Fluorescent Probes

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Engineering the Stereochemistry of Cephalosporin for Specific Detection of Pathogenic Carbapenemase-Expressing Bacteria**

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Dedicated to Professor George M. Whitesides on the occasion of his 75th birthday

Abstract: *Reported herein is the design of fluorogenic probes specific for carbapenem-resistant Enterobacteriaceae (CRE) and they were designed based on stereochemically modified cephalosporin having a 6,7-trans configuration. Through experiments using recombinant β-lactamase enzymes and live bacterial species, these probes demonstrate the potential for use in the specific detection of carbapenemases, including metallo-β-lactamases in active bacterial pathogens.*

Antibiotic resistance among Gram-negative bacteria such as *Escherichia coli*, *Klebsiella* species, and other *Enterobacteriaceae* is emerging worldwide at an alarming rate because of the misuse and overuse of antibiotics.[1] Particularly, the resistance against broad-spectrum β-lactam antibiotics has become a major public health concern. Of the many antibiotics, carbapenems have the broadest spectrum of activity and greatest potency against different types of bacteria, and have become the last resort in the treatment of serious bacterial infections.[2] However, carbapenem-resistant *Enterobacteriaceae* (CRE) are now frequently being observed and the number of cases is steadily growing, mostly as a consequence of acquired carbapenemases.[1a,3] Carbapenemases are a group of β-lactamases with the ability to hydrolyze almost all β-lactam antibiotics, including the Ambler class A β-lactamases of the KPC-type, the metallo-β-lactamases (MBLs; Ambler class B) VIM, IMP, New Delhi metallo-β-lactamase (NDM), and the relatively rare class D carbapenemase of the OXA-48 type.[1a,3,4] Carbapenemases have been increasingly reported as the cause of therapeutic failures both in hospital- and community-acquired infections, especially for MBLs because of its transmission between different strains and poor susceptibility to clinically available inhibitors.[1,5] Therefore, rapid and accurate detection of carbapenemase producers is critically important for appropriate antibacterial chemotherapies and rigorous infection control.

Currently, the standard diagnostic methods for the detection of β-lactamases, such as the Modified Hodge Test (MHT)[6] and double-disk synergy test (DDST),[7] lack good specificity and sensitivity, and are time-consuming because they require at least 24–48 hours for accurate detection of bacteria, and days for those bacteria with a slow growth rate. PCR[8] and mass spectrometry[9] based methods have high accuracy and sensitivity, but are associated with disadvantages such as high cost, expensive instruments, and the inability to detect new carbapenemase genes. The Carba NP test recently reported by Nordmann et al.[10] provided an inexpensive approach for rapid CRE detection, but still lacks specificity for MBLs. Fluorescence-based bioanalytical assays offer high sensitivity, ease of use, rapid detection, low cost, and little or no need for biological sample processing before analysis. A number of fluorescent probes that enable the detection of the activity of β-lactamases with high sensitivity have been developed.[11,12] However, most probes lack specificity, and some have been used for detecting MBLs, but only when combined with inhibitors.[12b] Herein, we report the design of a series of fluorogenic probes, based on stereochemistry-controlled cephalosporin with a 6,7-*trans* configuration, for specific detection of carbapenemases, including MBLs, in pathogenic bacteria.

Our design rationale for the probe is based on the fact that carbapenem is highly resistant to most β-lactamases except carbapenemases. A close examination of the structure of carbapenem reveals an *S* configuration at C6 and an *R* configuration at C5 (Figure 1). This *trans* configuration of the

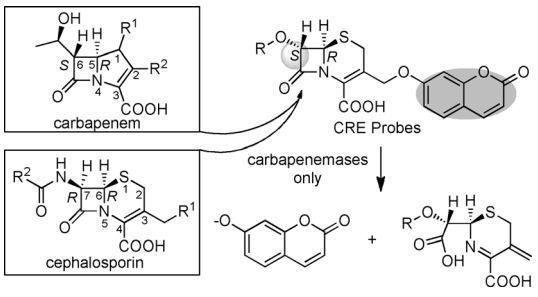

Figure 1. The proposed design of fluorogenic probes for targeting carbapenemases.

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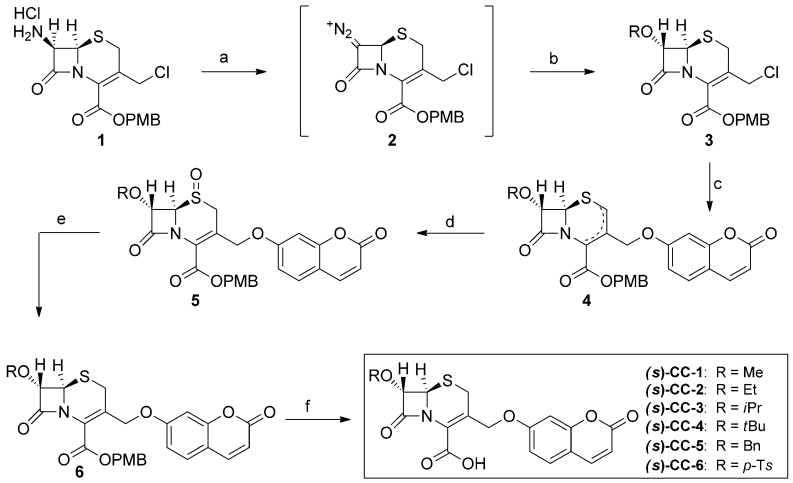
β-lactam ring at C5 and C6 provides antibiotics with good stability against most β-lactamases.[13] In comparison, other lactams such as cephalosporin, a class of β-lactam antibiotics originally derived from the *fungus Acremonium* and commonly used as the scaffold to develop probes for β-lactamases,[11] generally possess an *R* configuration at the corresponding C7. We hypothesized that the conversation of the *R* configuration in the cephalosporin to *S* might render it specific for carbapenemases. Figure 1 outlines this concept with a designed carbapenemase-specific probe containing the caged umbelliferone at the 3′-position. It should initially show no fluorescence and once it is hydrolyzed by a carbapenemase, the free fluorophore would be released, thus leading to the fluorescence emission.

A number of fluorogenic probes, each of which contains a cephalosporin moiety with the *S* configuration at C7, were synthesized as depicted in Scheme 1. The inversion of the configuration in cephalosporin was achieved by an S_N2 reaction between alcohols (R) and a diazo ketone intermediate,[14] which was prepared from the *para*-methoxybenzyl (PMB) ester of 7-aminocephalosporanic acid (**1**) and $NaNO_2$ under acidic conditions, in a quantitative yield. The R group was varied to probe the substrate preference of carbapenemases. All the probes were finally purified by HPLC and characterized by NMR spectroscopy and high-resolution mass spectroscopy (HRMS) before use in the biological assays.



Scheme 1. Synthesis of fluorogenic probes for targeting carbapenemases. a) $NaNO_2$, 2 N H_2SO_4, CH_2Cl_2, 0 °C, 1 h. b) ROH, *p*-TsOH, CH_2Cl_2, 0 °C to RT, overnight. c) 1. NaI, acetone, RT, 1 h; 2. 7-hydroxycoumarin, K_2CO_3, CH_3CN, RT, 2.5 h. d) 1.0 equiv. *m*-CPBA, CH_2Cl_2, 0 °C, 0.5 h. e) NaI, TFAA, acetone, 0 °C, 1 h. f) CH_2Cl_2/TFA/TIPS/H_2O = 65:30:2.5:2.5, 30 min, 0 °C. PMB = *para*-methoxybenzyl, *m*-CPBA = *meta*-chloroperbenzoic acid, TFAA = trifluoroacetic anhydride, TFA = trifluoroacetic acid, TIPS = triisopropylsilane, Ts = 4-toluenesulfonyl.

We first investigated their specificity to recombinant β-lactamases. Four of the most clinically prevalent carbapenemases, including three metallo-β-lactamases (VIM-27, IMP-1, NDM-1) and one class A β-lactamase (KPC-3) were prepared using the reported protocol.[2b,15] Two class A β-lactamases (TEM-1 Bla and BlaC) were also expressed as negative controls. The fluorescence of the probes was measured before and after enzyme treatment for a range of 1 to 200 fmols. All probes showed distinct specificity and sensitivity to six β-lactamase enzymes (Figure 2, and Figures S2 and S3 in the Supporting Information). As expected, CDC-**1** (its structure shown in Figure S1 in the Supporting Information), a previously reported nonspecific probe for β-lactamases, was used as a positive control[11e] and consistently gave positive fluorescence enhancement with all β-lactamases. However, the rest of the probes, to an extent, mainly exhibited fluorescence increases in the presence of the carbapenemases. For instance, high fluorescence signals were recorded for (*S*)-CC-**1**, (*S*)-CC-**2** (Figure S2), and (*S*)-CC-**5** when treated with the carbapenemases VIM-27, IMP-1, NDM-1, and KPC-3, but nearly no fluorescence enhancement was observed for TEM-1 Bla and BlaC. The compounds (*S*)-CC-**1** and (*S*)-CC-**5** showed high sensitivity for all four carbapenemases with a detection limit as low as a 5 fmol, thus indicating that (*S*)-CC-**1** and (*S*)-CC-**5** may be specific for carbapenemases. In contrast, (*S*)-CC-**3** and (*S*)-CC-**6** showed fluorescence enhancement only for three MBLs (VIM-27/IMP-1/NDM-1) when a low amount of enzyme (< 200 fmols) was used, thus suggesting specificity for metallo-β-lactamases. The compound (*S*)-CC-**6** showed better sensitivity than (*S*)-CC-**3**: fluorescence enhancement was clearly observed for (*S*)-CC-**6**

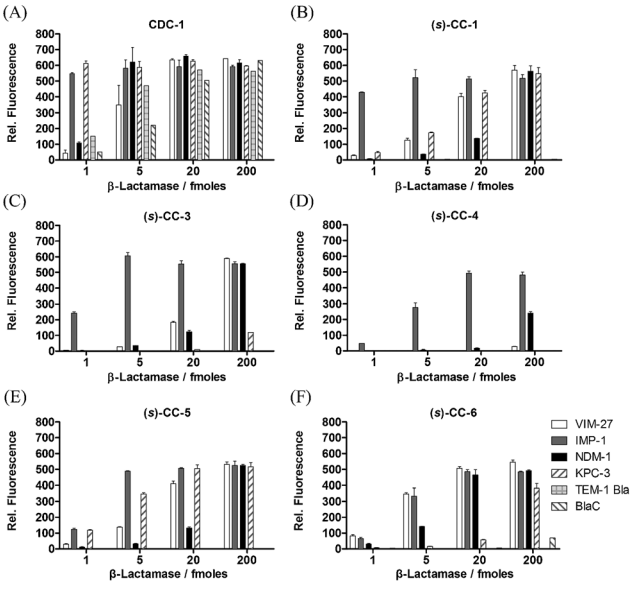


Figure 2. β-Lactamase selectivity of the designed probes. Relative fluorescence of indicated amounts of β-lactamases after incubation with probes (10 μM) at room temperature in 25 μL of 1 × PBS buffer (*p*H 7.4) for 2 h. A) CDC-**1**, B) (*S*)-CC-**1**, C) (*S*)-CC-**3**, D) (*S*)-CC-**4**, E) (*S*)-CC-**5**, and F) (*S*)-CC-**6**. From left to right at each concentration in each figure: VIM-27, IMP-1, NDM-1, KPC-3, TEM-1 Bla, BlaC. Relative fluorescence represents the difference in the fluorescence intensity with and without β-lactamase incubation. Fluorescence was measured with excitation at $\lambda = 363$ nm (band width 5 nm) and emission at $\lambda = 454$ nm (band width 7.5 nm). Error bars are ± SD.

with as low as 1.0 fmol of the enzyme. However, when the amount of KPC-3 used in the assay went up to 200 fmols, both probes produced enhanced fluorescence signals (Figure 2C,F).

Interestingly, (*S*)-CC-**4** (Figure 2D) displayed high specificity for IMP-1, and the fluorescence signal gradually increased in a concentration-dependent manner, however, NDM-1 produced a fluorescence increase at about 200 fmols. To further confirm the specificity of the probes, enzyme-blocking experiments were performed both for recombinant β-lactamases and β-lactamase-expressing bacteria with (*S*)-CC-**5**, which showed good specificity for carbapenemases (see Figure S7). Only weak fluorescence was observed when the enzyme/bacteria were pre-treated with β-lactamase inhibitors (EDTA[16] for VIM-27, IMP-1, and NDM-1; phenylboronic acid[17] for KPC-3), thus suggesting that the increased fluorescence emission is due to the hydrolysis of the probes by specific carbapenemases. Collectively, these results suggest that reversing the stereochemistry of the C7 position of cephalosporin is sufficient to render new specificity for carbapenemases.

The kinetic parameters of our probes, with different β-lactamases, were determined from Lineweaver–Burk plots and summarized in Table 1 and Figure S4 in the Supporting Information. All probes exhibited high stability with low spontaneous hydrolysis rates ranging from 0.7 to 5.9×10^{-7} s^{-1} (see Figure S5). A careful comparison of the kinetic parameters resulted in several important findings: firstly, all probes displayed the best kinetic efficiencies ($k_{cat}/K_m = 0.2–2.5\times10^6$ $s^{-1}M^{-1}$) for IMP-1 which is one of most clinically important metallo-β-lactamases and exhibits a worldwide distribution.[18] Among them, (*S*)-CC-**4** shows high specificity and kinetic efficiency (k_{cat}/K_m) for IMP-1 (2.2×10^5 $s^{-1}M^{-1}$), values which are over 50 to 100 times higher than that for other carbapenemases, and no hydrolysis by noncarbapenemases was detected. Secondly, a smaller R group is generally preferred for VIM-27 and KPC-3, but less so for IMP-1 and NDM-1. With a bulky *tert*-butyl group, (*S*)-CC-**4** shows decreased kinetic efficiency but high specificity for IMP-1. Thirdly, (*S*)-CC-**5** exhibits much higher kinetic efficiency for IMP-1 and KPC-3 (1.2×10^6 $s^{-1}M^{-1}$ and 2.2×10^5 $s^{-1}M^{-1}$, respectively) compared to VIM-27 and NDM-1 (2.6×10^4 $s^{-1}M^{-1}$ and 1.0×10^4 $s^{-1}M^{-1}$, respectively), and no hydrolysis by other enzymes was observed. In contrast, (*S*)-CC-**6** shows high kinetic efficiency for IMP-1, NDM-1, and VIM-27 (6.6×10^5 $s^{-1}M^{-1}$, 4.0×10^5 $s^{-1}M^{-1}$, and 2.8×10^5 $s^{-1}M^{-1}$), values which are over 14 to 33 times higher than that for KPC-3 (2.0×10^4 $s^{-1}M^{-1}$). These kinetic measurements are consistent with the results with recombinant β-lactamases in Figure 2.

We further sought to evaluate the feasibility of detecting live bacteria expressing carbapenemase using our probes. Eleven known bacteria strains that produce carbapenemases and other clinically prevalent β-lactamases were evaluated, including two class A β-lactamases (KPC-3 and IMI), three class B MBLs (VIM-27, IMP-1, and NDM-1), one class D β-lactamase OXA-48, and five clinically important non-carbapenemases (CTX-M, SHV-18, TEM-1 Bla, BlaC, and AmpC). We first examined the fluorescence response of the probes (10 μM unless indicated) to varying numbers (range from 10^4 to 10^6 c.f.u.) of live bacteria, as shown in Figure 3. As

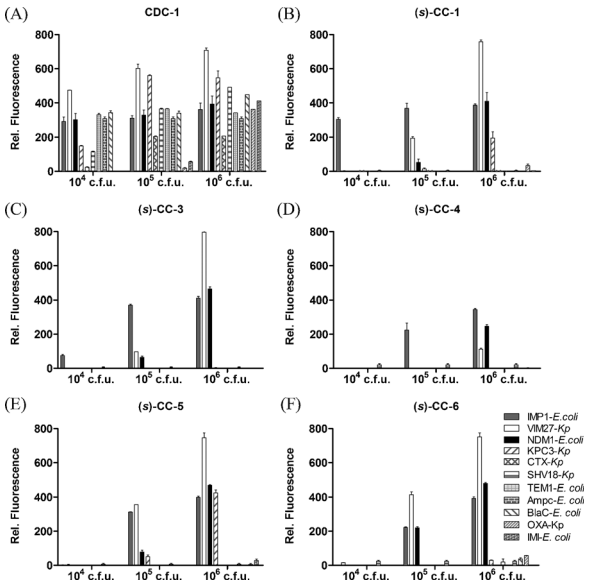


Figure 3. Relative fluorescence of β-lactamase-expressing bacteria after incubation with CRE-specific probes (10 μM) at room temperature in 1xPBS buffer (pH 7.4) for 2 h. A) CDC-**1**, B) (*S*)-CC-**1**, C) (*S*)-CC-**3**, D) (*S*)-CC-**4**, E) (*S*)-CC-**5**, F) (*S*)-CC-**6**. From left to right in each figure: *E. coli* with IMP-1 (IMP1-*E. coli*), *K. pneumoniae* with VIM-27 (VIM27-*Kp*), *E. coli* with NDM-1 (NDM1-*E. coli*), *K. pneumoniae* with KPC-3 (KPC3-*Kp*), K. *pneumoniae* with CTX-M (CTX-*Kp*), *K. pneumoniae* with SHV-18 (SHV18-*Kp*), *E. coli* with TEM-1 Bla (TEM1-*E. coli*), *E. coli* with AmpC (AmpC-*E. coli*), *E. coli* transformed with BlaC (BlaC-*E. coli*), *K. pneumoniae* with OXA-48 (OXA48-*Kp*), *E. coli* with IMI (IMI-*E. coli*). All bacteria numbers are as indicated except for *E. coli* expressing AmpC where 10^7 c.f.u. were used instead. Relative fluorescence represents the difference in fluorescence intensity with and without β-lactamase incubation. Fluorescence was measured with excitation at $\lambda = 363$ nm (band width 5 nm) and emission at $\lambda = 454$ nm (band width 7.5 nm). Error bars are ± SD.

Table 1: Kinetic parameters of fluorescent probes for β-lactamases.

Name	VIM-27[a]			IMP-1[a]			NDM-1[a]			KPC-3[a]			Spontaneous hydrolysis rate [s^{-1}]
	K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$s^{-1}M^{-1}$]	K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$s^{-1}M^{-1}$]	K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$s^{-1}M^{-1}$]	K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$s^{-1}M^{-1}$]	
CDC-**1**	3.8±2.7	2.3±0.3	7.5×10^5	4.0±0.1	7.9±0.1	2.0×10^6	5.04±3.2	4.6±0.3	1.1×10^6	21.0±1.34	35.4±0.04	1.7×10^6	2.4×10^{-7}[b]
(*S*)-CC-**1**	29.2±7.0	1.1±0.1	3.8×10^4	7.8±0.2	12.2±0.1	1.6×10^6	12.0±0.7	0.2±0.02	2.0×10^4	67.7±4.9	1.5±0.05	2.3×10^4	0.7×10^{-7}
(*S*)-CC-**2**	23.0±0.0	0.71±0.0	3.0×10^4	12.9±1.0	16.6±0.1	1.3×10^6	14.9±2.7	0.3±0.01	1.7×10^4	39.1±1.3	0.3±0.05	6.9×10^3	5.9×10^{-7}
(*S*)-CC-**3**	61.3±8.8	0.5±0.04	7.8×10^3	3.2±0.07	8.0±0.04	2.5×10^6	6.0±3.5	0.1±0.01	2.0×10^4	250±235	0.2±0.17	1.1×10^3	4.8×10^{-7}
(*S*)-CC-**4**	91.6±10.1	0.2±0.01	2.6×10^3	9.4±1.3	2.1±0.01	2.2×10^5	58.6±16.0	0.3±0.04	5.1×10^3	121±85	0.2±0.1	2.2×10^3	5.4×10^{-7}
(*S*)-CC-**5**	145.1±100.1	3.4±1.5	2.6×10^4	3.6±0.1	4.2±0.03	1.2×10^6	26.7±0.2	0.3±0.0	1.0×10^4	5.2±0.5	1.1±0.01	2.2×10^5	2.7×10^{-7}
(*S*)-CC-**6**	14.1±1.2	3.9±0.1	2.8×10^5	3.4±0.4	2.2±0.02	6.6×10^5	4.7±0.3	1.9±0.06	4.0×10^5	18.7±7.8	0.3±0.05	2.0×10^4	2.6×10^{-7}

[a] Kinetics measured in 1xPBS buffer (pH 7.4) at room temperature (22 °C) unless otherwise noted. [b] Ref. [11e]. All data indicate averages of three replicate experiments.

a positive control, CDC-**1** was consistently positive for all types of bacterial strains, thus indicating that all the bacteria are β-lactamase active. Compounds (*S*)-CC-**1** and (*S*)-CC-**5** showed fluorescence enhancement for all three MBLs (VIM-27, IMP-1, and NDM-1) and the most common carbapenemase, KPC-3-expressing bacteria ($\geq 10^5$ c.f.u.).[19] Upon treatment with 10^4 c.f.u of bacteria, only IMP-1-expressing bacteria can be detected by (*S*)-CC-**1**. The compounds (*S*)-CC-**3** and (*S*)-CC-**6** had intense fluorescence responses only to VIM-27, IMP-1, and NDM-1 expressing bacteria, which is in agreement with the results obtained from recombinant enzymes (Figure 2). About $1.0–10 \times 10^5$ c.f.u. of bacteria were required to reliably detect MBL bacteria with (*S*)-CC-**3** and (*S*)-CC-**6** after a 2 hour incubation. In comparison, (*S*)-CC-**6** apparently shows better specificity for MBLs than does (*S*)-CC-**3**. These results indicate that (*S*)-CC-**6** is a specific probe for the detection of MBLs. Additionally, (*S*)-CC-**4** showed a preference for IMP-producing strains, although interference was observed for 1×10^6 c.f.u. of VIM-27- and NDM-1-expressing bacteria. Further modification of (*S*)-CC-**4** may allow detection of IMP-expressing bacteria with high sensitivity and specificity.

In summary, by reversing the stereochemistry at the C7 position of cephalosporin, we have developed the first fluorogenic probes with specificity for carbapenemases, especially metallo-β-lactamases. The substituent at the C7 position modulates the selectivity among the carbapenemases: (*S*)-CC-**3** and (*S*)-CC-**6** demonstrate good specificity and sensitivity for MBLs; (*S*)-CC-**5** shows good specificity and sensitivity for MBLs, and the most common carbapenemase, KPC-3-expressing bacteria; and (*S*)-CC-**4** prefers IMP-1. While these probes use a blue fluorophore, fluorophores with longer emission wavelengths may be introduced to improve sensitivity.[11] With further refining, these new probes should facilitate rapid and accurate detection of carbapenem-resistant *Enterobacteriaceae* for the timely diagnosis and treatment of patients with CREs infections.

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