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Azadioxatriangulenium: Synthesis and Photophysical Properties of Reactive Dyes for Bioconjugation

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Abstract

Azadioxatriangulenium (ADOTA) is a fluorescent triangulenium dye with a long fluorescence lifetime, highly polarized transitions and emission in the red part of the visible spectrum. These properties make the chromophore suited for application in fluorescence polarization/anisotropy assay. To be useful for these applications, reactive forms of the dyes must be available in significant quantities. Here, the synthesis and photophysical properties of amine-reactive NHS esters and a thiol-reactive maleimide derivative of ADOTA are reported. The synthesis involves two steps of nucleophilic bridge forming reactions starting from tris(2,6-dimethoxyphenyl) methylum tetrafluoroborate, which can readily be made on 100 gram scale. In the third and final step the reactive NHS or maleimide groups are formed. The beneficial photophysical properties of the ADOTA chromophore are maintained in these derivatives, and we conclude that these systems are ideal to study protein motion and protein-protein interactions for systems of up towards 1000 kDa.

Keywords

Fluorescent dyes; Bioconjugate chemistry; Triangulenium; Nucleophilic aromatic substitution; Organic synthesis

Introduction

Fluorescent dyes are an essential tool in elucidating biological structure and function^[1] and an impressive catalogue of dyes is available for imaging and assay.^[2] However, among these dyes there are several limitations. One is the lack of red-emitting probes with fluorescence lifetimes in the range of 10 – 40 ns. This particular property is essential in monitoring the rotational motion of biomolecules with a molecular weight of 10 – 1000 kDa.^[3] While transition metal complexes with long emission lifetimes are available,^[4] these suffer from low brightness and an inherent low photon flux.^[5] Here, we report on the synthesis and optical properties of a series of azadioxatriangulenium (ADOTA) derivatives for

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bioconjugation with a fluorescence lifetime of up to 22 ns and a fluorescence quantum yield of up to 70 %.

ADOTA is a triangulenium dye,^[6] a group of cationic fluorescent dyes with unique properties.^[7] One characteristic property of the ADOTA chromophore is the rare combination of relatively low oscillator strengths of the emissive transition and high fluorescence quantum yields, which gives rise to fluorescence lifetime of 10 – 20 ns, despite the relatively low energy gap between the ground state and the first excited state.^[8] The result is an efficient long fluorescence lifetime fluorophore with emission in the red part of the visible spectrum.

We and others have used the long fluorescence lifetime of ADOTA to study metal enhanced emission,^[9] and to demonstrate the power of time-gated or fluorescence lifetime imaging (FLIM).^[10] In either application, the long fluorescence lifetime enables efficient suppression of autofluorescence and clear identification of probe signal. Similarly, the long fluorescence lifetime allows for lifetime based sensing of biorelevant species and determination of material related properties.^[11] The electronic transitions in the azaoxatriangulenium dyes are highly polarized,^[8] which in combination with the long fluorescence lifetime make the dyes ideally suited for fluorescence polarization (FP) or fluorescence anisotropy (FA) assays, or simply ideally suited to monitor rotational motion of large biomolecules.^[12] In order to fully explore these applications of ADOTA dyes, reactive derivatives for bioconjugation have to be readily available. We have previously reported an amino-reactive NHS ester of ADOTA, with the reactive NHS group linked via a flexible propyl linker, **1-NHS** (Scheme 1).^[10a, 12] Here, we report the synthesis of three new reactive derivatives, two amino-reactive NHS esters with semi-rigid and rigid linkers and one derivative with a rigid linked thiol-reactive maleimide group. In particular for FP and FA assays rigidity of the linker may play a critical role, since flexible linkers may cause loss of anisotropy due to fast local motions.^[13] NHS esters and maleimides constitute two of the most important groups of reagents for bioconjugation and will allow application of the unique long fluorescence lifetime ADOTA dye in a very broad range of bioimaging and assays.^[14] The reactive ADOTA derivatives were made in overall yields of up to 45 %, and it was documented that the attractive photophysical properties are conserved in the reactive derivatives.

Results and Discussion

Synthesis

Four derivatives of azadioxatriangulenium were synthesized following optimized procedures based on the original synthesis of azaoxatriangulenium salts.^[7b] The tris(2,6-dimethoxyphenyl) methylum tetrafluoroborate (**5**·BF₄) starting material was synthesized as reported by Martin and Smith,^[15] a synthetic approach amenable to up-scaling to 250 gram by replacing recrystallization of the carbinol intermediate from pure ether with recrystallization from 1:1 dichloromethane/ether. Reacting **5**·BF₄ with primary amines in acetonitrile with a suitable base catalyst result in quantitative formation of the tetramethoxy-acridine structures **6–9**·BF₄. The primary amine reacts with **5**·BF₄ in two subsequent nucleophilic aromatic substitutions resulting in the formation of an aza bridge. The purification of the resulting acridinium salts can be tedious, causing varying yields of the

isolated products.^[7b, 16] For primary amines carrying functional groups, the purification may be complicated by reaction of these. In strongly ether cleaving conditions, such as molten pyridinium chloride, the tetramethoxy-acridinium salts **6-9·BF₄** undergo two additional intramolecular ring-closing reactions, thus generating the azadioxatriangulenium chromophores **1-4·BF₄**.

Scheme 2 shows the synthesis of *N*-(3-carboxypropyl)- azadioxatriangulenium tetrafluoroborate **1·BF₄** and the corresponding NHS ester: **1-NHS·BF₄**. While these compounds were previously reported in account reporting of bio-relevant experiments,^[10a, 12] the synthesis was repeated and optimized as part of this work. The first reaction is between **5·BF₄** and the methyl ester of 4-aminobutanoic acid. It is worth noting that the methyl ester is used due to the low solubility of the free acid. The product: 9-(2,6-dimethoxyphenyl)-10-(3-carboxypropyl)-acridinium tetrafluoroborate **6·BF₄** was isolated as red flaky crystals by recrystallization from methanol in a yield of 67 %. By dissolving **6·BF₄** in molten pyridinium chloride at 200°C the target molecule *N*-(3-carboxypropyl)- azadioxatriangulenium tetrafluoroborate **1·BF₄** was synthesized, and isolated as a red powder upon recrystallization from acetonitrile/methanol in a yield of 60 %. The active ester **1-NHS·BF₄** was synthesized by reaction of **1·BF₄** with *N,N,N',N'*-Tetramethyl-*O*-(*N*-succinimidyl)uranium tetrafluoroborate (TSTU) in dimethyl sulfoxide in the presence of diisopropylethylamine. Repeated precipitations of **1-NHS·BF₄** with diethyl ether from acetonitrile solution afforded the desired product as a red powder in a yield of 83 %, corresponding to a total overall yield of 33 %.

Scheme 3 shows the synthesis of *N*-(2-(4-carboxyphenyl)-ethyl)- azadioxatriangulenium tetrafluoroborate **2·BF₄** and *N*-(4-carboxyphenyl)-azadioxatriangulenium tetrafluoroborate **3·BF₄**, which proceed analogous to the synthesis of **1·BF₄**. However, while the primary amine used in the synthesis of **7·BF₄** react instantaneously with **5·BF₄** to produce **7·BF₄** in 78 % isolated yield, the synthesis of **3·BF₄** requires elaborate reaction temperatures and the use of 2,6-lutidine as base as reported by Krebs^[17] due to the lower nucleophilicity of the aniline as compared to the primary amines used in the synthesis of **1·BF₄** and **7·BF₄**. The compound 9-(2,6-dimethoxyphenyl)-10-(4-carboxyphenyl)-acridinium tetrafluoroborate **8·BF₄** was isolated in a low yield of 18 %.

The ring closing reactions of the acridinium salts to the ADOTA dyes are performed in molten pyridinium chloride as described by Laursen and Krebs.^[7] For the methyl ester substituted acridinium salts (**6·BF₄** and **8·BF₄**), quenching of the pyridinium chloride reactions with water causes ester hydrolysis, yielding the desired free acid functionalized ADOTA dyes. Reaction of the free acids with TSTU in dimethyl sulfoxide results in the formation of the NHS esters in yields of 70–80 %.

The maleimide substituted ADOTA derivative *N*-(4-maleimido-phenyl)- azadioxatriangulenium tetrafluoroborate **4·BF₄** was synthesized from an amino functionalized dye. Compound **5·BF₄** was reacted with 1,4-phenylenediamine as shown in Scheme 4. Despite the low reactivity of the aniline, 9-(2,6-dimethoxyphenyl)-10-(4-aminophenyl)-acridinium tetrafluoroborate **9·BF₄** was isolated in 89 % yield, by using a procedure where **5·BF₄** is added drop-wise to a concentrated (1M) solution of the aniline in

acetonitrile. The ring-closing reaction of **9**·BF₄ to yield **10**·BF₄ was performed in molten pyridinium chloride followed by neutralization with NaOH solution and proceeded in 91 % yield after consecutive recrystallizations. The maleimide was prepared using the standard procedure of reacting an amine with maleic anhydride,^[18] in this case in acetonitrile and the presence of triethylamine. The resultant intermediate maleanilic acid is then heated in a mixture of acetic acid anhydride and sodium acetate to form the maleimide derivative **4**·BF₄ in 76 % yield (Scheme 4).

Photophysics

A long fluorescence lifetime is important when probing rotational motions, as a dye has to emit photons for the duration of the rotation that is to be followed. Using the Perrin equation (Eq. 1) this notion can be translated to the observed fluorescence anisotropy (*r*), see Figure 1.^[4a, 19] The Perrin equation describes the relationship between: the observed anisotropy (*r*), the fundamental anisotropy (*r*₀), the rotational correlation time (*θ*), and the fluorophore luminescence lifetime (*τ*). The second half of Eq. 1 describes the relationship between the rotational correlation time (*θ*), the rotational volume (*V*) and the molecular mass (*M*).

$$r_0/r = 1 + \tau/\theta, \text{ where } \theta = \eta V / RT = \eta M / RT \cdot (\nu + h) \quad \text{Eq. 1}$$

A short (1 ns) fluorescence lifetime probe will be able to monitor the rotational motion of a biomolecule with a total molecular weight of 10 kDa, while standard rhodamine fluorophores with 4 ns fluorescence lifetimes are useful for monitoring the rotational motion of a biomolecule with a weight of 100 kDa. A dye with a 20 ns fluorescence lifetime will be able to extend this range to 1000 kDa (Figure 1). In a FP/FA assay the change in molecular weight of the bound and free state should be on the order of $| \Delta r | = 0.2$, for the optimal detection efficiency. For a dye with a 20 ns fluorescent lifetime this could be achieved by a 10 kDa conjugate binding to a > 100 kDa receptor or a 50 kDa conjugate binding to a > 1000 kDa receptor. Red emitting dyes have not been available up to this point to study this highly important interval biomolecule interactions using fluorescence polarization/fluorescence anisotropy.^[4–5, 20] In order to verify that the linkers and functional groups did not perturb the attractive photophysical properties of the reactive dyes, extensive photophysical characterization was performed.

The absorption, fluorescence excitation, and fluorescence emission spectra were recorded for the ADOTA dyes **1–4**·BF₄ in acetonitrile, dimethyl sulfoxide and phosphate buffered saline (PBS, pH = 7.3). Acetonitrile was chosen as it is the benchmark solvent used for triangulenium dyes,^[7, 8] while both DMSO and PBS are highly relevant for bioconjugate chemistry. The spectral characteristics in the three solvents are compiled in Table 1, and the absorption and fluorescence emission spectra are shown in figure 2. For the reactive NHS esters **1**-NHS·BF₄, **2**-NHS·BF₄ and **3**-NHS·BF₄, the properties of the free acids were determined. No differences in the photophysical properties as compared to ADOTA is expected in the presence of the ester, amide or free acid groups, as these are substituted at a remote positions in the molecular structure, where substitutions do not alter the electron donor properties of the aryl substituent. The maleimide **4**·BF₄, is a different case as a free aniline (as found in compound **10**) is expected to quench the ADOTA fluorescence. The two

decoupled electronic systems undergo photoinduced electron transfer (PET) quenching. The complete decoupling is a result of the $\sim 90^\circ$ twist angle between the systems.^[17, 21]

Therefore, the photophysical properties of the maleimide are characterized carefully to establish if PET quenching affects its fluorescence properties. The specific reactivity of the maleimide towards thiolate groups is easy to control compared to the hydrolysis of the NHS ester, and did not pose a problem in the photophysical studies.

When comparing the absorption (λ_{max}) and emission (λ_{fl}) maxima of the ADOTA derivatives **1–4**·BF₄ (Table 1), it is found that they change only a few nanometre when comparing the four derivatives and that only a very limited solvatochromism is observed. There is a slight difference in the vibronic progression seen in the fluorescence excitation and absorption spectra, which are otherwise identical. The spectral differences between the derivatives are primarily seen in the vibronic progression and a slight shift in the emission maximum (Figure 2). The molar absorption coefficients (ϵ) are within the experimental error the same for all derivatives (Table 1).

The very small solvatochromic effects and minor spectral variations between the ADOTA dyes with different linker/reactive groups implies that the chromophore can be expected to provide similar optical output in very different bioconjugates and environments. It is noticed that **2**·BF₄ has limited solubility in acetonitrile. In all three solvents the fluorescence quantum yields (ϕ_{fl}) are high (55–70 %), and the fluorescence lifetime decays (τ_{fl}) are monoexponential and long (17 – 23 ns). The time-resolved fluorescence decay curves are shown in Figure 3 and the numbers are compiled in Table 1. All data can be found in Supporting Information. In DMSO and PBS solutions the quantum yields and fluorescence lifetimes are reduced slightly as compared to that in acetonitrile, indicating a moderate increase in the rate of internal conversion in the most polar solvents. These observations confirm that the ADOTA chromophore is quite robust to perturbations from the environment.

The reactive ADOTA derivatives behave as very good and robust fluorophores. The unique long fluorescence lifetime (~ 20 ns) is maintained in all the reactive derivatives and across different solvents. Therefore, the reactive forms ADOTA should be excellent molecular probes for fluorescence polarization based experiments.

Conclusions

Four reactive forms of the azadioxatriangulenium (ADOTA) chromophore were synthesized and their photophysical properties were characterized. Amino-reactive NHS esters were made from carboxylic acids with three linkers of variable rigidity. A thiol-reactive maleimide was made from a rigid aniline derivative of ADOTA. The optical properties of all four derivatives were characterized in three different solvents. The absorption and fluorescence properties were found to be practically invariant in all the solvents and with the presence of the linkers. Most importantly, the high quantum yields and long fluorescent lifetime were found to be conserved for all compound in all solvents. We conclude that the new reactive ADOTA dyes are ideally suited as molecular probes for fluorescence polarization based experiments and time-gated imaging.

Experimental Section

Unless otherwise stated, all starting materials were obtained from commercial suppliers and used as received. Solvents were of HPLC grade for reactions and recrystallizations and technical grade for column chromatography and were used as received. ^1H NMR and ^{13}C NMR spectra were recorded on a 500 MHz or a 400 MHz instrument (500/400 MHz for ^1H NMR and 126/101 MHz for ^{13}C NMR). Proton chemical shifts are reported in ppm downfield from tetramethylsilane (TMS) and carbon chemical shifts in ppm downfield of TMS, using the resonance of the residual solvent peak as internal standard. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were recorded on a vertical reflector instrument using dithranol as matrix. Dry column vacuum chromatography was performed on Kieselgel 60 (0.015–0.040 mm particle size). Thin layer chromatography was carried out using aluminum sheets pre-coated with silica gel 60F. Absorption spectroscopy was routinely recorded with a double-beam spectrophotometer using the pure solvent as baseline. Steady state fluorescence spectra were recorded with a standard L-configuration fluorimeter equipped with single grating monochromators. The absorbance at the excitation wavelength was kept below 0.1 to avoid inner filter effects and intermolecular interactions. All solvents used for spectroscopic experiments were of spectroscopic grade. Molar absorption coefficients were determined for each of the ADOTA dyes **1–4·BF₄** using Lambert-Beers law by measuring the absorption spectrum of three stock solutions with different concentrations of the same dye.

Quantum yields were determined using the relative method,^[22] using rhodamine 6G as standard.^[23] For all measurements the absorption was kept below 0.1 over the long wavelength absorption band, with respect to the excitation wavelength. The settings for both absorption and emission were the same for every fluorescence quantum yield measurement to ensure comparability. The emission signal was measured with a PMT detector with a spectral range of 300–900 nm. Compounds **1–3·BF₄** were excited at 510 nm using a solid-state laser excitation source, while **4·BF₄** was excited at 500 nm using a Xe-lamp excitation source. Magic angle settings were used for the emission measurements and the emission spectra were corrected for the wavelength dependent response of the detection system before use in the fluorescence quantum yield determinations. The fluorescence quantum yields were finally determined from the slopes of the linear fits, when the integrated fluorescence intensity was plotted as a function of the fraction of absorbed light. All quantum yield plots are shown in Supporting Information.

Fluorescence lifetimes were measured using FluoroTime 300 (PicoQuant, Berlin, Germany) system. The emission signal was measured with a PMT detector with a spectral range of 300–900 nm. Compounds **1–4·BF₄** were excited at 510 nm using a solid-state laser excitation source. The instrument response function was recorded at the excitation wavelength using dilute solution of Ludox®. The fluorescence decays were analyzed using the FluoFit software package version. The decay data were all found to be monoexponential and was fitted by iterative reconvolution with a single exponential

$$I_f(t) = \alpha \exp(-t/\tau) \quad (1)$$

In Eq. 1 α is the amplitude and τ is the fluorescence lifetime. All time-resolved emission decay profiles and fits are shown in Supporting Information.

N-(3-carboxypropyl)-azadioxatriangulenium tetrafluoroborate **1.BF₄** Pyridinium chloride (15 g) was stirred in a round bottom flask (250 ml) at 200 °C for 30 minutes. When sublimation on the inside of the flask was observed **6.BF₄** (1.01 g, 1.84 mmol) was added and stirred for 30 min. The reaction was quenched with sodium tetrafluoroborate solution (0.2 M, 0.2 L) and cooled to ambient temperature. The formed precipitate was filtered off and washed thoroughly with water, cold 2-propanol and diethyl ether to yield a crude product. Recrystallisation from methanol/acetonitrile, washing with cold methanol and drying in vacuum yielded the pure product **1.BF₄** as red crystals (0.495g 60%). **¹H NMR** (500 MHz, DMSO-*d*₆) δ 12.37 (s, 1H), 8.44 (t, J = 8.9 Hz, 2H), 8.17 (t, J = 8.4 Hz, 1H), 8.13 (d, J = 8.9 Hz, 2H), 7.69 (d, J = 8.1 Hz, 2H), 7.62 (d, J = 8.4 Hz, 2H), 4.88 – 4.82 (m, 2H), 2.63 (t, J = 6.9 Hz, 2H), 2.11 – 2.04 (m, 2H). **¹³C NMR** (126 MHz, DMSO-*d*₆) δ 174.08, 152.54, 151.75, 140.76, 140.42, 140.12, 139.71, 111.50, 110.40, 109.10, 108.61, 105.50, 47.39, 29.98, 21.46. **Anal. Calcd.** for C₂₃H₁₆BF₄NO₄: C, 60.42; H, 3.53; N, 3.06; found: C, 60.46; H, 3.29; N, 2.98 **HRMS (MALDI-TOF)**: m/z calcd. for C₂₃H₁₆NO₄⁺, 370.1074; found, 370.1075.

N-(3-carboxypropyl)-azadioxatriangulenium tetrafluoroborate NHS ester **1-NHS.BF₄**: (0.09 g, 0.2 mmol) and *N,N,N,N*-Tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU) (0.09 g, 0.3 mmol) were dissolved in DMSO (6 mL) and triethylamine (0.05 mL, 0.3 mmol) was added. The solution was stirred at ambient temperature overnight. The product was precipitated with sodium tetrafluoroborate solution (0.2 M, 0.15 L), filtered off and washed with water to give a crude material. Threefold precipitation of the compound from a solution of acetonitrile with diethyl ether and drying in vacuum yielded pure **1-NHS.BF₄** as red powder (0.09 g, 83%). **¹H NMR** (500 MHz, Acetonitrile-*d*₃) δ 8.35 (t, J = 8.3 Hz, 2H), 8.12 (t, J = 8.4 Hz, 1H), 7.88 (d, J = 8.9 Hz, 2H), 7.57 (d, J = 8.2 Hz, 2H), 7.50 (d, J = 8.4 Hz, 2H), 4.84 – 4.80 (m, 2H), 3.02 (t, J = 6.9 Hz, 2H), 2.81 (s, 4H), 2.40 – 2.33 (m, 2H). **¹³C NMR** (126 MHz, Acetonitrile-*d*₃) δ 171.10, 169.68, 154.28, 153.40, 142.05, 142.01, 141.58, 140.93, 112.60, 111.06, 110.46, 109.84, 106.59, 48.19, 28.58, 26.44, 22.07. **Anal. Calcd.** for C₂₇H₁₉BF₄N₂O₆: C, 58.51; H, 3.46; N, 5.05; found: C, 57.99; H, 3.03; N, 4.97 **MS (MALDI-TOF)**: m/z calcd. for C₂₇H₁₉N₂O₆⁺: 467.12; found 467.1

N-(2-(4-carboxyphenyl)-ethyl)-azadioxatriangulenium tetrafluoroborate **2.BF₄**: Pyridinium chloride was melted at 200 °C and **7.BF₄** (0.3 g, 0.429 mmol) was added to the solution which was stirred for 90 min. The product was precipitated by addition of sodium tetrafluoroborate solution (0.2 M, 0.3 L) and after cooling to ambient temperature filtrated, washed with water and dried. The crude material was recrystallized from acetonitrile and dried in vacuum to give pure **2.BF₄** as red crystals (0.187 g, 84% yield). **¹H NMR** (500 MHz, DMSO-*d*₆) δ 12.95 (s, 1H), 8.37 (t, J = 8.5 Hz, 2H), 8.21 (t, J = 8.4 Hz, 1H), 8.02 (d, J = 9.1 Hz, 2H), 7.84 (d, J = 7.9 Hz, 2H), 7.69 (d, J = 8.2 Hz, 2H), 7.66 (d, J = 8.4 Hz, 2H),

7.51 (d, $J = 7.9$ Hz, 2H), 5.15 (t, $J = 7.8$ Hz, 2H), 3.30 – 3.27 (m, 2H). **^{13}C NMR** (126 MHz, DMSO- d_6) δ 167.18, 152.56, 151.83, 140.67, 139.93, 139.76, 129.52, 129.38, 111.49, 110.62, 109.13, 108.57, 105.66, 103.22, 103.16, 99.49, 48.36, 32.03. **Anal. calcd.** for $\text{C}_{28}\text{H}_{18}\text{BF}_4\text{NO}_4$: C, 64.77; H, 3.49; N, 2.70; found, C, 65.06; H, 3.42; N, 2.56 **HRMS (MALDI-TOF)**: m/z calcd. for $\text{C}_{28}\text{H}_{18}\text{NO}_{4+}$: 432.1230; found, 432.1230

N-(2-(4-carboxyphenyl)-ethyl)-azadioxatriangulenium tetrafluoroborate NHS ester **2-NHS.BF₄**: (0.1 g, 0.2 mmol) and *N,N,N,N*-Tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU) (0.083 g, 0.275 mmol) were dissolved in DMSO (10 mL). Diisopropylethylendiamine (0.09 mL, 0.49 mmol) was added and the solution was stirred at ambient temperature overnight. The product was precipitated by addition of sodium tetrafluoroborate solution (0.2 M, 0.2 L), filtered off and washed with water. The crude was dissolved in CH_2Cl_2 and filtered. Finally it was precipitated twofold from a solution of acetonitrile solution with diethyl ether to yield pure **2-NHS.BF₄** as orange powder (0.084 g, 71% yield). **^1H NMR** (400 MHz, DMSO- d_6) δ 8.38 (t, $J = 8.5$ Hz, 2H), 8.21 (t, $J = 8.5$ Hz, 1H), 8.05 (d, $J = 9.0$ Hz, 2H), 8.00 (d, $J = 8.0$ Hz, 2H), 7.68 (q, $J = 8.1$ Hz, 6H), 5.20 (t, $J = 7.7$ Hz, 2H), 3.40 – 3.35 (m, 2H), 2.90 (s, 4H). **^{13}C NMR** (101 MHz, DMSO- d_6) δ 170.37, 161.63, 152.61, 151.86, 145.29, 140.75, 139.96, 139.85, 130.63, 130.09, 123.05, 111.55, 110.69, 109.23, 108.61, 105.70, 48.08, 32.20, 25.56. **MS (MALDI-TOF)**: m/z calcd. for $\text{C}_{30}\text{H}_{17}\text{N}_2\text{O}_6+$: 529.1; found, 529.1

N-(4-carboxyphenyl)-azadioxatriangulenium tetrafluoroborate **3.BF₄**: Pyridinium chloride (15 g) was melted at 200 °C and **8.BF₄** (0.6 g, 1.0 mmol) was added and stirred for 1 h. After completion of the reaction the product was precipitated by addition of sodium tetrafluoroborate solution (0.2 M, 0.25 L) and cooled to ambient temperature. The precipitate was filtered off and washed with water. The product was dissolved in acetonitrile and an insoluble material, which was highly soluble in methanol, was filtered off. The material was dissolved in CH_2Cl_2 and dried over sodium sulfate. Precipitation of the product from an acetonitrile solution with diethyl ether gave the crude material (0.27 g). Recrystallization from methanol and drying in vacuum gave the pure product as red crystals (0.241 g, 49%). **^1H NMR** (500 MHz, DMSO- d_6) δ 13.56 (s, 1H), 8.44 (d, $J = 8.4$ Hz, 2H), 8.29 (t, $J = 8.5$ Hz, 1H), 8.25 (t, $J = 8.5$ Hz, 2H), 7.83 (d, $J = 8.5$ Hz, 2H), 7.75 (d, $J = 8.4$ Hz, 4H), 6.92 (d, $J = 8.8$ Hz, 2H). **^{13}C NMR** (126 MHz, DMSO- d_6) δ 166.38, 152.42, 152.11, 141.70, 141.23, 141.06, 140.49, 140.07, 133.42, 132.90, 128.69, 111.96, 111.06, 109.53, 108.26, 105.71. **Anal. Calcd.** for $\text{C}_{26}\text{H}_{14}\text{BF}_4\text{NO}_4$: C, 63.58; H, 2.87; N, 2.85; found, C, 61.28; N, 2.45; N, 2.54 **HRMS (MALDI-TOF)**: m/z calcd. for $\text{C}_{26}\text{H}_{14}\text{NO}_{4+}$, 404.0917; found, 404.0896.

N-(4-carboxyphenyl)-azadioxatriangulenium tetrafluoroborate NHS ester **3-NHS.BF₄**: (0.15 g, 0.31 mmol) and *N,N,N,N*-Tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU) (0.14 g, 0.46 mmol) were dissolved in DMSO (10 mL) and diisopropylethylamine (0.05 mL, 0.3 mmol) was added and stirred at ambient temperature overnight. The product was precipitated by addition of sodium tetrafluoroborate solution (0.2 M, 0.15 L), filtered off and washed with water and diethyl ether. Precipitation from a solution of acetonitrile with diethyl ether followed by column chromatography over silica (CH_2Cl_2 /methanol 9/1) yielded pure **3-NHS.BF₄** as orange powder (0.14 g, 78%). **^1H NMR** (500 MHz,

Acetonitrile- d_3) δ 8.61 (d, J = 8.8 Hz, 2H), 8.21 (t, J = 8.6 Hz, 1H), 8.15 (t, J = 8.9 Hz, 2H), 7.82 (d, J = 8.8 Hz, 2H), 7.59 (d, J = 8.7 Hz, 4H), 6.96 (d, J = 8.8 Hz, 2H), 2.91 (s, 4H). **^{13}C NMR** (126 MHz, Acetonitrile- d_3) δ 171.07, 162.30, 154.02, 153.77, 143.27, 143.24, 142.61, 141.99, 141.57, 134.99, 130.61, 128.80, 112.95, 112.13, 110.84, 109.41, 106.88, 26.56. **MS (MALDI-TOF)**: m/z calcd. for $\text{C}_{30}\text{H}_{17}\text{N}_2\text{O}_{6+}$: 501.1; found, 501.1

N-(4-malimidophenyl)-azadioxatriangulenium tetrafluoroborate **4.BF₄**: (0.30 g, 0.65 mmol) and maleic anhydride (0.07g, 0.72 mmol) were placed in a round bottom flask (100 mL) equipped with a stirring bar and dissolved in acetonitrile (35 mL). Triethylamine (0.1 mL, 0.7 mmol) was added and the solution was stirred at 80 °C for 3 h. The solvent was then evaporated and the resulting mixture was dissolved in a mixture of acetic anhydride (20 mL) and acetonitrile (5 mL) and sodium acetate (0.064 g, 0.78 mmol) was added to the solution. The reaction was stirred at 120 °C for 1 h. After cooling sodium tetrafluoroborate solution (0.2 M, 0.3 L) was added and the product was extracted into CH_2Cl_2 . After drying over sodium sulfate the solvent was evaporated. The material was precipitated from a solution of acetonitrile with diethyl ether to yield **4.BF₄** as an orange powder (0.27 g, 76%). **^1H NMR** (500 MHz, Acetonitrile- d_3) δ 8.22 – 8.16 (m, 3H), 7.90 (d, J = 8.7 Hz, 2H), 7.70 (d, J = 8.6 Hz, 2H), 7.58 (d, J = 8.6 Hz, 4H), 7.04 (s, 2H), 6.94 (d, J = 8.8 Hz, 2H). **^{13}C NMR** (126 MHz, Acetonitrile- d_3) δ 170.58, 153.96, 153.74, 143.05, 142.96, 141.95, 141.41, 136.37, 135.78, 135.50, 130.31, 129.85, 112.89, 112.18, 110.64, 109.47, 106.83. **HRMS (MALDI-TOF)** m/z : calcd. for $\text{C}_{29}\text{H}_{15}\text{N}_2\text{O}_{4+}$; 455.1026; found, 455.1013.

1,8-dimethoxy-10-(2,6-dimethoxyphenyl)-9-(3-carboxypropyl)-acridinium **6.BF₄**: **5BF₄** (2.00 g, 4.72 mmol) and 4-amino-butyric acid methyl ester (0.87 g, 5.67 mmol) were placed in a round bottom flask (250 mL) and dissolved in acetonitrile (30 mL). Triethylamine (1 mL, 7 mmol) was added and the solution was stirred for 30 minutes at ambient temperature. Sodium tetrafluoroborate solution (0.2 M, 0.1 L) was added to the reaction mixture to precipitate the product which was filtered off and washed with water. The crude product was recrystallized from methanol and dried under vacuum to yield the pure product as red crystals (1.5 g, 67 %). **^1H NMR** (500 MHz, Acetonitrile- d_3) δ 8.23 (d, J = 8.0 Hz, 1H), 8.21 (d, J = 8.0 Hz, 1H), 8.08 (d, J = 10.0 Hz, 2H), 7.43 (t, J = 8.4 Hz, 1H), 7.10 (d, J = 7.9 Hz, 2H), 6.77 (d, J = 8.4 Hz, 2H), 5.13 – 5.08 (m, 2H), 3.75 (s, 3H), 3.54 (s, 12H), 2.79 (t, J = 6.5 Hz, 2H), 2.47 – 2.39 (m, 2H). **^{13}C NMR** (126 MHz, Acetonitrile- d_3) δ 173.05, 160.26, 157.14, 141.45, 139.66, 129.07, 119.53, 119.27, 108.84, 106.16, 103.34, 56.45, 55.29, 51.31, 51.25, 29.56, 21.95. **Anal. Calcd.** for $\text{C}_{28}\text{H}_{30}\text{BF}_4\text{NO}_6$: C, 59.70; H, 5.37; N, 2.49; found: C, 59.72; H, 5.29; N, 2.32 **HRMS (MALDI-TOF)**: m/z calcd. for $\text{C}_{28}\text{H}_{30}\text{NO}_6+$: 476.2068; found, 486.2041.

1,8-dimethoxy-10-(2,6-dimethoxyphenyl)-9-(2-(4-carboxyphenyl)-ethyl)-acridinium **7.BF₄**: 4-(2-aminoethyl)benzoic acid hydrochloride (0.37 g, 1.83 mmol) was suspended in a mixture of methanol (30 mL) and triethylamine (20 mL). A solution of **5.BF₄** (0.85 g, 1.67 mmol) in acetonitrile (40 mL) was added drop-wise to the suspension over 15 minutes. Immediate change of color from purple to red indicated the formation of the product. After 30 minutes sodium tetrafluoroborate solution (0.2 M, 0.25 L) was added together with tetrafluoroboric acid (5 mL, 48 w% in water) in water (50 mL) to precipitate the product. Filtration and subsequent washing with water gave a crude product. Repeated

recrystallization from methanol yielded red crystals of the pure product (0.792 g, 78%). **¹H NMR** (500 MHz, DMSO-*d*₆) δ 12.99 (s, 1H), 8.27 (d, *J* = 7.9 Hz, 1H), 8.26 (d, *J* = 7.9 Hz, 1H), 8.13 (d, *J* = 9.2 Hz, 2H), 7.90 (d, *J* = 8.2 Hz, 2H), 7.55 (d, *J* = 8.3 Hz, 2H), 7.44 (t, *J* = 8.4 Hz, 1H), 7.22 (d, *J* = 8.0 Hz, 2H), 6.81 (d, *J* = 8.5 Hz, 1H), 5.54 – 5.48 (m, 2H), 3.54 (s, 6H), 3.52 (s, 6H), 3.49 (d, *J* = 8.2 Hz, 2H). **¹³C NMR** (126 MHz, DMSO-*d*₆) δ 167.09, 159.96, 156.37, 155.23, 141.78, 140.94, 140.14, 129.59, 129.43, 129.38, 129.28, 119.10, 119.05, 109.70, 106.85, 103.68, 57.13, 55.85, 51.77, 33.05. **Anal. Calcd** for C₃₂H₃₀BF₄NO₆: C, 62.86; H, 4.95; N, 2.29; found: C, 62.85; H, 4.86; N, 2.26 **HRMS (MALDI-TOF)**: *m/z* calcd. for C₃₂H₃₀NO₆⁺: 524.2068; found, 524.2046.

1,8-dimethoxy-10-(2,6-dimethoxyphenyl)-9-(4-carboxyphenyl)-acridinium **8.BF₄**: **5.BF₄** (4.0 g, 7.8 mmol) and methyl 4-aminobenzoate (1.78 g, 11.76 mmol) were dissolved in a mixture of acetonitrile (50 mL) and 2,6-lutidine (0.84 g, 7.75 mmol). The mixture was stirred under reflux for 6 h. When the solution had cooled down to ambient temperature sodium tetrafluoroborate solution (0.2 M, 0.4 L) was added to precipitate the red product. Filtration and repeated precipitation from CH₂Cl₂ with Heptane and further from Acetonitrile with Ether and Heptane gave the crude product (3.8 g). Twofold recrystallisation from ethanol/methanol yielded the pure product as monoethanolate in the form of a red powder (0.86 g, 18%). **¹H NMR** (500 MHz, Methanol-*d*₄) δ 8.54 (d, *J* = 8.6 Hz, 2H), 8.04 (dd, *J* = 9.1, 8.0 Hz, 2H), 7.84 (d, *J* = 8.5 Hz, 2H), 7.52 (t, *J* = 8.4 Hz, 1H), 7.18 (d, *J* = 7.4 Hz, 2H), 6.93 (d, *J* = 9.9 Hz, 2H), 6.88 (d, *J* = 8.5 Hz, 2H), 4.07 (s, 3H), 3.67 (s, 6H), 3.63 (s, 6H). **¹³C NMR** (126 MHz, Methanol-*d*₄) δ 166.98, 162.03, 161.15, 157.17, 143.88, 143.75, 141.16, 134.46, 133.68, 131.04, 129.80, 120.97, 120.52, 111.72, 107.66, 104.73, 57.65, 56.40, 53.30. **Anal. Calcd** for C₃₃H₃₄BF₄NO₇ + 1C₂H₅OH: C, 61.60; H, 5.33; N, 2.18; found: C, 61.46; N, 2.19; H, 5.20 **HRMS (MALDI-TOF)**: *m/z* calcd. for C₃₁H₂₈NO₆⁺: 510.1911; found, 510.1911.

1,8-dimethoxy-10-(2,6-dimethoxyphenyl)-9-(4-aminophenyl)-acridinium **9.BF₄**: **5BF₄** (4.53 g, 8.88 mmol) dissolved in acetonitrile (75 mL) was slowly dropped to p-phenylenediamine (1.92 g, 17.76 mmol) dissolved in acetonitrile (20 mL) and 2,6-lutidine (2 mL, 17 mmol) and stirred at ambient temperature for 90 min. After completion of the reaction the product was precipitated by addition of sodium tetrafluoroborate solution (0.2 M, 0.3 L), filtered off, washed with water and dried. Precipitation twice from a solution of acetonitrile with diethyl ether gave **9.BF₄** as fine red powder (4.83 g, 98%). 1.87 g of the material was recrystallized from methanol to yield big crystals (1.66 g, 89%) **¹H NMR** (500 MHz, Acetonitrile-*d*₃) δ 7.97 (d, *J* = 7.9 Hz, 1H), 7.95 (d, *J* = 8.0 Hz, 1H), 7.46 (t, *J* = 8.4 Hz, 1H), 7.22 (d, *J* = 8.7 Hz, 2H), 7.11 (dd, *J* = 9.1, 0.9 Hz, 2H), 7.05 (dd, *J* = 8.1, 0.9 Hz, 2H), 7.01 (d, *J* = 8.7 Hz, 2H), 6.80 (d, *J* = 8.5 Hz, 2H), 4.83 (s, 2H), 3.59 (s, 6H), 3.55 (s, 6H). **¹³C NMR** (126 MHz, Acetonitrile-*d*₃) δ 161.01, 159.08, 156.85, 151.56, 144.76, 140.10, 130.53, 129.32, 128.57, 120.71, 120.45, 116.40, 112.41, 107.38, 104.69, 64.09, 57.77, 56.64, 25.61. **Anal. calcd.** for C₂₉H₂₇BF₄N₂O₄, C, 62.83; H, 4.91; N, 5.05 found: C, 63.15; H, 4.73; N, 5.08 **MS (MALDI-TOF)**: *m/z* calcd. for C₂₉H₂₇N₂O₄⁺ 467.20, found, 467.2

N-(4-aminophenyl)-azadioxatriangulenium tetrafluoroborate **10.BF₄**: Pyridinium chloride (30 g) was melted in a round bottom flask (250 mL) at 200 °C for 45 minutes until the material was resubliming on the inside of the flask. **9.BF₄** (1.63 g, 2.94 mmol) was added as

powder and stirred for 1 h. After removing the heat sodium hydroxide solution (2 M) was added until pH ~9 was reached followed by addition of sodium tetrafluoroborate solution (0.2 M, 0.1 L) to precipitate the product. The material was filtered off, washed with sodium tetrafluoroborate solution (0.2 M) and water and dried. It is then taken up in hot acetonitrile and filtered through a paper filter. Thereafter diethyl ether was added to precipitate a fine red powder. The crude material was recrystallized three times from methanol to yield the pure compound as dark crystals (1.24 g, 91%). **¹H NMR** (500 MHz, DMSO-*d*₆) δ 8.24 (dt, *J* = 11.7, 8.4 Hz, 3H), 7.69 (dd, *J* = 8.3, 1.6 Hz, 4H), 7.21 (d, *J* = 8.6 Hz, 2H), 7.03 (d, *J* = 8.8 Hz, 2H), 6.93 (d, *J* = 8.6 Hz, 2H), 5.87 (s, 2H). **¹³C NMR** (126 MHz, DMSO-*d*₆) δ 152.30, 152.07, 150.98, 142.33, 140.94, 140.61, 140.05, 128.16, 123.78, 115.41, 111.81, 111.38, 109.10, 108.42, 105.68. **Anal. Calcd.** for C₂₅H₁₅BF₄N₂O₂: C, 64.96; H, 3.27; N, 6.06 found; C, 64.61; H, 3.17; N, 6.04 **MS (MALDI-TOF)**: *m/z* calcd. for C₂₅H₁₅N₂O₂+ 375.11, found, 375.1

Supplementary Material

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Acknowledgments

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Figure 1.
Modelled anisotropy of biomolecule labelled with a fluorophore with a luminescence lifetime of 1 ns, 4 ns, 20 ns or 400 ns.

Figure 2.
Absorption and emission spectra of ADOTAs **1–4**·BF₄ in acetonitrile and DMSO.

Figure 3.

Time-resolved fluorescence decay profiles (black) for **1–4**.BF₄ in acetonitrile, instrument response function (grey) and the fit to the data (light grey).

Scheme 1.

NHS esters of ADOTAs **1–3** and ADOTA-maleimide **4**.

Scheme 2.

Synthesis of ADOTA 1·BF₄ and 1-NHS·BF₄, which serves as an illustration of the general procedure for preparation of the ADOTA-NHS esters 1–4-NHS·BF₄.

Scheme 3.
Synthesis of **2·BF₄** and **3·BF₄**.

Scheme 4.
Synthesis of **4·BF₄**.

Table 1

Photophysical properties of **1–4-BF₄** measured in acetonitrile, DMSO and PBS solutions. λ_{abs} is the wavelength of the longest wavelength absorption maximum in nm. ν_{abs} is the frequency of the longest wavelength absorption maximum in cm^{-1} . ϵ is the molar absorption coefficient in the longest wavelength absorption maxima in $\text{cm}^{-1}\text{M}^{-1}$. λ_{em} and ν_{em} are the wavelength and the frequency of the emission maximum given in nm and cm^{-1} , respectively. τ_{Stokes} is the Stokes' shift in cm^{-1} . τ is the fluorescence lifetime in ns, ϕ_{fl} is the fluorescence quantum yield measured using rhodamine 6G as a reference and τ_0 is the radiative lifetime in ns, calculated from $\tau_0 = \tau / \phi_{\text{fl}}$.

	1-BF ₄			2-BF ₄			3-BF ₄			4-BF ₄		
	MeCN	DMSO	PBS	MeCN	DMSO	PBS	MeCN	DMSO	PBS	MeCN	DMSO	PBS
$\lambda_{\text{abs}}/\text{nm}$	538	540	540	539	541	541	536	539	539	539	539	540
$\nu_{\text{abs}}/\text{cm}^{-1}$	18590	18520	18520	18550	18480	18480	18660	18550	18550	18550	18550	18520
$\epsilon/\text{cm}^{-1}\text{M}^{-1}$	11000	11200	10600	[a]	11800	11600	12200	11900	12200	12300	12200	11800
$\lambda_{\text{em}}/\text{nm}$	558	565	556	562	571	564	562	570	560	562	569	560
$\nu_{\text{em}}/\text{cm}^{-1}$	17920	17700	17990	17790	17510	17730	17790	17540	17860	17790	17570	17860
$\tau_{\text{Stokes}}/\text{cm}^{-1}$	670	820	530	760	970	750	870	1010	690	760	980	660
τ/ns [c]	22.8	19.3	19.5	22.3	17.0	17.9	21.0	17.1	17.9	20.6	14.8	16.4
ϕ_{fl} [b]	0.66	0.66	0.56	0.63	0.53	0.60	0.64	0.64	0.57	0.67	0.52	0.57
τ_0 experimental/ns	32.6	33.3	30.9	33.3	27.9	31.4	30.9	25.5	31.4	30.7	28.4	28.8
τ_0 Strickler-Berg/ns	[d]	[d]	[d]	[d]	[d]	[d]	28.1	23.5	27.6	[d]	[d]	[d]

[a] Due to low solubility of **2-BF₄** in MeCN the molar absorption coefficient (ϵ) could not be determined.

[b] Determined using multipoint determination, the relative error is estimated at $\pm 5\%$.

[c] χ^2 1.05 in all fits.

[d] Not determined.