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New Latent Fluorophore for DT Diaphorase

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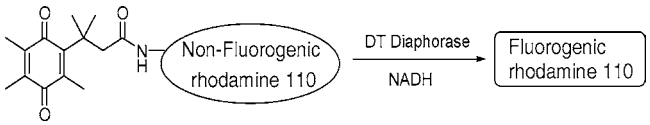
ABSTRACT



This study describes the design and synthesis of a novel latent fluorophore 3 for DT diaphorase based on the trimethyl lock effect and characterization of its enzymatic kinetics. Fluorophore 3 is also a sensitive fluorimetric reagent for detecting glucose when coupled with DTD and glucose dehydrogenase.

DT diaphorase (DTD) (EC 1.6.99.2) is a homodimeric, cytosolic flavoprotein widely distributed and ubiquitously present in all of the tissues of nearly all animal species.[1] It catalyzes the two-electron reduction of various quinones, quinone epoxide, and aromatic nitrocompounds, using NADH or NADPH as an electron donor.[2] DTD is generally regarded as a protective enzyme. The reduction of electrophilic, redox-active quinones by the enzyme is believed to result in detoxification.[3] Recent reports have indicated that DTD activity maintains the reduced states of ubiquinones and α-tocopherolquinone, thereby promoting their antioxidant function in membranes.[4] On the other hand, DTD is overexpressed in many cancerous tissues compared to normal tissue, especially nonsmall cell lung carcinoma, colorectal carcinoma, liver cancers, and breast carcinomas.[5] Because of the difference in the DTD expression between a tumor and normal tissue, this enzyme has attracted considerable attention as a potential candidate for targeted anticancer therapy.[6] In addition, DTD and the DTD-targeted fluorescent chromogen coupled with dehydrogenase have been broadly used for inspecting and diagnosing morbid states, instead of conventional chemical reagents.[7]

Fluorescence has long been viewed as a powerful tool for basic research in the biological sciences, the development of new drugs, the assurance of food safety and environmental quality, and the clinical diagnosis of diseases.[7] Currently, employing a redox reaction and a fluorimetric redox indicator is a sensitive approach for glucose diagnostics. In this case, an oxidizing or reducing with the presence of glucose results in the reduction or oxidation of the fluorimetric redox indicator or via a mediator, which allows for a qualitative or quantitative determination. The known fluorimetric detection reagents for determining glucose have some drawbacks. Oxygen-dependent fluorescent indicators for glucose are determined by detecting H_2O_2 generated by the glucose oxidase. This reaction is catalytically supported by the

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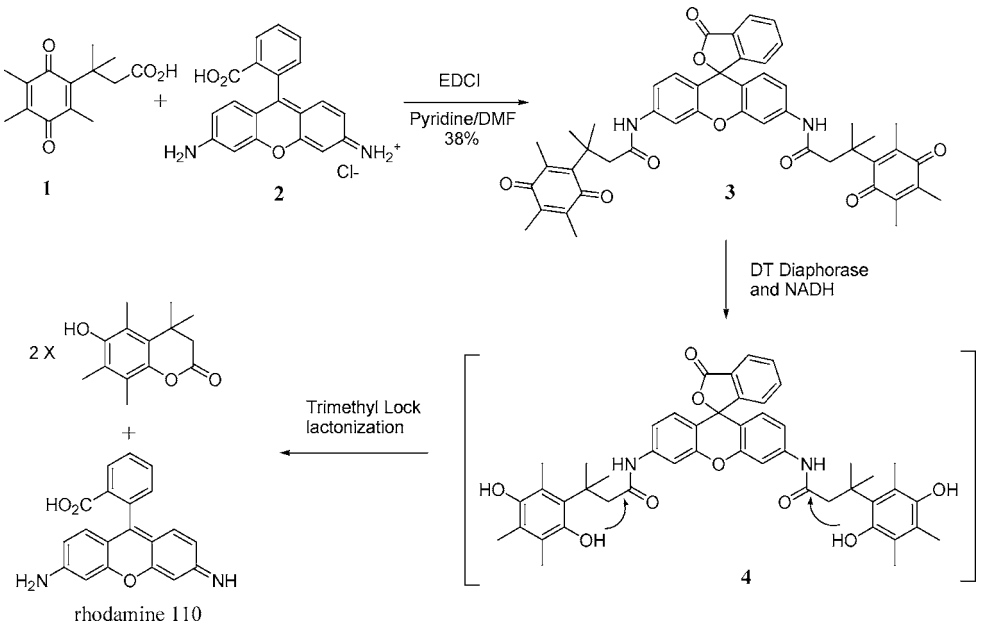
Scheme 1



peroxidase and is prone to interference by electron donors, such as urea or bilirubin.[8] In contrast, redox indicators that directly accept an electron from an oxidizing enzyme instead of oxygen are preferred. The resazurin,[9] transition metal Os, and Ru complexes[10] are the oxygen-independent fluorescent indicators for glucose. In the case of transition metals, their fluorescence efficiency varies with the oxygen content of the sample. On the other hand, the nonfluorescent resazurin is converted into fluorescence-developing resorufin by DTD, β-nicotinamide adenine dinucleotide (NAD^+), and glucoses dehydrogenase (GDH) in the presence of glucose.[9] However, the resorufin can be further reduced by DTD to yield a nonfluorescent product,[11] and the emission bands of resorufin formed by the redox reaction strongly overlap the absorption bands of the nonreacted resazurin, which significantly reduces the sensitivity of the glucose determination.

Latent fluorophores are stable probes that unmask their intense fluorescence only by a user-designated chemical reaction, and they are especially useful tools for basic research in the biological sciences. Recently, there are great interests in developing latent fluorophores due to their unique selectivity and minimal interference from the probe concentration, excitation intensity, and emission sensitivity.[12] We were inspired by the trimethyl lock effect as a means to reveal the cloaked fluorescence, which had been demonstrated by Raines's group.[12c] The rapid formation of 4,4,5,7-tetramethylhydrocoumarin from the corresponding *o*-hydroxycinnamic acid is a result of the trimethyl lock effect.[13] The unfavorable steric interactions between three methyl groups enhance the nucleophilicity of the phenolic oxygen and lead to a rapid lactone formation, and this is an example of the use of strain to enhance the reactivity. Our strategy for the optical detection of the DTD activity also relies on the trimethyl lock effect (Scheme 1). The chemistry of the trimethyl lock effect of quinone as a masked phenol has been thoroughly studied[14] and utilized in the pro-drug strategies;[15] however, there have been no reports of this chemistry for designing latent fluorophores. We envisioned that the trimethyl lock effect would be suitable for the design of a novel latent fluorophore for DTD. We selected rhodamine 110 (**2**) as the fluorophore to be masked because of its high quantum yield, long emission, excitation wavelength, and popularity for basic research in the biological sciences.[16] We reasoned that the installation of quinone acid **1** at the 3′ and 6′ positions of a xanthenone scaffold would allow this platform to adopt a closed and nonfluorescent lactone form. Upon selective reduction of the quinone moiety in **3**, the highly reactive phenol **4** was generated followed by rapid lactone formation with the concomitant release of the open and fluorescent rhodamine 110 (**2**).

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The design and synthesis of the bioreductive quinone-based cytotoxic analogue is an ongoing research effort in our group.[17] We are interested in developing a latent fluorophore which is based on the trimethyl lock effect, with the quinone as a masked phenol for detecting the activity of DTD. In addition, glucose diagnostics could still benefit from a latent fluorimetric reagent because the DTD and DTD-targeted fluorescent chromogen coupled with GDH are ideal oxygen-independent fluorimetric reagents for glucose diagnostics. Hence an objective of the present study was to provide an alternative redox-active latent fluorophore as the detection reagent for the fluorimetric determination of glucose, which can at least partially eliminate the disadvantage of the prior art. This study involves the synthesis and characterization of the novel DTD latent fluorophore **3**. In addition, we established the latent fluorophore **3** as a sensitive fluorimetric reagent for detecting glucose.

The synthesis of **3** was accomplished in one step by coupling of the known acid **1**[15] with rhodamine 110 (**2**) with moderate yield (Scheme 1). The excitation and emission spectra of **3** exhibited a near baseline even with co-incubation with DTD alone (Figure 1), 1 mM NADH alone (data not shown), or after months of storage in phosphate buffer (PBS) at pH 7.3 (data not shown). The introduction of one unit of human recombinant DTD and 1 mM NADH to the solution resulted in a large increase in the fluorescence characteristic of rhodamine 110 (**2**) after 4 h (Figure 1). The presence of mild reduction agent dithiothreitol (1 mM) alone with **3** (10 μM) in PBS also resulted in an increase in the fluorescence characteristic of rhodamine 110 after 4 h incubation at 37 °C. The quinone moiety in **3** is commonly found in various DTD substrates,[18a] and one molecular rhodamine 110 in **3** requires two 4,4,5,7-tetramethylhydrocoumarins to be formed; therefore, we estimated the yield of the 4,4,5,7-tetramethylhydrocoumarin based on fluorescence intensity of rhodamine 110 generated. Employing the fluorescence calibration curve of rhodamine 110, this enzymatic reaction synthesized the 4,4,5,7-tetramethylhydrocoumarin in 32% yield based on the fluorescence intensity of rhodamine 110. The solution containing **3** with DTD and NADH in PBS became fluorescent within 1 min (Figure 2A). In contrast, the incubation of rhodamine 110 with DTD (1 unit) and NADH (1 mM) exhibited no reaction, thus the rhodamine 110 (**2**) is not the substrate of DTD (Figure 2A). The apparent kinetic parameters of **3** with DTD have also been determined. The value of k_{cat}/K_m is 2.35×10^5 M^{-1} s^{-1}, and the K_m value is 26.8 μM (Figure 2B).[19] The fluorescence signal results from two steps, quinone reduction followed by the intramolecular lactonization. The rate-determining step for the fluorescence signal seems to be the quinone reduction step because the intramolecular lactonization induced by the trimethyl lock effect has an effective rate of 10^6 M^{-1} s^{-1}.[13b] The fluorescence signal that resulted from quinone reductions designed herein is 120-fold faster than the hydrolysis counterpart, which was designed by Raines's group.[12] The affinity of the quinone moiety in **3** toward DTD seems to be lower when compared to that of other similar DTD target quinone-based anticancer therapeutic agents;[18] this might be due to the steric interference of the bulky xanthenone scaffold in **3**. This novel DTD-targeted fluorogenic substrate **3** is a useful latent fluorophore, a stable molecule with an intense fluorescence that is unmasked by a user-designated chemical reaction.

We next assessed the ability of **3** as a fluorimetric reagent for determining glucose. NADH is the necessary cofactor

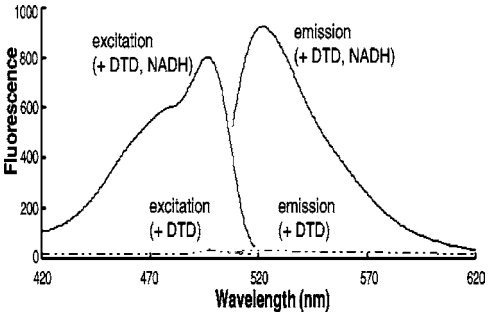

Figure 1. Excitation and emission spectra of **3** (10 μM) incubated for 4 h at 37 °C in PBS with or without NADH.

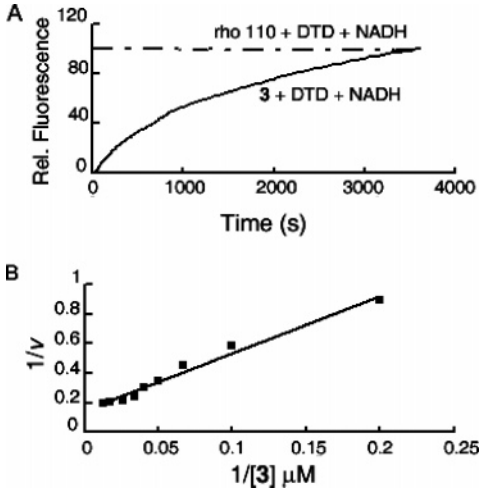

Figure 2. (A) Time course of the generation of fluorescence (λ_{ex} = 492 nm, λ_{em} = 520 nm) by rhodamine (dashed line, 5 μM) and **3** (solid line, 5 μM) in PBS at 25 °C. Data are normalized at the maximum emission. (B) Double-reciprocal plot of reduction of **3** per molecule per second (v) versus substrate concentration ([**3**]).

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(19) The kinetic data were obtained in PBS containing one unit of DTD and 1 mM NADH. Unmasking of the second amino group leads to the manifestation of a ca. 90% fluorescence.

for DTD to reduce the quinone moiety in **3**, and NADH is also the product of many dehydrogenases.

The GDH oxidizes glucose in the presence of NAD^+ to yield glucono-δ-lactone and NADH. The NADH generated from the oxidation reaction coupled with DTD selectively reduced the quinone moiety in **3**, and the highly reactive phenol **4** was generated followed by rapid lactone formation with the concomitant release of the open and fluorescent rhodamine 110 (**2**). Thus, **3** can be a useful fluorimetric reagent for monitoring the GDH activities and glucose concentration. As expected, the mixture of **3** (5 μM) with DTD (1 unit), GDH (1 unit), and NAD^+ (40 μM) was nearly nonfluorescent even after 1 h of incubation at 25 °C. The addition of glucose with a 1 h incubation triggers a prompt fluorescence increase that is characteristic of rhodamine 110 (**2**). A plot of the fluorescence intensity and the concentration of glucose reveals a linear relationship at a glucose concentration between 1 and 6 μM (Figure 3). The latent fluorophore **3** coupled with DTD and GDH is a sensitive system to detect glucose in the low micromolar range, and this sensitivity is comparable to that of other fluorimetric reagent systems, such as resazurin coupled with glucose oxidase.[20] The fluorescence efficiency of the redox reaction and a fluorimetric redox indicator developed herein is not influenced by the oxygen content of the sample. Since the fluorophore rhodamine 110 (**2**) is not the substrate for DTD (Figure 2A), the fluorescence intensity of rhodamine 110 is not affected, and the sensitivity of the fluorimetric redox indicator for the glucose determination is not reduced. The study herein demonstrates that the DTD-targeted latent fluorophore **3** is a fluorescent indicator for glucose upon coupling with the NADH generating GDH.

Figure 3. Relationship of glucose concentration with fluorescence intensity observed after a 1 h incubation of a mixture of **3** (5 μM), glucose, DTD (1 unit), GDH (1 unit), and NAD^+ (40 μM).

In conclusion, this report describes the design of a new class of fluorogenic substrates of DTD and the characterization of its enzymatic kinetics, and it demonstrates its applicability as a fluorimetric reagent for detecting glucose. The new fluorogenic substrate is easy to make, simple to use for detecting DTD activity, and has a high sensitivity for determining the glucose concentration. Use of this latent fluorophore to identify a high-affinity DTD substrate is underway in our laboratory.

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Supporting Information Available: Procedures for the syntheses and analyses reported herein (PDF). This material is available free of charge via the Internet http://pubs.acs.org.

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