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Polarization and Symmetry of Electronic Transitions in Long Fluorescence Lifetime Triangulenium Dyes

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Abstract

To fully exploit the capabilities of fluorescence probes in modern experiments, where advanced instrumentation is used to probe complex environments, other photophysical properties than emission color and emission intensity are monitored. Each dye property can be addressed individually as well as collectively to provide in-depth information unavailable from the standard intensity measurements. Dyes with long emission lifetimes and strongly polarized transitions enable the monitoring of lifetime changes as well as emission polarization (or anisotropy). Thus experiments can be designed to follow slow dynamics. In this article the UV and visible electronic transitions of a series of red emitting dyes based on the triangulenium motif are investigated. We resolve overlapping features in the spectra and assign transition moment of the molecular axes. The result is the complete Jablonski diagram for the UV and visible spectral region. The symmetries of the studied dyes are shown to have a large influence on the optical response and they are clearly separated into two groups of symmetry by their photophysical properties. The C_{2v} symmetric dyes: azadioxatriangulenium (ADOTA⁺) and diazaoxatriangulenium (DAOTA⁺) have high emission anisotropies, fluorescence lifetimes around 20 ns, and fluorescence quantum yields of ~50%. The trioxatriangulenium (TOTA⁺) and triazatriangulenium (TATA⁺) dyes—nominally of D_{3h} symmetry—have fluorescence lifetimes around 10 ns lifetimes and fluorescence quantum yields of 10–15%. However, the D_{3h} -symmetry is shown to be lowered to a point group, where the axes transform uniquely such that the degeneracy of the E'-states is lifted.

Keywords

Fluorescence; polarization; triangulenium dyes; fluorescence lifetime; advanced optical spectroscopy

Introduction

The synthesis of new fluorescent organic dyes and their applications are fields of significant importance in modern chemistry.¹ However, dye and sensor development is almost

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Supporting Information **Available:** Synthetic procedures, absorption and emission spectra, labeling protocol and experimental procedures are included as supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

exclusively based on functionalization of—and variations on—a quite limited number of well-known basic chromophore structures.² As transition energy can often be tuned considerably within the same basic molecular structure, strong chromophores covering almost the entire UV/Vis/NIR region can be created. In contrast, tuning other fundamental photophysical characteristics such as polarization and lifetimes is much harder to achieve, as these properties are largely bound by the fundamental chromophore structure.³ The most common classes of dyes such as rhodamines, fluoresceins, coumarins and the bodipy, the rylene and the cyanine dyes are all high oscillator strength and consequently short fluorescence lifetime dyes.^{1a, 1g} The hydrocarbon based aromatic fluorophores such as coronene and pyrene may have long fluorescence lifetimes, but suffer from low oscillator strengths, emission in the blue and from low water solubility.^{1g, 4} The dansyl-type dyes have so far been the most used long-lifetime-probes,⁵ but their blue/green absorption and emission, solvent sensitivity, and low oscillator strengths put severe restrictions on their general applicability.

There are several parameters that are of interest when choosing a chromophore. Strong absorption and efficient emission is generally desirable, but are not necessarily sufficient for high signal-to-noise ratios. Note that high oscillator strength of the primary electronic transition is fundamentally incompatible with a long emission lifetime. In practical applications, in biological experiments particularly, long wavelength emission is of interest because it avoids issues related to spectral overlap with background emission and scattering phenomena. A long fluorescence lifetime is a property that can be exploited in a multitude of ways. The most direct application is removal of the short-lived background fluorescence observed in complex systems such as protein solutions or cells by time-gated detection.⁶ Long emission lifetimes are also advantageous in fluorescence quenching experiments: as the emission lifetimes of typical dyes at high degrees of quenching is very short, they are difficult to accurately resolve using the common diode/PMT set-ups found in non-specialized labs. On the other hand, a long-lifetime dye will still have lifetimes in the nanosecond range under heavily quenching conditions. High fundamental anisotropies, *i.e.* strongly polarized transitions, are a necessity in polarization loss measurements.^{1e} These measurements are complicated by the fact that the rotational correlation time^{1e} of the dye must be approximately the same as the emission lifetime of the dye in order to have the highest possible dynamic range. This makes dyes with both long lifetimes *and* strongly polarized transitions indispensable, when measuring the slow dynamics of large biomolecules. The azaoxatriangulenium dyes are unique as several derivatives that show this highly unusual and desirable combination of highly polarized and long-lived emission in the red, while still having moderate absorption strength. Dyes such as rhodamines and cyanines have strong absorption, efficient fluorescence, high emission anisotropies, but with short lifetimes they are not suited for probing of moderate or slow dynamics.

The triangulenium dyes (Figure 1) were introduced with the synthesis of the parent compound trioxatriangulenium (TOTA⁺) by Martin and Smith in 1964^{2b}. The photophysics and photochemistry of TOTA⁺ was investigated thoroughly, and its use in photoinduced electron transfer (PET)⁷ and as a DNA intercalator⁸ has been studied. Decades later the aza-analogues were prepared in our lab⁹ and their PET properties were investigated by Dileesh and Gopidas.^{7b, 9b, 10} The triangulenium dyes are extremely stable cations, and are unreactive towards most nucleophiles; the diazaoxa- and triazatriangulenium (DAOTA⁺ and TATA⁺) in particular. Due to their high stability the compounds have found use as phase transfer catalyst¹¹ and in functional materials.¹² More recently TOTA⁺ has been synthesized with peripheral aminogroups leading to high brightness fluorophores, which can be considered a blueshifted rhodamine-analogue.¹³ The same synthetic approach was used to functionalize azadioxatriangulenium (ADOTA⁺) with peripheral amino groups, yielding a fluorophore that is even further blueshifted, making it one of the only high brightness

fluorophores with emission at shorter wavelengths than fluorescein.¹⁴ High emission yield chromophores based on the TATA⁺ motif were recently prepared by introducing steric constraints in the system.¹⁵ The basic triangulene motif has also recently been modified to produce the pH sensitive fluorophore H-TOTA¹⁶, which by itself covers the range from approximately pH 2 to 8 due to multiple protonation stages with differing optical properties. The triangulenium ions are of high symmetry, and can be classified accordingly. However, it is relevant to make a distinction between compounds with and without donor substituents in the para positions.

The substituted class of compounds such as the A-TOTA⁺ and A-ADOTA⁺ series, (Figure 1, bottom row) are chromophores where the lowest electronic transitions to a large extent are defined by the donor groups, leading to optical properties largely similar to those of Rhodamine derivatives.^{13b, 13c, 17} They have high oscillator strengths ($\epsilon \approx 80,000$ -130,000 M⁻¹cm⁻¹) and quantum yields ($\Phi \approx 0.7$), lifetimes in the 3-5 ns range, and feature very narrow bands in both absorption and emission.

The unsubstituted triangulenium compounds TOTA⁺, ADOTA⁺, DAOTA⁺, and TATA⁺ (Figure 1, top row) show remarkable different optical properties which are the subject of the current paper. These red emitting dyes have unusually long fluorescence lifetimes (10-25 ns), medium strong absorption ($\epsilon \approx 8,000$ -15,000 M⁻¹cm⁻¹) and high quantum yields (≈ 0.5). As such they are effectively filling the lifetime gap between the typical high brightness, short lifetime organic fluorophores and the metallo-complexes with lifetimes in the microsecond range.¹⁸ In recent work, these special optical properties have been used to demonstrate: excited state lifetime engineering by silver nanoparticles¹⁹, time-gated bio-imaging,²⁰ time-resolved fluorescence correlation spectroscopy,²¹ and a fluorescence anisotropy assay for large protein complexes.²² In this work we provide a detailed study of the electronic properties of all four aza-oxa-triangulenium chromophores and in particular the fundamental emission anisotropy and orientation of the lower electronic transitions.

Methods and Materials

The BF₄⁻ salts of trioxatriangulenium (TOTA⁺), *N*-propyl-azadioxatriangulenium (ADOTA⁺), *N,N*-dipropyl-diazaoxatriangulenium (DAOTA⁺), and *N,N,N'*-tripropyl-triazatriangulenium (TATA⁺) were synthesised and purified according to the methods in ref. ^{2b, 9b}. PVA and solvents (Sigma-Aldrich) were of HPLC grade and used as received. Water was used from a Milli-Q purification apparatus.

Steady State Spectroscopy

Absorption measurements were done on solutions in the 10-50 μM range, resulting in absorbances around 0.5 in the 1 cm pathlength cuvettes used. Emission spectra and lifetimes were measured in 1 cm cuvettes with absorbances below 0.05 at the excitation wavelengths.

The solution emission and excitation spectra were measured on a Horiba-Yvon Fluorolog 3 emission spectroscopy fitted with polarizers and double grating monochromators. The UV-vis spectra were recorded on a Perkin-Elmer Lambda 1050 UV/Vis/NIR spectroscopy with automatic polarizers, or a Varian Cary 50 equipped with a rotatable film holder and a Glan-Thompson polarizer prism.

Low temperature spectra were recorded by cooling glycerol solutions of the dyes in 1×1 cm quartz cuvettes to 190 K in a heater controlled liquid nitrogen cryostat, resulting in clear and defect-free glasses. All measurements were performed at 90 degree angle between excitation and emission, and the excitation light entered the sample perpendicular to the cuvette.

Lifetime Measurements

Fluorescence lifetimes were measured using FluoroTime 200 (PicoQuant GmbH, Berlin, Germany) equipped with a multichannel plate detector (MCP-PMT from Hamamatsu) or a FluoroTime 300 (PicoQuant GmbH, Berlin, Germany) system. The excitation was from a pulsed solid state laser 470nm (65 ps pulse width) driven by a PDL800-B driver with repetition rate of 5MHz. The fluorescence decays were analysed using the FluoFit software package version 4.2.1. The decay data were fitted by iterative reconvolution with a sum of exponentials:

$$I(t) = \sum_i \alpha_i \exp\left[-\frac{t}{\tau_i}\right] \quad \text{eq 1.}$$

In eq 1 α_i is the amplitude and τ_i is the fluorescence lifetime of the component respectively.

PVA Film Preparation

PVA films were prepared from 10% w/w aqueous solution. The triangulenium dyes dissolved in methanol were added to the PVA solution. The PVA solutions containing different dyes were poured into Petri dishes and dried. PVA films were stretched under a hot-air gun to approximately 450% of the original length and mounted in a custom made holder to avoid shrinkage and warping of the film post stretching.

Results

The triangulenium dyes and their derivatives are of high symmetry, and their optical properties are determined by the specific point group symmetry of the molecular skeleton. The azatriangulenium dyes ADOTA⁺ and DAOTA⁺ belong to the point group C_{2v}, dictating that all optically allowed transitions are orthogonal and in-plane (the two possible transition dipole directions are at a 90 degree angle in the flat π -system). The C_{2v} symmetric dyes have long lifetimes and high emission yields. TOTA⁺ and TATA⁺ belong to the point group D_{3h}, implying degenerate electronic transitions. Only transitions to the doubly degenerate E' symmetric excited states are dipole allowed in these dyes, a maximum fundamental emission anisotropy of $r = 0.1$ (instead of $r = 0.4$ for regular fluorophores) is expected.^{1e, 7b, 23} The D_{3h} symmetric dyes have approximately 10 ns lifetimes and moderate emission yields.

Solution Photophysics

The low-energy part of the absorption and the emission spectra of the triangulenium dyes measured in acetonitrile are shown in Figure 2. The four triangulenium dyes have medium strength absorption bands in the green, with weaker bands in the blue/near UV region. Spectra, fluorescence quantum yields and fluorescence lifetimes for the four derivatives were measured in a series of solvents. From these measurements the optical properties of the triangulenium dyes were found not to be solvent sensitive, with transition energies, band shapes, and quantum yields only weakly affected, see the supporting information. The band shapes of the D_{3h} symmetric dyes are sensitive to solvent. However, the main observation is a narrowing of the width of the vibrational peaks in lower polarity environments. As the properties change little with solvent, only the properties of the dyes in acetonitrile solution are listed in Table 1. Spectra and emission properties of the triangulenium dyes in methanol, glycerol and dichloromethane are provided as supporting information.

The C_{2v} symmetric derivatives: ADOTA⁺ and DAOTA⁺, show S₀→S₁ transitions of medium strength in the visible, followed by a S₀→S₂ transition with considerably lower oscillator strength. Both the oscillator strength of the primary transition and the sum of the two visible transitions are included in Table 1. The absorption and emission spectra of the

C_{2v} symmetric dyes (Figure 2) do not follow the mirror image rule. This is seen as considerably less pronounced vibrational structure in emission compared to absorption. In glasses at low temperature the Stokes' shift decreases and the mirror image rule is considerably improved as the vibrational structure gets more pronounced in the emission. The low temperature spectra in glycerol can be found in the supporting information.

The D_{3h} symmetric dyes: $TOTA^+$ and $TATA^+$, show transitions in the visible range of comparable strength to $ADOTA^+$ and $DAOTA^+$, however, the spectra are of unusual shape, broad and lacking clear vibrational progressions. By inspection of the spectrum an obvious deviation from the mirror image rule is seen. That is: the emission spectra are almost entirely without vibrational structure and far narrower than the absorption bands.²⁴

The structure and grouping of the isotropic absorption bands are similar for all the triangulenium dyes. The general structure going from the red to the blue is: a medium strong main absorption in the visible followed by a blue-shifted absorption of half the oscillator strength. In the $29,000\text{ cm}^{-1}$ (345 nm) region a number of low intensity bands is followed by a high intensity band around $33,000\text{ cm}^{-1}$ (300 nm). For $TOTA^+$ the low intensity bands seen around $28,000\text{--}30,000\text{ cm}^{-1}$ in the azaoxatrianguleniums are replaced by two high intensity bands around $30,000$ and $35,000\text{ cm}^{-1}$. In the high-energy region around $40,000\text{ cm}^{-1}$ (250 nm) a number of narrow and intense bands are observed for all species. Solution absorption spectra over the entire spectral range as well as specific transition energies and intensities can be found in the supporting information.

Transition moments of C_{2v} Symmetric Triangulenium dyes

Full characterization of the electronic transitions requires information about transition moment directions in addition to the strengths and energies available from isotropic solution spectra. In the determination of the transition moment directions and the state symmetries of the dyes, polarized absorption and emission spectroscopy was used. To measure the fundamental emission anisotropy r_0 the dyes are immobilized in low temperature glycerol glasses. The absolute direction of absorption dipoles are determined from oriented ensembles of molecules in stretched polymer films at room temperature.

Figure 3 shows the fluorescence excitation and emission anisotropies of $ADOTA^+$ and $DAOTA^+$ superimposed onto the excitation and emission spectra. The fluorescence excitation anisotropies show that the excitation spectra consist of highly polarized and well-separated bands. The anisotropy of the $S_0 \rightarrow S_1$ transition is almost flat over the bands and reaches an r_0 value of 0.38, indicating parallel emission and absorption dipoles. The $S_0 \rightarrow S_2$ transition has the opposite polarization of the emission; because of the overlap with the lower energy transition it only reaches anisotropy values of approximately -0.1 rather than the limiting value of -0.2 . Interestingly the band at $28,000\text{ cm}^{-1}$ has opposite polarization for the two molecules. For $ADOTA^+$ it is polarized parallel to the emission, while for $DAOTA^+$ it is perpendicular. The emission anisotropy of both molecules is well-behaved, with high anisotropy values close to the 0-0 transition that decrease slightly with emission wavelength.

While the emission and excitation anisotropies provide the necessary information for use in depolarization assays, they only give the transition moment directions relative to the emission dipole. In order to determine the absolute direction of the transition moments relative to the molecular framework, it is necessary to perform measurements on an oriented ensemble of molecules. Because of the substituents on the nitrogen atoms (propyl chains in the present case) the dyes have a long axis, which invites the use of the stretched-polymer method for alignment of the molecules. The in-plane long axis is not radically different from the in-plane short axis and perfect orientation cannot therefore be expected. However, the

C_{2v} point group symmetry dictates that dipole-allowed transitions can only be in-plane and either parallel or perpendicular to the C_2 axis. Thus, any degree of preferential orientation of the molecules is sufficient to resolve the spectra. To separate transitions based on their orientation we adopt the approach of Thulstrup *et al.*²⁵. The reduced spectra, i.e. the purely z- and y-axis polarized components A_z and A_y of the absorption, can be calculated according to eq 2.

$$A_z = E_{\parallel} - d_{\parallel}^0 E_{\perp} \quad \text{and} \quad A_y = n_0 (E_{\perp} - d_{\perp}^0 E_{\parallel}) \quad \text{eq 2.}$$

The components $E_{\parallel}$ and $E_{\perp}$ in eq 2 are the experimental spectra recorded with polarizers oriented parallel and perpendicular to the stretching direction respectively, the d^0 components are the reduction factors that can be read directly from the polarized spectra in this case as the transitions are well-separated, and n_0 is a constant of proportionality which for a C_{2v} symmetric molecule can be calculated from the reduction factors if uniaxial stretching of the polymer can be assumed. The details of the procedure for reducing the spectra can be found in a series of articles by Thulstrup, Michl and Eggers.²⁵⁻²⁶

The reduced spectra of ADOTA⁺ and DAOTA⁺ (Figure 4) reveal two clearly resolved and purely polarized spectra, *i.e.* all peaks appear as either z- or y-polarized. The near-UV and Vis region of these spectra shows the same structure as suggested by the excitation anisotropy spectra – two series of well-separated orthogonal transitions. The spectra of the oriented molecules show that in ADOTA⁺ the $S_0 \rightarrow S_1$ transition dipole is oriented along the C_2 axis, while for DAOTA⁺ the transition dipole is perpendicular to this. This finding suggests that the $S_0 \rightarrow S_1$ transition in both molecules involve movement of charge from the nitrogen atoms to the center of the molecule. Disregarding the 600 cm⁻¹ red shift of the DAOTA⁺ spectra relative to those of ADOTA⁺, the main differences between the spectra are in the UV region.

Transition moments of D_{3h} Symmetric Triangulenium dyes

The emission and excitation spectra with corresponding fundamental anisotropies of immobilized TOTA⁺ and TATA⁺ in glycerol glass are shown in Figure 5. The emission anisotropy of TOTA⁺ is constant over the entire band, and with excitation at 485 nm has a value of 0.13. The emission anisotropy of TATA⁺ is not constant, and decreases from 0.21 to 0.15 over the band with 535 nm excitation. The shape of the excitation anisotropies of the two dyes is largely similar. There are overlapping bands of opposite polarization in the UV, while the low energy band features anisotropies of approximately 0.1 at the blue edge, which rapidly increases towards the maximum possible value of 0.4 at the red edge. Note that the shape of the excitation anisotropy spectra is independent of the emission wavelength detected, and the increase in anisotropy at low energy is therefore not caused by scattered light as one might suspect given the shape of the spectrum. As dyes with D_{3h} symmetry can never have anisotropies above 0.1, and the excitation anisotropy spectrum is expected to be flat and featureless because of the state degeneracy, these anisotropies are conclusive evidence that the electronic state symmetry of the dyes is lower than the formal symmetry of the molecular framework.

Unlike ADOTA⁺ and DAOTA⁺ the D_{3h} symmetric dyes do not have a well-defined long axis, as we must assume that the changes in the geometry of the molecular framework are minute even though the symmetry of the electronic states is lowered. Thus, we cannot use the stretched film approach to orient the molecules since the molecules would still be randomly oriented in the zy plane and it would only be possible to distinguish in-plane from out-of-plane transitions in a stretched polymer film, the latter of which are forbidden by

symmetry both in the point groups D_{3h} and C_{2v} . We can therefore only characterize the polarization of the transitions in these dyes relative to the emission dipole.

As the fluorescence anisotropy of the molecules is higher than 0.1 the symmetry of the electronic states is necessarily lowered to a point group where the axes transform uniquely, and we can attempt to separate the excitation spectra into the individual polarized components by use of the approach of Albrecht.²⁷ In order to make use of this method we have to make certain assumptions. We have to assume that the absorption and emission dipoles of the absorbing state are parallel in order to extract any useful information from the polarized spectra. We find it likely that this is at least approximately correct since the excitation anisotropy rapidly approaches the maximum value of a random ensemble of 0.4 at low energies where the overlap of the transitions is minimal. Further, we have to assume that the transition moments are orthogonal and oriented along the in-plane molecular axes i.e. that the electronic symmetry is at least C_2 or higher. An earlier computational study of $TOTA^+$ indicates that the point group is C_{2v} .^{7a}

We assume that the molecules in the glass are immobile during the excited state lifetime, and use the fundamental anisotropy eq 3 to calculate the component spectra.

$$r_0 = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad \text{eq 3.}$$

Expressing the results of Albrecht using the fundamental anisotropy rather than the polarization (see SI for derivations) we can calculate the reduced excitation spectra from the expressions in eq 4.

$$F_y = (I_{VV} + 2GI_{VH}) \times \frac{(1 + 5r_0)}{3} \quad \wedge \quad F_z = (I_{VV} + 2GI_{VH}) \times \frac{(2 - 5r_0)}{3} \quad \text{eq 4.}$$

The first factor on the right side in these equations is the isotropic excitation spectrum, and the second is essentially a unit vector along the relevant axis. Here, the z-axis is set to be parallel to the emission. The resulting purely polarized reduced spectra are shown in Figure 6. The reduced spectra of both $TOTA^+$ and $TATA^+$ show two closely overlapping spectra with a splitting of approximately $300\text{--}500\text{ cm}^{-1}$. The lower energy spectra obeys the mirror image rule when considering the observed emission, as one would expect of an emitting state without considerable structural reorganization in the excited state. The perpendicular oriented higher energy spectra show higher intensity of the second vibronic peak in the progression compared to the lower energy spectra. Note that using the Albrecht procedure on $ADOTA^+$ and $DAOTA^+$ results in reduced excitation spectra identical to the stretched film absorption spectra. These spectra can be found in the supporting information.

Discussion

Comparison of transition strengths of the triangulenium dyes is complicated by the fact that $TOTA^+$ and $TATA^+$ in principle feature degenerate transitions; although in practice they are non-degenerate but heavily overlapping. We find that the straightforward comparison of the absorption strength of these dyes is between the sums of the two lowest transitions. By integrating the relevant absorption spectra we find that these oscillator strength sums are $f_{TOTA} = 0.15$, $f_{ADOTA} = 0.17$, $f_{DAOTA} = 0.19$ and $f_{TATA} = 0.20$, i.e. a trend of increasing total oscillator strength with the number of nitrogen atoms is observed. This trend is also observed for the ca. $28,000\text{ cm}^{-1}$ band in the azatriangulenes. The energy of the weak $28,000\text{ cm}^{-1}$ band is in the range of internal charge transfer absorptions of donor substituted

anilines, and we assign the transitions in the azaoxatrianguleniums to excitations of this type. In TOTA^+ these transition cannot take place, instead the first set of strong UV transitions are red-shifted approximately $2,000\text{ cm}^{-1}$ compared to the azaoxatriangulenium dyes.

It is clear that the symmetry of the molecular framework to a large extent determines the optical properties of the dyes. The fundamental anisotropy spectra (Figure 3) show that the C_{2v} symmetric dyes ADOTA^+ and DAOTA^+ are well behaved, with a close to ideal maximum emission anisotropy of 0.38 and highly polarized orthogonal bands in excitation. The absolute directions of the transition moments in the near-UV and Vis region relative to the molecular skeleton were determined for these dyes by use of polarized absorption spectroscopy on stretched polymer films. As dictated by the molecular point group the transition moments are along the C_2 axis to a state of A_1 symmetry or in-plane perpendicular to this to a state of B_2 symmetry. In ADOTA^+ the lowest energy transition is parallel to the C_2 axis while for DAOTA^+ it is perpendicular, showing that this transition involves movement of charge from the nitrogen atoms towards the centre of the molecule. Oscillator strengths of transitions typically increase with introduction of stronger electron donors, and as nitrogen is a stronger electron donor than oxygen this also explains the considerably higher oscillator strength of the $\text{S}_0 \rightarrow \text{S}_1$ transition than the $\text{S}_0 \rightarrow \text{S}_2$ transitions which involves movement of charge from oxygen towards the centre.²⁸ The moderate oscillator strengths of the transitions in the visible range compare favourably to e.g. Dansyl, but are smaller than typical strong chromophores such as rhodamines. As the radiative deactivation rate of a chromophore is directly related to the oscillator strength of the transition moderate absorptivity is a necessary feature for long fluorescence lifetimes.

In practical applications the strong polarization and long lifetimes of ADOTA^+ and DAOTA^+ are a great advantage in providing a good dynamic range in time resolved and quenching experiments. In e.g. an 80% quenched FRET pair a 20 ns lifetime azaoxatriangulenium dye acting as a donor will have a fluorescence lifetime of 4 ns. This is easily and accurately measured even on low-end equipment. For comparison a typical rhodamine or cyanine in the same situation will have their lifetimes of approximately 4 ns and 1 ns will be reduced to 800 ps and 200 ps, respectively. Both measuring and especially differentiating such sub-nanosecond lifetimes with the typical pulsed diode/PMT set-ups found in non-specialized labs is both time-consuming and highly inaccurate. Similar observations can be made in gated detection where an azaoxatriangulenium dye would still have approximately 80% intensity with a 5 ns gate, and 60% with a 10 ns gate. For the rhodamines this would drop to approximately 25% and 8%, while the cyanine would have less than 1% emission intensity at 5 ns gate and would be almost undetectable with a 10 ns gate. We therefore find that both of the C_{2v} symmetric azaoxatriangulenium salts have great potential as biolabels and as probes for slow dynamics.

The total oscillator strength of the visible transition in the D_{3h} symmetric dyes is comparable to that of ADOTA^+ and DAOTA^+ , but they have lower emission yields and shorter lifetimes. Additionally, the absorption spectra have unusual shapes and do not obey the mirror image rule observed for most fluorophores. From group theoretical observations we expect doubly degenerate HOMO orbitals and a non-degenerate LUMO in these dyes, which would result in doubly degenerate transitions. If the D_{3h} symmetry is maintained in the dyes this degeneracy of transitions would result in maximum observed anisotropies of 0.1. Experimentally we find that this formal expectation is inconsistent with the data, as the observed anisotropies considerably exceed 0.1.

Comparison of the reduced excitation spectra of TOTA^+ and TATA^+ with those of ADOTA^+ and DAOTA^+ reveals that the band structure is fundamentally different even

though the symmetry is demonstrably lowered in the former. While the transitions in ADOTA⁺ and DAOTA⁺ are well-separated with the lower transition carrying the majority of the oscillator strength, TOTA and TATA have closely spaced transitions with comparable oscillator strengths.

The knowledge of the transition moment directions and the molecular point group allows us to build a Jablonski diagram of the observed transitions containing the symmetries of all states. The state symmetries of ADOTA and DAOTA of the four lowest energy transitions in the triangulenium dyes are shown in the diagram Figure 7.

Conclusion

We have characterized the fundamental photophysical properties of the triangulenium and azaoxatriangulenium class of fluorophores. The optical properties of the triangulenium dyes are solvent independent in both absorption and emission; however, symmetry has a large influence. The C_{2v} symmetric triangulenium dyes ADOTA⁺ and DAOTA⁺ have high quantum yields and long lifetimes, showing well-separated transitions with clear vibrational structure. The electronic transitions of these dyes are highly polarized, which combined with the long fluorescence lifetime makes them uniquely suited as e.g. labels in polymer and biology studies.^{12c, 29} The long fluorescence lifetime makes the triangulenium dyes ideal probes for studies involving gated detection and fluorescence life time microscopy (FLIM),^{6a, 6b, 30} with applications to Förster resonance energy transfer (FRET)^{1d, 31} and the high polarization as probes for studies of molecular motion of large biomolecules using anisotropies.^{6a, 32} The red emission of ADOTA⁺ and DAOTA⁺ is a further advantage in various bio-assays relative to e.g. dansyl dyes in that it is at lower energy than the typical background fluorescence seen in *in-vivo* experiments. ADOTA⁺ and DAOTA⁺ with short alkyl side chains or longer side chains with hydrophilic functional groups are water soluble with even hydrophobic counterions such as hexafluorophosphate. More lipophilic ADOTA⁺ and DAOTA⁺ derivatives are water soluble only as chloride salts.

The two formally D_{3h} symmetric triangulenium dyes TOTA⁺ and TATA⁺ have moderate emission yields and lifetimes. While the transitions are presumably orthogonal the resulting bands are heavily overlapping and therefore not practical for use in most polarization studies. Given the molecular point group they are expected to have a degenerate HOMO and should, in theory, have degenerate transitions only. In practice, it was shown that the symmetry is broken, as evidenced by the breakdown of the mirror image rule and the anisotropy spectrum of the transitions. We speculate that the symmetry is broken to either C₂ or C_{2v}, and we have classified the transitions and states under the assumption that this is the case. While TOTA⁺ and TATA⁺ are less obviously useful than ADOTA⁺ and DAOTA⁺ in fields such as biology and biochemistry, we note that TATA⁺ is highly stable towards quenching by electron donors such as amines^{7b}, and as such could find use as a red label in donor-rich environments where fluorescence quenching would otherwise preclude acceptable signal to noise ratios. Further they provide formally degenerate electronic systems where the breakdown of symmetry by e.g. solvent interactions is easily monitored by absorption and emission spectroscopy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Notes and References

- (a) Schäfer, FP.; Drexhage, KH. Dye lasers. Springer-Verlag; Berlin, New York: 1973. p. 285p x(b) Johnson I. Fluorescent probes for living cells. *Histochem.J.* 1998; 30(3):123–140. [PubMed: 10188922] (c) Stephens DJ, Allan VJ. Light microscopy techniques for live cell Imaging. *Science.* 2003; 300(5616):82–86. [PubMed: 12677057] (d) Giepmans BNG, Adams SR, Ellisman MH, Tsien RY. Review - The fluorescent toolbox for assessing protein location and function. *Science.* 2006; 312(5771):217–224. [PubMed: 16614209] (e) Lakowicz JR. Principles of Fluorescence Spectroscopy. (f) Loudet A, Burgess K. BODIPY dyes and their derivatives: Syntheses and spectroscopic properties. *Chem. Rev.* 2007; 107(11):4891–4932. [PubMed: 17924696] (g) Lavis LD, Raines RT. Bright ideas for chemical biology. *ACS Chem. Bio.* 2008; 3(3):142–155. [PubMed: 18355003] (h) McDonagh C, Burke CS, MacCraith BD. Optical chemical sensors. *Chem. Rev.* 2008; 108(2):400–422. [PubMed: 18229950] (i) Sreejith S, Carol P, Chithra P, Ajayaghosh A. Squaraine dyes: a mine of molecular materials. *J. Mater. Chem.* 2008; 18(3):264–274. (j) Ulrich G, Ziessel R, Harriman A. The chemistry of fluorescent bodipy dyes: Versatility unsurpassed. *Angew. Chem. Int. Ed.* 2008; 47(7):1184–1201.
- (a) Lewis G, Calvin M. The Color of Organic Substances. *Chem. Rev.* 1939; 25(2):273–328. (b) Martin JC, Smith RG. Factors Influencing Basicities of Triarylcarbinols Synthesis of Sesquioxanthidrol. *J. Am. Chem. Soc.* 1964; 86(11):2252–2256. (c) Seybold G, Wagenblast G. New Perylene and Violanthrone Dyestuffs for Fluorescent Collectors. *Dyes and Pigments.* 1989; 11:303–317. (d) Turro, NJ. *Modern Molecular Photochemistry.* 1 ed.. University Science Books; 1991. p. 627
- (a) Whitaker JE, Haugland RP, Prendergast FG. Spectral and Photophysical Studies of Benzo[c]xanthene Dyes: Dual Emission pH Sensors. *Anal. Biochem.* 1991; 194:330–344. [PubMed: 1862936] (b) Umezawa K, Matsui A, Nakamura Y, Citterio D, Suzuki K. Bright, Color-Tunable Fluorescent Dyes in the Vis/NIR Region: Establishment of New “Tailor-Made” Multicolor Fluorophores Based on Borondipyrromethene. *Chem. Eur. J.* 2009; 15(5):1096–1106. [PubMed: 19117043] (c) Haugland, RP.; Spence, MTZ.; Johnson, ID. *Handbook of fluorescent probes and research chemicals.* 6th ed.. Molecular Probes; Eugene, OR, USA: 1996. p. 6804849 Pitchford Ave., Eugene 97402p xii
- Seybold G, Wagenblast G. New Perylene and Violanthrone Dyestuff for Fluorescent Collector. *Dyes and Pigment.* 1989; 11(4):303–317.
- (a) Doody MA, Baker GA, Pandey S, Bright FV. Affinity and mobility of polyclonal anti-dansyl antibodies sequestered within sol-gel-derived biogels. *Chem. Mater.* 2000; 12(4):1142–1147. (b) Michl J, Eggers JH. Polarization Spectra in Stretched Polymer Sheets 4: Benzo[k]Fluoranthene and its Aza Analogs. *Tetrahedron.* 1974; 30:813–817.
- (a) Suhling K, Siegel J, Lanigan PMP, Leveque-Fort S, Webb SED, Phillips D, Davis DM, French PMW. Time-resolved fluorescence anisotropy imaging applied to live cells. *Opt. Lett.* 2004; 29(6): 584–586. [PubMed: 15035478] (b) Squire A, Bastiaens PIH. Three dimensional image restoration in fluorescence lifetime imaging microscopy. *J. Microsc.-Oxf.* 1999; 193:36–49. (c) Mohandessi S, Rajendran M, Magda D, Miller LW. Cell-Penetrating Peptides as Delivery Vehicles for a Protein-Targeted Terbium Complex. *Chem. Eur. J.* 2012; 18(35):10825–10829. [PubMed: 22807190] (d) Beeby A, Botchway SW, Clarkson IM, Faulkner S, Parker AW, Parker D, Williams JAG. Luminescence imaging microscopy and lifetime mapping using kinetically stable lanthanide(III) complexes. *J. Photochem. Photobio. B-Bio.* 2000; 57(2-3):83–89.
- (a) Reynisson J, Wilbrandt R, Brinck V, Laursen BW, Norgaard K, Harrit N, Brouwer AM. Photophysics of trioxatriangulenium ion. Electrophilic reactivity in the ground state and excited singlet state. *Photochem. Photobio. Sci.* 2002; 1(10):763–773. (b) Dileesh S, Gopidas KR. Photoinduced electron transfer in azatriangulenium salts. *J. Photochem. Photophys. A-Chem.* 2004; 162(1):115–120.

8. (a) Reynisson J, Schuster GB, Howerton SB, Williams LD, Barnett RN, Cleveland CL, Landman U, Harrit N, Chaires JB. Intercalation of trioxatriangulenium ion in DNA: Binding, electron transfer, X-ray crystallography, and electronic structure. *J. Am. Chem. Soc.* 2003; 125(8):2072–2083. [PubMed: 12590535] (b) Pothukuchy A, Mazzitelli CL, Rodriguez ML, Tuesuwan B, Salazar M, Brodbelt JS, Kerwin SM. Duplex and quadruplex DNA binding and photocleavage by trioxatriangulenium lone. *Biochem.* 2005; 44(6):2163–2172. [PubMed: 15697242]
9. (a) Laursen BW, Krebs FC. Synthesis of a triazatriangulenium salt. *Angew. Chem. Int. Ed.* 2000; 39(19):3432–3434. (b) Laursen BW, Krebs FC. Synthesis, structure, and properties of azatriangulenium salts. *Chem. Eur. J.* 2001; 7(8):1773–1783. [PubMed: 11349920]
10. Dileesh S, Gopidas KR. Photophysical and electron transfer studies of a stable carbocation. *Chem. Phys. Lett.* 2000; 330(3-4):397–402.
11. Nicolas C, Lacour J. Triazatriangulenium cations: Highly stable carbocations for phase-transfer catalysis. *Org. Lett.* 2006; 8(19):4343–4346. [PubMed: 16956222]
12. (a) Bando Y, Sakamoto S, Yamada I, Haketa Y, Maeda H. Charge-based and charge-free molecular assemblies comprising pi-extended derivatives of anion-responsive acyclic oligopyrroles. *Chem. Commun.* 2012; 48(17):2301–2303. (b) Baisch B, Raffa D, Jung U, Magnussen OM, Nicolas C, Lacour J, Kubitschke J, Herges R. Mounting Freestanding Molecular Functions onto Surfaces: The Platform Approach. *J. Am. Chem. Soc.* 2009; 131(2):442–+. [PubMed: 19113847] (c) Inglese J, Johnson RL, Simeonov A, Xia MH, Zheng W, Austin CP, Auld DS. High-throughput screening assays for the identification of chemical probes. *Nature Chem. Bio.* 2007; 3(8):466–479. [PubMed: 17637779] (d) Haketa Y, Sasaki S, Ohta N, Masunaga H, Ogawa H, Mizuno N, Araoka F, Takezoe H, Maeda H. Oriented Salts: Dimension-Controlled Charge-by-Charge Assemblies from Planar Receptor-Anion Complexes. *Angew. Chem. Int. Edit.* 2010; 49(52):10079–10083.
13. (a) Laursen BW, Krebs FC, Nielsen MF, Bechgaard K, Christensen JB, Harrit N. 2,6,10-Tris(dialkylamino)trioxatriangulenium ions. Synthesis, structure, and properties of exceptionally stable carbenium ions. *J. Am. Chem. Soc.* 1998; 120(47):12255–12263. (b) Laursen BW, Reynisson J, Mikkelsen KV, Bechgaard K, Harrit N. 2,6,10-tris(dialkylamino)trioxatriangulenium salts: a new promising fluorophore. Ion-pair formation and aggregation in non-polar solvents. *Photochem. Photobio. Sci.* 2005; 4(8):568–576. (c) Sørensen TJ, Laursen BW. Synthesis and Optical Properties of Trioxatriangulenium Dyes with One and Two Peripheral Amino Substituents. *J. Org. Chem.* 2010; 75(18):6182–6190. [PubMed: 20738142]
14. Laursen BW, Sørensen TJ. Synthesis of Super Stable Triangulenium Dye. *J. Org. Chem.* 2009; 74(8):3183–3185. [PubMed: 19281195]
15. Hammershoj P, Sørensen TJ, Han BH, Laursen BW. Base-Assisted One-Pot Synthesis of N,N',N''-Triaryltriaxatriangulenium Dyes: Enhanced Fluorescence Efficiency by Steric Constraints. *J. Org. Chem.* 2012; 77(13):5606–5612. [PubMed: 22616844]
16. Westerlund F, Hildebrandt CB, Sørensen TJ, Laursen BW. Trihydroxytrioxatriangulene-An Extended Fluorescein and a Ratiometric pH Sensor. *Chem-Eur J.* 2010; 16(10):2992–2996. [PubMed: 20146272]
17. Westerlund F, Elm J, Lykkebo J, Carlsson N, Thyrhaug E, Akerman B, Sørensen TJ, Mikkelsen KV, Laursen BW. Direct probing of ion pair formation using a symmetric triangulenium dye. *Photoch Photobio Sci.* 2011; 10(12):1963–1973.
18. (a) Browne WR, Vos JG. The effect of deuteration on the emission lifetime of inorganic compounds. *Coordin Chem Rev.* 2001; 219:761–787. (b) Lo KKW, Hui WK, Chung CK, Tsang KHK, Lee TKM, Li CK, Lau JSY, Ng DCM. Luminescent transition metal complex biotin conjugates. *Coordin. Chem. Rev.* 2006; 250(13-14):1724–1736.
19. Sørensen TJ, Laursen BW, Luchowski R, Shtoyko T, Akopova I, Gryczynski Z, Gryczynski I. Enhanced fluorescence emission of Me-ADOTA(+) by self-assembled silver nanoparticles on a gold film. *Chem. Phys. Lett.* 2009; 476(1-3):46–50. [PubMed: 20161182]
20. Rich RM, Stankowska DL, Maliwal BP, Sørensen TJ, Laursen BW, Krishnamoorthy RR, Gryczynski Z, Borejdo J, Gryczynski I, Fudala R. Elimination of autofluorescence background from fluorescence tissue images by use of time-gated detection and the AzaDiOxaTriAngulenium (ADOTA) fluorophore. *Anal. Bioana.l Chem.* 2013; 405(6):2065–2075.

21. Rich RM, Mummert M, Gryczynski Z, Borejdo J, Sørensen TJ, Laursen BW, Foldes-Papp Z, Gryczynski I, Fudala R. Elimination of autofluorescence in Fluorescence Correlation Spectroscopy by using the AzaDiOxaTriAngulenium (ADOTA) fluorophore in combination with time correlated single photon counting (TCSPC). *Analytical and Bioanalytical Chemistry*, in review.
22. Sørensen TJ, Thyrhaug E, Szabelski M, Gryczynski I, Gryczynski Z, Laursen BW. Azadioxatriangulenium (ADOTA⁺): A long fluorescence lifetime fluorophore for large biomolecule binding-assay. *Methods Appl. Fluoresc.* accepted.
23. Thulstrup, EW.; Michl, J. *Elementary Polarization Spectroscopy*. VCH Publishers, Inc.; New York: 1989.
24. Note that although the azaoxatriangulenium cations do not have solvent sensitive transitions, the high-symmetry para-substituted triangulenium derivatives can exhibit large solvent induced spectral changes. However, these changes are attributed to ion pairing between the chromophore and its counter-ion and aggregation, rather than solvent sensitivity of the solvated chromophore itself. While some changes in the spectral profiles on solvent change can be observed for both TATA⁺ and TOTA⁺, these are modest compared to what is observed for the para-substituted triangulenium dyes and is indicative of changes in specific solvent-solute interactions rather than ion pairing.
25. Thulstrup EW, Michl J, Eggers JH. Polarization Spectra in Stretched Polymer Sheets .2. Separation of Pi-Pi* Absorption of Symmetrical Molecules into Components. *J. Phys. Chem.* 1970; 74(22): 3868.
26. (a) Michl J, Thulstrup EW, Eggers JH. Polarization Spectra in Stretched Polymer Sheets .3. Physical Significance of Orientation Factors and Determination of Pi-Pi* Transition Moment Directions in Molecules of Low Symmetry. *J. Phys. Chem.* 1970; 74(22):3878.(b) Langkilde FW, Thulstrup EW, Michl J. The Effect of Solvent Environment on Molecular Electronic-Transition Moment Directions - Symmetry Lowering in Pyrene. *J. Chem. Phys.* 1983; 78(6):3372–3381.
27. Albrecht AC. Polarizations and Assignments of Transitions - Method of Photoselection. *J. Mol. Spectrosc.* 1961; 6(1):84.
28. Griffiths, J. *Colour and Constitution of Organic Molecules*. Academic Press; 1976.
29. (a) Pope AJ, Haupts UM, Moore KJ. Homogeneous fluorescence readouts for miniaturized high-throughput screening: theory and practice. *Drug Discov. Today*. 1999; 4(8):350–362. [PubMed: 10431145] (b) Owicki JC. Fluorescence polarization and anisotropy in high throughput screening: Perspectives and primer. *J. Biomol. Screen.* 2000; 5(5):297–306. [PubMed: 11080688]
30. Brismar H, Trepte O, Ulfhake B. Spectra and Fluorescence Lifetimes of Lissamine Rhodamine, Tetramethylrhodamine Isothiocyanate, Texas Red, and Cyanine 3.18 Fluorophores: Influences of Some Environmental Factors Recorded with a Confocal Laser Scanning Microscope. *Journal of Histochemistry and Cytochem.* 1995; 43:699–707.
31. (a) Forster T. Zwischenmolekulare Energiewanderung und Fluoreszenz. *Ann. Phys. Berlin.* 1948; 2(1-2):55–75.(b) Zhuang XW, Kim H, Pereira MJB, Babcock HP, Walter NG, Chu S. Correlating structural dynamics and function in single ribozyme molecules. *Science*. 2002; 296(5572):1473–1476. [PubMed: 12029135] (c) Adams SR, Harootunian AT, Buechler YJ, Taylor SS, Tsien RY. Fluorescence Ratio Imaging of cyclic AMP in Single Cells. *Nature*. 1991; 349:694–697. [PubMed: 1847505]
32. Forkey JN, Quinlan ME, Shaw MA, Corrie JET, Goldman YE. Three-dimensional structural dynamics of myosin V by single-molecule fluorescence polarization. *Nature*. 2003; 422(6930): 399–404. [PubMed: 12660775]

Figure 1.

The molecular structure of the nitrogen and oxygen containing triangulenium dyes. Top row, from left: **TOTA⁺**, **ADOTA⁺**, **DAOTA⁺**, and **TATA⁺**. Bottom row from left: **A-TOTA⁺**, **A-ADOTA⁺**, and **H-TOTA** (all triangulenium dyes with donor substituents in the para positions).

Figure 2. Absorption (full line) and emission (dashed line) spectra of the triangulenium dyes in acetonitrile solution.

Figure 3.

Excitation and emission spectra of ADOTA⁺ (top) and DAOTA⁺ (bottom) in a glycerol glass at 190 K. Excitation anisotropy spectra (565 nm and 590 nm detection wavelength respectively) and emission anisotropy spectra (525 nm and 540 nm excitation wavelength respectively) are superimposed on the excitation and emission spectra.

Figure 4. Reduced absorption spectra of ADOTA⁺ (top) and DAOTA⁺ (bottom) in stretched PVA film. The molecular z-axis is defined to be parallel to the stretching direction.

Figure 5. Isotropic excitation and emission spectra and fundamental anisotropies of TOTA⁺ (top) and TATA⁺ (bottom) in glycerol glass at 190 K.

Figure 6. Reduced excitation spectra of TOTA⁺ (top) and TATA⁺ (bottom) in glycerol glass at 190 K. The emission dipole is arbitrarily set to be parallel to the z-axis.

Figure 7.

Jablonski diagram describing the first 4 transitions in the triangulenium dyes. The degenerate transitions of TOTA⁺ and TATA⁺ are split, and the states are here designated under the assumption that the nitrogen/oxygen on the remaining axis of symmetry is the stronger electron donor (ADOTA-like). If the donor strength is weaker the labels on the states will be reversed (DAOTA-like)

Table 1

Collected optical properties of the triangulenium dyes in acetonitrile solution

	TOTA⁺	ADOTA⁺	DAOTA⁺	TATA⁺
$\sim_{abs} \nu$	20800 cm ⁻¹	18490 cm ⁻¹	17950 cm ⁻¹	19200 cm ⁻¹
λ_{abs}	481 nm	541 nm	557 nm	521 nm
ϵ_{max}	8580 M ⁻¹ cm ⁻¹	9840 M ⁻¹ cm ⁻¹	15940 M ⁻¹ cm ⁻¹	15200 M ⁻¹ cm ⁻¹
f^b	0.15/0.08	0.17/0.12	0.19/0.14	0.20/0.10
$\sim_{fl} \nu$	19080 cm ⁻¹	17870 cm ⁻¹	16960 cm ⁻¹	17940 cm ⁻¹
λ_{em}	524 nm	560 nm	590 nm	557 nm
Φ_{fl}^a	0.11	0.49	0.44	0.16
Δ_{Stokes}	1720 cm ⁻¹	620 cm ⁻¹	990 cm ⁻¹	1260 cm ⁻¹
τ_{fl}	13.8 ns	23.2 ns	19.4 ns	7.5 ns
k_f^c	0.80	2.11	2.27	2.13
k_{nr}^c	6.44	2.20	2.89	11.2

^aQuantum yield measured against Rhodamine 101 and TOTA.^bIntegrated over the two lowest absorption bands (left), and integrated over the z-polarized absorption only (right). See text for details^cin units of 10⁷ s⁻¹