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Design, Synthesis, and Characterization of Small-Molecule Reagents That Cooperatively Provide Dual Readouts for Triaging and, When Necessary, Quantifying Point-of-Need Enzyme Assays

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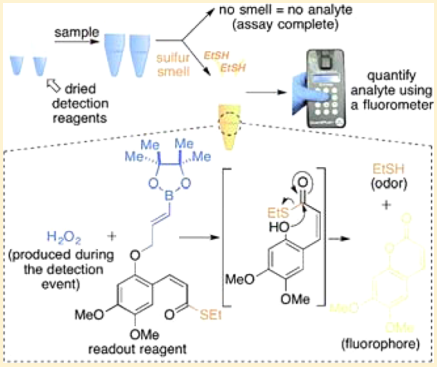
Supporting Information

ABSTRACT: A newly designed small molecule reagent provides both qualitative and quantitative readouts in assays that detect enzyme biomarkers. The qualitative readout enables rapid triaging of samples so that only samples that contain relevant concentrations of the target analyte must be quantified. The reagent is accessible in essentially three steps and 34% overall yield, is stable as a solid when heated to 44 °C for >1 month, and does not produce background signal when used in an assay. This paper describes the design and synthesis of the reagent, characterizes its response properties, and establishes the scope of its reactivity.



INTRODUCTION

This paper describes a small molecule assay reagent that provides the dual readouts of fluorescence (a coumarin derivative) and smell (ethanethiol) (Figure 1), which enables rapid triaging of samples for the presence of a target analyte. This assay strategy requires only one aliquot of a sample and minimizes the frequency of time-consuming quantitative measurements. This article focuses on the organic chemistry of this new reagent, whereas future studies will detail the analytical performance of the reagent.

The design of this new small molecule reagent addresses an unrealized goal in analytical chemistry, which is the development of high-throughput, low-cost, quantitative assays that operate in low-resource point-of-need settings.[1−4] Example scenarios where such assays are needed include (i) testing multiple sources of recreational water for the presence of *Escherichia coli* (*E. coli*) derived from fecal contamination, and if present, then measuring the quantity of the contamination; (ii) evaluating streams, lakes, and rivers for specific pollutants from mining or fracking; or (iii) screening fruits and vegetables or other food products for the presence and quantity of specific bacterial pathogens. In these scenarios, not only is information needed about the presence (and if present, the abundance) of a contaminant to make an informed decision, but the tests often must be rapid so that tens or hundreds of samples can be measured easily and quickly without the resources of a laboratory.

Designing tests that meet these criteria has proven difficult, particularly since the assay reagents must be stable, the assay components must be portable and inexpensive, and the assay itself must proceed smoothly with little input by users, including those with minimal training. While no current assay satisfies all of the requirements for an effective high-throughput, low-cost, quantitative point-of-need assay, new approaches are being developed to address this deficiency. These strategies include assays based on cell phones,[5−17] repurposing glucose meters to detect analytes other than glucose,[18−25] using a voltage meter to measure the results of an electrochemical assay,[26−35] assays based on relative measurements of time,[36−41] detection based on the release of odorous compounds,[42−45] as well as assays based on the distance that a sample travels on an assay platform.[46−48]

These approaches, however, all require that every sample be analyzed quantitatively. Performing quantitative assays on every sample typically is slow, expensive, and operationally involved, which is not an ideal scenario when many of the analytical samples to be tested may lack relevant levels of the analyte of interest and therefore do not require a quantitative measurement (a qualitative assay would suffice for these samples).

An alternative approach to running quantitative assays on all samples is to use a triaging assay first, followed by quantitative assays on samples that test positive for the target analyte. Of course, this approach requires two separate assays, likely two aliquots of a sample, and twice as many manipulations by the user. An approach that provides both quantitative and qualitative readouts simultaneously, so that only one test is performed, offers a practical alternative. We demonstrated an example of a dual readout approach (Figures 1a and 2a) in a recent report in which only samples with relevant levels of the

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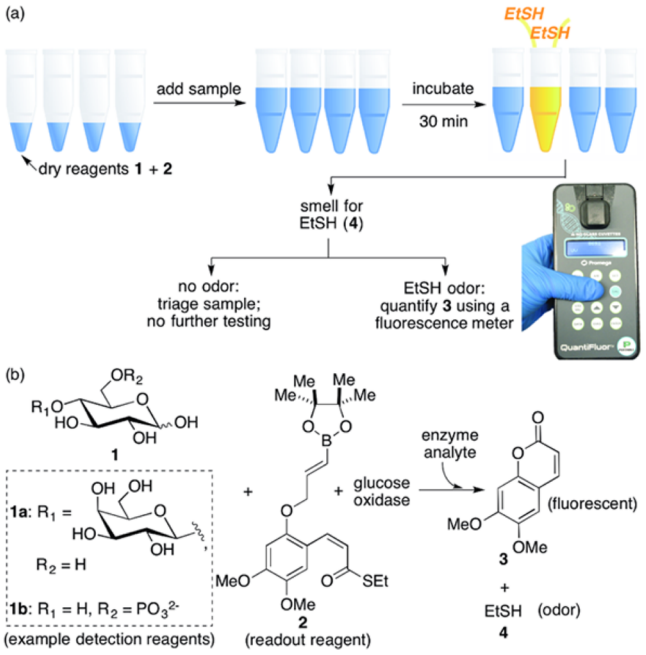

Figure 1. Design for a triaging assay where odor (ethanethiol) signals which samples require quantitative measurements. (a) Schematic representation of the assay. (b) Specific reagents used in the assay. These reagents include detection reagents (**1**) that select for a target enzyme analyte; glucose oxidase, which produces hydrogen peroxide upon oxidation of glucose that is released during the detection event; and readout reagent **2** that provides the dual readouts of fluorescence (**3**) and smell (**4**).

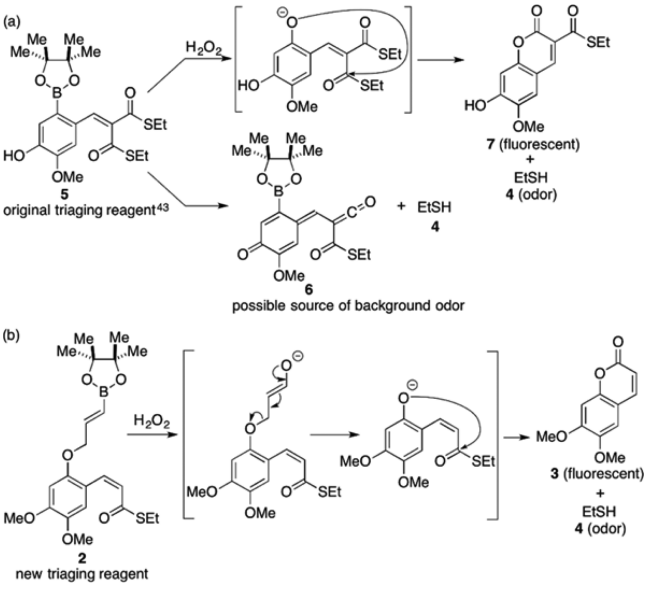

Figure 2. (a) Structure and proposed mechanism for the desired response and possible degradation pathway for first-generation triaging reagent **5**.[43] (b) Structure of **2** as well as a possible mechanism by which **2** releases ethanethiol.

analyte (as determined from the qualitative readout) required quantitative measurements of the second readout.[43] The advantage of this dual readout approach over other methods is that time-consuming quantitative measurements are saved for those samples that contain relevant concentrations of the target analyte, thus reducing consumption of time when conducting the assays. Herein, we describe a small-molecule reagent with substantially improved properties (compared with our initial design) for this type of dual readout assay (Figure 1b).

Our approach uses reagent **2** (Figure 1), which provides the dual readout—one qualitative (**4**) and one quantitative (**3**)—all in a single-step assay. If the qualitative readout (odor of **4**) indicates that the analyte is in the sample, then a hand-held fluorescence meter is used to measure the quantity of **3**. We chose ethanethiol (**4**) for the qualitative readout because humans have a keen ability to smell this compound.[49] In fact, ethanethiol is in a class of compounds to which the human nose is most sensitive, thus providing the required sensitivity to match the sensitivity of the quantitative readout using **3**.[49] A match in sensitivity for detecting **4** vs **3** is critical for the triaging portion of the assay in order to provide information that is relevant to deciding whether to run the quantitative assay.

The complete triaging assay requires three reagents that operate simultaneously in a single-pot reaction (Figure 1b). These reagents include the following: (i) a detection reagent (**1**) that selectively reacts with the target analyte (enzymes are the targets in this work) and releases glucose; (ii) glucose oxidase, which converts glucose into D-gluconic acid δ-lactone and simultaneously generates hydrogen peroxide; and (iii) a readout reagent (**2**) that responds to the hydrogen peroxide to produce the dual readout. We use two reagents (i.e., **1** and **2**) instead of a single reagent for detecting the analyte and providing readouts because it is easier to prepare two simple reagents than one complex reagent and because we envisage assays in which the detection reagent (**1**) is varied easily to target different enzymes, while the readout reagent (**2**) remains constant.

RESULTS AND DISCUSSION

Advantages of Reagent 2 vs 5. Our first-generation reagent for the triaging assay (**5**, Figure 2a) was limited in (i) sensitivity (particularly matching the sensitivity of the qualitative and quantitative readouts for the assay), (ii) reproducibility during an assay, and (iii) stability (which gave rise to background signal). In contrast, our new design, reagent **2** (Figure 2b), is thermally stable, readily accessible synthetically, responds to a target analyte faster than previous reagent **5**, eliminates background smell that limited the sensitivity of **5**, and provides a nearly perfect match in sensitivity between the qualitative and quantitative readouts for triaging assays (which is the ideal scenario for such an assay). These improvements are essential for enabling fast, sensitive, and reproducible triaging assays, and thus represent a critical synthetic step toward evaluating the feasibility of smell-based triaging assays in practical applications.

Considerations for Reagent 2. The advantages of reagent **2** vs **5** result from modifications to reagent **5**, such as replacing the phenol in **5** with a methyl ether, which we believe limits nonspecific release of ethanethiol via ketene **6** (Figure 2a).[50] Background release of thiol was further minimized by installing a cis-α,β-unsaturated thioester in **2** (Figure 2b) versus the dithioester of **5** (Figure 2a). This modification reduces the number of thioesters that are available for background hydrolysis, either upon storage or during the assay. Finally, we installed the vinyl boronate with the goal of increasing the rate of the oxidative cleavage reaction of the boronate with hydrogen peroxide that is generated during the assay (Figure 1).[51] We reasoned that the aryl boronate in **5** likely suffers from

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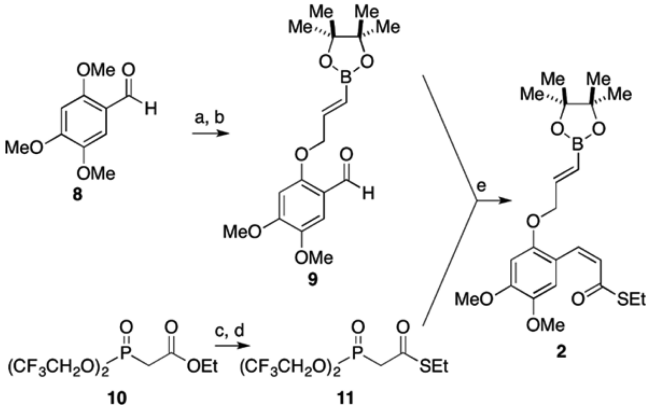
(i) unfavorable nonbonding interactions that slow the reaction of hydrogen peroxide with boron, (ii) a conformation around the aryl carbon−boron bond that slows the bond rearrangement steps during the oxidative cleavage reaction, and (iii) dative bonding between the lone pairs on the thioester and the empty p-orbital on boron that could hinder the ability of hydrogen peroxide to bind to the empty p-orbital on boron to initiate the oxidative cleavage reaction.[52] This type of dative bonding also might contribute to hydrolysis of the thioester via Lewis acid-mediated activation of the thioester.[52,53] Moving the boronate away from the aromatic ring (i.e., installing a vinyl boronate instead of an aryl boronate) in **2** minimizes the likelihood of these unfavorable interactions.

Synthesis of reagent 2. We prepared reagent **2** in three primary steps and 34% overall yield (Scheme 1) using a scalable synthetic strategy that makes available substantial quantities of **2**.

Scheme 1. Synthesis of Reagent 2[a]



[a]Reagents and conditions: (a) BBr_3, DCM, (94%); (b) vinyl boronate allyl chloride, TBAI, Cs_2CO_3, acetone (53%); (c) porcine liver esterase, phosphate buffer, acetone (99%); (d) EtSH, EDCI, DMAP, DCM (88%); (e) K_2CO_3, THF, −40 °C (68%).

Response of Reagent 2 to Hydrogen Peroxide. As expected, treatment of **2** with hydrogen peroxide in aqueous solutions quickly and cleanly (i.e., no observable intermediates) produces fluorescent product **3** and ethanethiol (**4**) (Figure 3). (The presence of **3** was confirmed using LCMS (Figure 3a), and **4** was detected by smell.) Time-dependent UV−vis spectra of the same reaction reveal isosbestic points at 310 and 358 nm (Figure 3b), which further supports conversion of **2** to **3** without proceeding through observable intermediates. Moreover, the intensity of the fluorescent signal for **3** increases over time when **2** is exposed to hydrogen peroxide (Figure 3c), thus indicating that fluorescence measurements can be used effectively to quantify assays.

Demonstration of Reagent 2 in Model Assays for Specific Enzyme Biomarkers. The ability of reagent **2** to operate in simulated assays is depicted in Figure 4 for detecting two model enzyme analytes: β-D-galactosidase (a general marker for fecal coliforms in water) (**1a**, Figure 1, was used as its substrate during the assay) and alkaline phosphatase (a reporter of liver function) (**1b** was used as the substrate). The assays involved dissolving **2** (final concentration = 230 μM) and either **1a** or **1b** (final concentration = 57 mM) in a sample containing the desired enzyme target, followed by incubation at 20 °C for 30 min. The linear calibration curves in Figure 4 were

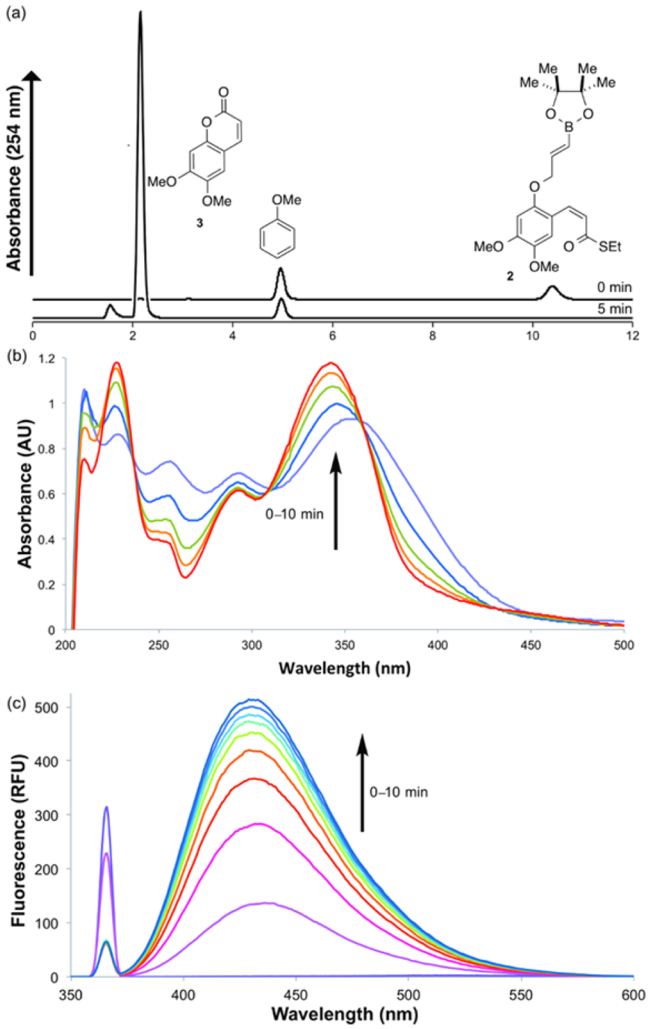


Figure 3. Response of **2** to hydrogen peroxide. (a) Liquid chromatograms (LC) (obtained from LCMS experiments) before and 5 min after exposure of **2** to hydrogen peroxide. (b, c) Time-dependent UV−vis and fluorescent spectra, respectively, after exposure of **2** (2.3 mM) to hydrogen peroxide (6.0 mM).

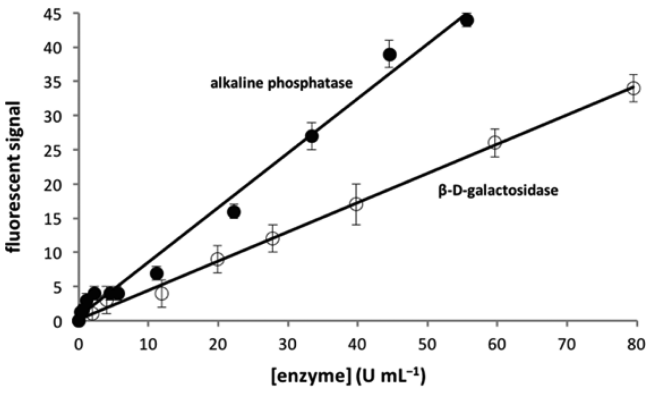


Figure 4. Calibration curves based on the fluorescent signal of **3** when either **1a** (for β-D-galactosidase) or **1b** (for alkaline phosphatase) combined with **2** were exposed to samples of enzyme at different initial concentrations. The data points are the average of three measurements, and the error bars represent the standard deviation from the averages.

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obtained using a hand-held, portable fluorescence meter that contains an LED that emits at 365 nm and measures absorbance from 440−470 nm (for reference, coumarin product **3** absorbs maximally at 345 nm and emits maximally at 430 nm). The limits-of-detection[54] for 30 min assays are 3.5 nM (0.4 U·mL^{-1}) and 8.5 nM (8 U·mL^{-1}) for the two model enzymes, respectively.

Comparison of Reagent 2 to Reagent 5. Reagent **5**, in comparison, provides a limit-of-detection for β-D-galactosidase that is 6× worse than **2**.[43] This improvement in limit-of-detection provided by **2** is impressive when considering that (i) the sensitivity for **2** was determined using a simple, fixed-intensity, fixed-gain, low-cost hand-held fluorescence meter, whereas the value for **5** was determined using a high-powered benchtop UV/vis spectrometer with perfectly tuned emission and excitation wavelengths to maximize the sensitivity of the assay.[43] This improvement in sensitivity for **2** compared to **5** likely is the result of the resistance of **2** to undergo nonspecific background reactions.

When compared to standard fluorescent reagents (such as 4-methylumbelliferyl β-D-galactopyranoside (4-MU); λ_{ex} = 365 nm; λ_{em} = 455 nm) for detecting β-D-galactosidase, **2** enables assays that are only 3.5× less sensitive (using the hand-held fluorescence meter and 30 min assay time), but it also enables rapid triaging based on smell, which is a capability that 4-MU and other standard reagents do not provide.[55]

Evaluation of the Practical Benefits of Reagent 2. Even more important than the absolute sensitivity of the fluorescence assay, however, is the match in sensitivity between the qualitative (smell) and quantitative (fluorescent) readouts. The limits-of-detection for the qualitative assays for β-D-galactosidase and alkaline phosphatase were determined using the recommended protocol provided by the American Society for Testing and Measurements (ASTM) (i.e., "forced-choice ascending concentration series method of limits").[56]

Briefly, this method involves panelists correctly identifying the most odorous sample in a set of three samples, where only one sample contains the analyte, while the other two are control samples that lack the analyte.[56] This procedure was repeated using six sets of samples that increase sequentially in the concentration of the analyte. Based on this protocol (and the responses of 10 panelists), the limits of detection for the qualitative assays using **2** were calculated as 6.2 nM (0.6 U·mL^{-1}) for β-D-galactosidase and 15 nM (14 U·mL^{-1}) for alkaline phosphatase. These values both are only 1.8× higher than the limits-of-detection of the fluorescence assays for the same enzymes. This close match in limits-of-detection between the qualitative and quantitative readouts demonstrates that **2** is a highly effective reagent for enabling down-selection assays.

An additional practical consideration is the stability of **2** (Figure 5). In the assay solution, reagent **2** does hydrolyze to the boronic acid within ∼5 min, but this hydrolysis product undergoes the oxidative cleavage reaction with hydrogen peroxide essentially as readily as the boronate, so the hydrolysis reaction does not appear to affect the assay. Other than this hydrolysis reaction, reagent **2** is stable for at least 5 days in the assay solution (which is far longer than is needed for an assay), with <8% isomerization of the cis alkene in the α,β-unsaturated thioester to form the trans alkene in **9** (Figure 6a). Formation of trans-α,β-unsaturated thioester **9** from **2** over the course of 5 days does not affect the sensitivity of assays using **2**, since no detectable quantity of **9** is present in a solution of **2** that is stored for 1 h (which is 30 min longer than the duration of the current assays). Our original reagent (**5**), in contrast, degraded to several byproducts in less than 20 min under solution-phase conditions, which decreased the quantity of **5** that was available to react with hydrogen peroxide generated during an assay (Figure 2a). Reagent **5** also generated a noticeable thiol smell within this short period of time, which negatively affected the

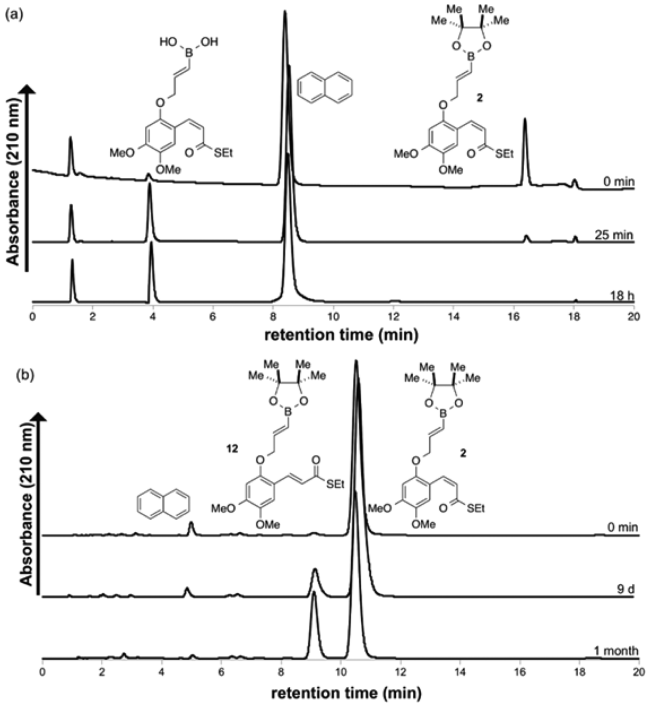

Figure 5. LC chromatographs that reveal the stability of **2**. (a) Solution-phase stability study (1.2 mM in 1:1 MeOH−HEPES buffer (pH 7.9)) at 31 °C. Naphthalene was used as an internal standard. (b) Solid-state stability study at 44 ± 3 °C, open to the air. The LC solvent gradient was different in (b) vs (a) to separate the cis and trans isomers of **2** that develop after heating solid **2** for days at 44 °C (b).

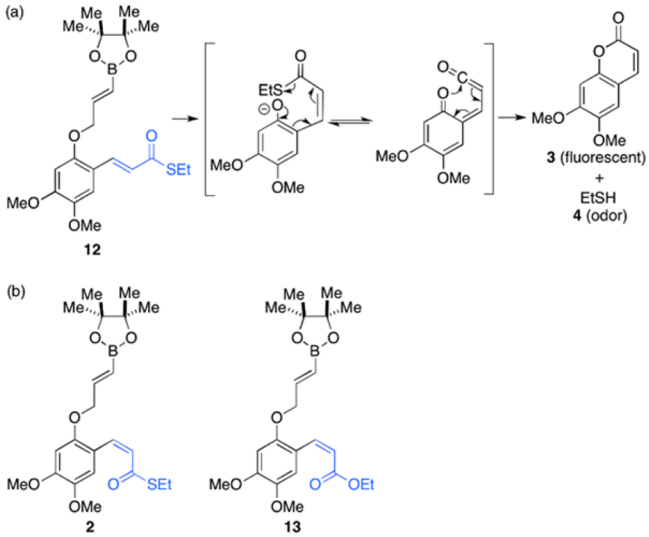

Figure 6. Structural variants of reagent **2** for studying the mechanism of coumarin production and ethanethiol release. (a) Possible cyclization with a ketene intermediate that would allow the trans alkene to generate **3** and **4**. (b) Comparison of the structure of cis ester **13** to the cis thioester triaging reagent **2**.

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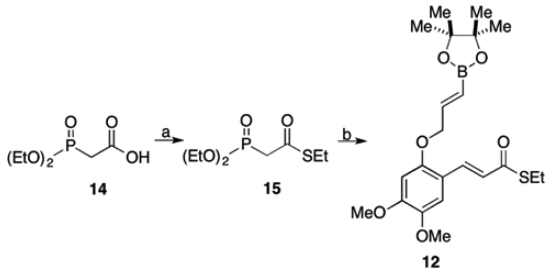
sensitivity of the qualitative down-selection portion of the assay when **5** was used instead of **2**.

Reagent **2** also is stable when stored as a solid (as it would be supplied before use in an assay; Figure 1). Solid **2** showed little decomposition when heated to 40 °C, open to the air for 1 month (this was the duration of our stability study) (Figure 5b). In fact, the only decomposition reaction of **2** involved isomerization of the α,β-unsaturated thioester to form **12**. Most importantly, no background release of thiol was detected by smell. Furthermore, when **2** was stored in the solid state under inert atmosphere at 8 °C, there was no isomerization or decomposition observed after more than 1 year (Figure S16).

Evaluation of Structural Variants of Reagent 2. Reagent **2** clearly is effective for use in triaging assays, particularly since it is readily accessible synthetically, thermally stable, and provides qualitative readouts that are almost as sensitive as the quantitative readout. Because of these promising results, we tested possible limitations of **2**, such as (i) whether the trans α,β-unsaturated thioester **12** could function in the assay and (ii) if side reactions with the released thiol reduce the overall sensitivity of the assay.

To address the first topic, we prepared **12** (Scheme 2) and then tested whether **12** responds to hydrogen peroxide to form

Scheme 2. Synthesis of Reagent 12[a]



[a]Reagents and conditions: (a) EtSH, EDCI, DMAP, DCM (34%); (b) **9**, Cs_2CO_3, THF, 50 °C (20%).

3 and **4**. Reagent **12** indeed undergoes the oxidative cleavage reaction when exposed to hydrogen peroxide, but no cyclization or other subsequent reactions occur within 4 d under the assay conditions (Figure 7). While the stability of the oxidative cleavage product of **12** obviates the use of **12** in down-selection assays, it does suggest that the direct cyclization

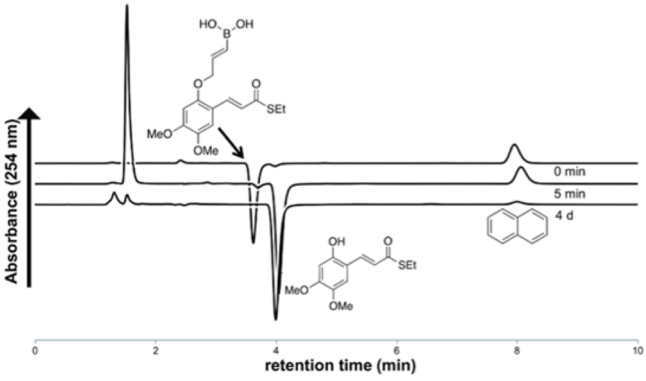


Figure 7. Exposure of trans alkene **12** (2.3 mM in 1:1 MeOH–HEPES buffer (pH 7.9)) at 31 °C to H_2O_2 (6 mM) yields the phenol, but none of **3**. Naphthalene was used as an internal standard.

mechanism from **2** (pathway in Figure 2b) likely is operative over an electrocyclization pathway (pathway in Figure 6a).

The second concern involved losing ethanethiol through oxidation–reduction reactions between ethanethiol and hydrogen peroxide that is generated during the detection event. To test the effects of this scenario, we prepared control compound **13** (Figure 6b) that contains an ethyl ester instead of an ethyl thioester. Exposure of **13** to hydrogen peroxide leads to formation of **3** and release of ethanol rather than ethanethiol, the former of which does not undergo rapid oxidation–reduction reactions with hydrogen peroxide. Comparison of emission intensities (Table 1) for **3**, when **2** and **13** are exposed

Table 1. Normalized Fluorescent Signal for 13 vs 2 upon Treatment of Each Reagent with H_2O_2

$[H_2O_2]$ (μM)	normalized fluorescent signal for **2** (thioester)	relative fluorescent signal for **13** (ester)
10	1	1.4 ± 0.1
20	1	1.8 ± 0.1
50	1	2.4 ± 0.2

to hydrogen peroxide under identical conditions, reveals that the impact of the proposed oxidation–reduction on the sensitivity of the quantitative and qualitative assays is substantial (i.e., **13** produces 2.5× more fluorescence signal than **2**) when high concentrations of hydrogen peroxide (>0.08 equiv relative to **2** or **13**) are present. High concentrations of hydrogen peroxide, however, are unlikely to form during an assay. In an assay, the hydrogen peroxide that is generated via the detection event with **1** likely is consumed immediately via the oxidative cleavage reaction with **2**; therefore, the loss in signal as a result of this oxidation–reduction reaction between hydrogen peroxide and ethanethiol is presumably small (<28%), as suggested by the limited loss in signal when **2** and **13** are treated with only 0.04 equiv (10 μM) of hydrogen peroxide. Therefore, the down-selection strategy described in Figure 1 should remain a viable strategy so long as the rate of oxidative cleavage of reagents like **2** remain competitive with the combined rates with which hydrogen peroxide is generated from reaction of the analyte with **1**, followed by reaction of the released glucose with glucose oxidase to generate hydrogen peroxide (Figure 1).

CONCLUSIONS

In conclusion, we designed a readout reagent that is capable of providing rapid, sensitive, qualitative, and quantitative results for triaging a large number of samples for a target analyte. In principle, this reagent can be paired with a variety of detection reagents for use in a wide range of enzyme assays. While the approach is not yet compatible with all classes of analytes, it does offer the potential to simplify quantitative point-of-need assays for detecting and measuring enzyme biomarkers, such as the various extracellular enzymes indicative of bacterial contamination, including necrotizing enzymes, kinases, hyaluronidase, hemolysins, hydrolases, and laccases.

Perhaps more importantly, the design of the readout reagent minimizes background reaction, enables rapid assays, and provides matching of the intensities of the qualitative and quantitative readouts for the assay. The straightforward synthesis of the readout reagent adds to these useful qualities and thus should provide a starting point for others to explore various analytical applications of this reagent.

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EXPERIMENTAL SECTION

General Experimental Methods. All reactions requiring inert atmosphere were performed in flame-dried glassware under a positive pressure of argon. Air- and moisture-sensitive liquids were transferred by syringe or stainless steel cannula. Organic solutions were concentrated by rotary evaporation (25−40 mmHg) at ambient temperature, unless otherwise noted. D-Lactose, D-glucose-6-phosphate, β-D-galactosidase, alkaline phosphatase, porcine liver esterase, and all other reagents were purchased commercially and used as received. Dry solvents were purified by the method developed by Pangborn et al.[57] Flash column chromatography was performed as described by Still et al.[58] Fluorescence assays were performed in UV-transparent ultramicro UV cuvettes, and smell-based assays were performed in microcentrifuge tubes (0.6 mL), unless otherwise noted.

Instrumentation. Assay-related fluorescence data was obtained using a Promega QuantiFluor-P hand-held fluorometer (λ_{ex} = 365 nm, λ_{em} = 440−470 nm). Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 25 °C. Proton chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to residual protium in the NMR solvent ($CDCl_3$, δ 7.26 ppm). Data are represented as follows: integration, chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and/or multiple resonances, br s = broad singlet, dd = doublet of doublet), and coupling constant (*J*) in hertz. Carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded at 25 °C. Carbon chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to the carbon resonance of the NMR solvent ($CDCl_3$, δ 77.16 ppm). LC−MS data were obtained on an analytical reversed-phase HPLC coupled to a quadrupole mass spectrometer using a phenyl hexyl column (150 mm × 5 mm, 5 μm particle size) for separation. The column was equilibrated with the initial acetonitrile−water ratio of the solvent gradient selected for column separation at 1.0 mL min^{-1} flow rate. Solvent gradients after injection are reported with each experiment. A portion of the HPLC stream was automatically injected into the mass spectrometer. The mass spectrometer (APCI) settings were as follows: gas temperature = 350 °C, drying gas flow = 11 L min^{-1}, nebulizer pressure = 35 psi, and voltage = 3000 V.

2-Hydroxy-4,5-dimethoxybenzaldehyde (16). This procedure was modified from a method reported by Gündüz et al.[59] 2,4,5-Trimethoxybenzaldehyde (**8**) (1.00 g, 5.09 mmol, 1 equiv) was dissolved in CH_2Cl_2 (50 mL) under argon and cooled to −78 °C. To this solution was added 1 M BBr_3 in CH_2Cl_2 (8 mL) and the mixture stirred for 30 min before the cooling bath was removed and the reaction mixture stirred overnight. The brown reaction solution was then cooled to 0 °C, acidified with concd HCl (∼5 mL), diluted with water, and extracted. The organic layer was washed with 1 M HCl (3 × 50 mL), water (3 × 50 mL), and brine (3 × 50 mL). The aqueous layers were combined and back-extracted with 100 mL of EtOAc. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated to give 2-hydroxy-4,5-dimethoxybenzaldehyde (**16**) as an off-white solid that was used without further purification (873 mg, 4.79 mmol, 94%): mp 101−103 °C; ^{1}H NMR (360 MHz, $CDCl_3$) δ 11.4 (1H, s), 9.70 (1H, s), 6.90 (1H, s), 6.47 (1H, s), 3.93 (3H, s), 3.88 (3H, s). This spectral data is consistent with data for the known compound.[59]

2-((2*E*)-3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolanyl)-2-propenoxy)-4,5-dimethoxybenzaldehyde (9). (*E*)-2-Chloromethylvinylboronic acid pinacol ester (515 mg, 2.54 mmol, 1.55 equiv), **16** (298 mg, 1.64 mmol, 1 equiv), and tetrabutylammonium iodide (61 mg, 0.17 mmol, 0.10 equiv) were added to a round-bottom flask and dissolved in DMA (6 mL) under argon. Anhydrous K_2CO_3 (374 mg, 2.71 mmol, 1.65 equiv) was added and the solution heated at 80 °C for 8 h. This solution was diluted with 50 mL of Et_2O and 50 mL of water. The aqueous layer was removed and extracted once more with 50 mL of Et_2O. The organic layers were combined, washed with water (2 × 100 mL) and brine (3 × 100 mL), dried with $MgSO_4$, filtered, and concentrated in vacuo. The yellow oil was purified using column chromatography (30% EtOAc/hexanes) to obtain 2-((2*E*)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolanyl)-2-propenoxy)-4,5-dimethoxybenzaldehyde (**9**) as a white solid (305 mg, 0.876 mmol, 53%): mp 115−118 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.4 (1H, s), 7.33 (1H, s), 6.76 (1H, d of t, *J* = 18, 4.5 Hz), 6.45 (1H, s), 5.85 (1H, d, *J* = 17 Hz), 4.71 (2H, d of d, *J* = 4.3, 1.7 Hz), 3.93 (3H, s), 3.87 (3H, s), 1.28 (12H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.9, 157.6, 155.7, 146.2, 143.9, 120.7 (br.), 117.9, 109.0, 97.4, 83.7, 70.7, 56.3, 24.9; IR (neat) 3216, 2984, 2536, 2161, 2027, 1657, 1603 cm^{-1}; (TOF MS APCI+, *m*/*z*): 349.3 (M + H$^+$); HRMS (TOF MS ES+, *m*/*z*) calcd for $C_{18}H_{26}O_6B$ (M + H$^+$) 349.1822, found 349.1818.

(Bis(2,2,2-trifluoroethoxy)phosphinyl)acetic Acid (17). This procedure was adapted from the work of Sano et al.[60] Porcine liver esterase (1000 units/mm0l) was added to a solution of 9:1 1 M phosphate buffer (pH 7.8) and acetone (50 mL). Ethyl [bis(2,2,2-trifluoroethoxy)phosphinyl]acetate (**10**) (0.62 mL, 2.62 mmol, 1 equiv) was added to the solution in one portion and stirred in a round-bottom flask for 16 h. Concentrated HCl was added to reduce the pH to 1. The solution was saturated with NaCl, and 20 mL of EtOAc was added with vigorous stirring. The excess NaCl was removed by vacuum filtration and the organic elutant separated from the aqueous. The aqueous layer was extracted with EtOAc (3 × 75 mL), and the combined organic layers were washed once with an equal volume of saturated brine. The organic layer was then dried over $MgSO_4$, filtered, and concentrated under reduced pressure to a viscous brown oil. The oil was dried overnight to yield [bis(2,2,2-trifluoroethoxy)phosphinyl]-acetic acid (**17**) as brown solid that was used without further purification (795 mg, 2.61 mmol, 99%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.95 (1H, broad s), 4.48 (4H, quin, *J* = 7.9 Hz), 3.22 (2H, d, J_{P-H} = 21 Hz). This spectral data is consistent with data for the known compound.[60]

***S*-Ethyl (Bis(2,2,2-trifluoroethoxy)phosphinyl)ethanethioate (11).** DMAP (54 mg, 0.442 mmol, 0.11 equiv) and **17** (1.26 g, 4.14 mmol, 1.0 equiv) were mixed in CH_2Cl_2 (16 mL) under argon and cooled to 0 °C. To this solution was added ethanethiol (3.2 mL, 43.2 mmol, 10.4 equiv) followed by EDCI (940 mg, 4.90 mmol, 1.2 equiv). The reaction mixture was stirred for 5 min, at which point the argon inlet was removed and the reaction stirred for 6 h at 0 °C in a tightly sealed vessel. The mixture was concentrated under reduced pressure to an oil and redissolved in Et_2O (50 mL). The organic layer was washed with $NaHCO_3$ (2 × 50 mL), 0.5 M HCl (2 × 50 mL), DI H_2O (1 × 50 mL), and brine (3 × 50 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo to a brown oil. This oil was passed through a silica plug with 25% EtOAc/hexanes to yield *S*-ethyl (bis(2,2,2-trifluoroethoxy)phosphinyl)ethanthioate (**11**) as a golden oil (1.27 g, 3.66 mmol, 88%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.48 (4H, quin, *J* = 8.1 Hz), 3.44 (2H, d, J_{P-H} = 21 Hz), 2.95 (2H, q, *J* = 7.4 Hz), 1.28 (3H, t, *J* = 7.4 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 189.4, 128.0 (q), 124.3 (q), 120.6 (q), 116.9 (q), 63.1 (q), 62.6 (q), 62.1 (q), 61.6 (q), 42.8 (d), 41.0 (d), 24.2, 13.9; IR 2975, 2937, 1679, 1455, 1419 cm^{-1}; MS (TOF MS APCI+, *m*/*z*) 348.9 (M + H$^+$); HRMS (TOF MS ES+, *m*/*z*) calcd for $C_8H_{12}F_6O_4PS$ (M + H$^+$) 349.0098, found 349.0092.

(2*Z*)-*S*-Ethyl 3-(2-((2*E*)-3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolanyl)-2-propenoxy)-4,5-dimethoxyphenyl)-propenethioate (2). This procedure was modified from a similar method reported by Touchard.[61] 18-Crown-6 ether (115 mg, 0.435 mmol, 2.02 equiv) and anhydrous K_2CO_3 (115 mg, 0.871 mmol, 4.05 equiv) were dissolved in 5 mL of THF under argon and allowed to equilibrate overnight. The cloudy solution was cooled to −40 °C, and then **9** (88 mg, 0.253 mmol, 1.18 equiv) was added in one portion. Compound **11** (75 mg, 0.215 mmol, 1 equiv) in 1 mL of THF was then added, and additional **1** and 0.5 mL portions of THF were used for quantitative transfer of **11**. The vibrant yellow solution was stirred in the dark under argon at −40 °C for 8 h (or until reaction completion). This mixture was concentrated in vacuo in the dark, redissolved in the Et_2O (50 mL), and washed with 1 M phosphate buffer pH 6.8 (2 × 50 mL), DI H_2O (1 × 50 mL), and brine (2 × 50 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated to an oil that was purified by column chromatography (30% EtOAc/hexanes). The product was dried in vacuo to a canary yellow solid, (2*Z*)-*S*-ethyl 3-(2-((2*E*)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolanyl)-2-propenoxy)-4,5-dimethoxyphenyl)propenethioate

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(**2**) (64 mg, 0.146 mmol, 68%): ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.79 (1H, s), 7.05 (1H, d, J = 12.7 Hz), 6.74 (1H, d of t, J = 18.3, 4.1 Hz), 6.41 (1H, s), 6.07 (1H, d, J = 12.7 Hz), 5.83 (1H, d, J = 18.2 Hz), 4.60 (2H, d of d, J = 3.9, 1.4 Hz), 3.88 (6H, s), 2.92 (2H, q, J = 7.4 Hz), 1.26 (15H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 190.4, 153.0, 152.1, 147.6, 143.0, 135.3, 123.9, 120 (br.), 116.4, 114.5, 98.1, 84.1, 71.3, 56.9, 56.6, 25.4, 24.3, 15.4; IR (neat) 2974, 2932, 2839, 2531, 2161, 2029, 1655 cm^{-1}; (TOF MS APCI+, m/z) 435.2 (M + H$^+$); HRMS (TOF MS ES+, m/z) calcd for $C_{22}H_{32}O_6BS$ (M + H$^+$) 435.2013, found 435.1994.

***S*-Ethyl (Diethoxyphosphinyl)ethanethioate (15).** A solution of diethylphosphonoacetic acid (**14**) (420 μL, 2.61 mmol, 1 equiv) and DMAP (31 mg, 0.253 mmol, 0.097 equiv) were mixed in CH_2Cl_2 (9 mL) under argon and cooled to 0 °C. To this solution were added ethanethiol (1 mL, 13.5 mmol, 5.17 equiv) and EDCI (600 mg, 3.13 mmol, 1.20 equiv). The reaction was stirred for 10 min, at which point the argon inlet was removed, and the reaction was stirred for 8 h at 0 °C in a tightly sealed vessel. The mixture was concentrated under reduced pressure to a clear oil and redissolved in Et_2O (50 mL). The organic layer was washed with $NaHCO_3$ (2 × 50 mL), 0.5 M HCl (2 × 50 mL), DI H_2O (1 × 50 mL), and brine (3 × 50 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo to yield *S*-ethyl (diethoxyphosphinyl)ethanethioate (**15**) as a clear oil (0.216 g, 0.90 mmol, 34%): ^{1}H NMR (360 MHz, $CDCl_3$) δ 4.17 (4H, quin., J = 7.2 Hz), 3.21 (2H, d, J_{P-H} = 21.3 Hz), 2.92 (2H, q, J = 7.5 Hz), 1.34 (5H, t, J = 7.1 Hz), 1.27 (3H, t, J = 7.5 Hz). This spectral data is consistent with data for the known compound.[62]

(2*E*)-*S*-Ethyl 3-(2-((2*E*)-3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolanyl)-2-propenoxy)-4,5-dimethoxyphenyl)-propenethioate (12). This procedure was adapted from the work of Mandal et al.[63] Cs_2CO_3 (29 mg, 0.135 mmol, 1.62 equiv) and **9** (29 mg, 83 μmol, 1 equiv) were dissolved in 2.5 mL of THF under argon. To this solution was added **15** (22 mg, 0.112 mmol, 1.35 equiv) in 2.5 mL of THF. The solution was heated at 50 °C for 16 h and then cooled to room temperature. This mixture was concentrated in vacuo, redissolved in the Et_2O (50 mL), and washed with 1 M phosphate buffer pH 6.8 (2 × 50 mL), DI H_2O (1 × 50 mL), and brine (2 × 50 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated to an oil that was purified by column chromatography (30% EtOAc/hexanes). The product was dried in vacuo overnight to give a vibrant yellow oil, (2*E*)-*S*-ethyl-3-(2-((2*E*)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolanyl)-2-propenoxy)-4,5-dimethoxyphenyl)-propenethioate (**12**) (7 mg, 17 μmol, 20%): ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.95 (1H, d, J = 15.9 Hz), 6.99 (1H, s), 6.75 (1H, d of t, J = 18.3, 4.1 Hz), 6.63 (1H, d, J = 15.9 Hz), 6.43 (1H, s), 5.79 (1H, d, J = 18.2 Hz), 4.66 (2H, d, J = 2.3 Hz), 3.87 (6H, s), 2.99 (2H, q, 7.2 Hz), 1.30 (15H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 190.2, 153.4, 152.4, 147.1, 143.6, 135.3, 123.1, 120.6 (br), 115.1, 110.5, 98.5, 84.8, 71.0, 56.5, 56.2, 24.9, 23.3, 15.0; IR (neat) 2975, 2932, 2543, 2163, 2030, 1649, 1593 cm^{-1}; (TOF MS APCI+, m/z) 435.2 (M + H$^+$); HRMS (TOF MS ES+, m/z) calcd for $C_{22}H_{32}O_6BS$ (M + H$^+$) 435.2013, found 435.2007.

(2*Z*)-Ethyl 3-(2-((2*E*)-3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolanyl)-2-propenoxy)-4,5-dimethoxyphenyl)acrylate (13). This procedure was modified from the procedure reported by Touchard.[61] 18-Crown-6 ether (70 mg, 0.265 mmol, 1.91 equiv) and anhydrous K_2CO_3 (75 mg, 0.543 mmol, 3.91 equiv) were dissolved in 5 mL of THF under argon and allowed to equilibrate overnight. The cloudy solution was cooled −40 °C, and **9** (50 mg, 0.144 mmol, 1.04 equiv) was added in one portion. Compound **10** (33 μL, 0.139 mmol, 1 equiv) was then added, and the pale yellow solution was stirred in the dark under argon at −40 °C for 5 h. This mixture concentrated in vacuo in the dark, redissolved in the Et_2O (50 mL), and washed with 1 M phosphate buffer pH 7.1 (2 × 50 mL), water (1 × 50 mL), and brine (2 × 50 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated to an oil that was purified by column chromatography (20% EtOAc/hexanes). The product was concentrated dried in vacuo overnight to a white solid, (2*Z*)-ethyl 3-(2-((2*E*)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolanyl)-2-propenoxy)-4,5-dimethoxyphenyl)acrylate (**13**) (35 mg, 83 μmol, 59%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (1H, s), 7.23 (1H, d, J = 12.8 Hz), 6.74 (1H, d of t, J = 18.2, 4.3 Hz), 6.43 (1H, s), 5.84 (1H, d, J = 12.8 Hz), 5.81 (1H, s), 4.59 (2H, d of d, J = 4.2, 1.7 Hz), 4.16 (2H, q, 3.88, J = 7.12 Hz), 3.87 (3H, s), 3.83 (3H, s), 1.29 (15H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.5, 152.0, 151.2, 147.3, 142.7, 138.2, 120 (br.), 117.3, 116.1, 114.5, 98.10, 83.6, 70.9, 60.2, 56.6, 56.2, 25.0, 14.4; IR (neat) 2973, 2921, 2852, 2531, 2162, 2030, 1670, 1647 cm^{-1}; (TOF MS APCI+, m/z) 419.2 (M + H$^+$); HRMS (TOF MS ES+, m/z) calcd for $C_{22}H_{32}O_7B$ (M + H$^+$) 419.2241, found 419.2213.

Experimental Procedure for LC/MS Monitoring of Reagent 2 Response to Hydrogen Peroxide (Figure 3b). A hydrogen peroxide solution (3 μL, 1.0 M in water) was added to a solution of **2** (500 μL, 2.3 mM in 1:1 MeOH−40 mM HEPES buffer, pH 7.9) and naphthalene (0.5 mM). The mixture was agitated by vortex for ∼5 s. An aliquot of the resulting mixture was injected into an analytical reversed-phase HPLC coupled to a mass spectrometer, and additional aliquots were injected at regular intervals. After injection of the sample, an isocratic solvent gradient was run as 3:2 acetonitrile−water for 20 min.

Procedure for Monitoring the Time-Dependent UV−vis Response of 2 to Hydrogen Peroxide (Figure 3b). A hydrogen peroxide solution (690 μL, 0 and 3 μM in 40 mM phosphate buffer, pH 7.0) was added to a solution of **2** (20 μL, 4 mM in MeOH) in a 1.5 mL centrifuge tube, agitated with a vortex mixer for ∼7 s, and then transferred to a quartz cuvette. The UV−vis spectrum was then monitored until no further changes were detected (∼10 min).

Procedure for Monitoring the Time-Dependent Fluorescence Response of 2 to Hydrogen Peroxide (Figure 3c). A hydrogen peroxide solution (330 μL, 0 and 3 μM in 40 mM phosphate buffer, pH 7.0) was added to a solution of **2** (20 μL, 4 mM in MeOH) in a 1.5 mL centrifuge tube, agitated with a vortex mixer for ∼7 s, and then transferred to a quartz cuvette. The fluorescence spectrum was then monitored until no further changes were detected (∼10 min).

General Experimental for Hydrogen Peroxide Fluorescence Emission Calibration Curve. A hydrogen peroxide solution (330 μL, 0−100 μM in 40 mM HEPES buffer, pH 7.9) was added to a solution of **2** (20 μL, 4 mM in MeOH) in an ultramicro cuvette, capped, and agitated with a vortex mixer for ∼7 s. After 30 min, the fluorescence emission (I) of the mixture was measured, and the average fluorescence of the 0 μM hydrogen peroxide sample (I_0) was subtracted ($I - I_0$); this difference was then plotted to obtain a calibration curve. (I = fluorescence intensity after 30 min from the initial mixing of solutions; I_0 = fluorescence intensity after 30 min from the initial mixing of the solutions with 0 μM hydrogen peroxide.)

General Procedure for Enzyme Assays (Figure 4). To a premixed solution of **2** (20 μL, 4 mM in MeOH), glucose oxidase (100 μL, 15 U·mL^{-1} in pH 7.01 20 mM phosphate buffer and 400 mM NaCl), and glucose-6-phosphate or lactose (100 μL, 200 mM in DI H_2O) was added a solution of alkaline phosphatase or β-D-galactosidase (130 μL, 0−100 nM, in pH 7.01 20 mM phosphate buffer and 400 mM NaCl) respective of enzyme substrate. This solution was vortex mixed for ∼10 s and the fluorescence measured at 30 min and 1 h. The fluorescence emission (I) of the mixture was measured, and the average fluorescence of the 0 μM enzyme sample (I_0) was subtracted ($I - I_0$), this difference was plotted to obtain a calibration curve. (I = fluorescence intensity after 30 min or 1 h from the initial mixing of solutions; I_0 = fluorescence intensity after 30 min from the initial mixing of the solutions with 0 μM enzyme.)

Procedure for Forced-Choice Ascending Concentration Series Method for Limit of Detection by Smell Using ASTM Protocol. Six sample sets of three mixed solutions of **2** (20 μL, 4 mM in MeOH), glucose oxidase (100 μL, 15 U·mL^{-1} in pH 7.01, 20 mM phosphate buffer and 400 mM NaCl), and glucose-6-phosphate or lactose (100, μL, 200 mM in DI H_2O) were prepared in 600 μL microcentrifuge tubes. Out of each set, two of the samples were blanks and had buffer (130 μL, pH 7.01 20 mM phosphate buffer and 400 mM NaCl) added. The remaining sample contained increasing concentrations of alkaline phosphatase or β-D-galactosidase (130 μL, 0−100 nM, in pH 7.01 20 mM phosphate buffer and 400 mM NaCl). These solutions were mixed by vortex for ∼10 s and then timed for 30

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min. When a set reached 30 min of incubation, a panelist was asked to identify which of the samples had the strongest odor. The geometric mean for each panelist was calculated between the last incorrectly and first correctly identified sample.

Procedure for Solution-Phase Stability of 2 by LC/MS (Figure 5a). A solution of **2** (500 μL, 1.2 mM in 1:1 MeOH−40 mM HEPES buffer, pH 7.9) and naphthalene (1.5 mM) was prepared in a brown glass vial. An aliquot of the resulting mixture was injected into an analytical reversed-phase HPLC coupled to a mass spectrometer, and additional aliquots were injected at regular intervals. The temperature of the autosampler chamber remained between 30 and 32 °C. After injection of the sample, a solvent gradient was run as follows: time, 0 min, 1:1 acetonitrile−water; time, 10 min, 1:1 acetonitrile−water; time, 15 min, 4:1 acetonitrile−water; time, 20 min, 4:1 acetonitrile−water.

Procedure for Solid-Phase Stability of 2 by LC/MS (Figure 5b). A 50 μL aliquot of **2** (10.2 mM) and naphthalene (10.4 mM) in acetonitrile was added to a 1.5 mL centrifuge tube. The samples were allowed to dry under a flow of air at room temperature, after which the lids were closed and placed in heating block at 44 ± 3 °C. At regular intervals, a sample was removed from heat, cooled to room temperature, and diluted with acetonitrile (125 μL). An aliquot of the resulting mixture was injected into an analytical reversed-phase HPLC coupled to a mass spectrometer. After injection of the sample, an isocratic solvent gradient was run as 3:2 acetonitrile−water for 20 min.

Experimental Procedure for LC/MS Monitoring of Reagent 12 Response to Hydrogen Peroxide (Figure 7). A hydrogen peroxide solution (3 μL, 1.0 M in water) was added to a solution of **12** (500 μL, 2.3 mM in 1:1 MeOH−40 mM HEPES buffer, pH 7.9) and naphthalene (0.5 mM). The mixture was agitated by vortex ∼5 s. An aliquot of the resulting mixture was injected into an analytical reversed-phase HPLC coupled to a mass spectrometer, and additional aliquots were injected at regular 25 min intervals and then 1 d later. After injection of the sample, a solvent gradient was run as follows: time, 0 min, 1:1 acetonitrile−water; time, 10 min, 1:1 acetonitrile−water; time, 15 min, 4:1 acetonitrile−water; time, 20 min, 4:1 acetonitrile−water.

Procedure for Comparing Fluorescent Output of Thioester (2) vs ester 13 (Table 1). A hydrogen peroxide solution (330 μL, 0, 10, 20, or 50 μM in 40 mM HEPES buffer, pH 7.9) was added to a solution of **2** or **13** (20 μL, 4 mM in MeOH) in an ultramicro cuvette, capped, and agitated with a vortex mixer for ∼7 s. After 30 min, the fluorescence emission (I) of the mixture was measured, and the average fluorescence of the 0 μM hydrogen peroxide sample (I_0) was subtracted ($I - I_0$); this difference was plotted to obtain a graph (I = fluorescence intensity after 30 min from the initial mixing of solutions; I_0 = fluorescence intensity after 30 min from the initial mixing of the solutions with 0 μM hydrogen peroxide).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b02013.

Tables of data and reproductions of NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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