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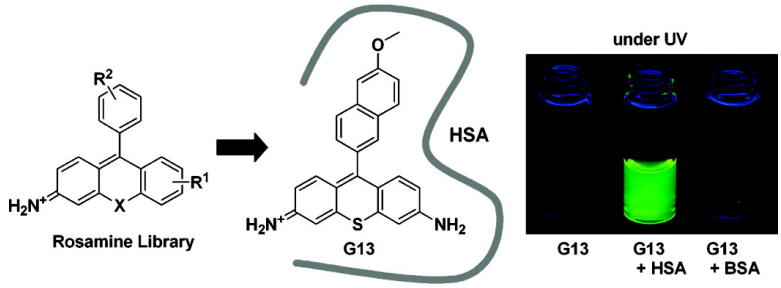

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Article

Selective Human Serum Albumin Sensor from the Screening of a Fluorescent Rosamine Library

Young-Hoon Ahn,[†] Jun-Seok Lee,[†,‡,§] and Young-Tae Chang*[,†,‡,§,||]

Department of Chemistry, New York University, New York, New York 10003, Department of Chemistry and NUS MedChem Program of the Office of Life Sciences, National University of Singapore, Singapore 117543, and Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium, Agency for Science, Technology and Research (ASTAR), Biopolis, Singapore 138667

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A fluorescent dye library approach for the development of a bioanalyte sensor was sought. The screening of a rosamine dye library against diverse macromolecules led to the discovery of a highly sensitive human serum albumin binder, **G13**, with ~36-fold fluorescence intensity change. **G13** showed a highly selective response to HSA over other macromolecules including albumins from other species. The potential use of **G13** for the detection of HSA in biofluids is described.

Introduction

A small molecule fluorescent probe that detects a specific bioanalyte can provide lots of information including the dynamic monitoring of bioanalytes in a cellular context[1] or selective detection and quantification of the analyte in a biosample.[2] While many fluorescent probes have been designed individually by modulating the structural features to control a fluorescence property,[3] a diversity oriented fluorescence library approach (DOFLA), where structurally and spectrally diverse fluorescent small molecules are synthesized and screened against a broad range of bioanalytes, has led to the discovery of many potential bioanalyte probe/sensors: for example, β-amyloid plaque and in vivo RNA probes from a styryl dye library,[4] a GTP sensor from a benzimidazolium dye library,[5] and an albumin binder from a dapoxyl dye library.[6]

Recently, our group has sought a fluorescent dye library with improved photophysical properties, and we have successfully constructed a library of rosamine derivatives (Figure 1).[7] While both rhodamine and fluorescein carry a 2′-carboxylic acid group, which plays a role in constraining the rotation of the 9-phenyl ring and thus modulating the fluorescence change in fluorescein,[8] rosamine derivatives that lack the 2′-carboxylic acid group were envisioned to contain an increased flexibility (Figure 1) and thus were expected to be potential sensor candidates in which fluorescence properties could be changed by a macromolecule binding event. Here we report the screening of a rosamine library against diverse macromolecules, leading to the discovery of a potential human serum albumin (HSA) sensor, **G13**, which shows a highly selective and sensitive response to HSA over other analytes.

Serum albumin is the most abundant protein in blood plasma and plays a role in maintaining the osmotic pressure of the blood compartment and also in the transport and deposition of many endogenous and exogenous substances.[9] The remarkably broad range of ligands binding to serum albumin, which results in a significant impact on the pharmacokinetics of drugs, have elicited high attention to this protein. Many studies have been directed toward the study of its binding sites[10] and structural characterizations[11] and also for the development of a specific and selective fluorescent binder.[6,12] In particular, the detection of albumin has a clinical importance because its concentration in body fluids such as blood or urine is a reliable indicator of various diseases including liver or kidney disease, malnutrition, and microalbuminuria.[12a,13] Although many fluorescent binders to HSA do exist, there is only one fluorescent probe that selectively detects albumin (albumin blue 580).[12a] Other fluorescent binders are mostly environmentally sensitive probes and have quite low excitation (<400 nm) and emission wavelengths (<500 nm),[12b–d] giving the possibility of interference with other proteins.

Results and Discussion

The rosamine library was designed to contain an increased flexibility because of the 9-phenyl ring rotation when

* To whom correspondence should be addressed. Phone: +65-6516-6774. Fax: +65-6779-1691. E-mail: chmcyt@nus.edu.sg.
[†] Department of Chemistry, New York University.
[‡] Department of Chemistry, National University of Singapore.
[§] NUS MedChem Program of the Office of Life Sciences, National University of Singapore.
[||] Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium.

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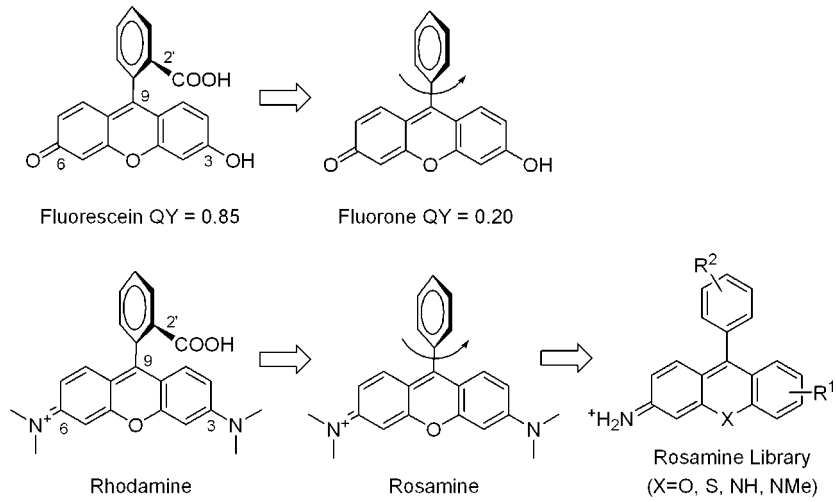

Figure 1. Structures and quantum yields of fluorescein derivatives[8] and the design of a rosamine library on the basis of the structural similarity.

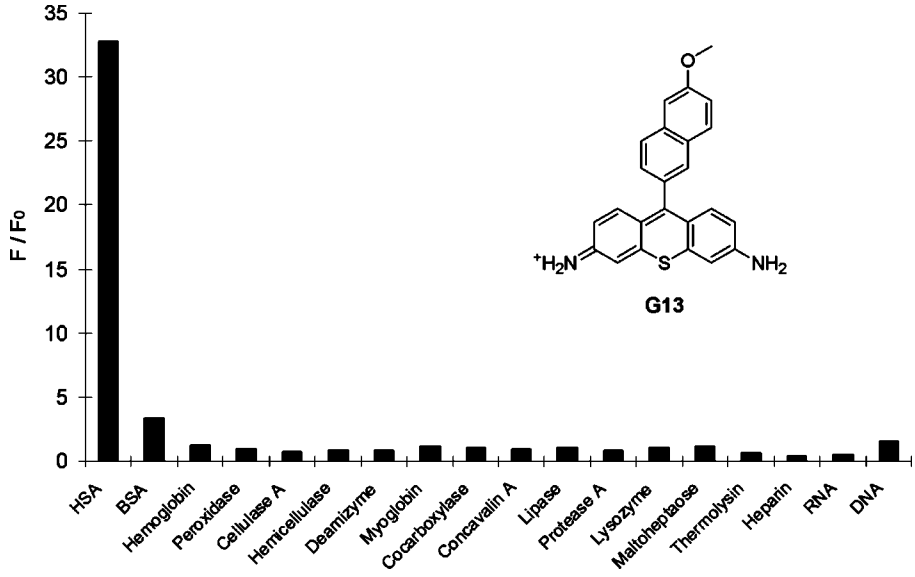

Figure 2. Fluorescence emission responses of **G13** toward 18 macromolecules. In 10 mM PBS (pH 7.4), the fluorescence intensity of **G13** (4 μM) in the presence of each analyte (0.5 mg/mL) (F) was compared with fluorescence intensity of **G13** in buffer (F_0). Bars represent the ratio of F/F_0.

compared to rigid rhodamine derivatives. A specific macromolecule binding event may lock this flexibility and induce a fluorescence enhancement. For primary screening, a library of 240 rosamine derivatives was tested for fluorescence intensity changes against a set of macromolecules including proteins, polysaccharides, and nucleic acids in a 384-well plate by using a fluorescence plate reader (Supporting Information). While many of the library compounds showed nonselective changes of fluorescence intensity, **G13** exhibited a highly selective fluorescence enhancement with HSA. **G13** was further tested in 10 mM PBS buffer (pH 7.4) with a collection of 18 different macromolecules (Figure 2), showing its distinguishing selectivity to HSA with ~30-fold enhancement of fluorescence intensity. The second biggest increase was found with BSA with a 3-fold change, while others showed practically no change.

It would be challenging to achieve this kind of selective response between HSA and BSA because of their structural similarity, in particular, of the domains responsible for ligand−protein interaction.[14] To confirm species selectivity, a set of albumins from different species including human, bovine, sheep (SSA), rat (RSA), and porcine (PSA) were compared for their fluorescence responses in a dose-dependent manner (Figure 3a). At a high concentration of albumins (30 μM), HSA induced the most dramatic intensity change (~36-fold), while BSA, SSA, and PSA showed small changes (~5−7 fold), and RSA gave a medium change (~15-fold). These differences were visually observed under 365 nm UV light (Figure 3b), allowing for the selective detection of HSA among albumins from different species. Three-dimensional structures of albumins from different species are assumed to be very similar because of the highly homologous primary structure (about 75% sequence homology). No major species difference was found in some of X-ray crystallographic structures of albumins that have been analyzed to date.[11] However, several literature reports indicated a species-dependent difference in ligand binding to albumins, and these ligands have been useful in gaining structural information of the binding site of the albumins.[15] In contrast to several ligands that displayed a small difference

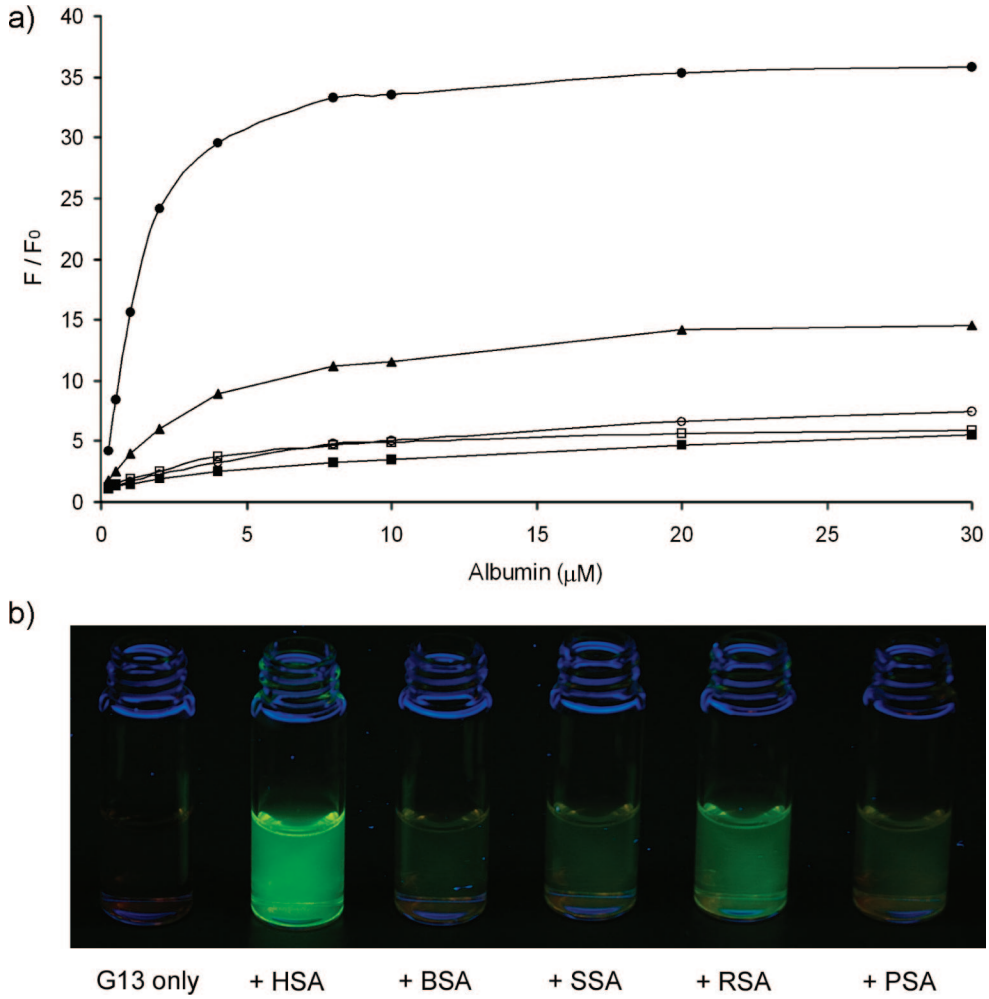

Figure 3. Fluorescence emission responses of **G13** toward albumins from different species. (a) Titration curves of **G13** (4 μM) with (●) HSA, (■) BSA, (□) SSA, (▲) RSA, and (○) PSA. (b) Picture showing solutions of **G13** (8 μM) and albumins (10 μM) from different species in 10 mM PBS (pH 7.4).

in species dependent bindings,[15] **G13** showed a highly selective response to HSA among albumins from different species.

The binding property of **G13** to albumins was further investigated. Compound **G13** (4 μM) in a buffer (10 mM PBS, pH 7.4) displayed a weak fluorescence emission (QY = 0.019) with an excitation maximum of 522 nm and an emission maximum of 544 nm (Figure 4a) The addition of HSA (30 μM) induced a marked increase of fluorescence intensity (~36-fold, QY = 0.20) with a slight red shift in excitation (540 nm) and emission (554 nm) (Figure 4). To verify specific binding of **G13** to HSA, the displacement of **G13** from HSA was measured with three site-specific binding drugs including warfarin (site I), ibuprofen (site II), and digitoxin (site III). **G13** (4 μM) with HSA (10 μM) was treated with differing concentrations of the above drugs. At a ratio of HSA to drug (1:20), both warfarin and ibuprofen displaced **G13** by ~33−35%, while digitoxin induced almost no displacement (Figure 5). Furthermore, various concentrations of **G13** were titrated with HSA or BSA to determine the relative binding affinity. A Scatchard plot of **G13** with HSA gave an association constant of 8.0×10^5 M^{-1} (n = 2.20), while BSA showed an association constant of 7.2×10^4 M^{-1} (n = 1.93) (Figure 6). This ~11-fold difference in binding affinity highlights the selective fluorescence detection of HSA over BSA by **G13**.

The selective and sensitive response of **G13** to HSA would be useful in detection or quantification of HSA in biofluids. To demonstrate its potential use, a series concentration of HSA was mixed with 10% urine from normal healthy people in PBS buffer. The data in Figure 7 shows a good linear correlation between the emission response and the total HSA content over the studied range of 0−150 mg/L, establishing that **G13** is capable of detecting different levels of HSA in a urine sample.

In conclusion, we pursued DOFLA to develop a selective bioanalyte sensor using a rosamine library. The compound, **G13**, which displayed high selectivity and sensitivity over other proteins, including albumins from other species, was discovered. A linear correlation in the emission response of **G13** versus the total HSA content in a urine sample suggests the potential for the use of **G13** in the detection of HSA in biofluids.

Experimental Section

Materials and Methods. G13 was prepared by procedures reported previously.[7] Albumins from different species, human, bovine, sheep, rat, and porcine were obtained from

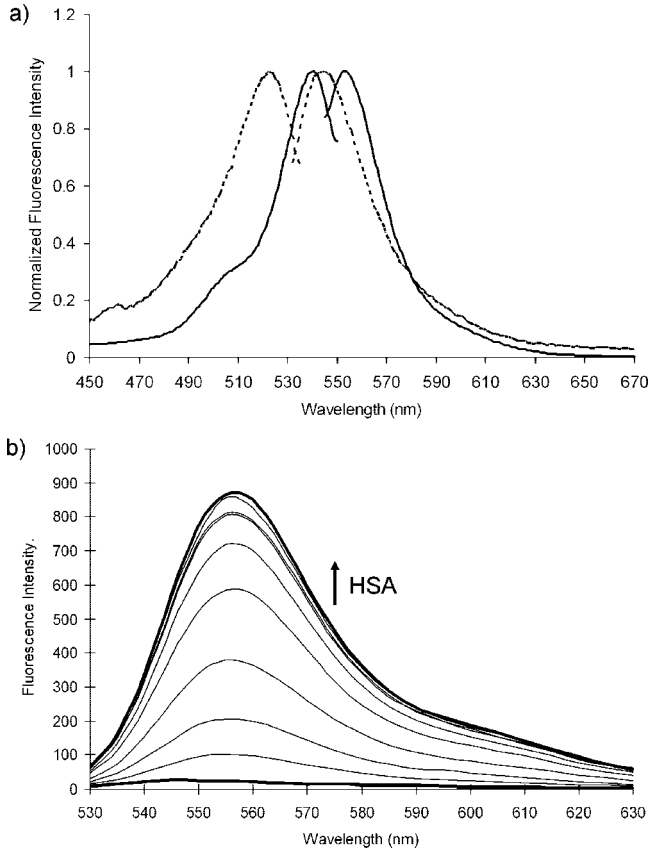

Figure 4. (a) Fluorescence excitation and emission spectra of **G13**: dotted (···) and solid (—) lines are spectra of **G13** before and after addition of HSA, respectively. (b) Titration of **G13** (4 μM) with HSA at concentrations of 0, 0.5, 1, 2, 4, 8, 10, 20, and 30 μM. Spectra were taken in 10 mM PBS (pH 7.4) with excitation at 490 nm.

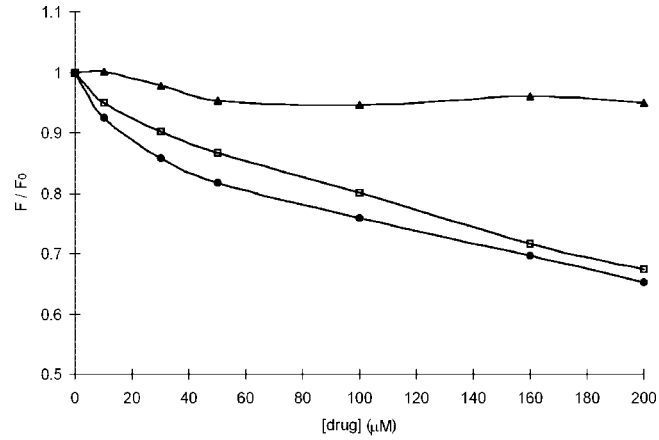

Figure 5. Displacement of **G13** from HSA−**G13** complex by the addition of site-specific drugs. To **G13** (4 μM) and HSA (10 μM) were added a series concentration of drugs to give final concentrations of 0, 10, 30, 50, 100, 160, and 200 μM: (▲) digitoxin (site III), (□) ibuprofen (site II), (●) warfarin (site I). F is the fluorescence intensity at the indicated concentration of each drug, and F_0 is the fluorescence intensity at drugs (0 μM). F/F_0 indicates the ratio of F to F_0.

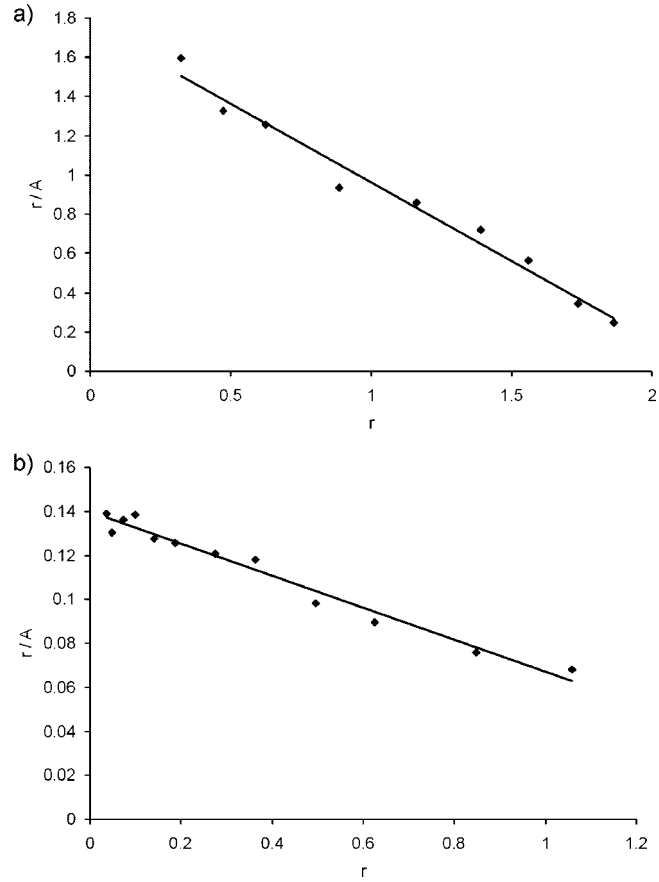

Figure 6. Scatchard plots for **G13** binding to albumins. HSA (4 μM) or BSA (8 μM) was titrated with **G13** (1−20 μM). The concentrations of free and bound **G13** were calculated from the fluorescence intensity, and a curve was generated to give the association constant: (a) HSA with $K_a = 8.0 \times 10^5$ M^{-1} and $n = 2.20$, (b) BSA with $K_a = 7.2 \times 10^4$ M^{-1} and $n = 1.93$. r is the number of moles of **G13** bound per mole of albumin, and A is the concentration of free dye.

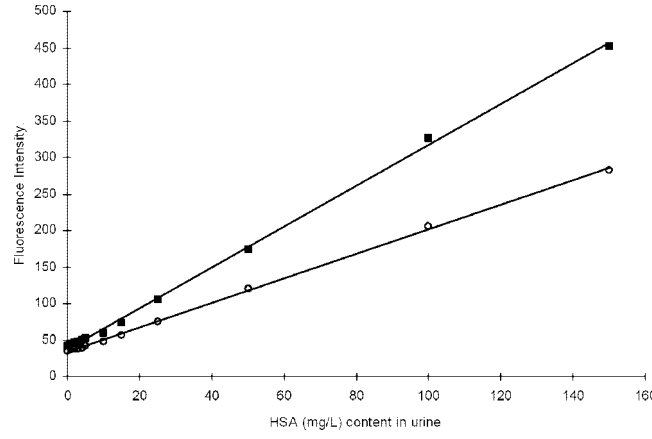

Figure 7. Fluorometric analysis of HSA content in a urine sample. A series of HSA concentrations were mixed with 10% urine in 10 mM PBS buffer (pH 7.4) to give the final concentration of HSA in a range of 0−150 mg/L, and the emission intensities of **G13** at 555 nm with excitation at 490 nm were plotted versus total HSA content: (■) **G13** (8 μM) and (○) **G13** (4 μM).

Sigma as essentially fatty acid-free grade and used without further purification. The concentration of HSA and BSA were determined by UV absorption at 279 nm ($E^{1\%}_{1cm} = 5.30$ for HSA and 6.67 for BSA).[15e] All other analytes were purchased from Sigma and Amano Enzyme Inc. Urine samples were collected from consenting normal healthy individuals. The fluorescence excitation and emission spectra of **G13** were determined by a Hitachi F-2500 fluorescence spectropho-

tometer. All other fluorescence assays were performed using a Spectra Max Gemini XS plate reader. Fluorescence emission was measured from 530 to 630 nm with excitation at 490 nm. Fluorescence quantum yields were determined with a standard of rhodamine 6G (QY = 0.95 in H_2O).[16]

Measurement of Binding Affinity.[12c] HSA (4 μM) or BSA (8 μM) solution was titrated by a series concentration of **G13** (1−20 μM) and the fluorescence intensity was measured with excitation at 490 nm and emission at 555 nm. The bound fraction (X) of **G13** at each concentration was determined using the equation

$$X = \frac{F_c - F_o}{F_{sat} - F_o} \quad (1)$$

where F_c and F_o are the fluorescence intensities of a given concentration of **G13** with and without albumin, respectively. F_{sat} is the fluorescence intensity at the same concentration of **G13** when fully bound. F_{sat} was determined by fluorescence titration of **G13** at each concentration with a series concentration of HSA. The results were plotted according to the Scatchard equation

$$r = \frac{nK_aA}{1 + K_aA} \quad (2)$$

where r is the number of moles of **G13** bound per mole of albumin, n is the number of binding site, K_a the association constant, and A the concentration of the free dye.

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Supporting Information Available. Characterization of **G13** and the primary screening data of 240 rosamine compounds against various bioanalytes are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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