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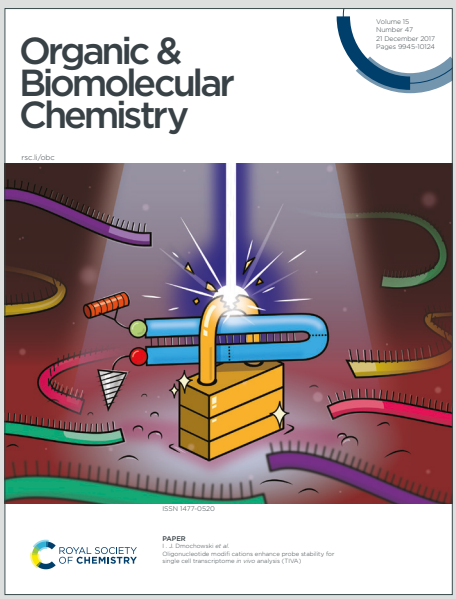

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Minor structure modification serendipitously leads to a highly carbapenemase-specific fluorogenic probe

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Jie Wang,‡ Weipan Xu,‡ Shuyuan Xue, Tao Yu and Hexin Xie*

Reported herein is a fluorogenic probe for the detection of carbapenemase activity. This reagent features with carbapenem as enzyme recognition motif and a carbon-carbon double bond between carbapenem and fluorophore, exhibiting high specificity to all carbapenemases, including metallo carbapenemases and serine carbapenemases, over other β-lactamases.

Bacterial resistance to β-lactam antibiotics, the most widely used antibiotics in clinical practice over the past several decades, has posed a severe threat to public health globally.[1] The major mechanism for bacteria to survive in the presence of β-lactam antibiotics involves the production of a class of highly efficient β-lactam-degrading enzymes, named as β-lactamases (blas), in bacteria.[2] These enzymes, on the basis of Ambler classification, are divided into four classes, class A, class B, class C, and class D β-lactamases.[3] Class A, C, and D β-lactamases are known as serine-β-lactamases (SBLs) while class B β-lactamases are metallo-β-lactamases (MBLs).

Carbapenems (e.g., Imipenem, Meropenem, Doripenem, and Ertapenem), with the unusual *trans*-substituents on the β-lactam rings, were relatively resistant to the hydrolysis by most β-lactamases and thus remained effective to these β-lactamase-expressing bacterial pathogens; carbapenems are considered as "antibiotics of last resort".[4] Nevertheless, carbapenem-hydrolyzing β-lactamases (carbapenemases), which include all MBLs and a number of SBLs, for instance, *Klebsiella pneumoniae* carbapenemase (KPC), have been identified in pathogenic bacteria.[5] The increasing occurrence of these carbapenemase-producing organisms (CPOs) has significantly undermined the efficacy of these life-saving molecules.[4]

Early identification is crucial to prevent rapid spreading of CPOs.[6] Taking advantage of the high catalytic efficiency of β-lactamases, a large number of β-lactamases fluorescent probes have been developed for the detection of resistant bacteria.[7] In spite of these, fluorescent reagents with high specificity to carbapenemase were less explored. Rao and colleagues employed *trans*-substituted cephalosporin as carbapenemase recognition moiety and developed a number of carbapenemase-selective fluorogenic probes.[8] With the core structure of carbapenem as enzyme recognition motif and alkenyl-linked boron dipyrromethene (BODIPY) dye as activatable fluorophore, we have reported a highly carbapenemase-specific reagent for the rapid detection of CPOs.[9] Moreover, we recently integrated carbapenem and umbelliferone to develop a fluorogenic probe CPC-1.[10] However, to our surprise, this carbapenem-based reagent is selective to metallo carbapenemases, yet barely responses to serine carbapenemases. Following our continuing interest on β-lactamase fluorescent probe,[7h, 7j, 9-11] we herein present a structurally similar carbapenem-based probe, but with unexpectedly high specificity to both metallo carbapenemases and serine carbapenemases.

Inspired by the cephalosporin-based fluorescent probes CC1,[12] which equips with an extra carbon-carbon double bond between enzyme recognition motif and fluorophore compared

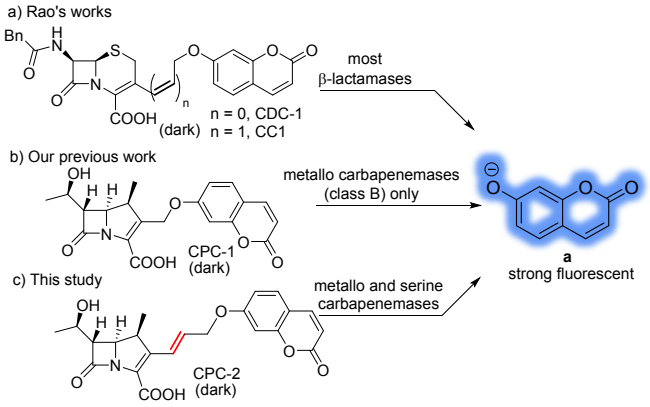

Figure 1 Design of fluorogenic probe for the detection of carbapenemase activities.

[a] State Key Laboratory of Bioreactor Engineering, Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, Shanghai 200237, China. E-mail: xiehexin@ecust.edu.cn

‡ These authors contributed equally.

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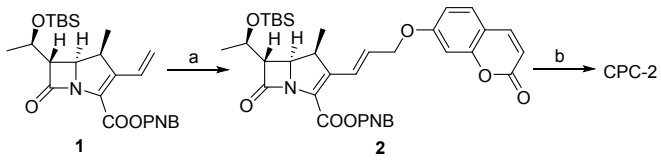

Scheme 1 Preparation of CPC-2. a) 7-allyloxycoumarin, Hoveyda-Grubbs 2nd catalyst, $CHCl_3$, reflux; b) (i) (NH_4) HF_2, DMF/NMP, rt; (ii) Zn, THF/PB (0.35M, pH 6.0), rt. TBS: *tert*-butyldimethylsilyl; PNB: *p*-nitrobenzyl.

to CDC-1,[7c] we designed a carbon-carbon double bond-containing carbapenem-based probe CPC-2 in order to simplify the synthesis of carbapenem-based probe (Fig. 1). As the double bond is far away from the enzyme recognition site and the double bonding-bearing CC1 exhibits very similar enzyme kinetics as CDC-1, we expected CPC-1 and CPC-2 would likely have very close enzymatic hydrolysis profile.

As outlined in Scheme 1, starting from readily available carbapenem **1**, CPC-2 was easily prepared after a cross-metathesis reaction in presence of second generation of Hoveyda-Grubbs catalyst, followed by removal of TBS- and PNB-protecting groups, subsequently. This fluorogenic molecule was purified by reverse-phase high-performance liquid chromatography (RP-HPLC) and the structure was unambiguously confirmed by nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HR-MS).

With CPC-2 available, we first examined its absorbance spectra before and after incubation with metallo

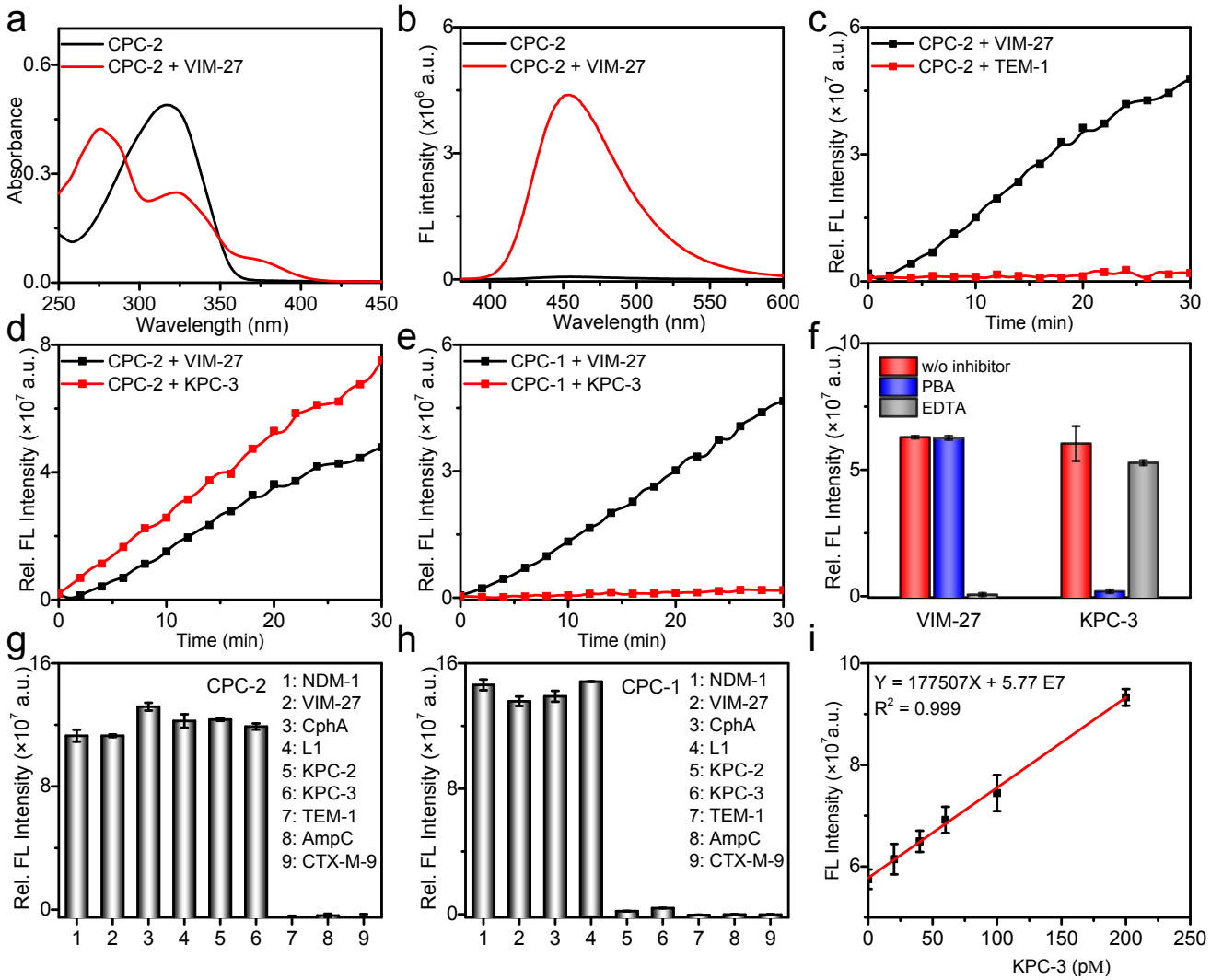

Figure 2 Detection of β-lactamase activities with CPC-2. UV/Vis spectra (a) and fluorescent spectra (b) of CPC-2 (10 μM in PBS, pH 7.4) before and after incubation with VIM-27 β-lactamase (500 nM) for 3 min. (c-e) Time course fluorescence intensity of CPC-2 (10 μM in PBS, pH 7.4) with indicated β-lactamases (0.5 nM). (f) Fluorescent enhancement of CPC-2 (10 μM in PBS, pH 7.4) after incubation of indicated β-lactamases (0.5 nM) for 30 min in the presence of PBA (2 mM) or EDTA (400 μM). (g, h) Fluorescent enhancement of CPC-2 and CPC-1 (10 μM in PBS, pH 7.4) upon incubation with series of β-lactamases (1 nM) for 30 min. (i) Linear correlation between fluorescent intensity of CPC-2 (10 μM in PBS, pH 7.4) and concentration of KPC-3 β-lactamase. $\lambda_{ex/em}$ =365/460 nm; error bars represent standard deviation of three experiments.

carbapenemase VIM-27.[13] As expected, these spectra are similar to that of CPC-1. In addition, the fluorescence spectra of CPC-2 were also recorded with or without incubation with same enzyme and, again, these show high similarity with those of CPC-1: both probes alone are barely fluorescent and turn strong fluorescent after treatment with VIM-27.

Having demonstrated the high fluorescent response of CPC-2 to metallo carbapenemase, we thus turned our attention to the selectivity of this reagent to different β-lactamases. TEM-1 (a narrow-spectrum class A β-lactamase commonly found in Gram-negative bacteria)[14] and VIM-27 were first selected in this study. As depicted in Fig. 2c, VIM-27 elicited massive enhancement in fluorescence intensity while TEM-1 hardly turned on the fluorescence of CPC-2, indicating the carbapenem-based CPC-2 is highly selective to VIM-27 over TEM-1.

Moreover, KPC-3,[15] a class A serine carbapenemase, was also investigated and, to our surprise, this enzyme is highly efficient in the hydrolysis of CPC-2, significantly increasing fluorescence intensity. By contrast, CPC-1 was only activated by VIM-27; this molecule is obviously a poor substrate of KPC-3 and led to almost no fluorescence enhancement, which is in agreement with previous observations.[10a] We further applied HPLC and LC-MS to analyze the KPC-3-incubated CPC-2 and it turned out umbelliferone (**a**) and hydrolyzed β-lactam (**b**) were formed as the major products, which are identical with those from VIM-27-treated CPC-2 (Fig. S1).

Additionally, we performed an inhibition assay to validate whether the fluorescence enhancement of CPC-2 is attributed solely to the activity of enzyme. We employed metal ion chelator ethylenediaminetetraacetic acid (EDTA) as inhibitor for metallo β-lactamase and phenylboronic acid (PBA) as inhibitor for serine β-lactamase (PBA). As illustrated in Fig. 2f, EDTA selectively suppressed the fluorescence enhancement by VIM-27, leaving the KPC-3-containing sample unaffected. On the other hand, PBA inhibited only the fluorescence enhancement mediated by KPC-3, having little impact on the activity of VIM-27. These results further demonstrate the fluorescence enhancement of CPC-2 was mediated only by β-lactamase, which thus allows to monitor the activity of carbapenemase by simply measuring the increasement in fluorescence intensity.

To further reveal the specificity of CPC-2, we tested more β-lactamases with this imaging reagent, including class A β-lactamases (KPC-2,[16] KPC-3, TEM-1, and CTX-M-9,[17] etc), class B β-lactamases (NDM-1,[18] VIM-27, L1,[19]and CphA,[20] etc), and class C β-lactamases (AmpC[21]). Among these β-lactamases, all class B β-lactamases (or metallo β-lactamases) and KPC-2 and KPC-3 are known as carbapenemases, whereas AmpC (class C) and CTX-M-9 (class A) belong to extended-spectrum β-lactamase (ESBL). As shown in Fig. 2g, all carbapenemases, including metallo carbapenemases and serine carbapenemases (KPC-2 and KPC-3) are capable of activating CPC-2 and lead to strong fluorescence signal; all of other β-lactamases, even ESBLs (AmpC and CTX-M-9), are ineffective to turn on fluorescence of CPC-2. In other word, CPC-2 is a highly carbapenemase-specific fluorogenic sensor. Under identical

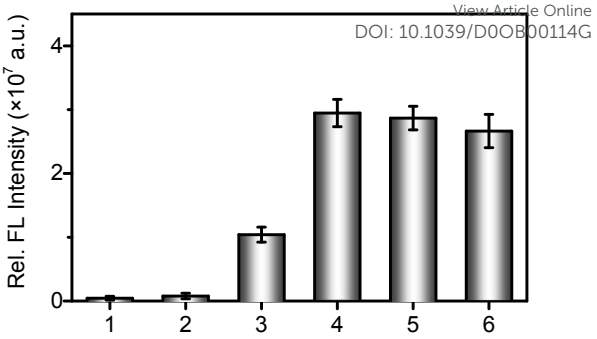

Figure 3 Enhanced fluorescence intensity of CPC-2 (15 μM in PBS, pH 7.4) upon incubation at room temperature for 60 min with β-lactamase-negative and clinically important antibiotic-resistant bacteria. 1: bla-negative *E. coli* (ATCC 25922, 4×10^5 CFU); 2: TEM-1 *E. coli* (ATCC 35218, 4×10^5 CFU); 3: KPC-2 *E. coli* (ATCC BAA 2340, 4×10^5 CFU); 4: VIM-1 *K. pneumoniae* (NCTC 13440, 4×10^5 CFU); 5: VIM-1 *K. pneumoniae* (NCTC 13440, 4×10^5 CFU) + TEM-1 *E. coli* (ATCC 35218, 4×10^4 CFU); 6: VIM-1 *K. pneumoniae* (NCTC 13440, 4×10^5 CFU) + TEM-1 *E. coli* (ATCC 35218, 4×10^5 CFU); $\lambda_{ex/em}$ = 365/460 nm; error bars represent standard deviation of three experiments.

circumstance, CPC-1 was activated only by metallo β-lactamases whereas all of the others serine β-lactamases, including serine carbapenemases (KPC-2 and KPC-3) are much less efficient to trigger the enhancement in fluorescence intensity; CPC-1 is a metallo β-lactamase-selective fluorogenic reagent. Given the high structural similarity of CPC-1 and CPC-2, their totally different responses to serine carbapenemases (KPC-2 and KPC-3) suggest divergent mechanisms may involve in this serine carbapenemase-mediated hydrolysis process though exact reason remains unknown at current stage.

In general, carbapenems are less stable compared to cephalosporin-based antibiotics. To assess the stability of carbapenem-based CPC-2, we measured its spontaneous hydrolysis rate in PBS (pH 7.4) at room temperature and it was estimated to be 4.7 × 10^{-5} s^{-1} (Fig. S2). This result indicates the introduction of extra carbon-carbon double bond decreases its aqueous stability. Nevertheless, the effect of the spontaneous hydrolysis on the detection of carbapenemases might somehow be compensated by the incubation of CPC-2 alone at the same time.

To obtain information on the detection sensitivity of CPC-2, we performed further investigation on the determination of limit of detection (LOD). We incubated CPC-2 with a range concentration of carbapenemases (VIM-27, KPC-2, and KPC-3) in PBS for 30 minutes before measuring their fluorescence intensity with microplate reader. After plotting fluorescence intensity versus concentrations of enzymes, the LOD (*3N/k*; *N*: standard deviation of blank sample; *k*: slope) to VIM-27, KPC-2, and KPC-3 were calculated to be 18.6, 40.6, and 34.4 pM, respectively. These data support CPC-2 as a sensitive reagent for the detection of carbapenemases in spite of its relatively low aqueous stability.

With the detection sensitivity and specificity of CPC-2 to carbapenemases validated, we moved to apply this molecule in the detection of resistant pathogenic bacteria. As proof of concept, we selected four strains of clinically occurring pathogenic bacteria for this test, including TEM-1-expressing *Escherichia coli* (*E. coli*, ATCC 35218), KPC-2-expressing *E. coli* (ATCC BAA 2340), and VIM-1-expressing *Klebsiella pneumonia* (*K. pneumonia,* NCTC 13440), along with β-lactamase-negative *E. coli* (ATCC 25922). We incubated CPC-2 with these bacteria respectively for 60 minutes and then measured fluorescence intensity with a microplate reader. As depicted in Fig. 3, all of the CPOs (KPC-2-expressing *E. coli* and VIM-1-expressing *K. pneumonia*) led to significant enhancement in fluorescence intensity whereas other bacteria, susceptible *E. coli* or TEM-1-expressing *E. coli*, remained barely fluorescent. These results demonstrate CPC-2 as a potential sensor in the identification of carbapenemase-producing bacterial pathogens, even though the detection sensitivity of this reagent is somewhat limited by its relatively low stability. Moreover, to investigate the interference of carbapenemase-negative bacteria in the test, we incubated CPC-2 with VIM-1-expressing *K. pneumonia* ($4x10^5$ CFU) in the presence of TEM-1-expressing *E. coli* ($4x10^4$ CFU or $4x10^5$ CFU). It turned out the enhancement of these samples in fluorescence intensity is comparable with the sample without *E. coli*, suggesting the presence of *E. coli* has insignificant impact on the test of carbapenemase-producing bacteria with CPC-2.

In summary, with a carbon-carbon double bond-conjugated carbapenem as enzymatic recognition motif, we have developed a fluorogenic reagent to visualize the activity of carbapenemases. This probe can be selectively activated by all carbapenemases, including metallo carbapenemases and serine carbapenemases, and massively enhances fluorescence intensity but remains non-response to other β-lactamases, even extended-spectrum β-lactamases. Further test of this reagent with carbapenemase-producing bacteria has demonstrated this compound as a promising probe in the rapid identification of carbapenem-resistant microbes.

Acknowledgements

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