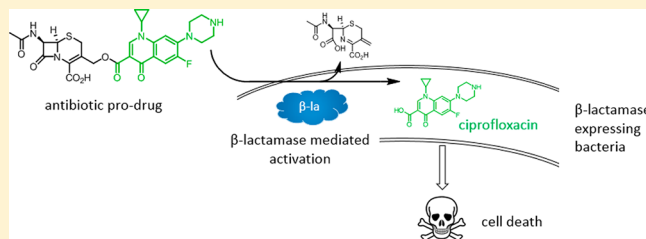


Exploitation of Antibiotic Resistance as a Novel Drug Target:
Development of a β -Lactamase-Activated Antibacterial ProdrugLindsay E. Evans,^{*,†,‡,§} Aishwarya Krishna,[†] Yajing Ma,[‡] Thomas E. Webb,[§] Dominic C. Marshall,^{§,⊥} Catherine L. Tooke,^{||} James Spencer,^{||} Thomas B. Clarke,[†] Alan Armstrong,^{‡,§} and Andrew M. Edwards^{*,†}[†]MRC Centre for Molecular Bacteriology and Infection, Imperial College London, SW7 2AZ London, United Kingdom[‡]Department of Chemistry, Molecular Sciences Research Hub, Imperial College London, W12 0BZ London, United Kingdom[§]Department of Medicine, Imperial College London, SW7 2AZ London, United Kingdom^{||}School of Cellular and Molecular Medicine, University of Bristol, Biomedical Sciences Building, University Walk, BS8 1TD Bristol, United Kingdom

Supporting Information

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 prodrug that combines ciprofloxacin with a β -lactamase-cleavable motif. The prodrug 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 prodrug 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 preterm infants.^{1,2} The most prevalent and important resistance determinant is the β -lactamase enzyme, which hydrolyzes 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 gastrointestinal 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 difficile* 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 tendinitis 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}

Received: December 7, 2018

Published: April 22, 2019

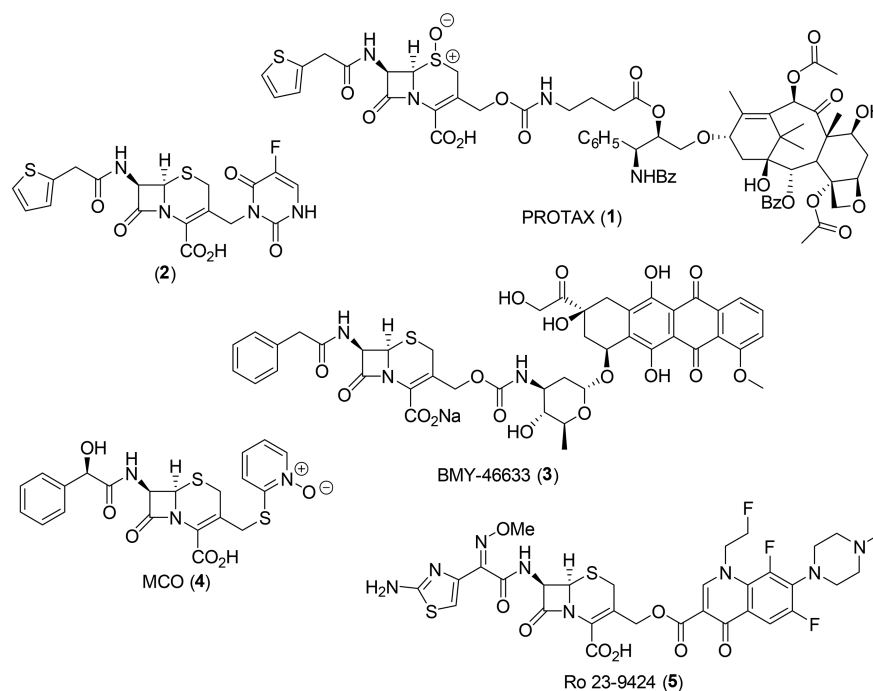


Figure 1. Selected representative examples of cephalosporin prodrugs (PROTAX 1,^{33, 34} and BMY-46633 3³⁵) and co-drugs (MCO 4³⁸ and Ro 23-9424 5³⁹).

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 antibacterial target. Furthermore, β -lactamase expression is prevalent among 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 prodrug 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, Figure 1).^{31–35} Additionally, β -lactam–fluoroquinolone conjugates have been proposed as a co-drug strategy to treat bacterial infections (4 and 5, Figure 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, while having minimal effect on bacteria that 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 prodrug, 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 prodrug with selective activity against drug-resistant bacteria.

RESULTS AND DISCUSSION

Prodrug Design. In order to create a prodrug 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 nonbactericidal intact molecule and enabled the rapid and efficient release of the antibiotic upon activation by β -lactamase. The success of this strategy required a prodrug motif that would enable efficient substrate turnover rather than inhibition of the β -lactamase enzyme. The cephalosporin class of β -lactams are efficiently hydrolyzed 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 (Figure 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 (Figure 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 While this choice of attachment site was selected to remove the ciprofloxacin activity from the intact prodrug, it remained likely that the

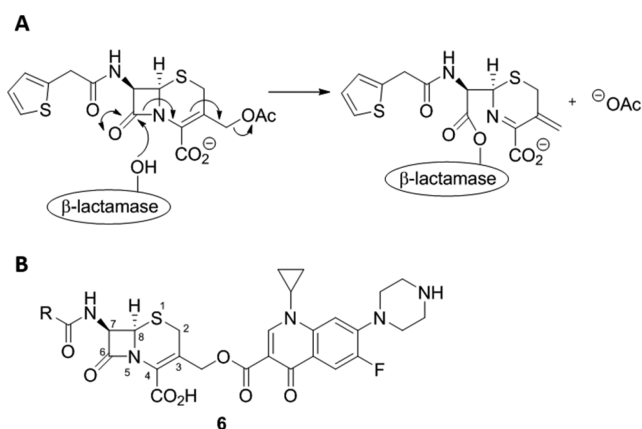


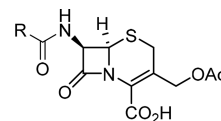
Figure 2. (A) Mechanism of β -lactamase triggered cephalothin hydrolysis. (B) General structure of proposed cephalosporin–ciprofloxacin prodrug **6**.

prodrug 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 prodrug.

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. By use of 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 (Figure S1 in Supporting Information).^{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 DH5 α \pm 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

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 ($\mu\text{M/S}^{-1}$)
		DH5 α	DH5 α + TEM-116	
Ceph 7		12.5	100	2.86 \pm 0.33
8		50	200	2.50 \pm 0.28
9		50	200	2.36 \pm 0.10
10		400	>800	0.87 \pm 0.15
11		50	100	1.86 \pm 0.09
12		100	400	1.77 \pm 0.19
13		100	200	1.34 \pm 0.20
14		100	400	4.78 \pm 0.13
15		200	800	5.06 \pm 0.32
16		>800	>800	3.99 \pm 0.88
17		>800	>800	7.33 \pm 1.72
18		200	400	9.13 \pm 1.66
19		>800	>800	31.14 \pm 2.67
20		>400	>400	0.52 \pm 0.18
21		>400	>400	0.26 \pm 0.10
22		>800	>800	0.31 \pm 0.38
23		>400	>400	0.88 \pm 0.15
24		>400	>400	ND
25		>400	>400	0.17 \pm 0.09
26		200	400	0.53 \pm 0.16
27		100	400	0.64 \pm 0.04
28		200	>800	0.81 \pm 0.09

activity; an increase in k_{cat}/K_m was observed with increasing chain length.

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* DH5 α 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* DH5 α 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 into, 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 ^1H NMR signals, which can be detected using whole bacterial cells in real time by ^1H NMR spectroscopy (Figure S2). As hydrolysis occurred within the bacterial periplasm, only compounds with sufficient intracellular accumulation were hydrolyzed. Compounds 16 and 17 were selected as representative examples of 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 DH5 α \pm TEM-116 (Table 2). After 90 min, 16 was 69%

Table 2. Percentage Hydrolysis of Cephalothin (Ceph) 7 and Compounds 16 and 17 by DH5 α Cells \pm β -Lactamase in Whole-Cell NMR Hydrolysis Assay

compd	concn (μM)	incubation time (min)	strain	% hydrolysis by NMR	
				$-\beta\text{la}$	$+\beta\text{la}$
Ceph 7	50	60	DH5 α \pm TEM-116		61
16	100	90		14	69
17	100	90		13	53
Ceph 7	100	60	DH5 α \pm CTX-M-1	0	100
16	100	60		0	100
17	100	60		0	95

hydrolyzed in DH5 α + TEM-116 compared to 14% hydrolyzed in DH5 α – TEM-116 and 17 was 53% hydrolyzed in DH5 α + TEM-116 compared to 13% hydrolyzed in DH5 α – 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* DH5 α without β -lactamase was due to an absence of PBP engagement and not due to poor permeability or efflux activity.

Biological Evaluation in Uropathogenic *E. coli*. Initial assessment of compound activity was performed in the laboratory *E. coli* strain DH5 α . 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, 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 \pm β -lactamase. The primary β -lactamase used in this work was CTX-M-1 because CTX-M enzymes are the most prevalent β -lactamases among 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

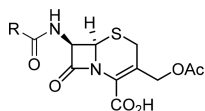
In the first instance, MIC values were determined for selected compounds against CFT073 + pSU18 \pm CTX-M-1 (Table 3). For all the compounds tested, the MIC values for CFT073 + pSU18 were within 2-fold of those determined against DH5 α . Next the hydrolytic activity of these compounds was assessed in the whole cell NMR assay (Table 3). For the majority of the compounds tested, a low level of hydrolysis, <20% after 60 min, was detected. However, levels of hydrolysis comparable to that observed for cephalothin (68%) were observed only for 24 and 26 (64% and 67%, respectively).

A clear feature of the SAR was that CTX-M-1 mediated hydrolytic activity in whole CFT073 cells correlated with lipophilicity. Plotting the calculated log *P* (cLogP) values for compounds against the log of percentage hydrolysis revealed that moderate–high levels of hydrolysis (>30%) were only observed for compounds with cLogP values below 0.1 (Figure 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.

Interestingly, compounds 16 and 17 were hydrolyzed rapidly (69% and 54% after 90 min, respectively) in DH5 α expressing the TEM-116 β -lactamase (Table 2) but only 4% hydrolyzed after 60 min 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 DH5 α expressing CTX-M-1 was determined for cephalothin 7, 16, and 17 (Table 2). After 60 min complete hydrolysis for cephalothin 7 and 16 and 94% hydrolysis for 17 were 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 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 \pm CTX-M-1 ($\geq 400 \mu\text{M}$) and high hydrolytic activity in whole CFT073 cells (67% after 60 min), was selected for incorporation into the full prodrug molecule. Compound 24, which also possessed a high MIC value in both CFT073 \pm CTX-M-1 ($\geq 400 \mu\text{M}$) and high hydrolytic activity in whole CFT073 cells (64% after 60 min), 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

Prodrug Preparation. Preparation of the prodrug derived from compound 26 required coupling of an activated 26 derivative, iodocephalosporin 30, to ciprofloxacin derivative 33 (Scheme 1). The iodocephalosporin 30 was prepared in three steps from commercially available 7-aminocephalosporanic

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^b



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
24		>400	>400	64
25		ND	ND	0
26		400	>400	67
27		200	>400	25
28		100	>400	7

^aPercentage hydrolysis determined for 100 μM compound at 1 h by whole-cell NMR assay. ^bND = not done.

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 -cephem to the biologically inactive Δ^2 -cephem.^{67–69} Iodination at the 3'-position with TMSI gave the activated iodocephalosporin **30** ready for coupling.³⁶ The ciprofloxacin component was prepared by BOC protection of the piperazine

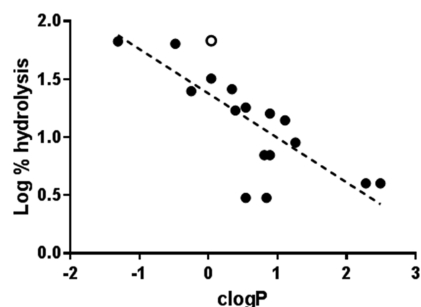


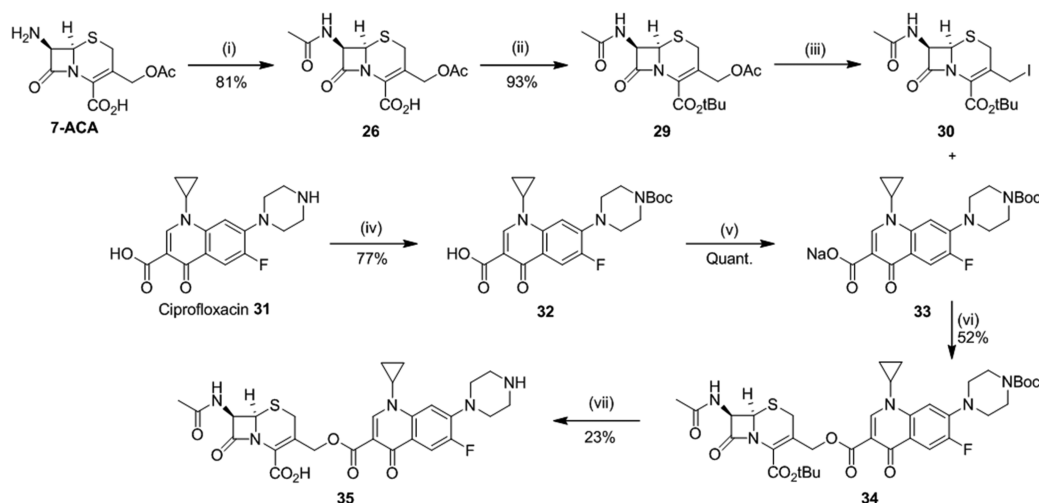
Figure 3. Plot of log of percentage hydrolysis in CFT073 + CTX-M-1 cells against calculated log *P* (cLogP) for synthesized compounds (filled circle) and cephalothin **7** (open circle): linear regression (dashed line), $R^2 = 0.60$ (GraphPad Prism 7).

NH to give **32** and subsequent conversion of the carboxylic acid to the sodium salt **33**.⁷⁰ 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 deprotection with TFA to remove the BOC and *tert*-butyl ester afforded the final prodrug **35**.⁷¹ Synthesis of **35** was achieved in seven steps from commercially available materials without the requirement for toxic metal reagents.

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 prodrug **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 prodrug **35** and ciprofloxacin to inhibit recombinant DNA gyrase enzyme activity in the absence and presence of the purified recombinant β -lactamase CTX-M-15 (Figure 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 prodrug **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*.

Selective Prodrug Activity against Uropathogenic *E. coli* Expressing β -Lactamase. Next, the activity of prodrug **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 (Figure 5). NDM-1 is an example of an increasingly prevalent β -lactamase that is capable of hydrolyzing carbapenems, usually considered the last line of defense against β -lactamase expressing bacteria,⁷³ while KPC enzymes are class A β -lactamases and the most common carbapenemases globally.^{74,75}

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

Scheme 1. Synthesis of Prodrug 35^a

^aReagents and conditions: (i) acetic anhydride, NaHCO₃, H₂O, acetone, 0 °C, 30 min; (ii) TBTA, DCM, 60 °C, 16 h; (iii) TMSI, DCM, rt, 2 h; (iv) Boc₂O, 1 M NaOH, THF, rt, 16 h; (v) 0.1 M NaOH, MeOH, rt, 30 min; (vi) 3:1 1,4-dioxane/DMF, rt, 4 h; (vii) 1:1 TFA/DCM, anisole, 0 °C to rt.

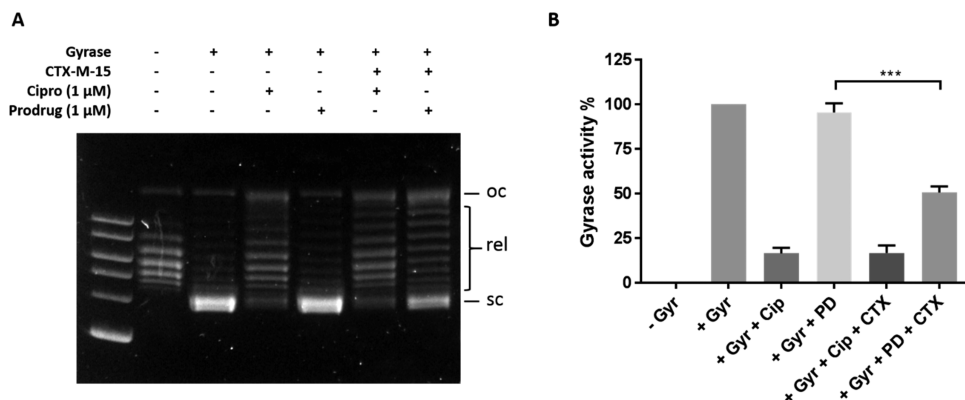


Figure 4. Activity of prodrug 35 and ciprofloxacin 31 against recombinant DNA gyrase \pm CTX-M-15. (A) DNA was separated by agarose gel electrophoresis with 2 log DNA ladder: oc, open circle DNA; rel, relaxed DNA; sc, supercoiled DNA. (B) Quantification of gel bands corresponding to supercoiled DNA and normalized to no gyrase and gyrase only activity (ImageJ 1.52a): Gyr, DNA gyrase; cip, ciprofloxacin 31; PD, prodrug 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).

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 prodrug cleavage and active antibiotic release, resulting in arrest of bacterial cell growth at concentrations comparable to that with free ciprofloxacin.

The activity of prodrug 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 prodrug 35 was confirmed against the three ciprofloxacin sensitive bacterial strains, while no arrest in bacterial growth was observed for either ciprofloxacin 31 or prodrug 35 for strain EC12, EC13,

or EC19 (Figure 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 prodrug by testing its activity against two representative *E. faecalis* strains that did not express β -lactamase. Prodrug 35 showed reduced activity compared to ciprofloxacin, indicating that our approach could minimize undesirable damage to the microbiota caused by fluoroquinolones (Figure 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 prodrug by cleaving the ester linkage. However, data from MIC assays performed in the presence of human serum (Figure S4) were equivalent to those obtained in the absence of serum (Figure 5). Combined, these

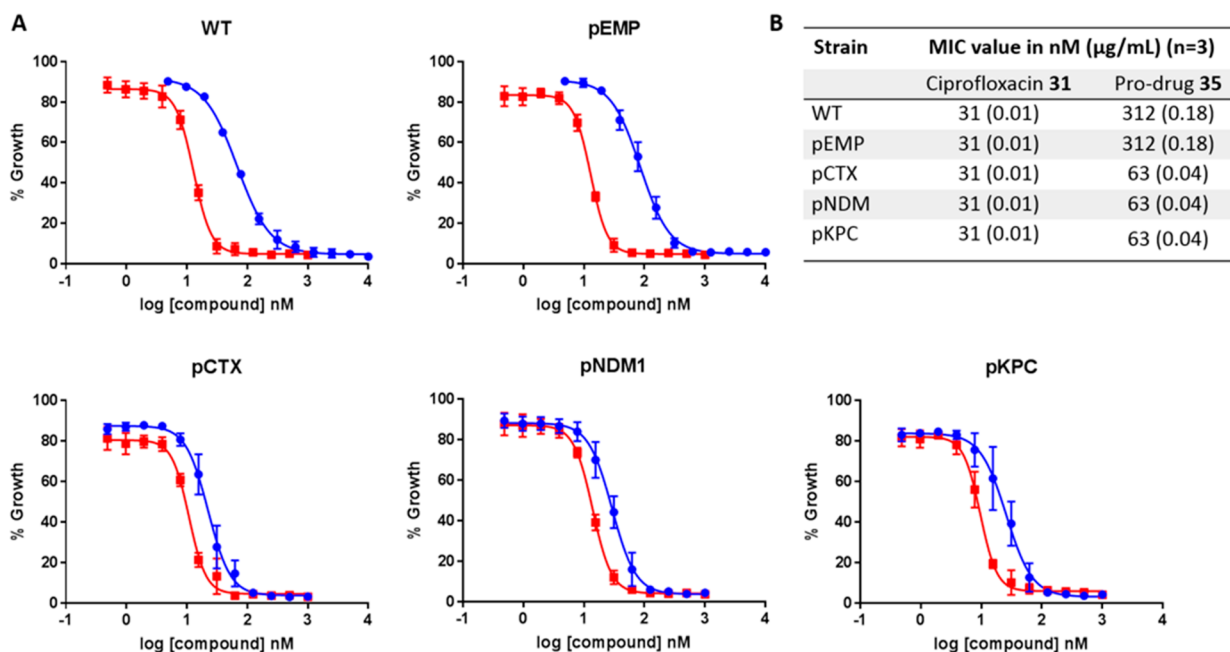


Figure 5. Antibacterial activities for prodrug 35 (blue) and ciprofloxacin 31 (red) against CFT073 *E. coli* cells WT and expressing empty plasmid (pEMP), CTX-M-15 (pCTX), NDM1 (pNDM1), and KPC (pKPC): (A) dose–response curves, where each point represents the mean \pm SEM, $n = 3$; (B) summary of MIC values.

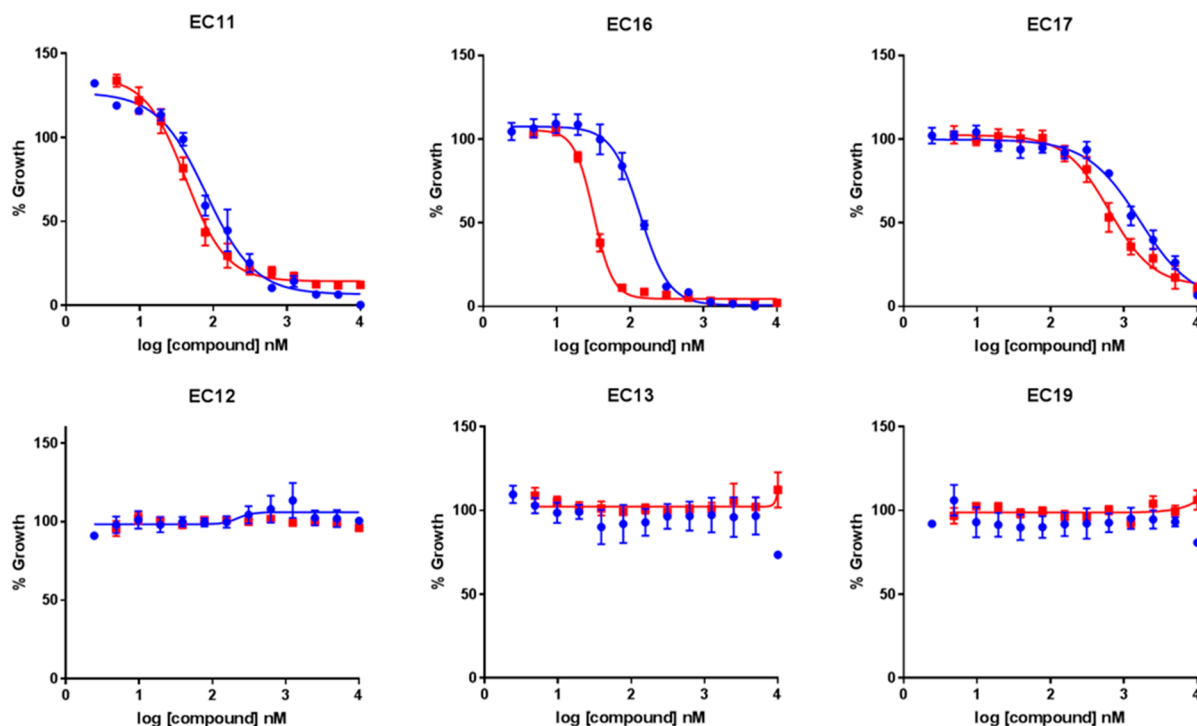


Figure 6. Effect of prodrug 35 (blue) or ciprofloxacin 31 (red) against six uropathogenic *E. coli* clinical isolates. Each point represents the mean \pm SEM, $n = 3$.

findings provided further confidence in the selectivity of prodrug 35 and its stability in the host environment.

Selective Bactericidal Activity against β -Lactamase Expressing Bacteria. Finally, the ability of prodrug 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 prodrug 35 was determined over time by CFU counts (Figure 7). After 6 h incubation with

prodrug 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, while 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 prodrug approach,

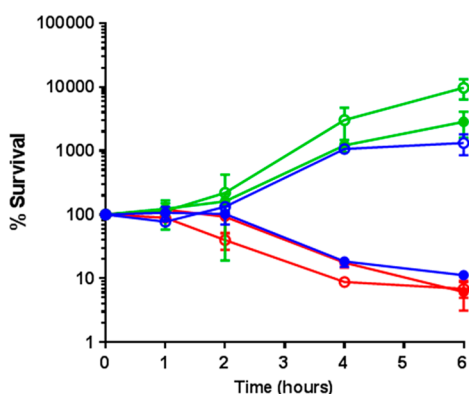


Figure 7. Survival of CFT073 pEMP (open circle) and pCTX (filled circle) with no treatment (green), ciprofloxacin 31 (78 nM) (red), or prodrug 35 (78 nM) (blue): Cipro, ciprofloxacin; PD, prodrug 35.

without adversely affecting bacteria that do not produce β -lactamase.

CONCLUSIONS

A novel cephalosporin–fluoroquinolone antibiotic prodrug has been designed, synthesized, and evaluated for biological activity. A program of optimization was successfully undertaken to reduce the antibacterial activity of the intact prodrug through modification to the cephalosporin component. Prodrug 35 exhibits similar growth inhibitory activity to ciprofloxacin against uropathogenic *E. coli* expressing the diverse ESBLs CTX-M-1, NDM-1, and KPC but little activity against strains that did not express β -lactamases. The selectively observed bactericidal activity was even greater, with prodrug 35 killing β -lactamase expressing bacteria at the same rate as free ciprofloxacin while not affecting the growth of bacteria that did not express β -lactamases.

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

Together, these studies demonstrate that our prodrug 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. First, increased selectivity of fluoroquinolones will enable maintenance of the microbiota leading to reduced secondary infection rate and subsequent antibiotic use. Second, there is 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 is 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 prodrug 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 prodrug approach could be used to 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 while minimizing selection for resistance and without collateral damage to the microbiota. This complements ongoing 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-workers⁷⁹ 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, while 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). ¹H 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: CDCl₃ (δ_{H} 7.26), D₂O (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. ¹³C 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: CDCl₃ (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 Acquity UPLC I-class system operating in ES+ or ES− mode. Analytical separation was performed using a Waters BEH Acquity C18, 50 mm × 2.1 mm column using a flow rate of 0.5 mL/min in a 4 min 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 enkephalin or sulfadimethoxine. All tested compounds were ≥95% pure 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. NaHCO₃ (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 (×3).⁵⁵ The organic extracts were combined, dried over Na₂SO₄, evaporated and the resulting solid was triturated with ice-cold Et₂O (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% NaHCO₃ (aq). The aqueous layer was separated and the organic layer washed with 3% NaHCO₃ (aq) (×2). 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-dimethylamino-propyl)-*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 H₂O and extracted with EtOAc (×3). The organic extracts were combined, washed with 1 M LiCl (aq) and brine, and dried over Na₂SO₄. Solvent was removed under reduced pressure and the resulting oil triturated with Et₂O. The precipitate was collected by vacuum filtration and washed with DCM to afford the product as beige amorphous solid (36 mg, 8%). IR (solid): ν_{\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). ¹³C 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 C₁₆H₁₇BrN₂O₆S₂ (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 μ L, 0.88 mmol), and DMF (1 drop) were reacted 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 μ L) according to method B. The aqueous layer was extracted with DCM (×3), and the organic layers were combined, dried over Na₂SO₄, 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): ν_{\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). ¹³C 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 C₁₆H₁₆N₂O₆S₂ (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 μ L, 0.74 mmol), oxalyl chloride (76 μ L, 0.88 mmol), and DMF (1 drop) were reacted 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 μ L) according to method B. The aqueous layer was extracted with DCM (×3), and the organic layers were combined, dried over Na₂SO₄ 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): ν_{\max} 3571, 3317, 1785, 1759, 1718, 1662, 1517, 1238 cm⁻¹. ¹H NMR (400 MHz, CDCl₃) 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). ¹³C 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 C₂₄H₂₄N₂O₆S (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 μ L, 0.73 mmol) were reacted in sat. NaHCO₃(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): ν_{\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). ¹³C 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 C₁₈H₁₉N₂O₆S (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 μ L, 0.88 mmol), and DMF (1 drop) were reacted 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 μ L) 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): ν_{\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). ¹³C 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 C₁₉H₂₁N₂O₆S (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 μ L, 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 μ L) according to method B. The resulting mixture was cooled to 4 °C 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): ν_{\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). ¹³C 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 C₁₈H₁₈FN₂O₆S (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 μ L) 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): ν_{\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). ¹³C 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 C₁₈H₁₈ClN₂O₆S (M + H)⁺ 447.0394, found 447.0414.

(6R,7R)-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 μ L) 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): ν_{\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 $\text{C}_{18}\text{H}_{18}\text{BrN}_2\text{O}_6\text{S}$ ($\text{M} + \text{H}$) $^+$ 469.0069, found 469.0076.

(6R,7R)-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 μ L, 0.88 mmol), and DMF (1 drop) were reacted 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 μ L) according to method B. The resulting precipitate was collected by vacuum filtration and washed with ice-cold Et $_2$ O to afford the product as a white amorphous solid (112 mg, 65%). IR (solid): ν_{\max} 3302, 1756, 1737, 1654, 1621, 1536, 1237 cm^{-1} . ^1H 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 $\text{C}_{24}\text{H}_{23}\text{N}_2\text{O}_6\text{S}$ ($\text{M} + \text{H}$) $^+$ 467.1277, found 467.1287.

(6R,7R)-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 μ L, 0.88 mmol), and DMF (1 drop) were reacted 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 μ L) according to method B. The aqueous layer was extracted with DCM (\times 3), 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): ν_{\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 $\text{C}_{24}\text{H}_{23}\text{N}_2\text{O}_7\text{S}$ ($\text{M} + \text{H}$) $^+$ 483.1226, found 483.1212.

(6R,7R)-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 μ L, 0.88 mmol), and DMF (1 drop) were reacted 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 μ L) according to method B. The aqueous layer was extracted with DCM (\times 3), 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): ν_{\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 $\text{C}_{19}\text{H}_{21}\text{N}_2\text{O}_6\text{S}$ ($\text{M} + \text{H}$) $^+$ 405.1120, found 405.1126.

(6R,7R)-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 μ L, 0.88 mmol), and DMF (1 drop) were reacted 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 μ L) according to method B. The aqueous layer was extracted with DCM (\times 3), 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): ν_{\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 $\text{C}_{24}\text{H}_{23}\text{N}_2\text{O}_7\text{S}$ ($\text{M} + \text{H}$) $^+$ 483.1226, found 483.1233.

(6R,7R)-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 μ L, 0.44 mmol) were reacted in sat. 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): ν_{\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 $\text{C}_{17}\text{H}_{17}\text{N}_2\text{O}_6\text{S}$ ($\text{M} + \text{H}$) $^+$ 377.0807, found 377.0807.

(6R,7R)-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 μ L, 0.74 mmol) were reacted in sat. 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): ν_{\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 $\text{C}_{18}\text{H}_{19}\text{N}_2\text{O}_6\text{S}$ ($\text{M} + \text{H}$) $^+$ 391.0964, found 391.0972.

(6R,7R)-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 μ L, 0.74 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 μ L) according to method B. The aqueous layer was extracted with DCM (\times 3), and the organic layers were combined, dried over Na_2SO_4 , and evaporated. The resulting solid was triturated with ice-cold Et $_2$ O to afford the product as a white amorphous solid (36 mg, 24%). IR (solid): ν_{\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 C₁₈H₁₉N₂O₈S (M + H)⁺ 407.0913, found 407.0919.

(6R,7R)-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 sat. NaHCO₃ (aq) (10 mL) and acetone (5 mL) according to method A. Several drops of MeOH were added prior to the addition of ice-cold DCM to afford the product as a white amorphous solid (54 mg, 35%). IR (solid): ν_{\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). ¹³C 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 C₁₇H₁₆N₃O₈S (M + H)⁺ 422.0658, found 422.0660.

(6R,7R)-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 μ L, 0.74 mmol) were reacted in sat. NaHCO₃ (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): ν_{\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). ¹³C 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 C₁₅H₁₅N₂O₇S (M + H)⁺ 367.0600, found 367.0609.

(6R,7R)-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 μ L, 0.44 mmol) were reacted in sat. NaHCO₃ (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): ν_{\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). ¹³C 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 C₁₇H₂₃N₂O₆S (M + H)⁺ 383.1277, found 383.1264.

(6R,7R)-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 H₂O (8 mL), NaHCO₃ (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 μ L, 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, and the resulting material was diluted in H₂O and neutralized with sat. NaHCO₃ (aq). The aqueous solution was washed with EtOAc, acidified to pH 2 with 1 M HCl and extracted with EtOAc (\times 3). The organic layers were combined, washed with brine, dried over Na₂SO₄, and evaporated to afford the product as a colorless foam (471 mg, 81%). IR (solid): ν_{\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). ¹³C 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 C₁₂H₁₅N₂O₆S (M + Na)⁺ 337.0470, found 337.0479.

(6R,7R)-3-(Acetoxymethyl)-7-butiramido-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (27). Butyryl chloride (76 μ L, 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 μ L) according to method B. The aqueous layer was extracted with DCM (\times 3), and the organic layers were combined, dried over Na₂SO₄, 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): ν_{\max} 3265, 2960, 1774, 1751, 1715, 1654, 1539, 1223 cm⁻¹. ¹H 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). ¹³C 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 C₁₄H₁₉N₂O₆S (M + H)⁺ 343.0964, found 343.0959.

(6R,7R)-3-(Acetoxymethyl)-7-hexanamido-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (28). Hexanoyl chloride (103 μ L, 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 μ L) 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): ν_{\max} 3283, 3183 2930, 1774, 1752, 1711, 1651, 1536, 1223 cm⁻¹. ¹H 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). ¹³C 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 C₁₆H₂₃N₂O₆S (M + H)⁺ 371.1277, found 371.1290.

tert-Butyl (6R,7R)-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 μ L, 0.96 mmol) was added, and the reaction was heated to 60 °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 10 g 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): ν_{\max} 3291, 2982, 1774, 1718, 1670, 1528, 1368, 1223 cm⁻¹. ¹H NMR (400 MHz, CDCl₃) 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). ¹³C NMR (101 MHz, CDCl₃) 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 C₁₆H₂₃N₂O₆S (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 dropwise addition of Boc₂O (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 H₂O and neutralized with sat. NH₄Cl (aq). The precipitate was collected by vacuum filtration and washed with H₂O to afford the product as a white amorphous solid (502 mg, 77%). IR (solid): ν_{\max} 2971, 1733, 1688, 1629, 1249 cm⁻¹. ¹H NMR (400 MHz, CDCl₃) 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). ¹³C NMR (101 MHz, CDCl₃) 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 C₂₂H₂₇N₃O₅F (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 was stirred at 30 °C for 30 min. Solvent was removed under reduced pressure and resulting material suspended in H₂O (5 μ L) and EtOH (5 mL) and evaporated to dryness ($\times 3$). Then, the solid was suspended in DCM and evaporated to afford the product as a cream amorphous solid (111 mg, quant.). IR (solid): ν_{\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 μ L, 0.82 mmol) added dropwise.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) Na₂SO₃ (aq). The organic layer was dried over Na₂SO₄ and evaporated to 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 dropwise. The reaction mixture was stirred in the dark for 2 h before the solvent was removed under a stream of N₂. 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): ν_{\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). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 171.5, 170.0, 164.9, 164.2, 160.5, 153.7, 152.6 (d, ¹*J*_{C–F} = 247.5 Hz), 148.4, 143.8 (d, ²*J*_{C–F} = 10.1 Hz), 138.0, 126.2, 123.5, 122.1 (d, ³*J*_{C–F} = 7.1 Hz), 111.6 (d, ²*J*_{C–F} = 22.2 Hz), 108.6, 106.7 (d, ³*J*_{C–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 C₃₆H₄₄FN₅O₉S (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 dropwise 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 N₂ and the resulting gum triturated with ice-cold EtOAc. The precipitate was collected, diluted in H₂O and DCM, and basified to pH 9 with 3% NaHCO₃ (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 H₂O). Fractions containing product were freeze-dried to afford the product as a white solid (2.5 mg, 23%). ¹H NMR (500 MHz, D₂O) 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 C₂₇H₂₉FN₅O₇S (M + H)⁺ 586.1772, found 586.1794.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.8b01923.

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

Molecular formula strings (CSV)

■ AUTHOR INFORMATION

Corresponding Authors

*L.E.E.: e-mail, lindsay.evans@imperial.ac.uk.

*A.M.E.: phone, +44 (0) 20 7594 2072; e-mail, a.edwards@imperial.ac.uk.

ORCID

Lindsay E. Evans: 0000-0002-6950-9329

James Spencer: 0000-0002-4602-0571

Alan Armstrong: 0000-0002-3692-3099

Present Address

¹D.C.M: Critical Care Research Group, Nuffield Department of Clinical Neurosciences, University of Oxford, OX3 9DU Oxford, United Kingdom.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health

■ ACKNOWLEDGMENTS

We thank the Imperial Confidence in Concept scheme and the Wellcome Trust (Pathfinder Award 204337/Z/16/Z) for funding this work. A.M.E. also acknowledges support from the National Institute for Health Research (NIHR) Imperial Biomedical Research Centre (BRC). J.S. acknowledges funding the Engineering and Physical Sciences Research Council (Grant EP/M027546/1) and the Biotechnology and Biological Sciences Research Council-funded South West Biosciences Doctoral Training Partnership (Training Grant Reference BB/J014400/1, studentship to C.L.T.). T.B.C. is a Sir Henry Dale Fellow jointly funded by the Wellcome Trust and Royal Society (Grant 107660/Z/15Z). We thank Peter Haycock from the Imperial College Department of Chemistry NMR Facility and Lisa Haigh from the Imperial College Department of Chemistry Mass Spec Facility for their support with NMR and mass spectrometry data collection, respectively. We thank Prof. Matthew B. Avison, University of Bristol, for providing the pSU18 vectors used in this study. We also thank Ali Abdolrasouli, Charing Cross Hospital, for providing the clinical isolates used in this study.

■ ABBREVIATIONS USED

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.

■ REFERENCES

- (1) *Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations*, Review on Antimicrobial Resistance; O'Neill, J., Chair; HM Government, Wellcome Trust, 2014.
- (2) Rossolini, G. M.; Arena, F.; Pecile, P.; Pollini, S. Update on the Antibiotic Resistance Crisis. *Curr. Opin. Pharmacol.* **2014**, *18*, 56–60.
- (3) Kong, K.-F.; Schneper, L.; Mathee, K. Beta-Lactam Antibiotics: From Antibiosis to Resistance and Bacteriology. *APMIS.* **2010**, *118* (1), 1–36.

- (4) Fernandes, R.; Amador, P.; Prudêncio, C. β -Lactams. *Rev. Med. Microbiol.* **2013**, *24* (1), 7–17.
- (5) Bush, K. Proliferation and Significance of Clinically Relevant β -Lactamases. *Ann. N. Y. Acad. Sci.* **2013**, *1277* (1), 84–90.
- (6) Cantón, R.; González-Alba, J. M.; Galán, J. C. CTX-M Enzymes: Origin and Diffusion. *Front. Microbiol.* **2012**, *3*, 110.
- (7) Shaikh, S.; Fatima, J.; Shakil, S.; Rizvi, S. M. D.; Kamal, M. A. Antibiotic Resistance and Extended Spectrum Beta-Lactamases: Types, Epidemiology and Treatment. *Saudi J. Biol. Sci.* **2015**, *22* (1), 90–101.
- (8) Flores-Mireles, A. L.; Walker, J. N.; Caparon, M.; Hultgren, S. J. Urinary Tract Infections: Epidemiology, Mechanisms of Infection and Treatment Options. *Nat. Rev. Microbiol.* **2015**, *13* (5), 269–284.
- (9) Peach, B. C.; Garvan, G. J.; Garvan, C. S.; Cimiotti, J. P. Risk Factors for Urosepsis in Older Adults: A Systematic Review. *Gerontol. Geriatr. Med.* **2016**, *2*, 2333721416638980.
- (10) Tandogdu, Z.; Wagenlehner, F. M. E. Global Epidemiology of Urinary Tract Infections. *Curr. Opin. Infect. Dis.* **2016**, *29* (1), 73–79.
- (11) Kabbani, S.; Hersh, A. L.; Shapiro, D. J.; Fleming-Dutra, K. E.; Pavia, A. T.; Hicks, L. A. Opportunities to Improve Fluoroquinolone Prescribing in the United States for Adult Ambulatory Care Visits. *Clin. Infect. Dis.* **2018**, *67* (1), 134–136.
- (12) Scheld, W. M. Maintaining Fluoroquinolone Class Efficacy: Review of Influencing Factors. *Emerging Infect. Dis.* **2003**, *9* (1), 1–9.
- (13) Becattini, S.; Taur, Y.; Pamer, E. G. Antibiotic-Induced Changes in the Intestinal Microbiota and Disease. *Trends Mol. Med.* **2016**, *22* (6), 458–478.
- (14) Stewardson, A. J.; Gaia, N.; Francois, P.; Malhotra-Kumar, S.; Delémont, C.; Martinez de Tejada, B.; Schrenzel, J.; Harbarth, S.; Lazarevic, V. Collateral Damage from Oral Ciprofloxacin versus Nitrofurantoin in Outpatients with Urinary Tract Infections: A Culture-Free Analysis of Gut Microbiota. *Clin. Microbiol. Infect.* **2015**, *21* (4), 344.e1–344.e11.
- (15) Dethlefsen, L.; Relman, D. A. Incomplete Recovery and Individualized Responses of the Human Distal Gut Microbiota to Repeated Antibiotic Perturbation. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (Suppl. 1), 4554–4561.
- (16) Sullivan, Å.; Edlund, C.; Nord, C. E. Effect of Antimicrobial Agents on the Ecological Balance of Human Microflora. *Lancet Infect. Dis.* **2001**, *1* (2), 101–114.
- (17) Jernberg, C.; Lofmark, S.; Edlund, C.; Jansson, J. K. Long-Term Impacts of Antibiotic Exposure on the Human Intestinal Microbiota. *Microbiology* **2010**, *156* (11), 3216–3223.
- (18) Brown, K. A.; Khanafer, N.; Daneman, N.; Fisman, D. N. Meta-Analysis of Antibiotics and the Risk of Community-Associated Clostridium Difficile Infection. *Antimicrob. Agents Chemother.* **2013**, *57* (5), 2326–2332.
- (19) Chellat, M. F.; Raguž, L.; Riedl, R. Targeting Antibiotic Resistance. *Angew. Chem., Int. Ed.* **2016**, *55* (23), 6600–6626.
- (20) Lewis, K. Platforms for Antibiotic Discovery. *Nat. Rev. Drug Discovery* **2013**, *12* (5), 371–387.
- (21) FDA. Highlights of prescribing information (ciprofloxacin hydrochloride). www.fda.gov/medwatch (accessed Oct 25, 2018).
- (22) Press Announcements. FDA updates warnings for fluoroquinolone antibiotics. <https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm513183.htm> (accessed Oct 24, 2018).
- (23) Marchant, J. When Antibiotics Turn Toxic. *Nature* **2018**, *555* (7697), 431–433.
- (24) Dingle, K. E.; Didelot, X.; Quan, T. P.; Eyre, D. W.; Stoesser, N.; Golubchik, T.; Harding, R. M.; Wilson, D. J.; Griffiths, D.; Vaughan, A.; Finney, J. M.; Wyllie, D. H.; Oakley, S. J.; Fawley, W. N.; Freeman, J.; Morris, K.; Martin, J.; Howard, P.; Gorbach, S.; Goldstein, E. J. C.; Citron, D. M.; Hopkins, S.; Hope, R.; Johnson, A. P.; Wilcox, M. H.; Peto, T. E. A.; Walker, A. S.; Crook, D. W.; Del Ojo Elias, C.; Crichton, C.; Kostiou, V.; Giess, A.; Davies, J. Effects of Control Interventions on Clostridium Difficile Infection in England: An Observational Study. *Lancet Infect. Dis.* **2017**, *17* (4), 411–421.
- (25) Stewardson, A. J.; Vervoort, J.; Adriaenssens, N.; Coenen, S.; Godycki-Cwirko, M.; Kowalczyk, A.; Huttner, B. D.; Lammens, C.; Malhotra-Kumar, S.; Goossens, H.; Harbarth, S.; Vervoort, J.; Lammens, C.; Malhotra-Kumar, S.; Goossens, H.; Adriaenssens, N.; Coenen, S.; Kowalczyk, A.; Godycki-Cwirko, M.; Stewardson, A. J.; Huttner, B.; Harbarth, S.; Brossier, C.; Delemont, C.; de Tejada, B. M.; Renzi, G.; Schrenzel, J.; Van Bylen, S.; Vanbergen, J.; Koeck, P.; Leysen, P.; Vandercam, K.; Kluijtmans, J.; Borkiewicz, A.; Heyvaert, F.; Michels, N.; Deswaef, G.; Beckx, T.; Declerck, H.; Embrechts, K.; Verheyen, N.; Bauwens, T.; Beghin, J.; Verpooten, L.; Bombeke, K.; Vandenaabeele, T.; Muras, M.; Swistak, J.; Wesolowska, A.; Sterniczuk, E.; Brzozowska, L.; Cichowska, K.; Szewczyk, J.; Krupinska, G.; Blaszczyk, H.; Stawinska, U.; Szyler, M.; Kasielski, M.; Rydz, R.; Myszkowska, A.; Zebrowska, L. Effect of Outpatient Antibiotics for Urinary Tract Infections on Antimicrobial Resistance among Commensal Enterobacteriaceae: A Multinational Prospective Cohort Study. *Clin. Microbiol. Infect.* **2018**, *24* (9), 972–979.
- (26) Klein, E. Y.; Van Boeckel, T. P.; Martinez, E. M.; Pant, S.; Gandra, S.; Levin, S. A.; Goossens, H.; Laxminarayan, R. Global Increase and Geographic Convergence in Antibiotic Consumption between 2000 and 2015. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115* (15), E3463–E3470.
- (27) *Rapid Diagnostics: Stopping Unnecessary Use of Antibiotics*, Review on Antimicrobial Resistance; O'Neill, J., Chair; HM Government, Wellcome Trust, 2015.
- (28) Mota, R.; Pinto, M.; Palmeira, J.; Gonçalves, D.; Ferreira, H. Intestinal Microbiota as a Reservoir of Extended-Spectrum β -Lactamase-Producing Escherichia Coli: An Exploratory Study in Healthy University Students. *J. Glob. Antimicrob. Resist.* **2018**, *14*, 10–11.
- (29) de Lastours, V.; Goulenok, T.; Guérin, F.; Jacquier, H.; Eyma, C.; Chau, F.; Cattoir, V.; Fantin, B. Ceftriaxone Promotes the Emergence of AmpC-Overproducing Enterobacteriaceae in Gut Microbiota from Hospitalized Patients. *Eur. J. Clin. Microbiol. Infect. Dis.* **2018**, *37* (3), 417–421.
- (30) Ríos, E.; López, M. C.; Rodríguez-Avil, I.; Culebras, E.; Picazo, J. J. Detection of Escherichia Coli ST131 Clonal Complex (ST705) and Klebsiella Pneumoniae ST15 among Faecal Carriage of Extended-Spectrum β -Lactamase- and Carbapenemase-Producing Enterobacteriaceae. *J. Med. Microbiol.* **2017**, *66* (2), 169–174.
- (31) Shepherd, T. A.; Jungheim, L. N.; Meyer, D. L.; Starling, J. J. A Novel Targeted Delivery System Utilizing a Cephalosporin-Oncolytic Prodrug Activated by an Antibody β -Lactamase Conjugate for the Treatment of Cancer. *Bioorg. Med. Chem. Lett.* **1991**, *1* (1), 21–26.
- (32) Jungheim, L. N.; Shepherd, T. A.; Meyer, D. L. Synthesis of Acylhydrazido-Substituted Cephems. Design of Cephalosporin-Vinca Alkaloid Prodrugs: Substrates for an Antibody Targeted Enzyme. *J. Org. Chem.* **1992**, *57* (8), 2334–2340.
- (33) Rodrigues, M. L.; Carter, P.; Wirth, C.; Mullins, S.; Lee, A.; Blackburn, B. K. Synthesis and β -Lactamase-Mediated Activation of a Cephalosporin-Taxol Prodrug. *Chem. Biol.* **1995**, *2* (4), 223–227.
- (34) Phelan, R. M.; Ostermeier, M.; Townsend, C. A. Design and Synthesis of a β -Lactamase Activated 5-Fluorouracil Prodrug. *Bioorg. Med. Chem. Lett.* **2009**, *19* (4), 1261–1263.
- (35) Hudyma, T. W.; Bush, K.; Colson, K. L.; Firestone, R. A.; King, H. D. Synthesis and Release of Doxorubicin from a Cephalosporin Based Prodrug by a β -Lactamase-Immunoconjugate. *Bioorg. Med. Chem. Lett.* **1993**, *3* (2), 323–328.
- (36) Demuth, T. P.; White, R. E.; Tietjen, R. A.; Storrin, R. J.; Skuster, J. R.; Andersen, J. A.; McOsker, C. C.; Freedman, R.; Rourke, F. J. Synthesis and Antibacterial Activity of New C-10 Quinolonyl-Cephem Esters. *J. Antibiot.* **1991**, *44* (2), 200–209.
- (37) Bryskier, A. Dual β -Lactam-Fluoroquinolone Compounds: A Novel Approach to Antibacterial Treatment. *Expert Opin. Invest. Drugs* **1997**, *6* (10), 1479–1499.
- (38) O'Callaghan, C. H.; Sykes, R. B.; Staniforth, S. E. A New Cephalosporin with a Dual Mode of Action. *Antimicrob. Agents Chemother.* **1976**, *10* (2), 245–248.
- (39) Georgopapadakou, N. H.; Bertasso, A.; Chan, K. K.; Chapman, J. S.; Cleeland, R.; Cummings, L. M.; Dix, B. A.; Keith, D. D. Mode of

Action of the Dual-Action Cephalosporin Ro 23-9424. *Antimicrob. Agents Chemother.* **1989**, *33* (7), 1067–1071.

(40) O'Callaghan, C. H.; Kirby, S. M.; Morris, A.; Waller, R. E.; Duncombe, R. E. Correlation between Hydrolysis of the β -Lactam Bond of the Cephalosporin Nucleus and Expulsion of the 3-Substituent. *J. Bacteriol.* **1972**, *110* (3), 988–991.

(41) Smyth, T. P.; O'Donnell, M. E.; O'Connor, M. J.; St Ledger, J. O. β -Lactamase-Dependent Prodrugs—Recent Developments. *Tetrahedron* **2000**, *56* (31), 5699–5707.

(42) Albrecht, H. A.; Beskid, G.; Chan, K. K.; Christenson, J. G.; Cleeland, R.; Deitcher, K. H.; Georgopapadaku, N. H.; Keith, D. D.; Pruess, D. L. Cephalosporin 3'-Quinolone Esters with a Dual Mode of Action. *J. Med. Chem.* **1990**, *33* (1), 77–86.

(43) Correia, S.; Poeta, P.; Hébraud, M.; Capelo, J. L.; Igrejas, G. Mechanisms of Quinolone Action and Resistance: Where Do We Stand? *J. Med. Microbiol.* **2017**, *66* (5), 551–559.

(44) Dunn, G. L. Ceftizoxime and Other Third-Generation Cephalosporins: Structure-Activity Relationships. *J. Antimicrob. Chemother.* **1982**, *10* (Suppl. C), 1–10.

(45) Kaushik, D.; Rathi, S.; Jain, A. Ceftaroline: A Comprehensive Update. *Int. J. Antimicrob. Agents* **2011**, *37* (5), 389–395.

(46) Fung-Tomc, J. C. Fourth-Generation Cephalosporins. *Clin. Microbiol. Newsl.* **1997**, *19* (17), 129–136.

(47) Sader, H. S.; Jones, R. N. Historical Overview of the Cephalosporin Spectrum: Four Generations of Structural Evolution. *Antimicrob. Newsl.* **1992**, *8* (12), 75–82.

(48) García-Rodríguez, J. A.; Muñoz Bellido, J. L.; García Sánchez, J. E. Oral Cephalosporins: Current Perspectives. *Int. J. Antimicrob. Agents* **1995**, *5* (4), 231–243.

(49) Turck, M. Cephalosporins and Related Antibiotics: An Overview. *Clin. Infect. Dis.* **1982**, *4* (Suppl.2), S281–S287.

(50) Snyder, N. J.; Tabas, L. B.; Berry, D. M.; Duckworth, D. C.; Spry, D. O.; Dantzig, A. H. Structure-Activity Relationship of Carbacephalosporins and Cephalosporins: Antibacterial Activity and Interaction with the Intestinal Proton-Dependent Dipeptide Transport Carrier of Caco-2 Cells. *Antimicrob. Agents Chemother.* **1997**, *41* (8), 1649–1657.

(51) Sonawane, V. C. Enzymatic Modifications of Cephalosporins by Cephalosporin Acylase and Other Enzymes. *Crit. Rev. Biotechnol.* **2006**, *26* (2), 95–120.

(52) Oberdorf, C.; Schepmann, D.; Vela, J. M.; Diaz, J. L.; Holenz, J.; Wunsch, B. Thiophene Bioisosteres of Spirocyclic σ Receptor Ligands. 1. *N*-Substituted Spiro[piperidine-4,4'-thieno[3,2-*c*]pyrans]. *J. Med. Chem.* **2008**, *51* (20), 6531–6537.

(53) Brown, N. *Bioisosteres in Medicinal Chemistry*; Wiley-VCH: Weinheim, Germany, 2012.

(54) Essack, S. Y. The Development of β -Lactam Antibiotics in Response to the Evolution of β -Lactamases. *Pharm. Res.* **2001**, *18* (10), 1391–1399.

(55) Buckwell, S. C.; Page, M. I.; Waley, S. G.; Longridge, J. L. Hydrolysis of 7-Substituted Cephalosporins Catalysed by β -Lactamases I and II from *Bacillus Cereus* and by Hydroxide Ion. *J. Chem. Soc., Perkin Trans. 2* **1988**, *0* (10), 1815–1821.

(56) Rao, W.-H.; Zhan, B.-B.; Chen, K.; Ling, P.-X.; Zhang, Z.-Z.; Shi, B.-F. Pd(II)-Catalyzed Direct Sulfonylation of Unactivated C(Sp³)-H Bonds with Sodium Sulfinates. *Org. Lett.* **2015**, *17* (14), 3552–3555.

(57) Pai, H.; Lyu, S.; Lee, J. H.; Kim, J.; Kwon, Y.; Kim, J. W.; Choe, K. W.; Jeong, B. C.; Lee, S. H. Survey of Extended-Spectrum Beta-Lactamases in Clinical Isolates of *Escherichia coli* and *Klebsiella pneumoniae*: Prevalence of TEM-52 in Korea. *J. Clin. Microbiol.* **1999**, *37* (6), 1758–1763.

(58) Bush, K.; Sykes, R. B. Methodology for the Study of Beta-Lactamases. *Antimicrob. Agents Chemother.* **1986**, *30* (1), 6–10.

(59) Sanders, C. C. Chromosomal Cephalosporinases Responsible for Multiple Resistance to Newer β -Lactam Antibiotics. *Annu. Rev. Microbiol.* **1987**, *41* (1), 573–594.

(60) Ma, J.; Cao, Q.; McLeod, S. M.; Ferguson, K.; Gao, N.; Breeze, A. L.; Hu, J. Target-Based Whole-Cell Screening by ¹H-NMR Spectroscopy. *Angew. Chem., Int. Ed.* **2015**, *54* (16), 4764–4767.

(61) Ma, J.; McLeod, S.; MacCormack, K.; Sriram, S.; Gao, N.; Breeze, A. L.; Hu, J. Real-Time Monitoring of New Delhi Metallo- β -Lactamase Activity in Living Bacterial Cells by ¹H-NMR Spectroscopy. *Angew. Chem., Int. Ed.* **2014**, *53* (8), 2130–2133.

(62) Welch, R. A.; Burland, V.; Plunkett, G.; Redford, P.; Roesch, P.; Rasko, D.; Buckles, E. L.; Liou, S.-R.; Boutin, A.; Hackett, J.; Stroud, D.; Mayhew, G. F.; Rose, D. J.; Zhou, S.; Schwartz, D. C.; Perna, N. T.; Mobley, H. L. T.; Donnenberg, M. S.; Blattner, F. R. Extensive Mosaic Structure Revealed by the Complete Genome Sequence of Uropathogenic *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99* (26), 17020–17024.

(63) Mobley, H. L.; Green, D. M.; Trifillis, A. L.; Johnson, D. E.; Chippendale, G. R.; Lockatell, C. V.; Jones, B. D.; Warren, J. W. Pyelonephritogenic *Escherichia coli* and Killing of Cultured Human Renal Proximal Tubular Epithelial Cells: Role of Hemolysin in Some Strains. *Infect. Immun.* **1990**, *58* (5), 1281–1289.

(64) Ghai, I.; Ghai, S. Understanding Antibiotic Resistance via Outer Membrane Permeability. *Infect. Drug Resist.* **2018**, *11*, 523–530.

(65) Peterson, L. A. Reactive Metabolites in the Biotransformation of Molecules Containing a Furan Ring. *Chem. Res. Toxicol.* **2013**, *26* (1), 6–25.

(66) Wang, Y.; Yuan, H.; Wright, S. C.; Wang, H.; Larrick, J. W. Synthesis and Preliminary Cytotoxicity Study of a Cephalosporin-CC-1065 Analogue Prodrug. *BMC Chem. Biol.* **2001**, *1* (1), 4.

(67) Lee, H. W.; Kang, T. W.; Kim, E. N.; Cha, K. H.; Shin, J.; Cho, D. O.; Choi, N. H.; Kim, J. W.; Hong, C. Preparation Of Ceph-3-Em Esters Unaccompanied By δ^3 To δ^2 Isomerization Of The Cephalosporin Derivatives. *Synth. Commun.* **1999**, *29* (11), 1873–1887.

(68) Kaiser, G. V.; Cooper, R. D. G.; Koehler, R. E.; Murphy, C. F.; Webber, J. A.; Wright, I. G.; Van Heyningen, E. M. Chemistry of Cephalosporin Antibiotics. XIX. Transformation of Δ^2 -Cephem to Δ^3 -Cephem by Oxidation-Reduction at Sulfur. *J. Org. Chem.* **1970**, *35* (7), 2430–2433.

(69) Popa, E.; Huang, M.-J.; Brewstera, M. E.; Bodora, N. On the Mechanism of Cephalosporin Isomerization. *J. Mol. Struct.: THEOCHEM* **1994**, *315*, 1–7.

(70) Tanaka, K. S. E.; Houghton, T. J.; Kang, T.; Dietrich, E.; Delorme, D.; Ferreira, S. S.; Caron, L.; Viens, F.; Arhin, F. F.; Sarmiento, I.; Lehoux, D.; Fadhil, I.; Laquerre, K.; Liu, J.; Ostiguy, V.; Poirier, H.; Moeck, G.; Parr, T. R.; Rafai Far, A. Bisphosphonated Fluoroquinolone Esters as Osteotropic Prodrugs for the Prevention of Osteomyelitis. *Bioorg. Med. Chem.* **2008**, *16* (20), 9217–9229.

(71) Chan, M. F.; Castillo, R. S.; Li, Q.; Doppalapudi, V. R.; Hixton, M. S.; Lobl, T. J. Improved Beta-Lactam Antibiotics. WO 01/83492 A1, 2001.

(72) Aldred, K. J.; Kerns, R. J.; Osheroff, N. Mechanism of Quinolone Action and Resistance. *Biochemistry* **2014**, *53*, 1565–1574.

(73) Khan, A. U.; Maryam, L.; Zarrilli, R. Structure, Genetics and Worldwide Spread of New Delhi Metallo- β -Lactamase (NDM): A Threat to Public Health. *BMC Microbiol.* **2017**, *17* (1), 101.

(74) Yigit, H.; Queenan, A. M.; Anderson, G. J.; Domenech-Sanchez, A.; Biddle, J. W.; Steward, C. D.; Alberti, S.; Bush, K.; Tenover, F. C. Novel Carbapenem-Hydrolyzing Beta-Lactamase, KPC-1, from a Carbapenem-Resistant Strain of *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **2001**, *45* (4), 1151–1161.

(75) Queenan, A. M.; Bush, K. Carbapenemases: The Versatile Beta-Lactamases. *Clin. Microbiol. Rev.* **2007**, *20* (3), 440–458.

(76) Remington, T.; Jahnke, N.; Harkensee, C. Oral Anti-Pseudomonal Antibiotics for Cystic Fibrosis. *Cochrane Database Syst. Rev.* **2016**, No. 10, CD005405.

(77) Langton Hewer, S. C.; Smyth, A. R. Antibiotic Strategies for Eradicating *Pseudomonas aeruginosa* in People with Cystic Fibrosis. *Cochrane Database Syst. Rev.* **2017**, No. 11, CD004197.

(78) Atkin, S. D.; Abid, S.; Foster, M.; Bose, M.; Keller, A.; Hollaway, R.; Sader, H. S.; Greenberg, D. E.; Finklea, J. D.;

Castanheira, M.; Jain, R. Multidrug-Resistant *Pseudomonas aeruginosa* from Sputum of Patients with Cystic Fibrosis Demonstrates a High Rate of Susceptibility to Ceftazidime-Avibactam. *Infect. Drug Resist.* **2018**, *11*, 1499–1510.

(79) Liu, R.; Miller, P. A.; Vakulenko, S. B.; Stewart, N. K.; Boggess, W. C.; Miller, M. J. A Synthetic Dual Drug Sideromycin Induces Gram-Negative Bacteria To Commit Suicide with a Gram-Positive Antibiotic. *J. Med. Chem.* **2018**, *61* (9), 3845–3854.

(80) Baell, J. B.; Holloway, G. A. New Substructure Filters for Removal of Pan Assay Interference Compounds (PAINS) from Screening Libraries and for Their Exclusion in Bioassays. *J. Med. Chem.* **2010**, *53* (7), 2719–2740.

(81) Irwin, J. J.; Duan, D.; Torosyan, H.; Doak, A. K.; Ziebart, K. T.; Sterling, T.; Tumanian, G.; Shoichet, B. K. An Aggregation Advisor for Ligand Discovery. *J. Med. Chem.* **2015**, *58* (17), 7076–7087.

(82) Quotadamo, A.; Linciano, P.; Davoli, P.; Tondi, D.; Costi, M.; Venturelli, A. An Improved Synthesis of CENTA, a Chromogenic Substrate for β -Lactamases. *Synlett* **2016**, *27* (17), 2447–2450.

(83) Reaction was performed under strict anhydrous conditions. Increasing the equivalents of TMSI or extending the reaction time led to decomposition of the product and a reduction in recovered material.