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Synthesis of cephalosporin-3′-diazeniumdiolates: biofilm dispersing NO-donor prodrugs activated by β-lactamase

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Keywords
3, cephalosporin, biofilm, dispersing, no, donor, prodrugs, activated, lactamase, synthesis, diazeniumdiolates, CMMB

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Synthesis of Cephalosporin-3′-Diazeniumdiolates: Biofilm Dispersing NO-Donor Prodrugs Activated by β-Lactamase

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Abstract

Use of biofilm-dispersing NO-donor compounds in combination with antibiotics has emerged as a promising new strategy for treating drug-resistant bacterial biofilm infections. This paper details the synthesis and preliminary evaluation of six cephalosporin-3′-diazeniumdiolates as biofilm-targeted NO-donor prodrugs. Each of the compounds is shown to release NO following reaction with the bacteria-specific enzyme β-lactamase and to trigger dispersion of *Pseudomonas aeruginosa* biofilms *in vitro*.

The discovery of clinically useful therapeutics and novel treatment strategies against bacterial biofilm infections is one of the greatest challenges in modern infectious disease control.[1] In 2006 we reported that low concentrations of the biologically ubiquitous gas nitric oxide (NO) trigger biofilm dispersion in the important human pathogen *Pseudomonas aeruginosa* and demonstrated that the spontaneous NO-donor sodium nitroprusside (SNP) greatly enhances the efficacy of antibacterial compounds (e.g. tobramycin) in the removal of *P. aeruginosa* biofilms.[2] Follow-up studies showed that NO levels in the pM and low nM range mediate dispersal in several other single- and multi-species bacterial and yeast biofilms[3] and that the effects correlate with increases in bacterial phosphodiesterase activity and associated decreases in cyclic-di-GMP levels.[4] These combined results have unveiled a putative new anti-biofilm strategy which uses low concentrations of NO-donor compounds in combination with antibiotics to clear bacterial biofilm infections.

Scheme 1. Proposed mechanism of β-lactamase-triggered NO release and biofilm dispersion by cephalosporin-3′-diazeniumdiolates (e.g. **1**).

Targeted prodrugs capable of releasing NO only after reaction with a bacteria-specific enzyme represent ideal NO-donors for clinical anti-biofilm applications (as opposed to spontaneous NO donors) since they should localise NO to biofilm infection sites and

minimise exposure of host tissues to NO (and associated side-effects). We recently described a rationally designed cephalosporin-3′-diazeniumdiolate NO-donor prodrug **1** which selectively releases NO upon contact with the bacterial enzyme β-lactamase (proposed mechanism shown in Scheme 1).[5] Compound **1** was shown to effect NO-dependent *P. aeruginosa* biofilm dispersal and to act synergistically with front-line antibiotics (tobramycin and ciprofloxacin) in clearing bacterial biofilms. This paper details synthetic efforts towards **1** and demonstrates the versatility of the chemistry by producing five analogues carrying variations in the cephalosporin 7-acylamido side chain and 3′-diazeniumdiolate NO-donor portions. Each of the analogues is shown to release NO upon reaction with β-lactamase and to disperse *P. aeruginosa* biofilms *in vitro*.

Alkylation of sodium diazeniumdiolate salts with ester protected 3′-chloro-cephalosporins appeared as a viable route towards cephalosporin-3′-diazeniumdiolates since terminal oxygen alkylation of sodium diazeniumdiolates with alkyl halides was well established.[6] Initial investigations with diphenylmethyl (DPM)- or *p*-methoxybenzyl (PMB)-protected 3′-chloro-cephalosporin esters **2** showed that alkylation reactions with sodium diazeniumdiolate **3** did not proceed. Finkelstein conversion of **2** to the corresponding allylic iodides (NaI/acetone) followed by purification and reaction with **3** did produce the desired alkylation products **4** under several conditions (Electronic Supplementary Information, Scheme S1), however the yields were consistently low due to double bond migration and formation of inseparable mixtures containing the undesired Δ2-isomers **4a** as the major product.[7] Addition of 1.0 mol eq of solid **3** *in situ* to freshly prepared allylic iodide/acetone solutions of **2** afforded mixtures wherein the Δ3-isomer predominated (i.e., **4**:**4a** = 80:20 for DPM-protected **2**, 90:10 for PMB-protected **2**). The low solubility of **3** in acetone may have reduced solution basicity minimising deprotonation at the cephalosporin 2-position. It is well established that

deprotonation here triggers double bond migration during substitution reactions with basic nucleophiles and cephalosporins carrying leaving groups at the 3′-position.[7]

The optimised alkylation conditions were used to prepare two analogues of **4** carrying variations in the 7-acylamido side chain (R^1: compounds **6**, **8**, Figure 1). Compound **4**, bearing a thiophenacetyl group at R^1 (as found in Cefalotin and other cephalosporins that have been used clinically), was obtained pure in 85% isolated yield from **2** following silica gel chromatography and recrystallisation from MeOH or EtOH. Compound **6**, carrying a phenacetyl group at R^1 (as found in Cephaloram), was obtained in 75% yield from **5**, while the yield of **8** (R^1 = 1-tetrazolylacetyl, as found in Cefazolin and others) was lower at 14% (from **7**) due to appreciable formation of the Δ2-isomer **8a** (42% isolated yield).

It was of particular interest to explore whether diazeniumdiolate salts other than **3** (NO release $t_{1/2}$ = 2.0 min)[8] could be attached to the cephalosporin scaffold, especially those with similarly short NO-release half-lives. We predict that diazeniumdiolates with short half-lives should work best in clinical anti-biofilm applications since rapid NO release from the diazeniumdiolate anions following expulsion from the cephalosporin prodrug would be necessary to limit their diffusion away from biofilms before releasing NO. Reaction of 3′-chloro-cephalosporin PMB ester **5** with sodium diazeniumdiolate **9** ($t_{1/2}$ = 2.8 sec)[8] under the optimised conditions afforded alkylated product **10** in 66% yield. Reactions of **5** with salts **11** ($t_{1/2}$ = 1.9 min)[9] and **13** ($t_{1/2}$ = 2.0 min)[9] similarly produced the desired adducts **12** and **14** in 39% and 80% yields, respectively.

PMB-deprotection using neat trifluoroacetic acid/anisole at 0 ºC proceeded smoothly for all analogues affording the pure cephalosporin-3′-diazeniumdiolate free carboxylic acids **1**, **15**-

19 in 36-93% yields (Figure 1). The free acids **1**, **15**, **17** and **18** could be converted to their water soluble potassium salts (**21**, **20**, **23** and **24**, respectively) by stirring with 1.0 mol eq aqueous KOH at 0 ºC followed by freeze-drying. Applying the same procedure with acids **16** and **19** led to decomposition.

Fig. 1 Synthesis of cephalosporin-3′-diazeniumdiolates.

Release of NO from cephalosporin-3′-diazeniumdiolate free acids **1**, **15**-**19** in the presence of a commercial β-lactamase (penicillinase, Sigma) was studied amperometrically at *p*H 7 in

100 mM Tris buffer (Figure 2). Compound **18** was found to be stable over 15 min following addition to buffer, as indicated by an absence of detectable NO. Compounds **1** and **17** showed evidence of slight decomposition producing low but detectable levels of NO, presumably through β-lactam hydrolysis and expulsion of the NO donor. Compounds **15**, **16** and **19** appeared to be less stable generating 2-3 fold higher levels of NO than **1** or **17** upon addition to buffer. Treatment with β-lactamase (0.1 U/mL) triggered immediate release of NO from each of the acids. Compounds **1** and **18** reached steady-state NO concentrations of ~2 μM within 15 min, while acids **15**, **16**, **17** and **19** produced higher NO levels, reaching steady-state concentrations of ~3-4 μM over the same period. Adding a further 0.2 U/mL of β-lactamase to each of the acids led to additional ~2-fold increases in NO and reestablishment of steady-state NO concentrations within 15 min. Formation of steady-state NO concentrations and release of NO upon addition of a second aliquot of enzyme are consistent with the reaction of the β-lactamase with cephalosporin-3′-diazeniumdiolates leading to enzyme inhibition. Quenching of the amperometric response upon addition of the free radical scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) confirmed that the observed signals were due to NO.

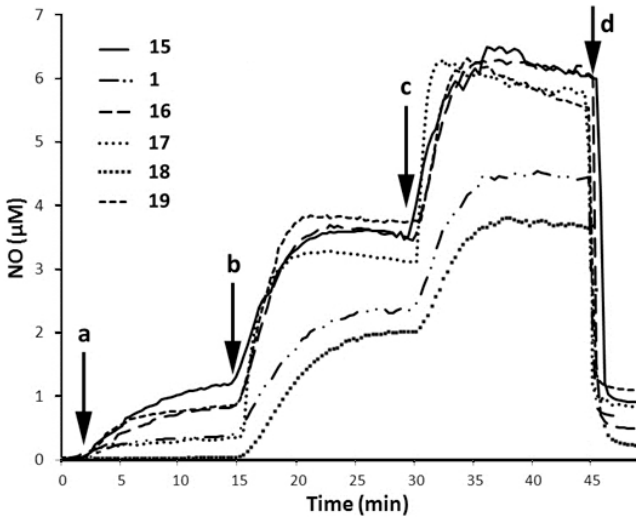

Fig. 2 Amperometric characterisation of NO release from cephalosporin-3′-diazeniumdiolate free acids **1**, **15**-**19** in the presence of β-lactamase. Arrows indicate addition of the following to reaction vials containing 10 mL Tris buffer (100 mM) at *p*H 7.0: (a) 10 μL of 100 mM cephalosporin-3′-diazeniumdiolate free acid, (b) 10 μL 100 U/mL β-lactamase, (c) 20 μL 100 U/mL β-lactamase, (d) 80 μL of 10 mM free radical scavenger PTIO.

Dispersion of *P. aeruginosa* biofilms by cephalosporin-3′-diazeniumdiolate free acids **1**, **15**-**19**, along with the Δ2-isomer **16a**, was examined *in vitro* using microtiter plate biofilm assays (Figure 3).[5] Briefly, *P. aeruginosa* PAO1 wild type biofilms were grown in 24-well plates containing sub-inhibitory imipenem (0.3 μg/mL) to induce β-lactamase expression. After 6 h, the biofilms were treated with test compounds and incubated for 15 min before washing, staining with crystal violet and quantifying the remaining biofilms by measuring OD550 of the homogenized suspensions. Compounds **1** and **15**-**19** all showed dose-dependent biofilm dispersion responses in the range 5−100 μM. Compounds **1** and **17** appeared as the most potent members of the series reducing biofilm mass at 5 μM by 31% and 28%, respectively. The other compounds showed no significant effect at this concentration. At 10 μM, compound **1** reduced biofilm mass by 78%, with only slight further reductions being observed at higher concentrations (87% at 50 μM, 91% at 100 μM). A similar dose dependency was observed with **17**. While less potent, the other four analogues **15**, **16**, **18** and **19** all reduced biofilms by more than 70% at 100 μM. As expected, the Δ2-isomer of **16** (i.e., **16a**) showed no dispersion effect at any concentration, consistent with its inability to undergo the conjugate elimination reaction to expel the diazeniumdiolate anion following β-lactam cleavage.

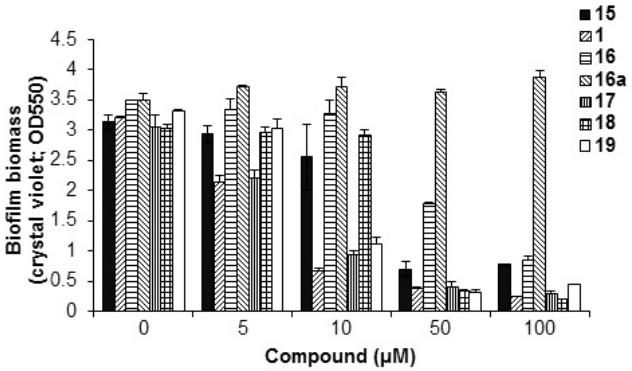

Fig. 3 Dispersal of *P. aeruginosa* biofilms by cephalosporin-3′-diazeniumdiolate free acids **1**, **15-19**. *P. aeruginosa* PAO1 biofilms grown in microtiter plates were pre-treated with imipenem (0.3 μg/mL) before exposing to various concentrations of compounds (15 min) and quantifying the remaining biofilm mass by crystal violet staining and measurement of OD550. (n = 2) Control biofilms treated with imipenem alone produced OD550 readings of ~ 3-3.5.

Conclusions

Bacteria encased in biofilms are known to exhibit upwards of 10−1,000-fold higher resistance to biocides and traditional antibiotics and to be less susceptible to host immune defences than their free-swimming planktonic counterparts.[10] As a result, chronic bacterial infections tend to be biofilm-based. In our anti-biofilm strategy, low concentrations of NO-donors are used to first trigger biofilm dispersion such that the more vulnerable planktonic bacteria can be cleared by conventional antibiotics working in concert with host immune defences.[2-4] It is important to note that NO at the concentrations used are not toxic to bacteria, but rather induce a genetically programmed response. The current work has advanced the strategy significantly towards clinical utility in describing a novel class of drug-like biofilm-targeted NO-donor prodrugs (i.e., cephalosporin-3′-diazeniumdiolates) that make use of the known tendency of cephalosporins to eject leaving groups from the 3′-position via conjugate elimination[12] following β-lactam cleavage (in this case diazeniumdiolate NO donors). Six

examples from the class were prepared using similar chemistry and each was shown to release NO upon contact with β-lactamase and to trigger biofilm dispersion in *P. aeruginosa*, an important pathogen responsible for recalcitrant and often fatal bronchopulmonary biofilm infections, especially in cystic fibrosis (CF) patients.[11] Studies aimed at identifying optimal cephalosporin-3′-diazeniumdiolate development candidates for these and other types of biofilm-based chronic infections are on-going in our laboratories.

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†Electronic Supplementary Information (ESI) available: (a) Experimental procedures, characterisation data and ^{1}H NMR spectra for all compounds. (b) Experimental procedures for amperometric NO release measurements and microtitre plate biofilm dispersion assays. See DOI: 10.1039/b000000x/

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