Fluorescence Imaging

DOI: 10.1002/ange.201005705

Self-Immobilizing Fluorogenic Imaging Agents of Enzyme Activity**

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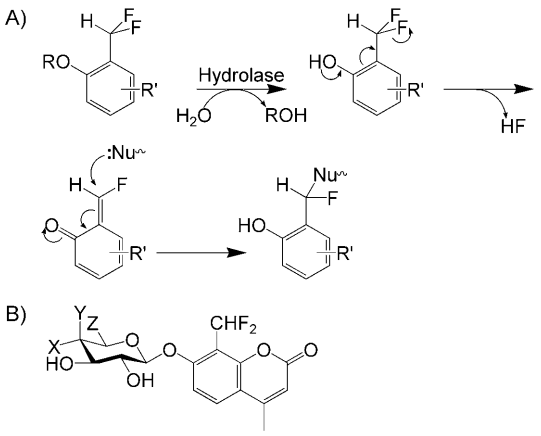
Chromogenic and fluorogenic substrates are valuable tools for locating endogenous- and reporter-enzyme activities, and thus for visualizing gene expression within cells and tissues. They therefore find use in applications ranging from histological analyses to fluorescence-activated cell sorting (FACS).[1] A limiting factor in such studies is the tendency of the colored dye product to diffuse from the site of cleavage, whereby resolution and utility are decreased. This problem was addressed many years ago for visible dyes with the introduction of bromochloroindolyl conjugates, such as 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), which, when cleaved, dimerize to form an intensely blue product and precipitate; thus, diffusion is minimized. Few such systems[2] have been developed for fluorescent reagents, largely because their precipitation results in fluorescence quenching. An alternative strategy for the reduction of product diffusion would be for the enzyme to release a highly reactive fluorescent species that could covalently derivatize nucleophilic sites in the nearby medium. Site-specific immobilization of the fluorophore would result, without precipitation, and quenching would therefore be minimized. For example, tyramide signal amplification (TSA) takes advantage of this strategy.[3] Herein, we report the development of simply modified derivatives of coumarin glycosides that are not innately fluorescent, but when cleaved release a fluorescent aglycone that readily forms a quinone methide, which rapidly reacts with a nearby nucleophile. Their use in histological studies and in FACS sorting of cell types is demonstrated.

Enzyme substrates that generate latent quinone methides have been developed in the past for a range of enzymes, including proteases, esterases, phosphatases, sulfatases, and glycosidases.[4–10] The most commonly employed are those containing *ortho*- or *para*-(di)halomethyl phenols, which, when liberated, generate reactive quinone methides (Scheme 1). These compounds were originally developed as mechanism-based inactivators for the selective inhibition of specific activities or the labeling and identification of active-site residues.[11,12] However, the time taken for the (di)halomethyl phenol to decompose, and for the quinone methide thereby generated to react, is often sufficiently long for the reagent to leave the active site and react with other nearby nucleophiles, including water.[12–14] This problem is further aggravated when, as in most cases, the aryl moiety has no specific affinity for the active site. This behavior has significantly reduced the utility of such compounds and, despite numerous reports,[15–17] has rendered them essentially useless as probes for activity-based proteomics studies, since, although generated by the specific enzyme activity, the quinone methides react indiscriminately.



1 (D-glucurono): X = OH, Y = H, Z = COOH
2 (D-gluco): X = OH, Y = H, Z = CH_2OH
3 (D-galacto): X = H, Y = OH, Z = CH_2OH

Scheme 1. A) Generation of and nucleophilic addition to a quinone methide. B) Fluorogenic quinone methide generating glycoside substrates synthesized in this study.

However, this behavior is ideal for imaging agents of the type proposed. Indeed, the perfect reagent would never react at the active site, since that may inactivate the enzyme, but rather would react on the exterior of the protein, or with cellular components in the immediate vicinity. To test this approach, we synthesized glycosides **1**, **2**, and **3** (Scheme 1). The choice of the difluoromethyl over the monofluoromethyl derivative was based upon the greater stability of the parent compound towards solvolysis[18] and upon the longer anticipated "lifetime" of the dihalomethyl phenol; the longer lifetime improves its chances of diffusing out of the active site.[14] The simple synthetic route employed (see the Experimental Section and the Supporting Information) could be

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201005705.

used to generate latent quinone methide versions of a range of known fluorogenic substrates. The reactivity of each compound as an enzyme substrate was demonstrated by exposure to its cognate glycosidase in buffered solutions. Visual and TLC analysis confirmed cleavage in each case.

To assess the utility of these compounds as histological agents and thereby ascertain the attainable resolution, we tested the β-glucuronide **1** as an imaging agent to identify the site of expression of a β-glucuronidase (GUS) gene marker in an *Arabidopsis thaliana* plant line. The *MUTE* gene was chosen as the target, since its expression triggers the differentiation of certain specialized plant cells to form stomata.[19] These stomata, which consist of a pair of guard cells, are epidermal pores specialized for gas exchange.[20] The MUTE protein is thus localized within developing guard cells and their immediate progenitors. Five-day old transgenic *Arabidopsis* (Col-0) plants carrying *pMUTE*:*GUS*, in which the β-glucuronidase reporter gene has been placed under transcriptional regulation by the upstream putative promoter sequence of the *MUTE* gene, were incubated for 1 or 2 days with **1** or with a 5-bromo-4-chloro-3-indoyl β-D-glucuronide, XGlcU. The leaf samples were imaged after epidermal peels had been dissected from them to reduce the signal from chlorophyll autofluorescence (Figure 1). Treatment with

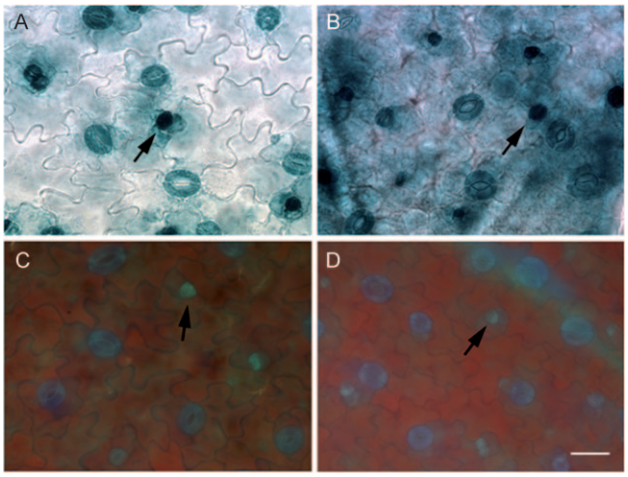

Figure 1. Promoter activity of the *MUTE* gene with different GUS substrates. A,B) Staining of *pMUTE*:GUS transgenic plants with XGlcU: A) after 1 day and B) after 2 days in the staining solution. C,D) Staining of *pMUTE*:GUS transgenic plants with **1**: C) after 1 day and D) after 2 days in the staining solution. Arrows show examples of meristemoids most strongly expressing GUS. Scale bar: 15 μm.

glucuronide **1** indeed resulted in highly specific fluorescence labeling of the guard cells of stomata and their differentially committed progenitor meristemoid cells. This pattern of labeling is consistent with that observed when nonfluorescent XGlcU was used (Figure 1 A,C). However, prolonged staining with the bromochloroindoyl glucuronide led to nonspecific staining of nearby tissue as a result of diffusion (Figure 1 B), whereas treatment with glucuronide **1** over the same prolonged exposure did not lead to degradation of the image to the same extent (Figure 1 D). Similar results can be obtained in plant cells with the commercial reagent ImaGene, which contains an aglycone (a fluorescein derivative) that becomes membrane-impermeable upon cleavage. However, this fluorescein derivative was more sensitive to photobleaching. Treatment with methylumbelliferyl glucuronide, an analogue of **1** containing an unreactive aglycone, yielded no useful images as a consequence of diffusion of the released coumarin, as also noted previously.[21] The greater sensitivity attainable through fluorescence imaging and the temporal stability of the signal should prove extremely valuable in the measurement of expression levels of genes in localized cells and tissues at specific stages of growth. Such compounds may thus be particularly useful in studies of the developmental biology of organisms beyond the plant kingdom.

A second potential use for such reagents is in the activity-based analysis and separation of cells by FACS sorting. Existing fluorogenic substrates have proved to be of limited utility for activity-based FACS sorting of single-cell organisms, such as bacteria and yeast, owing to diffusion of the fluorescent product from the cells. The glucoside and galactoside conjugates **2** and **3**, both of which are hydrolyzed by the *Agrobacterium* sp. β-glucosidase/galactosidase (Abg),[22] were thus incubated separately for 1 h with *Escherichia coli* R1360 ($lacZ^-$) cells that had been transformed either with a plasmid (pTKNd-Abg) encoding Abg or, as controls, with an empty plasmid (pTKNd) or a plasmid (pGSV3-AbgE586) encoding a hydrolytically inactive mutant (AbgE358G). Fluorescence (λ_{ex}: 330 nm, λ_{em}: 450 nm) was observed only in the cultures expressing active Abg that were treated with either **2** or **3** (Figure 2 A). These cells remained fluorescent after multiple washes, which indicated that the cells had been successfully labeled through a covalent linkage. In contrast, when Abg-expressing cells were treated with 4-methylumbelliferyl β-glucoside (4MUβGlc) or 4-methylumbelliferyl β-galactoside (4MUβGal), the fluorescence was completely removed by washing (Figure 2 B). Thus, stable fluorescence labeling of cells expressing the enzyme of interest was observed.

We similarly tested the latent quinone methides **2** and **3** with yeast cells bearing the more specific (PNPGlc: $k_{cat}/K_M = 57\ s^{-1}\,mM^{-1}$, PNPGal: $k_{cat}/K_M = 3\ s^{-1}\,mM^{-1}$) Thai rosewood β-glucosidase (TRBG).[23,24] *Pichia pastoris* Y11430 cells either carrying plasmid-encoded TRBG (pPICZB-TRBG) or, as a negative control, containing no plasmid were incubated with either **2** or **3**. As with *E. coli* expressing Abg, the *P. pastoris* cells expressing TRBG hydrolyzed **2** and **3** and were fluorescently labeled by covalent attachment of the quinone methide generated from the aglycone. Importantly, with **2** or **3**, TRBG-expressing cells could be specifically labeled amongst a larger population of cells that contained no TRBG and could be sorted by FACS; this process enabled specific enrichment. As a test of the potential of this approach, mixtures containing either 1 or 10% TRBG-encoding cells in an excess amount of cells lacking TRBG were sorted in this way (Figure 3). The fluorescently labeled, TRBG-expressing *P. pastoris* cells that were collected by FACS were grown up on agar plates. Of the ten randomly picked colonies grown from cells sorted from each of the mixtures treated with **2** that contained 1 and 10% TRBG-encoding cells, TRBG activity was present in all clones,

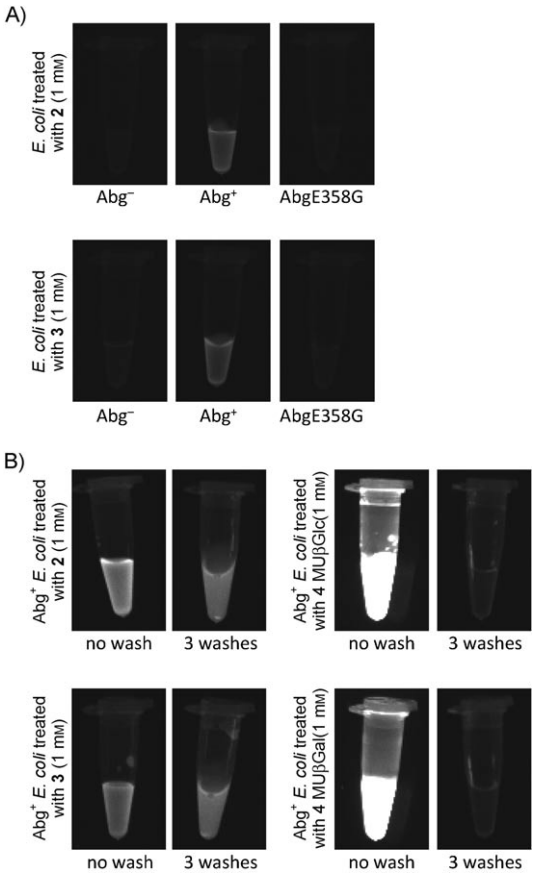

Figure 2. A) *E. coli* cells treated with **2** or **3**. B) Abg-expressing *E. coli* cells treated with **2**, **3**, 4MUβGlc, or 4MUβGal before and after washing.

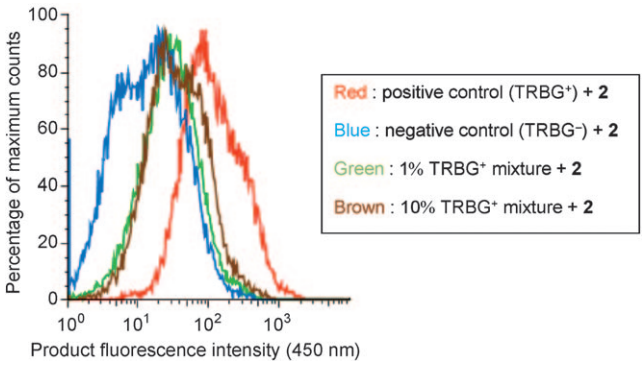

Figure 3. FACS histogram of *P. pastoris* cells treated with **2**.

whereas for the mixtures treated with **3** that contained 1 or 10% TRBG-encoding cells, two and four of the ten clones had TRBG activity, respectively. This result clearly demonstrates the enrichment of TRBG-encoding cells and also demonstrates the sensitivity of this screening strategy to substrate specificity. The approach therefore provides a very powerful means of screening in directed-evolution experiments. We are currently studying the application of the method in this context.

Similar attempts to sort *E. coli* cells expressing Abg met with limited success. When a mixture containing Abg-expressing cells and a 100-fold excess of Abg-lacking cells was treated with glucoside **2**, and the sample was then washed, it was possible to sort and collect the small portion of fluorescently labeled cells by FACS. However, when the collected cells were plated, far fewer colonies grew (ca. 10%) than the number of cells collected. The quinone methide generated from **2** or **3** appears to have a negative effect on the viability of *E. coli* expressing Abg (see the Supporting Information). The greater apparent tolerance of *P. pastoris* cells towards the toxic effects exhibited by **2** and **3** in *E. coli* is probably either a consequence of the compartmentalization of eukaryotic organelles, which may protect yeast cells from damage caused by the nucleophilic addition of reactive quinone methides to vital cellular components, or due to higher concentrations of "trapping" nucleophiles in the yeast cells. This toxicity can probably be avoided by the use of scavenging nucleophiles or further design of the reagent.

In this study, we have demonstrated a novel application for enzyme substrates that generate quinone methides. Such substrates have previously been used as enzyme inactivators and abused as proteomics probes; however, these compounds may find greater value as histological or cell-labeling agents for the investigation of a wide variety of organisms, as illustrated by our results with plants, yeast, and bacteria.

Experimental Section

Glycosides **1**, **2**, and **3** were synthesized from the corresponding protected glycosyl bromides by treatment with 8-formyl-7-hydroxy-4-methylcoumarin[25] to yield the corresponding protected 8-formyl-4-methylcoumarin glycosides, which were fluorinated with diethylaminosulfur trifluoride and then deprotected. Details of the synthesis and characterization are presented in the Supporting Information.

The *A. thaliana* Col-0 *pMUTE:GUS* transgenic plant was the kind gift of Dr. Keiko Torii. Plants were grown on AT-medium (*A. thaliana* minimal medium) plates for at least 5 days under long-day conditions (18 h day/6 h night) at 21 °C. Samples were then incubated in one of the following GUS staining solutions under the following conditions: 2.5 mM **1** for 1–2 days at 37 °C, 1 mM XGlcU (Gold Biotech) for 1–2 days at 37 °C, or 0.05 mM ImaGene (a lipophilic analogue of fluorescein di-β-D-glucuronic acid containing a 12-carbon aliphatic chain; Invitrogen) for 40 min at room temperature. Epidermal peels were manually dissected from leaf tissue to remove background noise due to chlorophyll red autofluorescence from underlying tissue. Samples were viewed with a Leica DMR microscope by using the bright-field technique for XGlcU-stained samples, Leica filter cube A4, commonly used for DAPI, UV excitation at 340–380 nm and emission at 440–470 nm for samples treated with **1**, and excitation at 450–490 nm and emission at 515 nm for samples treated with ImaGene.

E. coli cells were grown in M9 medium supplemented with 0.2% casamino acids and trace minerals. After growth overnight at 20 °C, small cultures (1–2 mL) were centrifuged, and the cell pellet was resuspended in M9 medium (0.15 mL; without supplements) and incubated with **2** or **3** (0.2 or 1 mM) for 1 h at 37 °C. Cultures of *P. pastoris* grown in BMMH medium (composition of BMMH: yeast nitrogen base 0.34% + ammonium sulfate 1% + 1 M potassium phosphate, pH = 6.0 1/10 volume + casamino acids 0.5%; 5 mL) were induced with 0.5% methanol and grown for 5 days at 30 °C. The cells were then washed twice with phosphate-buffered saline (PBS; pH 7.4) and then resuspended in 100 mM aqueous NaOAc (5 mL,

pH 5). Aliquots of 0.5 mL were taken and incubated with **2** or **3** (0.2 mM) for 30 min at 30 °C. Before sorting by FACS, the *P. pastoris* or *E. coli* cells were washed with PBS (pH 7.4) until fluorescence was removed from the supernatant.

Received: September 12, 2010

Keywords: enzymes · fluorescence-activated cell sorting · histology · imaging agents · quinone methides

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