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Novel Fluorogenic Substrates for Imaging β-Lactamase Gene Expression

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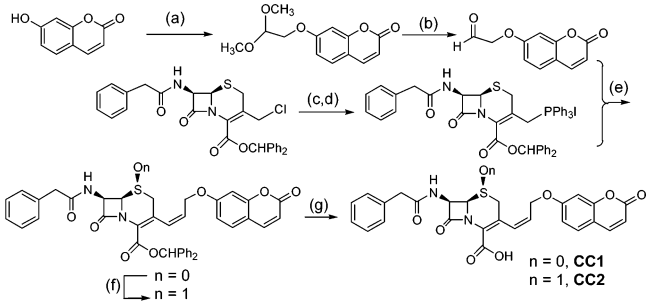
β-Lactamases are a family of bacterial enzymes that cleave penicillins and cephalosporins with high catalytic efficiency and render these bacteria resistant to β-lactam antibiotics.[1] Rapid and sensitive detection of β-lactamase activity in biological samples is thus of large clinical importance. While β-lactamases are the biochemical markers for identification of β-lactam antibiotics-resistant bacterial pathogens, the β-lactamase activity can also serve as a "reporter" or "sensor" for monitoring biological processes and interactions of interest. TEM-1 β-lactamase (Bla), the 29 kDa isoform product of the ampicillin resistance gene (*amp*^{r}), is especially useful because it is relatively small and monomeric, does not exist in eukaryotes, but can be easily expressed in eukaryotic cells without any noticeable toxicity.[2,3] TEM-1 Bla has been developed as a reporter for examining the promoter/regulatory elements activity in living mammalian tissue culture cells.[4−6] Protein fragment complementation assays based on Bla have been successfully applied to monitor constitutive and inducible protein interactions both in vitro and in vivo.[7−9] Incorporation of Bla into HIV-1 virions allows convenient detection of HIV-1 virion fusion in primary T lymphocytes in complex cell populations.[10]

Substrates that detect Bla activity with high sensitivity are critical for the successful implementations of Bla assays. Fluorogenic substrates are superior to chromogenic substrates (such as well-known nitrocefin and PADAC)[11−13] in detecting enzyme activity because of the high sensitivity of fluorescence detection. The first reported fluorogenic Bla substrate shown to work with cells was CCF2, which consists of a donor 7-hydroxycoumarin linked via a cephalosporin to an acceptor fluorescein.[4] CCF2 fluoresces green because of fluorescence resonance energy transfer from the coumarin donor to the fluorescein acceptor. Hydrolysis of the cephalosporin by Bla splits off the fluorescein, disrupts energy transfer, and shifts the emission to blue.[4] As the only fluorogenic Bla substrate currently available, CCF2 has many successful applications in tissue culture,[4−10] but its high molecular weight and low aqueous solubility have prevented applications in intact mammalian tissues or in cells with thick walls such as in yeast or plants. More fluorogenic substrates would expand the usefulness of Bla as a biosensor. Here, we report a new class of small fluorogenic substrates that work by releasing a phenolic dye from a vinylogous cephalosporin.

Cleavage of the β-lactam ring of a cephalosporin creates a free amino group, which triggers spontaneous elimination of any leaving group previously attached to the 3′ position.[14] Umbelliferone is a widely used fluorophore with maximal excitation at 360 nm and emission at 460 nm. When its 7-hydroxy group is alkylated, the compound becomes essentially nonfluorescent. We thus designed and synthesized a β-lactam called **CC1** in which the 3′ position of the cephalosporin is linked to the 7-hydroxy group of umbelliferone alkylated through an allylic ether bond (Scheme 1).[15] With the 7-hydroxy group of umbelliferone alkylated, **CC1** should be essentially nonfluorescent, and if Bla hydrolysis leads to spontaneous release of umbelliferone, in this case through an extended conjugation system, fluorescent signals will be produced (Figure 1A). The complete synthesis of **CC1** is illustrated in Scheme 1. The Wittig coupling reaction exclusively afforded the *Z*-isomer, as shown by its ^{1}H NMR spectrum.

Scheme 1. Synthesis of **CC1** and **CC2**[a]



[a] (a) $BrCH_2CH(OCH_3)_2$, DMF, K_2CO_3, 115 °C, 12 h, 82%; (b) concentrated HCl/$CHCl_3$, 0 °C, 30 min, 63%; (c) NaI, acetone, 1 h; (d) PPh_3, EtOAc, o/n, 81% in two steps; (e) CH_2Cl_2, 1 M NaOH, room temperature, o/n, 28%; (f) *m*-CPBA, CH_2Cl_2, 20 min, 93%; (g) TFA, CH_2Cl_2, anisole, 1 h, 0 °C, 71% (for $n = 0$) and 81% (for $n = 1$).

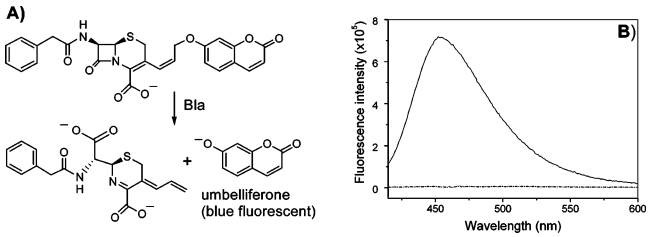


Figure 1. (A) Hydrolysis of **CC1** by β-lactamase releases the fluorophore umbelliferone. (B) Emission of **CC1** (10 nM in PBS) before (dash dot line) and after (solid line) treatment of β-lactamase (excitation at 400 nm).

We first examined the emission spectrum of **CC1** before and after the Bla treatment and observed a 153-fold increase in the intensity at the wavelength of 460 nm upon complete hydrolysis of **CC1** (Figure 1B). This result validates that Bla cleaves **CC1** and releases the fluorophore umbelliferone. The increase in the fluorescent emission at 460 nm is considerably larger than the 20-fold enhancement of CCF2 at similar wavelengths and affords a convenient means to measure the Bla activity. In the phosphate-buffered saline (PBS) at pH 7.1, **CC1** is hydrolyzed by TEM-1 Bla with a catalytic constant (k_{cat}) of 52 ± 1 s^{-1} and a Michaelis constant (K_m) of 70 ± 7 μM (values were obtained from weighted least-squares fit of a double-reciprocal plot of the hydrolysis rate

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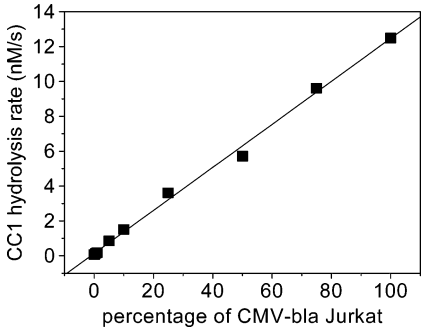

Figure 2. Linear dependence of the **CC1** hydrolysis rate on the β-lactamase concentration. Wild-type and CMV-*bla* Jurkat cells were mixed at different ratios (with the percentage of CMV-*bla* Jurkat cells from 0, 0.5, 1, 5, 10, 25, 50, and 75 to 100%), lysed, and diluted for **CC1** assay with excitation at 365/42 nm and emission at 465/35 nm.

versus **CC1** concentration); its catalytic efficiency (k_{cat}/K_m) is 7.4 $\times$ 10^5 M^{-1} s^{-1}. The spontaneous hydrolysis rate constant of **CC1** in the PBS is $\sim$1.3 $\times$ 10^{-6} s^{-1}, and the enzymatic acceleration is $\sim$4 $\times$ 10^7 fold.

As compared to CCF2 (k_{cat} = 29 s^{-1} and K_m = 23 μM),[4] **CC1** has a 2-fold lower affinity for Bla, but its k_{cat} is nearly twice as fast. Because **CC1** itself is nonfluorescent, a higher concentration can be used to obtain a faster hydrolysis rate. The stability of **CC1** in the absence of Bla can be further improved by oxidation of the sulfide in the six-membered ring to sulfoxide **CC2**, which resulted in a 4-fold decrease in the spontaneous hydrolysis rate ($\sim$2.6 $\times$ 10^{-7} s^{-1}). **CC2** remains as a substrate with slightly less catalytic efficiency (k_{cat} = 10 s^{-1} and K_m = 0.35 mM). **CC2** may be preferred for experiments where a longer incubation is needed.

We tested the ability of **CC1** in detecting Bla activity in measuring the percentage of Bla-expressed cells in a cell mixture. Wild-type Jurkat cells do not express any Bla, and a clonal Jurkat cell line constitutively transfected with Bla gene under the cytomegalovirus (CMV) promoter control (CMV-*bla* Jurkat cells) expresses approximately 1.5 $\times$ 10^4 Bla/cell.[5] Two cell lines were mixed at different ratios, and the cell lysates were analyzed with **CC1** for the Bla activity. A plot of the apparent hydrolysis rate versus the percentage of CMV-*bla* Jurkat cells reveals a linear relationship (Figure 2). This assay can reliably detect 0.5% CMV-*bla* Jurkat cells in the background of wild-type cells, which corresponds to approximately 500 fM of Bla.

The importance of the inserted double bond is further exemplified in making a red fluorescent substrate, which is preferred due to a longer excitation and emission wavelength. Resorufin fluoresces maximally at 585 nm when excited at 550 nm, and a structure with resorufin directly linked to the 3′-position of cephalosporin has been made but found to spontaneously hydrolyze rapidly in water.[16] However, an analogue of **CC2** with umbelliferone replaced by resorufin (**CR2**) is stable in PBS buffer with a half-life of $\sim$182 h. **CR2** displays a 42-fold increase in the fluorescence intensity upon the hydrolysis by Bla with the catalytic parameters k_{cat} = 17 $\pm$ 3 s^{-1} and K_m = 114 $\pm$ 12 μM. A membrane-permeable acetoxymethyl ester of **CR2** (**CR2/AM**) was able to image the Bla activity in Bla-stably transfected C6 glioma cells (Figure 3).

This report describes a design of a new class of fluorogenic substrates of β-lactamase and characterization of their enzymatic kinetics, and it demonstrates their applicability in detecting β-lactamase activity in biological samples. These new fluorogenic substrates are easy to make, simple to use, have high sensitivity for detecting β-lactamase activity, and should facilitate applications

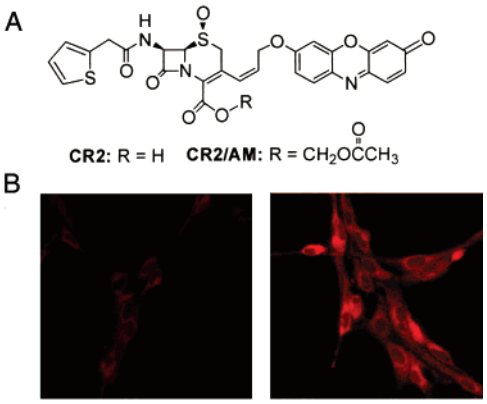

Figure 3. (A) Structures of **CR2** and **CR2/AM**. (B) Fluorescence images of wild-type (left) and Bla-stably transfected (right) C6 glioma cells loaded with **CR2/AM** in Hank's Balanced Salts Solution for 25 min at room temperature. Excitation filter, 540/25; emission filter, 635/55; and 40$\times$ magnification.

with β-lactamase as a biosensor. The design reported here is not limited to umbelliferone and resorufin and could be extended to other molecules containing phenolic leaving groups. It may serve as a general strategy to create a wide variety of fluorogenic, chromogenic, and lumogenic substrates for β-lactamase.

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Supporting Information Available: Synthesis of **CC1**, **CC2**, and **CR2**, and procedures for the measurements of kinetic parameters and Bla activity in cell lysates (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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