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# "Singapore Green": A New Fluorescent Dye for Microarray and Bioimaging Applications

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# “Singapore Green”: A New Fluorescent Dye for Microarray and Bioimaging Applications

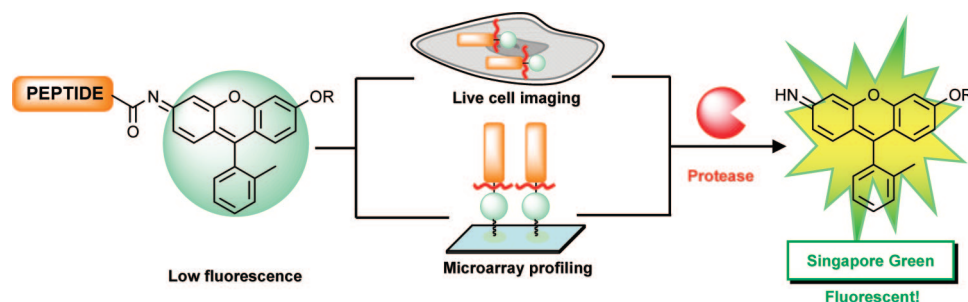
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## ABSTRACT



We have synthesized Singapore Green (SG), a structural hybrid of Tokyo Green and Rhodamine 110. This new dye is a green light-emitting substitute for 7-aminocoumarin, a blue fluorescent dye widely used in enzymatic assays. SG-conjugated peptide substrates were successfully synthesized and used in microarray-based protease substrate profiling and live-cell imaging experiments.

Fluorogenic peptide substrates, including those employing latent fluorophores, internally quenched and fluorescence resonance energy transfer (FRET)-based substrates, have emerged as indispensable tools in the profiling and visualization of protease activities both *in vitro* and *in vivo*.<sup>1</sup> They are also widely used in high-throughput screening of protease inhibitors. Among the various types of reagents used, fluorogenic peptide substrates containing a C-terminally capped coumarin derivative (i.e., 7-amino-4-methylcoumarin (AMC) or 7-amino-4-carbamoylmethylcoumarin (ACC)) are arguably the most useful for substrate specificity profiling experiments, as only cleavage at the amide bond between the peptide and the coumarin moiety will release the highly fluorescent coumarin.<sup>2</sup> Consequently, coumarin-based fluorogenic peptide libraries have been employed to study the

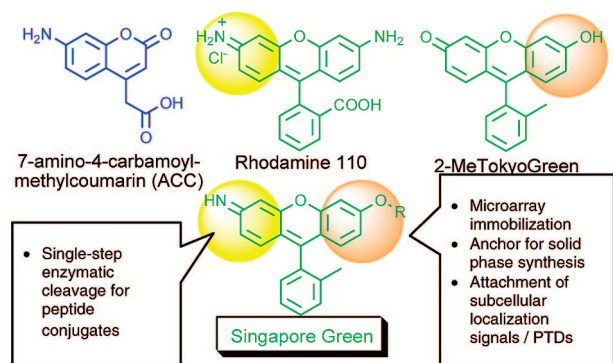
substrate specificities of numerous therapeutically important proteases, including caspases, thrombin, cathepsins, and many others.<sup>2</sup> In recent years, several attempts have been made to introduce these substrate libraries to microarray-based applications where further miniaturization and higher throughput of enzymatic assays can be achieved.<sup>3,4</sup> We and others recently reported the immobilization of coumarin-

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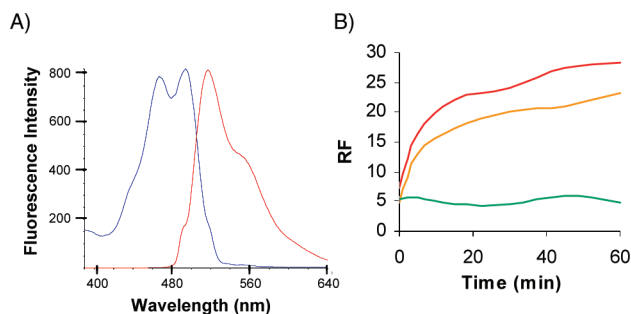
based enzyme substrates onto microarray platforms and used them to profile substrate specificities of proteases.<sup>4</sup> Since coumarin dyes are excited in the UV region (maximum  $\lambda_{\text{ex}}$  ~350 nm), these strategies have not been effective due to high fluorescence backgrounds and the lack of microarray scanners with UV light sources. For similar reasons, coumarin-based peptide substrates are rarely used in live-cell imaging experiments.<sup>1b</sup>



**Figure 1.** Structures of common fluorophores used in fluorogenic peptide substrates (ACC and Rhodamine 110) and fluorophores from which Singapore Green was derived (Rhodamine 110 and Tokyo Green).

We sought to replace coumarin with a new fluorophore having excitation and emission wavelengths in the visible range, so that it is suitable for both microarray and live-cell imaging applications. We turned to other fluorescein and rhodamine fluorophores that have been used for labeling reagents and enzymatic assays.<sup>5</sup> Of these, Rhodamine 110 (R110)-based substrates are well-established peptide probes for serine and cysteine proteases.<sup>6,7</sup> Despite the desirable fluorescence properties of R110, several drawbacks hinder the direct use of these substrates: (1) R110-conjugated peptides require both peptide groups to be cleaved in order to generate maximum fluorescence and thus are not suitable for quantitative studies of linear enzyme kinetics;<sup>5,6a</sup> (2) “asymmetric” versions of these dyes containing a single peptide cleavage site lack an immobilization handle which is essential for both solid-phase peptide synthesis and microarray applications;<sup>7b-d</sup> (3) equilibrium between the

quinone and the nonfluorescent spirolactone forms of R110 reduces fluorescence output.<sup>7b,c</sup> Our new fluorophore, Singapore Green (SG), is a hybrid of R110 and the fluorescein analogue 2-Me TokyoGreen (Figure 1),<sup>8</sup> with a phenolic group on one end providing a chemical handle (for solid-phase peptide synthesis, microarray immobilization, and potentially other applications in cell-based experiments) and an amino group on the other end serving as the point of conjugation to a peptide sequence. We reasoned that amidation at the amino group of SG by a peptide should suppress the fluorescence of the dye. Similar fluorescence-quenching effects have been observed in other coumarin- and rhodamine-based peptide conjugates.<sup>5</sup> Cleavage of the amide bond by a protease should release the highly fluorescent SG, thus reporting protease activity accordingly. Herein, we report the synthesis and characterizations of SG, the solid-phase synthesis of SG-conjugated peptides, as well as their applications in microarray-based and live-cell imaging experiments.



**Figure 2.** (A) Fluorescence spectra of SG1. Fluorescence increase from cleavage of Ac-DEVD-SG1 by caspase-3 (blue line) and caspase-7 (red line) over the background fluorescence (orange line).

As shown in Scheme 1, the synthesis of SG started with the formation of the asymmetric xanthone **1**, which was generated by Ullman-type coupling between 3-acetamidophenol and 2-chloro-4-nitrobenzoic acid based on a published procedure.<sup>9</sup> Diazotization of the amino group and hydrolysis of the diazonium salt yields the phenol **2** which underwent methylation or alkylation with a linker unit to give **3**. The nitro group was subsequently reduced to give **4a** and **4b**. Subsequent trityl protection and Grignard addition of *o*-tolylmagnesium bromide followed by deprotection of the trityl group afforded the fluorophores SG1 and SG2, respectively. SG1 was found to have excitation maxima at 469 and 493 nm and emission maxima at 517 nm in ethanol (Figure 2a), similar to fluorescein (494 and 521 nm respectively, in water), and is thus compatible with the 488 nm argon-ion laser used in most microarray scanners and fluorescence microscopes. SG1 has a quantum yield of 0.50 and an extinction coefficient of 28500 M<sup>-1</sup>cm<sup>-1</sup>, which is

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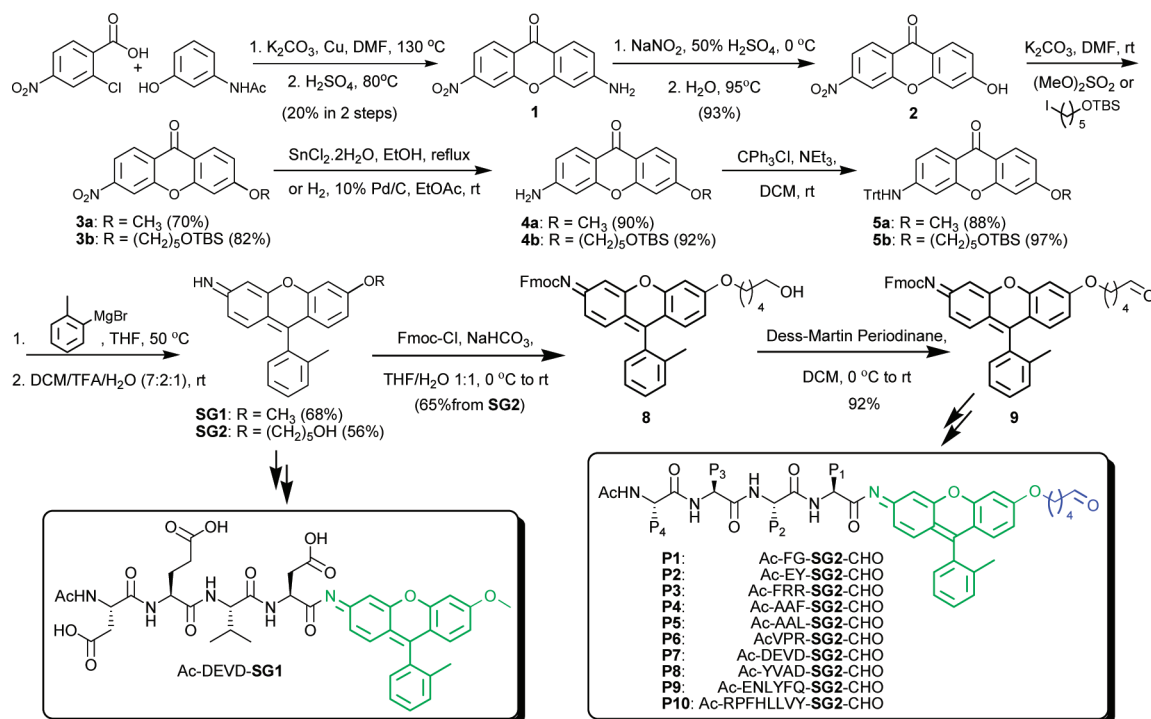
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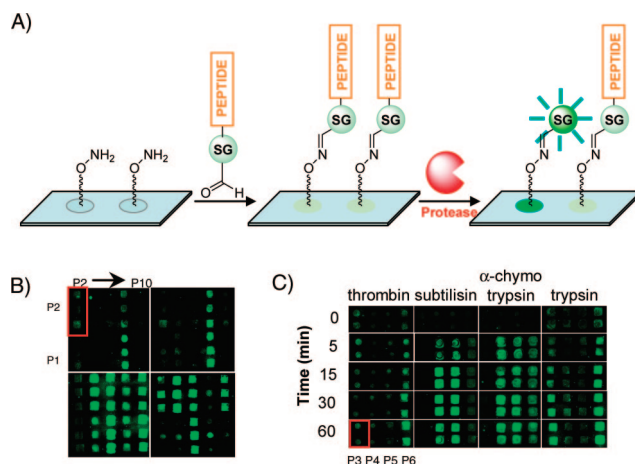
### Scheme 1. Synthesis of Peptide Conjugates of SG1 and SG2



reasonably bright for most applications. The photostability of **SG** was tested by irradiating the dye with a fluorimeter; results indicated that **SG** is at least as photostable as fluorescein. To test if peptide conjugates of **SG1** can be used as fluorogenic probes to detect enzymatic activity, Ac-DEVD-**SG1** was synthesized as an analogue of Ac-DEVD-AMC (a coumarin-based substrate for caspase-3 and -7 from commercial sources). Ac-DEVD-**SG1** was weakly fluorescent. Upon incubation with caspase-3 and -7, however, there was a time-dependent increase in fluorescence, which follows typical Michaelis–Menten curves (Figure 2b), indicating that **SG**-based peptide conjugates are indeed suitable green light-emitting substitutes of their coumarin counterparts.

We next sought to test the feasibility of using peptide conjugates of **SG2** for reporting protease activities on the microarray. We picked 10 peptide sequences from commercially available *p*-nitroanilide or coumarin-based substrates targeting various proteases and replaced the *p*-nitroaniline chromophore or coumarin fluorophore with **SG2**. To render the fluorophore compatible with Fmoc-based chemistry used in solid-phase peptide synthesis, the amino end of **SG2** was protected with Fmoc, giving **8**, and the hydroxyl-containing linker located on the other end was oxidized to an aldehyde with Dess–Martin periodinane to give **9**. The compound was subsequently loaded onto a threonine-functionalized aminomethyl polystyrene resin by acid-catalyzed oxazolidine formation (Supporting Information). Peptide synthesis then proceeded using standard Fmoc chemistry on solid phase. Final cleavage of the peptides from the solid support gave the desired aldehyde-functionalized peptides in good or excellent purity (most crude products except **P9** showed a

single major peak in LCMS profiles; see the Supporting Information), thus demonstrating the compatibility of **SG2** with standard Fmoc chemistry.



**Figure 3.** (A) Strategy for detecting protease activity on microarray. (B) Enzyme "fingerprints" obtained (clockwise from top left: caspase-3, caspase-7, α-chymotrypsin, subtilisin). Peptides were spotted in triplicate (vertically). (C) Time-dependent kinetic profiles obtained from the peptide microarray. Peptides were spotted in duplicate (vertically). See the Supporting Information for details of spotting patterns.

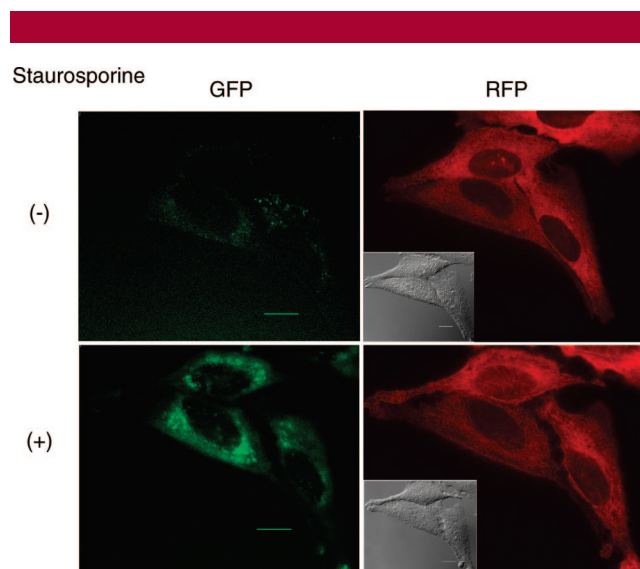
We next spotted the peptides onto alkoxyamine-functionalized glass slides to generate the corresponding peptide



microarray via chemoselective oxime bond formation (Figure 3a). As a proof-of-concept experiment, the immobilized peptides were treated with four different proteases (caspase-3, caspase-7,  $\alpha$ -chymotrypsin, and subtilisin). The microarray was subsequently scanned with a microarray scanner equipped with a green light source. As shown in Figure 3b, images obtained immediately revealed discerning “substrate fingerprints” of each enzyme. Of the proteases used, enzymes with broader substrate specificity (subtilisin and  $\alpha$ -chymotrypsin) cleaved multiple substrates to different extents, while highly specific proteases (caspase-3 and -7) cleaved only its optimal substrate sequence, Ac-DEVD-SG2. The fluorescence readouts generally correlated well with the corresponding enzymatic reactions carried out in microplate-based assays (data not shown). We next examined whether the SG-based peptide microarray could be used to obtain quantitative enzyme kinetic data by incubating four selected peptides with four different enzymes in a time-dependent experiment (Figure 3c). Fluorescence intensities were quantified and fitted to kinetic curves to obtain  $k_{\text{obs}}$  values which reflected the substrate preferences of the proteases (see the Supporting Information). Taken together, these experiments indicate the applicability of SG-based substrates in microarray-based protease profiling experiments.

To demonstrate that our SG-based substrates can be used for live-cell imaging, we tested the ability of Ac-DEVD-SG1 to image apoptosis in live cells (Figure 4). Caspase-3 and -7 are key mediators of this important biological process where improper regulation of caspase activity has detrimental pathological and physiological effects. To image apoptosis, numerous peptide- and protein-based probes (including R110 peptide conjugates) have been developed for the sensitive detection of caspase activity.<sup>10,11</sup> We thus evaluated Ac-DEVD-SG1 as a fluorogenic probe for reporting caspase-3 and -7 activity in apoptotic HeLa cells. As shown in Figure 4, cells treated with Ac-DEVD-SG1 developed a strong green fluorescence upon apoptosis induction with staurosporine (bottom vs top left panels). In contrast, no significant increase in green fluorescence was observed in nonapoptotic cells even after extended incubation time (Supporting Information). Nonapoptotic cells remained viable after treatment with the probe for more than 3 h, indicating that the probe was not cytotoxic (data not shown). These results suggest that SG-based substrates could serve as useful fluorogenic probes for live-cell imaging of protease activities. To accommodate future bioimaging applications, the phenolic handle in SG-

based probes may be conveniently modified with cellular localization sequences, protein transduction domains, etc.



**Figure 4.** Detecting caspase activity in live HeLa cells with Ac-DEVD-SG1. Bottom panel shows fluorescence increase from cleavage of Ac-DEVD-SG1 after apoptosis induction (with staurosporine). Cells were injected with 50  $\mu$ M of Ac-DEVD-SG1 (with tetramethylrhodamine-dextran as marker to identify injected cells in RFP channel) and imaged. Inset: DIC images of the cells. Scale bar = 15  $\mu$ m.

In summary, we have developed a new fluorophore that possesses desirable chemical and fluorescence properties suitable for biomedical applications. In this report, we have shown that peptide conjugates of this new fluorophore can be readily synthesized using standard solid-phase peptide chemistry, and conveniently immobilized on a microarray for high-throughput substrate specificity profiling of proteases. We further showed that these probes are equally amenable for live-cell imaging of protease activities. We anticipate that these probes will find wide applications in the emerging field of catalomics.<sup>12</sup>

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**Supporting Information Available:** Experimental procedures, characterization of new compounds, and additional images. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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