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Discovery of Cephalosporin-3'-Diazeniumdiolates That Show Dual Antibacterial and Antibiofilm Effects against Pseudomonas aeruginosa Clinical Cystic Fibrosis Isolates and Efficacy in a Murine Respiratory Infection Model

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Abstract

The formation of biofilms provides a formidable defense for many bacteria against antibiotics and host immune responses. As a consequence, biofilms are thought to be the root cause of most chronic infections, including those occurring on medical indwelling devices, endocarditis, urinary tract infections, diabetic and burn wounds, and bone and joint infections. In cystic fibrosis (CF), chronic *Pseudomonas aeruginosa* (*P. aeruginosa*) respiratory infections are the leading cause of morbidity and mortality in adults. Previous studies have shown that many bacteria can undergo a coordinated dispersal event in the presence of low concentrations of nitric oxide (NO), suggesting that NO could be used to initiate biofilm dispersal in chronic infections, enabling clearance of the more vulnerable planktonic cells. In this study, we describe efforts to create "all-in-one" cephalosporin-based NO donor prodrugs (cephalosporin-3'-diazeniumdiolates, C3Ds) that show both direct β -lactam mediated antibacterial activity and antibiofilm effects. Twelve novel C3Ds were synthesized and screened against a panel of *P. aeruginosa* CF clinical isolates and other human pathogens. The most active compound, AMINOPIP2 ((Z)-1-(4-(2-aminoethyl)piperidin-1-yl)-2-(((6R,7R)-7-((Z)-2-(2-aminothiazol-4-yl)-2-(((2-carboxypropan-2-yl)oxy)imino)acetamido)-1-oxide)-ceftazidime 12, showed higher antibacterial potency than its parent cephalosporin and front-line antipseudomonal antibiotic ceftazidime, good stability against β -lactamases, activity against ceftazidime-resistant *P. aeruginosa* in vitro biofilms, and efficacy equivalent to ceftazidime in a murine *P. aeruginosa* respiratory infection model. The results support further evaluation of AMINOPIP2-ceftazidime 12 for *P. aeruginosa* lung infections in CF and a broader study of "all-in-one" C3Ds for other chronic infections.

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The formation of biofilms provides a formidable defense for many bacteria against antibiotics and host immune responses. As a consequence, biofilms are thought to be the root cause of most chronic infections, including those occurring on medical indwelling devices, endocarditis, urinary tract infections, diabetic and burn wounds and bone and joint infections. In cystic fibrosis (CF), chronic *Pseudomonas aeruginosa* respiratory infections are the leading cause of morbidity and mortality in adults. Previous studies have shown that many bacteria can undergo a coordinated dispersal event in the presence of low concentrations of nitric oxide (NO), suggesting that NO could be used to initiate biofilm dispersal in chronic infections, enabling clearance of the more vulnerable planktonic cells. In this study, we describe efforts to create ‘all-in-one’ cephalosporin-based NO donor prodrugs (cephalosporin-3'-diazoniumdiolates, C3Ds) that show both direct β -lactam mediated antibacterial activity and antibiofilm effects. Twelve novel C3Ds were synthesized and screened against a panel of *P. aeruginosa* CF clinical isolates and other human pathogens. The most active compound, AMINOPIP2-ceftazidime **12**, showed higher antibacterial potency than its parent cephalosporin and front-line antipseudomonal antibiotic ceftazidime, good stability against β -lactamases, activity against ceftazidime-resistant *P. aeruginosa in vitro* biofilms and efficacy equivalent to ceftazidime in a murine *P. aeruginosa* respiratory infection model. The results support further evaluation of AMINOPIP2-ceftazidime **12** for *P. aeruginosa* lung infections in CF and broader study of ‘all-in-one’ C3Ds for other chronic infections.

Keywords: Biofilm, cephalosporin-3'-diazoniumdiolate, *Pseudomonas aeruginosa*, cystic fibrosis, chronic infection

Bacterial biofilms are high-density populations of cells encapsulated within a self-produced exo-polysaccharide matrix. The biofilm lifestyle protects many bacteria from a range of harmful stressors, with biofilm-encased bacteria known to be up to 1000-fold more tolerant of antibiotics than their planktonic counterparts¹ and less vulnerable to host immune defences.² It is estimated that more than 80% of all infections involve biofilms and they are thought to be the root cause of chronic infections, such as those occurring on medical indwelling devices, urinary tract infections, otitis media, endocarditis, diabetic wounds, bone and joint infections and more.³ In cystic fibrosis (CF), chronic *Pseudomonas aeruginosa* lung infections are the major cause of morbidity and mortality in adults.⁴ Few therapeutics effectively treat biofilm-mediated chronic infections and the development of new drugs that directly address biofilms remains a major challenge in infection control.³

Given appropriate cues, many bacteria can transition between the biofilm and planktonic growth modes via a coordinated dispersal event mediated by the second messenger dinucleotide cyclic-di-GMP.⁵ Accordingly, a promising antibiofilm strategy has emerged wherein agents are used to initiate biofilm dispersal and enable clearance of the more vulnerable planktonic cells by co-administered antibiotics and host immune defenses.⁶ Low concentrations of nitric oxide (NO, nM range) have been shown to signal biofilm dispersal in a broad range of bacteria, including *P. aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*, supporting use of NO-donor compounds (or NO gas)⁷ in combination with antibiotics as an antibiofilm treatment approach.⁸ While many compounds are known that spontaneously emit NO in neutral aqueous (physiological) solution,⁹ their use clinically against biofilms would be complicated by the need to direct NO to the infection site and the safety risks associated with systematic exposure of non-target host tissues to NO. These risks could potentially be mitigated by use of NO donor prodrugs that release NO upon reaction with bacteria-specific enzymes, thereby targeting NO exposure to biofilm infection sites.

Cephalosporin antibiotics have been used for many decades to treat diverse bacterial infections.¹⁰ Their primary mechanism of action involves inhibition of penicillin binding proteins (PBPs), which catalyze a crucial peptidoglycan cross-linking step during cell wall synthesis.¹¹ In Gram-positive bacteria, PBPs are generally located on the outer surface of the cytoplasmic membrane making them easily accessible to cephalosporins and other β -lactams.¹² In Gram-negative bacteria, β -lactams must penetrate the outer membrane to reach PBPs.¹³ Additionally, bacterial defence enzymes (e.g. β -lactamases) present in the periplasmic space can hydrolyze β -lactams, rendering the treatment of Gram-negative infections with β -lactams more challenging.¹⁴

Cephalosporin-3'-diazoniumdiolates (C3Ds) are a class of biofilm-targeted NO donor prodrugs that carry a chemically stable, O^2 -linked diazeniumdiolate at the cephalosporin 3'-position.^{15,16} We originally designed C3Ds to undergo reaction with bacterial β -lactamases, where ring opening of the β -lactam causes release (via the known conjugate elimination mechanism)¹⁷ of an unstable diazeniumdiolate anion (NONOate) that spontaneously emits NO, triggering biofilm dispersal (Figure 1). In principal, targeting NO to biofilm infection sites in this drug-like C3D form could serve to both optimize the NO-mediated dispersal signal and limit systemic exposure of host tissues to NO.

Our first generation of C3Ds incorporated the simple 7-aminoacyl side chain structures of early-generation cephalosporins to enhance β -lactamase susceptibility.¹⁵ For example, PYRRO-cefaloram **1** derived from the 1st-generation cephalosporin cefaloram (CFL) carried the 7-phenacetyl group (Figure 2). Thus, although armed with the cephalosporanic acid scaffold, 1st-generation C3Ds were not designed to elicit direct β -lactam/PBP-mediated antibacterial effects but rather serve as drug-like, β -lactamase-triggered NO carriers capable of targeting NO to infection sites. Their use clinically would require co-administration with antibiotics to control released planktonic and remaining biofilm cells (Figure 1). In our original

report, we showed that 1st-generation C3Ds act synergistically with ciprofloxacin and tobramycin against *P. aeruginosa* biofilms *in vitro*.¹⁶ We later showed that the combination of PYRRO-cefaloram **1** and azithromycin enhances the susceptibility of nontypeable *Haemophilus influenza* biofilms co-cultured on primary ciliated respiratory epithelial cells¹⁸ and primary ciliary dyskinesia cells.¹⁹ More recently, we reported that combining a 1st-generation C3D with colistin leads to almost complete eradication of *ex-vivo P. aeruginosa* biofilms grown from CF sputum isolates.²⁰

One limitation of 1st-generation C3Ds is that they could only be used against β -lactamase-producing bacteria. It is possible that a simpler, more effective and perhaps more general antibiofilm approach might be realized if C3Ds could be optimized to show increased β -lactamase stability and enhanced reactivity towards PBPs. In theory PBP-mediated β -lactam ring opening of the C3D in this case would be expected to kill a subpopulation of biofilm bacteria and at the same time release a diazeniumdiolate anion and NO (by the same mechanism as the β -lactamase-triggered reaction), potentially initiating biofilm dispersal. β -lactam-mediated PBP inactivation (by the same C3D) could then additionally serve to control released planktonic cells (Figure 1).

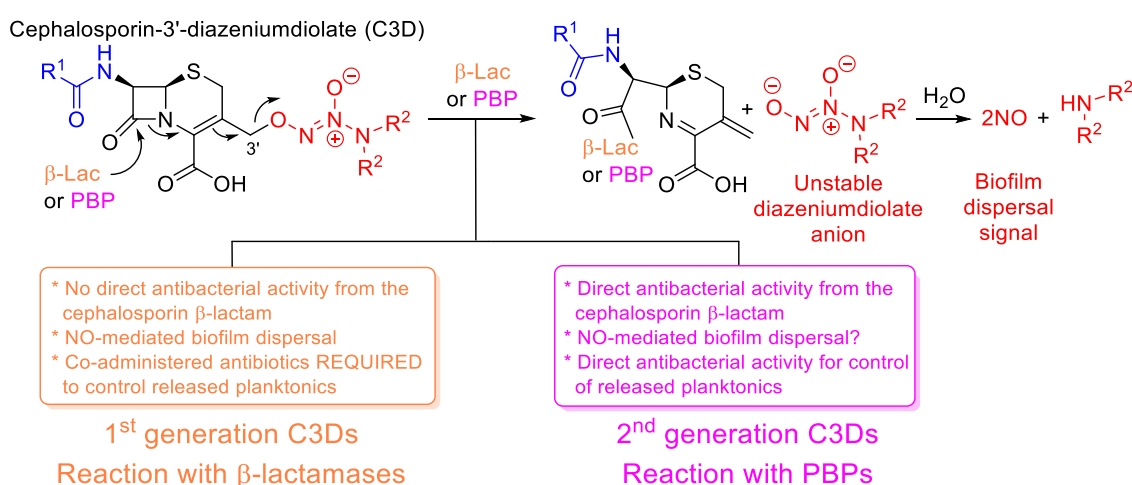


Figure 1. Proposed effects arising from 1st and 2nd generation C3Ds.

Early support for this 'all-in-one' C3D concept was obtained using PYRRO-cefaloram **1**, which showed potent β -lactam-mediated antibacterial activity (minimum inhibitory concentration, MIC = 900 nM) against planktonic cultures of a non- β -lactamase producing strain of *Streptococcus pneumoniae*.²¹ The parent antibiotic cefaloram containing a 3'-acetate group in place of the diazeniumdiolate (Figure 2) showed the same MIC, thus demonstrating that the presence of a diazeniumdiolate at the 3'-position of a cephalosporin is compatible with classical β -lactam-mediated PBP inactivation and antibacterial effects. These results motivated the current study, which aimed to explore the 'all-in-one' C3D concept using *P. aeruginosa* CF clinical isolates and identify leads for further study as new agents for chronic respiratory infections in CF.

'All-in-one' C3Ds for CF applications would require high reactivity towards *P. aeruginosa* PBPs and low susceptibility to β -lactamases. For cephalosporins, these properties can be inferred from low MICs in liquid culture assays. Guided by these principles, we conceived a 2nd-generation of C3Ds containing 7-aminoacyl side chains corresponding to the 7-oxyimino ether groups of later-generation, extended-spectrum cephalosporins (i.e. cefuroxime (CXM), ceftazidime (CAZ), cefotaxime (CTX)/ceftriaxone (CRO)/cefepime (FEP) and cefozopran (CZO), Figure 2); structures that had been optimized in past medicinal chemistry efforts to confer high PBP reactivity and increased β -lactamase stability.¹¹ Twelve C3Ds were synthesized and screened for direct antibacterial activity against a panel of *P. aeruginosa* CF clinical isolates, along with a panel of ESKAPE²² and other human pathogens. The most active compound to emerge from the screening, AMINOPIP2-ceftazidime **12**, showed good stability against β -lactamases and activity against other human pathogens, was able to reduce *P. aeruginosa in vitro* biofilms that were resistant to the parent antibiotic (ceftazidime) and showed efficacy equivalent to ceftazidime in an acute murine *P. aeruginosa* respiratory infection model.

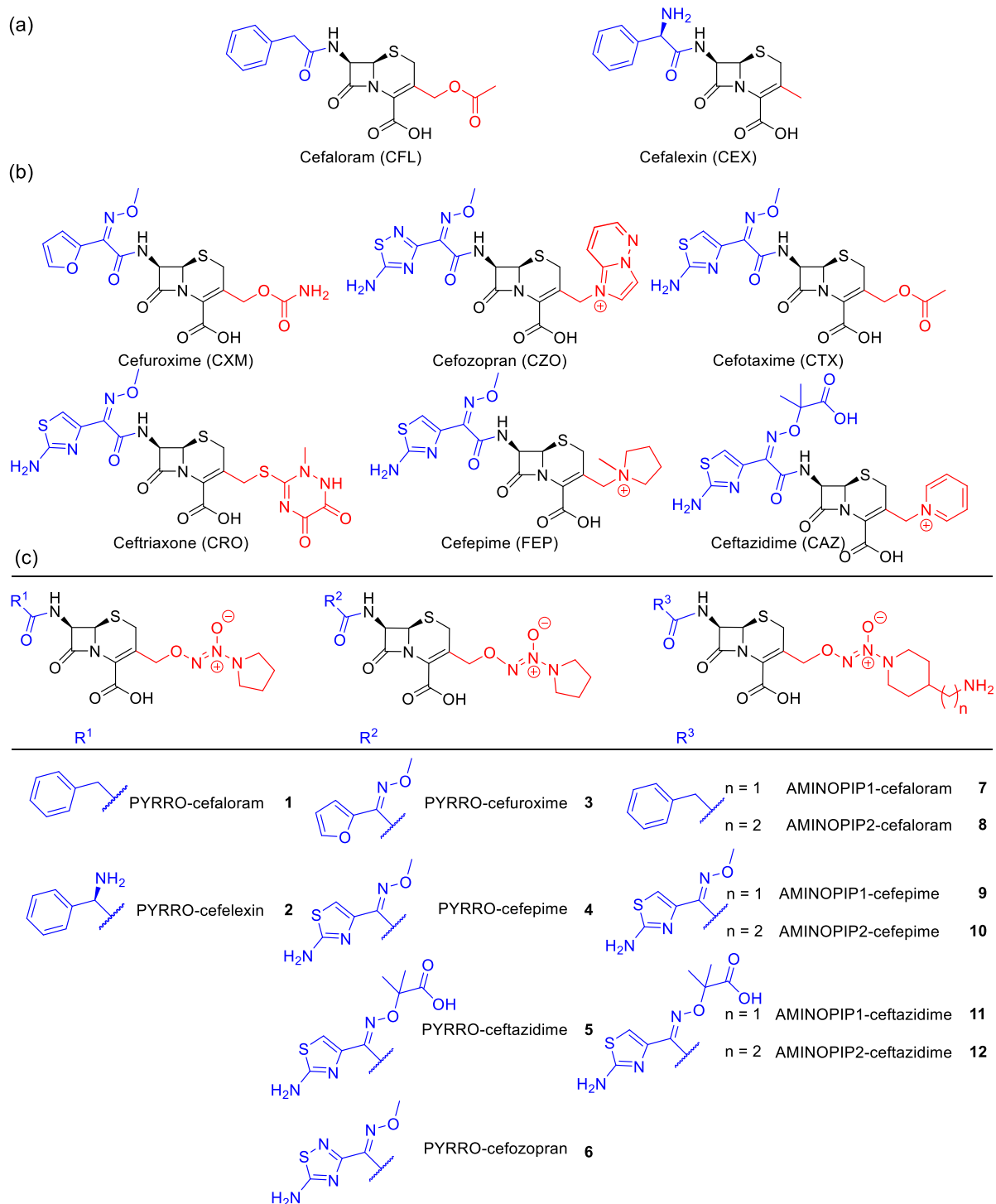


Figure 2. Compounds used in this study: (a) early generation cephalosporin antibiotics (b) later-generation oxyimino ether cephalosporin antibiotics and (c) C3Ds 1-12.

RESULTS

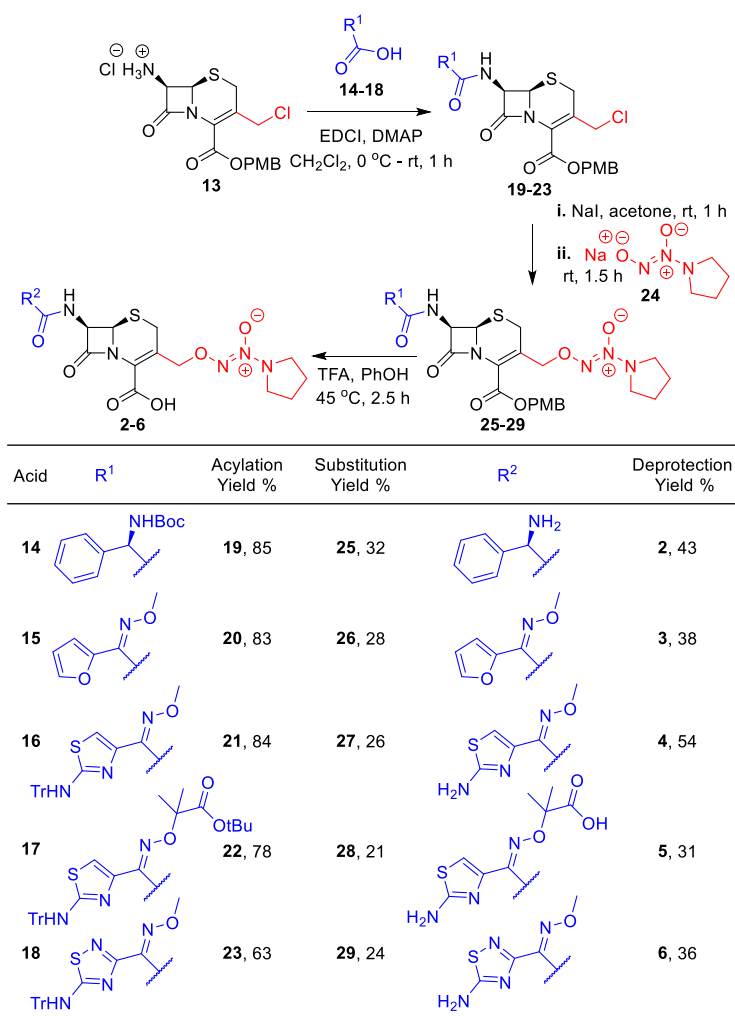
Chemistry

The first series of analogs (PYRRO-C3Ds **3-6**) carried the pyrrolidine-based diazeniumdiolate PYRRO/NO at the 3'-position. This NO donor appeared promising due to the rapid NO release properties of its anion ($t_{1/2} = 2$ s),²³ which we predict may be important *in vivo* as diffusion of an expelled diazeniumdiolate anion away from infection sites before releasing NO would presumably reduce effectiveness and elevate NO-mediated safety concerns. PYRRO-cefaloram **1** and PYRRO-cefalexin **2** were included as early-generation, non-oxyimino ether-containing C3D controls.

Compound **1** was synthesised using our published procedure.¹⁵ For compounds **4-6**, the requisite 2-aminothia(dia)zole carboxylic acid side chains were *N*-trityl protected using the known method (Scheme 1).²⁴ The resulting carboxylic acids **16-18**, along with the commercially available cephalixin and cefuroxime carboxylic acid intermediates **14** and **15**, respectively, were coupled to the commercial 7-amino-*p*-methoxybenzyl (PMB)-protected 3'-chloro-cephalosporin ester.HCl salt **13** using EDCI and DMAP. The acylation reactions were typically finished after 1 h and gave 3'-chloro-cephalosporin PMB esters **19-23** in good yields (63-85%) with minimal formation of the Δ 2-isomer.²⁵⁻²⁷

In situ Finkelstein conversion of chlorides **19-23** to iodides using NaI/acetone followed by substitution with PYRRO/NO sodium salt **24** gave the penultimate intermediates **25-29** in 21-32% yields. Our reported method for PMB deprotection of C3Ds using neat TFA/anisole^{15,16} theoretically allowed for global deprotection of the Boc, PMB, trityl and *tert*-butyl groups in all analogs. However, trial reactions using these conditions invariably produced complex mixtures. Based on work by Torii *et al*,²⁸ we investigated varying concentrations of TFA in a phenol melt as a deprotection alternative. For deprotection of **26**, where PMB was the only protecting group, molten phenol at 45 °C (without TFA) gave PYRRO-cefuroxime **3**

in 38% yield. Use of 40 molar equivalents of TFA in phenol afforded deprotection of the Boc, PMB and trityl groups of **25**, **27** and **29**, giving targets **2**, **4** and **6** in 43, 54 and 36% yields, respectively. Use of 50 equivalents of TFA in molten phenol was required to remove the additional *tert*-butyl ester in **28**, giving **5** in 31% yield.

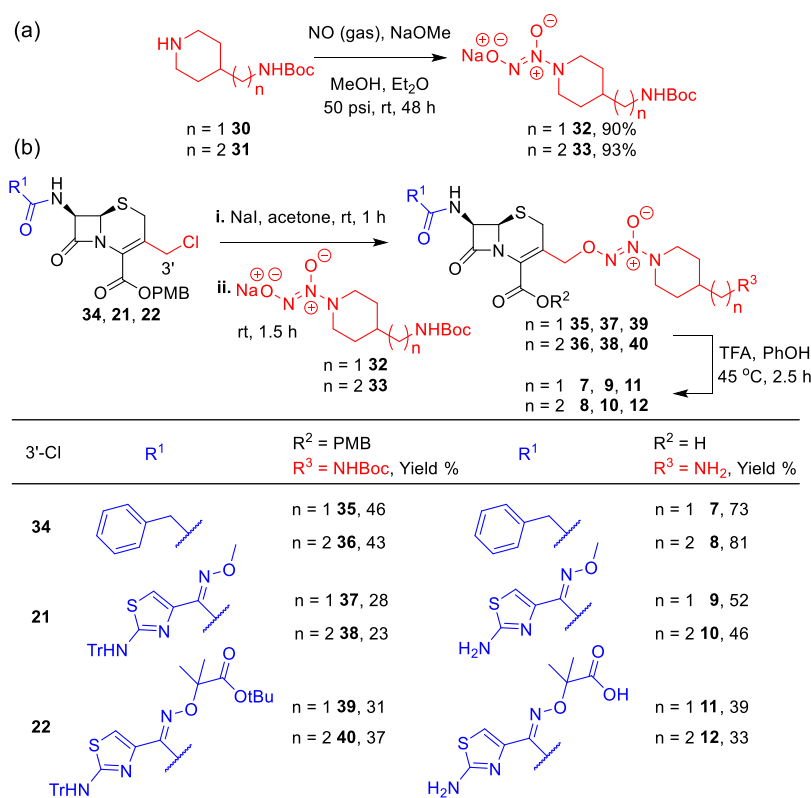


Scheme 1. Synthesis of PYRRO-C3Ds **2-6**.

Computational analysis of over 180 diverse antibacterials by Hergenrother and co-workers²⁹ revealed that basic, positively charged non-sterically hindered primary amino groups can aid penetration of antibiotics into Gram-negative cells and increase activity. Based on this, a second series of C3Ds was designed to include the oxyimino ether side chains of cefepime and ceftazidime while carrying an additional primary amino group in the diazeniumdiolate

moiety. Novel piperidine-based diazeniumdiolates (termed AMINOPIP/NO) were chosen for use due to their (predicted) short NO release half-lives and because non-chiral variants carrying 1- and 2-carbon linked primary alkyl amines at the piperidine 4-position could be prepared from commercially available *N*-Boc-4-alkylamino piperidine precursors (**30** and **31**). The target C3Ds, termed AMINOPIP1- and AMINOPIP2-C3Ds (depending on the number of methylene groups in the 4-alkylamino chain), included two cefepime-based analogs (AMINOPIP1-cefepime **9** and AMINOPIP2-cefepime **10**) and two ceftazidime-based analogs (AMINOPIP1-ceftazidime **11** and AMINOPIP2-ceftazidime **12**). AMINOPIP1-cefaloram **7** and AMINOPIP2-cefaloram **8** were prepared as 1st-generation (i.e. non-oxyimino ether-containing) AMINOPIP-C3D controls (Scheme 2).

To synthesize the required sodium diazeniumdiolate reagents **32** and **33**, *N*-Boc-4-alkylamino piperidines **30** and **31** were dissolved in MeOH/Et₂O mixtures containing sodium methoxide in a Paar-Knorr vessel and exposed to NO gas at 50 psi. Shaking at room temperature for 48 h delivered *N*-Boc-AMINOPIP1/NO **32** and *N*-Boc-AMINOPIP2/NO **33** in 90% and 93% yields, respectively (Scheme 2(a)).³⁰ Reactions of **32** and **33** with protected 3'-chloro-cephalosporin intermediates **21**, **22** and **34** using the standard alkylation procedure (see above) gave the penultimate C3D precursors **35-40** in 23-46% yields (Scheme 2(b)). Stirring **35** and **36** with 20 molar equivalents of TFA in molten phenol at 45 °C gave C3Ds **7** and **8** in 73% and 81% yields, respectively. Deprotection of **37** and **38** with 40 equivalents of TFA afforded **9** and **10** in 52% and 46% yields, respectively. Use of 50 equivalents of TFA was required to remove the *tert*-butyl, *N*-trityl, PMB and Boc groups from **39** and **40**, giving C3Ds **11** and **12** in 39% and 33% yields, respectively. Complete synthetic chemistry details, compound characterization data and ¹H and ¹³C NMR spectra are provided in the Supporting Information. All final compounds showed >95% purity, as determined by NMR and HPLC analyses.



Scheme 2. Synthesis of: (a) sodium diazeniumdiolate reagents *N*-Boc-AMINOPIP1/NO **32** and *N*-Boc-AMINOPIP2/NO **33** and (b) AMINOPIP-C3Ds **7-12**.

Antibacterial activity against *P. aeruginosa* Clinical Cystic Fibrosis Isolates

The antibacterial activities of C3Ds **1-12** and their parent cephalosporin antibiotic comparators were measured against *P. aeruginosa* reference strain PAO1 and ten clinical isolates obtained from the expectorated sputum of CF patients (see Supporting Information Table S1 for patient and strain information). The older cephalosporin antibiotics cefaloram (CFL) and cefalexin (CEX) showed no activity against any strain (MIC >128 µg/mL) and introduction of PYRRO/NO or AMINOPIP1/NO diazeniumdiolates onto the CFL (i.e. compounds **1**, **7** and **8**) or CEX (i.e. compound **2**) scaffolds did not improve activity (Supporting Information Table S2). Similarly, little or no activity was observed against any of the strains with the early oxyimino ether antibiotic cefuroxime (CXM) or its PYRRO/NO variant **3** (Table 1). Much greater activity was observed with ceftazidime (CZO), which showed MICs mostly in the range 1-16 µg/mL. However, introduction of the PYRRO/NO group onto

the CZO scaffold (i.e. compound **6**) caused a loss of potency in multiple strains. Cefepime (FEP) showed good activity across the panel (MIC = 0.5-16 µg/mL) but again introduction of the PYRRO/NO **4**, AMINOPIP1/NO **9** and AMINOPIP2/NO **10** diazeniumdiolates onto the FEP scaffold reduced potency. Similarly, ceftazidime (CAZ) was active against most strains but switching its 3'-pyridinium group to PYRRO/NO **5** reduced potency. In contrast, replacing the group with AMINOPIP1/NO and AMINOPIP2/NO diazeniumdiolates (compounds **11** and **12**) either maintained or improved potency (MIC = 0.5-16 µg/mL) against PAO1 and nine out of the ten clinical strains (relative to CAZ). In general, the strain-specific activities of **11** and **12** paralleled those of CAZ, where strains with higher resistance to CAZ also showed increased MICs against the C3Ds, but the losses in potency for the two C3Ds were lower (e.g. PA26 and PA37).

Table 1. Minimum inhibitory concentration (MIC) of oxyimino ether cephalosporin antibiotics cefuroxime (CXM), ceftazidime (CAZ), cefepime (FEP) and ceftazidime (CAZ) and their C3D variants **3-6** and **9-12** against *P. aeruginosa* lab strain PAO1 and ten CF clinical isolates.

Strain	MIC (µg/mL)											
	CXM	3	CZO	6	FEP	4	9	10	CAZ	5	11	12
PAO1	>128	>128	1	32	0.5	32	4	4	1	32	1	1
PA05	64	>128	8	4	4	4	8	8	1	2	1	1
PA10	128	>128	4	8	2	8	8	8	1	4	0.5	0.5
PA21	>128	>128	8	128	4	>128	64	128	16	64	16	8
PA26	>128	>128	16	>128	4	>128	32	128	64	>128	8	16
PA30	>128	>128	8	>128	4	128	32	32	4	128	4	4
PA37	>128	>128	16	16	4	32	16	32	128	>128	16	16
PA44	>128	>128	>128	>128	16	128	128	128	4	128	16	16
PA56	>128	>128	8	128	4	128	16	32	4	64	2	4
PA58	>128	>128	4	128	8	>128	64	128	16	128	4	8
PA68	>128	>128	8	>128	4	128	32	32	4	64	4	4

To inform structure-activity conclusions, the strains from the *P. aeruginosa* clinical isolate panel were characterised for β-lactamase activity (relative to PAO1) using a nitrocefin assay.³¹ All strains tested positive for β-lactamase activity, with PA5, PA30, PA44, PA56 and PA58 showing similar or slightly lower activity than PAO1 (Supporting Information Figure

S1). Despite strains PA10, PA21, PA26, PA37 and PA58 all showing several-fold higher β -lactamase activity (than PAO1), these increases had only a small effect on the antibacterial potency of **11** and **12**, suggesting the compounds show good stability against *P. aeruginosa* β -lactamases.

The β -lactamase stability of C3Ds **1** and **4-12** was examined using our reported amperometric method^{15,16} with two commercial enzymes; one from the Gram-positive bacterium *Bacillus cereus* and one from *P. aeruginosa*. As this technique directly measures NO concentrations in solution, these assays additionally served to confirm NO release from the compounds following enzyme-mediated β -lactam ring opening.

Addition to buffer solutions (PBS, pH 7.4) produced an immediate NO signal for some C3Ds, presumably due to a small amount of aqueous β -lactam hydrolysis and accompanying diazeniumdiolate release occurring upon dissolution (Figure 3). Addition of the first aliquot of β -lactamase produced a strong NO signal from susceptible C3Ds, reaching a plateau (steady-state) several minutes later when the rates of NO production and loss become equal. Further increases in NO were observed after adding a second aliquot of β -lactamase, indicating that exhaustion of C3D molecules had not occurred during the first β -lactamase addition.

As expected, 1st-generation C3D PYRRO-cefaloram **1** strongly released NO upon exposure to both *B. cereus* and *P. aeruginosa* β -lactamases, thus providing a benchmark for stability comparisons (Figure 3a). Introduction of methoxyimino ether side chains (PYRRO-cefepime **4** and PYRRO-cefzopran **6**) significantly increased β -lactamase stability (relative to **1**), while addition of the 2-methyl propionic acid moiety (PYRRO-ceftazidime **5**) afforded almost complete stability towards both enzymes. AMINOPIP2-cefaloram **8** underwent rapid hydrolysis in the presence of the *B. cereus* enzyme, while AMINOPIP1-cefaloram **7** and the AMINOPIP-cefepimes **9** and **10** were slightly more stable. Both AMINOPIP-ceftazidimes **11** and **12** were completely stable in the presence of this enzyme. AMINOPIP-cefalorams **7** and **8**

were highly susceptible to the *P. aeruginosa* β -lactamase but the four oxyimino ether AMINOPIP-C3Ds **9-12** all showed lower susceptibility. The generally higher β -lactamase stability of ceftazidime-based C3Ds **5**, **11** and **12** relative to other analogs aligns with the higher stability of ceftazidime compared to cephalosporins lacking the 2-methyl propionic acid oxyimino ether group.³²

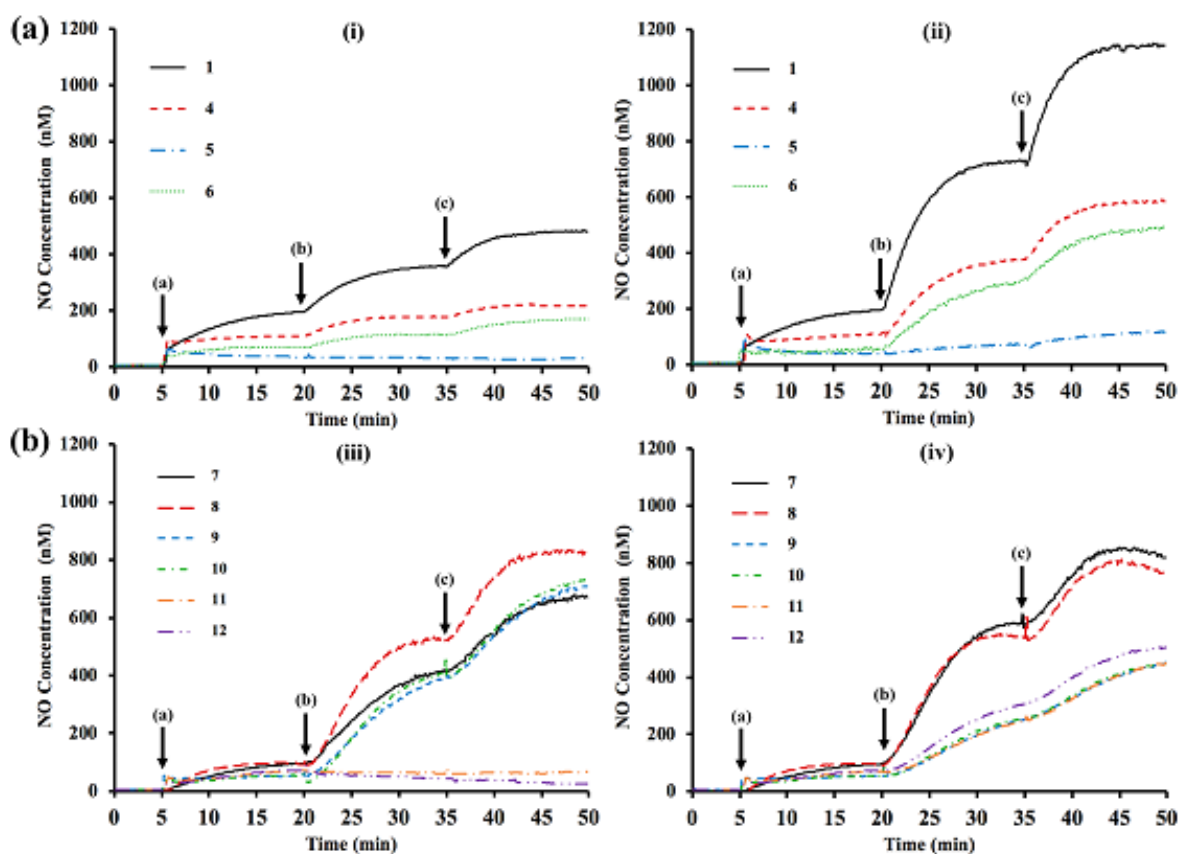


Figure 3. (a) Stability of PYRRO-C3Ds **1, 4-6** in the presence of β -lactamases from: (i) *B. cereus* and (ii) *P. aeruginosa*. (b) Stability of AMINOPIP-C3Ds **7-12** in the presence of β -lactamases from: (iii) *B. cereus* and (iv) *P. aeruginosa*. Arrows indicate addition of the following to a reaction vessel containing 20 mL PBS (pH 7.4) at 25 °C: a. C3Ds **1, 4-12** (final concentration 50 μ M), b. 100 μ L of 40 U.mL⁻¹ *B. cereus* β -lactamase or 25 μ L of 40 U.mL⁻¹ *P. aeruginosa* β -lactamase. Multiple trials of each compound were performed and a representative curve is presented for each.

Spectrum of antibacterial activity and eukaryotic cell cytotoxicity

C3Ds **1-12** were screened for activity against the major human pathogens *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *Mycobacterium tuberculosis*, *Neisseria Gonorrhoeae* and *S. pneumoniae* (Supporting Information Table S4). The MICs were obtained from multiple international laboratories using standard broth microdilution methods performed in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines.³³ The MIC of the parent cephalosporin antibiotic was included for comparison, where available.

PYRRO-C3D controls **1** and **2** showed little or no activity against multiple *E. coli* strains and PYRRO-cefuroxime **3** showed similarly poor activity. Introduction of aminothi(dia)zole oxyimino ether side chains yielded a significant boost in potency, with PYRRO-cefepime **4**, PYRRO-ceftazidime **5** and PYRRO-cefzopran **6** all showing much lower MICs than **1** and **2** against *E. coli*, including several below 1 µg/mL. While AMINOPIP1-cefaloram **7** and AMINOPIP2-cefaloram **8** both showed poor activity, their oxyimino ether counterparts **9-12** showed good potency across most strains. In general, active C3Ds showed MICs similar to their parent cephalosporin antibiotics against *E. coli*. In the case of AMINOPIP1-ceftazidime **11**, 8-16 fold higher activity was observed relative to ceftazidime. Collectively, these findings indicate that aminothia(dia)zole oxyimino ether side chains and AMINOPIP/NO donors confer C3Ds with the highest activity against *E. coli*.

C3Ds **1-12** showed moderate potency (MIC ≤ 16 µg/mL) against some strains of *S. aureus* and poor activity against others, with no clear structure-activity trends evident. For *K. pneumoniae*, all analogs were active against a strain that was sensitive to cephalosporin antibiotics (KP1). Oxyimino ether analogs **4-6** and **9-12** showed moderate potency against one other strain (KP3) but were poorly active against four others. Low potency was seen for all C3Ds against *A. baumannii*, consistent with the low activity of the parent cephalosporin antibiotics tested against this species. Six strains of *E. faecalis* were mostly resistant to C3Ds with the exception of AMINOPIP1-cefepime **9** and AMINOPIP2-cefepime **10**, which showed good potency (MIC = 2 µg/mL) against the EF1 strain. None of the compounds showed activity against *M. tuberculosis*.

PYRRO-C3Ds **1-5** showed low activity against *N. gonorrhoeae* but the activity of PYRRO-cefzopran **6** was markedly higher (MIC = 1.25 µg/mL). AMINOPIP-C3Ds **9-12** were more active than their PYRRO-C3Ds variants and AMINOPIP2-ceftazidime **12** (MIC = 0.6 µg/mL) showed only 2-fold lower activity than ceftriaxone (CRO, MIC = 0.3 µg/mL), the

standard-of-care cephalosporin used to treat gonorrhoea.³⁴ This represents a 67-fold increase in potency for AMINOPIP2-ceftazidime **12** relative to its parent antibiotic ceftazidime. The three *S. pneumoniae* strains tested were all highly sensitive to C3Ds, with AMINOPIP2-cefepime **10** showing excellent potency (MIC = 0.06 µg/mL).

C3Ds **1-12** were screened against seven additional *P. aeruginosa* lab strains held by the collaborating laboratories (Supporting Information Table S4). PYRRO-C3Ds **1-6** showed little or no activity across these strains and similarly poor activity was observed with AMINOPIP1-cefaloram **7** and AMINOPIP2-cefaloram **8**. Introduction of aminothiazole oxyimino ether side chains and AMINOPIP/NO diazeniumdiolates greatly enhanced activity, with AMINOPIP1-cefepime **9**, AMINOPIP2-cefepime **10**, AMINOPIP1-ceftazidime **11** and AMINOPIP2-ceftazidime **12** all showing equivalent or lower MICs than their parent antibiotics against some strains. In agreement with the clinical isolate testing (Table 1), AMINOPIP-ceftazidimes **11** and **12** appeared as the most active C3Ds against *P. aeruginosa*, returning MICs in the range 1-8 µg/mL against four of the seven strains.

When tested for cytotoxicity in HEK293 cells, compounds **3-12** showed no evidence of growth inhibition at 32 µg/mL (Supporting Information Table S5). AMINOPIP-ceftazidimes **11** and **12** were also tested for haemolysis of human red blood cells and neither showed any effect at 32 µg/mL.

Activity of **12 against *P. aeruginosa* ex-vivo biofilms grown from cystic fibrosis clinical isolates**

Having shown good antibacterial potency against the panel of *P. aeruginosa* clinical strains, broader activity against other human pathogens, good stability against β-lactamases and low eukaryotic cell cytotoxicity, AMINOPIP2-ceftazidime **12** was advanced to testing against *P. aeruginosa* CF clinical isolate biofilms. Preliminary screening of the isolate panel

using crystal violet assays³⁵ led to selection of PA30 and PA68 for the experiments after confirming that they reproducibly form *ex-vivo* biofilms and their biofilms disperse in response to low micromolar concentrations of the spontaneous NO donor sodium nitroprusside (SNP, Supporting Information Figure S2).

Confocal laser scanning microscopy (CLSM) experiments were used to compare the effects of AMINOPIP2-ceftazidime **12** and its parent antibiotic (ceftazidime) on PA30 and PA68 biofilms. Biofilms were grown from the two strains in 35 mm glass-bottom microwell plates and treated with compounds. Following incubation, the supernatant was removed and the remaining biofilms treated with fluorescent dyes that label cells as living (SYTO9, green) or dead (propidium iodide, red). Fluorescence images were obtained and processed using COMSTAT software to characterize treated biofilms.

Treatment of PA30 biofilms with ceftazidime (128 µg/mL) showed an unexpected effect, where statistically significant increases relative to untreated controls were observed in three out of four biofilm readouts (i.e. Total Biomass, Thickness Distribution and Total Surface Area Coverage but not Maximum Thickness; Figure 4(b)). No significant decreases were seen between controls and AMINOPIP2-ceftazidime **12** (128 µg/mL), however, all four biofilm readouts were lower relative to ceftazidime. Treatment of PA68 biofilms with **12** (Figure 4(d)) yielded a greater dispersion effect, where statistically significant reductions were seen in all four readouts for **12** relative to controls, whereas no differences were observed for ceftazidime relative to controls. Significant reductions were also evident in all biofilm readouts for **12** relative to ceftazidime, except Maximum Thickness.

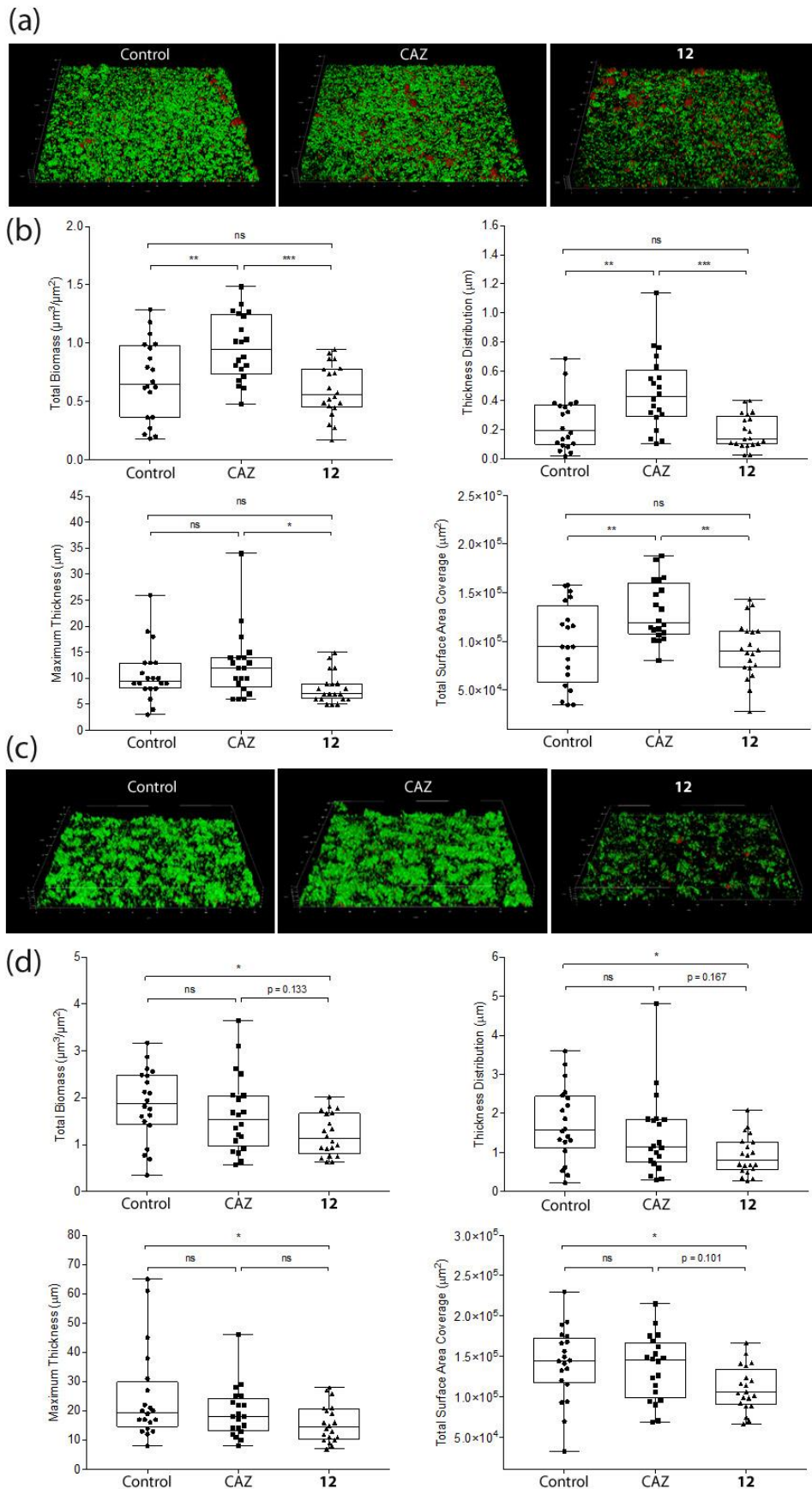


Figure 4. Activity of AMINOPIP2-ceftazidime **12** and ceftazidime (CAZ) against *P. aeruginosa* PA30 (a and b) and PA68 (c and d) biofilms. Biofilms were grown for 24 h and treated with compounds (128 $\mu\text{g}/\text{mL}$, 3 h) before staining remaining biofilms with SYTO9

(green; alive) and propidium iodide (red; dead). CLSM with COMSTAT analysis was performed to image and quantify: Total Biomass, Thickness Distribution, Maximum Thickness and Total Surface Area Coverage. Representative 3D CLSM images obtained after compound treatments are shown for: *P. aeruginosa* (a) PA30 and (c) PA68 biofilms. Error bars represent the mean \pm SEM. One-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis comparing compound treatment groups to controls and to each other. ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Activity of 12 in an acute murine *P. aeruginosa* respiratory infection model

The efficacy of AMINOPIP2-ceftazidime **12** was examined alongside ceftazidime in an acute *P. aeruginosa* lung infection model in neutropenic mice. In preparing for the model, a dose-escalation study was carried out (in mice) with **12** to investigate its tolerability. AMINOPIP2-ceftazidime **12** did not produce any adverse effects 30 minutes or 1 hour after a single subcutaneous (SC) injection of 50, 100 or 300 mg/kg (Supporting Information Table S6). All treated animals survived (Supporting Information Table S8) and showed no significant body weight changes over a 72 hour observation period (Supporting Information Table S7).

The pharmacokinetics of AMINOPIP2-ceftazidime **12** in mice were examined by monitoring concentrations of the drug in plasma and lung tissue homogenates over time following a single SC injection of 75 mg/kg. A mean peak plasma concentration of 104 $\mu\text{g/mL}$ was observed after 15 min, with levels lowering to 8 $\mu\text{g/mL}$ after 1 hour (Supporting Information Figure S3) and the compound still detectable in plasma after 6 hours (66 ng/mL). A peak lung concentration of 35 $\mu\text{g/g}$ was evident 15 min post-injection and after 1 hour the concentration was 4 $\mu\text{g/g}$. At the 6 hour mark the lung concentration was 236 ng/g. Interestingly, the lung:plasma ratio remained steady (0.26-0.33) until 45 min after injection but rose dramatically after 1 hour (0.49) and increased further with time (2.0-4.5, Supporting Information Table S9), indicating **12** is cleared more slowly from the lungs than the plasma.

The MIC of ceftazidime and AMINOPIP2-ceftazidime **12** against the *P. aeruginosa* strain used in the infection model (FDA-CDC AR-BANK#0264) was measured at 1 $\mu\text{g/mL}$

and 4 µg/mL, respectively, immediately prior to the experiment. Mice were inoculated intranasally with *P. aeruginosa* (9.1×10^5 CFU/mouse) and treatment commenced 2 hours later. Treatment consisted of subcutaneous injection of compounds at 50 mg/kg and 120 mg/kg every 4 hours for 24 hours (6 injections in total). The lungs were harvested and the bacterial burden measured. Two hours after inoculation the lung burden was 5.54 ± 0.12 log CFU/g in the vehicle control group, increasing to 9.50 ± 0.17 log CFU/g at the end of the 26 h period (Figure 5). Mice treated with 50 mg/kg ceftazidime showed a statistically significant reduction in lung bacteria at the end of the experiment (7.57 ± 0.39 log CFU/g) and the effect was increased at 120 mg/kg (6.40 ± 0.24 log CFU/g). Treatment with AMINOPIP2-ceftazidime **12** also significantly reduced bacteria at 50 mg/kg (8.06 ± 0.21 log CFU/g) and 120 mg/kg (7.22 ± 0.23). No statistically significant difference was observed between ceftazidime and AMINOPIP2-ceftazidime **12** treated mice at either dose.

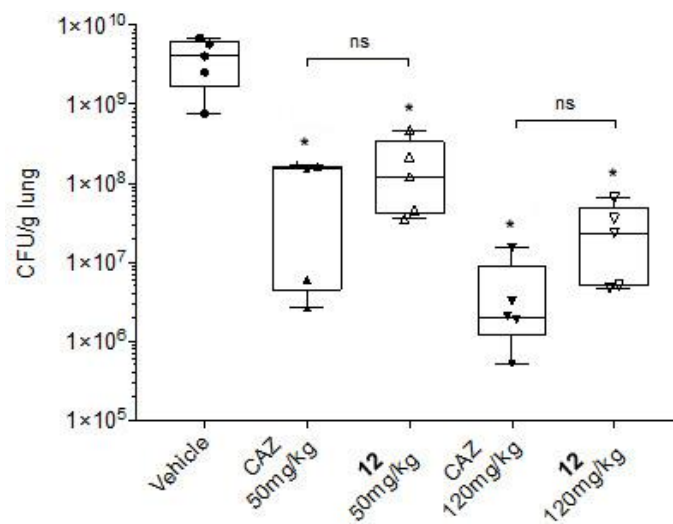


Figure 5. Effects of ceftazidime (CAZ) and AMINOPIP2-ceftazidime **12** in an acute *P. aeruginosa* lung infection model in neutropenic mice. Animals were inoculated intranasally (0.02 mL/lung) with 9.1×10^5 CFU per mouse. Ceftazidime and AMINOPIP2-ceftazidime **12** (50 and 120 mg/kg; $n = 5$ for each cohort) were administered SC every 4 hours starting 2 hours after infection. At 26 hours after inoculation, lung tissues were harvested and weighed and the lung bacterial burden measured (CFU/g). Error bars represent the mean \pm SEM. *Significant difference ($p < 0.05$) compared to the vehicle control was determined by one-way ANOVA followed by Dunnett's test. ns = not significant.

DISCUSSION AND CONCLUSIONS

In this study, we designed and synthesized a library of 2nd-generation C3Ds carrying 7-aminoacyl side chain structures corresponding to the oxyimino ether groups of later generation cephalosporin antibiotics; cefuroxime, ceftazidime, cefepime and ceftazopran. It was postulated that introduction of these groups, which had previously been optimized during historical medicinal chemistry campaigns, would confer C3Ds with increased β -lactamase stability and high PBP reactivity, and therefore potentially broad spectrum antibacterial activity. We posited that reaction of the new C3Ds with PBPs might not only kill biofilm bacteria but also trigger dispersal of remaining biofilm cells due to the resulting NO signal. If the same molecule were then also able to kill released planktonic cells, C3Ds could represent ‘all-in-one’ antibiofilm antibiotics for use in chronic infections.

P. aeruginosa was the primary organism of interest as it is the cause of chronic respiratory infections in CF; the most well-studied of all biofilm-mediated chronic infections and logical target indication for C3D clinical development. Testing of the library against a panel of *P. aeruginosa* CF clinical isolates showed that oxyimino ether side chains alone are not sufficient to confer C3Ds with good antibacterial potency against *P. aeruginosa* since PYRRO-cefepime **4**, PYRRO-ceftazidime **5** and PYRRO-ceftazopran **6** all showed significantly reduced potency relative to their parent cephalosporin controls. While PYRRO-ceftazidime **5** showed excellent stability towards β -lactamases (Figure 3), this did not translate into potent antibacterial activity across the panel. We speculated that poor penetration of C3Ds across Gram-negative cell walls might instead be responsible. Based on Hergenrother and co-workers study showing that positively charged, non-sterically hindered primary amino groups can aid antibiotic penetration across Gram-negative cell walls,²⁹ we added amines to C3Ds by replacing the PYRRO/NO diazeniumdiolates at the 3'-position with AMINOPIP/NO donors. Switching to this group on cefaloram (AMINOPIP-cefalorams **7** and **8**) had no effect as both

compounds, like PYRRO-cefaloram **1**, were totally inactive across the panel. Given that all of the *P. aeruginosa* clinical isolates expressed β -lactamase activity (Supporting Information Figure S1) and compounds **1**, **7**, and **8** showed high β -lactamase susceptibility (Figure 3), it is likely their poor activity was due to β -lactamase degradation. Although NO would be expected to be released from these compounds after reactions with β -lactamases, our previous work with *S. pneumoniae* suggests that NO delivered from C3Ds is unlikely to generate a direct antibacterial effect.²¹

AMINOPIP-C3Ds **9** and **10** containing the aminothiazole methoxyimino ether side chain of cefepime showed slightly better activity across the panel than their matched PYRRO-C3D counterpart **4**, but they showed poor activity relative to cefepime. In contrast, AMINOPIP1-ceftazidime **11** and AMINOPIP2-cetazidime **12** both showed much greater activity than PYRRO-ceftazidime **5**, along with superior activity to the parent ceftazidime. We conclude that potent antibacterial activity in 2nd-generation C3Ds against *P. aeruginosa* requires a suitable balance between PBP reactivity, β -lactamase susceptibility and cell wall penetration, with these parameters appearing to converge in structures **11** and **12**.

Spectrum of activity testing revealed excellent potency for many of the C3Ds against *E. coli* and *S. pneumoniae*. This, along with the proven capacity of NO to disperse *E. coli* biofilms,³⁶ motivates further exploration of C3Ds as possible treatments for chronic *E. coli* (e.g. urinary tract) infections. We previously reported that despite showing good antibacterial potency against *S. pneumoniae*, 1st-generation C3Ds alone are unable to disperse *S. pneumoniae* biofilms.²¹ This was in agreement with a separate report showing that NO modulates metabolic activity, but not dispersal, in *S. pneumoniae* biofilms.³⁷ Thus, further exploration of 2nd-generation C3Ds against *S. pneumoniae* biofilms would appear to not be warranted. AMINOPIP2-ceftazidime **12** showed excellent activity against *N. gonorrhoeae* (MIC = 0.6 μ g/mL), suggesting further study of C3Ds against biofilms and chronic infections

caused by this organism might yield interesting findings. Recent evidence showing that *N. gonorrhoeae* aggregates (similar to biofilms) are more resistant to ceftriaxone than planktonic cells,³⁸ along with knowledge that NO plays a role in *N. gonorrhoeae* biofilm biology,³⁹ support these studies.

To advance the ‘all-in-one’ hypothesis in *P. aeruginosa*, we tested the most active C3D AMINOPIP2-ceftazidime **12** for *in vitro* antibiofilm effects alongside ceftazidime. Preliminary screening of the *P. aeruginosa* clinical isolate panel for reproducible biofilm growth and the ability to undergo NO-mediated biofilm dispersal identified two strains suitable for the experiments, PA30 and PA68. Compound **12** showed the same MIC as ceftazidime against both strains (4 µg/mL) but only **12** produced statistically significant biofilm reductions in the PA68 strain relative to untreated controls. Ceftazidime was indeed found to enhance biofilm growth in the PA30 strain. Together, these results suggest that NO released from **12** after reaction with PBPs might be responsible for the biofilm reduction, which would support the ‘all-in-one’ C3D concept in *P. aeruginosa*. However, further experiments are required to conclusively demonstrate this.

To complete the study, we tested AMINOPIP2-ceftazidime **12** alongside ceftazidime in an acute *P. aeruginosa* respiratory infection model. Lead up experiments supported the safety of **12**, where no signs of eukaryotic cell cytotoxicity were evident at 32 µg/mL and no adverse effects, deaths or weight loss were observed in mice following SC administration at high dose (300 mg/kg). Pharmacokinetic studies revealed that the plasma levels of **12** in mice remained above 10 µg/mL for about 1 h post-SC dosing at 75 mg/kg, while the levels in lung homogenates remained above 10 µg/g for around 45 min. In terms of treating a respiratory infection, a positive feature was noted where clearance of **12** from the lung was found to be slower than the plasma. Based on these experiments, *P. aeruginosa*-infected mice were dosed with **12** at 50 and 120 mg/kg SC every 4 h for 24 h. Despite AMINOPIP2-ceftazidime **12**

showing 4-fold lower *in vitro* antibacterial potency against the *P. aeruginosa* strain used in the model, **12** was able to deliver reductions in bacterial lung burden that were equivalent to ceftazidime. While not a chronic or biofilm infection model, this finding demonstrates that C3Ds can produce *in vivo* efficacy equivalent to a standard-of-care antipseudomonal cephalosporin. Collectively, results from this study support further evaluation of **12** as a potential new antipseudomonal for chronic lung infections in CF and motivate broader study of ‘all-in-one’ C3Ds against other chronic infections.

METHODS

Chemistry - General

Carboxylic acids **14** and **15** were purchased from Sigma Aldrich. Carboxylic acid **16** was prepared by *N*-tritylation of the commercially available aminothiazole precursor (Sigma Aldrich),⁴⁰ as were **17** and **18** (AK Scientific).⁴¹ Cephalosporin hydrochloride salt **13** was purchased from AK Scientific. Sodium iodide, EDCI, trityl chloride and phenol were purchased from Sigma Aldrich. TFA was purchased from Auspep. DMAP was purchased from Alfa-Aesar. Piperidines **30** and **31** were purchased from Combi-Blocks. Nitric oxide gas was purchased from Asia Pacific Gas Enterprise Company Ltd. Anhydrous acetone was prepared by heating analytical reagent (AR) grade solvent at reflux overnight with Drierite granules (anhydrous CaSO₄) before distilling onto 4Å molecular sieves (pre-dried overnight at 400 °C) under N₂. Anhydrous methanol was prepared by distilling analytical reagent (AR) grade solvent onto 3Å molecular sieves (pre-dried overnight at 400 °C) under N₂. Anhydrous ether was prepared by distillation from sodium/benzophenone ketyl. The term petroleum spirit (pet. spirit) refers to petroleum spirit within the boiling range 40-60 °C. Analytical thin layer chromatography (TLC) was performed using SiliaPlate Aluminium Backed F₂₅₄, 200 µm plates and SiliaPlate Aluminium Backed C18 F₂₅₄, 150 µm plates from Silicycle. Normal phase

column chromatography was performed using SiliaFlash P60, 40-63 μm , 230-400 mesh silica gel. Reverse phase column chromatography was performed using SiliaBond C18 (carbon 17 %), 60 \AA , 40-63 μm , 230-400 mesh silica gel from Silicycle. Centrifugation was performed using a REMI R-8C BL centrifuge. ^1H and ^{13}C NMR spectra were recorded on Bruker 400 and 500 MHz spectrometers. Chemical shifts (δ) are relative to tetramethylsilane (TMS, 0 ppm). High resolution electrospray mass spectra were recorded on a Waters XEVO Ultima spectrometer. The final target compounds were lyophilised using a CHRIST Alpha 1-2 LD freeze dryer at $-53\text{ }^\circ\text{C}$ and 0.15 mbar. Compound **1** was prepared according to the reported method.¹⁵

General Procedure I (Compounds 19-23)

To a stirred solution of **14-18** (1 eq.), **13** (1 eq.) and 4-*N,N*-dimethylaminopyridine (DMAP) (0.5 eq.) in CH_2Cl_2 (15 mL) at $0\text{ }^\circ\text{C}$ was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (1.1 eq.) in small portions and the resulting mixture was stirred for 1 h. The reaction mixture was diluted with CH_2Cl_2 (150 mL) and washed with water (100 mL) and brine (50 mL). The organic fraction was dried over anhydrous MgSO_4 , filtered and evaporated to give a yellow residue that was purified by silica gel column chromatography affording the acylated products.

4-methoxybenzyl-7-((R)-2-((tert-butoxycarbonyl)amino)-2-phenylacetamido)-3-(chloromethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate 19

Compound **19** was prepared using General Procedure I from **14** (2.00 g, 7.96 mmol), **13** (3.23 g, 7.96 mmol), EDCI (1.36 g, 8.76 mmol) and DMAP (0.486 g, 3.98 mmol) to give **19** as a pale yellow solid (4.07 g, 85%). ^1H NMR (CDCl_3 , 400 MHz) δ 7.36-7.30 (m, 7H), 6.89 (d, 2H, $J = 8.7\text{ Hz}$), 6.74 (bs, 1H), 5.80 (dd, 1H, $J = 9.2\text{ Hz}$, $J = 4.9\text{ Hz}$), 5.66 (d, 1H, $J = 6.2\text{ Hz}$), 5.24-5.21 (m, 1H, overlapped), 5.23 (d, 1H, $J = 11.8\text{ Hz}$), 5.18 (d, 1H, $J = 11.8\text{ Hz}$), 4.89

(d, 1H, $J = 4.9$ Hz), 4.48 (d, 1H, $J = 11.8$ Hz), 4.37 (d, 1H, $J = 11.8$ Hz), 3.80 (s, 3H), 3.53 (d, 1H, $J = 18.3$ Hz), 3.35 (d, 1H, $J = 18.3$ Hz), 1.41 (s, 9H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 170.7, 164.2, 161.0, 160.0, 154.9, 137.3, 130.7, 129.2, 128.7, 127.4, 126.6, 126.1, 125.6, 114.0, 80.5, 68.2, 59.2, 58.8, 57.7, 55.3, 43.2, 28.3, 27.1.

4-methoxybenzyl(6R,7R)-3-(chloromethyl)-7-((Z)-2-(furan-2-yl)-2-(methoxyimino)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate **20**

Compound **20** was prepared using General Procedure I from **15** (2.00 g, 11.8 mmol), **13** (4.79 g, 11.8 mmol), EDCI (2.02 g, 13.0 mmol) and DMAP (0.722 g, 5.91 mmol) to give **20** as a pale yellow solid (5.10 g, 83%). ^1H NMR (CDCl_3 , 500 MHz) δ 7.60 (d, 1H, $J = 1.9$ Hz), 7.40 (d, 2H, $J = 8.4$ Hz), 7.21 (d, 1H, $J = 8.9$ Hz), 6.95 (m, 3H), 6.50 (dd, 1H, $J = 3.50$ Hz, $J = 1.5$ Hz), 5.95 (dd, 1H, $J = 8.9$ Hz, $J = 5.0$ Hz), 5.25 (d, 1H, $J = 12.5$ Hz), 5.22 (d, 1H, $J = 12.5$ Hz), 5.06 (d, 1H, $J = 5.0$ Hz), 4.54 (d, 1H, $J = 11.8$ Hz), 4.45 (d, 1H, $J = 11.8$ Hz), 4.10 (s, 3H), 3.82 (s, 3H), 3.68 (d, 1H, $J = 18.3$ Hz), 3.51 (d, 1H, $J = 18.3$ Hz). ^{13}C NMR (CDCl_3 , 125 MHz) δ 163.4, 161.6, 160.2, 160.1, 145.4, 144.8, 142.5, 130.8, 126.6, 126.1, 125.8, 114.9, 114.1, 111.9, 68.4, 63.7, 59.1, 57.5, 55.3, 43.2, 27.4. ESI-HRMS calcd. for 520.0940 $[\text{M}+\text{H}]^+$ $\text{C}_{23}\text{H}_{23}\text{O}_7\text{N}_3\text{SCl}$, found 520.0945.

4-methoxybenzyl-(Z)-3-(chloromethyl)-7-(2-(methoxyimino)-2-(2-(tritylamino)thiazol-4-yl)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate **21**

Compound **21** was prepared using General Procedure I from **16** (2.00 g, 4.51 mmol), **13** (1.83 g, 4.51 mmol), EDCI (0.770 g, 4.96 mmol) and DMAP (0.275 g, 2.25 mmol) to give **21** as a pale yellow solid (3.01 g, 84%). ^1H NMR (CDCl_3 , 400 MHz) δ 7.34-7.27 (m, 17H), 7.04 (s, 1H), 6.91-6.87 (m, 3H), 6.67 (s, 1H), 5.91 (dd, 1H, $J = 8.9$ Hz, $J = 4.9$ Hz), 5.23 (d, 1H, $J = 11.8$ Hz), 5.19 (d, 1H, $J = 11.8$ Hz), 5.02 (d, 1H, $J = 5.0$ Hz), 4.54 (d, 1H, $J = 11.8$ Hz), 4.42 (d, 1H, $J = 11.8$ Hz), 4.06 (s, 3H), 3.80 (s, 3H), 3.64 (d, 1H, $J = 18.4$ Hz), 3.46 (d, 1H, $J =$

18.3 Hz). ^{13}C NMR (CDCl_3 , 100 MHz) δ 168.6, 163.9, 162.3, 161.1, 160.1, 147.2, 143.2, 140.1, 130.8, 129.3, 128.3, 127.7, 126.7, 126.2, 125.8, 114.1, 113.1, 71.9, 68.4, 63.4, 59.1, 57.7, 55.4, 43.3, 27.4.

4-methoxybenzyl(6R,7R)-7-((Z)-2-(((1-(tert-butoxy)-2-methyl-1-oxopropan-2-yl)oxy)imino)-2-(2-(tritylamino)thiazol-4-yl)acetamido)-3-(chloromethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate **22**

Compound **22** was prepared using General Procedure I from **17** (2.00 g, 3.50 mmol), **13** (1.42 g, 3.50 mmol), EDCI (0.597 g, 3.84 mmol) and DMAP (0.214 g, 1.75 mmol) to give **22** as a pale yellow solid (2.52 g, 78%). ^1H NMR (CDCl_3 , 500 MHz) δ 8.21 (d, 1H, $J = 9.0$ Hz), 7.26-7.35 (m, 18H), 6.89 (d, 2H, $J = 8.5$ Hz), 6.72 (s, 1H), 5.98 (dd, 1H, $J = 8.8$ Hz, 5.0 Hz), 5.26 (d, 1H, $J = 12.0$ Hz), 5.19 (d, 1H, $J = 12.0$ Hz), 5.02 (d, 1H, $J = 5.0$ Hz), 4.53 (d, 1H, $J = 11.5$ Hz), 4.44 (d, 1H, $J = 11.5$ Hz), 3.81 (s, 3H), 3.61 (d, 1H, $J = 18.0$ Hz), 3.44 (d, 1H, $J = 18.0$ Hz), 1.63 (s, 3H), 1.59 (s, 3H), 1.41 (s, 9H). ^{13}C NMR (CDCl_3 , 125 MHz) δ 174.2, 168.2, 164.2, 163.4, 161.3, 160.0, 149.3, 143.3, 141.5, 130.8, 129.4, 128.3, 127.7, 126.8, 126.3, 125.9, 114.1, 112.6, 83.1, 82.3, 71.9, 68.3, 59.3, 57.9, 55.4, 43.5, 28.1, 27.5, 24.4, 23.8.

4-methoxybenzyl-(Z)-3-(chloromethyl)-7-(2-(methoxyimino)-2-(5-(tritylamino)-1,2,4-thiadiazol-3-yl)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate **23**

Compound **23** was prepared using General Procedure I from **18** (2.00 g, 4.50 mmol), **13** (1.82 g, 4.50 mmol), EDCI (0.768 g, 4.95 mmol) and DMAP (0.275 g, 2.25 mmol) to give **23** as a pale yellow solid (2.25 g, 63%). ^1H NMR (CDCl_3 , 400 MHz) δ 7.64 (s, 1H), 7.34-7.21 (m, 18H), 6.88 (d, 2H, $J = 8.7$ Hz), 5.94 (dd, 1H, $J = 8.8$ Hz, $J = 4.9$ Hz), 5.23 (d, 1H, $J = 11.8$ Hz), 5.18 (d, 1H, $J = 11.8$ Hz), 5.03 (d, 1H, $J = 4.9$ Hz), 4.52 (d, 1H, $J = 11.9$ Hz), 4.43 (d, 1H, $J = 11.8$ Hz), 4.12 (s, 3H), 3.80 (s, 3H), 3.63 (d, 1H, $J = 18.4$ Hz), 3.46 (d, 1H, $J = 18.3$ Hz).

¹³C NMR (CDCl₃, 100 MHz) δ 184.1, 163.9, 161.4, 161.2, 160.1, 160.0, 146.6, 142.1, 130.8, 129.3, 128.7, 128.3, 126.7, 126.1, 125.8, 114.2, 72.4, 68.4, 64.0, 59.2, 57.9, 55.4, 43.4, 27.5.

General Procedure II (Compounds 25-29, 35-40)

Sodium iodide (1 eq.) was added to a suspension of PMB-protected cephalosporin ester **19-23, 34** (1 eq.) in anhydrous acetone (10 mL) under argon and the mixture was stirred in the dark at room temperature for 1 h. Freshly prepared sodium diazeniumdiolates **24, 32, 33** (1 eq.) were added in one shot and the mixture was stirred at room temperature for a further 1.5 hrs. The solvent was removed under reduced pressure and the residue purified by silica gel column chromatography to give the desired adducts.

(Z)-2-(((7-((*R*)-2-((*tert*-butoxycarbonyl)amino)-2-phenylacetamido)-2-(((4-methoxybenzyl)oxy)carbonyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)-1-(pyrrolidin-1-yl)diazene 1-oxide **25**

Compound **25** was prepared using General Procedure II from **19** (500 mg, 0.830 mmol), NaI (124 mg, 0.830 mmol) and **24** (127 mg, 0.830 mmol) to give **25** as a yellow solid (185 mg, 32%). ¹H NMR (CDCl₃, 500 MHz) δ 7.39-7.30 (m, 7H), 6.88 (d, 2H, *J* = 8.5 Hz), 6.50 (bs, 1H), 5.79 (dd, 1H, *J* = 9.2 Hz, *J* = 4.8 Hz), 5.59 (bs, 1H), 5.24-5.14 (m, 4H), 4.89-4.84 (m, 2H), 3.81 (s, 3H), 3.50-3.45 (m, 5H), 3.37 (d, 1H, *J* = 18.6 Hz), 1.92 (m, 4H), 1.42 (s, 9H).

(Z)-2-(((6*R*,7*R*)-7-((*Z*)-2-(furan-2-yl)-2-(methoxyimino)acetamido)-2-(((4-methoxybenzyl)oxy)carbonyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)-1-(pyrrolidin-1-yl)diazene 1-oxide **26**

Compound **26** was prepared using General Procedure II from **20** (500 mg, 0.961 mmol), NaI (144 mg, 0.961 mmol) and **24** (147 mg, 0.961 mmol) to give **26** as a yellow solid (165 mg, 28%). ¹H NMR (CDCl₃, 500 MHz) δ 7.51 (d, 1H, *J* = 1.7 Hz), 7.35 (d, 2H, *J* = 8.3 Hz), 7.20

(d, 1H, $J = 9.0$ Hz), 6.91-6.89 (m, 3H), 6.48 (dd, 1H, $J = 3.5$ Hz, $J = 1.8$ Hz), 5.95 (dd, 1H, $J = 9.1$ Hz, $J = 5.0$ Hz), 5.26-5.19 (m, 3H), 5.04 (d, 1H, $J = 5.0$ Hz), 4.93 (d, 1H, $J = 14.1$ Hz), 4.09 (s, 3H), 3.82 (s, 3H), 3.68 (d, 1H, $J = 18.6$ Hz), 3.54-3.47 (m, 5H), 1.93 (t, 4H, $J = 6.5$ Hz). ^{13}C NMR (CDCl_3 , 125 MHz) δ 163.8, 161.3, 160.2, 160.0, 145.4, 144.8, 142.6, 130.7, 127.1, 126.7, 125.5, 114.9, 114.0, 111.9, 71.4, 68.2, 63.7, 59.0, 57.3, 55.3, 50.8, 26.4, 22.8. ESI-HRMS calcd. for $[\text{M}+\text{Na}]^+$ $\text{C}_{27}\text{H}_{30}\text{O}_9\text{N}_6\text{SNa}$ 637.1687, found 637.1686.

(Z)-2-((2-(((4-methoxybenzyl)oxy)carbonyl)-7-((Z)-2-(methoxyimino)-2-(2-(tritylamino)thiazol-4-yl)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)-1-(pyrrolidin-1-yl)diazene 1-oxide 27

Compound **27** was prepared using General Procedure II from **21** (500 mg, 0.629 mmol), NaI (94 mg, 0.629 mmol) and **24** (96 mg, 0.629 mmol) to give **27** as a yellow solid (145 mg, 26%). ^1H NMR (CDCl_3 , 400 MHz) δ 7.34-7.27 (m, 17H), 7.01 (s, 1H), 6.89 (d, 2H, $J = 8.7$ Hz), 6.78 (d, 1H, $J = 9.0$ Hz), 6.72 (s, 1H), 5.91 (dd, 1H, $J = 9.0$ Hz, $J = 4.9$ Hz), 5.25-5.16 (m, 3H), 5.01 (d, 1H, $J = 4.9$ Hz), 4.91 (d, 1H, $J = 14.1$ Hz), 4.07 (s, 3H), 3.81 (s, 3H), 3.59 (d, 1H, $J = 18.6$ Hz), 3.50-3.45 (m, 5H), 1.94-1.90 (m, 4H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 168.6, 163.8, 162.1, 161.3, 160.0, 147.1, 143.1, 140.8, 130.7, 129.3, 128.3, 127.7, 127.0, 126.7, 125.4, 114.0, 113.2, 71.8, 71.4, 68.1, 63.3, 58.9, 57.4, 55.3, 50.8, 26.4, 22.9. ESI-HRMS calcd. for 889.2796 $[\text{M}+\text{H}]^+$ $\text{C}_{45}\text{H}_{45}\text{N}_8\text{O}_8\text{S}_2$, found 889.2834.

(Z)-2-(((6R,7R)-7-((Z)-2-(((1-(tert-butoxy)-2-methyl-1-oxopropan-2-yl)oxy)imino)-2-(2-(tritylamino)thiazol-4-yl)acetamido)-2-(((4-methoxybenzyl)oxy)carbonyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)-1-(pyrrolidin-1-yl)diazene 1-oxide 28

Compound **28** was prepared using General Procedure II from **22** (500 mg, 0.542 mmol), NaI (81 mg, 0.542 mmol) and **24** (83 mg, 0.542 mmol) to give **28** as a yellow solid (116 mg,

21%). ¹H NMR (CDCl₃, 500 MHz) δ 8.13 (d, 1H, *J* = 9.5 Hz), 7.34-7.28 (m, 18H), 6.89 (d, 2H, *J* = 8.0 Hz), 6.72 (s, 1H), 5.98 (dd, 1H, *J* = 8.5 Hz, *J* = 5.0 Hz), 5.27-5.24 (m, 2H), 5.17 (d, 1H, *J* = 12.0 Hz), 5.00 (d, 1H, *J* = 5.0 Hz), 4.90 (d, 1H, *J* = 14.0 Hz), 3.81 (s, 3H), 3.55 (d, 1H, *J* = 18.5 Hz), 3.49-3.43 (m, 5H), 1.92 (m, 4H), 1.63 (s, 3H), 1.59 (s, 3H), 1.41 (s, 9H). ¹³C NMR (CDCl₃, 125 MHz) δ 174.0, 168.6, 164.2, 163.2, 161.6, 159.9, 148.9, 143.2, 141.2, 130.7, 129.4, 128.2, 127.6, 127.2, 126.9, 125.6, 114.0, 112.5, 83.0, 82.1, 71.5, 68.0, 59.1, 57.7, 55.3, 50.8, 28.0, 26.4, 24.2, 23.8, 22.9. ESI-HRMS calcd. for 1039.3453 [M+Na]⁺ C₅₂H₅₆O₁₀N₈S₂Na, found 1039.3455.

(Z)-2-((2-(((4-methoxybenzyl)oxy)carbonyl)-7-((*Z*)-2-(methoxyimino)-2-(5-(tritylamino)-1,2,4-thiadiazol-3-yl)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)-1-(pyrrolidin-1-yl)diazene 1-oxide **29**

Compound **29** was prepared using General Procedure II from **23** (500 mg, 0.629 mmol), NaI (94 mg, 0.629 mmol) and **24** (96 mg, 0.629 mmol) to give **29** as a yellow solid (134 mg, 24%). ¹H NMR (CDCl₃, 400 MHz) δ 7.60 (s, 1H), 7.34-7.22 (m, 18H), 6.88 (d, 2H, *J* = 8.7 Hz), 6.81 (d, 1H, *J* = 8.7 Hz), 5.94 (dd, 1H, *J* = 8.9 Hz, *J* = 4.9 Hz), 5.24-5.14 (m, 3H), 5.01 (d, 1H, 4.9 Hz), 4.91 (d, 1H, *J* = 14.0 Hz), 4.12 (s, 3H), 3.80 (s, 3H), 3.57 (d, 1H, *J* = 18.6 Hz), 3.49-3.45 (m, 5H), 1.92 (t, 4H, *J* = 6.8 Hz). ¹³C NMR (CDCl₃, 126 MHz) δ 184.1, 164.0, 161.5, 161.3, 160.1, 160.0, 146.6, 142.0, 130.8, 129.3, 128.7, 128.4, 127.0, 126.9, 125.5, 114.1, 72.4, 71.5, 68.2, 64.0, 59.3, 57.7, 55.4, 50.9, 26.5, 23.0. ESI-HRMS calcd. for 912.2568 [M+Na]⁺ C₄₄H₄₃O₈N₉S₂Na, found 912.2615.

(Z)-1-(4-(((*tert*-butoxycarbonyl)amino)methyl)piperidin-1-yl)-2-((2-(((4-methoxy benzyl)oxy)carbonyl)-8-oxo-7-(2-phenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)diazene 1-oxide **35**

Compound **35** was prepared using General Procedure II from **34** (500 mg, 1.03 mmol), NaI (154 mg, 1.03 mmol) and **32** (304 mg, 1.03 mmol) to give **35** as an off white solid (342 mg, 46%). ¹H NMR (CDCl₃, 500 MHz) δ 7.38-7.26 (m, 7H), 6.88 (d, 2H, *J* = 8.7 Hz), 6.19 (d, 1H, *J* = 8.7 Hz), 5.82 (dd, 1H, *J* = 9.0 Hz, *J* = 4.9 Hz), 5.21-5.15 (m, 3H), 4.96 (d, 1H, *J* = 14.0 Hz), 4.91 (d, 1H, *J* = 4.9 Hz), 4.62 (bs, 1H), 3.86 (d, 2H, *J* = 9.6 Hz), 3.80 (s, 3H), 3.67 (d, 1H, *J* = 16.0 Hz), 3.62 (d, 1H, *J* = 16.0 Hz), 3.49 (d, 1H, *J* = 18.5 Hz), 3.41 (d, 1H, *J* = 18.5 Hz), 3.01 (t, 2H, *J* = 6.2 Hz), 2.84 (t, 2H, *J* = 12.0 Hz), 1.78 (d, 2H, *J* = 12.5 Hz), 1.65-1.55 (m, 1H, overlapped by H₂O), 1.44 (s, 9H), 1.36 (t, 2H, *J* = 12.8 Hz). ¹³C NMR (CDCl₃, 125 MHz) δ 171.3, 164.8, 161.4, 160.1, 156.2, 133.8, 130.8, 129.6, 129.3, 127.9, 126.8, 126.4, 125.8, 114.1, 79.6, 71.8, 68.3, 59.4, 57.7, 55.4, 51.4, 45.6, 43.4, 35.9, 28.6, 28.3, 26.2. ESI-HRMS calcd. for 747.2783 [M+Na]⁺ C₃₅H₄₄O₉N₆SNa, found 747.2791.

(Z)-1-(4-(2-((*tert*-butoxycarbonyl)amino)ethyl)piperidin-1-yl)-2-((2-(((4-methoxy benzyl)oxy)carbonyl)-8-oxo-7-(2-phenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy) diazene 1-oxide **36**

Compound **36** was prepared using General Procedure II from **34** (500 mg, 1.03 mmol), NaI (154 mg, 1.03 mmol) and **33** (319 mg, 1.03 mmol) to give **36** as an off white solid (326 mg, 43%). ¹H NMR (CDCl₃, 500 MHz) δ 7.36-7.25 (m, 7H), 6.89 (d, 2H, *J* = 8.7 Hz), 6.13 (d, 1H, *J* = 9.1 Hz), 5.81 (dd, 1H, *J* = 9.1 Hz, *J* = 4.9 Hz), 5.24-5.10 (m, 3H), 4.93 (d, 1H, *J* = 14.1 Hz), 4.90 (d, 1H, *J* = 4.9 Hz), 4.52 (bs, 1H), 3.84-3.76 (m, 5H), 3.66 (d, 1H, *J* = 16.1 Hz), 3.61 (d, 1H, *J* = 16.1 Hz), 3.50 (d, 1H, *J* = 18.6 Hz), 3.40 (d, 1H, *J* = 18.6 Hz), 3.15 (d, 2H, *J* = 5.9 Hz), 2.82 (t, 2H, *J* = 11.6 Hz), 1.82 (d, 2H, *J* = 10.8 Hz), 1.46-1.33 (m, 14H). ¹³C NMR (CDCl₃, 125 MHz) δ 171.1, 164.7, 161.3, 159.9, 156.0, 133.7, 130.7, 129.5, 129.2, 127.8, 126.7, 126.4, 125.5, 114.0, 79.5, 71.7, 68.1, 59.2, 57.5, 55.3, 51.6, 43.3, 38.1, 36.1, 32.5, 30.7, 28.4, 26.1. ESI-HRMS calcd. for 761.2939 [M+Na]⁺ C₃₆H₄₆O₉N₆SNa, found 761.2957.

(Z)-1-(4-(((*tert*-butoxycarbonyl)amino)methyl)piperidin-1-yl)-2-((2-(((4-methoxy benzyl)oxy)carbonyl)-7-((*Z*)-2-(methoxyimino)-2-(2-(tritylamino)thiazol-4-yl)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)diazene 1-oxide **37**

Compound **37** was prepared using General Procedure II from **21** (500 mg, 0.629 mmol), NaI (94 mg, 0.629 mmol) and **32** (187 mg, 0.629 mmol) to give **37** as a yellow solid (182 mg, 28%). ¹H NMR (CDCl₃, 500 MHz) δ 7.33-7.24 (m, 18H), 7.04 (bs, 1H), 6.89 (d, 2H, *J* = 8.7 Hz), 6.68 (s, 1H), 5.90 (dd, 1H, *J* = 8.8 Hz, *J* = 4.9), 5.25-5.13 (m, 3H), 5.03-4.98 (m, 2H), 4.65 (bs, 1H), 4.06 (s, 3H), 3.87 (d, 2H, *J* = 11.3 Hz), 3.81 (s, 3H), 3.54 (d, 1H, *J* = 18.5 Hz), 3.46 (d, 1H, *J* = 18.4 Hz), 3.01 (t, 2H, *J* = 6.4 Hz), 2.85 (t, 2H, *J* = 12.0 Hz), 1.78 (d, 2H, *J* = 10.5 Hz), 1.56 (bs, 1H), 1.41 (s, 9H), 1.40-1.32 (m, 2H). ¹³C NMR (CDCl₃, 125 MHz) δ 168.7, 163.9, 162.2, 161.4, 160.1, 156.1, 147.3, 143.2, 141.0, 130.8, 129.4, 128.4, 127.8, 126.8, 126.5, 125.8, 114.2, 113.2, 79.6, 71.9, 71.7, 68.3, 63.4, 59.1, 57.6, 55.4, 51.4, 45.5, 35.9, 28.5, 28.3, 26.4. ESI-HRMS calcd. for 1032.3743 [M+H]⁺ C₅₂H₅₈O₁₀N₉S₂Na, found 1032.3762.

(Z)-1-(4-(2-(((*tert*-butoxycarbonyl)amino)ethyl)piperidin-1-yl)-2-((2-(((4-methoxy benzyl)oxy)carbonyl)-7-((*Z*)-2-(methoxyimino)-2-(2-(tritylamino)thiazol-4-yl)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)diazene 1-oxide **38**

Compound **38** was prepared using General Procedure II from **21** (500 mg, 0.629 mmol), NaI (94 mg, 0.629 mmol) and **33** (195 mg, 0.629 mmol) to give **38** as a yellow solid (151 mg, 23%). ¹H NMR (CDCl₃, 400 MHz) δ 7.34-7.28 (m, 17H), 7.03 (s, 1H), 6.89 (d, 2H, *J* = 8.7 Hz), 6.84 (d, 1H, *J* = 8.9 Hz), 6.70 (s, 1H), 5.91 (dd, 1H, *J* = 9.0 Hz, *J* = 4.9 Hz), 5.27 (d, 1H, *J* = 14.0 Hz), 5.22 (d, 1H, *J* = 11.8 Hz), 5.17 (d, 1H, *J* = 11.8 Hz), 5.01 (d, 1H, *J* = 5.0 Hz), 4.97 (d, 1H, *J* = 14.0 Hz), 4.50 (bs, 1H), 4.06 (s, 3H), 3.84-3.75 (m, 5H), 3.55 (d, 1H, *J* = 18.5 Hz), 3.46 (d, 1H, *J* = 18.6 Hz), 3.15 (d, 2H, *J* = 5.9 Hz), 2.83 (t, 2H, *J* = 11.1 Hz), 1.82 (d, 2H,

$J = 10.2$ Hz), 1.44-1.40 (m, 14H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 168.7, 163.9, 162.2, 161.4, 160.1, 156.1, 147.2, 143.2, 141.0, 130.8, 129.4, 128.4, 127.8, 126.8, 126.6, 125.7, 114.2, 113.2, 79.4, 71.9, 71.8, 68.3, 63.5, 59.1, 57.5, 55.4, 51.8, 38.2, 36.3, 32.6, 30.8, 28.5, 26.4. ESI-HRMS calcd. for 1046.3899 $[\text{M}+\text{H}]^+$ $\text{C}_{53}\text{H}_{60}\text{O}_{10}\text{N}_9\text{S}_2$, found 1046.3905.

(Z)-2-(((6*R*,7*R*)-7-((*Z*)-2-(((1-(*tert*-butoxy)-2-methyl-1-oxopropan-2-yl)oxy)imino)-2-(2-(tritylamino)thiazol-4-yl)acetamido)-2-(((4-methoxybenzyl)oxy)carbonyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)-1-(4-(((*tert*-butoxycarbonyl)amino)methyl)piperidin-1-yl)diazene 1-oxide **39**

Compound **39** was prepared using General Procedure II from **22** (500 mg, 0.542 mmol), NaI (81 mg, 0.542 mmol) and **32** (161 mg, 0.542 mmol) to give **39** as a yellow solid (195 mg, 31%). ^1H NMR (CDCl_3 , 500 MHz) δ 8.12 (d, 1H, $J = 8.7$ Hz), 7.34-7.26 (m, 17H), 6.91-6.88 (m, 3H), 6.72 (s, 1H), 5.99 (dd, 1H, $J = 8.8$ Hz, $J = 5.0$ Hz), 5.29 (d, 1H, $J = 14.0$ Hz), 5.24 (d, 1H, $J = 11.8$ Hz), 5.17 (d, 1H, $J = 11.8$ Hz), 5.00 (d, 1H, $J = 5.0$ Hz), 4.96 (d, 1H, $J = 14.1$ Hz), 4.63 (bs, 1H), 3.86-3.81 (m, 5H), 3.52 (d, 1H, $J = 18.5$ Hz), 3.43 (d, 1H, $J = 18.5$ Hz), 3.03 (t, 2H, $J = 6.3$ Hz), 2.84 (tt, 2H, $J = 12.0$ Hz, $J = 2.6$ Hz), 1.81 (d, 2H, $J = 13.0$ Hz), 1.62 (s, 3H), 1.58 (s, 3H), 1.60-1.54 (m, 1H, overlapped), 1.44 (s, 9H), 1.41 (s, 9H). 1.41-1.36 (m, 2H, overlapped). ^{13}C NMR (CDCl_3 , 125 MHz) δ 174.0, 168.2, 164.3, 163.4, 161.6, 160.0, 156.1, 149.3, 143.3, 141.5, 130.8, 129.5, 128.4, 127.7, 126.9, 126.8, 125.9, 114.2, 112.6, 83.1, 82.2, 79.6, 72.0, 68.2, 59.3, 57.8, 55.4, 51.5, 45.5, 35.9, 28.5, 28.4, 28.2, 26.5, 24.4, 23.9. ESI-HRMS calcd. for 1160.4580 $[\text{M}+\text{H}]^+$ $\text{C}_{59}\text{H}_{70}\text{O}_{12}\text{N}_9\text{S}_2$, found 1160.4546.

(Z)-2-(((6*R*,7*R*)-7-((*Z*)-2-(((1-(*tert*-butoxy)-2-methyl-1-oxopropan-2-yl)oxy)imino)-2-(2-(tritylamino)thiazol-4-yl)acetamido)-2-(((4-methoxybenzyl)oxy)carbonyl)-8-oxo-5-thia-1-

azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)-1-(4-(2-((tert-butoxycarbonyl)amino)ethyl) piperidin-1-yl)diazene 1-oxide **40**

Compound **40** was prepared using General Procedure II from **22** (500 mg, 0.542 mmol), NaI (81 mg, 0.542 mmol) and **33** (168 mg, 0.542 mmol) to give **40** as a yellow solid (236 mg, 37%). ¹H NMR (CDCl₃, 500 MHz) δ 8.11 (d, 1H, *J* = 8.8 Hz), 7.34-7.26 (m, 17H), 6.91-6.88 (m, 3H), 6.72 (s, 1H), 5.99 (dd, 1H, *J* = 8.8 Hz, *J* = 5.0 Hz), 5.30 (d, 1H, *J* = 14.1 Hz), 5.24 (d, 1H, *J* = 11.8 Hz), 5.17 (d, 1H, *J* = 11.8 Hz), 5.00 (d, 1H, *J* = 5.0 Hz), 4.95 (d, 1H, *J* = 14.1 Hz), 4.49 (bs, 1H), 3.83-3.78 (m, 5H), 3.52 (d, 1H, *J* = 18.4 Hz), 3.43 (d, 1H, *J* = 18.5 Hz), 3.16 (d, 2H, *J* = 5.9 Hz), 2.83 (tt, 2H, *J* = 11.6 Hz, *J* = 2.7 Hz), 1.82 (d, 2H, *J* = 10.0 Hz), 1.62 (s, 3H), 1.58 (s, 3H), 1.48-1.44 (m, 2H, overlapped, observed in gHSQC), 1.45-1.41 (m, 1H, overlapped, observed in gHSQC), 1.44-1.38 (m, 2H, overlapped, observed in gHSQC), 1.44 (s, 9H), 1.41 (s, 9H). ¹³C NMR (CDCl₃, 125 MHz) δ 174.1, 168.3, 164.3, 163.4, 161.7, 160.1, 156.1, 149.3, 143.4, 141.5, 130.9, 129.5, 128.4, 127.7, 127.0, 126.8, 125.8, 114.2, 112.6, 83.1, 82.2, 79.4, 72.0, 68.2, 59.3, 57.8, 55.4, 51.8, 38.2, 36.3, 32.6, 30.8, 28.6, 28.2, 26.5, 24.4, 23.9. ESI-HRMS calcd. for 1174.4742 [M+H]⁺ C₆₀H₇₂O₁₂N₉S₂, found 1174.4760.

General Procedure III (Compounds 2-12)

Mixtures containing **25-29**, **35-40** (1 eq.) and phenol (3-4 g) were heated to 45 °C in a dry flask under an atmosphere of argon. Upon complete melting of phenol, trifluoroacetic acid (0-50 eq.) was added and the mixtures stirred at 45 °C for 2.5 hrs. Addition of 50% pet.spirit/ether (100 mL) to the cooled reaction mixtures (room temperature) caused precipitation of a white solid that was collected by centrifugation (3000 rpm, 5 min). The pellets were washed with ice cold anhydrous diethyl ether and the crude solid was purified by C18-reverse phase silica gel column chromatography. Fractions containing the pure product (as indicated by C18-reverse phase silica gel TLC) were combined and lyophilised.

(Z)-2-(((7-((*R*)-2-amino-2-phenylacetamido)-2-carboxy-8-oxo-5-thia-1-azabicyclo [4.2.0]oct-2-en-3-yl)methoxy)-1-(pyrrolidin-1-yl)diazene 1-oxide (*PYRRO-cefalexin 2*)

Compound **2** was prepared using General Procedure III from **25** (200 mg, 0.287 mmol) and TFA (879 μ L, 11.5 mmol) to give **2** as a fluffy white solid (59 mg, 43%). ^1H NMR (CD_3OD , 400 MHz) δ 7.54-7.52 (m, 2H), 7.47-7.44 (m, 3H), 5.73 (d, 1H, $J = 4.8$ Hz), 5.20 (d, 1H, $J = 12.5$ Hz), 5.05 (s, 1H), 4.96 (d, 1H, $J = 4.8$ Hz), 4.81-4.79 (m, 1H, overlapped by H_2O , observed in gHSQC), 3.51-3.46 (m, 5H), 3.24 (d, 1H, $J = 17.7$ Hz), 1.94-1.91 (m, 4H). ^{13}C NMR (CD_3OD , 100 MHz) δ 169.7, 168.3, 164.6, 134.3, 133.8, 131.1, 130.4, 129.5, 117.8, 73.9, 60.2, 58.8, 57.8, 51.9, 26.8, 23.7. ESI-HRMS calcd. for 499.1370 $[\text{M}+\text{Na}]^+$ $\text{C}_{20}\text{H}_{24}\text{O}_6\text{N}_6\text{SNa}$, found 499.1392.

(Z)-2-(((6*R*,7*R*)-2-carboxy-7-((*Z*)-2-(furan-2-yl)-2-(methoxyimino)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)-1-(pyrrolidin-1-yl)diazene 1-oxide (*PYRRO-cefuroxime 3*)

Compound **3** was prepared using General Procedure III from **26** (200 mg, 0.325 mmol) to give **3** as a fluffy white solid (61 mg, 38%). No TFA was required. ^1H NMR (CD_3OD , 500 MHz) δ 7.64 (s, 1H), 6.76 (d, 1H, $J = 3.4$ Hz), 6.56 (dd, 1H, $J = 3.5$ Hz, $J = 1.8$ Hz), 5.86 (d, 1H, $J = 4.8$ Hz), 5.24 (d, 1H, $J = 13.2$ Hz), 5.18 (d, 1H, $J = 4.8$ Hz), 4.94 (d, 1H, $J = 13.2$ Hz), 3.97 (s, 3H), 3.68 (d, 1H, $J = 18.1$ Hz), 3.53-3.51 (m, 5H), 1.95 (m, 4H). ^{13}C NMR (CD_3OD , 125 MHz) δ 165.0, 164.4, 147.1, 146.2, 146.1, 128.6 (observed in gHMBC), 123.2 (observed in gHMBC), 114.1, 112.8, 73.1, 63.2, 58.9, 51.9, 27.2, 23.7. ESI-HRMS calcd. for 517.1112 $[\text{M}+\text{Na}]^+$ $\text{C}_{19}\text{H}_{22}\text{O}_8\text{N}_6\text{SNa}$, found 517.1133.

(Z)-2-((7-((*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetamido)-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)-1-(pyrrolidin-1-yl)diazene 1-oxide (PYRRO-cefepime **4**)

Compound **4** was prepared using General Procedure III from **27** (200 mg, 0.225 mmol) and TFA (689 μ L, 9.0 mmol) to give **4** as a fluffy white solid (64 mg, 54%). ^1H NMR (DMSO- d_6 , 400 MHz) δ 9.58 (d, 1H, 8.2 Hz), 7.22 (s, 2H), 6.73 (s, 1H), 5.72 (dd, 1H, $J = 4.76$ Hz, $J = 8.1$ Hz), 5.12 (d, 1H, $J = 5.0$ Hz), 5.09 (d, 1H, $J = 13.3$ Hz), 4.80 (d, 1H, $J = 12.4$ Hz), 3.83 (s, 3H), 3.54 (d, 1H, $J = 17.8$ Hz), 3.44-3.40 (m, 5H), 1.85 (t, 4H, $J = 6.6$ Hz). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 168.4, 163.2, 163.1, 163.0, 149.0, 142.6, 130.4 (observed in gHMBC), 119.7 (observed in gHMBC), 108.9, 71.6, 61.9, 58.4, 57.6, 50.5, 25.9, 22.2. ESI-HRMS calcd. for 525.0980 [M-H] $^-$ C₁₈H₂₁O₇N₈S₂, found 525.1072.

(Z)-2-(((6*R*,7*R*)-7-((*Z*)-2-(2-aminothiazol-4-yl)-2-(((2-carboxypropan-2-yl)oxy)imino)acetamido)-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)-1-(pyrrolidin-1-yl)diazene 1-oxide (PYRRO-ceftazidime **5**)

Compound **5** was prepared using General Procedure III from **28** (200 mg, 0.197 mmol) and TFA (752 μ L, 9.8 mmol) to give **5** as a fluffy white solid (37 mg, 31%). ^1H NMR (DMSO- d_6 , 500 MHz) δ 7.29 (s, 2H), 6.72 (s, 1H), 5.85 (dd, 1H, $J = 8.2$ Hz, $J = 4.8$ Hz), 5.20 (d, 1H, $J = 4.8$ Hz), 5.03 (d, 1H, $J = 12.7$ Hz), 4.82 (d, 1H, $J = 12.7$ Hz), 3.60 (d, 1H, $J = 18.0$ Hz), 3.52 (d, 1H, $J = 18.0$ Hz), 3.44-3.39 (m, 4H), 1.87-1.83 (m, 4H), 1.45 (s, 3H), 1.44 (s, 3H). ^{13}C NMR (DMSO- d_6 , 125 MHz) δ 175.0, 168.5, 164.0, 162.9, 149.3, 142.6, 127.2, 123.2, 109.8, 81.7, 70.9, 58.6, 57.6, 50.5, 26.1, 24.2, 23.8, 22.3. ESI-HRMS calcd. for 597.1191 [M-H] $^-$ C₂₁H₂₅N₈O₉S₂, found 597.1159.

(Z)-2-((7-((*Z*)-2-(5-amino-1,2,4-thiadiazol-3-yl)-2-(methoxyimino)acetamido)-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)-1-(pyrrolidin-1-yl)diazene 1-oxide
(PYRRO-cefzopran **6**)

Compound **6** was prepared using General Procedure III from **29** (200 mg, 0.225 mmol) and TFA (688 μ L, 9.0 mmol) to give **6** as a fluffy white solid (43 mg, 36%). ^1H NMR (DMSO- d_6 , 400 MHz) δ 9.56 (d, 1H, $J = 8.6$ Hz), 8.13 (s, 2H), 5.77 (dd, 1H, $J = 8.5$ Hz, $J = 4.8$ Hz), 5.12 (d, 1H, $J = 4.8$ Hz), 5.07 (d, 1H, $J = 12.5$ Hz), 4.80 (d, 1H, $J = 12.5$ Hz), 3.91 (s, 3H), 3.55 (d, 1H, $J = 17.8$ Hz), 3.47-3.33 (m 5H, overlapped by H₂O), 1.85 (t, 4H, $J = 6.5$ Hz). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 183.2, 163.5, 163.1, 162.0, 162.0, 148.1, 129.7 (observed in gHMBC), 120.2 (observed in gHMBC), 71.4, 62.5, 58.3, 57.5, 50.5, 25.9, 22.3. ESI-HRMS calcd. for 526.0933 [M-H]⁻ C₁₇H₂₀O₇N₉S₂, found 526.0927.

(Z)-1-(4-(aminomethyl)piperidin-1-yl)-2-((2-carboxy-8-oxo-7-(2-phenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)diazene 1-oxide (AMINOPIPI-cefaloram **7**)

Compound **7** was prepared using General Procedure III from **35** (200 mg, 0.276 mmol) and TFA (422 μ L, 5.5 mmol) to give **7** as a fluffy white solid (102 mg, 73%). ^1H NMR (DMSO- d_6 , 400 MHz) δ 9.03 (d, 1H, $J = 8.4$ Hz), 8.48 (bs, 2H), 7.31-7.20 (m, 5H), 5.54 (dd, 1H, $J = 8.4$ Hz, $J = 4.8$ Hz), 5.25 (d, 1H, $J = 11.7$ Hz), 4.98 (d, 1H, $J = 4.9$ Hz), 4.77 (d, 1H, $J = 11.7$ Hz), 3.76 (t, 2H, $J = 12.2$ Hz), 3.58 (d, 1H, $J = 13.9$ Hz), 3.50 (d, 1H, $J = 14.0$ Hz), 3.45 (d, 1H, $J = 17.4$ Hz), 3.30 (d, 1H, $J = 17.5$ Hz), 2.88-2.78 (m, 2H), 2.76-2.67 (m, 2H), 1.84 (d, 2H, $J = 13.2$ Hz), 1.80-1.70 (m, 1H), 1.40-1.24 (m, 2H). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 171.0, 164.3, 163.3, 135.9, 134.7, 129.0, 128.2, 126.4, 112.2 (observed in HMBC), 73.0, 58.6, 57.4, 50.25, 50.17, 42.9, 41.6, 32.8, 27.4, 27.3 25.5. ESI-HRMS calcd. for 505.1864 [M+H]⁺ C₂₂H₂₉N₆O₆S, found 505.1886.

(Z)-1-(4-(2-aminoethyl)piperidin-1-yl)-2-((2-carboxy-8-oxo-7-(2-phenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)diazene 1-oxide (AMINOPIP2-cefaloram **8**)

Compound **8** was prepared using General Procedure III from **36** (200 mg, 0.271 mmol) and TFA (414 μ L, 5.4 mmol) to give **8** as a fluffy white solid (114 mg, 81%). ^1H NMR (DMSO- d_6 , 500 MHz) δ 9.02 (d, 1H, $J = 8.6$ Hz), 8.24 (bs, 2H), 7.30-7.20 (m, 5H), 5.53 (d, 1H, $J = 9.2$ Hz), 5.23 (d, 1H, $J = 10.1$ Hz), 4.97 (s, 1H), 4.80 (d, 1H, $J = 10.2$ Hz). ^{13}C NMR (DMSO- d_6 , 126 MHz) δ 171.0, 164.0, 163.3, 136.0, 134.6 (observed in gHMBC) 129.0, 128.2, 126.4, 112.5 (observed in gHMBC) 73.0, 58.6, 57.4, 50.9, 41.6, 36.4, 32.9, 31.5, 29.9, 25.4. ESI-HRMS calcd. for 519.2020 $[\text{M}+\text{H}]^+$ $\text{C}_{23}\text{H}_{31}\text{N}_6\text{O}_6\text{S}$, found 519.2051.

(Z)-1-(4-(aminomethyl)piperidin-1-yl)-2-((7-((*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetamido)-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)diazene 1-oxide (AMINOPIP1-cefepime **9**)

Compound **9** was prepared using General Procedure III from **37** (200 mg, 0.194 mmol) and TFA (593 μ L, 7.8 mmol) to give **9** as a fluffy white solid (57 mg, 52%). ^1H NMR (DMSO- d_6 , 400 MHz) δ 9.54 (d, 1H, $J = 8.2$ Hz), 8.45 (bs, 2H), 7.22 (s, 2H), 6.73 (s, 1H), 5.65 (dd, 1H, $J = 4.8$ Hz, $J = 7.9$ Hz), 5.24 (d, 1H, $J = 11.7$ Hz), 5.06 (d, 1H, $J = 4.9$ Hz), 4.77 (d, 1H, $J = 11.8$ Hz), 3.83 (s, 3H), 3.76 (t, 2H, $J = 11.6$ Hz), 3.47 (d, 1H, $J = 17.4$ Hz), 3.30 (d, 1H, $J = 17.5$ Hz), 2.83 (t, 2H, $J = 10.9$ Hz), 2.72 (d, 2H, $J = 6.0$ Hz), 1.83 (d, 2H, $J = 12.0$ Hz), 1.75 (m, 1H), 1.32 (t, 2H, $J = 11.1$ Hz). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 168.4, 164.3, 163.0, 162.4, 149.1, 142.6, 134.6 (observed in gHMBC), 113.4 (observed in gHMBC), 108.9, 73.0, 61.9, 58.2, 57.5, 50.3, 50.2, 43.0, 32.8, 27.44, 27.38, 25.7. ESI-HRMS calcd. for 570.1548 $[\text{M}+\text{H}]^+$ $\text{C}_{20}\text{H}_{28}\text{O}_7\text{N}_9\text{S}_2$, found 570.1561.

(Z)-1-(4-(2-aminoethyl)piperidin-1-yl)-2-((7-((*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)

acetamido)-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)diazene 1-oxide (AMINOPIP2-cefepime 10)

Compound **10** was prepared using General Procedure III from **38** (200 mg, 0.191 mmol) and TFA (585 μ L, 7.6 mmol) to give **10** as a fluffy white solid (51 mg, 46%). ^1H NMR (DMSO- d_6 , 400 MHz) δ 9.54 (d, 1H, $J = 9.54$ Hz), 8.27 (bs, 2H), 7.22 (s, 2H), 6.73 (s, 1H), 5.65 (dd, 1H, $J = 7.9$ Hz, $J = 4.8$ Hz), 5.22 (d, 1H, $J = 11.7$ Hz), 5.05 (d, 1H, $J = 4.8$ Hz), 4.79 (d, 1H, $J = 11.6$ Hz), 3.83 (s, 3H), 3.77-3.70 (m, 2H), 3.46 (d, 1H, $J = 17.5$ Hz), 3.35-3.25 (m, 1H, overlapped by H_2O , observed in gHSQC), 2.85-2.77 (m, 4H), 1.76 (d, 2H, $J = 12.6$ Hz), 1.52-1.40 (m, 3H), 1.28-1.17 (m, 2H). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 168.4, 164.1, 163.0, 162.3, 149.1, 142.6, 135.3 (observed in gHMBC), 113.1 (observed in gHMBC), 108.9, 73.0, 61.8, 58.2, 57.5, 50.9, 50.8, 36.4, 32.9, 31.5, 30.0, 29.9. ESI-HRMS calcd. for 584.1704 $[\text{M}+\text{H}]^+$ $\text{C}_{21}\text{H}_{30}\text{O}_7\text{N}_9\text{S}_2$, found 584.1724.

(Z)-1-(4-(aminomethyl)piperidin-1-yl)-2-(((6R,7R)-7-((Z)-2-(2-aminothiazol-4-yl)-2-((2-carboxypropan-2-yl)oxy)imino)acetamido)-2-carboxy-8-oxo-5-thia-1-azabicyclo [4.2.0]oct-2-en-3-yl)methoxy)diazene 1-oxide (AMINOPIP1-ceftazidime 11)

Compound **11** was prepared using General Procedure III from **39** (200 mg, 0.172 mmol) and TFA (659 μ L, 8.6 mmol) to give **11** as a fluffy white solid (43 mg, 39%). ^1H NMR (DMSO- d_6 , 500 MHz) δ 10.17 (bs, 1H), 8.38 (bs, 2H), 7.25 (bs, 2H), 6.73 (s, 1H), 5.77 (dd, 1H, $J = 7.7$ Hz, $J = 5.0$ Hz), 5.11 (d, 1H, $J = 5.0$ Hz), 5.04 (d, 1H, $J = 12.0$ Hz), 4.97 (d, 1H, $J = 12.0$ Hz), 3.82 (d, 2H, $J = 11.2$ Hz), 3.50 (d, 1H, $J = 17.7$ Hz), 3.37 (d, 1H, $J = 17.9$ Hz), 2.92 (d, 1H, $J = 11.4$ Hz), 2.87 (d, 1H, $J = 11.4$ Hz), 2.68 (d, 2H, $J = 6.1$ Hz), 1.82-1.68 (m, 3H), 1.46 (s, 3H), 1.43 (s, 3H), 1.38-1.17 (m, 2H). ^{13}C NMR (DMSO- d_6 , 125 MHz) δ 176.3 (observed in gHMBC), 168.4, 163.7, 163.3, 163.2, 149.7, 142.9, 132.0 (observed in gHMBC), 117.2

(observed in gHMBC), 109.8, 82.3, 72.0, 58.4, 57.7, 49.9, 43.0, 32.8, 26.7, 25.5, 24.4, 23.9.

ESI-HRMS calcd. for 642.1759 [M+H]⁺ C₂₃H₃₂N₉O₉S₂, found 642.1789.

*(Z)-1-(4-(2-aminoethyl)piperidin-1-yl)-2-(((6R,7R)-7-((Z)-2-(2-aminothiazol-4-yl)-2-(((2-carboxypropan-2-yl)oxy)imino)acetamido)-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methoxy)diazene 1-oxide AMINOPIP2-ceftazidime **12**)*

Compound **12** was prepared using General Procedure III from **40** (200 mg, 0.170 mmol) and TFA (652 μ L, 8.5 mmol) to give **12** as a fluffy white solid (37 mg, 33%). ¹H NMR (DMSO-*d*₆, 500 MHz) δ 9.96 (bs, 1H), 8.27 (bs, 2H), 7.26 (bs, 2H), 6.74 (s, 1H), 5.75 (dd, 1H, *J* = 7.7 Hz, *J* = 5.0 Hz), 5.13 (d, 1H, *J* = 11.9 Hz), 5.09 (d, 1H, *J* = 5.0 Hz), 4.85 (d, 1H, *J* = 11.9 Hz), 3.79-3.73 (m, 2H), 3.49 (d, 1H, *J* = 17.5 Hz), 3.33 (d, 1H, *J* = 17.5 Hz), 2.83-2.76 (m, 4H), 1.72 (d, 2H, *J* = 11.6 Hz), 1.46 (s, 3H), 1.43 (s, 3H), 1.49-1.40 (m, 2H, overlapped, observed in gHSQC), 1.49-1.40 (m, 1H, overlapped, observed in gHSQC), 1.25-1.16 (m, 2H). ¹³C NMR (DMSO-*d*₆, 125 MHz) δ 176.0, 168.4, 164.0, 163.2, 163.1, 149.6, 142.8, 133.8 (observed in gHMBC), 115.1 (observed in gHMBC), 109.8, 82.2, 72.5, 58.4, 57.6, 50.8, 50.7, 36.4, 32.9, 31.5, 29.7, 25.7, 24.4, 23.9. ESI-HRMS calcd. for 678.1735 [M+Na]⁺ C₂₄H₃₃N₉O₉S₂Na, found 678.1727.

*General Procedure IV (Compounds **32** and **33**)*

N-Boc-4-alkylamino piperidines **30** and **31** (2.5 g) were dissolved in a mixture containing 10 mL of anhydrous 25% sodium methoxide/methanol and 40 mL of anhydrous diethyl ether in a Paar-Knorr reaction vessel. The mixture was purged with nitrogen (bubbling for 15 mins) before the reaction vessel was sealed and charged with nitric oxide gas at 50 psi. After shaking for 48 h the white precipitate was collected by vacuum filtration under argon, washed with copious dry diethyl ether (under argon) and dried under vacuum.

N-Boc-AMINOPIPI1/NO 32

Compound **32** was prepared using General Procedure IV from **30** (2.50 g, 11.7 mmol) to give **32** as a white solid (3.11 g, 90 %). ¹H NMR (DMSO-*d*₆, 500 MHz) δ 6.86 (bs, 1H), 2.94 (d, 2H, *J* = 10.7 Hz), 2.88-2.81 (m, 4H), 1.68 (d, 2H, *J* = 11.5 Hz), 1.37 (bs, 9H), 1.36-1.39 (m, 1H, overlapped), 1.26 (qd, 2H, *J* = 11.8 Hz, *J* = 3.5 Hz). ¹³C NMR (DMSO-*d*₆, 125 MHz) δ 155.8, 77.4, 51.9, 45.1, 35.3, 28.9, 28.3.

N-Boc-AMINOPIPI2/NO 33

Compound **33** was prepared using General Procedure IV from **31** (2.50 g, 10.9 mmol) to give **33** as a white solid (3.16 g, 93 %). ¹H NMR (DMSO-*d*₆, 500 Hz) δ 6.78 (t, 1H, *J* = 5.4 Hz), 2.97-2.84 (m, 6H), 2.88-2.81 (m, 4H), 1.76-1.69 (m, 2H), 1.37 (bs, 9H), 1.34-1.24 (m, 5H). ¹³C NMR (DMSO-*d*₆, 125 MHz) δ 155.6, 77.3, 51.8, 37.5, 35.9, 32.0, 31.1, 28.3.

P. aeruginosa strain history and growth conditions

P. aeruginosa laboratory strain PAO1 was obtained from the University of Washington, USA. Clinical isolates of *P. aeruginosa* were obtained from the University of Southampton's culture collection, originally isolated from the sputum of CF patients based at University Hospital Southampton NHS Foundation Trust, UK. The mean age at informed consent was 28, with a range of ages from 19 to 65 years (NHS Research Ethics Committee 08/H0502/126). Details relating to the patients are provided in the Supporting Information Table S1. CF physiotherapists assisted patients with expectoration of sputum into sterile containers. The sputum was digested with Mucolyse (Pro-lab Diagnostic) containing dithiotheritol (DTT) and phosphate buffer for 15 min at 37 °C. *P. aeruginosa* was selected using cetrimide agar (Sigma Aldrich); a sterile loop was then drawn across the plate for a random selection of strains, which

were frozen down to form the culture collection. Multiplex polymerase chain reaction (PCR) was conducted to confirm *P. aeruginosa* in the samples.

Samples from the culture collection were streaked onto selective ceftrimide agar (Sigma Aldrich) to confirm isolation of *P. aeruginosa* and single colonies were selected to prepare fresh bacterial stocks in cryovials containing beads (Fisher Scientific), kept at -80 °C. Overnight cultures were prepared by inoculating a single bead of the frozen stock into 10 ml of Luria Bertani (LB) broth (Formedium) and incubated at 37 °C with shaking at 180 rpm.

P. aeruginosa clinical strain susceptibility testing

The minimum inhibitory concentrations (MIC) of C3Ds and cephalosporin antibiotic controls against the *P. aeruginosa* clinical isolates were determined using the broth microdilution method in accordance with Clinical Laboratory Standards Institute guidelines.³³ Two-fold serial dilutions of test compounds were prepared in a 96 well plate. Compound dilutions were transferred to a new 96 well plate in triplicate, followed by addition of inoculated cation-adjusted Mueller Hinton broth (CAMHB) broth, prepared by diluting an overnight culture 1 in 500 to achieve approximately 1×10^5 to 10^6 cells. A CFU count was carried out to confirm the exact cell number of the inoculum. Blank samples were prepared using compound dilutions and uninoculated CAMHB broth. The 96 well plates were incubated overnight at 37 °C and the OD values read at 584 nm using the FLUOstar Omega Microplate Reader (BMG Labtech). The MIC was recorded as the lowest concentration that inhibited bacterial growth, as determined by visible turbidity.

β-lactamase activity of P. aeruginosa CF clinical isolates relative to PAO1

A nitrocefin assay was used to measure the relative β-lactamase activity in CF clinical isolates of *P. aeruginosa* compared to PAO1. In this assay, the chromogenic cephalosporin

nitrocefin undergoes a quantifiable yellow to red colour change when hydrolysed by β -lactamases.³¹ *P. aeruginosa* isolates were grown overnight in LB broth, subcultured into fresh broth and grown to mid exponential phase. Nitrocefin (Cambridge Bioscience) was prepared in DMSO/PBS and added to the cultures to a final concentration of 50 $\mu\text{g}/\text{mL}$. Absorbance at 490 nm was recorded at intervals during subsequent incubation, with an increase in OD490 reporting β -lactamase activity (Supporting information Figure S1).

NO Release from C3Ds in the presence of commercial β -lactamases

Nitric oxide (NO) was detected amperometrically using an Apollo 4000 free radical analyser (World Precision Instruments) fitted with an NO specific probe (ISO-NOP). A saturated solution of NO (1.91mM at 20 °C) was prepared by bubbling the gas into 10 mL of degassed distilled water for 10 min according to manufacturer's instructions. A 10-fold dilution was performed giving a 191 μM stock solution from which subsequent aliquots (10, 20, 40, 60, 80 and 100 μL) were diluted into 20 mL of PBS buffer (pH = 7.4, 2.7 mM KCl, 135 mM NaCl, 1.75 mM KH_2PO_4 , 10 mM Na_2HPO_4) to calibrate the instrument response. Baseline NO levels were monitored for 5 min in 20 mL PBS solution (pH = 7.4 at 25 °C) followed by injection of C3Ds **1**, **4-12** (50 mM). NO levels were monitored for 15 min. Cleavage of the C3Ds was promoted by addition of 25 μL 40 $\text{U}\cdot\text{mL}^{-1}$ *P. aeruginosa* β -lactamase (Sigma, L6170) or 100 μL 40 $\text{U}\cdot\text{mL}^{-1}$ *Bacillus cereus* β -lactamase (Sigma, 61305). After steady state NO concentrations were reached, a second aliquot of β -lactamase was added. NO concentrations (nM) were established using a calibration curve.

Cytotoxicity in HEK293 Cells

Growth inhibition of HEK293 cells was determined by measuring fluorescence at ex:530/10 nm and em:590/10 nm after the addition of resazurin (25 $\mu\text{g}/\text{mL}$ final concentration)

and incubation at 37 °C and 5% CO₂ for additional 3 h. The fluorescence was measured using a Tecan M1000 Pro monochromator plate reader. The percentage of growth inhibition was calculated for each well, using the Negative Control (media only) and Positive Control (cell culture without inhibitors) on the same plate as references. CC₅₀ (concentration at 50% cytotoxicity) were calculated by curve fitting the inhibition values vs. log(concentration) using a Sigmoidal dose-response function with variable values for bottom top and slope.

Biofilm formation by P. aeruginosa CF clinical strains PA30 and PA68

P. aeruginosa clinical isolates PA30 and PA68 were confirmed to form reproducible *ex-vivo* biofilms using a crystal violet (CV) assay in 96 well microtitre plates. Biofilms were cultivated in M9 minimal media (pH 7; Formedium) containing 48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 19 mM NH₄Cl, and supplemented with 2 mM MgSO₄ (Sigma Aldrich), 100 uM CaCl₂ (Sigma Aldrich) and 20 mM glucose (Sigma Aldrich). Overnight cultures were diluted 1 in 100 into freshly prepared M9 minimal media and added into the wells of a flat-bottom tissue culture-treated 96 well microtitre plate (Thermo Scientific) with a minimum of six replicate wells per strain. Well locations were altered such that, of the 6 replicate wells, 3 were located near the edge of the plate and 3 near the centre of the plate to account for the effects of well location on biofilm growth. Uninoculated M9 minimal media served as the negative control. Four 96 well microtitre plates were set up for each strain for sacrificial analysis on days 1, 2, 3 and 5. Plates were incubated at 37 °C with daily media changes for biofilms grown for 2 or more days. Following the incubation period, the planktonic culture suspensions were gently aspirated and discarded. The wells were washed twice with PBS to remove any non-adherent cells. Biofilms were stained with 0.1% CV (Sigma Aldrich) for 15 mins, and then rinsed with water 3 to 4 times, or until clear, and left to air dry overnight. CV stained biofilms were resolubilised in 30% acetic acid (Sigma Aldrich) for 20 mins and

transferred to a new 96 well microtitre plate, and the absorbance measured at 584 nm (FLUOstar Omega Microplate Reader, BMG Labtech). Consistent and high absorbance readings were obtained with PA30 and PA68 on all days, confirming reproducible biofilm growth by both strains.

Confirmation of NO-mediated biofilm dispersion by strains PA30 and PA68

The dispersal responses of biofilms grown from *P. aeruginosa* CF clinical strains PA30 and PA68 to the spontaneous nitric oxide donor sodium nitroprusside (SNP) were examined using the CV microtitre plate assay. Briefly, overnight cultures were diluted 1 in 100 into freshly prepared M9 minimal media and added into flat-bottom tissue culture-treated 96 well microtitre plates (Thermo Scientific) and incubated at 37 °C for 24 h. Uninoculated M9 minimal media served as the negative control. After incubation, the planktonic suspension was gently aspirated and discarded, and biofilms were washed once with M9 minimal media to remove non-adherent cells. SNP solution was prepared fresh in M9 minimal media, serially diluted, and added to wells containing biofilms. A minimum of 6 replicate wells were used for each treatment concentration, with the well locations randomized as previously described. Following incubation at 37 °C for a further 20 h, planktonic suspensions were transferred to a new 96 well plate and the absorbance measured at 584 nm. Biofilms were washed twice with PBS, stained with 0.1% CV for 15 mins, resolubilised in 30% acetic acid and the absorbance was measured at 584 nm. Absorbance readings confirmed dispersal responses of PA30 and PA68 *ex-vivo* biofilms to a range of SNP concentrations (Supporting Information Figure S2).

Confocal laser scanning microscopy experiments with PA30 and PA68 biofilms

Overnight cultures of the strains were diluted 1 in 100 in M9 minimal media, and 2.5 mL volumes were used to inoculate 35 mm glass-bottom microwell plates (MatTek

Corporation). The plates were incubated at 37 °C with shaking at 50 rpm for 24 h to allow biofilm development. Biofilms were treated with test compounds solubilised in M9 minimal media and re-incubated at 37 °C for 3 h. Post-treatment, biofilms were washed twice with Hank's balanced salt solution (HBSS) to remove non-adherent cells. Biofilm viability was assessed using fluorescent dyes SYTO9 and propidium iodide (PI), as part of the LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies). SYTO9 stains bacterial cells with intact membranes green, whereas PI stains dead cells or cells with compromised membranes red. Following the manufacturer's instructions, stock solutions of each dye were diluted to 2 µg/mL, added to the glass-bottom microwell plates and incubated in the dark at room temperature for 20 minutes. Biofilms were examined under an inverted Leica SP8 confocal laser scanning microscope using the x63 glycerol immersion lens and argon and DPSS laser lines at 488nm and 561 nm respectively, with scanning at 1 µm intervals. A total of five replicate images were taken from each biofilm grown in the microwell plates. Imaged locations were kept constant and mathematically chosen to avoid bias; one image was taken at the centre of the well, and four images were taken at the centre of each theoretical well quadrant. Images were obtained and analysed using the LAS X software (Leica Microsystems GmbH). COMSTAT 2.0 software was used for quantitative analysis of images. The parameters calculated by COMSTAT included: (1) Total Biomass ($\mu\text{m}^3/\mu\text{m}^2$) = volume of biological material occupied by the biofilm divided by the substratum area, (2) Thickness Distribution (μm) = average thickness of the biofilm calculated from a list of the thicknesses of biofilm structures and their prevalence. This measure ignores the presence of pores or voids within the biofilm, (3) Maximum Height (μm) and (4) Total Surface Area coverage (μm^2) are descriptors of the entire biofilm formed on the microwell plate substratum.

Murine P. aeruginosa lung infection model

Dose-escalation studies performed prior to the infection model

(i) Animals: Male Institute of Cancer Research (ICR) mice weighing 25 ± 5 g were provided by BioLasco Taiwan (under Charles River Laboratories License). Animals were acclimated for 3 days prior to use and were confirmed to be in good health. All animals were maintained in a hygienic environment with controlled temperature (20 – 24 °C), humidity (30% - 70%) and 12 hour light/dark cycles. Free access to sterilized standard lab diet [MFG (Oriental Yeast Co., Ltd., Japan)] and autoclaved tap water were made available. All aspects of this work, including housing, experimentation, treatments and disposal of animals, were performed in accordance with the Guide for the Care and Use of Laboratory Animals: Eighth Edition (National Academy Press, Washington, D. C., 2011) in an AAALAC-accredited animal laboratory facility. The animal care and use protocol was reviewed and approved by the Institutional Animals Care and Use Committee (IACUC) at Pharmacology Discovery Services Taiwan, Ltd.

(ii) Protocol: AMINOPIP2-ceftazidime **12** was administered via subcutaneous injection (SC) to groups of three male ICR mice. The compound was formulated in water for injection (WFI) at 10, 15, 20 and 60 mg/mL and administered at 5 mL/kg. Animals received an initial dose of 50 mg/kg. If the animals survived for 72 hours, the dose was increased for the next cohort to 100 mg/kg. If the animals survived for 72 hours after 100 mg/kg, the dose was increased to the highest tested dose 300 mg/kg for the next cohort. Full clinical examinations and body weight changes were assessed. At each dose level, animals were observed for the presence of acute toxic symptoms (mortality, convulsions, tremors, muscle relaxation, sedation, etc.) and autonomic effects (diarrhoea, salivation, lacrimation, vasodilation, piloerection, etc.) during the first 30 minutes, and again at 1 hour (Supporting Information Table S6). Body weights are recorded pre-dose and at 72 hours (Supporting Information Table S7). The animals were observed and mortality noted daily (Supporting Information Table S8).

Plasma and lung pharmacokinetics of AMINOPIP2-ceftazidime 12 in mice after subcutaneous dosing

(i) Protocol: AMINOPIP2-ceftazidime **12** was dissolved in water for injection and administered via SC injection at 75 mg/kg (5 mL/kg) to male ICR mice weighing 20-30 g (n = 3). Drug-free mice (n = 3) were included as controls. Blood aliquots (300-400 μ L) were collected via cardiac puncture from individual anesthetized mice 5, 15, 30, 45, 60, 180, 360 mins after injection in tubes coated with lithium heparin, mixed gently, then kept on ice and centrifuged at 2,500 \times g for 15 minutes at 4°C, (within 1 hour of collection). For control animals, blood was collected by cardiac puncture. The plasma was then harvested and kept frozen at -70 °C until further processing. Immediately after blood sampling, the whole lungs were quickly removed, rinsed with cold saline (0.9 % NaCl, g/mL), blotted with dry gauze, weighed, and kept at -70 °C until further processing. Lungs were homogenized in an appropriate volume of cold PBS (pH 7.4) for 10 seconds on ice. The lung homogenate was then stored at -70 °C until further processing.

The plasma samples were processed using acetonitrile precipitation and analyzed by LC-MS/MS. A plasma calibration curve was generated from aliquots of drug-free plasma spiked with AMINOPIP2-ceftazidime **12** at the specified concentration levels (Supporting Information Figure S3(a)). The spiked plasma samples were processed together with the unknown plasma samples using the same procedure. The processed plasma samples were stored at -70 °C until LC-MS/MS analysis, at which time peak areas were recorded and the concentrations of AMINOPIP2-ceftazidime **12** in the unknown plasma samples determined using the calibration curve.

Each lung homogenate was centrifuged at 5400 \times g for 15 minutes at 4 °C. Supernatants were subsequently processed using acetonitrile precipitation and analyzed by LC-MS/MS. A

lung calibration curve was generated from aliquots of drug-free lung homogenate spiked with AMINOPIP2-ceftazidime **12** the specified concentration levels (Supporting Information Figure S3(b)). The spiked lung homogenate samples were processed together with the unknown lung homogenate samples using the same procedure. The processed lung samples were stored at -70°C until the LC-MS/MS analysis, at which time peak areas were recorded and the concentrations of AMINOPIP2-ceftazidime **12** in the unknown lung samples were determined using the appropriate calibration curve. Plots of plasma and lung concentration of AMINOPIP2-ceftazidime **12** versus time were constructed (Figure S4(a) and S4(b), respectively) and lung:plasma ratios calculated (Supporting Information Table S9).

Infection model protocol

Pseudomonas aeruginosa strain FDA-CDC AR-BANK#0264 was received from the FDA-CDC AR Bank in 2017 and cryopreserved as single-use frozen working stock cultures, stored at -80 °C. The stock was thawed at room temperature prior to use. A 0.2 mL aliquot was inoculated into 20 mL Tryptic soy broth (TSB) and incubated at 35-37 °C with shaking (250 rpm) for 6 h. One mL of the 6 h culture was used to seed 99 mL TSB and incubated at 35-37 °C with shaking at 250 rpm for 16 h. Cells in 20 mL culture were pelleted by centrifugation ($3,500 \times g$) for 15 minutes and re-suspended in 10 mL cold PBS $>1 \times 10^9$ CFU/mL, OD₆₂₀ 1.2-1.6. The culture was diluted in PBS to a target inoculum of 5×10^7 CFU/mL. The actual colony counts were determined by plating dilutions onto nutrient agar (NA) plates followed by incubation for 20-24 h. The CFU counts were 4.55×10^6 CFU/mL.

Groups of 5 female BltW:CD1 (ICR) mice weighing 22 ± 2 g were used. Animals were immunosuppressed by two intraperitoneal injections of cyclophosphamide, the first 150 mg/kg 4 days before infection (Day -4) and the second 100 mg/kg 1 day before infection (Day -1). On day 0, animals were anesthetized with etomidate-lipuro emulsion (20 mg/10 mL; 20 mg/kg

IV) and inoculated intranasally with *P. aeruginosa* FDA-CDC AR-BANK#0264 suspension (0.02 mL/mouse). The target inoculation density was 1.0×10^6 CFU/mouse and the actual count was 9.1×10^5 CFU/mouse. Vehicle (5% DMSO in 0.9% NaCl), AMINOPIP2-ceftazidime **12** and ceftazidime (both at two doses; 50 and 120 mg/kg in 5% DMSO in 0.9% NaCl) were administered subcutaneously (SC) six times at 4 hr intervals (q4h) starting 2 hr after infection for a total dose of 300 and 720 mg/kg over 24 h. Animals were sacrificed 26 h after inoculation by CO₂ asphyxiation and the lung tissues were harvested and weighed for each of the test animals. The removed lung tissue was homogenized in 1 mL sterile PBS (pH 7.4) and stored on ice for up to 2 h. Serial 10-fold dilutions in PBS were generated. Aliquots of the dilutions (100 μ L) were separately plated onto MacConkey II agar plates for bacterial enumeration. The bacterial counts (CFU/g) in lung tissue homogenates were calculated and the percentage decrease in counts compared to the corresponding vehicle control was calculated using the formula:

$$\text{Decrease (\%)} = [(\text{CFU/g of vehicle} - \text{CFU/g of treatment}) / (\text{CFU/g of vehicle})] \times 100\%$$

One-way ANOVA with Tukey's multiple comparisons test was performed to assess statistical difference in the lung bacterial counts for the AMINOPIP2-ceftazidime **12** and ceftazidime-treated mice compared to vehicle control, and to each other (* = $p < 0.05$).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at

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^1H and ^{13}C NMR spectra of all compounds and supporting biological activity and pharmacokinetic data.

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Author Contributions

A.R, T.M and F.A synthesized the compounds under the direction of M.R.N-J and M.J.K. A.R and T.M performed β -lactamase-triggered NO release assays. O.S completed MIC measurements, nitrocefin β -lactamase activity assays and CV/CLSM biofilm experiments at the UK National Biofilms Innovation Centre, University of Southampton (UK). A.G.E, J.Z, M. A. T. B and M.A.C completed the spectrum of activity screening, eukaryotic cell cytotoxicity and haemolysis assays at CO-ADD (University of Queensland, Australia). V.R, W.H.P and S.A.R completed the spectrum of activity screening at Nanyang Technological University (Singapore). V.D and M.C completed the *Neisseria gonorrhoeae* screening at the University of Southampton (UK). J.C and G.M.C completed antibacterial screening at the University of Otago (NZ). S.A.R, S.N.F, J.S.W and M.J.K directed the study. All authors contributed to drafting and revision of the manuscript.

Notes

The authors declare no competing financial interests.

ABBREVIATIONS

Boc, *tert*-butoxycarbonyl; CAZ, ceftazidime; C3D, cephalosporin-3'-diazoniumdiolate; CF, cystic fibrosis; CFL, cefaloram; CFU, colony forming units; CLSI, Clinical Laboratory Standards Institute; CLSM, confocal laser scanning microscopy; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; CZO, cefozopran; DMAP, *N,N*-dimethylamino pyridine; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; FEP, cefepime; MIC, minimum inhibitory concentration; NONOate, diazeniumdiolate; NO, nitric oxide; PBP, penicillin-binding protein; PMB, *p*-methoxybenzyl; SC, subcutaneous; SNP, sodium nitroprusside; TFA, trifluoroacetic acid;

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