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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.

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 ~ 750 cm^{-1}) [1] and large extinction coefficients ($\varepsilon \geq 10^5$ cm^{-1} 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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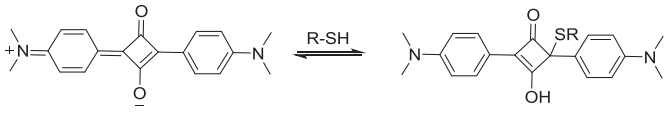


Scheme 1. Thiol addition to a squaraine.

[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 co-workers 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

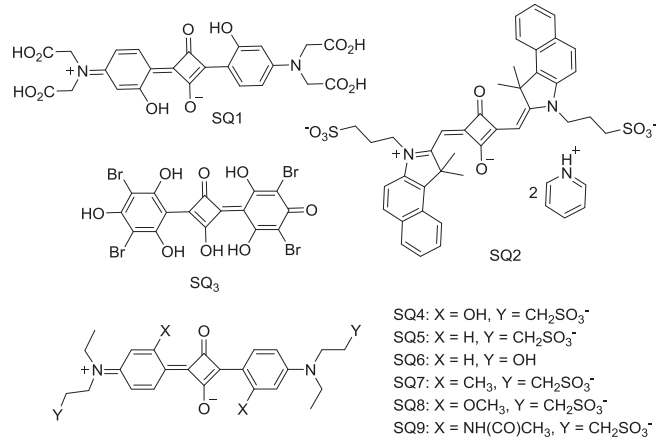
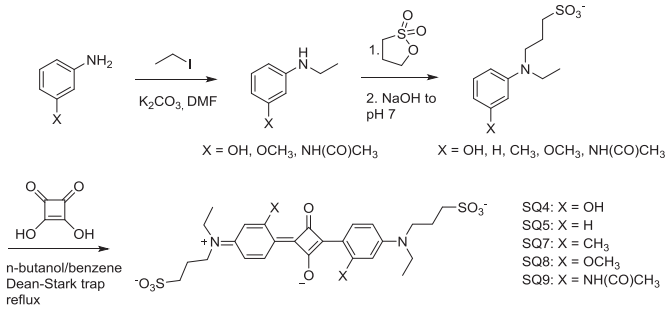


Fig. 1. Structures of squaraines (SQ1-SQ9).



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 anilinium 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 M^{-1} 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,

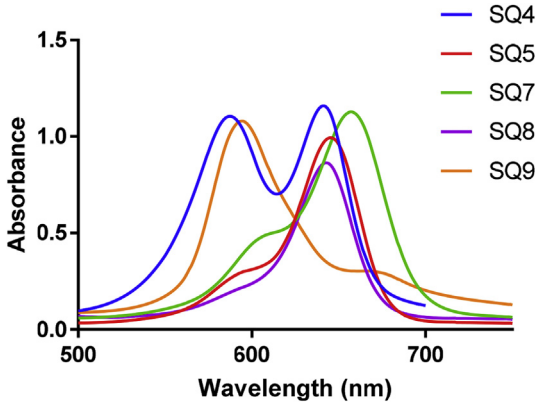


Fig. 2. Absorbance spectra of SQ4, 5, 7, 8, and 9. The squaraines (10 μM) were dissolved in 10 mM phosphate buffer, H_2O, pH 7.00, 0.02% NaN_3. 4 = OH, 5 = H, 7 = CH_3, 8 = OCH_3, and 9 = $NH(CO)CH_3$.

which are common for squaraines [3]. SQ5, SQ7, and SQ8 exist primarily as the monomer in aqueous solution. SQ4 is a mixture of the two forms, and SQ9 is mostly dimeric.

2.3. Thiol addition to squaraines

Evaluation of the addition of thiols to the fluorescent squaraines, was performed using *N*-acetylcysteine (NAC). Absorbance and fluorescence measurements were utilized to monitor thiol addition. The data for thiol addition to SQ2 (Fig. S1), SQ3 (Figs. S2 and S3), and SQ6 (Fig. S4) can be found in the SI.

2.3.1. SQ4, SQ5, SQ7, SQ8, and SQ9

We set out to investigate if we could tune the reactivity toward thiols through substitution at the *ortho* position on the benzene ring (X in Scheme 2). It has been demonstrated that squaraines with no *ortho* substitution (X = H, SQ5) will react readily with thiols. On the other hand, squaraines with a hydroxyl group in the *ortho* position (X = OH, SQ4) are essentially non-reactive to thiols, presumably because the hydroxyl group donates electron density to the squaraine core, decreasing electrophilicity. We hypothesized that placing other donating groups at the *ortho* position would result in intermediate reactivity between a hydrogen and a hydroxyl group. We synthesized squaraines with X = CH_3, OCH_3, and $NH(CO)CH_3$ (SQ7, SQ8, and SQ9) in order to test this hypothesis. NAC was titrated into each squaraine while monitoring the absorbance (Fig. 3 and Fig. S5-S9). From the isotherms, binding constants were calculated (Table 1).

From Fig. 3 and Table 1, it is clear that SQ5 and SQ7 react with thiols readily, while SQ8 and SQ9 do not react as readily. SQ4 is essentially not reactive, as expected. The reactivity of SQ7 appears to be slightly greater than for SQ5, which is unexpected because a methyl group is electron-donating. Furthermore, SQ8 is expected to be less reactive than SQ9 because a methoxy group is a better donor than an amide, but we observe the opposite order of reactivity. The observed trends do not agree well with Hammett σ values (Table S2) [50], although σ^+ values appear to be more appropriate than σ_{para}, which implies that resonance effects are more significant than inductive effects from the substituent. A methyl group does not provide resonance stabilization, which is consistent with the similar reactivity of SQ5 and SQ7. Furthermore, perhaps SQ7 is more reactive than SQ5 due to relief of steric strain between the methyl group and the oxygen when planarity is broken. The higher reactivity of SQ5 and SQ7 with thiols compared with SQ8 and SQ9 likely has a basis in a steric bulk effect. The larger methoxy and amide groups effectively block the site of thiol addition as compared with the minor steric hindrance of a methyl group or hydrogen, leading to stronger binding. While a methoxy group should be more donating than an amide group, it is possible that the monomer-dimer equilibrium for SQ9 can explain its extra stability compared to SQ8. SQ9 shows a strong tendency to dimerize compared to the other squaraines. A dimer would be less susceptible to nucleophilic attack statistically (i.e. two sides per two squaraines rather than two sides per one squaraine). Additionally, if the SQ9 dimer were more stable than or similar in stability to the SQ9-thiol complex, then the equilibrium would favor the SQ9 dimer. These additional factors could make σ values for substituents inapplicable in this case.

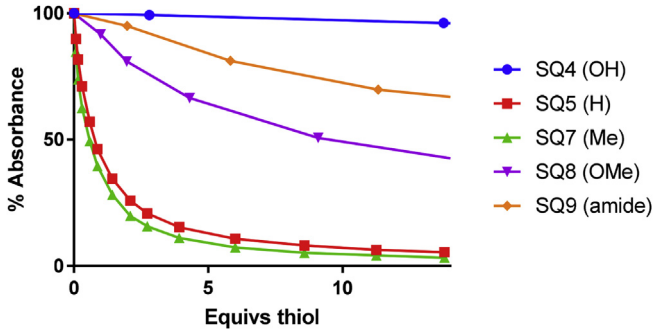


Fig. 3. Isotherms for thiol addition to SQ4, SQ5, SQ7, SQ8, and SQ9 overlaid. The decrease in absorbance for the monomer peak is described as a percentage of the initial absorbance of each squaraine. See Figs. S5–S9 for experimental details.

Table 1
Fitting of binding curves of NAC added to SQ to 1:1 regime.

Squaraine	Binding constant (M^{-1})	Relative standard error (%)	Covariance of fit	RMS of residuals
SQ7	8.57×10^5	9	0.017	0.032
SQ5	4.00×10^5	3	0.005	0.012
SQ8	1.92×10^4	11	0.003	0.006
SQ9	2.62×10^3	11	0.009	0.015
SQ4	6.44×10^2	12	0.038	0.019

2.3.2. ^{1}H NMR of thiol addition to squaraine

The desymmetrization of the squaraine upon thiol addition was observable by ^{1}H NMR (Figs. S10–S12). The ^{1}H NMR evidence is indicative of the reversibility of thiol addition to squaraines. After one equivalent of NAC was added, the solution decolored completely, and the ^{1}H NMR spectrum indicated complete conversion of SQ5 to SQ5-thiol. The SQ5-thiol peaks then diminished over time because the thiols were constantly leaving and re-adding to SQ5, and at the same time the free thiols in solution were also being oxidized to disulfides in a separate reaction. The thermodynamic sink for the system is the state in which the thiols are oxidized to disulfides and the squaraines are hydrolyzed.

2.4. Reactivity of squaraines to amines

The selectivity of squaraines for thiols over amines at pH 6 has been demonstrated by Ros-Lis et al. [17] However, we wanted to be sure that this selectivity was retained at pH 7. A large excess of Boc-α-lysine (175 equivalents of amine) was added to a solution of SQ7, the most reactive squaraine in our series (Fig. S13). The solution did not decolor, even after 1 h, indicating that at pH 7 amine nucleophiles are not reactive enough to add into our squaraines.

2.5. Squaraine binding to serum albumin (BSA)

We hypothesized that the squaraines would bind to serum albumin and that this binding would provide a positive or negative effect with respect to thiol addition to the dyes. In order to test the first part of this hypothesis, BSA was titrated into each solution of squaraine, while monitoring the absorbance and fluorescence. Furthermore, we used indicator displacement studies with dansyl amide (DNSA) and dansyl proline (DP) to probe the site-specificity of the binding of the squaraines. SQ2 [42] and SQ3 [34] have already been reported in the literature to bind to BSA., and the data for BSA binding to SQ1 (Fig. S14) and SQ6 (Fig. S15) can be found in the SI.

2.5.1. SQ4 binding to BSA

SQ4 dimerizes to a significant extent in aqueous solution (Fig. 2), and this monomer-dimer equilibrium leads to complex absorbance and fluorescence behavior of the squaraine upon addition of BSA (Fig. 4A and B). In the absorbance titration, the dimer peak at 585 nm consistently decreased, while a new peak (605 nm) grew in, which represents the bound dimer. The monomer peak first

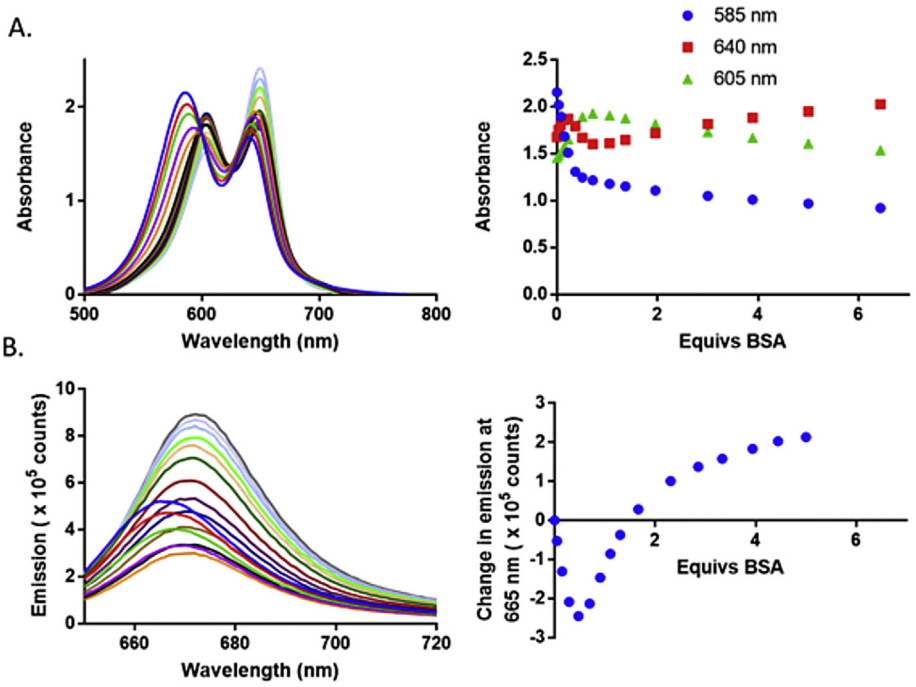


Fig. 4. A) Absorbance titration of BSA into SQ4: BSA (0-129 μM) to SQ4 (20 μM) in 10 mM phosphate buffer, H_2O, pH 7.00, 0.02% NaN_3. B) Fluorescence titration of BSA into SQ4: BSA (0-50 μM) to SQ4 (10 μM) in 10 mM phosphate buffer, H_2O, pH 7.00, 0.02% NaN_3, $\lambda_{ex} = 640$ nm.

increased up to about 0.2 equivalents of BSA, then decreased from 0.2 to 0.7 equivalents of BSA, and finally increased up to at least 6 equivalents of protein. The fluorescence titration also showed complex behavior: the emission decreased up to 0.5 equivalents of BSA and then increased. These results are indicative of a binding stoichiometry greater than 1:1, probably because both the monomer and dimer bind to serum albumin. Due to the complexity of the binding, we were unable to fit the data to obtain an association constant/s. As a comparison, we performed the same titration with Human serum albumin (HSA) (Fig. S16). Similar absorbance behavior to the addition of BSA was observed; however, small differences indicate that the binding of SQ4 is different to HSA than it is to BSA.

2.5.2. SQ5 binding to BSA

As BSA was added, the absorbance showed a decrease, while the fluorescence increased (Fig. 5A and B). We were puzzled by the observation of these apparently opposing phenomena. The absorbance decrease implies that the squaraine is becoming desymmetrized by reaction with a nucleophile, or the charge transfer band is otherwise being disrupted by steric or electronic factors upon binding. At the same time, the emission of the squaraine increased, which implies that binding improves the quantum yield of fluorescence. However, the squaraine was absorbing less light at the excitation wavelength, so the quantum yield increase must be quite large. For example, at three equivalents of BSA, the emission had increased by about five-fold, while the absorbance at the excitation wavelength had decreased by about five-fold. It is likely that we are observing the net effect of multiple factors. The data could be explained by some fraction of the SQ5 reacting with nucleophilic amino acids in the protein (e.g. lysine, cysteine), while at the same time some fraction of squaraine binds to the protein and experiences a large increase in its emission due to binding in the hydrophobic pocket/s.

Ajayaghosh and coworkers attribute the decrease in absorbance at 600-700 nm of their squaraine upon addition of serum albumin to reaction with the one free cysteine in BSA (Cys-34) [36]. To test this hypothesis in our system, we prepared a sample of BSA in which that cysteine had been blocked with iodoacetamide (c-BSA). The c-BSA was titrated into SQ5, and the binding isotherm was compared with that of BSA (Fig. S17). The two isotherms are nearly identical, which indicates that blocking the thiol does not significantly change the emission behavior of SQ5 in the presence of serum albumin. Of course, SQ5 could be reacting with other side chains. We found with SQ5 that nearly 200 equivalents of lysine did not decolorize the solution (Fig. S13), which indicates that amines will not react. However, the lysines in BSA have an average pK_a of 9.2 [51], and some individual lysines in the protein, particularly in the binding pocket, could have even lower pK_as.

To determine the binding stoichiometry, we used the method of continuous variation (Fig. S18). The Job plot indicates a 1:1 stoichiometry, which is consistent with the fluorescence binding isotherm. Thus, an association constant of 1.61×10^5 M^{-1} (±18%) was obtained from the data (Fig. 5A and B). To investigate which site SQ5 binds in, solutions of BSA with dansyl amide (DNSA, Site I-specific ligand) and with dansyl proline (DP, Site II-specific ligand) were titrated with SQ5 while measuring the emission of DNSA and DP (Fig. S19) [51]. Both probes experienced a decrease in their emission, which is indicative of their displacement from BSA. DP experienced a larger decrease than DNSA, and thus we concluded that SQ5 is primarily binding in Sudlow Site II, which is consistent with the results for SQ2 reported by Belfield et al. [42] Site II generally prefers aromatic molecules with peripherally-located negative charges [51], so it makes sense that these sulfonate-functionalized squaraines would bind in Site II.

Furthermore, a solution of BSA/SQ5 was titrated with DNSA and DP separately (Figs. S20–S23). We postulated that, if the absorbance behavior of SQ5 can be attributed to a non-covalent effect of being bound in the hydrophobic pocket, then displacing SQ5 would restore its absorbance and diminish its emission (Scenario 1). Conversely, if the absorbance behavior is due to SQ5 reacting with nucleophilic side chains of the protein, we would expect the probes

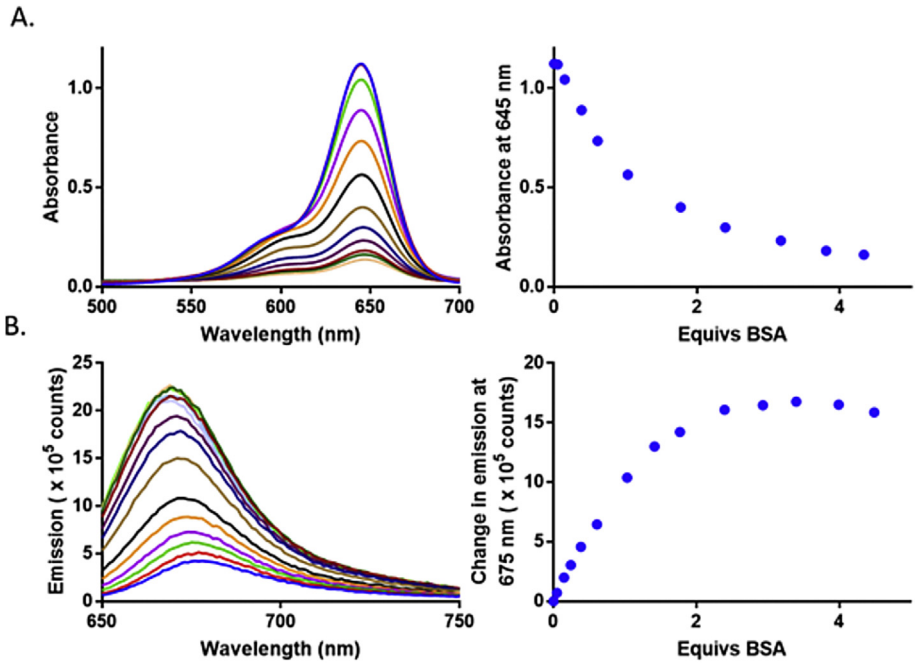


Fig. 5. A) Absorbance titration of BSA into SQ5: BSA (0-50 μM) to SQ5 (10 μM) in 10 mM phosphate buffer, H_2O, pH 7.00, 0.02% NaN_3. B) Fluorescence titration of BSA into SQ5: BSA (0-50 μM) to SQ5 (10 μM) in 10 mM phosphate buffer, H_2O, pH 7.00, 0.02% NaN_3, $\lambda_{ex} = 640$ nm.

to have no effect on this reaction, so the absorbance would not change (Scenario 2). While these were the scenarios we hypothesized, we did not observe either of these outcomes. The addition of DNSA slightly increased the emission of SQ5, which implies that DNSA has a small cooperative effect on SQ5 binding. From the other indicator displacement experiments (Fig. S19), we determined that SQ5 binds in Site II, so perhaps the binding of DNSA in Site I induces allosteric changes in BSA that render the Site II more hospitable to SQ5. The absorbance data echoed a cooperative effect by DNSA. The decrease in absorbance of SQ5 upon addition of DNSA suggests that the decrease observed upon SQ5 binding to BSA is not due to reaction with lysines, since DNSA would not affect that reaction. From both the absorbance and fluorescence data (Figs. S22 and S23), we observed that DP displaced SQ5 starting at about two equivalents, which is consistent with our Site II binding model and our Scenario 1 for indicator displacement. The increase in emission from 0 to 2 equivalents of DP matches with the decrease in the dimer peak in the absorbance spectrum. The presence of the DP appears to break up the dimer into monomer, which leads to the initial increase in emission upon addition of DP. These experiments provided insight into how SQ5 interacts with BSA; however, it is still unclear exactly to what phenomenon the decoloration of SQ5 in the presence of BSA can be attributed.

2.5.3. SQ7 binding to BSA

The evaluation of SQ7 again showed that absorbance decreased as BSA was added, while the emission increased to about 0.5 equivalents of BSA and then decreased (Fig. 6A and B). The shape is similar to the binding isotherm for SQ4, but with SQ7 the emission initially increased rather than decreasing as with SQ4. Thus, perhaps either the dimer or two separate monomers can bind to serum albumin. Due to the complexity of the binding, we were unable to fit the data to obtain an association constant.

2.5.4. SQ8 binding to BSA

The fluorescence and absorbance data for SQ8 showed features that were consistent with SQ5 (Fig. 7A and B). Fitting the data to a 1:1 binding regime yielded an association constant of 4.71×10^4 M^{-1} (±69%) from the absorbance and fluorescence titrations. Nevertheless, at < 0.2 equivalents of BSA, a small inflection is present in both isotherms. This phenomenon is due to a higher stoichiometry of binding occurring when very little BSA is present (e.g. dimer binding). Clearly, this effect was much smaller for SQ8 than for SQ7 or SQ4.

2.5.5. SQ9 binding to BSA

From SQ9's absorbance spectrum we found that it exists primarily as the dimer in solution (Fig. 8A and B). Perhaps the amide groups stabilize the dimer by hydrogen bonding, making it the dominant form for SQ9, but not for SQ5, SQ7, or SQ8. As BSA was added to SQ9, the dimer peak diminished and the monomer peak increased. This titration implies that the BSA only binds the monomer and so breaks up the dimer. Because SQ9 did not exhibit a decrease in its monomer peak upon BSA addition as did SQ5, SQ7, and SQ8, we used the same tests as we performed for SQ5 to probe the effect of displacement by DNSA and DP to compare to SQ5 (Figs. S24–27) and found a Site II binding model also applies to SQ9.

2.6. Thiol addition to squaraines in the presence of serum albumin

Finally, we measured whether the serum albumin exerts a protective or cooperative effect on thiol addition to squaraines. Solutions of BSA/SQ were titrated with thiol while monitoring the absorbance. In order to compare the effects of BSA on thiol addition to squaraine, the decrease in absorbance of the BSA/SQ solution was standardized by expressing it as the percent decrease of the initial absorbance (Fig. 9A and B). The original spectra and titration details for the experiments can be found in the SI (Figs. S28–S32) as well as plots comparing thiol addition to SQ and SQ/BSA for each dye (Figs. S33–S37). Table 2 shows the association constants calculated from the data. The order of reactivity from Fig. 3 was largely retained in the presence of serum albumin.

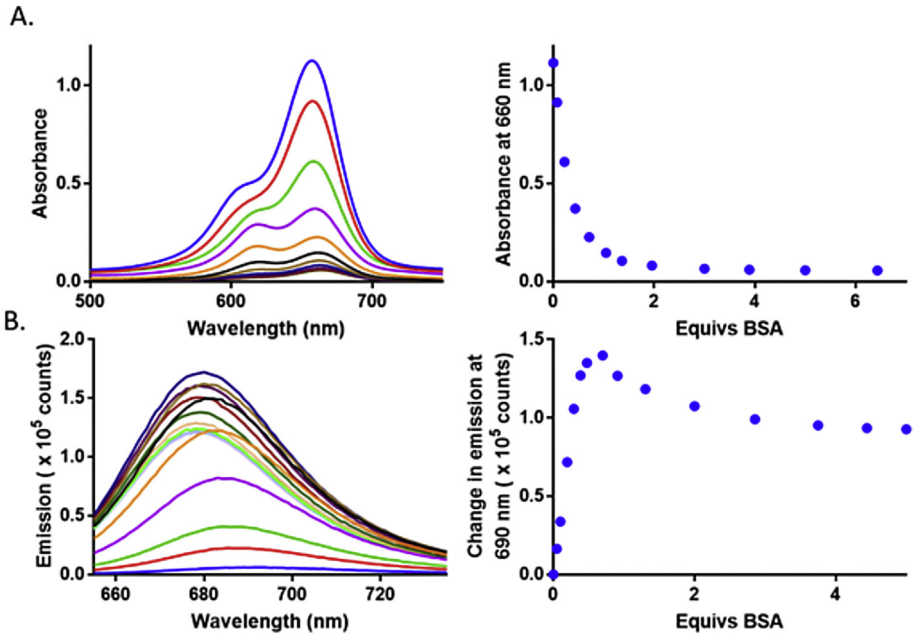


Fig. 6. A) Absorbance titration of BSA into SQ7: BSA (0-129 μM) to SQ7 (20 μM) in 10 mM phosphate buffer, H_2O, pH 7.00, 0.02% NaN_3. B) Fluorescence titration of BSA into SQ7: BSA (0-100 μM) to SQ7 (20 μM) in 10 mM phosphate buffer, H_2O, pH 7.00, 0.02% NaN_3, $\lambda_{ex} = 640$ nm, $\lambda_{em} = 690$ nm.

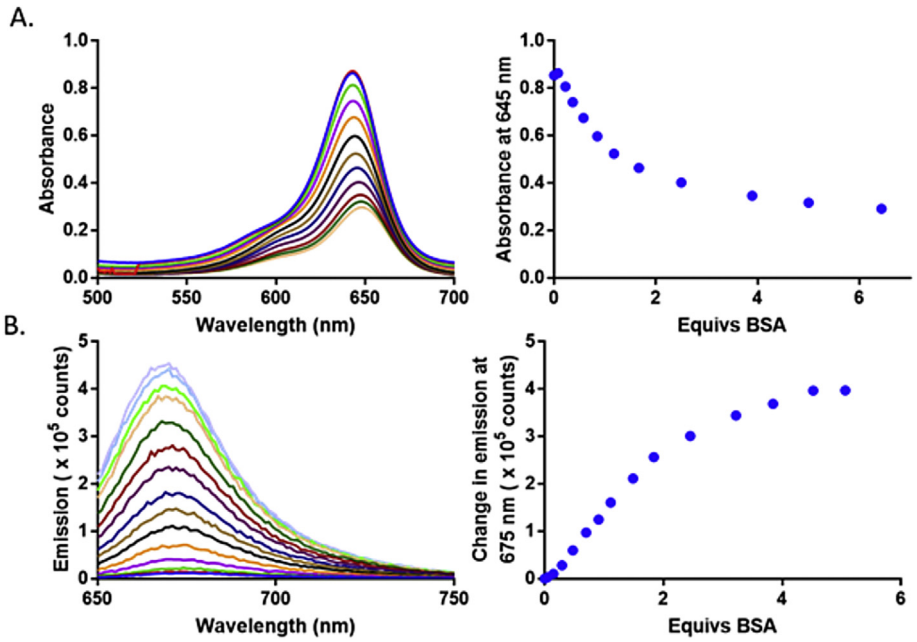


Fig. 7. A) Absorbance titration of BSA into SQ8: BSA (0-129 μM) to SQ8 (20 μM) in 10 mM phosphate buffer, H_2O, pH 7.00, 0.02% NaN_3. B) Fluorescence titration of BSA into SQ8: BSA (0-100 μM) to SQ8 (20 μM) in 10 mM phosphate buffer, H_2O, pH 7.00, 0.02% NaN_3, $\lambda_{ex} = 630$ nm, $\lambda_{em} = 675$ nm.

It was observed that BSA exerts a cooperative effect on thiol addition to the squaraines, and there are several possible mechanisms for this effect. The most clear-cut way for this to occur is that the SQ-thiol complex is bound more tightly by the protein than the squaraine alone, and this thermodynamically drives the thiol to add to the squaraine. However, because the equilibria involved in squarane dimerization, thiol addition, and SA binding are all interrelated (Scheme 3), we have considered two other possibilities. One alternative is that SA could have a cooperative effect by breaking up squaraine dimers that are resistent to thiol addition, thereby increasing the relative concentration of squaraine monomer, resulting in a larger amount of thiol addition. Another alternative is that the effect could be due to the squaraines reacting with nucleophilic side chains of the protein, which decreases the concentration of free squaraine in solution, showing the same resulting loss of fluoresence as would thiol addition. Thus, it would experimentally appear that less thiol is needed to bind the squaraine. However, the control studies described above indicate that the squaraine does not react with the SAs. Regardless of the cause, our results are consistent with those of Ramaiah and coworkers [29], in

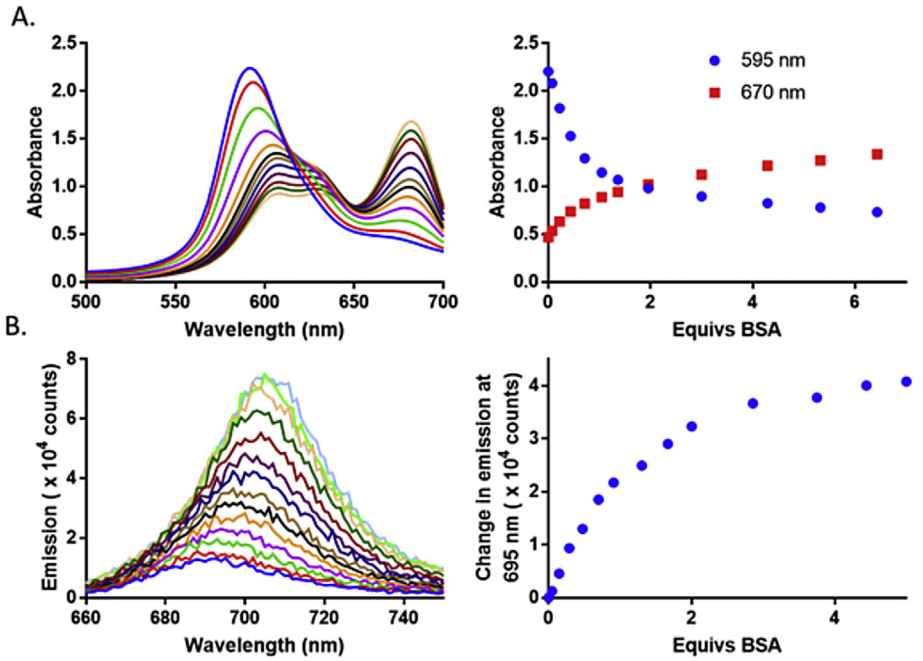


Fig. 8. A) Absorbance titration of BSA into SQ9: BSA (0-129 μM) to SQ9 (20 μM) in 10 mM phosphate buffer, H_2O, pH 7.00, 0.02% NaN_3. B) Fluorescence titration of BSA into SQ9: BSA (0-100 μM) to SQ9 (20 μM) in 10 mM phosphate buffer, H_2O, pH 7.00, 0.02% NaN_3, $\lambda_{ex} = 650$ nm, $\lambda_{em} = 695$ nm.

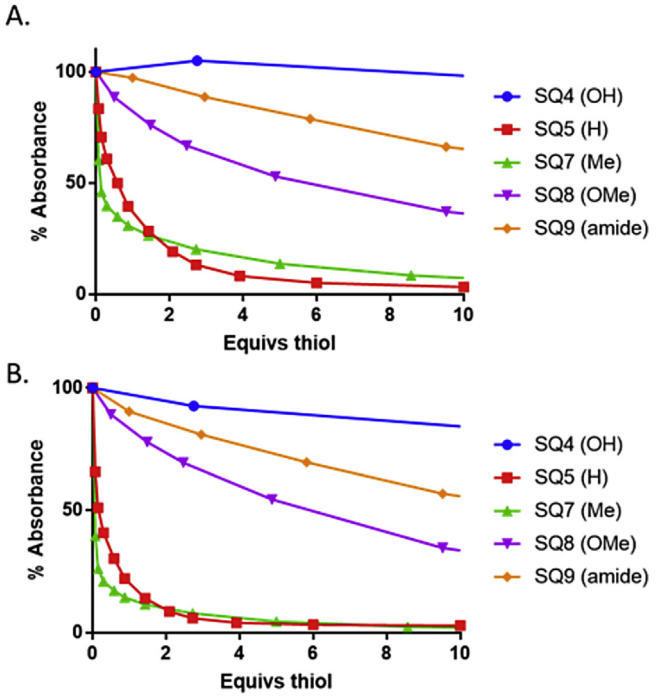
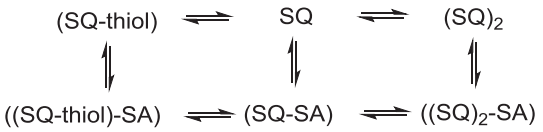


Fig. 9. A) Isotherms for *N*-acetylcysteine (NAC) addition to all of the SQ/BSA combinations overlaid (monomer). The decrease in absorbance for the monomer peak is described as a percentage of the initial absorbance of each squaraine. See Figs. S28–S32 for experimental details and original spectra. B) Isotherms for thiol addition to all of the SQ/BSA combinations overlaid (dimer). The decrease in absorbance for the dimer peak is described as a percentage of the initial absorbance of each squaraine. See Figures S28–S32 for experimental details and original spectra.



Scheme 3. Equilibria in a squaraine (SQ), thiol, and serum albumin (SA) system.

that they did not detect a protective effect of β-cyclodextrin on their aniline-derived squaraine, despite detecting binding. They did not find a cooperative effect by β-cyclodextrin either, which means that β-cyclodextrin does not influence the thiol addition reaction, unlike our study with BSA.

To elucidate among the possible mechanisms of the cooperativity with BSA, we performed indicator displacement experiments with DNSA and DP. A solution of BSA and DP (or DNSA) was titrated with SQ5 and an SQ5/NAC complex (Figs. S38 and S39). The emission of the DNSA or DP was monitored to measure displacement of these probes by the titrants. DP was displaced somewhat less by SQ5/NAC compared to SQ5 alone, while DNSA was displaced the same amount by all three titrants. These titrations indicate that the SQ5-thiol complex binds less well to serum albumin than SQ5 alone. Thus, we believe the mechanism involving perturbation of the squaraine dimerization by SA is the best explanation for the observed cooperativity in thiol addition. The thiol adds reversibly to the monomeric squaraine better than dimeric squaraine, and the squaraine is reversibly bound to BSA, an equilibrium which tends to prefer binding of the monomer. These two equilibria are functioning in parallel, and the increased fraction of monomeric squaraine leads to an increased amount of thiol addition. This mechanism is further supported by the finding that thiol addition to SQ8, which exists almost exclusively as the monomer in solution (Fig. 2), is essentially unaffected by the presence of SA, while the other squaraines exhibit 2- to 4-fold increases in their thiol affinity in the presence of SA (Table 2).

3. Conclusions

Squaraines are organic chromophores with absorption and emission in the red to near-IR region of the spectrum, making them

Table 2
Fitting of binding curves of NAC added to BSA/SQ to 1:1 regime.

Squaraine	Binding constant (M^{-1})	Relative standard error (%)	Covariance of fit	RMS of residuals
SQ7	2.64×10^6	14	0.017	0.010
SQ5	1.60×10^6	22	0.043	0.019
SQ8	2.15×10^4	11	0.002	0.006
SQ9	7.18×10^3	11	0.003	0.009
SQ4	1.35×10^3	11	0.007	0.015

useful dyes for a variety of applications. We synthesized a series of water-soluble squaraines and investigated tuning their reactivity toward thiols using variable *ortho* substitution and the addition of serum albumin. Aniline-derived squaraines with no *ortho* substitution are very reactive with thiols and other nucleophiles to the point that they are unstable in aqueous solution. On the other hand, squaraines with an *ortho* hydroxyl group are inert to nucleophiles. We hypothesized that we could tune access to intermediate reactivity with other donating groups in the *ortho* position. We found that this substitution did change the reactivity of the squaraine, and we achieved intermediate reactivity between the H-substituted squaraine and the OH-substituted squaraine, although the specific order of reactivity did not conform to σ values for these groups. We were able to rationalize these inconsistencies based on additional factors for the squaraines that the σ values would not take into account. Next, we investigated the binding of these squaraines to serum albumin as a mechanism by which the protein could encourage or inhibit thiol addition to the dyes. The sulfonated squaraines exhibit an increase in their emission upon binding to BSA. Some of the squaraines bind with greater than 1:1 stoichiometry to BSA, which could be due to the dimer binding in one site or multiple monomers in different sites. The dominant binding site for these squaraines seems to be Sudlow Site II, as evidenced by indicator displacement titrations with DNSA and DP. We detected a cooperative effect of the serum albumin on the thiol addition to the squaraines, which we attribute to perturbation of the squaraine monomer-dimer equilibrium by serum albumin.

4. Experimental section

General: Unless otherwise indicated, chemicals and reagents were obtained from Sigma Aldrich and used without further purification. Dansyl amide and dansyl proline piperidinium salt were obtained from TCI America. The fluorescence experiments were performed with a PTI fluorimeter using an 814 photomultiplier detection system and a 75W xenon short arc lamp. The absorbance experiments were performed with a Beckman Coulter DU 800 Spectrophotometer and an Agilent Cary 100 UV-VIS. The binding constants were calculated using Open Data Fit by the Centre for Bio-Nano Science (CBNS) at http://supramolecular.org/.

4.1. Synthesis

The syntheses for SQ1, SQ2, SQ3, and SQ6 were already reported in the literature as cited above. For the squaraines that had not previously been synthesized, ^{1}H NMR and high-resolution MS data were obtained. These squaraines have relatively low solubility in any solvent (<1 mM) and are unstable in their best solvent (water), decomposing over the course of several hours. Thus, it was not possible to obtain ^{13}C NMR data due to the high concentrations and long scan times necessary.

4.2. SQ4 [46]

To an oven-dried flask was added squaric acid (103 mg, 0.9 mmol, 1 eq), 3-(((3-hydroxyphenyl)(ethyl)ammonio)propane-1-sulfonate) (490 mg, 1.89 mmol, 2.1 eq), sodium bicarbonate (159 mg, 1.89 mmol, 2.1 eq), 10 mL butanol, 5 mL benzene, and 3 mL DMSO. The reaction was refluxed for 3 h. Ether was added to precipitate the product. The product was very wet, so it was taken up in methanol. The methanol was filtered to remove solid, and then the methanol was removed under reduced pressure to give a green solid. The crude product was purified by RP-HPLC in water/acetonitrile with 0.1% TFA. The product was a green iridescent solid (7.4 mg, 1.4% yield). MS (ESI): 596.2, 297.1 (M-1). HRMS (ESI): expected *m*/*z* 297.07, found *m*/*z* 297.07 ((M-1)/2); expected *m*/*z* 617.12, found *m*/*z* 617.13 (M-1).

4.3. SQ5

To an oven-dried flask was added squaric acid (64 mg, 0.6 mmol, 1 eq), sodium 3-(ethyl(phenyl)amino)propane-1-sulfonate [52] (303 mg, 1.2 mmol, 2.1 eq), 16 mL benzene, and 8 mL n-butanol. A Dean Stark trap was attached, and the reaction was refluxed at 100 °C for 30 h. The reaction was filtered, and the blue solid was washed with isopropanol. The crude product was purified on a C-18 RediSep column with a CombiFlash instrument eluting with water/acetonitrile. The product was a purple solid (5.51 mg, 1.6% yield). ^{1}H NMR (400 MHz, *d*-DMSO, ppm): δ 1.13 (t, 6H, CH_3), 1.85 (m, 4H, CH_2), 2.45 (m, 4H, CH_2), 3.53 (m, 4H, CH_2), 3.60 (t, 4H CH_2), 6.98 (d, 4H, Ar-H), 8.06 (d, 4H, Ar-H). LRMS (ESI): 281.2 ((M-1)/2). HRMS (ESI): expected *m*/*z* 281.07, found *m*/*z* 281.07 ((M-1)/2); expected *m*/*z* 585.13, found *m*/*z* 585.14 (M-1).

4.4. SQ7

To an oven-dried flask was added squaric acid (100 mg, 0.9 mmol, 1 eq), sodium 3-(ethyl(*m*-tolyl)amino)propane-1-sulfonate [52] (489 mg, 1.8 mmol, 2 eq), 16 mL benzene, and 8 mL n-butanol. A Dean-Stark trap was attached, and the reaction was refluxed at 100 °C for 24 h. The solvent was removed under reduced pressure. The crude product was purified on a C-18 RediSep column with a CombiFlash instrument eluting with water/acetonitrile. The product was a purple solid (6.8 mg, 1.2% yield). ^{1}H NMR (400 MHz, *d*-DMSO, ppm): δ 1.15 (t, 6H, CH_3), 1.88 (m, 4H, CH_2), 2.45 (m, 4H, CH_2), 2.76 (s, 6H, CH_3), 3.53 (m, 4H, CH_2), 3.58 (t, 4H CH_2), 6.77 (d, 2H, Ar-H), 7.09 (m, 2H, Ar-H), 8.06 (d, 2H, Ar-H). LRMS (ESI): 295.1 ((M-1)/2), 591.1 (M-1). HRMS (ESI): expected *m*/*z* 295.09, found *m*/*z* 295.09 ((M-1)/2); expected *m*/*z* 613.17, found *m*/*z* 613.17 ((M-1)+Na).

4.5. SQ8

To an oven-dried flask was added squaric acid (70 mg, 0.61 mmol, 1 eq), sodium 3-(ethyl(3-methoxyphenyl)amino)propane-1-sulfonate [52] (381 mg, 1.29 mmol, 2.1 eq), 10 mL benzene, and 5 mL n-butanol. A Dean-Stark trap was attached, and the reaction was refluxed at 100 °C for 48 h. The reaction mixture was filtered to collect the product, washing with ethyl acetate and allowing to dry thoroughly. The crude product was purified on a C-18 RediSep column with a CombiFlash instrument eluting with water/acetonitrile. The product was a purple solid (26.5 mg, 6% yield). ^{1}H NMR (400 MHz, *d*-DMSO, ppm): δ 1.12 (t, 6H, CH_3), 1.82 (m, 4H, CH_2), 2.45 (m, 4H, CH_2), 3.50 (m, 4H, CH_2), 3.65 (t, 4H CH_2), 3.83 (s, 6H, CH_3), 6.35 (s, 2H, Ar-H), 6.51 (d, 2H, Ar-H), 8.53 (d, 2H, Ar-H). LRMS (ESI): 311.2 ((M-1)/2), 623.2 (M-1). HRMS (ESI): expected *m*/*z* 311.08, found *m*/*z* 311.08 ((M-1)/2); expected *m*/*z* 645.16, found *m*/*z* 645.15 ((M-1)+Na).

4.6. Sodium 3-((3-acetamidophenyl)(ethyl)amino)propane-1-sulfonate

To a flask was added *N*-(3-(ethylamino)phenyl)acetamide [53] (0.5 g, 2.8 mmol, 1 eq), 1,3-propanesultone (0.3 mL, 3.4 mmol, 1.2 eq), and 10 mL acetonitrile. The reaction was refluxed for 18 h. The reaction mixture was concentrated. Water was added to the residue, and the solution was neutralized with 1M NaOH (aq). The solution was washed with DCM and then lyophilized. The product was a white solid (620 mg, 68% yield). ^{1}H NMR (400 MHz, d-DMSO, ppm): δ 1.02 (t, 3H, CH_3), 1.74 (m, 2H, CH_2), 1.96 (s, 3H, CH_3), 2.35 (m, 2H, CH_2), 3.23 (m, 4H, CH_2), 6.33 (d, 1H, Ar-H), 6.84 (m, 2H, Ar-H), 6.97 (t, 1H, Ar-H), 9.65 (s, 1H, NH). ^{13}C NMR (400 MHz, *d*-DMSO, ppm): δ 11.09 (CH_3), 22.55 (CH_3), 22.68 (CH_2), 27.82 (CH_2), 48.67 (CH_2), 60.41 (CH_2), 104.06 (Ar-C), 107.75 (Ar-C), 108.57 (Ar-C), 129.07 (Ar-C), 139.37 (Ar-C), 148.02 (Ar-C), 170.18 (C═O). LRMS (ESI): 299.2 (M-1). HRMS (ESI): expected *m*/*z* 299.11, found *m*/*z* 299.11 (M-1).

4.7. SQ9

To an oven-dried flask was added squaric acid (70 mg, 0.61 mmol, 1 eq), sodium 3-((3-acetamidophenyl)(ethyl)amino) propane-1-sulfonate (416 mg, 1.3 mmol, 2.1 eq), 5 mL benzene, and 5 mL n-butanol. A Dean-Stark trap was attached, and the reaction was refluxed at 100 °C for 48 h. The reaction mixture was filtered to collect the product, washing with ethyl acetate and isopropanol and allowing to dry thoroughly. The product was a green solid and required no further purification (212 mg, 49% yield). ^{1}H NMR (400 MHz, *d*-DMSO, ppm): δ 1.24 (t, 6H, CH_3), 1.86 (m, 4H, CH_2), 2.17 (s, 3H, CH_3), 2.39 (m, 4H, CH_2), 3.36 (m, 4H, CH_2), 3.55 (m, 4H CH_2), 6.76 (d, 2H, Ar-H), 8.06 (s, 2H, Ar-H), 8.28 (d, 2H, Ar-H) 11.94 (s, 1H, NH). LRMS (ESI): 338.2 ((M-1)/2). HRMS (ESI): expected *m*/*z* 338.09, found *m*/*z* 338.09 ((M-1)/2); expected *m*/*z* 699.18, found *m*/*z* 699.18 ((M-1)+Na).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dyepig.2016.11.060.

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