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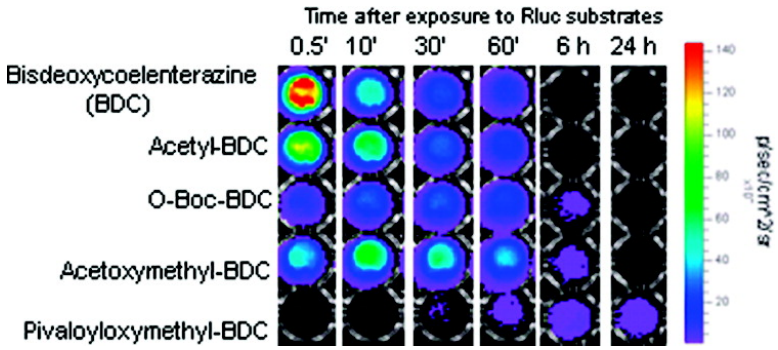

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Bisdeoxycoelenterazine Derivatives for Improvement of Bioluminescence Resonance Energy Transfer Assays

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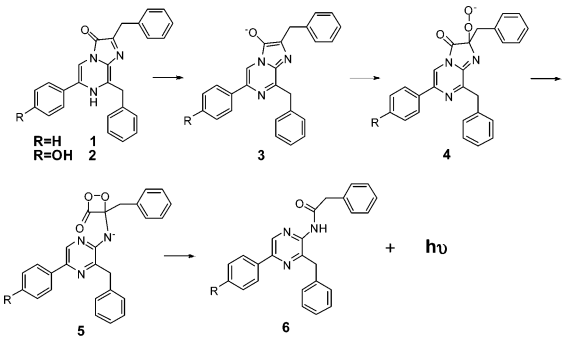
Protein−protein interactions are the basis of many important cellular functions. The ability to noninvasively image these interactions in living organisms provides a unique possibility of studying important biological processes in intact, minimally perturbed systems.[1] Molecular imaging assays based on bioluminescence resonance energy transfer (BRET) are one of the key strategies for investigating protein−protein and other interactions in live cells as well as in living subjects.[2−4] BRET is a phenomenon involving a transfer of energy, obtained from the oxidation of substrate by the energy donor, an oxygenase protein, and an energy acceptor, a fluorescent protein. Typically, BRET systems utilize *Renilla* luciferase (RLuc) as an energy donor and green fluorescent protein (GFP) and its mutants as an energy acceptor. Based on the specific *Renilla* substrate utilized, BRET systems are currently divided into BRET^1, BRET^2, and recently introduced eBRET.[5] Of the three, the BRET^2 system has the best spectral separation between the donor and the acceptor and thus higher signal to background ratio than all possible BRET^1 and eBRET combinations.[6−8] Despite the superior spectral resolution, the BRET^2 system has not been applied as broadly and as frequently as BRET^1, due to the poor quantum yield and very fast kinetics of oxidation of the substrate utilized, bisdeoxycoelenterazine (BDC, **1**). The fast oxidation of BDC results in a light signal that decays rapidly, limiting stable signal detection to only a few seconds.[5] We report here BDC derivatives that improve kinetics of BDC oxidation and consequently offer significantly longer lasting light signal. Considering that the half-times for protein−protein interactions vary from brief to long, this improvement in signal sustainability greatly expands the utility of BRET^2 in real-time protein−protein interaction imaging.

Oxidation of coelenterazine (**2**), the natural RLuc substrate, can generate light as a result of both enzymatic and chemical reaction, phenomena termed bioluminescence and chemiluminescence, respectively. Although the mechanism of coelenterazine oxidation is thought to be the same (Scheme 1) for both of these reactions,[9−11] the kinetics of light production differ quite remarkably. Bioluminescent reactions have very fast kinetics, leading to the production of intense, exponentially decaying light signal.[12,13] On the other hand, chemiluminescence caused by the reaction of coelenterazine with aprotic organic solvents in the presence of oxygen and base results in longer lasting light signal of lower intensity.[10] The rate-limiting step in chemiluminescence reactions seems to be the formation of a peroxide intermediate (**4**). Coelenterazine peroxide has been synthesized and detected at −80 °C by NMR.[14] At temperatures higher than −50 °C, the hydroperoxide intermediate spontaneously decomposes with emission of light. The decrease in the rate of the formation of the peroxide intermediate should therefore result in the decrease of the rate of the bioluminescent reaction and sustained production of light. We achieve this by introducing protecting groups at the reaction site, the carbonyl group of the imidazopyrazinone moiety, that have to be removed before the peroxide can be formed and oxidation can take place. The deprotection of the reaction site can be accomplished by the action of the cellular enzymes, such as esterases, which makes the derivatives suitable as live cell substrates.

Scheme 1. Mechanism of Coelenterazine Oxidation and Light Production



We have synthesized four BDC derivatives (Figure 1) with varying size and type of the groups at the carbonyl of the imidazopyrazinone moiety. BDC (**1**) was synthesized following a published procedure[10] by coupling 2-amino-3-benzyl-5-phenylpyrazine and 1,1-diethoxy-3-phenylacetone. Subsequent reaction with acetic anhydride, di-*tert*-butyl dicarbonate, bromomethyl acetate, and chloromethyl pivalate afforded acetyl bisdeoxycoelenterazine (**7**), *O*-Boc-bisdeoxycoelenterazine (**8**), acetoxymethyl bisdeoxycoelenterazine (**9**), and pivaloyloxymethyl bisdeoxycoelenterazine (**10**).

The kinetics of the bioluminescent reactions were evaluated by exposing the derivatives to HT1080 cells stably expressing the GFP^2-RLuc8 fusion protein[15] and detecting generated light over the course of 1 h using a cooled charge-coupled device camera (Figure 2). The luciferase used in this system is the mutant RLuc8 developed in our lab that in the reaction with BDC gives 59-fold higher light output than the native RLuc.[16] Of the four derivatives, only acetyl-BDC showed fast light signal decay, similar to BDC. Not surprisingly, the acetyl ester seems to be easily cleaved by cellular esterases, and the bioluminescent reaction is only slightly altered. The bulkier *tert*-butyloxycarbamyl group at the carbonyl of the imidazopyrazinone moiety results in the derivative that shows considerably slower kinetics. The peak light emission for *O*-Boc-BDC was observed 15 min after exposure to the RLuc8 expressing cells and remained fairly stable over 1 h. In comparison, at the 15 min time point, the parent BDC lost nearly 75% of its initial light emission. Bulkiness of the *tert*-butyl group appears to considerably slow the enzymatic ester hydrolysis and thus the bioluminescent reaction, providing a longer lasting light signal. The size of the protecting group had the same effect on the rate of the bioluminescent reaction in the case of the ether derivatives, acetoxymethyl-BDC and pivaloyloxymethyl-BDC. Acetoxymethyl group delayed the emergence of the maximum light signal by 5 min compared to

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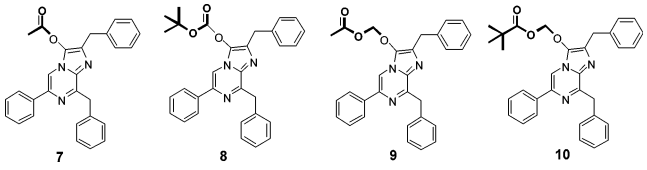

Figure 1. Synthesized bisdeoxycoelenterazine derivatives: acetyl bisdeoxycoelenterazine (**7**), *O*-Boc-bisdeoxycoelenterazine (**8**), acetoxymethyl bisdeoxycoelenterazine (**9**), pivaloyloxymethyl bisdeoxycoelenterazine (**10**).

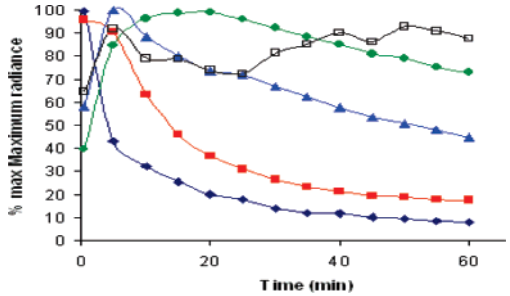

Figure 2. The rate of the bioluminescent reaction for BDC (♦), acetyl-BDC (■), *O*-Boc-BDC (●), acetoxymethyl-BDC (▲), and pivaloyloxymethyl-BDC (□) evaluated as the change of the maximum light signal over time.

the parent BDC, after which time the signal slowly decayed with time. Retardation of the enzymatic ether cleavage caused by the bulky *tert*-butyl group in the case of pivaloyloxymethyl-BDC resulted in the slowest bioluminescent reaction. In terms of the signal half-life, defined as the time required for the initial light flux to fall to its half-value, compared to BDC, derivatives **8**, **9**, and **10** have much improved characteristics. Compared to only 5 min half-life of the parent BDC, derivative **8** had a half-life of ~50 min, while half-lives of derivatives **9** and **10** were even longer than 1 h.

The rate of the bioluminescent reaction also depended on the type of the protecting group. Derivatives carrying ether protecting groups at the carbonyl of the imidazopyrazinone ring, **9** and **10**, showed slower bioluminescent reactions compared to their ester counterparts, derivatives **7** and **8**.

The carbonyl group protection inevitably affects the intensity of the light signal of the derivatives. Of the four derivatives, the highest light signal was observed with acetyl-BDC and the lowest with pivaloyloxymethyl-BDC (Figure 3a). Although the signal intensity of pivaloyloxymethyl-BDC never reached the intensity of the signal of any of the other three BDC derivatives, the only observable signal 24 h after exposure to the enzyme came from it (Figure 3b). Clearly, the decrease in the rate of the bioluminescent reaction is closely related to the decrease in signal intensity. It is important to point out here that the lower light signal does not render a BDC derivative inadequate for application in BRET^2 assays. Just as in the case of Enduren, the coelenterazine h derivative with prolonged lifetime, signal intensity can be increased by simply using higher concentrations of the substrate in BRET assays.[5] What makes a derivative have high utility in BRET^2 applications is the decelerated kinetics of its oxidation resulting in sustained emission of light.

In summary, protection of the carbonyl group of the imidazopyrazinone moiety in bisdeoxycoelenterazine led to derivatives with

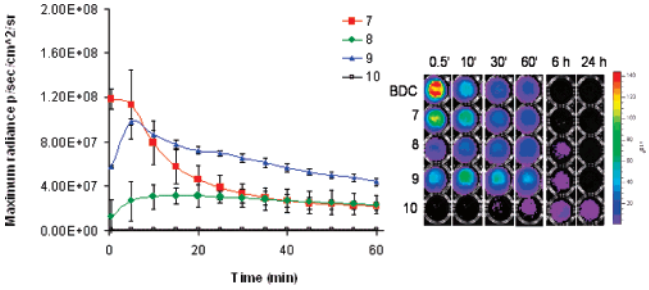

Figure 3. (a) The change in luminescence over time for acetyl bisdeoxycoelenterazine (**7**), *O*-Boc-bisdeoxycoelenterazine (**8**), acetoxymethyl bisdeoxycoelenterazine (**9**), and pivaloyloxymethyl bisdeoxycoelenterazine (**10**). Error bars represent standard deviation from the average value. (b) Bioluminescence imaging of HT1080 cells expressing GFP^2-RLuc fusion protein after exposure to bisdeoxycoelenterazine and its derivatives. Color bar units are p/s/cm^2/sr, where p is for photon and sr is for steradian.

improved kinetics of the bioluminescent reaction. Our results indicate that the extent of the effect the protecting group has on the rate of oxidation depends on the size and type of the protecting group. Of the four synthesized BDC derivatives, three (**8**, **9**, and **10**) show great promise for improving the existing BRET^2 assays in terms of light signal sustainability. The longer lasting signal offers a possibility of real-time imaging of protein−protein interaction in live cells in combination with unparalleled signal to background ratio. Additional testing of these in various applications including in small living subjects should help further characterize their utility.

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Supporting Information Available: Synthetic procedures and characterization of all four derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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