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Rational Drug Design

Cephalosporin-3′-diazeniumdiolates: Targeted NO-Donor Prodrugs for Dispersing Bacterial Biofilms**

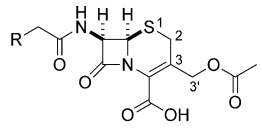
*Nicolas Barraud, Bharat G. Kardak, Nageshwar R. Yepuri, Robert P. Howlin, Jeremy S. Webb, Saul N. Faust, Staffan Kjelleberg, Scott A. Rice, and Michael J. Kelso**

Biofilms are sessile communities of microbial cells contained within a self-produced exopolymeric matrix. Bacteria encased in biofilms exhibit upwards of 10–1000-fold higher resistance to biocides and traditional antibiotics than their planktonic counterparts (i.e. floating, unattached), and they are less susceptible to host immune defenses.[1,2] Accordingly, chronic antimicrobial-tolerant bacterial infections are often biofilm-based (e.g. infections on indwelling medical devices and incurable *Pseudomonas aeruginosa* respiratory infections in cystic fibrosis (CF) sufferers).[3] Currently there are few effective therapeutics for clearing biofilm-based infections, and a critical need exists for new agents and treatment strategies.[2,4,5]

In recent years, it has emerged that many bacteria transition between the planktonic and biofilm states, and that given the correct environmental cues, biofilm bacteria can be induced to undergo coordinated dispersal and reversion to the planktonic form.[6] A putative anti-biofilm strategy has emerged that uses agents to first trigger biofilm dispersion, so that the more susceptible planktonic bacteria can be cleared by conventional antibiotics.[7] We previously reported that picomolar and low nanomolar concentrations of nitric oxide (NO) trigger biofilm dispersion in *P. aeruginosa* and multispecies biofilms,[8,9] and that the NO-donor sodium nitroprusside (SNP) greatly enhances the efficacy of antimicrobial compounds (e.g. tobramycin) in removing established *P. aeruginosa* biofilms.[10] While several studies have supported our findings,[11–14] other contradictory reports suggest that NO has no effect on biofilms or promotes biofilm formation.[15–18] These inconsistencies may arise from differences in experimental conditions[16] and from the fact that only low NO concentrations induce biofilm dispersal.[17] Nevertheless, mounting evidence supports using NO-induced dispersion in combination with antibiotics as a new strategy for clearing chronic biofilm infections.

Many compounds are known that spontaneously produce NO in aqueous solution (like SNP), and many of these may be useful as biofilm dispersants.[19] From a clinical perspective, however, prodrugs that release NO only upon reaction with biofilm-specific enzymes (e.g. β-lactamase) are more attractive because of their potential for targeting NO release to biofilms, thus limiting exposure of host tissues to exogenous NO. Herein, we report the synthesis of a cephalosporin-3′-diazeniumdiolate NO-donor prodrug and demonstrate its effectiveness as a β-lactamase-triggered *P. aeruginosa* biofilm dispersant.

Cephalosporins with leaving groups at the 3′-position (e.g. acetate in Cefaloram **1** and Cefalotin **2**, Figure 1) are known to spontaneously release the leaving group following β-lactam ring cleavage.[20,21] It was reasoned that prodrugs could be created which selectively release an NO-donor upon reaction with biofilm β-lactamases (and conceivably transpeptidases, the principal target of bactericidal cephalosporins).[22] Diazeniumdiolates (also known as NONOates) seemed to be the ideal NO-donor for inclusion in the prodrugs, because diazeniumdiolates alkylated at their terminal oxygen are



R = Ph Cefaloram **1**
= 2-thienyl Cefalotin **2**

Figure 1. Chemical formulas of cephalosporin antibiotics, Cefaloram **1** and Cefalotin **2**.

[*] Dr. B. G. Kardak, Dr. N. R. Yepuri, Dr. M. J. Kelso
School of Chemistry, University of Wollongong, 2522 (Australia)
E-mail: mkelso@uow.edu.au
Dr. N. Barraud, Prof. S. Kjelleberg, Dr. S. A. Rice
School of Biotechnology and Biomolecular Sciences and Centre for Marine Bio-Innovation, University of New South Wales (Australia)
Prof. S. Kjelleberg, Dr. S. A. Rice
The Singapore Centre on Environmental Life Sciences Engineering, Nanyang Technological University (Singapore)
Dr. R. P. Howlin, Dr. J. S. Webb, Dr. S. N. Faust
NIHR Respiratory Biomedical Research Unit, University of Southampton and
University Hospital Southampton NHS Foundation Trust (UK)
Dr. R. P. Howlin, Dr. J. S. Webb
Centre for Biological Sciences, University of Southampton (UK)
Dr. S. N. Faust
NIHR Wellcome Trust Clinical Research Facility, University of Southampton and University Hospital Southampton, NHS Foundation Trust (UK)

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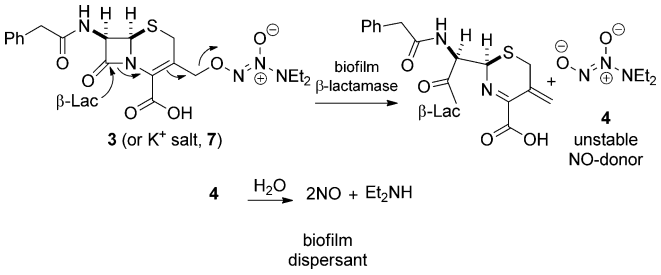
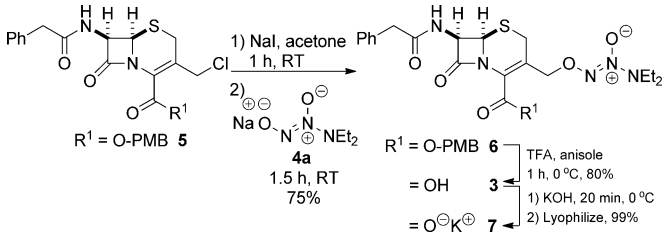
stable compounds that spontaneously produce NO only after cleavage of the O–C bond.[23] Cephalosporin-3′-diazeniumdiolate **3**, a composite of Cefaloram **1** and (*Z*)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate **4** (Scheme 1), was



Scheme 1. Proposed mechanism of β-lactamase-triggered NO release and biofilm dispersion by cephalosporin-3′-diazeniumdiolate **3**/**7**.

the prototype prodrug targeted for study. Diazeniumdiolate **4** was chosen for its short NO-release half-life at pH 7.4 ($t_{1/2}$ = 2.0 min),[24] because rapid NO release from the liberated NO-donor is crucial for clinical applications to ensure the donor does not diffuse away from the biofilm before releasing NO. Cefaloram was chosen as the base cephalosporin because it is more easily synthesized. A putative mechanism for β-lactamase-triggered release of NO from **3** (or its K^+ salt, **7**) and subsequent NO-mediated biofilm dispersion is outlined in Scheme 1.

The synthesis of **3**/**7** centered around a key nucleophilic displacement of the allylic 3′-chloride of the commercially available *p*-methoxybenzyl (PMB)-protected cephalosporin ester **5** with **4a** (sodium salt of **4**) to form the *O*-alkylated diazeniumdiolate adduct **6** (Scheme 2). A Finkelstein con-



Scheme 2. Synthesis of cephalosporin-3′-diazeniumdiolate free-acid **3** and K^+ carboxylate salt **7**.

version (NaI/acetone) of **5** to the allylic iodide, followed by in situ addition of freshly prepared solid **4a** was the optimal procedure, providing pure **6** in 75% yield of the isolated product. Deprotection of **6** using neat trifluoroacetic acid in the presence of anisole for 1 hour at 0 °C afforded the free acid **3** (80% yield), which was converted to the water-soluble potassium carboxylate salt **7** (99% yield) by stirring with 1.0 equivalent of KOH in water for 20 minutes at 0 °C followed by lyophilization.

Release of NO from **7** in the presence of a commercial β-lactamase (*Bacillus cereus* penicillinase, Sigma) was studied amperometrically at varying pH values (Figure 2 A). Com-

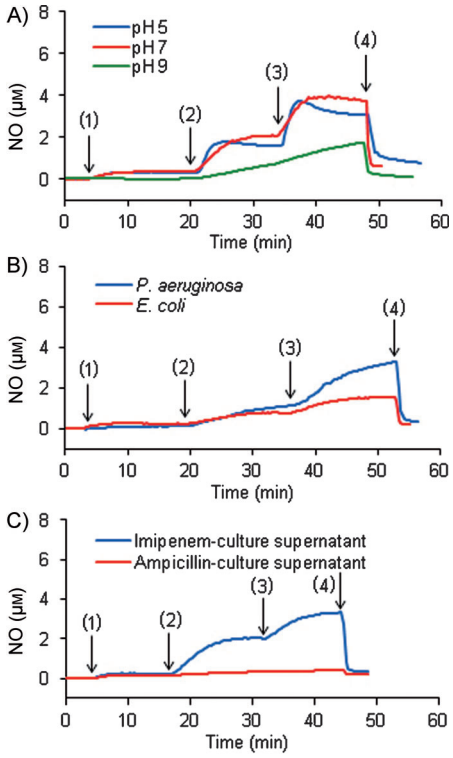


Figure 2. Amperometric characterization of NO release from **7**. A) NO release in the presence of penicillinase at varying pH values. Arrows indicate addition of the following to a reaction vial at 25 °C containing 10 mL tris buffer at pH 9.0, 7.0, or 5.0: 1) 10 μL of 100 mM **7**, 2) 5 μL of 0.1 U μL^{-1} penicillinase, 3) 10 μL of 0.1 U μL^{-1} penicillinase, 4) 80 μL of 10 mM PTIO. B) NO release in the presence of cell extracts of *P. aeruginosa* pretreated with 50 μg mL^{-1} ampicillin or non-β-lactamase-expressing *E. coli*. Arrows indicate addition of the following to a reaction vial containing 10 mL tris buffer at pH 7.0: 1) 10 μL of 100 mM **7**, 2) 100 μL cell extract, 3) 200 μL cell extract, 4) 80 μL of 10 mM PTIO. C) NO release in the presence of supernatants from *P. aeruginosa* cultures grown for 5 h in the absence of antibiotic then treated for 1 h with imipenem (0.5 μg mL^{-1}) or ampicillin (100 μg mL^{-1}). Arrows indicate addition of the following to a reaction vial containing 10 mL tris buffer at pH 7.0: 1) 10 μL of 100 mM **7**, 2) 500 μL supernatant, 3) 500 μL supernatant, 4) 80 μL of 10 mM PTIO.

pound **7** (100 μM) alone in Tris buffer (pH 5.0, 7.0, or 9.0) was stable and did not release NO. Addition of penicillinase (0.05 U mL^{-1}) at pH 7.0 led to rapid evolution of NO from **7**, reaching a steady-state concentration of approximately 2.0 μM NO within 15 minutes. The NO increased to approximately 4.0 μM upon addition of a further 0.1 U mL^{-1} penicillinase, and again the steady-state concentration was reached within 15 minutes. Quenching of this response with the free-radical scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) confirmed that the amperometric signal was from NO release. A similar release profile was measured at pH 5, while at pH 9 NO release was significantly decreased.

Release of NO from **7** was then studied in the presence of *P. aeruginosa* cell lysates, grown either in the presence of sub-inhibitory concentrations of ampicillin (which activates intracellular β-lactamases localized in the periplasmic space),[25] or non-β-lactamase-producing *Escherichia coli* cells. The *P. aer-*

uginosa extracts were found to generate steady-state NO concentrations of approximately 1.0 μM and 3.0 μM from **7** within 15 minutes, after successive additions of 100 μL and 200 μL cell extract, respectively (Figure 2B). A decreased, yet measurable, release of NO was detected with *E. coli* extracts, indicating that non-β-lactamase-producing bacteria can also cleave **3**, most likely by reaction with transpeptidases.

Release of NO from **7** was measured next in the presence of supernatants from *P. aeruginosa* cultures which had been pre-incubated with sub-inhibitory concentrations of either imipenem, an inducer of extracellular β-lactamases, or ampicillin (Figure 2C).[25] The imipenem-culture supernatants were found to trigger robust NO generation, and the amipicillin-culture supernatants produced no measurable NO. These findings, together with those from Figure 2B where NO release from **7** was demonstrated in the presence of ampicillin-treated *P. aeruginosa* extracts, are consistent with NO release being triggered by both extracellular and intracellular (periplasmic) β-lactamases.

The ability of *P. aeruginosa* cells to sense and genetically respond to NO released from **7** was tested using a strain of *P. aeruginosa* that expresses green fluorescent protein (GFP) under the control of the NO-responsive *nirS* promoter.[9] Compound **7** (150 μM) was found to induce approximately 25% more GFP expression than that of an equivalent concentration of SNP; this response was even greater in cells grown in the presence of a sub-inhibitory concentration of ampicillin (Figure 3). The enhanced response was equivalent to the expression when **7** was co-administered with penicillinase to cells grown in the absence of ampicillin.

Dispersal of *P. aeruginosa* biofilms by **7** was examined using microtiter plate biofilm assays. Established biofilms which had been pretreated with sub-inhibitory imipenem (0.5 μg mL^{-1}) for 1 hour showed a rapid and dose-dependent dispersal response to **7** in the range 2–100 μM (Figure 4), with 10 μM **7** removing 70% of the biofilm mass after treatment for 15 minutes. Pretreating biofilms with imipenem before exposure to **7**, produced similar dispersal effects to co-administering **7** with penicillinase to cells that had not been pretreated (Supporting Information, Figure S1).

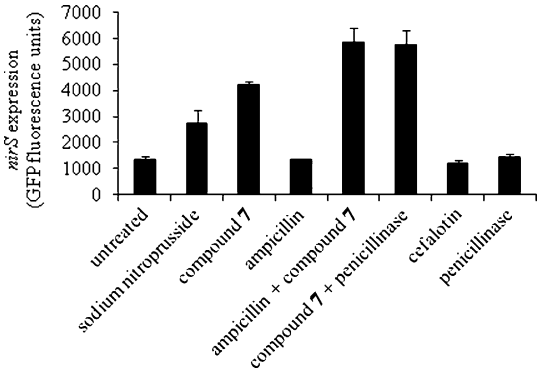


Figure 3. Compound **7** induces an NO-dependent genetic response (*nirS*) in a *P. aeruginosa* NSGFP reporter strain. NSGFP cells grown in the presence or absence of ampicillin (50 μg mL^{-1}) were exposed to SNP (150 μM), compound **7** (150 μM), compound **7** (150 μM) plus penicillinase (0.2 U mL^{-1}), cefalotin **2** (150 μM) or penicillinase (0.2 U mL^{-1}), or left untreated.

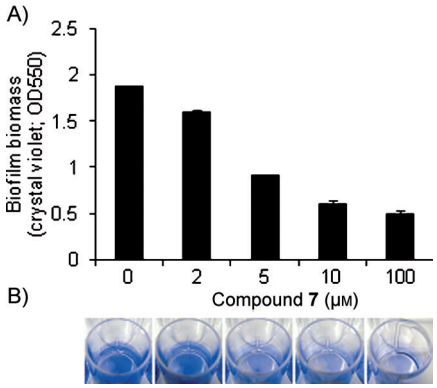


Figure 4. Dose-dependent dispersal of *P. aeruginosa* biofilms by **7**. A) *P. aeruginosa* biofilms grown in microtiter plates with shaking at 37°C were pretreated with imipenem (0.5 μg mL^{-1}) for 1 h and then exposed to various concentrations of **7** (15 min) before quantifying biofilm mass by crystal violet staining.[26] B) Stained biofilms treated with the indicated concentrations of **7**.

The biofilm dispersing properties of **7** were confirmed using a continuous-flow biofilm culture assay. *P. aeruginosa* PAO1 biofilms were established in glass microfermenters, receiving a continuous flow of fresh M9 minimal medium. The inlet was switched to vessels containing fresh medium, with or without **7** (100 μM), and OD_{600} measurements of the effluent were taken. A rapid and significant increase in released cells was observed after addition of **7**, while the amount of cells released from untreated biofilms remained unchanged (Supporting Information, Figure S2).

Our final experiments used microtiter-plate assays to examine the effects of **7** on *P. aeruginosa* biofilms when used in combination with two front-line antibiotics. We found that co-administration of **7** (10 μM) with tobramycin or ciprofloxacin resulted in further decrease of log 1.8 and log 1.5, respectively, in the number of colony-forming units (cfu) remaining in biofilms when compared to the equivalent antibiotic treatments in the absence of **7** (Table 1).

Table 1: Compound **7** potentiates the anti-biofilm efficacy of tobramycin and ciprofloxacin.[a]

Antibiotic	Compound **7** [μM]	log decrease [cfu]
Tobramycin	0	2.15 ± 0.16
80 μg mL^{-1}	10	3.92 ± 0.06
	100	3.58 ± 0.15
Ciprofloxacin	0	3.54 ± 0.09
5 μg mL^{-1}	10	5.06 ± 0.02
	100	4.90 ± 0.13

[a] Established *P. aeruginosa* biofilms grown in microtiter plates with shaking at 37°C were pretreated with sub-inhibitory imipenem (10 μg mL^{-1}) for 1 h. Tobramycin or ciprofloxacin were then added either with or without compound **7**, or biofilms were left untreated (control). Biofilm bacteria were further incubated for 1 h, followed by resuspension and quantitation of cfu. Values represent the decrease in cfu on a log scale, compared to control biofilms and are the mean of two independent experiments (± SEM). Bacteria in the supernatants were fully eradicated (below the detection limit; 10 cfu mL^{-1}) after treatment with either antibiotic alone or antibiotic in combination with **7**.

In summary, we have synthesized a novel cephalosporin-based NO-donor prodrug and demonstrated its potential for use in dispersing *P. aeruginosa* biofilms. Selective release of NO from the prodrug through contact with biofilm β-lactamases is an attractive feature, which should allow targeted enhancement of bacterial killing by conventional antimicrobials at sites of biofilm infections, while also minimizing NO-mediated toxicity. Given that cephalosporins have a long history of safe and effective use in humans, we anticipate that cephalosporin-3′-diazeniumdiolates will have acceptable drug-like properties, facilitating their development into clinically useful anti-biofilm therapeutics. Accordingly, compound **7** (and related analogues) are currently being tested as new treatments for chronic biofilm-mediated infections.

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Communications

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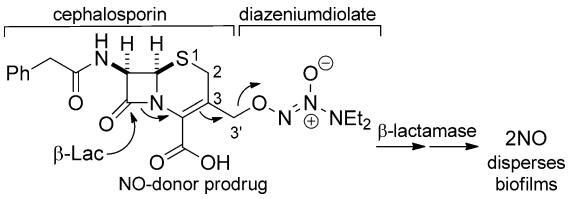
Cephalosporin-3′-diazeniumdiolates: Targeted NO-Donor Prodrugs for Dispersing Bacterial Biofilms



Just say NO to biofilms: NO-donors are used to disperse a bacterial biofilm so that co-administered antibiotics will kill the more susceptible unattached cells. The chemically stable cephalosporin-3′-diazeniumdiolate NO-donor prodrug (see scheme) is activated by bacterial β-lactamases and facilitates this two-step biofilm erradication.