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β-Galactosidase Fluorescence Probe with Improved Cellular Accumulation Based on a Spirocyclized Rhodol Scaffold

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Supporting Information

ABSTRACT: We identified a rhodol bearing a hydroxymethyl group (HMDER) as a suitable scaffold for designing fluorescence probes for various hydrolases. HMDER shows strong fluorescence at physiological pH, but phenolic *O*-alkylation of HMDER results in a strong preference for the spirocyclic form, which has weak fluorescence. As a proof of concept, we utilized this finding to develop a new fluorescence probe for β-galactosidase. This probe has favorable characteristics for imaging in biological samples: it has good cellular permeability, and its hydrolysis product is well-retained intracellularly. It could rapidly and clearly visualize β-galactosidase activity in cultured cells and in *Drosophila melanogaster* tissue, which has rarely been achieved with previously reported fluorescence probes.

Escherichia coli β-galactosidase is a well-characterized enzyme that is widely used as a reporter to examine transcriptional regulation or to evaluate transfection efficiency.[1,2] Several fluorogenic substrates for visualizing the activity of this enzyme in living cells and tissues have been developed, but most suffer from a variety of disadvantages. For example, fluorescein di-β-galactopyranoside (FDG)[3] can only be loaded into cells by hypotonic shock[4,5] or must be applied to fixed or permeabilized cells,[6] as it shows poor cellular permeability. In contrast, TG-βGal, our previously reported fluorescence probe based on the Tokyo-Green (TG) scaffold, can detect β-galactosidase activity in living cells[7] and proved to be useful for efficiently selecting clones expressing β-galactosidase on 96-well plates (unpublished data). However, when applied to cultured cells or tissues such as brain slices, it could not provide clear fluorescence images of β-galactosidase-expressing cells or regions, mainly because its intracellularly generated fluorescent hydrolysis product tends to diffuse across the cell membrane, resulting in a poorly defined signal (Figure S1 in the Supporting Information). A conventional solution to this problem would be to modify the probe to obtain improved cellular accumulation by introducing an esterase-reactive acetoxymethyl (AM) group.[8] However, this strategy cannot be employed *in vivo* or in living tissues because of the ubiquitous presence of esterase activity in extracellular fluid. Therefore, we aimed to develop an alternative approach that is independent of esterase activity. For this purpose, we reexamined available core fluorophores in order to identify one that would be more likely to favor intracellular accumulation. As a candidate scaffold, we focused on rhodol, which is a hybrid structure of fluorescein and rhodamine.[9,10] Rhodol derivatives are known to be highly fluorescent in aqueous solution and are more resistant to photobleaching in comparison with fluorescein.[9] They have been successfully used as scaffolds for fluorescence probes to detect hydrogen peroxide, zinc ion, peroxynitrite, and hypoxia.[11−15] Furthermore, from the viewpoint of sensor design, rhodols have favorable characteristics for our purpose: they contain a phenolic hydroxy group into which a β-galactopyranoside group can be incorporated, and more importantly, they are likely to be accumulated in living cells.[16] Thus, we decided to develop a new fluorescence probe for β-galactosidase based on rhodol. Among the series of rhodols, we focused on *N*,*N*-diethylrhodol (DER), which we found to have a pK_a of 5.7 (Figure S2); this should allow stable measurements near physiological pH. In order to control the fluorescence of DER-based probes, we first considered employing the same strategy used for our previously reported TG-βGal probe,[7] namely, precise control of fluorescence by means of a photoinduced electron transfer (PeT) mechanism. However, this strategy was unfortunately not suitable for DER-based hydrolase probes because DER shows only a small alteration in reduction potential in conversions between the phenolate form and the alkylated form (Table S1).

Thus, we focused on other mechanisms to control the fluorescence of the fluorophore. It has been reported that conversion of the carboxylate group of rhodamine derivatives to a mercaptomethyl or carbamoyl group stabilizes the spirocyclic structure, and this phenomenon has been utilized to control the fluorescence before/after reaction with targets such as hypochlorous acid[17,18] and cations.[19,20] It is also known that conversion to a hydroxymethyl group induces spirocycle formation of dichlorofluorescein under very basic conditions but not in the physiological pH range.[21] Since little is known to date about the effect of a hydroxymethyl group on the fluorescence properties of other fluorophores, including rhodol derivatives, we initially prepared hydroxymethyl-DER (HMDER) and examined the effect of the

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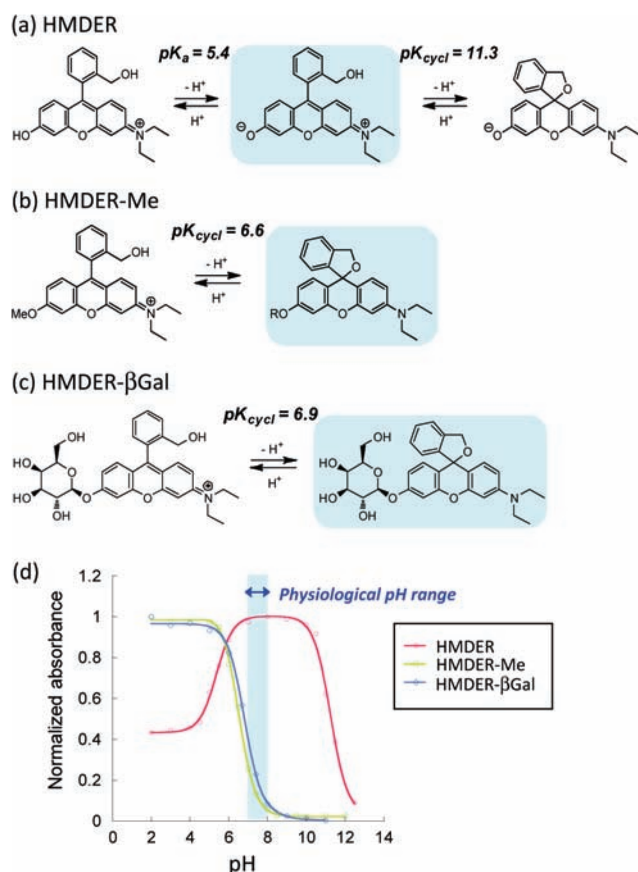

Figure 1. (a−c) Structures of (a) HMDER, (b) HMDER-Me, and (c) HMDER-βGal mainly present at various pH values. The highlighted structures are the putative forms predominantly present at physiological pH. (d) pH dependence of the absorbance of HMDER, HMDER-Me, and HMDER-βGal.

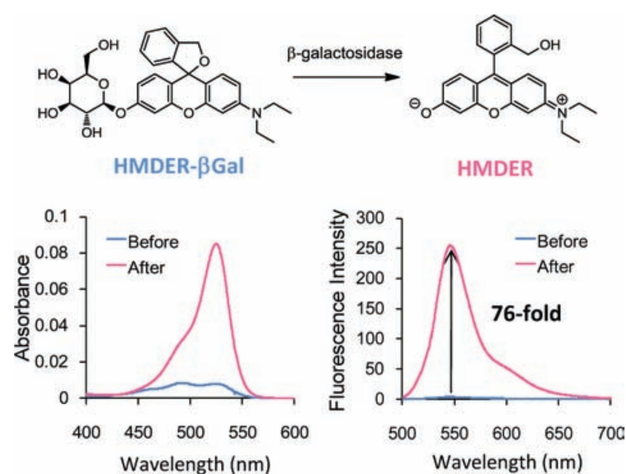

Figure 2. Enzymatic reaction of HMDER-βGal with β-galactosidase. Absorption and fluorescence spectra of a 1 μM solution of HMDER-βGal in PBS (pH 7.4) before and after addition of β-galactosidase (5 units) are shown. The excitation wavelength was 525 nm.

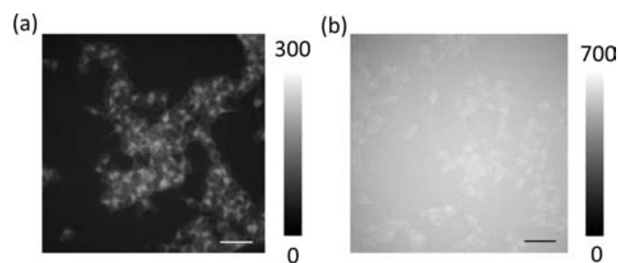

Figure 3. Detection of β-galactosidase activity in living cells. Fluorescence images of HEK/lacZ cells after incubation with a 10 μM solution of (a) HMDER-βGal or (b) TG-βGal for 30 min are shown. Scale bars represent 50 μm.

hydroxymethyl group by comparing the pH profiles of absorbance and fluorescence of DER and HMDER. The pK_a of the phenolic hydroxy group of HMDER was determined to be 5.4, which is close to that of DER (Figure 1a and Figures S2 and S3). The decreased absorption above pH 10 could be explained by the involvement of a nonfluorescent spirocyclic form. In this paper, we define pK_{cycl} as the pH value at which the absorbance of the compound decreases to half the maximum absorbance as a result of the spirocyclization. The pK_{cycl} of HMDER was determined to be 11.3 (Figure 1a and Figure S3). In contrast, a methyl ether derivative of HMDER (HMDER-Me) has a pK_{cycl} of 6.6 (Figure 1b and Figure S4). Interestingly, at the physiological pH of ~7.4, HMDER is present as a colored and fluorescent open form, while HMDER-Me is present in an equilibrium between a colorless nonfluorescent spirocyclic form (85%) and a weakly fluorescent open form (15%) (Figure 1d and Table S2). These results show that the hydroxymethyl group stabilizes the spirocyclic form of HMDER-Me but not that of HMDER at physiological pH. We considered that this remarkable difference in the pK_{cycl} values of HMDER and HMDER-Me, which might be due to the difference in their reduction potentials (Table S1), could be utilized to design a rhodol-based probe for β-galactosidase. That is, incorporation of an enzyme-reactive moiety at the phenolic hydroxy group of HMDER would result in suppression of the background fluorescence via spirocycle formation, while reaction with the enzyme would generate fluorescent HMDER.

To test this idea, we synthesized HMDER-βGal by incorporating a β-galactopyranoside group into HMDER (Figure 1c). We examined the pH profile and fluorescence properties of HMDER-βGal and obtained a pK_{cycl} value of 6.9, meaning that it would be present in an equilibrium between the spirocyclic form (75%) and the open form (25%) at pH 7.4, similar to the case of HMDER-Me (Figure 1c,d and Figure S5). The spirocyclic form has no absorption/fluorescence in the visible wavelength region, and the minor open form shows only weak fluorescence (Φ_{fl} = 0.009). In contrast, HMDER is predominantly present in the open form, which shows relatively strong fluorescence (Φ_{fl} = 0.141) (Table S2).

As a result of these effects, a fluorescence increase of up to 76-fold upon reaction with β-galactosidase was observed (Figure 2 and Figure S6). We next examined whether this new probe could visualize β-galactosidase activity in living cells. When HEK/lacZ cells were incubated with a 10 μM solution of HMDER-βGal, we observed a clear fluorescence increase within the cells (Figure 3a and Figure S7a), while no obvious fluorescence increase was seen with HEK cells, which do not express β-galactosidase (Figure S8a). In comparison with the fluorescence signal obtained with TG-βGal (Figure 3b and Figure S7b), the signal obtained with HMDER-βGal was confined within the cells and the surrounding

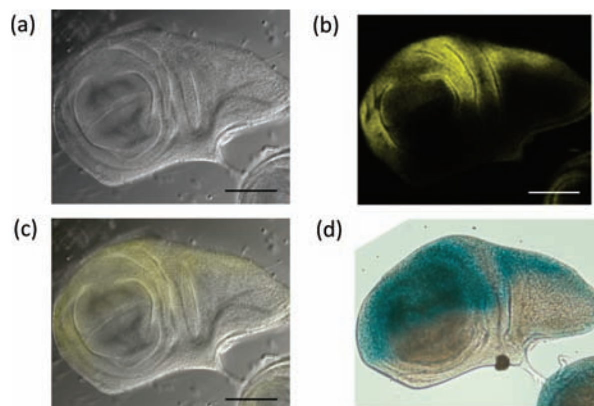

Figure 4. Fluorescence imaging of β-galactosidase activity in *D. melanogaster* wing disks with HMDER-βGal. Wing disks expressing β-galactosidase were incubated with HMDER-βGal (50 μM) in PBS(−) for 2 min. (a) Bright-field image. (b) Fluorescence image. (c) Merged image of (a) and (b). (d) Image after X-Gal staining. Scale bars represent 100 μm.

signal was suppressed to a low level, enabling us to achieve high-contrast imaging. We further examined the subcellular localization of the fluorescence signal produced by HMDER-βGal and found that the cleaved dye tends to accumulate mainly in the endoplasmic reticulum and partially in the Golgi apparatus, while the enzyme is expressed diffusely in the cytosol (Figures S9 and S10). Furthermore, the values of logP (the logarithm of the partition coefficient) calculated with the ALOGPS 2.1 program[22] were 2.88 ± 0.85 for HMDER-βGal (spirocyclic form) and 1.76 ± 0.68 for TG-βGal, while the logD values at pH 7.4 determined by an HPLC method were 2.0 for HMDER-βGal and 1.1 for TG-βGal (Table S3). In both cases, HMDER-βGal showed a value 1 unit larger than that of TG-βGal, suggesting that HMDER-βGal should have improved cellular permeability. We also examined the cytotoxicity of HMDER-βGal using an MTT assay and observed no cytotoxic effect at concentrations up to 100 μM (Figure S11).

Finally, we tried to visualize β-galactosidase activity in tissues of *Drosophila melanogaster*, which is one of the most commonly used model organisms in genetic studies.[23,24] We used wing discs (a precursor structure of the wing) taken from third-instar larvae, in which β-galactosidase (used as a marker enzyme) is expressed only at the posterior region. Soon after the start of incubation of the tissue with HMDER-βGal, a marked fluorescence increase was seen only in the β-galactosidase-expressing region (Figure 4). The rate of fluorescence increase was so rapid that an image could be obtained within just a few minutes (see the movie in the Supporting Information). The expression of β-galactosidase was confirmed by conventional X-Gal staining, which showed that the fluorescence signal was colocalized with the β-galactosidase activity. In contrast, when wing discs not expressing β-galactosidase were used, there was no clear fluorescence increase (Figure S12).

In conclusion, we have designed, synthesized, and evaluated HMDER-βGal, a new fluorescence probe for β-galactosidase. To our knowledge, this is the first demonstration that spirocyclization of a hydroxymethyl group can be effectively utilized to control fluorescence emission before/after reaction with the target enzyme at physiological pH. Furthermore, our use of the rhodol scaffold resulted in increased cellular accumulation of the probe, enabling us to perform clear *in situ* visualization of β-galactosidase activity in living cells and *D. melanogaster* tissues. Since β-galactosidase is one of the most widely used reporter enzymes in biological studies, the potential range of application of this new probe would be enormous. Finally, it should be easy to extend this simple but effective design strategy, which involves functionalization of the phenolic hydroxy group of HMDER, to develop fluorescent probes for a wide variety of target molecules, such as other glycosidases (e.g., sialidases/mannosidases), esterases (Figure S1), and reactive oxygen species. Such probes would be powerful tools for biological studies.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Synthesis and characterization of compounds, pH profiles, fluorescence imaging, cytotoxicity assay, experimental details, and a movie showing β-galactosidase activity in *D. melanogaster* tissues (AVI). This material is available free of charge via the Internet at http://pubs.acs.org.

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