



Tuning thiol addition to squaraines by *ortho*-substitution and the use of serum albumin



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ABSTRACT

Tuning the reactivity of squaraine dyes toward nucleophilic addition of thiols was investigated. A series of water soluble, aniline-derived squaraines were synthesized with various *ortho* substitutions to the squaraine ring. As hypothesized, we found that placing moderately electron donating groups in the *ortho* position conveyed intermediate reactivity to thiols between the essentially non-reactive hydroxyl-substituted squaraines and very reactive non-substituted squaraines. Furthermore, serum albumin was tested for its influence on the addition of thiols to the squaraines. The dyes bind in the hydrophobic cavities of the protein, and thus we expected serum albumin to affect the squaraines' reactivity. Rather than a protective effect by the protein, we found a cooperative effect for thiol addition.

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1. Introduction

Squaraines are fluorescent molecules prepared by the reaction of squaric acid with electron rich aryls, such as *N,N*-dialkylanilines, benzothiazoles, phenols, and pyrroles, with azeotropic removal of water [1]. The structure of a squaraine is a resonance-stabilized zwitterion. The central four-membered ring is electron deficient, while the oxygen atoms and the two aniline groups are electron donating, leading to a donor-acceptor-donor (D-A-D) structure. From theoretical calculations, Bigelow and Freund showed that during the $S_0 \rightarrow S_1$ transition there is a charge transfer that is largely from the oxygen atoms to the four-membered ring with some minor donation from the aniline groups [2]. This intramolecular charge transfer and the extended conjugation of the molecule lead to absorption in the visible to near-IR region with narrow bands (half bandwidth $\sim 750 \text{ cm}^{-1}$) [1] and large extinction coefficients ($\epsilon \geq 10^5 \text{ cm}^{-1} \text{ M}^{-1}$) [3]. Thus, squaraines have found application in imaging [1,4,5], photovoltaics [6–8], ion sensing [9–13], and other areas [14,15].

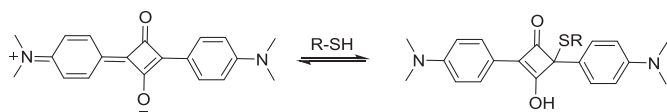
Martinez-Manez and coworkers demonstrated in 2002 that the electron deficient four-membered ring in a squaraine is susceptible to nucleophilic attack [16]. They used a squaraine as a cyanide probe in acetonitrile/water solutions buffered at pH 9.5. The

addition of a nucleophile to a squaraine (Scheme 1) breaks up the extended conjugation of the molecule, which switches off the distinctive absorbance and fluorescence of the molecule. Squaraines have been shown to be selective probes for thiols at physiological pH [17–28]. While the addition of thiols to squaraines is a well-known reaction, not all squaraines are susceptible to nucleophilic attack. Typically, the squaraines employed for addition are of the *N,N*-dialkylaniline type. Squaraines derived from indoles, benzothiazoles, and other heterocyclic compounds do not undergo nucleophilic attack and are stable in solution. Phenol-derived squaraines can be electrophilic if the phenol ring is substituted with electron withdrawing groups such as halogens [29]. For the aniline-derived squaraines, electron-donating hydroxyl groups *ortho* to the four-membered ring of the squaraine render the dye essentially non-electrophilic. [24,30]

Besides addition to thiols, squaraines bind to serum albumins (SAs). This has been investigated primarily for protein detection and non-covalent labeling [31–41], but also for imaging [42–44]. In these examples, when these squaraines bind to serum albumin, large increases in emission (10- to 200-fold) were observed and were accompanied by bathochromic shifts. The effect of serum albumin binding on the absorption spectra of squaraines is more variable. In the case of the 3-dicyanomethylene-substituted indolenine dye synthesized by Yarmoluk and coworkers, the absorption of the squaraine increased about three-fold in the presence of BSA [32]. However, Ramaiah and coworkers observed more complex absorbance behavior with their phloroglucinol-derived squaraine

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Scheme 1. Thiol addition to a squaraine.

[34]. The authors attributed this phenomenon to site selectivity of the binding. Similarly, Belfield and coworkers observed an initial decrease in absorption of their benzoindolenine-based squaraine upon addition of BSA, followed by an increase at higher equivalents of protein [42].

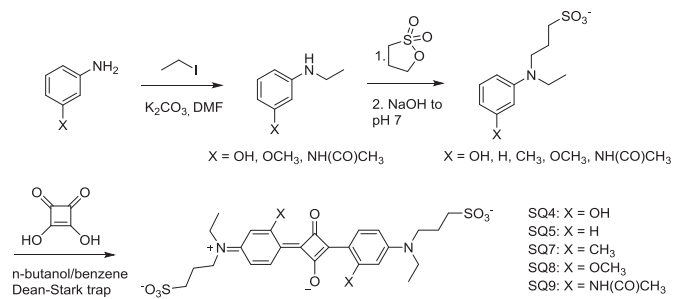
In this work, we synthesized water-soluble squaraines and used spectroscopic methods to study their thiol addition properties. We tested a series of aniline-derived, *ortho*-substituted squaraines to determine how different electron-donating groups affected the reactivity of the squaraines toward thiols. Although we expected to find that the reactivity would correlate directly with electron-donating ability of the *ortho* substituent, we instead found that the effect of the group is more complex than electronic factors alone. Furthermore, we investigated the effect of serum albumin on the reactivity of the squaraines toward thiols. Ramaiah and coworkers identified a protective effect on phloroglucinol-derived squaraines by β -cyclodextrin, although the cyclodextrin did not protect aniline-derived squaraines from thiol addition [29]. To this end, we first titrated the squaraines with serum albumin to investigate their binding behavior in the hydrophobic pockets of the protein. Then, we tested whether the presence of serum albumin would enhance or impede the thiol addition to the squaraines. We found that the presence of serum albumin generally enhanced thiol addition.

2. Results and discussion

2.1. Synthesis

The squaraines synthesized were all water soluble or able to be dissolved in water with a small amount (<10%) of acetonitrile, methanol, or dimethyl sulfoxide (DMSO). SQ1 [45], SQ2 [42], SQ3 [34,35], and SQ6 [20] (Fig. 1) were synthesized according to literature procedures.

We synthesized a series of squaraines with different substituents on the phenyl rings *ortho* to the cyclobutene ring (Scheme 2). To do so, the appropriate aniline derivative was monoalkylated and then sulfonated. Next, the squaraine was formed from the



Scheme 2. Synthesis of SQ4, 5, 7, 8, and 9.

aniline derivative and squaric acid in benzene/*n*-butanol via azeotropic removal of water. The procedure was based on a protocol described by Ghazarossian and coworkers to synthesize SQ4 [46]. We followed that procedure for the synthesis of SQ4 but modified it for the synthesis of the other squaraines. In that protocol, the sulfonated anilinium was used directly in the squaraine formation reaction along with sodium bicarbonate *in situ* to obtain the free base. The sodium bicarbonate interfered with the squaraine formation by deprotonating squaric acid (pK_a 1.5, 3.4) [47]. Thus, we obtained the free base of the sulfonated aniliniums by carefully adjusting an aqueous solution of the compound to pH 7 and then removing the water to isolate the sodium salt. The salt was used to form the squaraine. Furthermore, while Ghazarossian et al. successfully used ethylene glycol and DMSO to ameliorate the poor solubility of the sulfonated anilinium in benzene/butanol, we found that the presence of these solvents hindered the formation of squaraine rather than improving it. While their protocol yielded a small amount of SQ4, we could not make SQ5 in this manner.

2.2. Spectroscopic properties of the aniline-based squaraines

The absorbance spectra for SQ4, SQ5, SQ7, SQ8, and SQ9 are shown in Fig. 2, and the optical properties are summarized in Table S1. These compounds exhibit typical squaraine absorbance properties: bands between 500 and 700 nm and extinction coefficients near $10^5 \text{ M}^{-1} \text{ cm}^{-1}$. The squaraines are fluorescent in aqueous solution, but the intensity is relatively low. From the absorbance spectra, we can conclude that these squaraines exist as both monomers and dimers in solution [30,48,49]. The dimer peak is around 595 nm, while the monomer peak is around 650 nm. Only the monomer is emissive. These dimers are H-type aggregates,

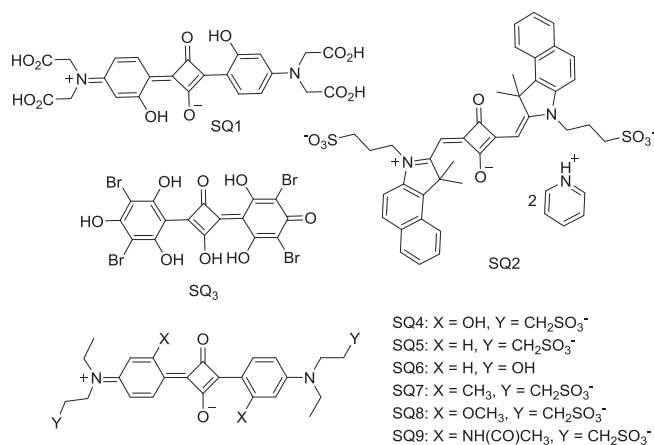


Fig. 1. Structures of squaraines (SQ1-SQ9).

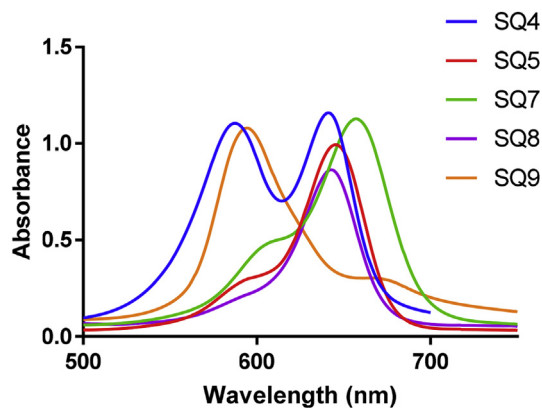


Fig. 2. Absorbance spectra of SQ4, 5, 7, 8, and 9. The squaraines (10 μM) were dissolved in 10 mM phosphate buffer, H₂O, pH 7.00, 0.02% NaNa₃. 4 = OH, 5 = H, 7 = CH₃, 8 = OCH₃, and 9 = NH(CO)CH₃.

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