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Design and Synthesis of a Long-Wavelength Latent Fluorogenic Substrate for Salicylate Hydroxylase: A Useful Fluorimetric Indicator for Analyte Determination by Dehydrogenase-Coupled Biosensors

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Salicylate hydroxylase (SHL) catalyzes the production of catechol (plus CO_2 and H_2O) from salicylate, NADH, and O_2. Coimmobilization of SHL with a NAD(P)$^+$-dependent dehydrogenase in front of a Clark-type oxygen electrode has been investigated in the development of a general type of dehydrogenase-based biosensors that can detect various biological analytes; however, currently, no fluorophores are available for these applications. We synthesized the first new long-wavelength latent fluorogenic substrate SHLF (3) for SHL. In the presence of NADH and under aerobic conditions, SHL catalyzes the decarboxylative hydroxylation of SHLF followed by a quinone−methide-type rearrangement reaction concomitant with the ejection of a fluorescence coumarin 2, which is spontaneous and irreversible at physiological temperatures in aqueous media. The fluorescence signal generated by this process is specific and, in the near red spectral region with an emission maximum at 595 nm, is suppressed by salicylic acid. The fluorescence response of SHLF is insensitive to various biological reactive oxygen species (ROS) and reductants. Furthermore, SHLF is a sensitive fluorimetric indicator for analyte determination in the SHL-coupled dehydrogenase assay in which NAD$^+$ is converted to NADH. This novel fluorescence assay detected 3-hydroxybutyrate and cholesterol in the nanomolar range and is more sensitive than the current SHL−dehydrogenase amperometric sensors, making it applicable to the construction of a fiber-optic fluorescence biosensor for clinical diagnostic uses.

Salicylate hydroxylase (SHL, EC 1.14.13.1), a flavoprotein, binds salicylate and an external reductant (NADH or NADPH) in random order to form a reduced enzyme−substrate complex, which upon binding of O_2 leads to the production of catechol, CO_2, and H_2O.[1] Production of this enzyme is induced in *P. putida* S-1 by salicylate as the sole carbon and energy source in the culture medium. SHL has been purified and crystallized, and its mechanistic−kinetic properties have been studied extensively.[1] SHL was used as a signal generator and for the electrochemical detection of NADH in electrochemical detection with high performance characteristics.[2a] Recently, the coimmobilization of an NAD(P)$^+$-dependent dehydrogenase with SHL in front of a Clark-type oxygen electrode has been investigated in the development of a general type of dehydrogenase-based biosensor that can detect formate,[2b] nitrate,[2c] alaine,[2d] glutamate,[2e] lactate,[2f] 3-hydroxybutyrate,[2g] and glucose-6-phosphate.[2h] At present, however, monitoring of SHL activity is limited to an electrochemical method, which requires a cumbersome enzyme immobilization procedure.

Due to its high resolution and sensitivity, fluorescence is 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.[3] Moreover, there is rapidly growing interest in the development of fluorophores with high selectivity for diagnostic applications. Latent fluorophores are stable molecules with intense fluorescence revealed by user-designated chemical reactions.[4] They are especially useful for diagnostic applications because of their unique selectivity and minimal interference from the probe concentration. Elegantly de-

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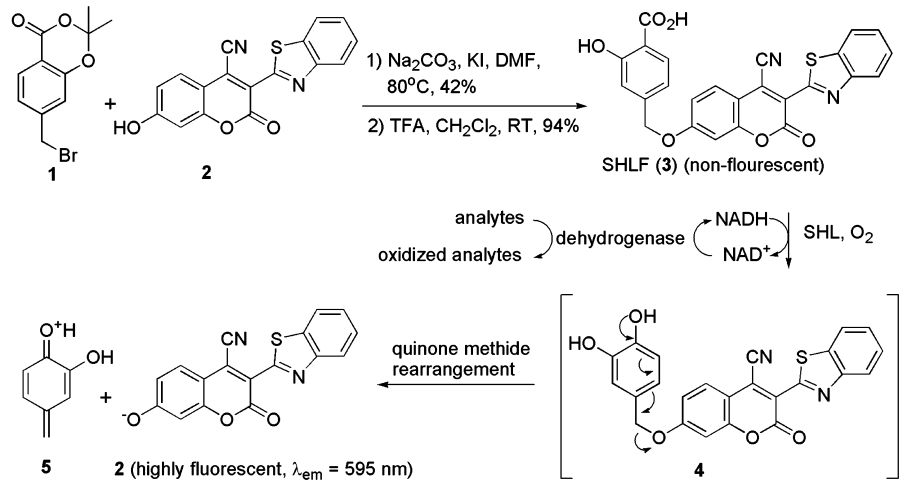
Scheme 1. Chemical Structure of SHLF (3), the Fluorescence Revealing Mechanism, and Illustration of the SHL Coupled Dehydrogenase Assay for the Analytes Determination



signed molecules that release fluorescent coumarin or rhodamine derivatives upon activation by enzymes are sensitive tools that can monitor specific enzymatic reactions for diagnostic applications.[4]

We have long attempted to design and synthesize new latent fluorophores for redox enzymes.[4d,e] We have described a new, long-wavelength, latent fluorophore that can detect biological thiols, with a fluorogenic coumarin releasing mechanism based on the quinone−methide rearrangement−elimination reaction (Scheme 1).[4e] This latter reaction has been utilized to release drugs from prodrugs,[5] in the selective cleavage of peptides from an enzyme-labile linker during solid-phase synthesis[6] and in the off−on fluorescence mechanism of a hydrogen peroxide latent fluorophore.[7] We hypothesized that this same fluorescence releasing mechanism could be adapted to the design of a latent fluorophore to monitor SHL activity. We, therefore, prepared the SHLF **3**, the first latent fluorogenic substrate for SHL (Scheme 1). The chemical architecture of **3** consists of a salicylate moiety directly linked to a fluorogenic coumarin **2** through an ether linkage (Scheme 1). We selected coumarin **2** as the fluorogenic dye to be masked because its anionic form is highly fluorescent in the near red region of the visible spectrum (λ_{em} = 595 nm and Stork shift = 90 nm), and it is an excellent leaving group ($pK_a = 6.08$), a desirable property for application as a latent fluorophore.[8] Many biological samples possess some degree of fluorescence, usually in the blue region of the spectrum, thus interfering with the measurement of the fluorescence of a fluorophore. It is, therefore, desirable to enhance the detection sensitivity using marker dyes that fluorescence in a low-energy region (≥600 nm) of the electromagnetic spectrum.[9] In the presence of NADH and under aerobic conditions, SHL catalyzes the decarboxylative hydroxylation of SHLF followed by a quinone−methide-type rearrangement reaction concomitant with the ejection of the fluorescence coumarin **2**, which is spontaneous and irreversible at physiological temperatures in aqueous media (Scheme 1). Here, we describe a concise synthesis of SHLF (**3**) and the mechanism by which its fluorescence is generated by SHL in the presence of NADH and O_2. We also show that coincubation of a NAD^+-dependent dehydrogenase with SHL in the presence of SHLF may be a general type of dehydrogenase-based fluorescence biosensor (Scheme 1).

RESULTS AND DISCUSSION

Design and Synthesis. A two-step synthesis of SHLF (**3**) is outlined in the scheme. Our previous experience suggested that alkylation of the hydroxyl moiety in coumarin **2** prevents its ionization at neutral pH and drastically reduces its intense fluorescence signal.[4f] Our synthesis began by masking the intense fluorescence signal emitted by coumarin **2**. The hydroxyl moiety of coumarin **2** was benzylated with benzyl bromide **1**[10] under basic conditions to yield the corresponding benzyl ether (not shown). Benzyl bromide **1** was selected because it is a protected salicylate that can be recognized by SHL after deprotection. The protected acid moiety in **1** is in the *para* position to the benzyl ether, the ideal position for a quinone−methide rearrangement−elimination reaction soon after SHL catalysis of decarboxylative hydroxylation of the acid moiety (Scheme 1). The final step of the synthesis was to deprotect the protected salicylate moiety with wet trifluoroacetic acid (TFA), yielding the desired latent fluorogenic substrate SHLF (**3**). Overall, this two-step synthesis had a 35% yield from coumarin (**2**). The chemical structures of the synthetic intermediate and final products were characterized by NMR and mass spectroscopy, and the results are listed in the Experimental Section.

Photochemical Characterization of the Optical Switch of SHLF by SHL. We first evaluated the optical switch of the latent fluorogenic substrate SHLF in the presence of SHL and NADH under

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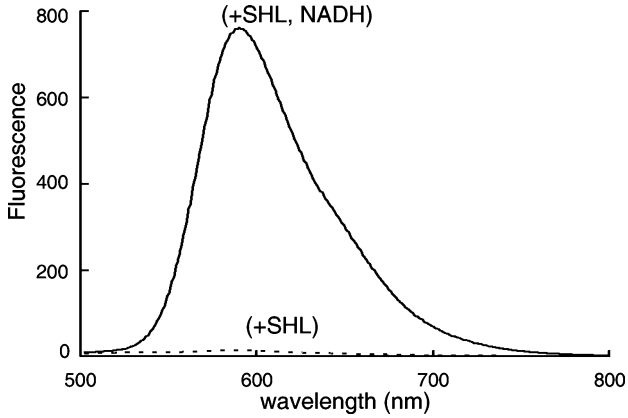

Figure 1. Normalized fluorescence emission spectra of 20 μM SHLF (**3**) and one unit of SHL with or without 1 mM NADH in Tris−HCl buffer, pH = 8.0.

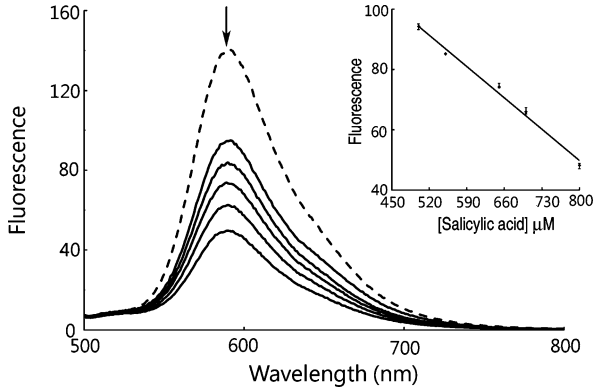

Figure 2. Fluorescence responses of SHLF (5 μM), SHL (1 unit), and 1 mM NADH coincubated with various concentrations of salicylic acid in Tris−HCl buffer, pH = 8.0. Inset: The fluorescence intensity (590 nm) plot vs concentration of salicylic acid (500−800 μM).

aerobic conditions. The emission spectrum of SHLF (20 μM) was baseline when incubated with SHL alone (Figure 1) or 1 mM NADH alone (data not shown) or after months of storage in Tris−HCl buffer at pH 8 (data not shown). The incubation of SHLF (20 μM) with 1 unit of SHL and 1 mM of NADH at 37 °C for 2 h, however, resulted in a 57-fold increase in the fluorescence characteristic of coumarin **2** (Figure 1). The fluorescence became detectable within seconds after the introduction of SHL (1 unit) and 1 mM NADH to a solution of SHLF in Tris−HCl buffer (Supporting Information, Figure III), and the fluorescence intensity of SHLF with only SHL (1 units) stayed alone at the baseline even after 10 min. The fluorescence intensity of coumarin **2** was not influenced by the presence of SHL and NADH even after 600 s of incubation (Supporting Information, Figure III). The pH dependence of fluorescence responses of SHLF in the presence of SHL, NADH, and O_2 was also characterized (Supporting Information, Figure IV), with the best signal to nose ratio observe at pH 8.

The fluorogenic conversion of SHLF by SHL in the presence of NADH under aerobic conditions could be suppressed by the addition of salicylic acid to the reaction media. The fluorescence intensities of SHLF (5 μM) incubated with SHL and NADH under aerobic conditions were reduced as the salicylic acid concentration increased (Figure 2). A plot of fluorescence intensity versus salicylic acid concentration revealed a linear relationship for the salicylic acid concentrations of 200−700 μM (Figure 2, inset). Salicylate is the main metabolite of aspirin. At blood concentrations higher than 2.2 mM, however, it may be toxic and blood concentration of 4.3 mM is lethal; thus, close monitoring of salicylate levels is of clinical interest.[11] The linear range of salicylate detected by SHLF plus SHL is within the tolerated concentration. The SHLF coupled SHL may be a useful fluorescence-off sensor for monitoring salicylic acid concentration.

HPLC Responses of SHLF in the Presence of SHL and NADH under Aerobic Condition. We also use high performance liquid chromatography (HPLC) to show that SHL in the presence of NADH under aerobic conditions catalyzes the release of coumarin **2** from SHLF (Supporting Information, Figure V). At a concentration of 150 μM, SHLF showed a peak with a retention time of 4.9 min (Supporting Information, Figure V, spectrum a). The introduction of 500 μM NADH and 1 unit of SHL to a solution of SHLF resulted in the reduction of this peak, together with the generation of new peaks, with a retention time of 6.3 min (Supporting Information, Figure V, spectra b−d), corresponding to coumarin **2** (Supporting Information, Figure V, spectra e). With prolong incubation, the peak area for SHLF decreased while the peak area for **2** increased, again showing that SHL in the presence of NADH under aerobic conditions catalyzed the release of the coumarin **2** from SHLF.

Quinone−methide-type rearrangement reaction sequences have been widely used as part of a molecular releasing mechanism in other applications.[4f,5−7] In designing the first latent fluorogenic substrate for real time monitoring of SHL activity, we successfully incorporated a quinone−methide-type rearrangement sequence into the elements of an optical switch design. On the basis of our design, we proposed a concise two-step synthesis of the latent fluorogenic substrate SHLF (**3**) incorporating a cloaked, long-wavelength, fluorogenic coumarin dye. We demonstrated that the uncloaking of this intense profluorogenic dye could be directed by a designated chemical process corresponding to our initial design. Furthermore, NADH was an essential electron donor for this SHL-triggered fluorescence unmasking process, although NADH exhibits its own fluorescence in the blue region of the spectrum. The fluorogenic chemical transformation of SHLF by SHL in the presence of NADH released the intensely fluorescent coumarin **2** with emission in the near red region of the spectrum. Thus, the quantitative monitoring of SHL by measuring the fluorescence of **2** was not disrupted by the presence of NADH.

Apparent Kinetic Parameters of SHLF with SHL in the Presence of NADH under Aerobic Condition. The apparent kinetic parameters of SHLF with SHL had been determined. A plot of the rate of appearance of the coumarin **2** ejected from SHLF versus various concentrations of SHLF (Supporting Information, Figure VI) showed that the K_m was 4.2 ± 0.4 μM, V_{max} was 13 ± 1.2 nM s^{-1} mg protein^{-1}, k_{cat} was 0.045 min^{-1}, and k_{cat}/K_m was 0.0087 μM^{-1} min^{-1}. The apparent K_m value of SHLF is about 3-fold higher than the K_m value of salicylic acid (1.6 μM).[1c] This reduction in affinity may have been due to steric interference from the nearby bulky coumarin **2**. To minimize this steric interference, we are currently designing a new fluorophore, in

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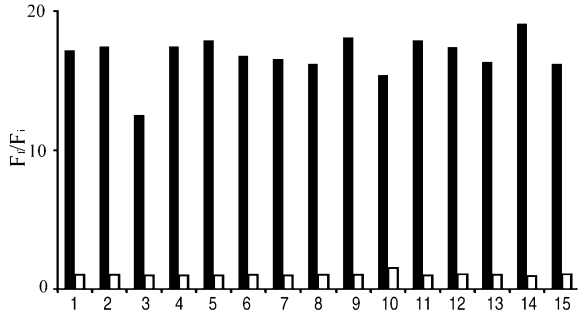

Figure 3. Fluorescence responses of SHLF (**3**; 20 μM) coincubated with various biological reductants and reactive oxygen species (ROS) in Tris−HCl buffer, pH 8.0, at 37 °C for 2 h. Bars represent the final fluorescence intensity (F_f) over the initial intensity F_i at λ_{em} = 595 nm (λ_{ex} = 500 nm). Light bars represent the addition of analytes (1) NADH, (2) dithiothreitol (1 mM), (3) $Fe(ClO_4)_2$ (300 μM), (4) glutathione (1 mM), (5) cysteine (1 mM), (6) urea (1 mM), (7) ascorbic acid (1 mM), (8) histidine (1 mM), (9) bilirubin (300 μM), (10) 5% bovine serum, (11) hydrogen peroxide (1 mM), (12) $^{\bullet}OH$ (generated by reaction of 100 μM Fe^{2+} and 500 μM H_2O_2), (13) $O_2^{\bullet-}$ (generated by addition of 1 mM KO_2), (14) ^{-}OCl (300 μM NaOCl), and (15) $ROO^{\bullet}$ (generated by addition of 1 mM 2,2′-azobis(2-amidinopropane)dihydrochloride). Dark bars represent the subsequent addition of SHL (1 unit) and NADH (1 mM) to the mixture.

which the separation between the salicylate moiety and the bulky fluorescent dye is extended.

Fluorescence Responses of Pro-Fluorophore SHLF to Various Biological Reductants and Reactive Oxygen Species. The selectivity of our approach was assessed by a specific redox reaction of the salicylate moiety in SHLF with the NADH−O_2−SHL complex to yield the catechol intermediate. Since SHL had been widely employed as the signal reporting enzyme in dehydrogenase-based biosensors,[2] the main concern was that other biological reductants or reactive oxygen species (ROS) may react directly with SHLF to yield a similar intermediate, resulting in erroneous amplification of the analytical signal. We, therefore, measured the fluorescence responses of SHLF to various biological reductants and ROS, each at either 15- or 100-fold greater concentration than the concentration of SHLF (Figure 3).[12] No obvious changes in or minimal responses of SHLF were observed upon the addition of reductants or ROS alone. When we assessed the effects of the reductants or ROS on the fluorescence responses of SHLF with SHL and NADH, we found that most had no significant effect on fluorescence intensities, although we found that one reductant, $Fe(ClO_4)_2$, showed slight fluorescence quenching. To determine the causes of this fluorescence signal reduction, we titrated coumarin **2** with various concentrations of $Fe(ClO_4)_2$ and found a linear relationship between quenching of the fluorescence intensity and $Fe(ClO_4)_2$ concentration (data not shown). These findings suggest that the $Fe(ClO_4)_2$-induced reduction of the fluorescence signal generated by the reaction of SHLF, SHL, NADH, and $Fe(ClO_4)_2$ was due to its fluorescence quenching of the released coumarin **2**. Conversely, the low reactivity of the other reductants and ROS toward SHLF indicate that the recognition selectivity of SHLF was sensitive and selective for monitoring SHL activities without interference from biological reductants and ROS.

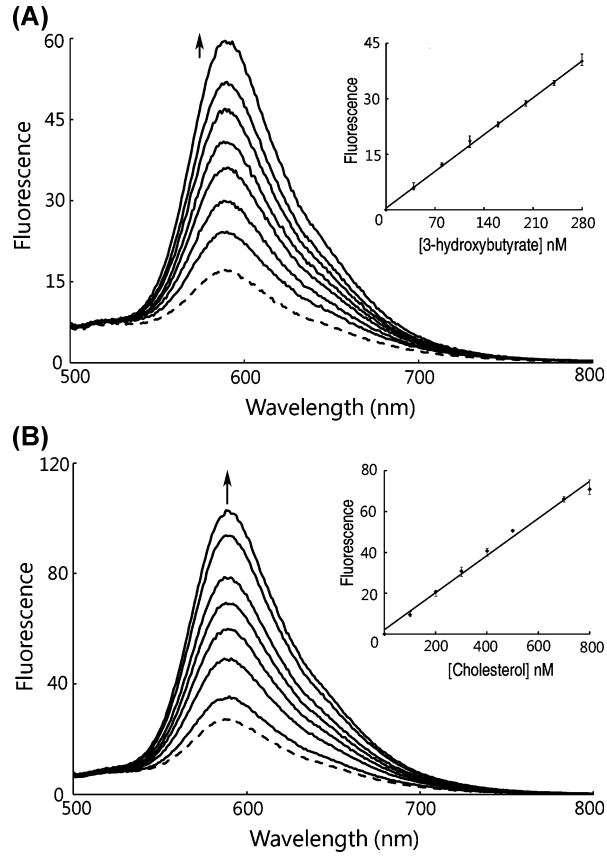

Figure 4. Fluorescence spectra changes of SHLF (10 μM) with SHL (1 unit), NAD^+ (50 μM), and (A) HBDH (2 unit) upon addition of 3-hydroxybutyrate (60 to 280 nM) (Inset: The fluorescence intensity (595 nm) plot vs concentration of 3-hydroxybutyrate (50−280 nM)) or (B) CDH (1 unit) upon addition of cholesterol (100−800 nM). Inset: The fluorescence intensity (595 nm) plot vs concentration of cholesterol (100−800 nM).

Dehydrogenase Coupling-Enzyme Assay for Analyte Determination. Finally, we assessed the utility of SHLF as a fluorimetric indicator in a dehydrogenase-coupling assay for analyte determination. In these experiments, SHLF was incubated with SHL, NAD^+, dehydrogenase, 3-hydroxybutyrate dehydrogenase[13] (HBDH, E.C. 1.1.1.30), and cholesterol dehydrogenase[14] (CDH) while omitting NADH, which is generated by reactions in the presence of the analyte (Scheme 1). In the presence of NAD^+, HBDH oxidizes 3-hydroxybutyrate to yield the acetoacetate and NADH,[13] whereas CDH catalyzes the oxidation of cholesterol to cholest-4-ene-3-one with NADH as the final electron acceptor.[14] To assess the utility of SHLF as a fluorimetric indicator, we used a bienzyme system containing SHL plus HBDH or CDH and NAD^+ as the electron transporter (Figure 4). The emission spectrum of SHLF (60 μM) was near baseline when coincubated with 50 μM NAD^+, 1 unit of SHL, and 2 units of HBDH or 1 unit of CDH (Figure 4A,B). The introduction of either 3-hydroxybutyrate or cholesterol to the solution resulted in a large increase in the fluorescence charac-

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teristics of coumarin **2** following incubation at 37 °C for 30 min. Plots of fluorescence intensity versus 3-hydroxybutyrate or cholesterol concentrations revealed linear relationships at 3-hydroxybutyrate concentrations between 50 and 280 nM and cholesterol concentrations between 100 and 800 nM, respectively (Figure 4A,B). These findings indicate that the SHLF is a sensitive fluorimetric indicator for detecting 3-hydroxybutyrate and cholesterol in the nanomolar range.

Numerous methods are available for measuring analytes for diagnostic applications. One of these methods employs a redox reaction with a redox indicator, in which an oxidizing or reducing system directly acts on the redox indicator or via a mediator. The presence of analytes results in the reduction or oxidation of the redox indicator allowing quantitative determinations. For example, the cholesterol oxidase−peroxidase chromogenic method is used to measure cholesterol;[15] however, this method has several drawbacks.[16] The chromogen indicating reactions in these methods are based on the detection of H_2O_2 generated by the cholesterol oxidase-catalyzed oxidation of cholesterol in the presence of O_2. H_2O_2 can be catalytically detected by peroxidase, which in the presence of H_2O_2, oxidizes the chromogenic indicators resulting in an increase or decrease of chromogens. These catalytic reactions, however, are prone to interference by electron donors such as urea, bilirubin, and glutathione.[16] Redox indicators that directly or indirectly accept an electron from an oxidizing enzyme instead of oxygen are preferred. NAD^+ is a stable electron carrier in biological systems and is insensitive to various electron donors. We have described the successful development of a low interference quantitative analyte determination platform by incorporating the stable electron transporter NAD^+ coupled with the fluorimetric indicator SHLF as the final electron acceptor in the SHL coupled dehydrogenase assay.

To our knowledge, there have been no reports in which the concentrations of 3-hydroxybutyrate or cholesterol have been measured using SHL coupled with the corresponding dehydrogenase and a fluorimetric indicator. The compound 3-hydroxybutyrate is an important marker for glycemic control in diabetes.[17] In a healthy individual, the blood concentration of 3-hydroxybutyrate is less than 0.5 mM, whereas in patients with diabetic ketoacidosis the concentration of this compound ranges from 4.3 to 6.0 mM.[18] Our method, using SHLF as the sensitive latent fluorimetric indicator for measuring low nM concentrations of 3-hydroxybutyrate was more sensitive than methods in which the bienzymes, HBDH and SHL, were coimmobilized on an amperometric probe, which detected a micromolar concentration of 3-hydroxybutyrate.[2g] In addition, cholesterol is an important indicator for monitoring cardiovascular disease. Colorimetric methods for assaying cholesterol-based measurements of NADH formation by CDH are sensitive only in the millimolar range.[19] In contrast, our method was a highly sensitive assay, determining cholesterol in the nanomolar range. Moreover, the assay system we have described may be applicable to the construction of fiber-optic biosensors in the future.

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CONCLUSION

In summary, we have successfully implemented the quinone−methide-type rearrangement reaction as the off−on optical switch into the design of the first long-wavelength latent fluorogenic substrate SHLF (**3**) for SHL. SHL in the presence of NADH under aerobic condition catalyzes the decarboxylative hydroxylation reaction of the salicylate moiety in SHLF (**3**), followed by a quinone−methide-type rearrangement reaction concomitant with the ejection of a long-wavelength fluorogenic coumarin **2**, which is spontaneous and irreversible at physiological temperatures in aqueous media. The fluorescence signal generated by this cascade reaction is specific and insensitive to various biological ROS and reductants. Furthermore, SHLF in the SHL-coupled HBDH or CDH assay reactions is a sensitive fluorimetric indicator for quantitatively measuring 3-hydroxybutyrate or cholesterol in the nanomolar ranges. This SHLF and SHL-coupled dehydrogenase assay platform is expected to be applicable to measure broad ranges of important physiological analyte for clinical diagnostics.

EXPERIMENTAL SECTION

General Considerations. ^1H and ^{13}C NMR were obtained on a Bruker AMX-500 spectrometer. Chemical shifts were reported in ppm relative to tetramethylsilane (δ units). High resolution electron ionization (HREI) and electrospray ionization (ESI) were performed at the Analytical Facility of The National Taiwan University and National Taiwan Normal University. The fluorescence measurements were made with the use of a fluorescence grade quartz cuvette and the Horiba Jobin Yvon Fluoromax-4 spectrofluorometer. High performance liquid chromatography (HPLC) was performed on a Thermo LOT 833-15 cm column (250 × 4.6 mm) with an ALPHA 10 Isocratic Pump; fractions were detected with a TOPAZ dual UV detector, and data was analyzed using Peak-ABC software. All other chemicals were purchased from Acros, Aldrich, TCI, or Sigma Chemical and used without future purification. The benzyl bromide **1** and coumarin **2** were prepared in lab according to the published procedures.[11,8] Salicylate hydroxylase (SHL, EC 1.14.13.1, from *Pseudomonas sp.*) was from GDS Technology Inc. (USA). *D*-3-Hydroxybutyrate dehydrogenase(III) was purchased from TOYOBO (product number HBD-301). Cholesterol dehydrogenase (from *Nocardia sp.*) was from Genzyme. All measurements were performed in 50 mM Tris−HCl (TRIZMA Base, Tris, pH 8). The stock solution of SHLF (**3**) was prepared in dimethylsulfoxide (DMSO) and added to Tris−HCl buffer before use, and the DMSO concentrations never exceeded 0.1% (v/v) in all the experiments.

Preparation of the Synthetic Intermediate: 3-Benzothiazol-2-yl-7-(2,2-dimethyl-4-oxo-4*H*-benzo[1,3]dioxin-7-ylmethoxy)-2-oxo-2*H*-chromene-4-carbonitrile. A solution of 7-bromomethyl-2,2-dimethyl-benzo[1,3]dioxin-4-one (**1**, 0.43 mmol), coumarin (**2**,

0.31 mmol), KI (0.042 mmol), and K_2CO_3 (0.37 mmol) in dry *N*,*N*-dimethylformamide (DMF; 2 mL) stirred overnight at rt under argon. The resulting mixture was diluted with water (50 mL). The organic layer was extracted with CH_2Cl_2 (3 × 50 mL), dried with $MgSO_4$, concentrated in vacuo, and then was purified by flash chromatography (CH_2Cl_2/toluene = 1/4) yielding the title compound (42%, 70 mg) as a orange solid. mp 187−190 °C. ^{1}H NMR (DMSO-d_6, 500 MHz, δ): 1.71 (s, 6H), 5.42 (s, 2H), 7.22 (s, 1H), 7.32 (m, 3H), 7.56 (t, J = 7.5 Hz, 1H), 7.62 (t, J = 7.5 Hz, 1H), 7.91 (d, J = 8.0 Hz, 1H), 7.96 (d, J = 8.0 Hz, 1H), 8.13 (d, J = 8.1 Hz, 1H), 8.21 (d, J = 8.1 Hz, 1H). MS(EI) $C_{28}H_{18}N_2O_6S$ calc. 510.5, m/z = 510.0. FT-IR (v/cm^{-1}): 3075, 2941, 2868, 2371, 1742, 1724, 1615, 1587, 1536, 1502, 1440, 1378, 1288, 1268, 1191, 1144, 1042, 953, 902, 867, 842, 772, 693, 672 cm^{-1}.

Preperaction of Latent Florogenic Probe 3: 4-(3-Benzothiazol-2-yl-4-cyano-2-oxo-2*H*-chromen-7-yloxymethyl)-2-hydroxy-benzoic acid (SHLF, 3). To a 50 mL round bottomd flask equipped with a magnetic stirring bar was added benzyl protected coumarin (0.38 mmol) and trifluoroacetic acid (TFA)−H_2O (1.75 mL, 9/1, v/v), and the solution was stirred for 12 h. Water (50 mL) was then added to the mixture, and the resulting mixture was stirred for 30 min. The precipitate was filtered and washed with cold water to obtain pure acid **3** (0.08 mg, 0.17 mmol, 94%) as orange solid. mp 176−178 °C. ^{1}H NMR (DMSO-d_6, 500 MHz, δ): 5.37 (s, 2H), 7.02 (d, J = 8.1 Hz, 1H), 7.06 (s, 1H), 7.30 (d, J = 8.1 Hz, 1H), 7.33 (s, 1H), 7.56 (t, J = 7.5 Hz, 1H), 7.62 (t, J = 7.5 Hz, 1H), 7.81 (d, J = 8.1 Hz, 1H), 7.93 (d, J = 8.1 Hz, 1H), 8.12 (d, J = 8.0 Hz, 1H), 8.23 (d, J = 8.1 Hz, 1H). MS (ESI) $C_{25}H_{14}N_2O_6S$ m/z = 469 (M − 1). MS (HESI) calc. 470.0573, m/z = 469.470. FT-IR (v/cm^{-1}): 3447, 2924, 2852, 2224, 1724, 1672, 1619, 1536, 1507, 1452, 1433, 1379, 1312, 1288, 1255, 1208, 1187, 1142, 1041, 1015, 948 cm^{-1}.

Photophysical and HPLC Characterization of SHLF with SHL and NADH. The solution of SHLF (20 μM) in 1 mL of Tris−HCl buffer containing 1 unit of SHL with or without 10 mM NADH was incubated at 37 °C for 2 h, or the same solution without SHL and only with 10 mM NADH was also incubated at 37 °C for 2 h; the fluorescence spectra (λ_{ex} = 500 nm and λ_{em} = 595 nm) of the solution were recorded. For the fluorescence suppression by salicylic acid experiments, the solutions of SHLF (5 μM) in 1 mL of Tris−HCl buffer containing 1 unit of SHL and 10 mM NADH with various concentration of salicylic acid (500−800 mM) were incubated at 37 °C for 1 h, and the fluorescence spectra (λ_{ex} = 500 nm and λ_{em} = 595 nm) of the solution were also recorded. For each HPLC experiment, the solution of SHLF (200 μM) in 1 mL of Tris−HCl buffer containing 5 units of SHL and 300 μM NADH was incubated at 37 °C. At each end point, aliquots (20 μL) were removed and injected into HPLC. The column was developed by a mobile phase of solvent (CH_3OH/H_2O/THF, 10:4:1, v/v) at a flow rate of 0.5 mL/min. The UV detector was set at a wavelength of 480 nm, and identification of the HPLC peaks was determined by their retention times and standards.

Kinetic Parameters Determination. All kinetic measurements were performed at room temperature with the excitation wavelength of λ_{ex} = 500 nm and the emission wavelength of λ_{em} = 595 nm in 1.0 mL of Tris−HCl buffer containing 1 unit of SHL and 10 mM NADH. The appearance rate of coumarin (**2**) released from SHLF triggered by SHL in the presence of 10 mM NADH was measured by adding a known concentration of SHLF (0−20 μM) to the reaction mixture and recording it as a function of time. The rate was calculated using the fluorescence calibration curve for coumarin (**2**) (λ_{ex} = 500 nm and λ_{em} = 595 nm). The kinetic parameters, Michaelis Menten constant (K_m) and catalytic rate constant (k_{cat}), were extrapolated from the Michaelis Menten plot. All assays were performed in triplicate, and the results reported were the average of at least three experiments.

Fluorescent Response of SHLF to Various Biological ROS and Reductants. All reductant stock solutions (10 mM) in buffer were prepared prior to use, and all of the ROS were generated following the published paper.[12] Typical reactions were carried out with a final concentration of 20 μM SHLF. (The final concentration of ROS and reductants are indicated in the figure legend.) An aliquot (10 μL) of a stock solution of SHLF (1 mM Tris buffer solution, pH = 7.0) was diluted in buffer to a final volume of 1 mL in a quartz cuvette. An aliquot of a stock solution of the reductants or ROS was then added, and the solution was agitated with a 1 mL pipet. All reactions were carried out at 37 °C for 2 h. The assays were carried out using fluorescence detection (λ_{ex} = 500 nm, λ_{em} =595 nm). The magnitude of the change in fluorescence intensity after 2 h was used to assign a qualitative extent of fluorescent signal, revealing the biological reductants and ROS.

SHL and Dehydrogenase Coupling Analytes Concentration Quantification Assay. All the fluorimetric measurements were carried out with an excitation wavelength of λ_{ex} = 500 nm and an emission wavelength of λ_{em} = 595 nm. The solutions containing SHLF (60 μM), SHL (1 unit), HBDH (2 unit) or CDH (1 unit), NAD^+ (50 μM), and 3-hydroxybutrate (0−0.28 μM) or cholesterol (0−0.8 μM) in Tris−HCl buffer with a final volume of 1 mL were incubated for 30 min at 37 °C. The fluorimetric intensity of each solution after subtracting the baseline intensity ([analytes] = 0) was plotted versus the analyte concentration.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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