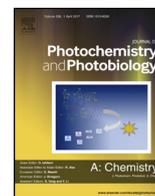




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Invited feature article

## On the origin of chloride-induced emission enhancement in ortho substituted squaramides



Ashwini Danao<sup>a</sup>, Vijayakumar Ramalingam<sup>b</sup>, Vaidhyanathan Ramamurthy<sup>a</sup>,  
Rajeev S. Muthyala<sup>c,\*</sup>

<sup>a</sup> Department of Chemistry, University of Miami, Coral Gables, FL 33146, USA<sup>b</sup> Department of Chemistry, Union College, Schenectady, NY 12308, USA<sup>c</sup> Center for Learning Innovation, University of Minnesota Rochester, 111 S. Broadway, Rochester, MN 55904, USA

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## ABSTRACT

There is significant current interest in the development of fluorescent sensors for the physiologically relevant chloride ion. In this study we report a new squaramide-based chloride sensor, derived from ethyl ortho aminobenzoate, whose emission intensity increases by nearly 40% upon chloride binding. The intramolecularly hydrogen bonded squaramide exhibited a blue-shifted excitation spectrum relative to its absorption spectrum, and its emission displayed excitation wavelength dependence. These unusual characteristics were attributed to the existence of two ground state conformers for the unbound squaramide; an intramolecular hydrogen bonded conformer which undergoes non-radiative decay and a non-hydrogen bonded non-planar conformer from which emission occurs. Using computational investigations we identified two nearly degenerate excited states arising from two different charge transfer pathways with the squaramide nitrogen atom as the donor and either the cyclobutene dione ring or the ortho substituted N-phenyl ring functioning as acceptors. In the unbound squaramide, this competition between the two charge transfer pathways decreases fluorescence intensity. Chloride binding, suppresses charge transfer into the cyclobutene dione ring thereby enhancing emission intensity. Our study contributes to an increased understanding of the excited states of squaramides and could facilitate their use in the development of new sensors.

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### 1. Introduction

Owing to the physiological importance of chloride ions [1], there is a growing need for new imaging agents to measure intracellular chloride concentration [2–12]. Currently used methods [4,13,14] have two significant disadvantages: first, there is no specific anion recognition site [15] and second, chloride binding induces fluorescence quenching which makes imaging difficult. Therefore, there is a need for new fluorophores with a specific anion binding site and whose sensing mechanism relies on an increase, rather than quenching, of the fluorescence.

We recently reported<sup>16</sup> a chloride sensing strategy in which the fluorescence intensity of ortho benzoyl squaramides increases, relative to the unbound receptor, upon binding chloride in acetonitrile. We proposed that the enhancement of fluorescence intensity is due to suppression of radiationless decay mediated by

proton transfer via intramolecular hydrogen bonding [17–20]. However, because the squaramide was derived from ortho aminobenzophenone which is a rarely used fluorophore, the origin of the emission was not clear. Therefore, we sought to prepare new squaramide sensors derived from well-known fluorophores. In this connection, we discovered chloride-induced enhancement of emission intensity in a squaramide derived from the widely used fluorophore – ortho aminobenzoate (henceforth referred to as anthranilate) [21–25]. We describe here the results of our study, combining experimental and computational investigations, aimed at uncovering the origin of the emission of the free squaramide receptor and the underlying reason for fluorescence enhancement in the chloride-bound form.

From a literature survey, it was apparent that little, if anything, is known about the excited states of squaramides. Not surprisingly, the use of squaramides in the design of fluorescent sensors has been sparse [8,26–29] despite widespread exploitation of their strong hydrogen bond donating ability [30] in anion supramolecular chemistry [31,32], and in a wide variety of other fields [33–35]. We anticipate that the findings from our study will facilitate the

\* Corresponding author.

E-mail address: [muthy004@r.umn.edu](mailto:muthy004@r.umn.edu) (R.S. Muthyala).

development of new squaramide-based fluorescent chloride sensors for intracellular imaging of chloride [36].

## 2. Materials and methods

Fluorescence emission and excitation spectra were recorded on an Edinburgh FS920CDT steady-state fluorimeter. A  $5 \times 10^{-4}$  M stock solution of the squaramides and  $5 \times 10^{-3}$  M tetrabutyl ammonium chloride (TBACl) were prepared in acetonitrile. Absorption spectra of  $5 \times 10^{-6}$  M squaramides were recorded before and after addition of 100 fold TBACl. Emission spectra were recorded in a quartz cell with excitation wavelength set at 320 nm. TBACl was added to the solution of squaramides gradually with the help of microsyringe and mixed for 2 min before recording emission and excitation spectra. All spectral measurements were carried out under ambient conditions. The samples were not degassed to exclude air.

### 2.1. Preparation of diethyl 2, 2'-(3, 4-dioxocyclobut-1-ene-1, 2-diyl) bis (azanediyl) dibenzoate (1b)

Diethyl squarate (0.170 g, 1 mmol) in 5 ml ethanol was cooled to 5 °C and Ethyl 2-amino benzoate (0.661 g, 4 mmol) was added to it. The reaction mixture was stirred at 60 °C for 18 h. The precipitated solid was filtered and washed with diethyl ether (2 × 100 ml) and dried under vacuum to get a light yellow solid obtained in 30% yield. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 11.38 (2H, s), 8.25(2H, d, J = 8 Hz), 8.09(2H, d, J = 8 Hz), 7.65(2H, t, J = 7.8 Hz), 7.1(2H, t, J = 8.0), 4.48(4H, q, J = 6.8 Hz), 1.47(6H, t, J = 7.2 Hz) ppm [13]. C NMR (CDCl<sub>3</sub>): δ 183.1, 168.6, 166.28, 141.0, 135.1, 131.1, 122.8, 119.9, 114.9, 61.8, 14.1 ppm HRMS calculated for C<sub>22</sub>H<sub>20</sub>N<sub>2</sub>O<sub>6</sub> (M<sup>+</sup>) = 408.1321 found (M+H) 409.1394. mp: 176–178 °C

### 2.2. Computational work

All calculations were performed in Spartan 14 (Wavefunction, Inc.) in vacuum. Geometry optimization of the ground states of squaramide **1a**, **1b** and **2** and acquisition of the calculated absorption spectra were carried out using density functional theory (DFT) with the B3LYP functional and 6-31G\* basis. Calculations using higher order basis sets such as 6-31G\*\* or 6-31G+\*\* yielded nearly identical results but took significantly longer to complete. For geometry optimization of the supramolecular complex between squaramide **1b** and chloride ion, a distance constraint of 3.1 Å between the squaramide N and Cl<sup>-</sup> ion was applied based on experimental data from reported crystal structures [37].

## 3. Results and discussion

Compounds **1a** and **2** were prepared as reported earlier [38]. Squaramide **1b**, hitherto unreported, was synthesized in a manner similar to compounds **1a** and **2** from diethyl squarate and ethyl anthranilate (**A1b**). A relatively strong intramolecular hydrogen bond in the ground state of **1b** was suggested by the deshielding of its NH signal (δ<sub>NH</sub> in CDCl<sub>3</sub> 11.87 ppm) which was more downfield compared to the *o*-benzoyl derivative **1a** (δ<sub>NH</sub> in CDCl<sub>3</sub> 11.49 ppm). In the case of compound **2**, the NH resonance is significantly upfield (δ<sub>NH</sub> in CDCl<sub>3</sub> 10.40 ppm) indicating a weaker intramolecular hydrogen bond [38,39] compared to **1a** or **1b**. Therefore the compounds listed in Table 1 are of varying intramolecular hydrogen bond strength.

The absorption and emission characteristics, in acetonitrile, of the compounds used in this study are summarized in Table 1. The absorption maximum of squaramide **1b** was red-shifted compared to ethyl anthranilate (**A1b** in Scheme 1), the underivatized

**Table 1**

Absorption and emission data of squaramides in acetonitrile.

Compound	λ <sub>abs</sub> (nm)	λ <sub>exc</sub> (nm)	λ <sub>em</sub> (nm)	Stokes Shift (nm)
<b>1a</b>	388	358	440	52 nm
<b>1b</b>	356	335	393	37 nm
<b>2</b>	417	415	490	73 nm

fluorophore, indicating extended delocalization via the vinylogous amide in the cyclobutene dione ring [29]. However, the absorption maximum of squaramide **2**, with its weaker intramolecular hydrogen bond, was nearly the same as that of 1-aminofluorenone (**A2** in Scheme 1, λ<sub>max</sub> 419) [40] from which it is derived.

In acetonitrile, squaramide **1b** exhibited fluorescence (Fig. 1) with an emission maximum of 393 nm. The Stokes shift was similar in magnitude to our earlier reported [16] *ortho* benzoyl derivative **1a** and is consistent with emission primarily from the locally excited (LE) state [41]. The emission was weakly dependent on solvent polarity (see Supporting information, Fig. S2) unlike the underivatized anthranilate esters [23,24]. For example, the emission maximum of squaramide **1b** in the non-polar cyclohexane and the relatively polar acetonitrile was identical (λ<sub>em</sub> 393 nm) whereas the emission maximum of anthranilate esters has been reported to be 380 nm in cyclohexane and 391 nm in acetonitrile [23]. In the hydrogen bond-disrupting solvent methanol, the emission was red-shifted relative to acetonitrile but the emission maximum (λ<sub>em</sub> 406 nm) was again identical to underivatized anthranilate esters (λ<sub>em</sub> 405 nm) in alcohol solvents [24]. Thus, while the fluorescence characteristics of squaramide **1b** are strikingly similar to underivatized ethyl anthranilate, its absorption spectral data suggest delocalization into the cyclobutene dione ring.

To address the seemingly divergent absorption and emission spectral data we examined the fluorescence excitation maximum of **1b** in acetonitrile and found that it was blue-shifted relative to the absorption maximum. Only the short wavelength part of the main absorption band was reproduced in the fluorescence excitation spectrum (Fig. 1). The excitation maximum of squaramide **1b** was the same regardless of solvent polarity and was identical to that of ethyl anthranilate. The emission of **1b** was also dependent on the excitation wavelength (see Supporting information, Fig. S6). When excited at the absorption maximum or at longer wavelengths, the compound was non-fluorescent but emission was observed when the sample was excited at wavelengths shorter than the absorption maximum.

The blue-shifting of the excitation spectra and the excitation wavelength dependence is likely attributable to two different ground state conformers: an intramolecularly hydrogen bonded conformer which undergoes radiationless decay, as for example, in ultraviolet stabilizers such as Tinuvin P [42], and a non-hydrogen bonded, and possibly non-planar (Note: There is literature precedence for the attribution of blue shifting of excitation spectra to non-hydrogen bonded or twisted ground state conformers [43,44]) conformer which is fluorescent. Evidence in support of this claim comes from: (i) the observation that the emission of **1b** in the intramolecular hydrogen bond-disrupting methanol was significantly stronger compared to chloroform, cyclohexane or acetonitrile (see Supporting information, Fig. S3). The fact that the blue shifting of the excitation spectrum of squaramide **1b**, relative to its absorption spectrum, persists even in methanol suggests ground state equilibrium between intramolecularly hydrogen-bonded as well as intermolecularly hydrogen-bonded conformers. (ii) Weakening the intramolecular hydrogen bond strength, as in squaramide **2**, leads to its excitation maximum being nearly identical to its absorption maximum. This latter observation also

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