β-Lactam Antibiotics

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Ratiometric Fluorescence Detection of Pathogenic Bacteria Resistant to Broad-Spectrum β-Lactam Antibiotics

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β-Lactam antibiotics have been the frontline drugs to fight bacterial infections because of their high efficacy and low toxicity ever since they were first discovered in the 1920s. These agents confer their antimicrobial ability by inhibiting transpeptidases, also known as penicillin binding proteins (PBPs), the activity of which is required for bacterial cell wall synthesis. To date, hundreds of β-lactam compounds have been synthesized and evaluated for their antibacterial properties, including derivatives of penicillin, cephalosporin, monobactam, and carbapenem.[1] As one of the primary strategies to survive in the presence of these antibiotics, bacterial cells can acquire β-lactamases, a family of highly efficient enzymes that are able to destroy β-lactam structural moieties before they interact with PBPs.[2] Hence, inactivation or inhibition of potential β-lactamases is a key consideration for the treatment of bacterial infections.

There are many types of β-lactamases, each with distinct expression patterns and catalytic mechanisms. One of the most commonly encountered β-lactamases is TEM-1.[3] Production of this plasmid-harboring serine hydrolase accounts for most cases of penicillin resistance in Gram-negative bacteria, such as Escherichia coli and Klebsiella pneumoniae. AmpC, also a serine hydrolase, is an inducible chromosomally encoding enzyme that is often observed in Enterobacter and Citrobacter species. On the other hand, metallo-β-lactamases, like the newly isolated NDM-1, require a zinc ion in their active site for enzymatic activity.[4]

It is of great medical importance to prevent β-lactamases from hydrolyzing β-lactam drugs. One way to counteract these enzymes is to concurrently administer both a β-lactam drug and a β-lactamase inhibitor. Several prescription drugs utilize this strategy, including Unasyn (ampicillin plus sulbactam) and Augmentin (amoxicillin plus clavulanate). Another general approach is to develop β-lactams that are not hydrolyzable by β-lactamases. Many third- and fourth-generation cephalosporins, in which a methoxyimino group attaches to the β-lactam ring, are too large to fit into the active site of TEM-1, thereby conferring resistance to TEM-1.[5] Unfortunately, these "next generation" antibiotics can still be efficiently cleaved by newly emerging β-lactamases such as NDM-1.[6] In addition, bacteria can readily develop antibiotic resistances by evolving novel mechanisms to extend the effectiveness of their current repertoire of β-lactamases. For example, as few as one or two point mutations to key residues of TEM-1 can noticeably improve its ability to degrade methoxyimino cephalosporins. These TEM-1 mutants with enhanced activity are often referred to as extend-spectrum β-lactamases (ESBLs).[7] Similarly, hyperexpression of AmpC also causes its hosts to become more refractory to broad-spectrum cephalosporins.[8]

Since the development of bacterial drug resistance is tightly associated with the misuse/overuse of antibiotics, it would be desirable to reserve broad-spectrum antibiotics (e.g. third- and fourth-generation cephalosporins) for multidrug-resistant infections only and avoid using them on bacterial strains sensitive to narrow-spectrum agents (e.g. ampicillin). To achieve this goal, a diagnostic must be capable of determining bacterial susceptibility toward various β-lactam drugs, so a clinical decision on the selection of antibiotics can be made accordingly. This task is currently achieved by standard methods like the E-test and the double-disc test.[9] These approaches usually take 48–72 h, or longer for slow-growing bacteria like Mycobacterium tuberculosis, which is often too slow to offer therapeutic guidance. A new Cica-β-test, based on a chromogenic β-lactam derivative, is able to shorten the diagnostic period to 24 h. However, its sensitivity has been reported to be low.[10] PCR and other DNA-based diagnostic methods are both rapid and sensitive, yet they are not functional assays and can only detect resistance mechanisms derived from well-characterized genes.[11] As a result, novel approaches with better sensitivity, requiring less time, and capable of detecting multidrug-resistant bacteria are still actively sought to prolong the useful lifespan of broad-spectrum β-lactam antibiotics.

Currently, many fluorogenic β-lactamase substrates are available, which allow the detection of β-lactamases with high sensitivity and in real time.[12] Yet, most of these molecules are hydrolyzable by all types of β-lactamases; only a few show selective cleavage toward different β-lactamases.[13] So existing β-lactamase substrates cannot distinguish enzymes with extended substrate profiles from those with narrow-spectrum. Herein, we describe the synthesis of a novel fluorescent β-lactamase substrate (C-2; Figure 1). This compound contains a methoxyimino side chain, so it is resistant to TEM-1, but should be susceptible to enzymes like NDM-1 and overexpressed AmpC. Indeed, C-2 (50 μM) only degraded slightly when incubated with highly purified TEM-1 (1 μg). By

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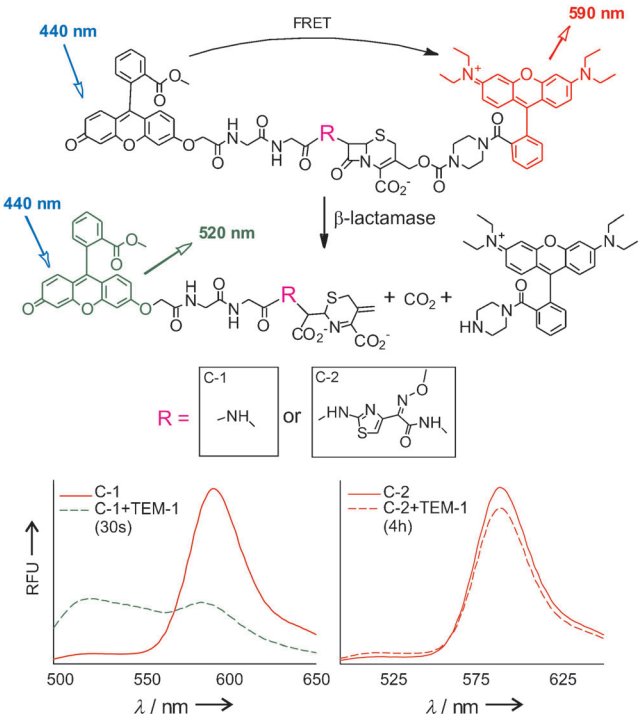
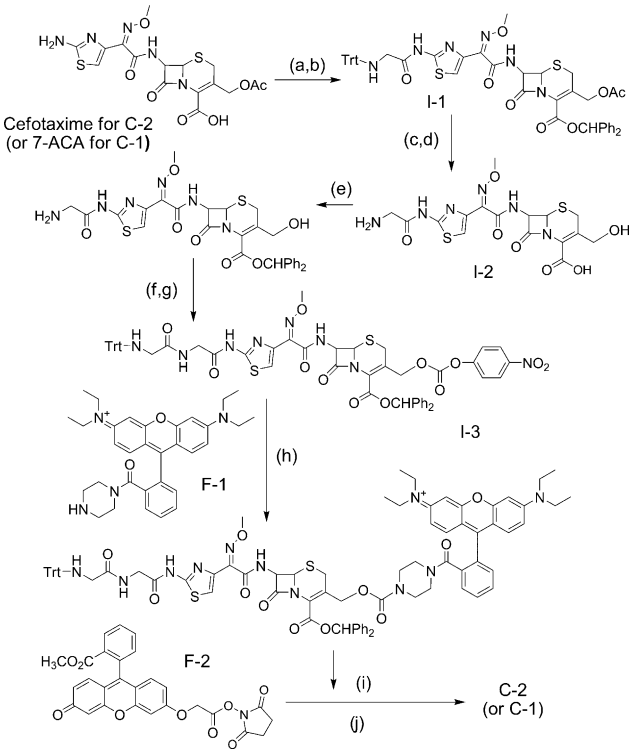


Figure 1. Structure of C-1, C-2, and their hydrolyzed products. C-1 (50 μM) is degraded by 1 μg TEM-1 completely within 30 s. By contrast, C-2 (50 μM) is only slightly hydrolyzed under the same conditions after 4 h. λ_{ex} = 440 nm. RFU = relative fluoresence unit.



Scheme 1. Synthesis of C-2 (or C-1). a) Ph_2CN_2, TsOH. b) Trt-Gly-OH, HBTU, HOBt. c) 5% TFA, 10% TIS. d) Orange peel acetylesterase, PBS buffer, pH 5.5. e) Ph_2CN_2, MeCN, pH 3. f) Trt-Gly-OSu. g) 4-nitrophenyl chloroformate. h) F-1, DIPEA. i) 5% TFA, 10% TIS. j) F-2, DIPEA. DIPEA = *N*,*N*-diisopropylethylamine, HBTU = 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphat, Bt = benzotriazole, TFA = trifluoroacetic acid, TIS = triisopropylsilane, Trt = trityl, Ts = 4-toluenesulfonyl.

contrast, its counterpart without this bulky methoxyimino group (C-1) was instantly degraded under the same condition. Consequently, C-2 should easily distinguish β-lactamases capable of hydrolyzing methoxyimino cephalosporins from those lacking this activity.

The synthesis of C-2 started from cefotaxime, a widely-used cephalosporin drug containing a characteristic 2-aminothiazol-4-yl-methoxyimino structure that is shared by many third-generation antibiotics (Scheme 1). Once its free carboxylate was masked by forming benzhydryl ester, cefotaxime was coupled with a trityl-protected glycine (Trt-Gly-OH) to give I-1. The benzhydryl and trityl groups on I-1 were removed by 5% TFA, and its acetyl was then removed by an acetylesterase fleshly prepared from orange peel to yield I-2. This cleavage of benzhydryl and trityl before enzymatic treatment is necessary because I-1 itself does not dissolve in aqueous solution required by the acetylesterase. The carboxylate on I-2 was masked again by benzhydryl and its amine was coupled to trityl-protected glycine N-hydroxysuccinimide ester (Trt-Gly-OSu) under mild conditions. Then, its free hydroxy group was activated by reacting with 4-nitrophenyl chloroformate to provide I-3. After I-3 was coupled with a fluorescent compound F-1, its benzhydryl and trityl groups were removed, which exposed an amine to couple with another fluorescent compound F-2 to offer the final product C-2. The synthesis of C-1 followed the same scheme, but started from 7-aminocephalosporanic acid (7-ACA) instead.

C-2 is a fluorescent molecule with a single emission peak at 590 nm when it is excited at 440 nm. This is caused by efficient fluorescence resonance energy transfer (FRET) between F-1 and F-2 fluorophores in its structure. When C-2 is hydrolyzed by a β-lactamase, this FRET will disappear as one fluorophore moves away from the other. As a result, the degradation of C-2 is accompanied by the decrease of fluorescence at 590 nm and the increase of fluorescence at 520 nm (Figure 2 A). This design allows us to follow the hydrolysis of C-2 based on the ratio of fluorescence intensity at two wavelengths. Compared to other methods that only measure absolute fluorescence intensity, the ratiometric approach represents unique advantages. The ratio of two fluorescence signals is independent on the amount of substrate present, but proportional to the activity of the enzymes. In addition, because fluorescence variations caused by instruments and other unpredictable factors are cancelled out, the quantitative determination of enzyme activity is more accurate and reliable, which in turn improves the sensitivity of detection. Using this substrate, we tested β-lactamase activity from several bacterial species, including Enterobacter cloacae (AmpC positive, inducible), Klebsiella pneumoniae (NDM-1 positive), and Enteric group 137 (ESBL positive). The enzymatic activity was monitored by measuring time-dependent fluorescence changes at 520 and 590 nm. We plotted $(F_{590}/F_{520})/(F_{590}/F_{520})_S$ (y axis) versus time (x axis) of these samples. Here F_{520} and F_{590} are the fluorescence intensities at 520 and 590 nm. F_{590}/F_{520} is the ratio of the sample containing

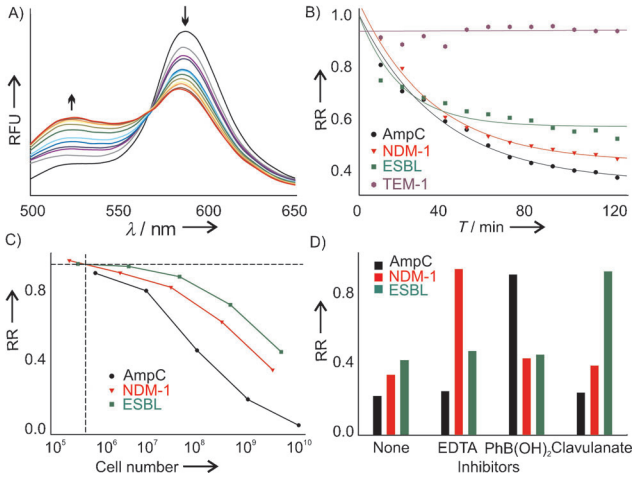


Figure 2. Quantitative determination of β-lactamase activity from different bacterial species. A) Time-dependent fluoresence change at a 10 min interval when C-2 was incubated with E. cloacae (inducible AmpC) cell lysate. B) Time course of the β-lactamase activity from Enterobacter cloacae (inducible AmpC), Klebsiella pneumoniae (NDM-1), Enteric group 137 (ESBL), and Escherichia coli (TEM-1). RR (relative ratio) = $(F_{590}/F_{520})/(F_{590}/F_{520})_S$. C) Minimal number of cells (5×10^5 to 1×10^6) required for detecting β-lactamase activity above background (RR < 0.95, horizontal dash line) dertermined by a set of serial dilution experiments. D) Inhibition profiles of different classes of β-lactamases against various inhibitors. EDTA, ethylenediaminetetraacetic acid; $PhB(OH)_2$, phenylboronic acid.

both the substrate and a β-lactamase of interest, whereas $(F_{590}/F_{520})_S$ is the ratio of the substrate itself under the same condition. By calculating $(F_{590}/F_{520})/(F_{590}/F_{520})_S$, we can compensate variations and compare results measured at different time points and on various instruments. The value of $(F_{590}/F_{520})/(F_{590}/F_{520})_S$ roughly ranges from 0.2 to 1.0, which corresponds to the degree of C-2 degradation. If all conditions are kept constant when testing the activity of several β-lactamases, a lower value means a more efficient enzyme to hydrolyze C-2. Clearly, C-2 was able to distinguish bacterial strains capable of degrading methoxyimino cephalosporin derivatives from those strains lacking this activity (Figure 2B).

Clinically, the time period between the specimen collection and the determination of drug resistance should be as short as possible. In this aspect, a more sensitive assay would certainly be favorable. To find out the detection limit of the C-2 based assay, we performed an experiment in which the β-lactamase activity was measured on a set of serial dilutions of bacterial cells (Figure 2C). The value of $(F_{590}/F_{520})/(F_{590}/F_{520})_S$ at 0.95 under this experimental condition was considered reflecting the minimal β-lactamase activity above the background. With this criterion, we concluded that 5×10^5 to 1×10^6 cells were required to reliably determine whether a bacterial strain is resistant to methoxyimino cephalosporins. The activity of β-lactamases in different bacteria is dependent on many factors such as the subfamily of β-lactamases present and their relative expression level. Therefore, our result here only serves as an example to show that C-2 can detect broad-spectrum β-lactamases from a small number of bacterial cells that we studied and only in a limited number of species. Whether this trend can be extended to all bacterial species at this level of sensitivity remains to be determined.

Even though AmpC (when hyper-expressed), NDM-1, and ESBLs all have high activity hydrolyzing C-2 and other methoxyimino cephalosporins, they use different mechanisms and can be inhibited by different small molecules. For example, phenylboronic acid is a potent inhibitor of AmpC. Likewise, EDTA can chelate zinc ions from NDM-1, thereby inactivating this metallo-β-lactamase. Most ESBLs derived from TEM-1 remain susceptible to clavulanate although some ESBL mutants also acquire new capabilities to resist clavulanate and other TEM-1 inhibitors. By combining C-2 with these substances, we should be able to determine the subclass of β-lactamase present to provide more information for therapeutic determination. As expected, different classes of β-lactamases showed distinct responses when incubated with these inhibitors, suggesting the type of a β-lactamase from an untested bacterial strain could be quickly assigned (Figure 2D).

Finally, we tested whether C-2 can be used to detect bacteria from blood directly. Microbial infection in the circulatory system, which is usually a sterile environment, can have serious consequences. Bacterial cells can easily spread to other parts of the body to establish new colonies and cause secondary infections. The immune system can also elicit whole-body inflammatory responses, leading to life-threatening sepsis and septic shock conditions. Therefore, detecting blood-borne bacterial cells is of clinical importance. As shown in Figure 3, we were able to detect the same bacterial species expressing various classes of β-lactamases in the presence of

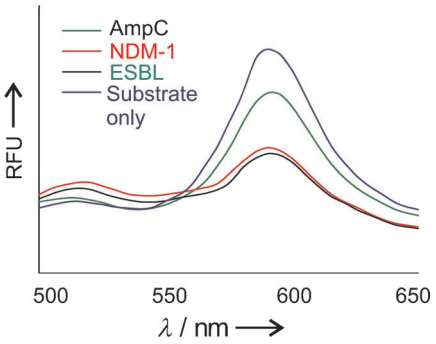


Figure 3. Detection of various β-lactamases in the presence of whole blood. For each β-lactamase, C-2 was incubated with a mixture of sheep blood and a bacterial cell lysate, then its fluorescence was recorded.

blood, but we can no longer use $(F_{590}/F_{520})/(F_{590}/F_{520})_S$ to compare the enzymatic reactivity from different samples. The oxy- and deoxy-hemoglobin in blood can absorb visible light strongly (up to 600 nm), so the fluorescence signal at 520 nm is almost completely suppressed and that at 590 nm is attenuated. Therefore, the decrease of fluorescent signal at 590 nm alone is used to estimate the β-lactamase activity in a sample, which loses the benefits offered by the ratiometric measurement. The sensitivity is also compromised owing to this absorption, as well as shorter half-life of the substrate in

blood. To improve the detection of bacterial cells in blood, we will develop β-lactam probes incorporating FRET fluorophores at the near-infrared region (800–1000 nm) in future studies since blood has minimal absorbance in this window. The Cica-β-test is not compatible with blood test because it relies on a chromogenic substrate whose color change upon enzymatic reaction is completely masked by blood.

We are losing the war to fight bacterial infections. The recently discovered NDM-1 and its global dissemination is an alarming example as most bacteria expressing NDM-1 are only susceptible to a few effective antibiotics that are usually reserved as a last resort.[6,14] Restricting the use of antibiotics will delay the emergence of multidrug-resistant superbugs. The ratiometric fluorescence measurement reported here is a quick and sensitive procedure for screening microbes resistant to methoxyimino cephalosporins. This assay would provide useful advice for antibiotic selection in a timely fashion and prevent unnecessary use of broad-spectrum antimicrobial agents. In conjunction with classic microbiological techniques, our approach would certainly help combat ever-evolving bacterial drug resistance.

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