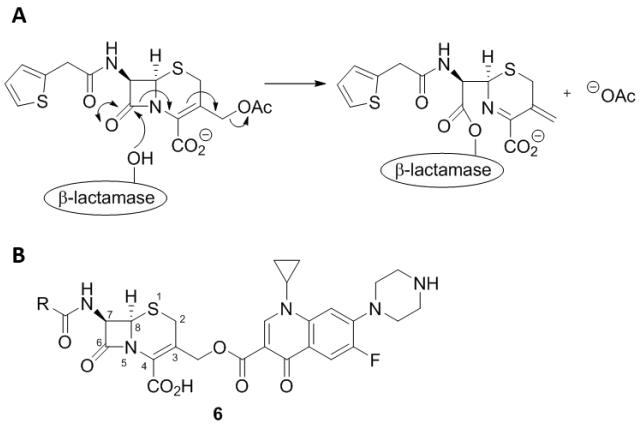


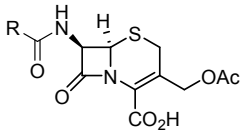
Figure 2. (A) Mechanism of β-lactamase triggered cephalothin hydrolysis; (B) General structure of proposed cephalosporin-ciprofloxacin pro-drug **6**.

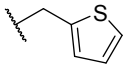
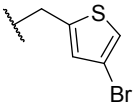
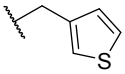
β-Lactam Analogue Preparation and Biological Evaluation

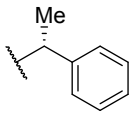
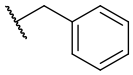
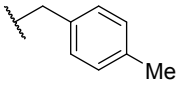
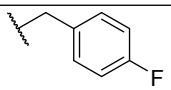
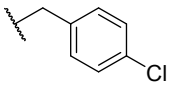
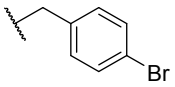
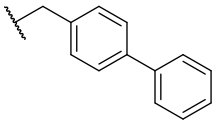
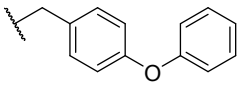
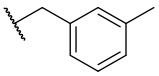
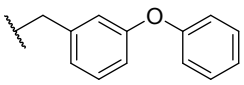
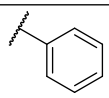
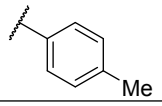
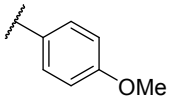
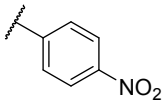
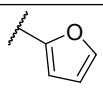
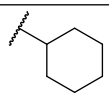
Analysis of the literature identified the amide functionality at C-7 of the cephem ring as central to PBP and β-lactamase activity[44–51] and therefore structural changes at this position provided the initial focus of investigation. Using cephalothin **7** (Table 1) as the starting point, analogues were prepared to explore bioisosteric replacement,[52,53] functionalities present in early generation β-lactam antibiotics, and to probe steric and electronic tolerance.[54,55] All compounds were synthesized according to the previously reported methods (Fig S1).[55,56] Antibacterial activity was assessed by determining the minimal concentration required to inhibit bacterial growth, known as the minimal inhibitory concentration (MIC), against the *E.coli* strain DH5α ± expression of the ESBL TEM-116.[57] The susceptibility to β-lactamase mediated hydrolysis was assessed by determining the physiological efficiency (k_{cat}/K_m) of hydrolysis by recombinant AmpC protein.[3,58,59]

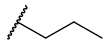
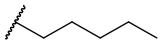
For all compounds (Table 1), a higher MIC value was determined for the *E. coli* strain expressing TEM-116 than the strain not expressing β-lactamase, indicating hydrolytic activity by the β-lactamase. Introduction of a substituent to the thiophene ring (**8**) or switching from a C-2 to a C-3 substitution (**9**) gave a modest increase in MIC values and a small decrease in k_{cat}/K_m compared to cephalothin **7**. Although no measurable MIC value could be determined for any of the phenyl analogues (**20-23**), this was accompanied by a >3-fold decrease in k_{cat}/K_m. In general, a quaternary carbon (**20-24**) or tertiary carbon (**10** and **25**) at the α-position relative to the amide carbonyl was not well tolerated by AmpC. This finding is consistent with prior reports and has previously been exploited to reduce β-lactamase activity in the development of later-generation β-lactams. Compounds containing straight-chain aliphatic groups (**26-28**) retained some antibacterial activity; an increase in k_{cat}/K_m was observed with increasing chain length.

Table 1. Antibacterial activities (MIC) and AmpC hydrolytic efficiency of synthesized compounds and reference compound cephalothin (ceph) **7**.



Compound	R group	MIC value (μM)		AmpC-catalysed hydrolytic efficiency k_{cat}/K_m (μM/S^{-1})
		DH5α	DH5α + TEM-116	
Ceph **7**		12.5	100	2.86 ± 0.33
8		50	200	2.50 ± 0.28
9		50	200	2.36 ± 0.10

10		400	>800	0.87 ± 0.15
11		50	100	1.86 ± 0.09
12		100	400	1.77 ± 0.19
13		100	200	1.34 ± 0.20
14		100	400	4.78 ± 0.13
15		200	800	5.06 ± 0.32
16		>800	>800	3.99 ± 0.88
17		>800	>800	7.33 ± 1.72
18		200	400	9.13 ± 1.66
19		>800	>800	31.14 ± 2.67
20		>400	>400	0.52 ± 0.18
21		>400	>400	0.26 ± 0.10
22		>800	>800	0.31 ± 0.38
23		>400	>400	0.88 ± 0.15
24		>400	>400	ND
25		>400	>400	0.17 ± 0.09
26		200	400	0.53 ± 0.16

27		100	400	0.64 ± 0.04
28		200	>800	0.81 ± 0.09

Examination of the tested analogues (Table 1) immediately revealed the importance of bulky benzylic substituents (**11-19**). Thiophene rings are frequently used as a bioisosteric replacement for a phenyl groups and it is therefore perhaps unsurprising that there was only a modest 4-fold increase in MIC value against *E. coli* DH5α and a slight decrease in k_{cat}/K_m for **11** compared to cephalothin.[52,53] However, introduction of substituents at the para-position (**12-17**) gave a further 2- to 4-fold increase in MIC against *E. coli* DH5α compared to unsubstituted benzyl **11**. Substitution at the para-position also affected hydrolysis by AmpC with the following order of activity observed F < Me = H < Cl = Br. Movement of the methyl substituent from the para- (**12**) to the meta-position (**18**) gave a 5-fold increase in k_{cat}/K_m, and a 3-fold increase compared to cephalothin. High k_{cat}/K_m values were determined for bisaryl **16** and the para- and meta-substituted biphenyl ethers **17** and **19** (3.99 ± 0.88, 7.33 ± 1.72 and 31.14 ± 2.67, respectively). In addition, no measurable MIC values could be determined for **16**, **17** or **19**. This led us to question if the results were indicative of no antibacterial activity, or simply a result of increased efflux activity out of, or a lack of permeability in to, the bacterial cell.

β-Lactamase hydrolytic activity in whole-cell NMR assay

To address the question of compound permeability/efflux, a whole-cell β-lactamase hydrolysis assay was used to detect the penetration of compounds into the periplasm.[60,61] Hydrolytic decomposition of β-lactam rings is associated with changes in ^{1}H NMR signals, which can be detected using whole bacterial cells in real time by ^{1}H NMR spectroscopy (Fig S2). As hydrolysis occurred within the bacterial periplasm, only compounds with sufficient intracellular

accumulation were hydrolysed. Compound **16** and **17** were selected as representative examples of a high lipophilicity compounds with no measurable antibacterial activity and moderate *in vitro* β-lactamase hydrolysis. We evaluated the hydrolysis of bisaryl **16**, biaryl ether **17** and cephalothin **7** in DH5α ± TEM-116 (Table 2). After 90 minutes, **16** was 69% hydrolyzed in DH5α + TEM-116 compared to 14% hydrolyzed in DH5α - TEM-116 and **17** was 53% hydrolyzed in DH5α + TEM-116 compared to 13% hydrolyzed in DH5α - TEM-116. These results indicated a high degree of *in vivo* β-lactamase mediated hydrolysis and that **16** and **17** accumulated in the bacterial cell. We therefore concluded that the lack of antibacterial activity of this compound against *E. coli* DH5α without β-lactamase was due to an absence of PBP engagement and not due poor permeability or efflux activity.

Table 2. Percentage hydrolysis of cephalothin (ceph) **7** and compounds **16** and **17** by DH5α cells ± β-lactamase in whole-cell NMR hydrolysis assay.

Compound	Conc. (μM)	Incubation time (mins)	Strain	% Hydrolysis by NMR	
				- βla	+ βla
Ceph **7**	50	60	DH5a ± TEM-116	-	61
16	100	90		14	69
17	100	90		13	53
Ceph **7**	100	60	DH5a ± CTX-M-1	0	100
16	100	60		0	100
17	100	60		0	95

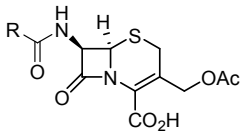
Biological evaluation in uropathogenic *E. coli*

Initial assessment of compound activity was performed in the laboratory *E. coli* strain DH5α. To assess the activity of the β-lactams against a clinically-relevant pathogenic strain of *E. coli* we selected the uropathogenic strain CFT073. This bacterium was isolated from the blood of a patient with acute pyelonephritis, is devoid of all virulence plasmids commonly associated with

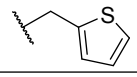
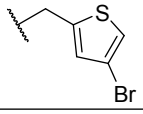
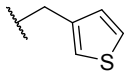
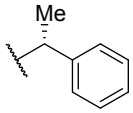
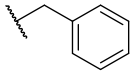
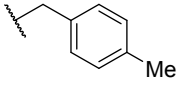
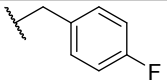
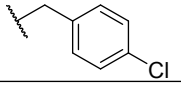
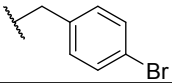
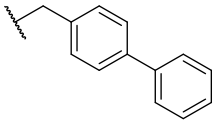
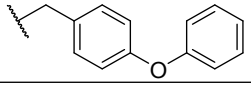
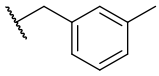
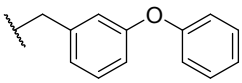
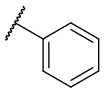
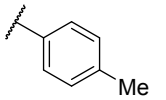
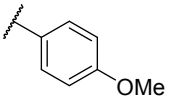
uropathogenic strains and proved tractable for genetic manipulation.[62,63] The plasmid pSU18, either without the coding sequence for β-lactamase (referred to here as pEMP) or encoding for the β-lactamase CTX-M-1 was introduced into CFT073, enabling comparison of compound activity in CFT073 and CFT073 + pSU18 ± β-lactamase. The primary β-lactamase used in this work was CTX-M-1 because CTX-M enzymes are the most prevalent β-lactamases amongst Enterobacteria such as *E. coli*. As part of a class of extended-spectrum β-lactamases (ESBL) it confers resistance to most β-lactam antibiotics, with the exception of carbapenems.[6]

In the first instance, MIC values were determined for selected compounds against CFT073 + pSU18 ± CTX-M-1 (Table 3). For all the compounds tested, the MIC values for CFT073 + pSU18 were within 2-fold of those determined against DH5α. Next the hydrolytic activity of these compounds was assessed in the whole cell NMR assay (Table 3). For the majority of the compounds tested, a low level of hydrolysis, <20% after 60 minutes, was detected. However, levels of hydrolysis comparable to that observed for cephalothin (68%) were observed only for **24** and **26** (64% and 67%, respectively).

Table 3. Antibacterial activities (MIC) against CFT073 ± CTX-M-1 and percentage hydrolysis in CFT073 + CTX-M-1 cells for selected compounds and reference compound cephalothin (ceph) **7**.



Compound	R group	MIC value (μM)		% hydrolysis in CFT073 + pSU18 + CTX-M-1[a]
		CFT073 + pSU18	CFT073 + pSU18 + CTX-M-1	

Ceph **7**		25	>400	68
8		50	>400	ND
9		50	>400	32
10		>400	>400	0
11		50	>400	17
12		100	>400	7
13		100	>400	18
14		100	>400	14
15		100	>400	9
16		>400	>400	4
17		>400	>400	4
18		200	>400	16
19		≥400	>400	0
20		>400	>400	26
21		>400	>400	3
22		>400	>400	3

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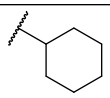
25		ND	ND	0
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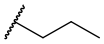
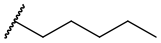
27		200	>400	25
28		100	>400	7

[a] Percentage hydrolysis determined for 100 μM compound at 1 hour by whole-cell NMR assay. ND = Not done.

A clear feature of the SAR was that CTX-M-1 mediated hydrolytic activity in whole CFT073 cells correlated with lipophilicity. Plotting the calculated LogP (cLogP) values for compounds against the log of percentage hydrolysis revealed that moderate-high levels of hydrolysis (>30%) were only observed for compound with cLogP values below 0.1 (Fig 3). Linear regression analysis revealed moderate correlation ($R^2 = 0.60$), despite the degree of hydrolysis reflecting both cellular penetration and β-lactamase activity, which are both sensitive to compound lipophilicity.

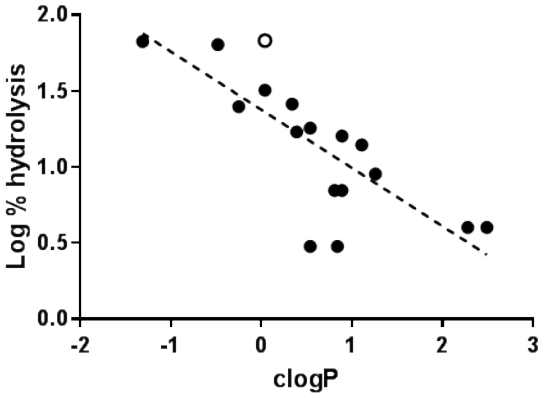


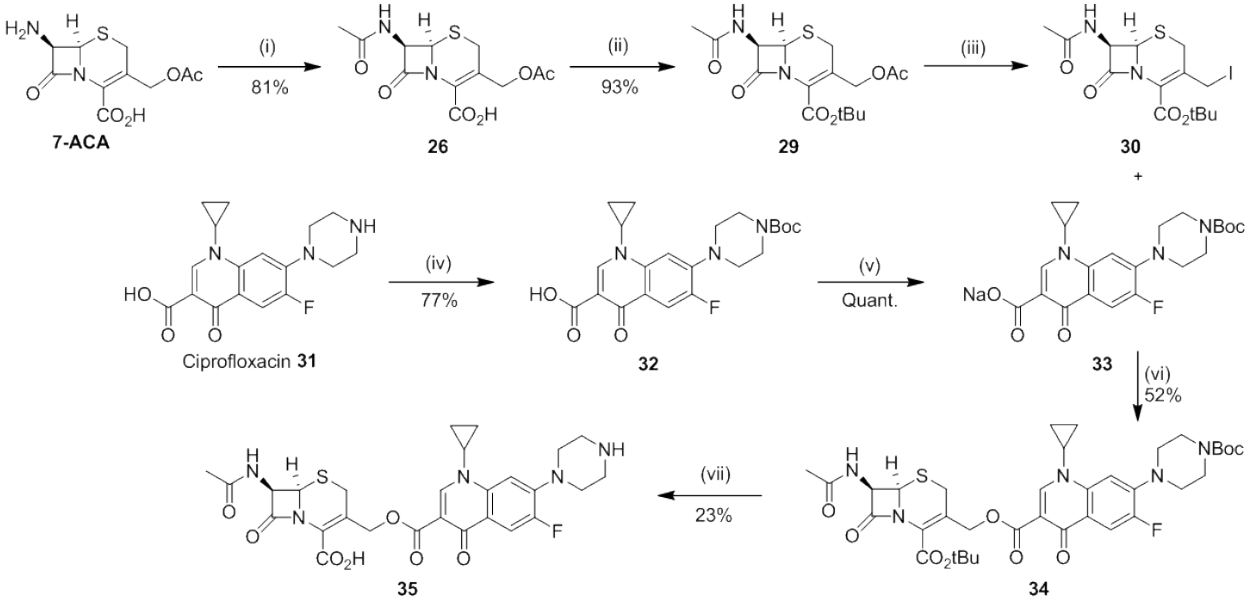
Figure 3. Plot of log percentage hydrolysis in CFT073 + CTX-M-1 cells against calculated logP (clogP) for synthesized compounds (filled circle) and cephalothin **7** (open circle). Linear regression (dashed line), $R^2 = 0.60$ (GraphPad prism 7).

Interestingly, compounds **16** and **17** were hydrolyzed rapidly (69% and 54% after 90 minutes, respectively) in DH5α expressing the TEM-116 β-lactamase (Table 2) but only 4% hydrolyzed after 60 minutes in CFT073 expressing the CTX-M-1 β-lactamase. We hypothesized that the low level of hydrolytic activity observed for many of the compounds could be a result of poor intracellular accumulation in CFT073 *E. coli* since clinical isolates often have reduced permeability to antibiotics.[64] To test this hypothesis, hydrolysis in DH5α expressing CTX-M-1 was determined for cepaholothin **7**, **16** and **17** (Table 2). After 60 minutes complete hydrolysis for cepaholothin **7** and **16** and 94% hydrolysis for **17** was observed, suggesting that the low level of hydrolysis observed in CFT073 + CTX-M-1 was not due to the inability of CTX-M-1 to hydrolyze this chemotype. Instead it is likely that either due to poor membrane permeability or increased efflux activity, lipophilic analogues were unable to engage with CTX-M-1 in CFT073. For compounds with low hydrolytic activity in the whole cell NMR assay with CFT073 we were unable to discern if a high MIC value in the absence of CTX-M-1 truly reflected a lack of antibacterial activity or a lack of permeability/high efflux activity. Therefore, compound **26**, with its high MIC value in both CFT073 ± CTX-M-1 (≥400 μM) and high hydrolytic activity in whole CFT073 cells (67% after 60 minutes), was selected for incorporation into the full prodrug molecule. Compound **24**, which also possessed a high MIC value in both CFT073 ± CTX-M-1 (≥400 μM) and high hydrolytic activity in whole CFT073 cells (64% after 60 minutes) was not progressed at this time as we wished to avoid the potential for toxicity problems arising from the furan ring, which has been identified as common toxicophore due to metabolic instability.[65]

Pro-drug preparation

Preparation of the pro-drug derived from compound **26** required coupling of an activated **26**-derivative, iodo-cephalosporin **30**, to ciprofloxacin-derivative **33** (Scheme 1). The iodo-cephalosporin **30** was prepared in three steps from commercially available 7-aminocephalosporanic acid (7-ACA). First, 7-ACA was reacted with acetic anhydride to give *N*-acetyl **26**.[55] Protection of the carboxylic acid as the *tert*-butyl ester was then performed using *tert*-butyl 2,2,2-trichloroacetimidate (TBTA) enabling formation of the *tert*-butyl ester **29** in the absence of base,[66] which has previously been reported to be associated with isomerization from the Δ^3- to the biologically inactive Δ^2-cephem.[67–69] Iodination at the 3'-position with TMSI gave the activated iodo-cephalosporin **30** ready for coupling.[36] The ciprofloxacin component was prepared by BOC protection of the piperazine NH to give **32** and subsequent conversion of the carboxylic acid to the sodium salt **33**.[69] Coupling of compounds **30** and **33** was performed in 3:1 1,4-dioxane/DMF to give the protected cephalosporin-ciprofloxacin conjugate **34**.[36,67] Finally, global de-protection with TFA to remove the BOC and *tert*-butyl ester afforded the final pro-drug **35**.[71] Synthesis of **35** was achieved in 7-steps from commercially-available materials without the requirement for toxic metal reagents.

Scheme 1. Synthesis of pro-drug **35**.[a]



[a]Reagents and conditions: (i) Acetic anhydride, $NaHCO_3$, H_2O, acetone, 0 °C, 30 min; (ii) TBTA, DCM, 60 °C, 16 hr; (iii) TMSI, DCM, rt, 2 hr; (iv) Boc_2O, 1 M NaOH, THF, rt, 16 hr; (v) 0.1 M NaOH, MeOH, rt, 30 min; (vi) 3:1 1,4-dioxane/DMF, rt, 4 hr; (vii) 1:1 TFA/DCM, anisole, 0 °C - rt.

In vitro DNA gyrase activity

Members of the fluoroquinolone antibiotic family, including Ciprofloxacin **31**, target the type II topoisomerase enzymes, DNA gyrase and topoisomerase IV. Inhibition of these enzymes results in the arrest of DNA replication and transcription preventing bacterial cell growth.[43,72] Having successfully prepared pro-drug **35**, we moved to testing our hypothesis that the intact prodrug would not inhibit DNA gyrase or topoisomerase IV but β-lactamase triggered hydrolysis would result in the release of free ciprofloxacin capable of engaging these targets. To test this hypothesis, we evaluated the ability of pro-drug **35** and ciprofloxacin to inhibit recombinant DNA gyrase enzyme activity both in the absence and presence of the purified recombinant β-lactamase CTX-M-15 (Fig 4). Compounds were incubated with relaxed pBR322 plasmid DNA

with and without recombinant CTX-M-15 and recombinant DNA gyrase. As predicted, inhibition of DNA gyrase by pro-drug **35** was not observed in the absence of CTX-M-15. However, in the presence of CTX-M-15, 1 μM **35** was capable of reducing DNA gyrase activity by >50%. Ciprofloxacin **31** activity was not affected by CTX-M-15. These results confirmed that β-lactamase-specific hydrolysis of **35** results in liberation of active antibiotic capable of DNA gyrase inhibition *in vitro*.

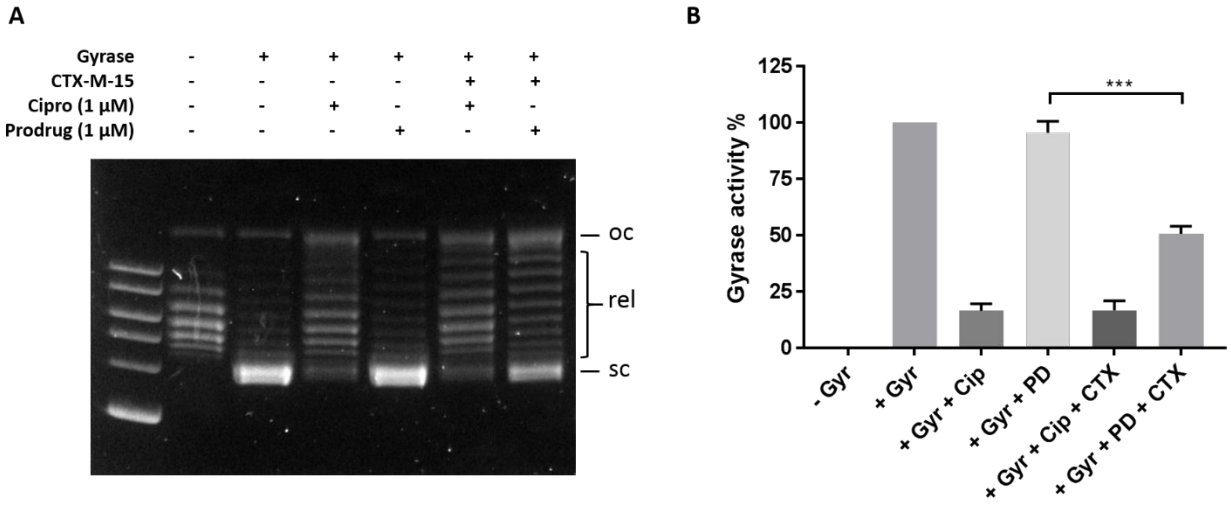
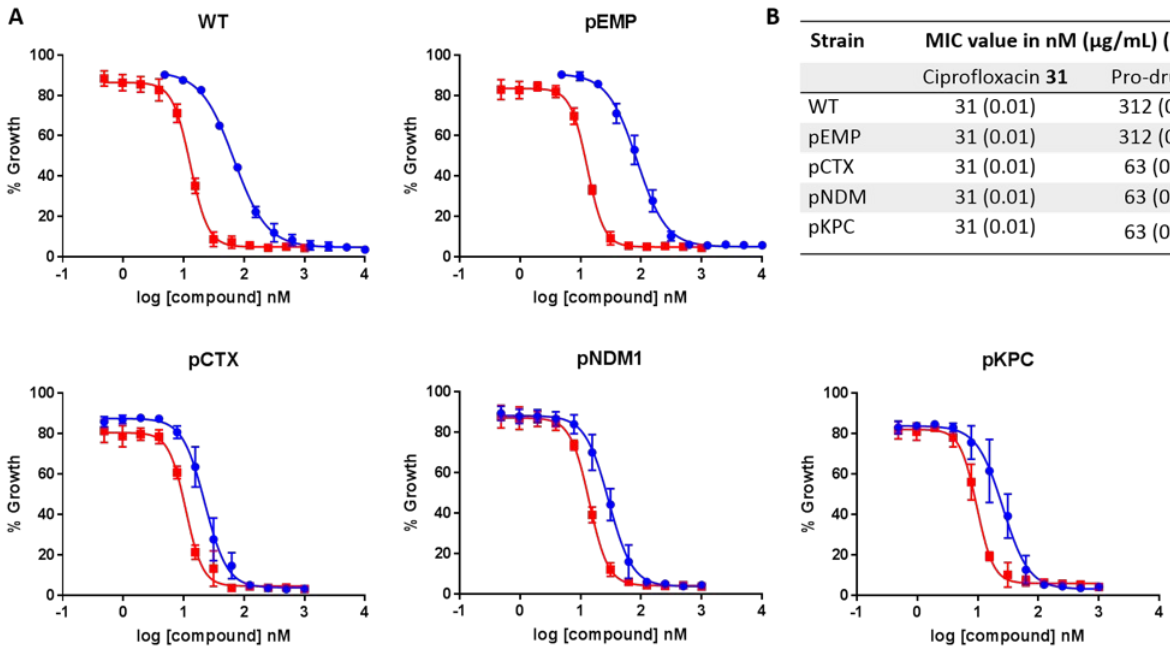


Figure 4. Activity of pro-drug **35** and ciprofloxacin **31** against recombinant DNA gyrase ± CTX-M-15. A) DNA was separated by agrose gel electrophoresis with 2-log DNA ladder. oc, open circle DNA; rel, relaxed DNA; sc, supercoiled DNA. B) Quantification of gel bands corresponding to supercoiled DNA and normalized to no gyrase and gyrase only activity (ImageJ 1.52a). Gyr, DNA gyrase; cip, ciprofloxacin **31**; PD, pro-drug **35**; CTX, CTX-M-15. Error bars represent SEM (n = 4); prodrug vs prodrug + CTX-M-15 was analyzed by unpaired *t*-test, $p = 0.0004$ (GraphPad Prism 7.03).

Selective pro-drug activity against uropathogenic *E. coli* expressing β-lactamase

Next, the activity of pro-drug **35** was evaluated using whole bacterial cells. MIC values for **35** and ciprofloxacin **31** were determined in *E. coli* CFT073 expressing the disease-relevant β-lactamases CTX-M-1, New Delhi metallo-β-lactamase 1 (NDM-1) and *Klebsiella pneumoniae* carbapenemase (KPC-3) β-lactamase (Fig 5). NDM-1 is an example of an increasingly prevalent β-lactamase that is capable of hydrolyzing carbapenems, usually considered the last line of defence against β-lactamase expressing bacteria[73], whilst KPC enzymes are class A β-lactamases and the most common carbapenemases globally.[74,75]



B

Strain	MIC value in nM (μg/mL) (n=3)	
	Ciprofloxacin **31**	Pro-drug **35**
WT	31 (0.01)	312 (0.18)
pEMP	31 (0.01)	312 (0.18)
pCTX	31 (0.01)	63 (0.04)
pNDM	31 (0.01)	63 (0.04)
pKPC	31 (0.01)	63 (0.04)

Figure 5. Antibacterial activities for pro-drug **35** (blue) and ciprofloxacin **31** (red) against CFT073 E.coli cells WT and expressing empty plasmid (pEMP), CTX-M-15 (pCTX), NDM1 (pNMD1) and KPC (pKPC). (A) Dose-response curves, each point represents the mean ± SEM, n = 3; (B) Summary of MIC values.

As expected, the MIC value determined for ciprofloxacin was consistent across all tested strains at 31 nM. The MIC determined for pro-drug **35** in *E. coli* CFT073 WT and expressing empty

plasmid (pEMP) was 310 nM, representing a 10-fold decrease in activity compared to ciprofloxacin **31** in the absence of β-lactamase. By contrast to bacteria without β-lactamase, the MIC value for **35** was 63 nM for *E. coli* CFT073 strains expressing CTX-M-1, NDM1 or KPC, only 2-fold higher than ciprofloxacin **31**. These data demonstrate efficient and selective β-lactamase mediated pro-drug cleavage and active antibiotic release, resulting in arrest of bacterial cell growth at comparable concentrations to that with free ciprofloxacin.

The activity of pro-drug **35** compared to ciprofloxacin was profiled further in six independently-isolated uropathogenic *E. coli* clinical isolates expressing the CTX-M-15 β-lactamase, which were obtained from Charing Cross Hospital, Imperial College NHS Trust. Three of the strains were ciprofloxacin sensitive (EC11, EC16 and EC17) and three were ciprofloxacin resistant (EC12, EC13 and EC19) as determined by diagnostic susceptibility testing. Activity of pro-drug **35** was confirmed against the three ciprofloxacin sensitive bacterial strains, whilst no arrest in bacterial growth was observed for either ciprofloxacin **31** or pro-drug **35** for strains EC12, EC13 or EC19 (Fig 6 and Table S1). These results demonstrate that the antibacterial activity of **35** observed in β-lactamase expressing strains is mediated through liberated ciprofloxacin and provide evidence for the clinical utility of **35.** The gut microbiota includes both Gram-negative bacteria such as *E. coli* and Gram-positive organisms such as *E. faecalis*. Since we had shown that **35** was inactive against *E. coli* we decided to further examine the potential clinical value of the pro-drug by testing its activity against two representative *E. faecalis* strains, that did not express β-lactamase. Pro-drug **35** showed reduced activity compared to ciprofloxacin, indicating that our approach could minimize undesirable damage to the microbiota caused by fluoroquinolones (Fig S3). We also assessed the activity of **35** against CFT073 pEMP or pCTX-M-1 in the presence of human serum, which can modulate drug activity via protein-binding and

also contains esterases that have the potential to activate the pro-drug by cleaving the ester linkage. However, data from MIC assays performed in the presence of human serum (Fig S4), were equivalent to those obtained in the absence of serum (Fig 5). Combined, these findings provided further confidence in the selectivity of prodrug **35** and its stability in the host environment.

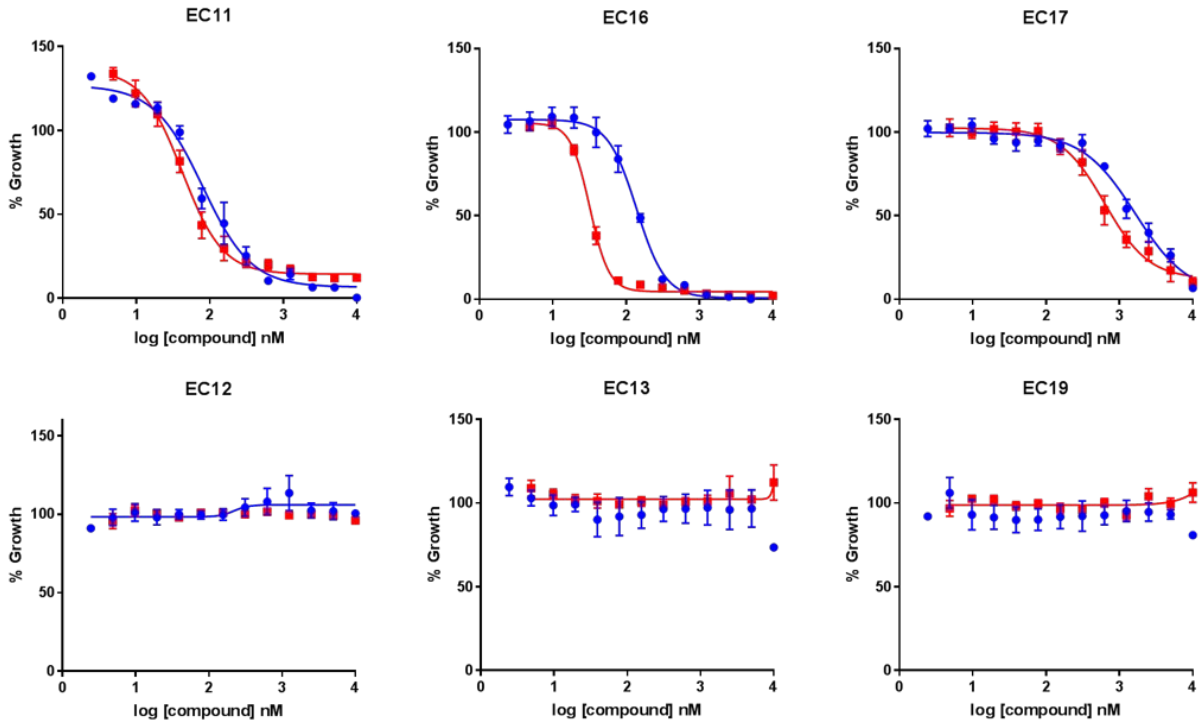


Figure 6. Effect of pro-drug **35** (blue) or ciprofloxacin **31** (red) against six uropathogenic *E.coli* clinical isolates. Each point represents mean ± SEM, n = 3.

Selective bactericidal activity against β-lactamase expressing bacteria

Finally, the ability of pro-drug **35** to kill bacteria, rather than arrest growth was evaluated. Survival of *E. coli* CFT073 pEMP or pCTX-M-1 with no treatment or exposed to ciprofloxacin **31** or pro-drug **35** were determined over time by CFU counts (Fig 7). After 6 hours incubation with pro-drug **35** there was >100-fold greater killing of *E. coli* expressing CTX-M-1, compared

with bacteria that did not express the enzyme. Killing activity of **35** in *E. coli* expressing CTX-M-1 was almost identical to free ciprofloxacin, whilst growth comparable to no treatment controls was detected for CFT073 expressing empty plasmid incubated with **35**. These findings demonstrate that it is possible to selectively kill β-lactamase-producing bacteria using our pro-drug approach, without adversely affecting bacteria that do not produce β-lactamase.

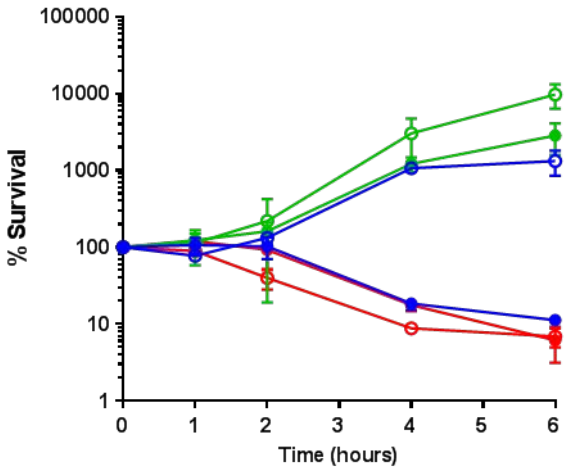


Figure 7. Survival of CFT073 pEMP (open circle) and pCTX (filled circle) with no treatment (green), ciprofloxacin **31** (78 nM) (red) or pro-drug **35** (78 nM) (blue). Cipro, ciprofloxacin; PD, pro-drug **35**.

CONCLUSIONS

A novel cephalosporin-fluoroquinolone antibiotic pro-drug has been designed, synthesized and evaluated for biological activity. A program of optimization was successfully undertaken to reduce the antibacterial activity of the intact pro-drug though modification to the cephalosporin component. Pro-drug **35** exhibits similar growth inhibitory activity to ciprofloxacin against uropathogenic *E. coli* expressing the diverse ESBLs CTX-M-1, NDM-1 or KPC, but little

activity against strains that did not express β-lactamases. The selectively observed for bactericidal activity was even greater, with pro-drug **35** killing β-lactamase expressing bacteria at the same rate as free ciprofloxacin, whilst not affecting the growth of bacteria that did not express β-lactamases.

Overall, the activity of pro-drug **35** is consistent with: 1) permeability to pathogenic gram-negative bacteria; 2) a low-level of antibacterial activity for the intact pro-drug; 3) β-lactamase mediated intracellular release of ciprofloxacin upon cleavage of the cephalosporin; 4) activation of the pro-drug by a broad range of β-lactamases.

Together, these studies demonstrate that our pro-drug approach can harness resistance as a therapeutic opportunity to selectively kill antibiotic-resistant bacteria. Since fluoroquinolones are a clinically useful, broad-spectrum antibiotic we envisage that increasing the selectivity profile will have two major advantages. Firstly, increased selectivity of fluoroquinolones will enable maintenance of the microbiota leading to reduced secondary infection rate and subsequent antibiotic use. Secondly, a decreased side-effect profile due to minimized exposure of host cells to fluoroquinolone antibiotic.

The focus of this work was uropathogenic *E. coli* (UPEC), which are a major cause of UTIs and frequently expresses β-lactamase. Our approach is expected to result in high concentrations of active ciprofloxacin at the site of infection (bladder and kidneys), without causing disruption to the host microbiota. However, it is important to consider that the primary reservoir of UPEC is the gut, and it is envisaged that our pro-drug approach would also enable the selective decolonization of ESBL-expressing *E. coli* from the GI tract of people who suffer from recurrent UTI. An additional use could be the treatment of lung infections caused by *P. aeruginosa* in

patients with cystic fibrosis, for which fluoroquinolones are the only available oral antibiotics.[76,77] Since >65% *P. aeruginosa* isolates express the AmpC β-lactamase,[78] it is possible that our pro-drug approach could be used treat the infection without the associated damage to the microbiota.

In summary, this study paves the way for the development and use of small molecule therapeutics that selectively target drug-resistant pathogens using broad-spectrum antibiotics, whilst minimizing selection for resistance and without collateral damage to the microbiota. This compliments on-going efforts to alter the spectrum of activity of existing antibiotics to enable them to be used in new ways. For example, recent work from Liu and co-workers[79] described an approach to broaden the spectrum of activity of the otherwise Gram-positive restricted oxazolidinone antibiotics to confer activity against Gram-negative bacteria. By contrast, our approach restricts the activity of the normally broad-spectrum agent ciprofloxacin to only those bacteria that express the β-lactamase resistance determinant. We anticipate that the modification of existing antibiotics will prolong and expand their clinical utility, whilst efforts to discover new antibiotic classes are underway.

EXPERIMENTAL SECTION

Experimental Procedures (Chemistry). Unless otherwise stated, reactions were conducted in oven-dried glassware under an atmosphere of argon using anhydrous solvents. All commercially obtained reagents and solvents were used as received. TLC analysis was performed on precoated aluminum sheets of silica (60 F254 nm, Merck) and visualized using short-wave UV light. Column chromatography was also performed on an Isolera™ Spektra Four purification system using Biotage Flash silica cartridges (SNAP KPSil, SNAP Ultra or SNAP KP-C18-HS). ^{1}H

NMR spectra were recorded on Bruker Av-400 spectrometers at 400 MHz using an internal deuterium lock. Chemical shifts are quoted in parts per million (ppm) using the following internal references: $CDCl_3$ (δ_H 7.26), D_2O (δ_H 4.79), and DMSO-d_6 (δ_H 2.50). Signal multiplicities are recorded as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), doublet of doublets (dd), triplet of triplets (tt), apparent (app), broad (br), or obscured (obs). Coupling constants, J, were measured to the nearest 0.1 Hz. ^{13}C NMR spectra were recorded on Bruker Av-400 spectrometers at 101 MHz using an internal deuterium lock. Chemical shifts are quoted to 0.1 ppm, unless greater accuracy was required, using the following internal references: $CDCl_3$ (δ_C 77.0) and DMSO-d_6 (δ_C 39.5). High resolution mass spectra were recorded on a Waters LCT with a Waters Aquilty UPLC I-class system operating in ES+ or ES- mode. Analytical separation was performed using a Waters BEH Acquity C18, 50mm x 2.1mm column using a flow rate of 0.5ml/min in a 4 minute gradient elution at 40 °C. The mobile phase was a mixture of 99.9% Water and 0.1% Formic Acid (solvent A), and 99.9% Acetonitrile and 0.1% Formic Acid (solvent B). Gradient elution was as follows: 95:5 (A/B) to 5:95 (A/B) over 3.2 min, and then reversion back to 95:5 (A/B) over 0.3 min, finally 95:5 (A/B) for 0.5 min. For accurate mass determination samples were referenced against Leucine Enkaphalin or Sulfadimethoxine. All tested compounds were ≥95% purity by LCMS analysis. All final compounds were screened through computational PAINS and aggregator filters and gave no structural alerts as potential assay interference compounds or aggregators.[80,81] All compounds were soluble at the concentrations used for biological evaluation.

General Synthetic Procedures. Method A. 7-Aminocephalosporanic acid (1 equiv) was dissolved in sat. $NaHCO_3$ (aq) and acetone added, followed by acid chloride (1.2 or 2 equiv). The reaction was stirred at room temperature for 30 min then washed with EtOAc. The aqueous

layer was acidified to pH 2 with 1 M HCl and extracted with DCM (x3).[55] The organic extracts were combined, dried over Na_2SO_4, evaporated and the resulting solid triturated with ice-cold Et_2O (unless otherwise stated) to afford the product.

Method B. 7-Aminocephalosporanic acid (1 equiv) and acid chloride (2 equiv) were dissolved in EtOAc and heated to reflux for 30 min. After cooling to room temperature, aniline (1.3 – 3 equiv) was added and stirred for 1 h before the reaction mixture was diluted with 3% $NaHCO_3$ (aq). The aqueous layer was separated and the organic layer washed with 3% $NaHCO_3$ (aq) (x2). The aqueous layers were combined, washed with EtOAc and acidified to pH 2 with 1 M HCl.[55] The desired product was isolated as described.

Method C. Carboxylic acid (1 equiv) was dissolved in DCM and oxalyl chloride (1.2 equiv) added followed by DMF (1 drop) and the reaction stirred for 16 h.[56] The solvent was removed under reduced pressure to afford the acyl chloride, which was used without further purification.

Preparation of compounds

6*R*,7*R*)-3-(Acetoxymethyl)-7-(2-(4-bromothiophen-2-yl)acetamido)-8-oxo-5-thia-1 azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (8). 2-(4-Bromothiophen-2-yl)acetic acid (203 mg, 0.92 mmol), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (193 mg, 1.01 mmol) and 7-aminocephalosporanic acid (250 mg, 0.92 mmol) were suspended in DMF (8 ml) and stirred at room temperature for 48 h.[82] The resulting mixture was filtered and the filtrate diluted with H_2O and extracted with EtOAc (x3). The organic extracts were combined, washed with 1 M LiCl (aq) and brine, and dried over Na_2SO_4. Solvent was removed under reduced pressure and the resulting oil triturated with Et_2O. The precipitate was collected by vacuum filtration and washed with DCM to afford the product as beige amorphous solid (36 mg, 8%). IR

(solid): υ_{max} 3273, 3101, 2837, 1774, 1748, 1707, 1662, 1539, 1223 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.15 (d, J = 8.1 Hz, 1H), 7.51 (d, J = 1.2 Hz, 1H), 6.93 (s, 1H), 5.68 – 5.59 (m, 1H), 5.06 (d, J = 4.8 Hz, 1H), 5.00 (d, J = 12.6 Hz, 1H), 4.70 (d, J = 12.6 Hz, 1H), 3.78 (d, J = 2.6 Hz, 2H), 3.58 (d, J = 18.0 Hz, 1H), 3.42 (d, J = 18.7 Hz, 1H), 2.02 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 170.3, 169.4, 162.8, 139.1, 128.5, 122.8, 107.7, 59.0, 57.2, 35.6, 25.4, 20.6. HRMS (ESI^+): calcd for $C_{16}H_{17}BrN_2O_6S_2$ $(M + H)^+$ 496.9453, found 496.9479.

(6*R*,7*R*)-3-(Acetoxymethyl)-8-oxo-7-(2-(thiophen-3-yl)acetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (9). 3-Thiopheneacetic acid (104 mg, 0.74 mmol), oxalyl chloride (76 μL, 0.88 mmol) and DMF (1 drop) were reaction in DCM (3 mL) according to method C. The resulting acid chloride and 7-aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 μL) according to method B. The aqueous layer was extracted with DCM (x3) and the organic layers were combined, dried over Na_2SO_4 and evaporated. The resulting solid was triturated with ice-cold DCM to afford the product as an off-white amorphous solid (48 mg, 33%). IR (solid): υ_{max} 3284, 1751, 1730, 1651, 1621, 1536, 1241 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 8.95 (d, J = 8.3 Hz, 1H), 7.46 (dd, J = 4.9, 3.0 Hz, 1H), 7.26 (dd, J = 2.9, 1.0 Hz, 1H), 7.03 (dd, J = 4.9, 1.2 Hz, 1H), 5.46 (dd, J = 8.3, 4.8 Hz, 1H), 4.99 (d, J = 11.9 Hz, 1H), 4.93 (d, J = 4.8 Hz, 1H), 4.73 (d, J = 11.9 Hz, 1H), 3.61 – 3.49 (m, 2H), 3.45 (d, J = 17.2 Hz, 1H), 3.19 (d, J = 17.3 Hz, 1H), 2.00 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.6, 170.5, 163.3, 162.8, 135.8, 135.6, 128.6, 125.7, 122.3, 64.7, 58.5, 57.2, 36.4, 25.1, 20.8. HRMS (ESI^+): calcd for $C_{16}H_{16}N_2O_6S_2$ $(M + H)^+$ 397.0528, found 397.0540.

(6*R*,7*R*)-3-(Acetoxymethyl)-8-oxo-7-((*R*)-2-phenylpropanamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (10). (*R*)-(−)-2-Phenylpropionic acid (101 μL,

0.74 mmol), oxalyl chloride (76 μL, 0.88 mmol) and DMF (1 drop) were reaction in DCM (3 mL) according to method C. The resulting acid chloride and 7-aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 μL) according to method B. The aqueous layer was extracted with DCM (x3) and the organic layers were combined, dried over Na_2SO_4 and evaporated. The resulting solid was triturated with ice-cold DCM to afford the product as a white amorphous solid (42 mg, 28%). IR (solid): υ_{max} 3571, 3317, 1785, 1759, 1718, 1662, 1517, 1238 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.94 (d, J = 8.4 Hz, 1H), 7.34 – 7.26 (m, 4H), 7.23 – 7.18 (m, 1H), 5.57 (dd, J = 8.4, 4.8 Hz, 1H), 4.99 – 4.91 (m, 2H), 4.68 (d, J = 12.3 Hz, 1H), 3.82 (q, J = 7.1 Hz, 1H), 3.44 (d, J = 17.6 Hz, 1H), 3.24 (d, J = 17.7 Hz, 1H), 1.99 (s, 3H), 1.34 (d, J = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.1, 170.3, 163.7, 163.1, 141.6, 128.1, 127.2, 126.6, 63.7, 58.5, 57.4, 44.2, 25.2, 20.7, 17.8. HRMS (ESI^+): calcd for $C_{24}H_{24}N_2O_6S$ $(M + H)^+$ 405.1120, found 405.1131.

(6*R*,7*R*)-3-(Acetoxymethyl)-8-oxo-7-(2-phenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (11). 7-Aminocephalosporanic acid (100 mg, 0.35 mmol) and phenylacetyl chloride (97 μL, 0.73 mmol) were reacted in satd $NaHCO_3$ (aq) (10 mL) and acetone (5 mL) according to method A to afford the product as a white amorphous solid (45 mg, 31%). IR (solid): υ_{max} 3254, 3034, 1778, 1737, 1707, 1654, 1536, 1223 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 13.71 (br s, 1H), 9.10 (d, J = 8.3 Hz, 1H), 7.35 – 7.18 (m, 5H), 5.68 (dd, J = 8.3, 4.8 Hz, 1H), 5.08 (d, J = 4.8 Hz, 1H), 5.00 (d, J = 12.8 Hz, 1H), 4.68 (d, J = 12.8 Hz, 1H), 3.66 – 3.47 (m, 4H), 2.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 170.9, 170.2, 164.7, 162.8, 135.8, 129.0, 128.2, 126.54, 126.48, 123.1, 62.7, 59.1, 57.4, 41.6, 25.5, 20.6. HRMS (ESI^+): calcd for $C_{18}H_{19}N_2O_6S$ $(M + H)^+$ 391.0964, found 391.0972.

(6*R*,7*R*)-3-(Acetoxymethyl)-8-oxo-7-(2-(*p*-tolyl)acetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (12). 4-Methylphenylacetic acid (111 mg, 0.74 mmol), oxalyl chloride (76 μL, 0.88 mmol) and DMF (1 drop) were reaction in DCM (3 mL) according to method C. The resulting acid chloride and 7-Aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 μL) according to method B. The resulting precipitate was collected by vacuum filtration and washed with DCM to afford the product as a white amorphous solid (63 mg, 42%). IR (solid): υ_{max} 3261, 3045, 1778, 1752, 1707, 1655, 1536, 1223 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 13.66 (br s, 1H), 9.04 (d, J = 8.3 Hz, 1H), 7.15 (d, J = 8.1 Hz, 2H), 7.10 (d, J = 8.0 Hz, 2H), 5.67 (dd, J = 8.3, 4.8 Hz, 1H), 5.08 (d, J = 4.8 Hz, 1H), 5.00 (d, J = 12.8 Hz, 1H), 4.69 (d, J = 12.8 Hz, 1H), 3.62 (d, J = 18.1 Hz, 1H), 3.54 – 3.41 (m, 3H), 2.26 (s, 3H), 2.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 171.1, 170.2, 164.8, 162.8, 135.5, 132.7, 128.9, 128.8, 62.7, 59.1, 57.4, 41.2, 25.5, 20.63, 20.57. HRMS (ESI^+): calcd for $C_{19}H_{21}N_2O_6S$ $(M + H)^+$ 405.1120, found 405.1119.

(6*R*,7*R*)-3-(Acetoxymethyl)-7-(2-(4-fluorophenyl)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (13). 4-Fluorophenylacetyl chloride (98 μL, 0.74 mmol) and 7-aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 μL) according to method B. The resulting mixture was cooled to 4 ^oC and the precipitate collected by vacuum filtration and washed with ice-cold DCM to afford the product as a white amorphous solid (61 mg, 41%). IR (solid): υ_{max} 3273, 1763, 1736, 1659, 1532, 1215 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 13.67 (br s, 1H), 9.10 (d, J = 8.2 Hz, 1H), 7.30 (dd, J = 8.4, 5.6 Hz, 2H), 7.12 (app t, J = 8.8 Hz, 2H), 5.67 (dd, J = 8.1, 4.8 Hz, 1H), 5.08 (d, J = 4.8 Hz, 1H), 5.00 (d, J = 12.8 Hz, 1H), 4.69 (d, J = 12.8 Hz, 1H), 3.65 – 3.46 (m, 4H), 2.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.8, 170.1, 164.6, 162.8, 131.9,

130.9, 130.8, 126.4, 123.3, 115.0, 114.8, 62.7, 59.1, 57.4, 40.6, 25.5, 20.5. HRMS (ESI^+): calcd for $C_{18}H_{18}FN_2O_6S$ $(M + H)^+$ 409.0870, found 409.0864.

(6*R*,7*R*)-3-(Acetoxymethyl)-7-(2-(4-chlorophenyl)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (14). Hexanoyl chloride (139 mg, 0.74 mmol) and 7-aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (75 μL) according to method B. The resulting precipitate was collected by vacuum filtration and washed with ice-cold DCM to afford the product as a cream amorphous solid (104 mg, 67%). IR (solid): υ_{max} 3265, 3056, 1778, 1748, 1707, 1643, 1536, 1223 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 13.69 (br s, 1H), 9.13 (d, J = 8.2 Hz, 1H), 7.36 (d, J = 8.5 Hz, 2H), 7.29 (d, J = 8.5 Hz, 2H), 5.67 (dd, J = 8.2, 4.8 Hz, 1H), 5.08 (d, J = 4.8 Hz, 1H), 5.00 (d, J = 12.8 Hz, 1H), 4.68 (d, J = 12.8 Hz, 1H), 3.65 – 3.45 (m, 4H), 2.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.6, 170.2, 164.6, 162.8, 134.8, 131.3, 130.9, 128.2, 62.7, 59.1, 57.4, 40.8, 25.5, 20.6. HRMS (ESI^+): calcd for $C_{18}H_{18}ClN_2O_6S$ $(M + H)^+$ 447.0394, found 447.0414.

(6*R*,7*R*)-3-(Acetoxymethyl)-7-(2-(4-bromophenyl)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (15). 3-Bromophenylacetyl chloride (171 mg, 0.74 mmol) and 7-aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (75 μL) according to method B. The resulting precipitate was collected by vacuum filtration and washed with ice-cold DCM to afford the product as a cream amorphous solid (131 mg, 76%). IR (solid): υ_{max} 3265, 3060, 1782, 1748, 1707, 1651, 1543, 1223 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 13.66 (br s, 1H), 9.14 (d, J = 8.2 Hz, 1H), 7.50 (d, J = 8.4 Hz, 2H), 7.23 (d, J = 8.4 Hz, 2H), 5.67 (dd, J = 8.2, 4.8 Hz, 1H), 5.08 (d, J = 4.8 Hz, 1H), 5.00 (d, J = 12.8 Hz, 1H), 4.68 (d, J = 12.8 Hz, 1H), 3.65 – 3.45 (m, 4H), 2.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.6, 170.2, 164.7, 162.8, 135.2, 131.3, 131.1, 126.3, 123.4,

119.7, 62.7, 59.1, 57.4, 40.8, 25.5, 20.6. HRMS (ESI$^+$): calcd for $C_{18}H_{18}BrN_2O_6S$ $(M + H)^+$ 469.0069, found 469.0076.

(6*R*,7*R*)-7-(2-([1,1'-Biphenyl]-4-yl)acetamido)-3-(acetoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (16). 4-Biphenylacetic acid (156 mg, 0.736 mmol), oxalyl chloride (76 μL, 0.88 mmol) and DMF (1 drop) were reaction in DCM (3 mL) according to method C. The resulting acid chloride and 7-aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 μL) according to method B. The resulting precipitate was collected by vacuum filtration and washed with ice-cold Et_2O to afford the product as a white amorphous solid (112 mg, 65%). IR (solid): υ_{max} 3302, 1756, 1737, 1654, 1621, 1536, 1237 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.02 (d, J = 8.3 Hz, 1H), 7.65 (d, J = 7.2 Hz, 2H), 7.60 (d, J = 8.2 Hz, 2H), 7.45 (app t, J = 7.6 Hz, 2H), 7.41 – 7.30 (m, 3H), 5.48 (dd, J = 8.3, 4.8 Hz, 1H), 5.00 (d, J = 11.9 Hz, 1H), 4.94 (d, J = 4.8 Hz, 1H), 4.75 (d, J = 12.0 Hz, 1H), 3.62 (d, J = 13.9 Hz, 1H), 3.54 (d, J = 13.9 Hz, 1H), 3.46 (d, J = 17.2 Hz, 1H), 3.20 (d, J = 17.2 Hz, 1H), 2.00 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.9, 170.4, 163.4, 162.8, 139.9, 138.3, 135.7, 135.2, 129.6, 128.8, 127.2, 126.5, 126.5, 64.7, 58.5, 57.2, 41.2, 25.1, 20.7. HRMS (ESI$^+$): calcd for $C_{24}H_{23}N_2O_6S$ $(M + H)^+$ 467.1277, found 467.1287.

(6*R*,7*R*)-3-(Acetoxymethyl)-8-oxo-7-(2-(4-phenoxyphenyl)acetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (17). 4-Phenoxyphenylacetic acid (170 mg, 0.74 mmol), oxalyl chloride (76 μL, 0.88 mmol) and DMF (1 drop) were reaction in DCM (3 mL) according to method C. The resulting acid chloride and 7-aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (75 μL) according to method B. The aqueous layer was extracted with DCM (x3) and the organic layers

were combined, dried over Na_2SO_4 and evaporated. The resulting solid was precipitated from hot DCM to afford the product as a cream amorphous solid (24 mg, 14%). IR (solid): υ_{max} 3280, 1774, 1730, 1655, 1532, 1223 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.05 (d, J = 8.3 Hz, 1H), 7.37 (app t, J = 7.9 Hz, 2H), 7.29 (d, J = 8.5 Hz, 2H), 7.12 (t, J = 7.4 Hz, 1H), 7.04 – 6.89 (m, 5H), 5.57 (dd, J = 8.2, 4.8 Hz, 1H), 5.03 – 4.95 (m, 2H), 4.72 (d, J = 12.4 Hz, 1H), 3.59 – 3.46 (m, 3H), 3.33 (d, J = 17.7 Hz, 1H), 2.01 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.0, 170.3, 163.7, 163.2, 156.9, 155.2, 131.0, 130.6, 130.0, 123.2, 118.6, 118.3, 63.7, 58.8, 57.3, 40.7, 25.3, 20.6. HRMS (ESI^+): calcd for $C_{24}H_{23}N_2O_7S$ $(M + H)^+$ 483.1226, found 483.1212.

(6*R*,7*R*)-3-(acetoxymethyl)-8-oxo-7-(2-(m-tolyl)acetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (18). 3-Methylphenylacetic acid (111 mg, 0.74 mmol), oxalyl chloride (76 μL, 0.88 mmol) and DMF (1 drop) were reaction in DCM (3 mL) according to method C. The resulting acid chloride and 7-aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (75 μL) according to method B. The aqueous layer was extracted with DCM (x3) and the organic layers were combined, dried over Na_2SO_4 and evaporated. The resulting solid was triturated with DCM to afford the product as a cream amorphous solid (36 mg, 24%). IR (solid): υ_{max} 3288, 1726, 1662, 1625, 1526, 1223 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.00 (d, J = 8.3 Hz, 1H), 7.17 (app t, J = 7.5 Hz, 1H), 7.12 – 6.98 (m, 3H), 5.51 (dd, J = 8.1, 4.7 Hz, 1H), 5.05 – 4.91 (m, 2H), 4.74 (d, J = 12.1 Hz, 1H), 3.56 – 3.41 (m, 3H), 3.26 (d, J = 17.5 Hz, 1H), 2.27 (s, 3H), 2.01 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.0, 170.5, 163.5, 163.3, 137.2, 135.8, 129.7, 128.1, 127.1, 126.1, 64.3, 58.6, 57.3, 41.5, 25.2, 21.0, 20.7. HRMS (ESI^+): calcd for $C_{19}H_{21}N_2O_6S$ $(M + H)^+$ 405.1120, found 405.1126.

(6*R*,7*R*)-3-(Acetoxymethyl)-8-oxo-7-(2-(3-phenoxyphenyl)acetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (19). 3-Phenoxyphenylacetic acid (170 mg,

0.74 mmol), oxalyl chloride (76 μL, 0.88 mmol) and DMF (1 drop) were reaction in DCM (3 mL) according to method C. The resulting acid chloride and 7-aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (75 μL) according to method B. The aqueous layer was extracted with DCM (x3) and the organic layers were combined, dried over Na_2SO_4 and evaporated. The resulting solid was precipitated from hot DCM to afford the product as a cream amorphous solid (22 mg, 12%). IR (solid): υ_{max} 3280, 3042, 1771, 1726, 1659, 1528, 1226 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.04 (d, J = 8.2 Hz, 1H), 7.39 (app t, J = 7.9 Hz, 2H), 7.31 (t, J = 7.9 Hz, 1H), 7.13 (t, J = 7.4 Hz, 1H), 7.07 – 6.95 (m, 4H), 6.87 (dd, J = 8.1, 2.2 Hz, 1H), 5.55 (dd, J = 8.1, 4.8 Hz, 1H), 5.02 – 4.94 (m, 2H), 4.71 (d, J = 12.3 Hz, 1H), 3.59 – 3.45 (m, 3H), 3.30 (d, J = 17.7 Hz, 1H), 2.01 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.6, 170.3, 163.6, 163.1, 156.6, 156.5, 138.0, 130.0, 129.69, 124.2, 123.3, 119.2, 118.6, 116.7, 63.7, 58.8, 57.2, 41.4, 25.3, 20.6. HRMS (ESI^+): calcd for $C_{24}H_{23}N_2O_7S$ $(M + H)^+$ 483.1226, found 483.1233.

(6*R*,7*R*)-3-(Acetoxymethyl)-7-benzamido-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (20). 7-Aminocephalosporanic acid (100 mg, 0.37 mmol) and benzoyl chloride (51 μL, 0.44 mmol) were reacted in satd $NaHCO_3$ (aq) (10 mL) and acetone (5 mL) according to method A to afford the product as a white amorphous solid (75 mg, 55%). IR (solid): υ_{max} 3250, 1774, 1752, 1710, 1651, 1520, 1223 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 13.69 (br s, 1H), 9.41 (d, J = 8.0 Hz, 1H), 7.91 (d, J = 7.7 Hz, 2H), 7.57 (t, J = 7.3 Hz, 1H), 7.48 (app t, J = 7.5 Hz, 2H), 5.88 (dd, J = 8.1, 4.8 Hz, 1H), 5.19 (d, J = 4.8 Hz, 1H), 4.99 (d, J = 12.8 Hz, 1H), 4.70 (d, J = 12.7 Hz, 1H), 3.64 (d, J = 18.0 Hz, 1H), 3.50 (d, J = 18.0 Hz, 1H), 2.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.2, 166.9, 164.0, 162.8, 133.0, 131.8, 128.3, 127.7, 123.1, 62.7, 59.8, 57.6, 25.5, 20.5. HRMS (ESI^+): calcd for $C_{17}H_{17}N_2O_6S$ $(M + H)^+$ 377.0807, found 377.0807.

(6*R*,7*R*)-3-(Acetoxymethyl)-7-(4-methylbenzamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (21). 7-Aminocephalosporanic acid (100 mg, 0.37 mmol) and 4-methylbenzoyl chloride (97 μL, 0.74 mmol) were reacted in satd $NaHCO_3$ (aq) (10 mL) and acetone (5 mL) according to method A to afford the product as a white amorphous solid (39 mg, 26%). IR (solid): υ_{max} 3258, 1774, 1730, 1648, 1525, 1223 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.29 (d, J = 8.1 Hz, 1H), 7.83 (d, J = 7.8 Hz, 2H), 7.29 (d, J = 7.9 Hz, 2H), 5.81 (dd, J = 8.1, 4.7 Hz, 1H), 5.14 (d, J = 4.8 Hz, 1H), 4.99 (d, J = 12.5 Hz, 1H), 4.72 (d, J = 12.5 Hz, 1H), 3.59 (d, J = 17.7 Hz, 1H), 3.43 (d, J = 17.7 Hz, 1H), 2.37 (s, 3H), 2.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 170.3, 166.8, 163.5, 163.0, 141.8, 130.3, 128.8, 127.8, 63.4, 59.5, 57.6, 25.4, 21.0, 20.6. HRMS (ESI^+): calcd for $C_{18}H_{19}N_2O_6S$ $(M + H)^+$ 391.0964, found 391.0972.

(6*R*,7*R*)-3-(Acetoxymethyl)-7-(4-methoxybenzamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (22). 7-Aminocephalosporanic acid (100 mg, 0.37 mmol) and 4-methoxybenzoyl chloride (100 μL, 0.74 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 μL) according to method B. The aqueous layer was extracted with DCM (x3) and the organic layers were combined, dried over Na_2SO_4 and evaporated. The resulting solid was triturated with ice-cold Et_2O to afford the product as a white amorphous solid (36 mg, 24%). IR (solid): υ_{max} 3254, 1774, 1752, 1705, 1640, 1528, 1223 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.22 (d, J = 8.1 Hz, 1H), 7.92 (d, J = 8.9 Hz, 2H), 7.01 (d, J = 8.9 Hz, 2H), 5.84 (dd, J = 8.1, 4.8 Hz, 1H), 5.16 (d, J = 4.8 Hz, 1H), 4.98 (d, J = 12.7 Hz, 1H), 4.70 (d, J = 12.7 Hz, 1H), 3.82 (s, 3H), 3.62 (d, J = 17.9 Hz, 1H), 3.46 (d, J = 17.9 Hz, 1H), 2.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.2, 166.3, 164.1, 162.9, 162.1, 129.7, 125.1, 113.6, 62.9, 59.7, 57.7, 55.4, 25.5, 20.6. HRMS (ESI^+): calcd for $C_{18}H_{19}N_2O_7S$ $(M + H)^+$ 407.0913, found 407.0919.

(6*R*,7*R*)-3-(Acetoxymethyl)-7-(4-nitrobenzamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (23). 7-Aminocephalosporanic acid (100 mg, 0.37 mmol) and 4-nitrobenzoyl chloride (136 mg, 0.74 mmol) were reacted in satd $NaHCO_3$ (aq) (10 mL) and acetone (5 mL) according to method A. Several drops of MeOH were added prior to the addition of ice-cold Et_2O to afford the product as a white amorphous solid (54 mg, 35%). IR (solid): υ_{max} 3265, 3064, 2971, 1785, 1748, 1711, 1640, 1595, 1524, 1223 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 13.72 (s, 1H), 9.80 (d, J = 7.8 Hz, 1H), 8.34 (d, J = 8.8 Hz, 2H), 8.13 (d, J = 8.8 Hz, 2H), 5.88 (dd, J = 7.8, 4.7 Hz, 1H), 5.22 (d, J = 4.8 Hz, 1H), 5.00 (d, J = 12.8 Hz, 1H), 4.71 (d, J = 12.8 Hz, 1H), 3.66 (d, J = 17.9 Hz, 1H), 3.51 (d, J = 18.0 Hz, 1H), 2.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.7, 166.0, 164.0, 163.3, 149.9, 139.0, 129.8, 124.1, 63.2, 60.4, 58.0, 26.0, 21.0, 15.6. HRMS (ESI^+): calcd for $C_{17}H_{16}N_3O_8S$ $(M + H)^+$ 422.0658, found 422.0660.

(6*R*,7*R*)-3-(Acetoxymethyl)-7-(furan-2-carboxamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (24). 7-Aminocephalosporanic acid (100 mg, 0.37 mmol) and 2-furoyl chloride (73 μL, 0.74 mmol) were reacted in satd $NaHCO_3$ (aq) (10 mL) and acetone (5 mL) according to method A to afford the product as a white amorphous solid (30 mg, 22%). IR (solid): υ_{max} 3243, 1793, 1718, 1710, 1632, 1595, 1223 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 13.71 (br s, 1H), 9.29 (d, J = 8.2 Hz, 1H), 7.90 (d, J = 1.7 Hz, 1H), 7.36 (d, J = 3.5 Hz, 1H), 6.65 (dd, J = 3.5, 1.7 Hz, 1H), 5.81 (dd, J = 8.2, 4.8 Hz, 1H), 5.16 (d, J = 4.8 Hz, 1H), 4.99 (d, J = 12.8 Hz, 1H), 4.70 (d, J = 12.8 Hz, 1H), 3.64 (d, J = 18.0 Hz, 1H), 3.50 (d, J = 18.0 Hz, 1H), 2.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.7, 164.4, 163.3, 158.4, 146.8, 146.5, 115.4, 112.4, 63.2, 59.6, 58.1, 26.0, 21.0. HRMS (ESI^+): calcd for $C_{15}H_{15}N_2O_7S$ $(M + H)^+$ 367.0600, found 367.0609.

(6*R*,7*R*)-3-(Acetoxymethyl)-7-(cyclohexanecarboxamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (25). 7-Aminocephalosporanic acid (100 mg, 0.37 mmol) and cyclohexanecarbonyl chloride (60 μL, 0.44 mmol) were reacted in satd $NaHCO_3$ (aq) (10 mL) and acetone (5 mL) according to method A to afford the product as a white amorphous solid (15 mg, 11%). IR (solid): υ_{max} 3261, 2926, 2851, 1778, 1737, 1711, 1648, 1532, 1215 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 13.67 (br s, 1H), 8.71 (d, J = 8.2 Hz, 1H), 5.63 (dd, J = 8.2, 4.8 Hz, 1H), 5.07 (d, J = 4.8 Hz, 1H), 4.99 (d, J = 12.8 Hz, 1H), 4.67 (d, J = 12.8 Hz, 1H), 3.61 (d, J = 18.1 Hz, 1H), 3.46 (d, J = 18.0 Hz, 1H), 2.34 – 2.24 (m, 1H), 2.03 (s, 3H), 1.76 – 1.57 (m, 5H), 1.39 – 1.14 (m, 5H). ^{13}C NMR (101 MHz, DMSO) δ 176.1, 170.2, 164.8, 162.9, 62.8, 59.0, 57.6, 43.3, 29.8, 28.6, 25.4, 25.4, 25.2, 25.0, 20.6. HRMS (ESI^+): calcd for $C_{17}H_{23}N_2O_6S$ $(M + H)^+$ 383.1277, found 383.1264.

(6*R*,7*R*)-7-Acetamido-3-(acetoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (26). 7-Aminocephalosporanic acid (500 mg, 1.84 mmol) was suspended in H_2O (8 mL), $NaHCO_3$ (387 mg, 4.60 mmol) was added and the resulting mixture stirred at room temperature for 10 min before being cooled to 0 °C. Acetic anhydride (347 μL, 0.368 mmol) in acetone (10 mL) was added and the reaction stirred at 0 °C for 30 min. Acetone was removed under reduced pressure, the resulting material was diluted in H_2O and neutralised with satd $NaHCO_3$ (aq). The aqueous solution was washed with EtOAc, acidified to pH 2 with 1 M HCl and extracted with EtOAc (x3). The organic layers were combined, washed with brine, dried over Na_2SO_4 and evaporated to afford the product as a colourless foam (471 mg, 81%). IR (solid): υ_{max} 3317, 2937, 1771, 1718, 1755, 1625, 1528, 1219 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 13.67 (br s, 1H), 8.84 (d, J = 8.4 Hz, 1H), 5.68 (dd, J = 8.3, 4.9 Hz, 1H), 5.08 (d, J = 4.9 Hz, 1H), 5.00 (d, J = 12.8 Hz, 1H), 4.68 (d, J = 12.8 Hz, 1H), 3.63 (d, J = 18.0 Hz, 1H), 3.48 (d,

J = 18.1 Hz, 1H), 2.03 (s, 3H), 1.91 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 170.2, 170.1, 165.0, 162.9, 126.4, 123.4, 62.7, 59.0, 57.4, 22.1, 20.6. HRMS (ESI$^+$): calcd for $C_{12}H_{15}N_2O_6S$ $(M + Na)^+$ 337.0470, found 337.0479.

(6*R*,7*R*)-3-(Acetoxymethyl)-7-butyramido-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (27). Butyryl chloride (76 μL, 0.74 mmol) and 7-aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 μL) according to method B. The aqueous layer was extracted with DCM (x3) and the organic layers were combined, dried over Na_2SO_4 and evaporated. The resulting solid was triturated with ice-cold DCM to afford the product as a white amorphous solid (15 mg, 12%). IR (solid): υ_{max} 3265, 2960, 1774, 1751, 1715, 1654, 1539, 1223 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.74 (d, J = 8.2 Hz, 1H), 5.61 (dd, J = 7.9, 4.8 Hz, 1H), 5.04 (d, J = 4.7 Hz, 1H), 4.99 (d, J = 12.6 Hz, 1H), 4.69 (d, J = 12.6 Hz, 1H), 3.57 (d, J = 17.8 Hz, 1H), 3.39 (d, J = 17.7 Hz, 1H), 2.21 – 2.13 (m, 2H), 2.02 (s, 3H), 1.52 (app h, J = 7.2 Hz, 2H), 0.86 (t, J = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 172.9, 170.3, 164.4, 163.1, 63.3, 58.9, 57.4, 36.6, 25.4, 20.6, 18.7, 13.5. HRMS (ESI$^+$): calcd for $C_{14}H_{19}N_2O_6S$ $(M + H)^+$ 343.0964, found 343.0959.

(6*R*,7*R*)-3-(Acetoxymethyl)-7-hexanamido-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (28). Hexanoyl chloride (103 μL, 0.74 mmol) and 7-aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 μL) according to method B. The resulting precipitate was collected by vacuum filtration and washed with ice-cold DCM to afford the product as a white amorphous solid (78 mg, 57%). IR (solid): υ_{max} 3283, 3183 2930, 1774, 1752, 1711, 1651, 1536, 1223 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.65 (br s, 1H), 8.78 (d, J = 8.2 Hz, 1H), 5.67 (dd, J = 8.2, 4.8 Hz, 1H), 5.08 (d, J = 4.8 Hz, 1H), 4.99 (d, J = 12.8 Hz, 1H), 4.68 (d, J = 12.8 Hz, 1H), 3.62 (d, J = 18.1 Hz, 1H), 3.48 (d,

J = 18.1 Hz, 1H), 2.23 – 2.13 (m, 2H), 2.03 (s, 3H), 1.56 – 1.46 (m, 2H), 1.32 – 1.18 (m, 4H), 0.86 (t, J = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.0, 170.1, 164.9, 162.8, 126.4, 123.2, 62.7, 59.0, 57.5, 34.6, 30.7, 25.5, 24.8, 21.8, 20.5, 13.8. HRMS (ESI$^+$): calcd for $C_{16}H_{23}N_2O_6S$ $(M + H)^+$ 371.1277, found 371.1290.

***tert*-Butyl (6*R*,7*R*)-7-acetamido-3-(acetoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (29).** Compound **26** (75 mg, 0.24 mmol) was dissolved in DCM (2 mL), *tert*-butyl 2,2,2-trichloroacetimidate (170 μL, 0.96 mmol) was added and the reaction heated to 60 ^{o}C for 24 h. After cooling to room temperature the reaction was diluted with MeOH. Solvent was removed under reduced pressure and the resulting solid triturated with cold DCM. The solute was loaded directly onto a 10g SNAP KPSil column and purified by column chromatography (0 – 10% MeOH in DCM) to afford the product as a cream glassy solid (82 mg, 93%). IR (thin film): υ_{max} 3291, 2982, 1774, 1718, 1670, 1528, 1368, 1223 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.39 (d, J = 9.0 Hz, 1H), 5.84 (dd, J = 9.0, 4.9 Hz, 1H), 5.09 (d, J = 13.3 Hz, 1H), 4.95 (d, J = 4.9 Hz, 1H), 4.80 (d, J = 13.2 Hz, 1H), 3.55 (d, J = 18.4 Hz, 1H), 3.36 (d, J = 18.4 Hz, 1H), 2.08 (s, 3H), 2.06 (s, 3H), 1.53 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.7, 170.4, 164.9, 160.5, 127.5, 123.8, 84.0, 63.3, 59.4, 57.5, 27.9, 26.6, 23.0, 20.9. HRMS (ESI$^+$): calcd for $C_{16}H_{23}N_2O_6S$ $(M + H)^+$ 371.1277, found 371.1276.

7-(4-(*tert*-Butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (32). Ciprofloxacin **31** (500 mg, 1.51 mmol) was dissolved in 1 M NaOH (aq) (5 mL) and THF (10 mL) added, followed by the drop-wise addition of Boc_2O (360 mg, 1.66 mmol) in THF (10 ml) and stirred at room temperature for 16 h. Solvent was removed under reduced pressure and the resulting material diluted in H_2O and neutralised with satd NH_4Cl (aq). The precipitate was collected by vacuum filtration and washed with H_2O

to afford the product as a white amorphous solid (502 mg, 77%). IR (solid): υ_{max} 2971, 1733, 1688, 1629, 1249 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 14.95 (s, 1H), 8.78 (s, 1H), 8.05 (d, *J* = 12.9 Hz, 1H), 7.37 (d, *J* = 7.1 Hz, 1H), 3.73 – 3.62 (m, 4H), 3.53 (tt, *J* = 7.3, 4.0 Hz, 1H), 3.34 – 3.25 (m, 4H), 1.50 (s, 9H), 1.43 – 1.37 (m, 2H), 1.24 – 1.17 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.1, 154.7, 153.1, 147.7, 113.0, 112.7, 108.5, 105.1, 80.5, 35.4, 28.6, 8.4. HRMS (ESI^+): Calcd for $C_{22}H_{27}N_3O_5F$ $(M+H)^+$ 432.1935, Found: 432.1951.

Sodium 7-(4-(*tert*-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (33). Compound **32** (105 mg, 0.240 mmol) was suspended in MeOH (2.44 mL), 0.1 M NaOH (aq) (2.44 mL) was added and the reaction mixture stirred at 30 °C for 30 min. Solvent was removed under reduced pressure and resulting material suspended in H_2O (5 μL) and EtOH (5 mL) and evaporated to dryness (x3). Then, the solid was suspended in DCM and evaporated to afford the product as a cream amorphous solid (111 mg, quant.). IR (solid): υ_{max} 1617, 1478, 1242 cm^{-1}.

***tert*-Butyl (6*R*,7*R*)-7-acetamido-3-(((7-(4-(*tert*-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carbonyl)oxy)methyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (34).** Compound **29** (198 mg, 0.54 mmol) was dissolved in DCM (8 mL) and TMSI (117 μL, 0.82 mmol) added drop-wise.[83] The reaction mixture was stirred in the dark for 2 h at room temperature, then diluted with DCM and washed with 10% (wt/v) Na_2SO_3 (aq). The organic layer was dried over Na_2SO_4 and evaporated to a give compound **30** as a yellow glassy solid. Compound **30** (120 mg, 0.27 mmol) and compound **33** (100 mg, 0.26 mmol) were suspended in anhydrous 1,4-dioxane (3.5 mL) and DMF (1.15 mL) was added drop-wise. The reaction mixture was stirred in the dark for 2 h before the solvent was removed under a stream of N_2. The resulting material was dissolved in minimal DCM and loaded

directly onto a 10 g SNAP Ultra cartridge and purified by column chromatography (0 – 6% MeOH in DCM) to afford the product as a pale yellow glassy solid (108 mg, 52%). IR (solid): υ_{max} 2974, 1782, 1685, 1618, 1250 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 8.86 (d, J = 8.5 Hz, 1H), 8.44 (s, 1H), 7.80 (d, J = 13.3 Hz, 1H), 7.47 (d, J = 7.4 Hz, 1H), 5.71 (dd, J = 8.4, 4.9 Hz, 1H), 5.15 – 5.09 (m, 2H), 4.86 (d, J = 13.1 Hz, 1H), 3.72 – 3.62 (m, 3H), 3.58 – 3.50 (m, 4H), 3.25 – 3.16 (m, 4H), 1.91 (s, 3H), 1.49 (s, 9H), 1.43 (s, 9H), 1.31 – 1.25 (m, 2H), 1.13 – 1.05 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.5, 170.0, 164.9, 164.2, 160.5, 153.7, 152.6 (d, $^1J_{C\text{-}F}$ = 247.5 Hz), 148.4, 143.8 (d, $^2J_{C\text{-}F}$ = 10.1 Hz), 138.0, 126.2, 123.5, 122.1 (d, $^3J_{C\text{-}F}$ = 7.1 Hz), 111.6 (d, $^2J_{C\text{-}F}$ = 22.2 Hz), 108.6, 106.7 (d, $^3J_{C\text{-}F}$ = 3.0 Hz), 82.8, 79.2, 62.6, 59.0, 57.4, 54.9, 49.50, 49.46, 34.9, 28.1, 27.5, 25.6, 22.1, 7.6, 7.5. HRMS (ESI^+): calcd for $C_{36}H_{44}FN_5O_9S$ $(M + H)^+$ 742.2922, found 742.2930.

(6*R*,7*R*)-7-Acetamido-3-(((1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carbonyl)oxy)methyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (35). Compound **34** (15 mg, 0.02 mmol) was dissolved in anhydrous DCM (0.3 mL) and cooled to 0 °C. Anhydrous anisole (3 drops) was added followed by the drop-wise addition of TFA (0.3 mL). The reaction mixture was stirred at 0 °C for 30 min, warmed to room temperature and stirred for a further 40 min. Solvent was removed under a stream of N_2 and the resulting gum triturated with ice-cold EtOAc. The precipitate was collected, diluted in H_2O and DCM and basified to pH 9 with 3% $NaHCO_3$ (aq). The aqueous phase was separated and loaded directly onto a 12 g SNAP KP-C18-HS cartridge and purified by reverse-phase column chromatography (0 – 100% MeCN in H_2O). Fractions containing product were freeze-dried to afford the product as a white solid (2.5 mg, 23%). ^1H NMR (500 MHz, D_2O) δ 8.58 (s, 1H), 7.73 (d, J = 13.0 Hz, 1H), 7.46 (d, J = 7.2 Hz, 1H), 5.58 (d, J = 4.6 Hz, 1H), 5.11 (d, J = 12.6 Hz, 1H),

5.07 (d, J = 4.7 Hz, 1H), 4.81 (d, J = 12.6 Hz, 1H), 3.68 – 3.31 (m, 11H), 2.00 (s, 3H), 1.27 (d, J = 6.9 Hz, 2H), 1.06 (d, J = 4.3 Hz, 2H). HRMS (ESI^+): calcd for $C_{27}H_{29}FN_5O_7S$ $(M + H)^+$ 586.1772, found 586.1794.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

Experimental procedures (biological testing), bacterial strains, protein production, assay procedures, ^1H NMR and ^{13}C NMR spectra of synthesized compounds (PDF)

Molecular formula strings (CSV)

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ABBREVIATIONS

E. coli, *Escherichia coli*; FDA, Food and Drug Administration; GI, gastrointestinal; GU, genitourinary; k_{cat}, catalytic constant for the conversion of substrate to product; *P. aeruginosa*, *Pseudomonas aeruginosa*; TMSI, trimethylsilyl iodide.

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Table of Contents graphic