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COMMUNICATION

Design of small molecule reagents that enable signal amplification *via* an autocatalytic, base-mediated cascade elimination reaction†

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This Communication describes three small molecule reagents that amplify the signal for a detection event *via* an autocatalytic reaction. Two signals are obtained from each reagent: (i) the dibenzofulvene chromophore and (ii) piperidine, which can be visualized using a pH indicator dye. The reagents are demonstrated in a model assay for palladium.

The developing world poses many challenges for diagnostic tests and the corresponding reagents.[1] Reagents used in these environments must be stable for prolonged periods at the elevated temperatures encountered in the developing world (refrigeration is often lacking)[2] and must be selective and sensitive enough to enable trace-level detection of biomarkers of disease. Herein, we describe the design of a new small molecule reagent that provides a starting point for addressing these issues. Our reagent is stable, is easily modified, and is capable of amplifying signal through an autocatalytic reaction (*i.e.*, one of the products of the signal amplification reaction catalyzes its own formation).

This reagent is similar conceptually to an autoinductive signal amplification reagent that we reported recently,[3] but also differs in the following important ways: (i) the signal that initiates and propagates the amplification reaction, (ii) the signal that is produced upon amplification, and (iii) the potential for rapid signal amplification. Small molecule reagents related to our autoinductive reagent also have been reported,[4–7] but none amplifies signal autocatalytically, which is, theoretically, a more efficient amplification process than autoinductive signal amplification. Silver reduction is one of few diagnostically relevant autocatalytic signal amplification processes, but it uses thermally-unstable silver(I) salts, and therefore, is of limited utility in remote environments that lack refrigeration.[8] The wide use of silver reduction in laboratory diagnostics, however, highlights the power of an autocatalytic signal amplification reagent, and points to the need for a similar reagent for use in resource-limited environments.

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† Electronic supplementary information (ESI) available: synthesis and characterization of reagents **1**, **2**, and **3**, supporting figures, and tables of data. See DOI: 10.1039/c2cc17566e

Herein, we demonstrate that the reagents shown in Fig. 1 amplify signal through an autocatalytic reaction (we describe kinetics that are characteristic of an autocatalytic reaction, as well as demonstrate that the quantity of piperidine is amplified during the reaction), and we show that reagent **1** can be used in combination with a second reagent (a detection reagent,[9] *e.g.*, reagent **4**, Fig. 2) to provide sensitive detection of a model analyte.[3] The goals of this Communication are to describe the design of these types of signal amplification reagents, to demonstrate that they can be paired with a detection reagent in a model assay, and to establish the physical-organic foundation that will be needed to modify and optimize the reagents for future use in specific analytical assays.

The design of amplification reagents **1**, **2**, and **3** involved several considerations that are worth noting. First, an autocatalytic amplification reaction requires that the signal propagating the reaction (*e.g.*, piperidine) must not be consumed, and therefore, we chose to employ acid–base chemistry that enables rapid recycling of the propagating signal. Second, we reasoned that the most general signal amplification reagent would provide more than one type of amplified signal to accommodate multiple strategies for reading the output of the assay. In the case of reagents **1**, **2**, and **3**, we included two output signals: the dibenzofulvene chromophore (which is easily quantified using UV/vis spectroscopy) and base (which can be quantified with a pH indicator; this readout will be particularly convenient for assays in resource-limited environments). Third, to enable flexibility

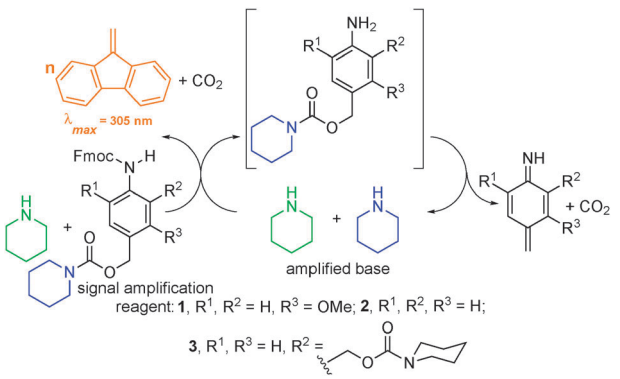


Fig. 1 Balanced autocatalytic reaction sequence for reagents **1–3** when exposed to a trace quantity of piperidine (green). Reagent **3** releases a second equivalent of piperidine (blue) through additional reactions that are not shown.

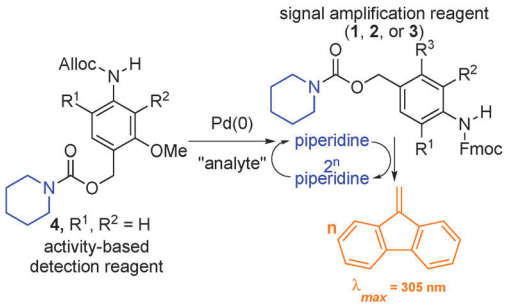
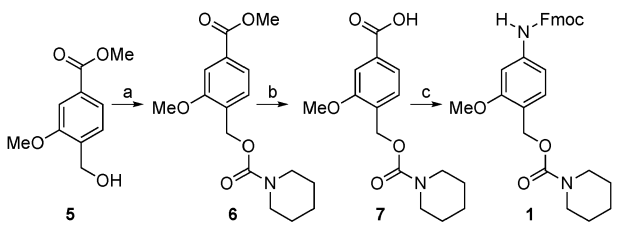


Fig. 2 Signal amplification reagents **1**, **2**, and **3** are designed to amplify signal after **4** releases piperidine in response to a specific activity-based detection event.[3,9,10] In the tandem reaction depicted above, the selectivity for the assay is provided by the activity-based detection reagent, and the sensitivity is provided by reagent **1**, **2**, or **3** (these reagents amplify both the dibenzofulvene chromophore and piperidine). The notation "n" refers to the number of cycles of the autocatalytic reaction.

in the degree of amplification, we connected the Fmoc group with piperidine through an aniline linker that accommodates the release of one (reagent **1**), two (reagent **3**), or even three (compound not shown) equivalents of piperidine per reaction with piperidine. Fourth, the rate of amplification must be made as fast as possible (without causing background reactions); therefore, we included a methyl ether at the 3-position of the aniline linker (reagent **1**) to enhance the rate of release of piperidine (*vide infra*). Finally, the design of the reagent must be coupled with a strategic synthetic plan since reagents that disassemble autocatalytically are especially sensitive to trace levels of decomposition (*i.e.*, release of piperidine) during the preparation of the reagent. An efficient synthetic route to reagent **1** is shown in Scheme 1; the routes to reagents **2** and **3** are shown in Schemes S1 and S2 (ESI†).

To determine whether our reagent design was effective, we first examined the products that are formed when **1** is exposed to piperidine. Time-dependent LC-MS analysis (Fig. S1, ESI†) of the reaction mixture obtained when **1** reacts with 0.02 equiv of piperidine in 50 : 1 DMSO–H_2O at 18 °C revealed clean, quantitative conversion of **1** to dibenzofulvene within 6 h; the LC-MS data also revealed an increase in the intensity of the mass signal for piperidine as the reaction progressed. The aniline intermediate resulting from deprotection of the Fmoc group was too short-lived to be observed using LC-MS; we presume that release of CO_2 and piperidine occurs quickly to form azaquinone methide. Although azaquinone methide was not present in the LC-MS spectra, we did observe masses



(a) (i) CDI, CH_2Cl_2, (ii) piperidine (90%); (b) LiOH, 3:1:1 THF-H_2O-MeOH (92%); (c) (i) EtOCOCl, DIEA, CH_2Cl_2, (ii) NaN_3, acetone-H_2O, (iii) toluene, reflux, (iv) 9-fluorenemethanol, reflux (75%).

Scheme 1 Synthesis of signal amplification reagent **1**.

consistent with short oligomers (1–4 repeating units; Fig. S2, ESI†), which likely arise from oligomerization of azaquinone methide under the reaction conditions. The LC-MS data accounts for all components of reagent **1**, and demonstrates that **1** is capable of releasing piperidine when the Fmoc group is cleaved. In contrast, when stored as a solid at 20 °C open to the air and in the absence of piperidine, reagent **1** showed no signs of decomposition for at least 14 days (14 days was the duration of the stability experiment).

The ability of reagent **1** to provide an amplified signal becomes apparent when **1** is exposed to various substoichiometric quantities of piperidine (Fig. 3). The graph in Fig. 3 reveals four key features of the reagent: (i) it is consumed completely, even when exposed to only 0.001 equivalents of piperidine; (ii) the background reaction in the absence of applied piperidine is negligible over the course of the experiment; (iii) it is capable of >1000× signal amplification; and (iv) the kinetics profile of its reaction with piperidine is characteristic of an autocatalytic reaction (*i.e.*, it displays a sigmoidal response curve;[11] in contrast, if each equivalent of piperidine were consumed when reacting with **1**, then the reaction would display a logarithmic response curve).

The design of reagent **1** includes a methyl ether at the 3-position on the aniline linker; we anticipated that the presence of this substituent would accelerate the rate of azaquinone methide formation and, therefore, increase the rate of the amplification reaction.[12] As predicted, when **1** is exposed to 0.01 equiv of piperidine, **1** is consumed 2.3× faster than **2** (which lacks the methyl ether) under identical reaction conditions (Fig. S3, ESI†).

A second method for increasing the rate of the amplification reaction is to release more than one copy of piperidine from the aniline linker. Reagent **3** releases two equivalents of piperidine per reaction with piperidine, and, when exposed to 0.01 equiv of piperidine (Fig. S3, ESI†), **3** is consumed in 16 h. In comparison, 18 h are necessary for complete

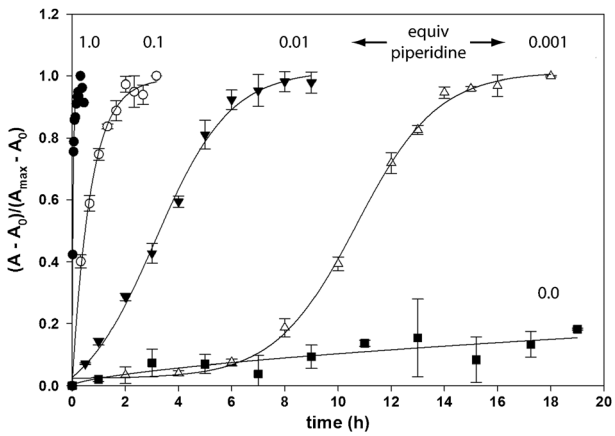


Fig. 3 Quantification of dibenzofulvene when **1** is exposed to substoichiometric quantities of piperidine. The graph provides the normalized absorbance of dibenzofulvene at 305 nm during the course of the signal amplification reaction, and as a function of the number of equivalents of piperidine that were exposed to **1**. The experiments were conducted in triplicate, and the error bars reflect the standard deviations from the average values. The concentration of **1** was 40 mM in 45 : 5 : 1 DMSO–THF–H_2O, and the experiments were conducted at 18 °C.

consumption of reagent **2** (which releases only one equivalent of piperidine) under the same reaction conditions. Furthermore, at the end of the amplification reaction **3** will have released twice the quantity of base as **2**. Ultimately, the ability to easily tune the rate of the amplification reaction by modifying the structure of the reagent is a useful characteristic of the general reagent design shown in Fig. 1.

Additional evidence that reagent **1** (and, by analogy, reagents **2** and **3**) amplifies signal autocatalytically is provided by the observation that the quantity of piperidine (the free base) is amplified during the course of the reaction. To measure the quantity of piperidine free base, we exposed aliquots of the reaction mixture to a solution of the pH indicator bromocresol green and measured the change in color of the solution. In the absence of base (t = 0 h), the solution of bromocresol green has negligible absorbance at 625 nm, but as the quantity of base increases due to the autocatalytic reaction, the absorbance of bromocresol green at 625 nm increases sigmoidally (Fig. S4, ESI†). In fact, the quantity of piperidine (as reflected by the normalized absorbance of bromocresol green at 625 nm) increases with a rate and absorbance–time profile that is superimposable on the rate and absorbance–time profile for the formation of dibenzofulvene under the same reaction conditions (Fig. S4, ESI†), thus demonstrating that both signals (base and dibenzofulvene) are generated with equal rates. Control experiments using either 3-methoxyaniline or 4-aminobenzyl alcohol (40 mM) in place of **1** reveal that neither aniline compound is sufficiently basic to deprotonate bromocresol green and cause the absorbance at 625 nm (Fig. S5, ESI†). Exposure of bromocresol green to 40 mM piperidine in 50 : 1 DMSO–H_2O, in contrast, induces a large absorbance at 625 nm (Fig. S5, ESI†).

Having established that reagents **1**, **2**, and **3** amplify signal *via* autocatalytic reactions, we next evaluated whether they could be used in tandem with an activity-based detection reagent to provide sensitive detection of an analyte (see Fig. 2 for a depiction of this concept).[3,7] We tested this idea using Pd(II) as a model analyte,[13] reagent **1** for signal amplification, and reagent **4** as the activity-based detection reagent (this compound incorporates an allyl group as the substrate for detecting palladium). The model assay was conducted as follows: a 100 μL solution of reagent **4** (0.2 M) and $PhSiH_3$ (438 mM) in THF was mixed in a 1 : 1 ratio with a solution of $Pd(OAc)_2$ (the model analyte) and Bu_3P (40.5 mM) in THF. After a 1-h incubation period (in which piperidine was released from reagent **4** *via* a catalytic reaction with Pd), the detection solution was diluted with water (40 μL), and a 50-μL aliquot was transferred to a solution of the amplification reagent **1** in DMSO (56.8 μM, 360 μL). The piperidine released from the detection event then initiated the signal amplification reaction with **1**.

To determine the effectiveness of this tandem sequence, we performed several experiments with various initial quantities of palladium. In all cases, the signal amplification reaction was allowed to proceed for 16 h, after which we measured the absorbance of dibenzofulvene at 305 nm. The dose–response curve shown in Fig. S6 (ESI†) reveals that (i) the limit of detection for this proof-of-concept assay is 12 ppm Pd, which is approximately the government-regulated threshold level of palladium permitted in drugs;[10,14] (ii) the assay is capable of distinguishing samples with Pd concentrations that differ by only 2 ppm; and (iii) amplification reagent **1** can be paired effectively with an activity-based detection reagent (*e.g.*, **4**) that releases piperidine in response to a specific analyte.

In conclusion, we demonstrated that reagents **1**, **2**, and **3** are capable of amplifying a signal *via* a base-catalyzed autocatalytic reaction. The pairing of reagents **1**, **2**, and **3** with different activity-based detection reagents opens the possibility of conducting future assays that are both selective and sensitive for a variety of analytes. The amplification reagents do not yet provide signal amplification with a rate that is ideal for point-of-care assays, but they do serve as a valuable starting point for further optimization. Alternatively, this type of autocatalytic amplification reagent may be useful more immediately in other contexts, including stimuli-responsive materials,[15,16] in which an amplified response to a specific chemical signal is needed, but where the time required to generate an amplified response is less important. Experiments in this direction are in progress.

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