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Synthesis of Beta-Lactamase Activated Nitric Oxide Donors

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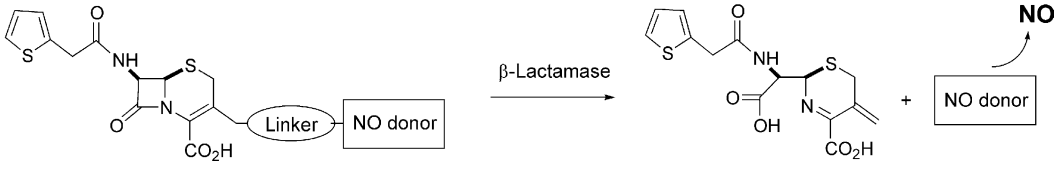
Abstract—In order to achieve site specific delivery of NO, we designed conjugates of cephalosporin with NO donors. NO donors such as cupferron and SIN-1 were evaluated as potential choices for conjugates. Cephalosporin conjugated with SIN-1 demonstrated promising beta-lactamase dependent NO releasing ability.

Nitric oxide (NO) plays important roles in a number of physiological processes including vasodilatation, antiplatelet effects, macrophage-induced cytotoxicity, and neurotransmission.[1,2] To deliver NO to specific targeted cells or sites, one can convert the spontaneous NO releasing agents to stable prodrugs by attaching them to carriers which can be selectively removed in certain target sites. As a result, NO dosage could be concentrated at a specific site even if the prodrugs were systemically distributed. Recently, we reported several classes of enzyme activated NO donors based on this concept. For example, peptide–diazeniumdiolate conjugates were synthesized by attaching diazeniumdiolate to prostate specific antigen (PSA) substrates, these conjugates demonstrated NO release upon activation by PSA.[3] In addition, glycosylated diazeniumdiolates could readily release NO in the presence of certain glycosidase.[4]

Antibacterial effects of NO have been well documented.[5] To address microbial resistance to current antibiotics, we present here a new type of NO prodrugs, conjugates of cephalosporin with NO donors. As shown in Scheme 1, this design is based on the knowledge that hydrolysis of cephalosporin's β-lactam bond by β-lactamase can result in the expulsion of NO donors at the C-3′ position. The released cephalosporin is nontoxic to mammalian cells and the NO donors would generate active NO to kill sensitive bacteria. In fact, the development of β-lactamase dependent prodrugs with the applications in antibody-directed enzyme prodrug therapy (ADEPT) has been an area of particular interest.[6,7] β-Lactamases are small, soluble monomeric enzymes without a mammalian counterpart. They are abundant, available in multiple forms, inexpensive, and retain their activities in vivo. Several studies have demonstrated that β-lactamase can release nitrogen mustard,[8–11] doxorubicin,[12,13] vinca alkaloid,[14] mitomycin C,[15] platinum compounds,[16] and paclitaxel[17] from cephalosporin prodrugs. Our designed β-lactamase activated NO donors may be also useful for ADEPT. If the bacteria antibody and β-lactamase conjugates were applied in vivo, and then our designed NO donors were used, NO would be released around the bacteria and demonstrate bactericidal activities.

Cupferron releases NO upon enzymatic,[18] electrochemical,[19] as well as chemical oxidation.[20] *O*-alkyl cupferron derivatives exhibited significantly improved



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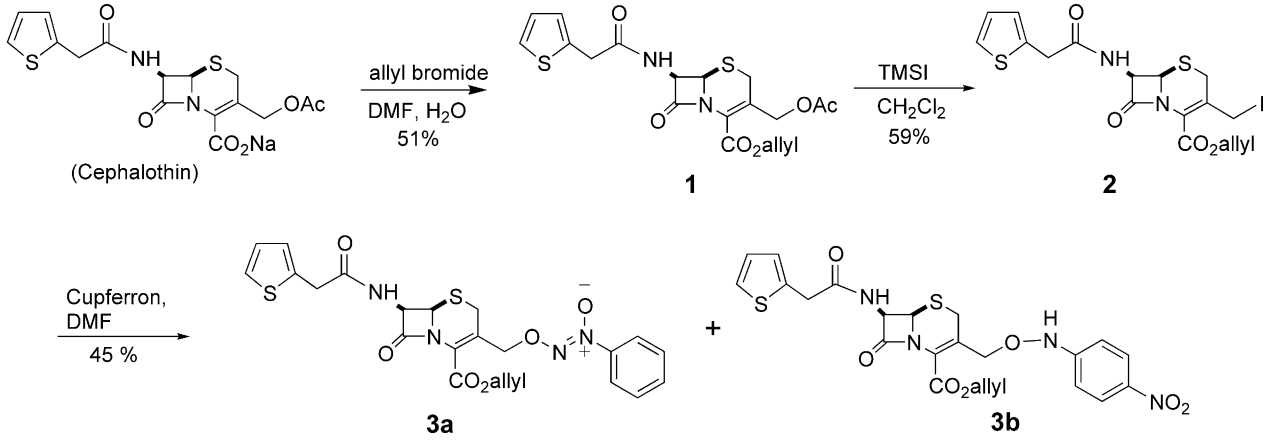
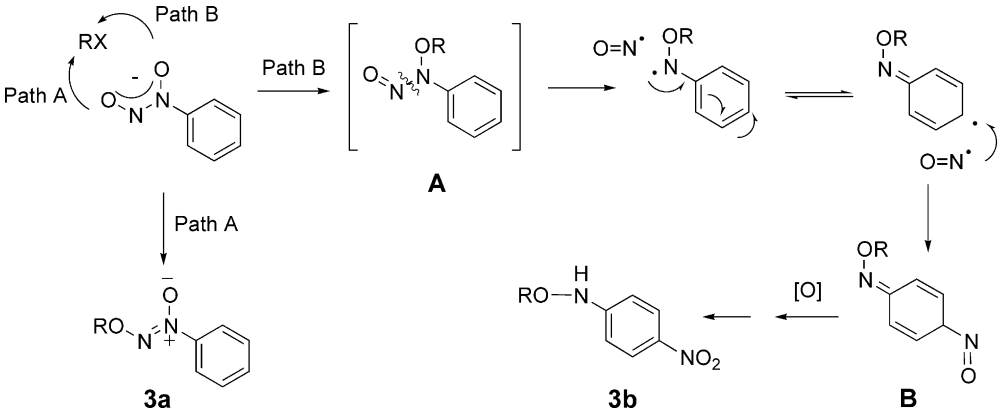
stability compared to their corresponding parent compounds.[21] We first synthesized a conjugate of cephalosporin with cupferron as a model compound. As illustrated in Scheme 2, esterification of cephalothin with allyl bromide gave the ester **1** in good yield.[14] Treatment of **1** with two equivalents of iodotrimethylsilane cleaved the acetoxyl moiety to provide the iodide **2**, which was immediately used in the following step without further purification. Subsequent addition of cupferron gave a mixture of compounds **3a** and **3b** in a molar ratio of 1:2. Separation of the two products was readily achieved by silica gel flash column chromatography. Both compounds **3a** and **3b** have been characterized by ^{1}H, ^{13}C NMR, as well as HRMS.[22] The presence of NO_2 group in **3b** was confirmed by its very strong IR stretches at 1530 and 1341 cm^{-1}. The major product was compound **3b** instead of the expected **3a**. A possible reaction mechanism is proposed in Scheme 3. The *O*-alkylation of cupferron can occur via pathway A and B. When the terminal oxygen attacks the electrophile, the *O*-alkylation reaction occurs via path A and product **3a** is obtained as the major product. In the case of path B, the interior oxygen reacts with the electrophile, which leads to the formation of an intermediate **A**. The N–N bond can be cleaved homolytically to form a NO radical. The addition of the NO radical to the *para* position of the benzene ring affords another intermediate **B**. This unstable intermediate undergoes oxidation to the final product **3b**. Similar observation has been reported in the *O*-alkylation chemistry of neocupferron.[23] Unfortunately, attempts to remove the allylic protecting group of the conjugate **3a** by palladium (0) catalysed hydrolysis[24] did not work, only starting material was recovered. Instead of using allylic protecting group for the acid, a variety of silyate protecting groups such as TBDMS, TMS or TPS were tested, but all of these approaches failed to provide the desired product.

3-Morphorlinosydnonimine (SIN-1) belongs to another important class of NO donors known as sydnonimines. In addition to its potent vasorelaxant and antiplatelet effects,[25] it also displays bactericidal activity.[26] We decided to synthesize a cephalosporin carbamate derivative of the SIN-1, because it has been known that a carbamate moiety at the C-3′ position of the cephem nucleus serves as a good leaving group upon enzyme-catalysed hydrolysis of the β-lactam.

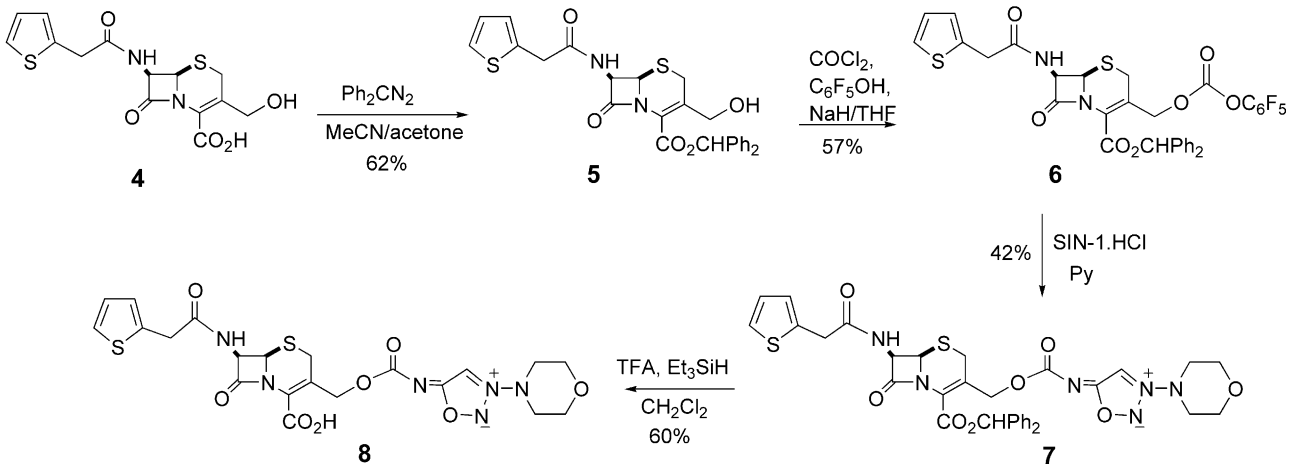
Scheme 4 illustrates the synthetic route undertaken. Selective hydrolysis of the C-3′ acetate of the commercially available cephalothin gave the (hydroxymethyl)cephem **4** in high yield.[27] Subsequent esterification of **4** with diphenyldiazomethane provided the benzhydryl ester **5**.[14] Chloroformate formation in situ (C_6F_5OH/NaH/$COCl_2$) followed by addition of **5** in



Scheme 2.



Scheme 3.



Scheme 4.

the presence of pyridine gave the pentafluorophenyl carbonate **6**. After purification, the carbonate **6** was reacted with one equivalent of SIN-1.HCl in anhydrous pyridine at 0 °C for 2 h to afford an inseparable 4:1 mixture of cephem-3′-carbamate **7** and, possibly, its corresponding 2′-isomer. Acid-catalyzed hydrolysis of the benzhydryl ester in the presence of triethylsilane, as a carbonium ion trap, provided the desired conjugate **8**.[28]

The NO releasing ability of the conjugate **8** was measured through the formation of nitrite (NO_2^-) in solution,[29] following incubation of the compound in the presence or the absence of β-lactamase. SIN-1 was used as control. A stock solution of **8** was prepared in 1.0 mL of methanol. Then 100 μL of the solution was diluted with 500 μL of Tris–HCl buffer (pH = 7.0) to a final concentration of 1.2 mM. Then 100 μL of the solution of penicillinase (0.5 units, SIGMA E.C. 3.5.2.6, Type III: from *Enterobacter cloacae*) in the Tris–HCl buffer (pH = 7.0) was added. The mixture incubated at room temperature for about 1 h before the addition of Griese reagent (150 μL of 1% sulfaniamide in 1 N HCl and 150 μL of 0.1% *N*-(1-naphthyl)-ethylenediamine in 1 N HCl). The absorbance of the solution was then measured at 548 nm. The calibration curves were made from known concentrations of $NaNO_2$ in the same buffer solution to determine the amount of NO_2^- formed in the reactions. The results are summarized in Table 1. As expected, the SIN-1 decomposes easily in the aqueous solution. The conjugate **8** is more stable than SIN-1 under the test conditions, and it releases NO in the presence of β-lactamase.

In summary, we have designed conjugates of cephalosporin with NO donors, creating a new class of NO donor prodrugs. The cephalosporin carbamate of SIN-1 was successfully synthesized and its β-lactamase dependent NO releasing ability was demonstrated. Using this established synthetic strategy, we plan to generate a series of cephalosporin carbamate derivatives. The synthesis of cephalosprin conjugates using other NO donors is also in progress. We hypothesize that this class of NO donors may be a potential drug in the field of ADEPT, as well as a new class of antibiotics.

Table 1. Nitrite concentration in the incubation of compound **8** with or without the β-lactamase, with SIN-1 as control

Substrate (1.0 mM)	$[NO_2^-]$ μM
SIN-1	15.5
8	2.2
8 + penicillinase	10.2

Acknowledgements

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22. Compund **3a**: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.94 (m, 2H), 7.54–7.46 (m, 3H), 7.25 (m, 1H), 7.00–6.96 (m, 2H), 6.38 (d, $J=9.0$ Hz, 1H), 5.98–5.87 (m, 1H), 5.85 (dd, $J=5.0$, 9.0 Hz, 1H), 5.50 (d, $J=13.5$ Hz, 1H), 5.38 (d, $J=16$ Hz, 1H), 5.30 (d, $J=12$ Hz, 1H), 5.18 (d, $J=14$ Hz, 1H), 4.98 (d, $J=5.0$ Hz, 1H), 4.76 (m, 2H), 3.85 (s, 2H), 3.62 (d, $J=18.5$ Hz, 1H), 3.48 (d, $J=18$ Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.95, 164.65, 161.00, 142.98, 134.60, 131.64, 131.07, 129.08, 127.90, 127.56, 126.11, 125.89, 125.70, 121.13, 119.70, 72.70, 67.03, 59.20, 57.43, 37.08, 26.24; MS (ESI): *m/z* 537 (M + Na), 553 (M + K); HRMS (FAB) calcd for $C_{23}H_{22}N_4O_6S_2Na$ [M + Na]: 537.0878, found: 537.0887. Compound **3b**: ^{1}H NMR (acetone-d_6, 500 MHz) δ 9.34 (s, 1H), 8.10 (dd, $J=9.0$ Hz, 2H), 7.32 (d, $J=5.0$ Hz, 1H), 7.07–6.95 (m, 4H), 5.96–5.86 (m, 2H), 5.36 (d, $J=17.5$ Hz, 1H), 5.18 (m, 2H), 4.88 (d, $J=12.5$ Hz, 1H), 4.71 (m, 3H), 3.85 (m, 3H), 3.71 (d, $J=18$ Hz, 1H); ^{13}C NMR (acetone-d_6, 125 MHz) δ 170.58, 165.70, 162.20, 155.54, 137.52, 132.77, 127.42, 127.35, 127.22, 126.18, 126.02, 125.66, 118.82, 112.88, 112.78, 74.21, 66.94, 60.48, 58.65, 37.02, 27.27. MS (ESI) 553 (M + Na), 569 (M + K); HRMS (FAB) calcd for $C_{23}H_{22}N_4O_7S_2Na$ [M + Na]: 553.0828, found: 553.0828.
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28. Compound **8**: ^{1}H NMR (CD_3OD, 500 MHz) δ 8.58 (s, 1H), 7.26 (m, 1H), 6.94 (m, 2H), 5.74 (d, $J=5.0$ Hz, 1H), 5.30 (d, $J=13.0$ Hz, 1H), 5.07 (d, $J=5.0$ Hz, 1H), 5.00 (d, $J=14.0$ Hz, 1H), 3.97 (m, 4H), 3.80 (d, $J=5.0$ Hz, 2H), 3.69 (m, 4H), 3.54 (d, $J=18.5$ Hz, 1H); ^{13}C NMR (CD_3OD, 125 MHz) δ 136.20, 126.58, 124.72, 65.16, 59.57, 57.71, 54.04, 35.93, 25.90; MS (ESI) 551 (M + H), 573 (M + Na); HRMS (FAB) calcd for $C_{21}H_{22}N_6O_8S_2Na$ [M + Na]: 573.0838, found: 573.0851.
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