Computational and Experimental Characterization of a Fluorescent Dye for Detection of Potassium Ion Concentration

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Abstract

The fluorescence of the SKC-417 (N-(9-(4-(1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl)phenyl)-6-(dimethylamino)-3H-xanthen-3-ylidene)-N-methylmethanaminium) dye is shown to have high sensitivity to binding of K^+ ion. Computations are used to explore the potential origins of this sensitivity and to make some suggestions regarding structural improvements. The lowest-energy excitation is associated with two nearly degenerate excited states, one of which is optically bright, while the other is optically dark. The sensitivity of the quantum yield to the ion's presence can be attributed to an inversion of the bright and dark states which, according to Kasha's rule, would convert the non-fluorescent to a fluorescent molecule. The near degeneracy of these excited states can be understood by considering SKC-417 as a reduced symmetry version of a parent molecule with three-fold symmetry. Computations show that acceptor-donor substituents can be used to alter the relative energies of the bright and dark states, and potentially enhance fluorescence sensitivity to the presence of an ion. Lengthening the bridge by one methylene group (between the heterocycle and phenylene group of SKC-417) enhances coupling between the bright and dark excited states, which could enhance sensitivity as well.

Keywords: Fluorescent probes, photoinduced-electron-transfer sensors

I Introduction

Florescence imaging provides a set of powerful techniques for monitoring biological processes in living organisms[1–6]. These techniques rely on dye molecules that change their fluorescence behavior under varying environments. For neural processes, it is useful to have dyes whose fluorescence tracks the concentration of K^+ ion. The experimental data presented below for the dye SKC-417 shows a substantial change in quantum yield as K^+ concentration varies between 0 and 1000 ppm. The dye has a crown ether portion that binds the potassium ion, and a chromophore that signals the presence of the ion. We use quantum chemical calculations to explore the mechanism through which the ion alters the fluorescence of the chromophore.

Computations show that SKC-417 is a photoinduced electron transfer (PET) sensors, a class of dyes whose fluorescence quantum yield is known to be sensitive to ion binding[4–6]. In these dyes, the change in quantum yield is attributed to the competition between a radiative pathway (emission from the neutral excited state created on photoexcitation) and a non-radiative pathway, which is mediated by a charge-separated excited state. Ion binding stabilizes the charge-separated state and alters the branching ratio between the radiative and non-radiative pathways. Our computations show that this mechanism is active in SKC-417. The lowest-energy excitations of SKC-417 correspond to a fluorescent state and a dark charge-transfer state. The potassium ion preferentially destabilizes the charge-transfer state, thereby altering the quantum yield, consistent with the mechanism implicated in other PET sensors.

A near degeneracy of the fluorescent and charge-transfer states of SKC-417 contributes to the sensitivity of ion binding. In fact, our calculations suggest that the charge-transfer state lies

below the fluorescent state in the absence of potassium, and rises above the fluorescent state with ion binding. Kasha's rule[7] suggests that a reversal of bright and dark states is expected to have a large impact on the quantum yield. SKC-417 has only a two-fold symmetry axis, such that this degeneracy appears to be accidental. However, comparison to a three-fold symmetric parent reveals a systematic origin. The three-fold symmetry of the parent molecule leads to a doubly-degenerate lowest excited state. In addition, the molecular orbitals involved in these degenerate excitations have very low density in parts of the molecule that must be altered to convert the parent molecule to SKC-417. The degeneracy of excited states in SKC-417 is therefore not accidental, but can be traced back to degeneracies expected for this three-fold symmetric parent.

Our calculations also find that replacing the potassium ion with a point charge has little effect on the nature of the relevant excited states. This suggests that changes in the excited states of the chromophore in SKC-417 arise from field effects of the potassium ion, as opposed to more specific interactions (such as ligation with the nitrogen atom that connects the crown ether to the chromophore).

II Synthesis of SKC-417

The procedure (Figure 1) for the synthesis of SKC-417 is:

Synthesis of 16-phenyl-1,4,7,10,13-pentaoxa-16-azacyclooctadecane (1):

In a 2 L 3-neck round bottom flask fitted with a reflux condenser, 8 g NaH (60% in mineral oil, 2 mmol) was added to 400 mL anhydrous THF and refluxed under argon. 18.17g of N-phenyldiethanolamine (1 mmol) and 50.631g of tetra-(ethylene glycol)-di–p-tosylate (1 mmol) dissolved in 400 ml anhydrous THF were added to the refluxed NaH solution over a period of 8

hours and refluxed for another 24 hours. After cooling and filtering the reaction mass, the solid was washed with 100 mL of THF. The combined organic filtrate was concentrated and the oily residue was subjected to silica gel column chromatography using diethyl ether as eluent, providing a light yellow oil (10.361g, 31% yield) as product.

^{1}H NMR: δ (CDCl$_3$): 3.702 (m, 24H), 6.697 (m, 2H), 7.22 (m, 1H), 7.37 (m, 1H), 7.805 (d, 1H); EI MS: m/z 339.73 (M+)

Synthesis of 4-(1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl)benzaldehyde (2):

Compound (1) (3.414g) (1 mmol) was dissolved in 45 ml DMF and the solution was cooled to -5 ^{o}C using a salt ice bath under stirring. 18.93g POCl$_3$ was added to the reaction solution over a period of 1 hour while maintaining the reaction temperature between -5 and 0°C. The solution was removed from the ice bath, stirred at room temperature for 20 hours, and then warmed to 70°C and stirred for an additional 2 hours. The solution was then cooled and added to 450 g of ice water, basified with Na$_2$CO$_3$ to pH 7.5 and extracted with 3x300 mL CHCl$_3$. The combined organic layer was then washed with 3x200 ml water, dried over anhydrous MgSO$_4$, and concentrated to provide 5.31g crude product. This was purified by silica gel column chromatography using CH$_2$Cl$_2$ to give 3.687g product (100% yield) as light yellow oil.

^{1}H NMR: δ (CDCl$_3$): 3.67(m, 24H), 6.743 (d, 2H), 7.73 (d, 2H), 9.73(s, 1H, CHO); EI MS: m/z 367.92 (M+)

Synthesis of SKC-417:

Compound (2) (551 mg, 0.15mmol), 3-dimethyl aminophenol (452mg, 0.32 mmol) and *p*-toluene sulfonic acid (PTSA, 29mg) were dissolved in 25 ml propionic acid and the mixture was stirred at 70 ^{0}C for 20 hours. After concentrating, 150 ml of 3M NaOAc aqueous solution was added to the residue and stirring continued for 1 hour at room temperature, providing gummy residue

which was used immediately for subsequent reaction with tetrachloro-1,4-benzoquinone (453 mg, 0.185 mmol) in 200 mL of methanol:chloroform (1:1) mixture at ambient temperature for 20 hours. Excess tetrachloro-1,4-benzoquinone was removed by filtration and the reaction mixture was concentrated under reduced pressure. The residue was purified twice by silica gel column chromatography using $CHCl_3$:MeOH:AcOH (9:1:0.1) as eluent to get a crimson to dark violet solid as product (158mg, 17.4 % yield).

^{1}H NMR: δ (CD_3OD): 3.01 (s, 6H), 3.68-3.77(m, 24H), 6.87-7.72 (m, 10H), EI MS: m/z 604.98 (M+)

III   Experimental Characterization

The fluorescence intensity of SKC-513 as a function of ion concentration is shown in Figure 2 and Figure 3. (The structure of SKC-513, a derivative of SKC-417, is shown in Figure 4.) Figure 3 highlights how fluorescence intensity increases with K^+ concentration, saturating at about 1000 ppm, giving an enhancement of nearly an order of magnitude in the fluorescence intensity. Figure 2 shows that the binding is selective over the biologically-relevant range of concentrations, with no change in fluorescence intensity found for Ca^{+2}, Mg^{+2}, or Na^+. Also shown is the binding of K^+ in the presence of the sarcoplasmic reticulum (SR). The experiment was conducted in a solution composed of 5µM ion (KCl, NaCl, $CaCl_2$ or $MgCl_2$) in 100 mM dye, 20 mM HEPES and 1mM Gluconic Acid. Fluorescence was measured in the presence of the isolated SR. The isolation procedure of SR is described in *Salama et al.*[8]

IV   Computational Methods

We used the SAM1 semiempirical method to obtain optimized ground state structures.[9] Excited states were obtained with the INDO Hamiltonian and direct singles configuration-interaction (S-CI).[10,11] The S-CI calculations included excitations between all filled and empty orbitals. Note that only vertical excitations were considered, with geometric relaxation in the excited state being ignored. In cases of scans over a structural coordinate, the geometries were obtained by optimizing the ground electronic state with a constraint applied to the scanned coordinate within the SAM1 method. Comparison to density functional theory is discussed in Section V-E.

The structure of the SKC-417 dyes is shown in Figure 1. The ion binds to the crown ether at the top of the dye. To facilitate the calculations, the structure was reduced to contain only the optical chromophore by replacing the crown ether with methyl groups. This reduced structure is shown in Figure 5, which will be referred to as Simplified-SKC-417 (SSKC-417). Ion effects were modeled by including the potential of a point charge placed at a location corresponding to the center of the crown ether. The charge was positioned at a distance of 2.8 Å from the nitrogen of the crown ether, along an axis connecting the nitrogen to the oxygen of the heterocycle. Experimental and theoretical studies on potassium crown ether complexes suggest that bound potassium is approximately this distance into the center of the crown ether[12–14]. The magnitude of this charge corresponded to that of the bare ion divided by the dielectric constant, q/ε. For pure water, $\varepsilon = 80$, while for organic media $\varepsilon \approx 2$. Since the ionic charge is partially screened by water, the value of q/ε is likely below 1/3 for this organic framework. Results are shown for q/ε between -1/3 and 1/3.

V Computational Results

V-A Torsional energy profile

The barrier height of the SSKC-417 was examined to find the optimized structure of the ground electronic state. Semiempirical and *ab initio* calculations indicate that the minimum energy structure has a torsional angle, θ of Figure 5, in the range of 50° to 70° (see Supplemental Materials), with the range from 30° to 80° being populated at room temperature. Below, we present results either as a function of torsional angle or a fixed angle of 60°, using the SAM1 method to optimize the remaining structural coordinates.

V-B Observation of nearly-degenerate excited states

The excited states of the SSKC-417 dye in Figure 5, obtained from INDO/SCI theory, are shown as a function of torsional angle in Figure 6. The radius of the circles is proportional to the optical intensity for transitions from the electronic ground state. The results exhibit two low-lying excited states, with the upper state carrying most of the intensity in the thermally populated region between 30° and 80°. Following Kasha's rule[7], we expect the molecule to fluoresce only when the lowest excited electronic state carries significant optical intensity. The underlying assumption of this rule is that an excitation to the optically intense excited state is followed by a rapid relaxation to the lowest excited state. If this state has an optical transition to the ground state, fluorescence will occur; otherwise, the molecule will decay non-radiatively. The results indicate two nearly degenerate excited states in this molecule, with the lower one carrying much less optical intensity. The molecule should therefore be only weakly fluorescent. The near degeneracy of these states suggests that small perturbations, such as a nearby ion, may alter the relative ordering of the states, and thus substantially alter the fluorescence quantum yield.

Figure 7 shows the effect of a point charge with magnitude $q/\varepsilon = 1/3$, placed at a location corresponding to the center of the crown ether in Figure 1. The charge has a significant effect on the splitting between the lowest-lying excited states, and inverts the bright and dark states. The lowest excited state is therefore changed from non-fluorescent to fluorescent. These effects depend only weakly on torsional angle, so we consider results obtained at a torsional angle of 60° for the remainder of this paper.

Figure 8 shows the dependence of the two lowest excited states of SSKC-417 on the magnitude of the point charge. For a positive charge, the lowest state is bright, and the molecule is predicted to be highly emissive. In the absence of a charge or in the presence of a negative charge, the molecule is non-fluorescent.

Due to the polarization induced in the solvent, the effects of the ion may be better modeled as a uniform electric field, as opposed to a bare point charge[15,16]. Results obtained from the application of a uniform electric field are in qualitative agreement with those from the point charge (see Supplemental Materials).

V-C   Origin of the degenerate excited states in SKC-417

Examination of the frontier molecular orbitals reveals that the near degeneracy of the lowest excited states arises from a near degeneracy of the highest occupied molecular orbitals (HOMOs). The lowest unoccupied molecular orbital (LUMO) resides on the heterocycle (Figure 9). One of the nearly degenerate HOMOs resides primarily on the phenylene ring, such that excitation to this orbital corresponds to a charge-transfer (CT) state. Since there is little overlap between this HOMO and the LUMO, this CT state carries little optical intensity. The other HOMO resides on the heterocycle, and excitation to this neutral (N) state has strong optical

intensity. A positive charge bound to the crown ether is closer to the phenylene ring than to the heterocycle. As such, the charge destabilizes the CT state. This is in agreement with Figure 8, which shows that a positive charge causes the dark CT state to rise above the bright N state.

V-D Three-fold symmetric parent molecule

The degenerate excited states observed in SSKC-417 may be unexpected, in that the excitations have a largely different character, one being a neutral excitation on the heterocycle and the other being a charge transfer from the heterocycle to the phenylene ring. Furthermore, the molecule appears to have at most two-fold symmetry, and degeneracies are not expected for C_2 symmetry groups. However, doubly degenerate states are expected for molecules with three-fold symmetry, since the C_3 symmetry groups have doubly degenerate representations. We consider the molecule of Figure 10 and Figure 11, which uses bridging oxygen atoms to convert SSKC-417 to a three-fold symmetric system. The orbitals of this symmetric parent molecule are shown in Figure 10. The HOMO is again doubly degenerate, while the LUMO is non-degenerate. The nodal pattern of the HOMOs also shows why removal of oxygen and rotation of the upper phenylene ring to form SSKC-417 does not lift the degeneracy: the HOMOs have little amplitude in the regions of these couplings. The degenerate HOMOs of SSKC-417 are therefore not an accidental degeneracy, but rather a result of the three-fold symmetry of this parent molecule.

The effects of a point charge on this three-fold symmetric molecule are shown in Figure 11. The point charge lifts the degeneracy, as also seen for SSKC-417 in Figure 8. However, both excited states carry optical intensity, so the field is not expected to alter the fluorescence quantum yield. The three-fold symmetric molecule is therefore useful for understanding the origin of the

degenerate excited states of SSKC-417, but is not expected to be a useful chromophore for ion detection.

V-E   *Ab Initio* Calculations

Some key conclusions from our semiempirical calculations can be verified by comparison to results of DFT calculations. We optimized the geometry of SSKC-417 with B3LYP/6-31G[17–2018–20], yielding a torsion angle of about 52° (see Supplemental Material). The Kohn-Sham orbitals show a pattern similar to that of Figure 9. There are two nearly degenerate HOMOs with the higher-energy orbital residing primarily on the phenylene group and the lower-energy orbital residing primarily on the heterocycle. The gap between these orbitals is 0.0135eV, similar to the 0.0187 eV obtained from INDO theory. Time-dependent DFT (TD-DFT) at the B3LYP/6-31G level is used to calculate the excited states. The lowest-energy excitations reveal a dark state at 2.214 eV, with a bright state at 2.7588 eV, similar to the behavior seen in INDO.

To confirm the accuracy of the INDO calculations, a set of TD-DFT calculations were performed using a point charge to model the effects of the K^+ ion (Figure 12). The point charge is positioned at a distance of 2.8 Å from the nitrogen on the phenylene ring, along an axis connecting the nitrogen to the oxygen of the heterocycle, as described in Section IV. As the magnitude of the point charge increases, a drop in the dark excited state energy is observed similar to the INDO predictions. For a point charge of magnitude 0.3, the bright state is 0.431 eV below the dark state, near the value of 0.478 eV seen in INDO for a charge of magnitude 1/3. The crossing of bright and dark states in TD-DFT occurs when the magnitude of the point charge is just under 0.2, which is somewhat higher than the 0 to 0.1 range found from INDO (see Figure 9).

To explore the possible presence of specific interaction with the K+ ion, TD-DFT calculations were performed with a potassium ion located at the position described in Section IV (2.6-3.0 Å from the nitrogen of the crown ether, along the axis connecting nitrogen to oxygen of the heterocycle)[12–14]. The presence of a potassium ion stabilizes the orbital residing primarily on the phenylene ring, leading to a drop in energy for that orbital. This drop makes the orbital residing on the heterocycle the sole HOMO and, as above, causes the lowest-energy excited state to become a bright state. Since the distance between the nitrogen on the phenylene ring and potassium ion is not precisely known, the distance was varied from 2.2-3.6 Å. The frontier Kohn-Sham orbitals show little density on the potassium ion, suggesting there is no specific interaction with potassium for the orbitals that contribute to the excited states. We consider the degree to which the effects of the potassium ion arise solely from the charge on the ion, as opposed to more specific chemical interactions. We do this by comparing TD-DFT results obtained with a potassium ion with those obtained by replacing the potassium with a point charge at the same location. In both cases, the ordering of the bright and dark states is the same. In addition, the energy of the excited states changes little as the distance to the nitrogen is varied from 2.2 Å to 3.6 Å. Even at a close distance of 2.2 Å, where specific interactions would be expected to play a strongly enhanced role, there is little change in the excitation energies. The frontier Kohn-Sham orbitals also do not show density on the K^+ ion, even at 2.2 Å. Finally, at the expected distance of 2.8 Å, the gap between the HOMO and the orbital on the phenylene groups is similar between the K^+ ion and a point charge, being 0.1732 eV and 0.1769 eV, respectively. Taken together, this is strong evidence that the influence of the potassium ion on

the fluorescence arises from the electric field of the ion, as opposed to a more specific chemical interaction, and that this field effect is well-modeled by a point charge.

V-F   Effects of electronic substituents

The suggested mechanism for the effects of ion binding on the fluorescence properties is that the electric field of the ion causes the molecule to move between the non-emissive regime on the left of Figure 8 to the emissive regime on the right. The computations performed are approximate, not only in the methods for the electronic structure of the chromophore, but also regarding the lack of solvent effects and the use of a simplified structure for the dye. While such approximations likely do not invalidate the proposed mechanism, they do suggest that the precise location of the crossing point between the non-emissive and emissive regimes may not be highly reliable. A reasonable target for synthetic modification to SSKC-417 would be the use of substituents to alter the transition point between emissive and non-emissive regimes. This can be done by using electronic acceptors or donors to alter the energy of the HOMOs in Figure 9.

The molecule shown in Figure 13 has fluorine atoms on the phenylene group. The fluorines should stabilize the HOMO located on the phenylene ring (the HOMO on the right in Figure 9), and thereby raise the energy of the dark charge-transfer state, relative to the energy of the bright, neutral excited state. The resulting excited states, as a function of the magnitude of the point charge, are also shown in Figure 13. The substituents move the crossing point between bright and dark states to a more negative value of the point charge. Similarly, placing fluorine substituents on the heterocycle stabilizes the LUMO to the left of Figure 9, and moves the crossing point to a more positive value of the point charge, as shown in Figure 14.

These results suggest that electronic substituents provide a handle that may be used to optimize the sensitivity of SSKC-417 fluorescence to ion binding. The precise location of the crossing point between emissive and non-emissive regimes may not be accurately determined by quantum chemical computations. These results should therefore be viewed as only suggesting the use of electronic substituents to optimize dye performance, as opposed to dictating the best choice for substituents.

Figure 15 shows the effects of a charge on the excited states when an extra methylene group is placed between the heterocycle and the phenylene group. The calculations are done for the optimized geometry, in which the phenylene ring is nearly perpendicular to the heterocycle. The avoided crossing between bright and dark states suggests a stronger electronic coupling between these states than is seen in Figure 8 for SSKC-417. A more detailed examination of the curve crossings suggests a coupling of 0.166eV for this system, compared to 0.007eV for SSKC-417. The proposed mechanism for ion sensitivity assumes rapid relaxation between bright and dark states, such that Kasha's rule applies. A stronger coupling between bright and dark states may enhance this relaxation. In this case, the addition of a bridge methylene group, as in Figure 15, may be expected to enhance ion sensitivity.[21] Lengthening the bridge to two methylene groups essentially eliminates the coupling (see Supplemental Materials).

VI   Summary

The quantum chemical calculations presented here suggest a mechanism for the sensitivity of SSKC-417 to ion binding, and a possible means for enhancing this sensitivity. The mechanism relates to the near degeneracy of a bright excitation, corresponding to a neutral excitation on the heterocycle of SSKC-417, and a dark excitation corresponding to electron transfer from the phenylene group to the heterocycle. Binding of an ion destabilizes the dark excited state, raising it above the neutral state, and following Kasha's rule, greatly enhances the fluorescence quantum yield. Electronic substituents may be used to alter the relative location of the bright and dark state, providing a synthetic handle for optimizing the sensitivity to ion binding. Lengthening the bridge between the heterocycle and phenylene group by one methylene group enhances the electronic coupling between the bright and dark state, which may also improve sensitivity.

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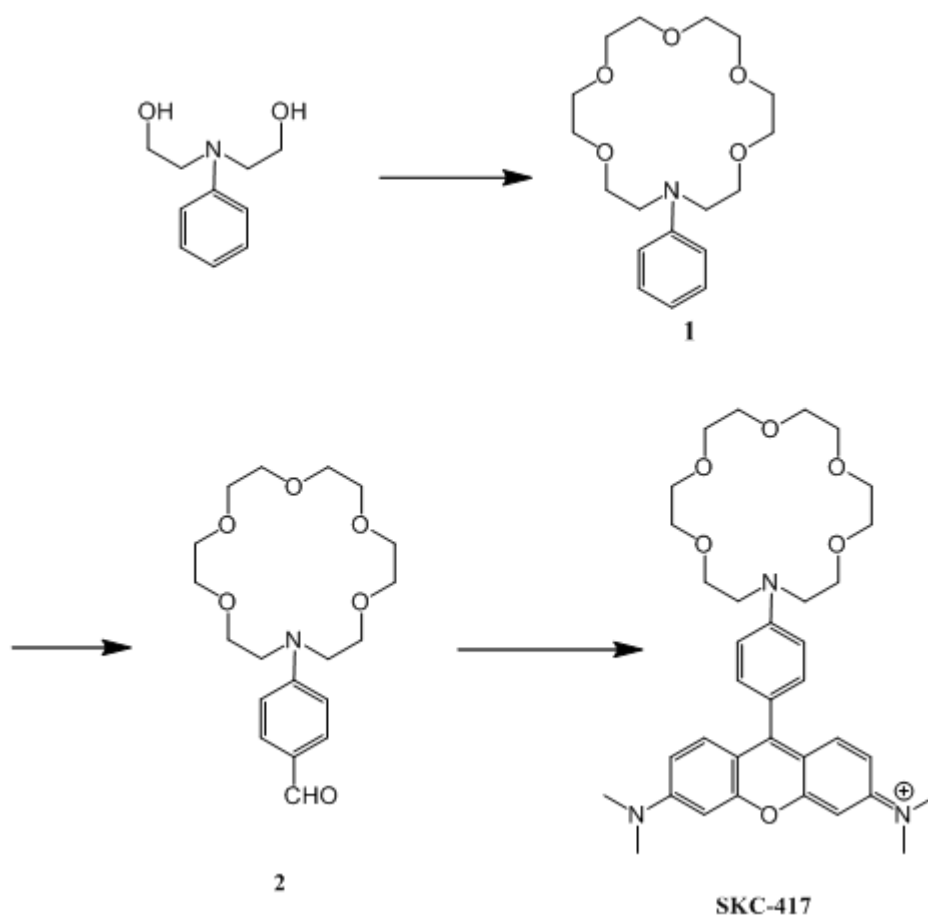
Figure 1 The three main steps for the synthesis of SKC-417
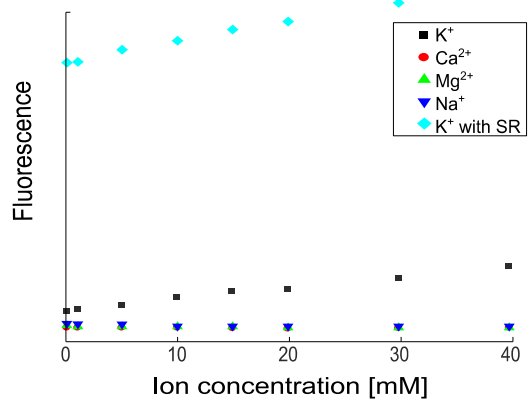
Figure 2 Experimental data for SKC-513 showing the fluorescence intensity as a function of ion concentration from 0-40 ppm. SR refers to experiments done in the presence of a sarcoplasmic reticulum.
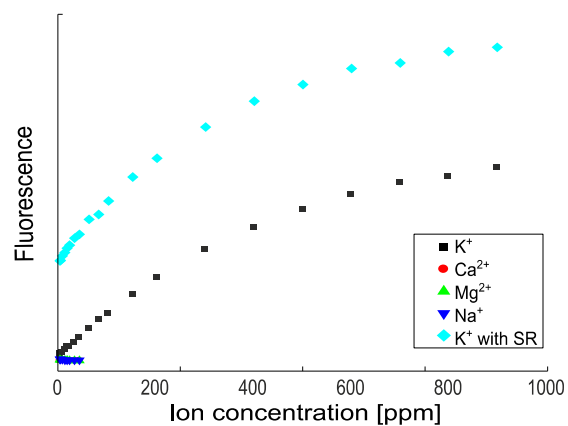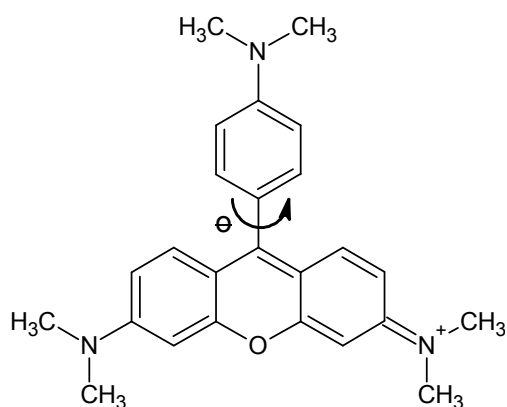

Figure 3 Experimental data similar to that of Figure 2 over an ion concentration of 0-1000 ppm
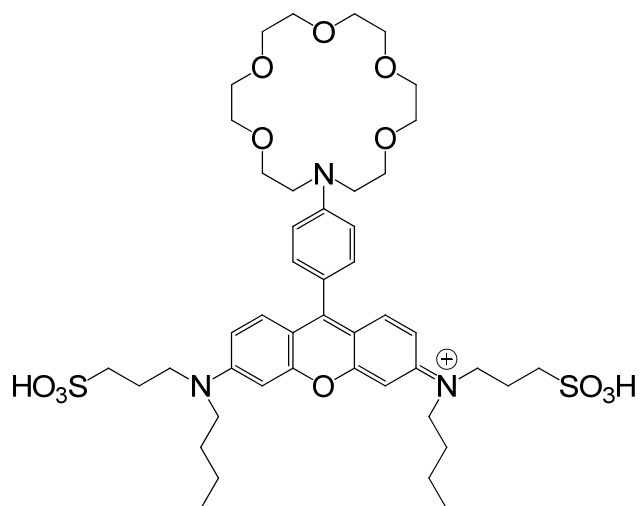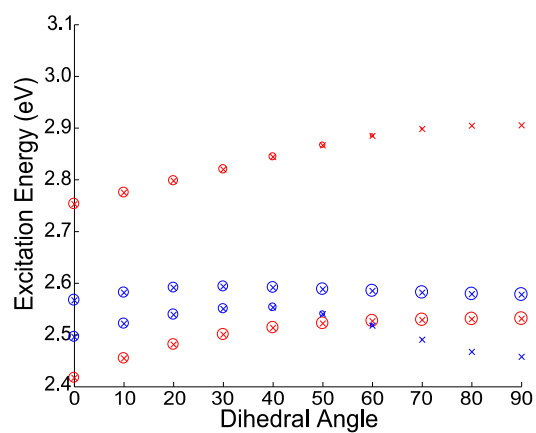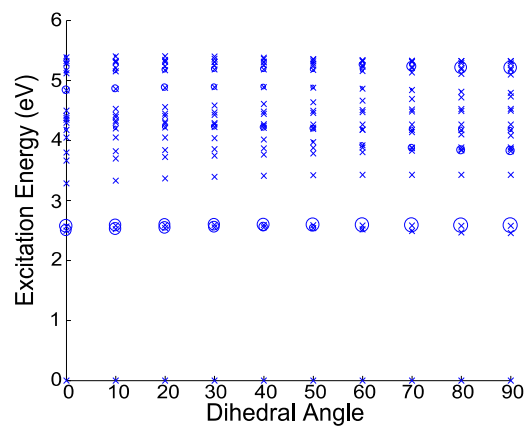
Figure 4 Chemical structure of SKC-513

**Figure 5** Optimized structure of the simplified SKC-417 dye, obtained from the SAM1 model.
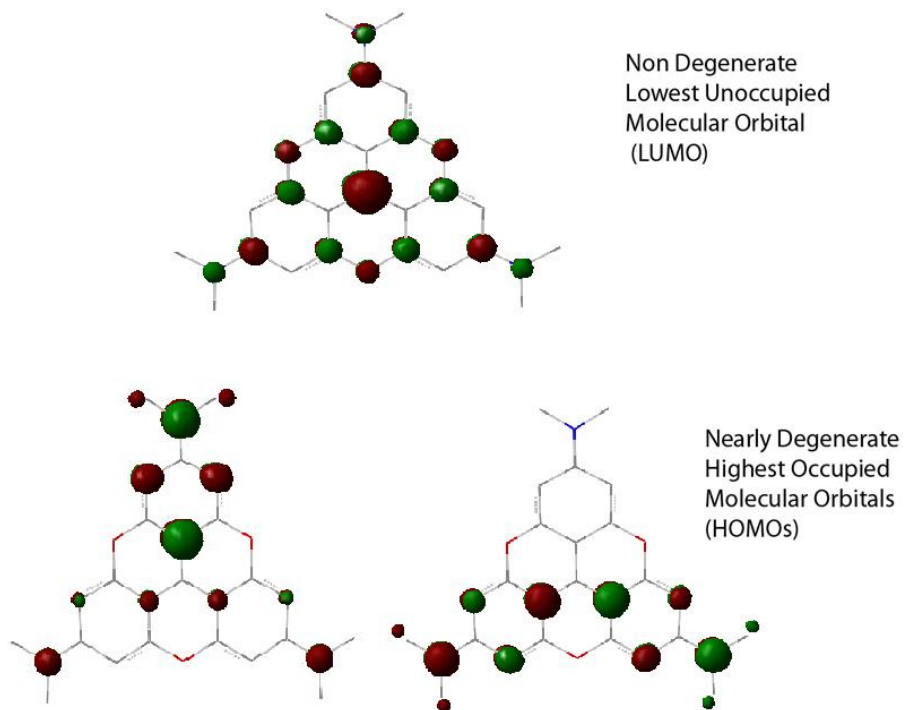

Figure 6 Excited states of the SSKC-417 dye as a function of torsional angle. The radius of the circles indicates the optical intensity of the transition from the ground electronic state to the given excited state.


Figure 7 Two lowest excited states of the SSKC-417 dye as a function of torsional angle with a point charge of magnitude 1/3 (red) and without a point charge (blue) . (Circles indicate optical intensity.)
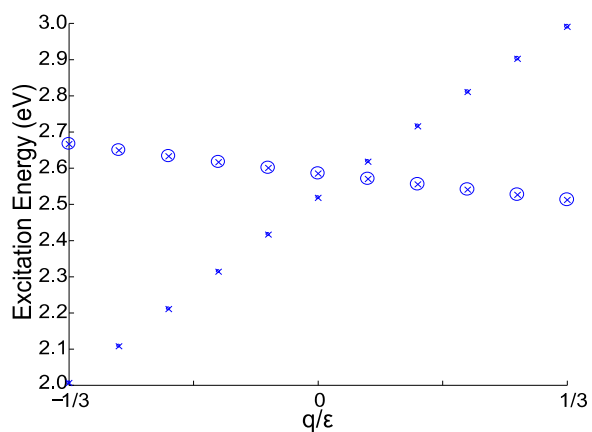
Figure 8 Effects of a point charge of magnitude q/ε placed at a location corresponding to the center of the crown ether on the two lowest excited states of SSKC-417.
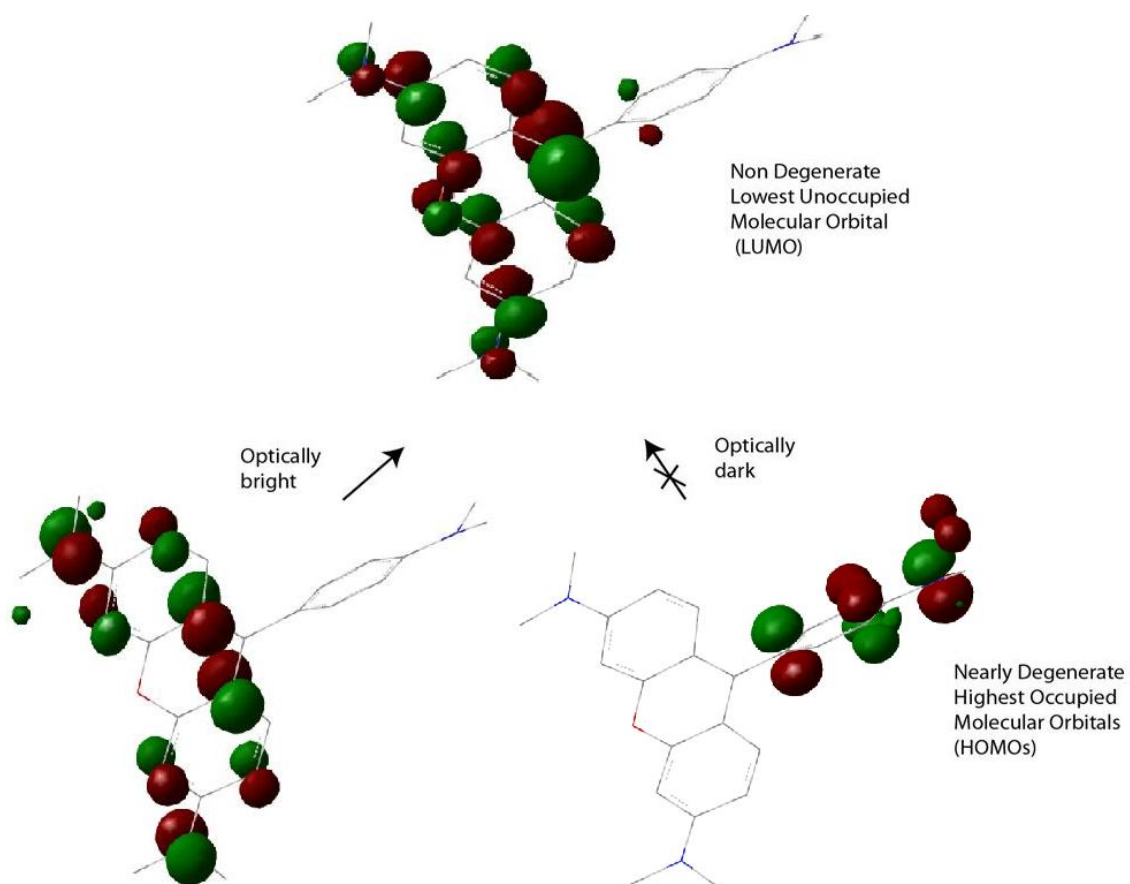
Figure 9 Frontier orbitals of SSKC-417. There are two nearly degenerate HOMOs, one located on the heterocycle and the other on the phenylene ring. The LUMO is non-degenerate and located on the heterocycle. Optical intensity relies on good overlap between orbitals, and only the transition from the HOMO on the heterocycle to the LUMO carries optical intensity.

Non Degenerate
Lowest Unoccupied
Molecular Orbital
(LUMO)

Nearly Degenerate
Highest Occupied
Molecular Orbitals
(HOMOs)

**Figure 10** Frontier molecular orbitals of the three-fold symmetric parent of SSKC-417.
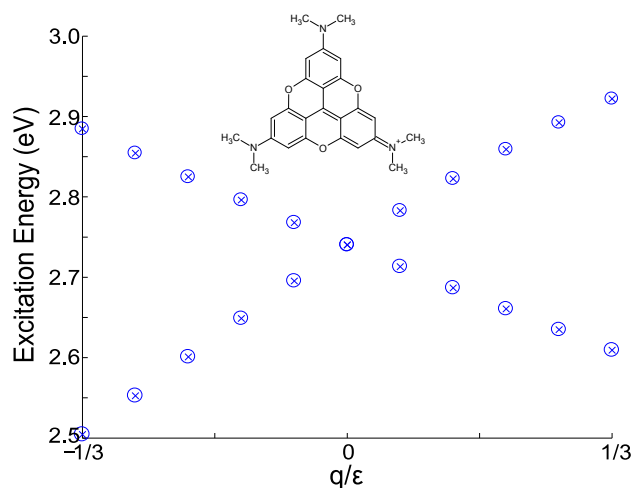
Figure 11 Effects of a point charge on the excited states of the three-fold symmetric parent of SSKC-417. (The radius of the circles show the optical intensity.)
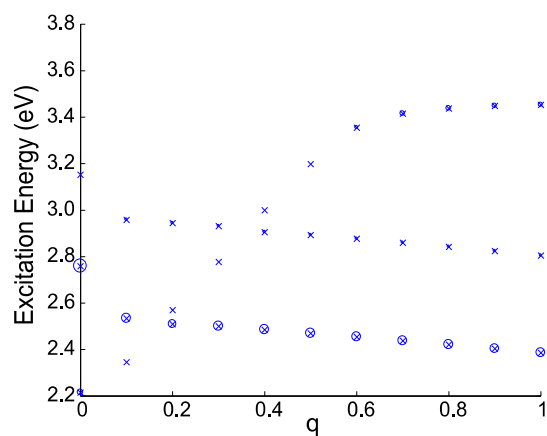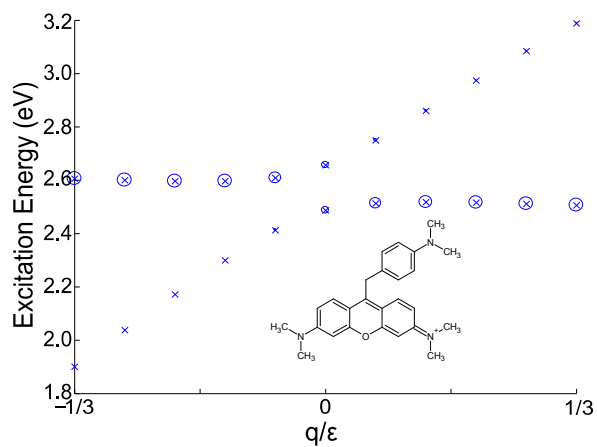

Figure 12 TDDFT calculations on SSKC-417 showing the effect of a point charge of magnitude q on the three lowest excitation states.
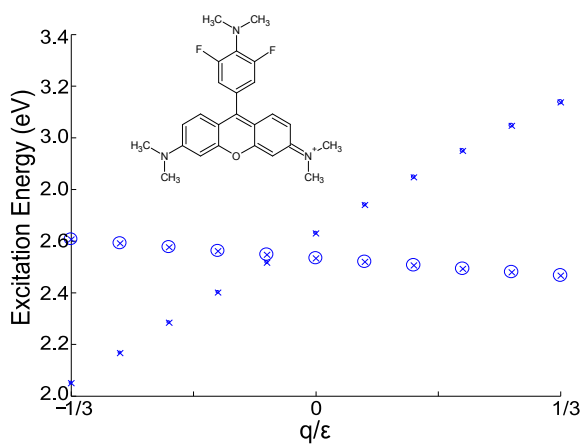
Figure 13 The two lowest excited states of SSKC-417 with two fluorine substituents attached to the phenylene group.
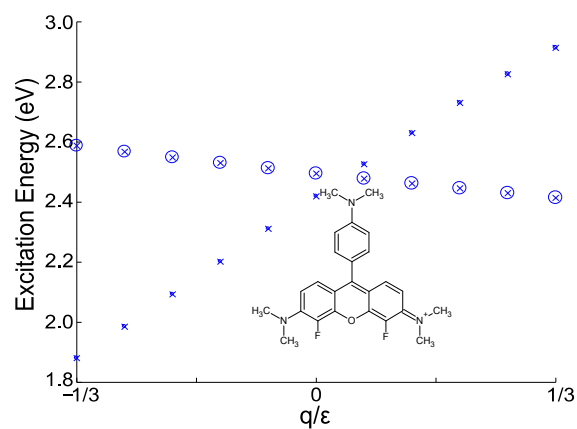
Figure 14 The two lowest excited states of SSKC-417 with two fluorine substituents attached to the heterocycle.

Figure 15 The two lowest excited states of a modified SSKC-417 with a methylene bridge between the phenylene group and the heterocycle.