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COMMUNICATIONS

Published on Web 03/23/2007

Combinatorial Rosamine Library and Application to in Vivo Glutathione Probe

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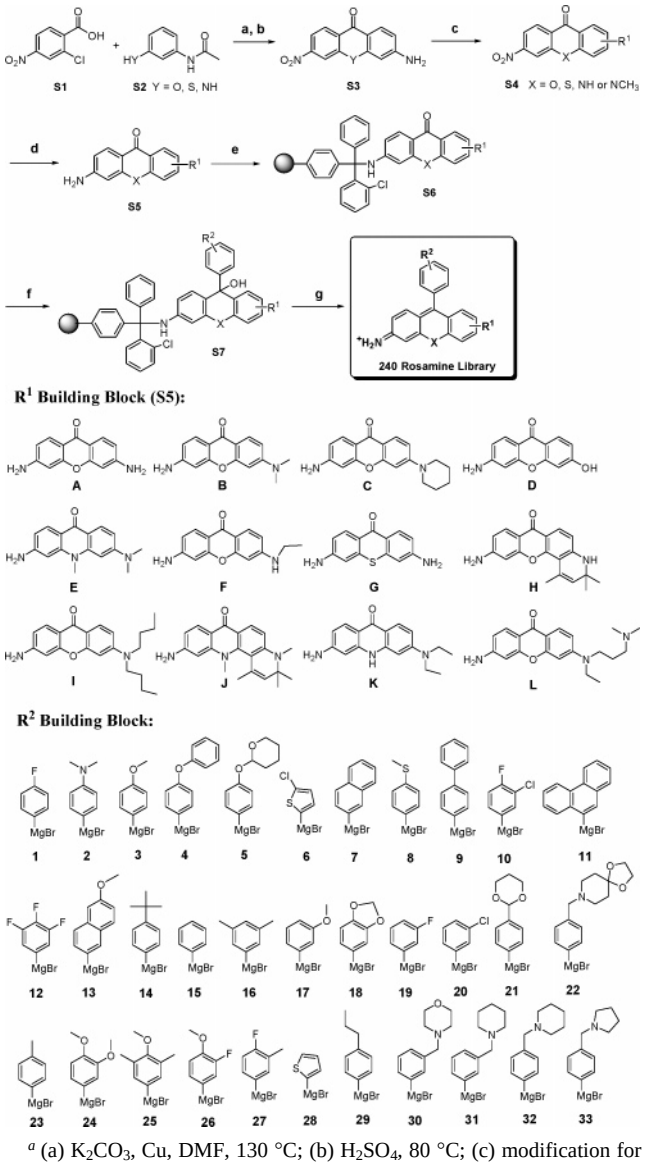
Fluorescent compounds have been excellent tools for the sensitive and specific detection of a variety of analytes.[1] As an alternative approach to conventional target-oriented rational design, we have previously introduced combinatorial fluorescent styryl libraries and successfully demonstrated the power of the diversity-oriented approach by generating specific sensors for various bioanalytes (DNA, RNA, β-amyloid protein, and organelles).[2] Encouraged by these results, we are expanding the general approach to different fluorophores for better properties.

Rhodamine is a highly favorable fluorescence scaffold because of its excellent photophysical properties such as high extinction coefficient, high quantum yield (often near 1), high photostability, pH-insensitivity (unlike fluorescein), and relatively long emission wavelength (>500 nm).[3] These superior and constant optical properties of rhodamine partially originate from its rigid-core structure. In addition to the fused xanthene structure, rhodamine carries 2′-carboxylic acid, which constrains the rotation of the 9-phenyl ring.[4] Therefore, we envisioned that removal of the 2′-carboxylic acid group from rhodamine (called rosamine) will introduce flexibility onto the rhodamine scaffold and thus would generate a possible sensor candidate whose fluorescence properties can be changed by an analyte environment. Here we report the first combinatorial synthesis of a rosamine library and an in vivo fluorescent probe for monitoring cellular glutathione (GSH) levels.

Most rhodamine derivatives including rosamine have been prepared by condensation reactions under strong acidic conditions,[5] requiring difficult or tedious purification. We have sought to incorporate solid-phase chemistry to generate the final product rosamine, circumventing acidic reflux conditions and time-consuming purification steps (Scheme 1). Initially three different 3-amino-6-nitro-9*H*-xanthone derivatives (**S3**, Y = O, NH, S) were synthesized. The first diversity was introduced by modifying the 3-amino group utilizing the 6-nitro group as a linker after reduction to an amino group. Twelve different unsymmetrical xanthone derivatives (**S5**, R^1 building blocks, **A**−**L**) containing oxygen, sulfur, and nitrogen bridges were synthesized (synthetic details for each intermediate are in Supporting Information (SI)). Each intermediate was loaded on the 2-chlorotrityl chloride resin (**S6**), and reacted with 33 different Grignard reagents (R^2 building block) for the second diversity.[6] An acidic cleavage from the resin resulted in the dehydration, giving the fully conjugated rosamine derivatives. All the compounds in the library were characterized by HPLC−MS and 240 relatively pure compounds (average purity is 93%, measured at 250 nm (see SI)) were collected for further study. The library compounds have a wide range of spectral diversities (excitation ranges from 480 to 545 nm and emission ranges from 530 to 605 nm). The quantum yield of each molecule highly varies from 0.00025 to 0.89 reflecting diverse structural and electronic characteristics.

For primary screening, the library compounds were screened for fluorescence intensity change toward a variety of biorelevant analytes. Notably, **H22** exhibited a highly selective response toward

Scheme 1. Synthesis Generating Rosamine Library and Building Blocks[a]



[a] (a) K_2CO_3, Cu, DMF, 130 °C; (b) H_2SO_4, 80 °C; (c) modification for R1 (see Supporting Information for each reaction); (d) $SnCl_2$, EtOH, 90 °C; (e) 3-chloro-trityl chloride resin, Pyr, CH_2Cl_2/DMF; (f) R^2 Grignard reagent, THF, 62 °C; (g) 1%TFA, CH_2Cl_2.

reduced glutathione (GSH) compared to other analytes (SI). In physiological condition (pH 7.4, 50 mM HEPES), **H22** (3 μM) was further evaluated and showed a marked fluorescence increase upon addition of GSH (5 mM) by ca. 11-fold in 30 min (Figure 1). The quantum yield of **H22** was 0.033 and 0.28 before and after addition of GSH. It is noteworthy that **H22** did not show any fluorescence response to GSSG (5 mM) while several thiol-containing analytes such as DTT, β-mercaptoethanol, and cysteine showed modest

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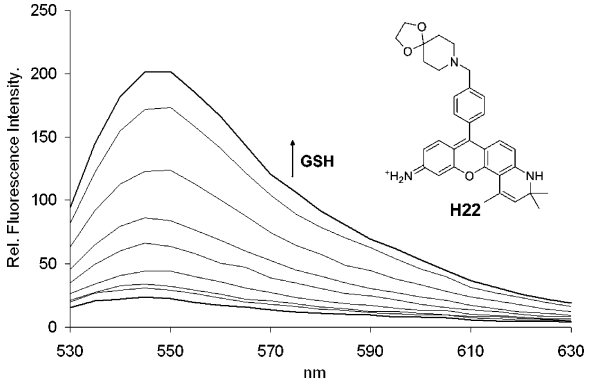

Figure 1. Fluorescence responses of **H22** (3 μM) toward GSH in 0, 0.01, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5 mM. **H22** was incubated with GSH at room temperature for 30 min in 50 mM HEPES, pH 7.4. Spectra were obtained with excitation at 500 nm.

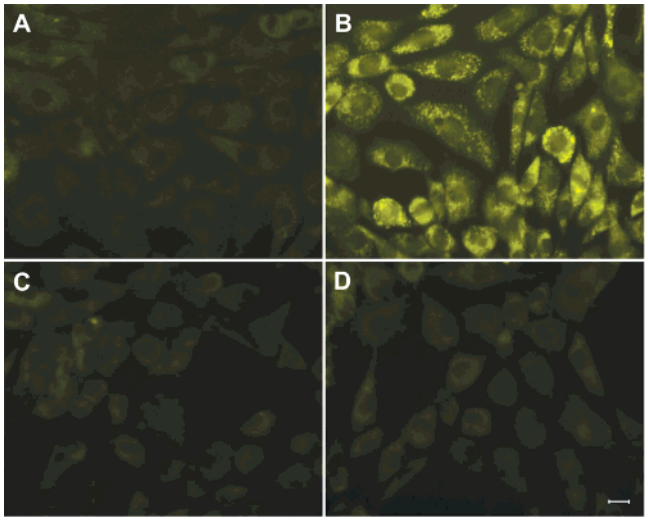

Figure 2. Fluorescence microscopic images of live 3T3 cells: (A) cells stained with **H22** (3 μM) for 15 min; (B) cells supplemented with lipoic acid (250 μM) for 48 h and stained with **H22** for 15 min. Subsequently, α-lipoic acid-supplemented cells stained with **H22** were incubated with (C) NMM (1 mM) or (D) diamide (50 μM) for 20 min at room temperature. Scale bar = 20 μm.

responses to **H22** (SI). To test its dynamic response toward GSH, the preincubated **H22** with GSH (0.5 mM) for 30 min was treated either with a thiol reactive reagent (*N*-methylmaleimide; NMM) or a thiol-oxidant (diamide) for another 30 min. The fluorescence intensity was decreased upon addition of either NMM or diamide in a dose-dependent manner (SI).

Next, we tested the capability of **H22** to monitor GSH in a living cell. α-Lipoic acid is known to enhance the reduced GSH level in a variety of cells,[7] thus 3T3 cells were supplemented with α-lipoic acid (250 μM) for 48 h. Subsequent staining of cells with **H22** (3 μM) showed a clear increase in the intracellular fluorescence intensity in α-lipoic acid treated cells (Figure 2B) compared to nontreated cells (Figure 2A). When NMM (Figure 2C) or diamide (Figure 2D) was supplemented to α-lipoic acid-treated cells stained with **H22**, a distinct decrease of fluorescence intensity was observed. In a similar manner, incubation of the cells with BSO (buthionine sulfoximine; GSH synthesis inhibitor)[8] for 60 min also showed a similar intensity decrease (SI), while untreated cells did not show such a decrease in the same experiment duration. Taken together, these experiments clearly demonstrate that **H22** is able to sense changes in the cellular GSH level in living cells.

GSH is the most abundant cellular thiol and plays the central role in maintaining redox homeostasis, existing in a redox equilibrium between the reduced (GSH) and oxidized (GSSG) form.[8] With a high concentration of GSH (~5 mM) in a cell, the ratio of GSH over GSSG (ranging from 1 to 100)[7d] is of critical importance in regulating the redox potential in a living cell. Although many thiol detecting fluorescent dyes have been utilized for measuring GSH,[3,9] there are few cellular applications of such small molecule probes for monitoring changes of the cellular GSH concentration.

In summary, we have developed the first rosamine library using solid-phase chemistry as a potential fluorescence sensor set, and described the potential of **H22** as a glutathione probe in living cells.

Acknowledgment. We gratefully acknowledge the National Institutes of Health for financial support (Grant P20GM072029) and for equipment grants for the NMR (Grant MRI-0116222) and the capillary LC-ion trap mass spectrometer (Grant CHE-0234863). Components of this work were conducted in a Shared Instrumentation Facility constructed with support from Research Facilities Improvement Grant C06 RR-16572 from the NCRR/NIH. J.S.L. was supported by a Korea Research Foundation Grant funded by the Korean government (MOEHRD, Basic Research Promotion Fund: KRF-2005-C00088)

Supporting Information Available: Synthesis and experimental procedures and characterization data of all library compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA068230M