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Diazaoxatriangulenium: synthesis of reactive derivatives and conjugation to bovine serum albumin†

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The azaoxa-triangulenium dyes are characterised by emission in the red and a long fluorescence lifetime (up to 25 ns). These properties have been widely explored for the azadioxatriangulenium (ADOTA) dye. Here, the syntheses of reactive maleimide and NHS-ester forms of the diazaoxatriangulenium (DAOTA) system are reported. The DAOTA fluorophore was conjugated to bovine serum albumin (BSA) and investigated in comparison to the corresponding ADOTA-BSA conjugate. It was found that the fluorescence of DAOTA experienced a significantly higher degree of solvent quenching if compared to ADOTA as non-conjugated dyes in aqueous solution, while the fluorescence quenching observed upon conjugation to BSA was significantly reduced for DAOTA when compared to ADOTA. The differences in observed quenching for the conjugates can be explained by the different electronic structures of the dyes, which renders DAOTA significantly less prone to reductive photoinduced electron transfer (PET) quenching from e.g. tryptophan. We conclude that DAOTA, with emission in the red and inherent resistance to PET quenching, is an ideal platform for the development of long fluorescence lifetime probes for time-resolved imaging and fluorescence polarisation assay.

Introduction

Fluorescence technology is prevalent in high-tech applications from diagnostics,1,2 point-of-care devices,3,4 and display technology to DNA sequencing,5 drug-discovery,6 and imaging.7,8 Each application relies on dye development, be it bioengineered fluorescent proteins,9 emissive nanoparticles,10 inorganic luminophores,11,12 or organic dyes.13–16 Organic dye development is limited by fluorophore design, where many of the existing molecular frameworks have been known for more than a century.17 Many dye systems have been synthesised using these well-known scaffolds,18–21 and have been optimised to show significantly enhanced performance.22–31 In particular, photostability, absorption cross-section, fluorescence quantum yield, and emission wavelength have been enhanced to the extent possible for the available fluorophores.

In the group of organic dyes, the triangulenium dyes are different.12 In these molecules, a small absorption cross-section, and the resulting low fluorescence rate constant, does not infer low photostability and low quantum yield. While donor-substituted triangulenium dyes are bright emitters similar to rhodamine and fluorescein dyes,33–36 the azaoxa-triangulenium dyes are highly photostable, highly emissive, long fluorescence lifetime dyes.37–41 This group of triangulenium dyes includes azadioxatriangulenium (ADOTA) and diazaoxatriangulenium (DAOTA) shown in Chart 1 (for details on the triangulenium nomenclature see ESI†). The aza-bridges are readily functionalised with groups compatible, with the reaction conditions used to form the aromatic core.

The triangulenium dyes are synthesised from common precursors using sequences of highly selective nucleophilic
aromatic substitution reactions (S$_{N}$Ar) \cite{33-35,38,42}. For the azaoxa-
triangulenium dyes, each substitution step occurs in a cascade of
two S$_{N}$Ar-reactions forming a heteroatom bridge\cite{37,38}. A similar
approach has recently been used to form the aza-bridge in
carbazoles\cite{43}. Alternatively, ether-cleaving reaction conditions
can be used to initiate an intramolecular S$_{N}$Ar-reaction with
the formation of an oxygen bridge\cite{44}. Here, we elaborate on the
synthesis of diazaoxatriangulenium (DAOTA) in an effort to
make DAOTA derivatives, with reactive linkers for conjugation
of the DAOTA fluorophore to biomolecules.

We chose to use bovine serum albumin (BSA) as a demonstra-
ctor for bioconjugation, although native BSA is not an ideal
model system\cite{26,29,45}, the results allow for a direct comparison
between DAOTA and ADOTA BSA-conjugates\cite{46}. We have pre-
viously investigated bioconjugates of ADOTA to BSA and IgG,
and used both of the azaoxa-triangulenium dyes in
bioimaging\cite{46-49}. When conjugated to a biomolecule, the
DAOTA fluorescence is significantly quenched. This has also
been observed for other fluorophores and rationalised as
photoinduced electron transfer (PET) quenching by tryptophan.

As DAOTA is less electron deficient than
ADOTA\cite{38,53}, it was expected that DAOTA would be much less
prone to reductive PET quenching by tryptophan. This differ-
ce in PET activity between ADOTA and DAOTA was recently
highlighted in a study of a DAOTA based DNA G-quadruplex
fluorescence lifetime probe\cite{41}. Here, we report a significant
reduction in fluorescence quenching of DAOTA upon bio-con-
jugation, but also that the DAOTA dyes undergo significant
solvent quenching, which leaves room for further improve-
ments of this long fluorescent lifetime fluorophore. In parti-
cular, the solubility of DAOTA in water needs to be improved
for several applications, in close analogy to what was achieved
with the Alexa dyes\cite{34-37}.

### Experimental

#### Materials and methods

Absorption spectroscopy was 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 monochro-
mators. All solvents used for spectroscopic experiments were
of HPLC grade and used as received. Phosphate buffered saline
(PBS) was prepared from slats according to common protocols.
The pH value of the buffer was determined and subsequently
adjusted to 7.4. Molar absorption coefficients were determined
for each of the dyes using Lambert–Beers law by measuring the
absorption spectrum of three stock solutions with different
concentrations of the dye. Quantum yields were determined using
the relative method\cite{38}, using rhodamine 6G as standard ($\phi_n$
= 0.95)\cite{59}. Details on the quantum yield measurements are given
in the ESI. Fluorescence lifetimes were measured using a Flu-
Time 300 (PicoQuant, Berlin, Germany) system. The emission
signal was measured with a Hybrid-PMT detector with a spectral
range of 220–650 nm. The dyes were excited at 510 nm using a
solid-state laser excitation source. The instrument response
function was recorded at the excitation wavelength using a
dilute solution of Ludox®. The fluorescence decays were ana-
yzed using the FluorFit software package. The decay data were
all found to be monoexponential and was fitted by iterative
reconvolution with a single exponential

$$I(t) = a \exp(-t/\tau)$$

In eqn (1) $\alpha$ is the amplitude and $\tau$ is the fluorescence life-
time. All time-resolved emission decay profiles and fits are
shown in the ESI.$^\dagger$

#### Synthetic procedures

Unless otherwise stated, all starting materials were obtained
from commercial suppliers and used as received. Solvents were
of HPLC grade for reactions and recrystallisations and techni-
cal grade for column chromatography and were used as
received. \textsuperscript{1}H NMR and \textsuperscript{13}C NMR spectra were recorded on a
500 MHz or a 300 MHz instrument (500/300 MHz for \textsuperscript{1}H NMR
and 126 MHz for \textsuperscript{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.
High-resolution mass spectra (HRMS) were recorded with an
ESP-MALDI-FT-ICR spectrometer equipped with a 7 T magnet
(prior to experiments, the instrument was calibrated using
NaTFA cluster ions) using dithanol as matrix. Column
chromatographic purifications were performed on Kieselgel 60
(0.040–0.063 mm particle size). Dry column vacuum chromato-
graphy 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.

#### Synthesis of 2 and 4

Compound 1 was prepared according
to the published procedure, see ref. 37. The compounds 2a–d
and 4a–d were prepared as reported in ref. 47.

**N**-(3-Carboxypropyl)-**N**-methyl-1,13-dimethoxy-quin[2,3,4-k]a-
lacridinium tetrafluoroborate 3a. 1,8-Dimethoxyl-10-(2,6-
dimethoxyphenyl)-9-(3-carboxypropyl)-lacridinium methyl ester
tetrafluoroborate 2a (0.5 g, 0.9 mmol) was placed in a sealable
tube and dissolved in acetonitrile (5 mL) and excess methyl-
amine (15 mL, 33 wt% in ethanol) was added. The solution
was stirred at 60 °C for five days. After it had cooled to
ambient temperature it was poured into diethyl ether (500 mL)
and the precipitate was filtered. After washing with ethanol,
the product was dried in vacuo.

**N**-(3-Carboxypropyl)-**N**-methyl-1,13-dimethoxy-quin[2,3,4-k]-
acridinium tetrafluoroborate 3a.

$$\text{Compound 1 was prepared according
to the published procedure, see ref. 37. The compounds 2a–d
and 4a–d were prepared as reported in ref. 47.}$$

N-(3-Carboxypropyl)-N-methyl-1,13-dimethoxy-quin[2,3,4-k]-
lacridinium tetrafluoroborate 3a. 1,8-Dimethoxy-10-(2,6-
dimethoxyphenyl)-9-(3-carboxypropyl)-lacridinium methyl ester
tetrafluoroborate 2a. (50 wt% in water) was used to acidify the solution when a dark
precipitate was formed, which was filtered off. The crude
compound was dissolved in warm acetonitrile, filtered through a
paper filter, and precipitated twice from a solution of aceto-
nitrile with diethyl ether. Recrystallisation from t-propanol/
acetonitrile and yielded dark green crystals, which were washed
with dichloromethane and dried in vacuum (0.315 g, 69%).

\textsuperscript{1}H NMR (500 MHz, Acetonitrile-$d_3$) $\delta$ 8.10 (t, $J = 8.4$ Hz, 1H),
7.91 (t, $J = 8.5$ Hz, 1H), 7.86 (t, $J = 8.4$ Hz, 1H), 7.72 (d, $J = 8.6$ Hz,
1H), 7.67 (d, J = 8.9 Hz, 1H), 7.43–7.34 (m, 2H), 6.93–6.84
(m, 2H), 4.78–4.68 (m, 1H), 4.53–4.43 (m, 1H), 4.02 (s, 3H),
3.72 (s, 3H), 3.71 (s, 3H). 2.68 (t, J = 6.3 Hz, 2H), 2.34–2.19
(m, 2H). \(^{13}C\) NMR (126 MHz, Acetonitrile-\(d_3\)) \(\delta \) 175.2, 160.5,
160.2, 143.7, 143.3, 143.1, 140.6, 139.7, 138.1, 137.8, 137.3,
120.1, 113.9, 113.9, 108.6, 108.5, 106.0, 105.8, 104.1, 104.0,
56.5, 38.2, 31.2, 21.9. HRMS (MALDI-TOF): m/z calcd for
\(\text{C}_{26}\text{H}_{25}\text{N}_2\text{O}_4\): 429.1809; found, 429.1811.

N-[2-(4-Carboxyphenyl)ethyl]-N-methyl-1,13-dimethoxy-quin-
[2,3,4-\(k\)]acridinium tetrafluoroborate 3b. 1H-Dimethoxy-10-
(2,6-dimethoxyphenyl)-9-[2-(4-carboxyphenyl)ethyl]-acridinium
tetrafluoroborate 2b (0.35 g, 0.60 mmol) was placed in a seal-
able tube and dissolved in acetonitrile (10 mL) and methyl-
amine (12 mL, 33 wt% in ethanol) was added. The tube was
sealed and the mixture was stirred at 65 °C for three days.
After cooling to ambient temperature the solution was poured
into diethyl ether to precipitate the compound. It was then dis-
solved in sodium hydroxide solution (1 M, 50 mL) and extracted
three times with dichloromethane. Then the pH value was adjusted to ~3 with tetrafluoroboric acid (50 wt% in water) to precipitate the product. The material was dissolved in dichloromethane and filtered. The solvent was removed in vacuum.
The material was subsequently precipitated twice from a solution of acetonitrile with diethyl ether to give the compound as blue powder which was washed with dichloro-
methane, and dried in vacuum (0.271 g, 78%). \(^{1}H\) NMR (500 MHz, Methanol-\(d_4\)) \(\delta \) 7.98–7.86
(m, 2H), 7.80–7.67 (m, 3H), 7.60 (d, J = 8.5 Hz, 1H), 7.57 (d, J =
4.0 Hz, 1H), 7.54 (d, J = 4.0 Hz, 1H), 7.23 (s, 2H), 7.00 (d, J =
8.1 Hz, 1H), 6.96 (d, J = 8.0 Hz, 1H), 5.20–4.99 (m, 2H), 4.18
(s, 3H), 3.78 (s, 3H), 3.76 (s, 3H), 3.42–3.34 (m, 2H). \(^{13}C\) NMR
(126 MHz, Methanol-\(d_4\)) \(\delta \) 160.4, 150.9, 145.3, 144.4, 144.3,
142.0, 139.5, 137.5, 136.8, 133.7, 136.9, 130.7, 129.3, 127.9, 120.0,
117.3, 116.7, 114.2, 113.6, 110.2, 108.7, 107.5, 106.0, 104.2,
104.0, 56.6, 38.2. HRMS (MALDI-TOF): m/z calcd for
\(\text{C}_{30}\text{H}_{26}\text{N}_3\text{O}_3\): 434.1863; found, 434.1849.

N-(3-Carboxypropyl)-N-methyl-diazaoctatrienilium tetra-
fluoroborate 5a. N-(3-Carboxypropyl)-N-methyl-1,13-dimethoxy-
quin[2,3,4-\(k\)]acridinium tetrafluoroborate 3a (3.0 g, 5.8 mmol)
dissolved in pyridine (4 mL) was added to molten pyridinium
chloride (20 g) at 170 °C and stirred for 35 min. The product was
precipitated by addition of sodium tetrafluoroborate solution
(0.2 M, 0.5 L) and filtered off. The crude compound was
dissolved in hot acetonitrile and filtered through a paper filter.
Twofold precipitation of the product from a solution of aceto-
nitrile with diethyl ether and recrystallisation from acetonitrile
gave the compound as red solid (2.26 g, 83%). \(^{1}H\) NMR
(500 MHz, Acetonitrile-\(d_3\)) \(\delta \) 8.22 (t, J = 8.6 Hz, 1H), 8.08–8.04
(m, 1H), 8.04–8.01 (m, 1H), 7.64 (d, J = 8.8 Hz, 1H), 7.56 (d, J =
8.6 Hz, 1H), 7.49 (d, J = 8.8 Hz, 1H), 7.40 (d, J = 8.6 Hz, 1H),
7.28–7.19 (m, 2H), 4.84–4.42 (m, 2H), 3.89 (s, 3H), 2.65 (t, J =
6.6 Hz, 2H), 2.11–2.09 (m, 2H, overlap with residual water in
CD<sub>3</sub>CN). \(^{13}C\) NMR (126 MHz, Acetonitrile-\(d_3\)) \(\delta \) 173.4, 152.1,
141.2, 140.4, 140.0, 139.3, 139.3, 138.2, 138.1, 117.0, 109.0,
108.8, 108.1, 108.0, 107.0, 105.7, 104.5, 46.9, 35.1, 29.1, 19.8.
HRMS (MALDI-TOF): m/z calcd for \(\text{C}_{26}\text{H}_{22}\text{N}_2\text{O}_4\): 438.1390;
found, 438.1394.

N-[2-(4-Carboxyphenyl)ethyl]-N-methyl-diazaoctatrienilium tetra-
fluoroborate 5b. N-(2-(4-Carboxyphenyl)ethyl)-azdia-
octatrienilium tetrafluoroborate 4b (0.09 g, 0.17 mmol) was
placed in a sealable tube and dissolved in N-methyl-2-pyrrolidone
(4 mL) and dimethylaniline (1 mL, 8.7 mmol, 33 wt% in ethanol). The solution was stirred at 85 °C overnight. After 15 h the solution was colored red and a precipitate formed, which was filtered off. The solution was poured into sodium tetrafluoroborate solution (0.2 M, 0.1 L) and the pH was adjusted to ~3 with tetrafluoroboric acid (50 wt% in water) to precipitate the remaining product. The crude product was washed with water, dissolved in hot acetonitrile and filtered through a paper filter. After cooling the material was precipitated with diethyl ether. Repeated precipitation from a solution of acetonitrile with diethyl ether yielded the pure compound as a fine red powder, which was dried under vacuum (0.04 g, 43%).

**8.** H NMR (500 MHz, DMSO-d_6) δ 8.25 (t, J = 8.4 Hz, 1H), 8.08 (m, 2H), 7.87 (d, J = 8.0 Hz, 2H), 7.70–7.65 (m, 2H), 7.60–7.53 (m, 4H), 7.34–7.29 (m, 2H), 4.8–4.74 (m, 2H), 3.95 (s, 3H), 3.22–3.16 (m, 2H).

**13C NMR** (126 MHz, DMSO-d_6) δ 167.2, 151.8, 151.7, 142.1, 141.0, 139.9, 139.8, 139.6, 139.0, 139.0, 138.5, 138.4, 129.8, 129.5, 118.1, 110.8, 109.6, 109.5, 108.3, 108.1, 107.0, 106.3, 106.0, 47.6, 35.6, 31.1.

**HRMS (MALDI-TOF):** m/z calcd for C_{29}H_{21}N_{2}O_{3}^−, 445.1547; found, 445.1549.

**N-(4-Carboxyphenyl)-N'-methyl-diazaoxatriangulenium tetrafluoroborate 5c.** N-(4-Carboxyphenyl)-azadiacoxatriangulenium tetrafluoroborate 4c (0.11 g, 0.23 mmol) and benzoic acid (0.6 g, 4.9 mmol) were placed in a round bottom flask and dissolved in ethanol (5 mL) and methylamine (0.57 mL, 4.6 mmol, 33 wt% in ethanol). The mixture was heated to reflux overnight. After 18 h additional methylamine (0.5 mL) was added and reflux of the mixture was continued for another day. Sodium tetrafluoroborate solution (0.2 M, 0.1 L) was added to precipitate the product. The red material was dissolved in dichloromethane, dried over sodium sulfate, and filtered. Repeated precipitation from a solution of acetonitrile with diethyl ether and drying in vacuum gave the pure product as a dark red powder (0.1 g, 86%).

**H NMR** (500 MHz, DMSO-d_6) δ 8.41 (d, J = 8.1 Hz, 2H), 8.20 (t, J = 8.5 Hz, 1H), 8.12 (t, J = 8.5 Hz, 1H), 7.95 (t, J = 8.4 Hz, 1H), 7.85 (d, J = 8.8 Hz, 1H), 7.74 (d, J = 8.1 Hz, 2H), 7.68 (d, J = 8.7 Hz, 1H), 7.49 (d, J = 8.1 Hz, 1H), 7.45 (d, J = 8.2 Hz, 1H), 6.60 (d, J = 8.6 Hz, 1H), 6.53 (d, J = 8.4 Hz, 1H), 4.09 (s, 3H).

**13C NMR** (126 MHz, DMSO-d_6) δ 166.5, 152.1, 151.9, 144.7, 141.7, 141.0, 140.5, 140.5, 140.3, 140.1, 139.4, 138.9, 138.4, 133.04, 128.9, 110.8, 110.1, 108.6, 108.5, 107.3, 107.0, 106.7, 106.7, 35.7.

**HRMS (MALDI-TOF):** m/z calcd for C_{27}H_{17}N_{2}O_{3}, 417.1234; found, 417.1236.

**N-(4-Aminophenyl)-N'-methyl-diazaoxatriangulenium tetrafluoroborate 5d.** Pyridinium chloride (36 g) was heated to 185 °C and N-(4-aminophenyl)-N'-methyl-1,13-dimethoxyquin [2,3,4-k]acridinium tetrafluoroborate 3d (0.94 g, 1.84 mmol) was added and stirred for 45 minutes. After completion of the reaction sodium tetrafluoroborate solution (0.2 M, 0.4 L) was added (pH adjusted to 9 with sodium hydroxide solution) to precipitate the product. After cooling to ambient temperature the material was filtered off and washed with additional sodium tetrafluoroborate solution and water. Precipitation of the compound from a solution of acetonitrile with diethyl ether gave a fine powder which was recrystallized from i-propanol/methanol to yield the pure compound as dark crystals which are washed with cold acetonitrile and methanol (0.15 g, 17.5%).

**1H NMR** (500 MHz, Acetonitrile-d_3) δ 8.17–8.09 (m, 2H), 7.96 (t, J = 8.4 Hz, 1H), 7.77 (d, J = 8.9 Hz, 1H), 7.59 (d, J = 8.6 Hz, 1H), 7.44–7.37 (m, 2H), 7.15 (d, J = 8.6 Hz, 2H), 6.93 (d, J = 8.6 Hz, 2H), 6.73 (d, J = 8.7 Hz, 1H), 6.67 (d, J = 8.5 Hz, 1H), 5.78 (s, 2H), 4.03 (s, 3H).

**13C NMR** (126 MHz, DMSO-d_6) δ 152.1, 151.8, 150.6, 142.8, 141.6, 141.0, 140.9, 140.3, 139.7, 139.2, 138.6, 138.1, 128.5, 124.5, 115.8, 111.0, 110.5, 109.9, 108.3, 108.2, 107.3, 107.1, 107.0, 106.1, 35.6.

**Anal. Calcld:** For C_{29}H_{31}BF_{4}N_{3}O: C, 65.71%; H, 3.82%; N, 8.84%; Found: C, 65.94%; H, 3.54%; N, 9.10.

**HRMS (MALDI-TOF):** m/z calcd for C_{29}H_{18}BF_{4}N_{3}O, 438.1444; found, 388.1455.
4.66 (t, J = 7.8 Hz, 2H), 3.88 (s, 3H), 3.29 (t, J = 7.7 Hz, 2H), 2.85 (s, 4H). $^{13}$C NMR (126 MHz, Acetonitrile-$d_3$) δ 171.2, 153.4, 153.3, 146.2, 142.5, 141.4, 141.2, 140.6, 140.4, 139.6, 139.5, 124.7, 112.1, 110.3, 110.2, 109.6, 109.4, 108.3, 107.2, 106.9, 48.8, 36.5, 32.4, 26.5. HRMS (MALDI-TOF): m/z calcd for C$_{33}$H$_{28}$N$_3$O$_7$: 542.1710; found, 542.1709.

$N$-(4-Carboxyphenyl)-N'-methyl-diazaoxatriangulenium NHS ester tetrafluoroborate 6c $N$-(4-Carboxyphenyl)-N'-methyl-diazaoxatriangulenium tetrafluoroborate 5c [0.04 g, 0.08 mmol] was placed in a flask and dissolved in DMSO (10 mL) and precipitated with diethyl ether twice to yield the product as a fine material was dissolved in warm acetonitrile, filtered and precipitated overnight. After confirmation of product formation by MALDI-TOF analysis the material was precipitated with addition of sodium tetrafluoroborate solution (0.2 M, 0.1 L) and filtered off. The crude red material was dissolved in dichloromethane, dried over magnesium sulfate, filtered, and the solvent was evaporated in vacuum. Then the material was precipitated from a solution of acetonitrile with diethyl ether and dried in vacuum to give a red powder (0.016 g, 34%). $^1$H NMR (500 MHz, Acetonitrile-$d_3$) δ 8.17 (t, J = 8.9 Hz, 1H), 8.06–8.00 (m, 3H), 7.97 (t, J = 8.5 Hz, 1H), 7.53 (d, J = 8.2 Hz, 2H), 7.48 (d, J = 8.8 Hz, 1H), 7.42 (d, J = 8.8 Hz, 1H), 7.40–7.35 (m, 2H), 7.21 (d, J = 3.7 Hz, 1H), 7.19 (d, J = 3.8 Hz, 1H), 4.66 (t, J = 7.8 Hz, 2H), 3.88 (s, 3H), 3.29 (t, J = 7.7 Hz, 2H), 2.85 (s, 4H). $^{13}$C NMR (126 MHz, Acetonitrile-$d_3$) δ 140.2, 139.8, 139.2, 135.0, 130.9, 111.1, 110.6, 109.8, 109.6, 107.9, 107.5, 36.5, 26.4. HRMS (MALDI-TOF): m/z calcd for C$_{33}$H$_{28}$N$_3$O$_7$: 541.1397; found, 514.1399.

$N$-(4-Malimidophenyl)-N'-methyl-diazaoxatriangulenium tetrafluoroborate 7. $N$-(4-Aminophenyl)-N'-methyl-diazaoxatriangulenium tetrafluoroborate 5d [0.1 g, 0.2 mmol] was placed in a round bottom flask and dissolved in acetonitrile (25 mL) and 2,6-lutidine (0.2 mL). After the compound dissolved completely maleic anhydride (0.1 g, 1 mmol) was added and the solution was refluxed for 4 h until all starting material was converted to the acid. The solvent was removed by evaporation and the acid was dissolved in acetic anhydride (8 mL) and stirred at 100°C. After 30 min the reaction was completed and the solution was cooled to ambient temperature. The crude product was precipitated by addition of sodium tetrafluoroborate solution (0.2 M, 0.3 L). After filtration the material was dissolved in warm acetonitrile, filtered and precipitated with diethyl ether twice to yield the product as a fine red powder (0.08 g, 66%). $^1$H NMR (500 MHz, DMSO-$d_6$) δ 8.24–8.20 (m, 1H), 8.20–8.15 (m, 1H), 8.01 (t, J = 8.4 Hz, 1H), 7.92–7.88 (m, 2H), 7.88–7.86 (m, 1H), 7.78 (d, J = 8.9 Hz, 2H), 7.70 (d, J = 8.7 Hz, 1H), 7.52 (d, J = 8.2 Hz, 1H), 7.48 (d, J = 8.2 Hz, 1H), 7.32 (s, 2H), 6.60 (d, J = 8.7 Hz, 1H), 6.53 (d, J = 8.4 Hz, 1H), 4.11 (s, 3H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 169.7, 152.1, 151.9, 142.0, 141.0, 140.8, 140.1, 135.9, 135.0, 133.6, 129.7, 129.7, 129.7, 129.2, 129.2, 110.8, 110.1, 108.5, 108.5, 107.6, 107.1, 106.7, 106.7, 106.6, 35.7. HRMS (MALDI-TOF): m/z calcd for C$_{20}$H$_{18}$N$_3$O$_7$: 468.1343; found, 468.1359.

**Labelling procedure.** Labelling of bovine serum albumin (BSA) was achieved either by activating the free carboxylic acid substituted dyes with O-$\{\text{N-succinimidyld}-\}N,N,N',N'$-tetramethyluronium tetrafluoroborate (TSTU) in the presence of diisopropylethylamine (DIPEA), resulting in in situ formation of the N-hydroxysuccinimide (NHS) ester, which was subsequently reacted with BSA. Alternatively, NHS esters of the dyes were used directly. The BSA conjugates were subsequently purified by dialysis. Consult ref. 46 for the full labelling protocols and procedures. Despite the tendency of BSA to bind small molecules electrostatically, we did not observe unbound dye in the optical experiments.

**Results and discussion**

**Synthesis**

The synthesis of azaoxa-triangulenium dyes was developed in our lab, based on the work of Martin and Smith, who first synthesised the trioxatriangulenium (TOTA) system. In our early work, azadioxo-, diazaoxo-, and triaza-triangulenium (ADOTA, DAOTA, and TATA) was reported. Lacour and coworkers have later expanded the series of triangulenium salts to also include derivatives with a single sulfur bridge. The incorporation of more than one sulfur atom in the triangulenium core seems not to be possible due to the distortion enforced by the significantly different C–S bond length as compared to C–N and C–O bonds. The aza-triangulenium dyes are all made from a common precursor; tris(2,6-dimethoxyphenyl)-carbenium tetrafluoroborate (1) which can be reacted stepwise with primary amines to form between one and three azo-bridges. Each or intermediate may be reacted under ether cleaving conditions at elevated temperatures to form the fully ring closed triangulenium core with one, two, or three oxo-bridges. The oxo-bridges are themselves reactive towards primary amines. However, this substitution reaction is slow compared to attack on the methoxy groups of the open precursors, but it is still highly selective.

Post-functionalisation of the substituents on the azo-bridges have previously been reported and we have synthesised and explored reactive derivatives of ADOTA for bioconjugation.

The syntheses of ADOTA with reactive NHS esters and maleimide groups (Scheme 1) are straightforward, and proceed by a $S_\text{Ar}$ reaction between 1 and a suitable amino acid or diamine. The primary amine attack one of the methoxy substituted carbon atoms in 1 followed by elimination of methanol upon formation of a transient intermediate set up for an intramolecular $S_\text{Ar}$ reaction, eliminating methanol and forming an azo-bridge. The product is an N-substituted tetramethoxy-acridinium salt (2). Reacting 2 in molten pyridinium chloride yields the fully ring closed azadioxa-triangulenium (4, ADOTA).

Alternatively, a second azo-bridge can be introduced by reacting 2 with a second primary amine forming a 1,4Helicenium ion. The product is an N,N'-substituted 1,13-dimethoxyquinacridinium (DMQA) salt (3, for details on this nomenclature see...
ESI†), which in molten pyridinium chloride yields the fully ring closed diazaoxa-triangulenium core (5, DAOTA).

The steps to ADOTA-derivatives suitable for bioconjugation as active esters and maleimides follow the direct path from 1 over 2 to 4.47 Similarly, the 3-carboxypropyl derivative of DAOTA (2a) could be synthesised by introducing first one (2a), and then a second aza-bridge to give the DMQA derivative (3c). Subsequent reaction of the DMQA compounds in molten pyridinium chloride followed by basic hydrolysis of the intermediate amide yielded the desired N-(3-carboxypropyl)-N'-methyl-DAOTA tetrafluoroborate (5a, Scheme 1) as a red powder in a good yield.49 The synthesis of the 4-aminophenyl DAOTA derivative (5d) was also performed via the route through the DMQA derivative (3d, Scheme 1). This yielded 3d in a high yield, while the ring closure reaction for conversion of 3d into 5d yielded 5d in a low yield. In attempts to prepare the 2-(4-carboxyphenyl)-ethyl and 4-carboxyphenyl DAOTA derivatives (5b and 5c, respectively) via the same synthetic route as described for 5a and 5d, we found that this was associated with difficulties. The compound 3b was isolated in a high yield. However, the following ring-closure reaction in molten pyridinium chloride yielded the desired product (5b) in combination with impurities, which were inseparable from 5b. Similarly, the DMQA derivative 3c could not be prepared as the basic hydrolysis of the intermediate amide derivative (3c', Scheme 2) was not successful.
Thus, 5b and 5c were synthesised via an alternative route, which to our knowledge has only been reported for the N,N'-dipropyl-ADOTA salt by Laursen and Krebs.38 The compounds 5b and 5c were obtained in good yields via reaction of the ADOTA derivatives (4b and 4c) with methylvamine in acetonitrile/ethanol or NMP/ethanol mixture. The acid derivatives 5a–c were reacted with N,N,N',N'-Tetramethyl-O-(N-succinimidyld BALIXI Iconium tetrafluoroborate (TSTU) in acetonitrile or DMSO solution to form the reactive NHS esters (6a–c) in good yields. The NHS ester functional group is used to conjugate fluorescent dyes to biomacromolecules via coupling to a primary amine residue of the biomacromolecule.64

Table 1 Photophysical properties of N-(4-carboxyphenyl)-N'-methyl-DATA core of tetrafluoroborate (ADOTA, 4c) and N-(4-carboxyphenyl)-N'-methyl-diazaoxatriangulium tetrafluoroborate (ADOTA, 5c) in acetonitrile (MeCN), dimethyl sulfoxide (DMSO), and phosphate buffered saline at pH = 7.4 (PBS) solutions

<table>
<thead>
<tr>
<th>Solvent</th>
<th>ADOTA 4c</th>
<th>ADOTA 5c</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>λabs/nm</td>
<td>λem/nm</td>
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<tr>
<td>MeCN</td>
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<tr>
<td>DMSO</td>
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</tbody>
</table>

Optical spectroscopy

In the following N-(4-carboxyphenyl)-N'-methyl-ADOTA tetrafluoroborate 5c is used to demonstrate the spectroscopic properties of the reactive ADOTA derivatives. The properties of 5c are compared to those of the corresponding N-(4-carboxyphenyl)-ADOTA tetrafluoroborate 4c.46,47 The spectra displayed in Fig. 1 show that DAOTA (5c) has more desirable absorption and emission properties. These are in the red region of spectrum, which is better for measurements on biological systems as compared to ADOTA (4c).49,53,65 Table 1 summarises the photophysical properties of 4c and 5c in acetonitrile, dimethyl sulfoxide (DMSO), and phosphate buffered saline at pH = 7.4 (PBS). The differences between 4c and 5c in organic solvents are closely related to the oscillator strengths of the lowest energy transition of the two parent chromophores. The higher molar absorption coefficient at the lowest energy transition of DAOTA as compared to that of ADOTA (ε = 16 000 vs. 10 000 M⁻¹ cm⁻¹ in acetonitrile solution),65 results in a shorter fluorescence lifetime (τfl = 19 ns vs. 21 ns in MeCN).65,66 In PBS solution, DAOTA 5c exhibits a significant change in fluorescence lifetime (τfl) and fluorescence quantum yield (ϕfl), where both are reduced; ϕfl from 55% to 35% and τfl from 19 ns to 14 ns. We have found that chloride is not a specific quencher of DAOTA, and suggest that the observed 30% reduction in ϕfl must be due to unspecific solvent quenching, likely due to the high hydrophobicity of the DAOTA core of 5c. ADOTA 4c is less hydrophobic and does not show more than 18% reduction in ϕfl in PBS solution as compared to the organic solvents.

The fluorescence lifetime (τfl) for DAOTA 5c is exceptionally long for a red emitting organic dye, even when reduced to 14 ns by unspecific solvent quenching in PBS solution. The combination of a long fluorescence lifetime (>10 ns) and emission properties in the red region (600 nm) makes DAOTA ideally suited to monitor rotational correlation times of biomolecules and as a fluorescent probe for fluorescence polarisation based assays.8,47–49

DAOTA-BSA conjugates

To test the ability of DAOTA as a probe for measuring the rotational motion of proteins the reactive ester of DAOTA 5c, N-(4-carboxyphenyl)-N'-methyl-diazaoxatriangulium NHS ester tetrafluoroborate 6c, was conjugated to BSA. This reaction is expected to predominantly result in conjugation of the triangulium based probe to the N-terminus of the protein.46 The labelling protocol was optimised to give a low degree of labelling (DOL) to ensure that complications arising from multiple labels, such as energy transfer between labels, was minimal. When developing a fluorescence polarisation assay, these effects can be probed by looking for Weber’s red-edge effect.57,68 A DOL of 0.9 DAOTA dyes per BSA was used to obtain the results presented below.

The Perrin equation (eqn (2)) describes the ideal relationship between: the observed fluorescence anisotropy (r), the fundamental anisotropy of the dye (r0), the rotational correlation time of the rotating volume (θ), and the fluorescence lifetime (τfl). The rotational correlation time (θ) is directly related (eqn (3)) to the rotational volume (V) and the viscosity of the surrounding medium.8,47–49
medium ($\eta$). For proteins, the rotational correlation time can be related (eqn (3)) to the molecular mass ($M$), the specific density ($\nu$), and the average hydration ($h$) of the protein:  
\[
\frac{r_0}{\tau_0} = 1 + \frac{\tau_0}{\theta} 
\]

Two factors related to the fluorescent probe used are found in the equations: the fluorescence lifetime ($\tau_0$) and the fundamental fluorescence anisotropy ($r_0$). The fluorescence lifetime determines the range of rotational correlation times that may be probed, in other words the range of molecular weights that can be investigated. The fundamental anisotropy, ranging from $0.4$ to $-0.2$, determines the dynamic range of the experiments. Dyes with a low $r_0$ value are poor probes for fluorescence polarisation-based methods. DAOTA has $r_0 = 0.38$, which is very close to the maximal value ($0.4$), while the fluorescence lifetime allows for probing biomolecules with a molecular weight up towards $1000$ kDa.

Fig. 2 shows a Perrin plot of $5c$-BSA, where the steady-state fluorescence anisotropy ($r$) measured at four different temperatures ($T$) are plotted as $1/r$ against $T/\eta$ and used to determine the $\theta/V$, and the apparent anisotropy ($r_{app}$) by extrapolation of $T/\eta$ to zero. The latter is a measure for the flexibility of the dye label, when conjugated to the biomolecule. Ideally, if the label upon conjugation loses all degrees of freedoms, except co-rotation with the biomolecule, the apparent and fundamental anisotropy will be identical. Any local flexibility of the dye label will induce a pathway for fast scrambling of the photoselection not related to the motion of the biomolecule. The result is a lowering of the apparent anisotropy $r_{app}$ in a Perrin plot, and a loss of dynamic range in any fluorescence polarisation based experiment. For $5c$-BSA the apparent anisotropy is at $r_{app} = 0.36$ surprisingly high, clearly indicating that the DAOTA label is immobilised on the surface of BSA.

While the effect of the long fluorescence lifetime of $5c$ may be hard to identify in the steady state spectra, it is directly visible in time-resolved experiments. Fig. 3 (top) shows a time-resolved emission decay profile for $5c$ and $5c$-BSA measured in PBS solution, obtained using time-correlated single photon counting (TCSPC). Cursory inspection of the fluorescence decays profiles in Fig. 3 (top) shows that the $5c$-BSA has a longer fluorescence lifetime than $5c$ in PBS solution ($\tau_{fl,5c} = 14.0$ ns vs. $\tau_{fl,5c}$-BSA $21.2$ ns, see ESI†), and that photons can be detected well beyond $150$ ns when using the standard settings of TCSPC with a maximum count of $10.000$. By using longer acquisition times (higher maximum number of counts), photons arising from emission of the $5c$-BSA conjugates may be detected up towards $250$ ns after excitation, this is without equal when considering organic dyes with emission in the red.

Fig. 3 (bottom) shows the time-resolved anisotropy decay for $5c$-BSA. The data allow for direct determination of the rotational correlation time of the rotating volume ($\theta$). The long fluorescence lifetime is important in obtaining these data, as photons must be emitted in a time interval long enough to describe the rotational motion of the biomolecule. Fig. 3b
shows that in the case of 5c-BSA the photoselection is fully scrambled by rotational motion in ~100 ns. That is, the anisotropy has decayed to zero. The long rotational correlation time determined for BSA from these data is \( \tau_{\text{BSA}} = 40 \text{ ns} \) (see ESI for details†), a number identical to the average literature value of \( \tau_{\text{BSA}} = 40 \text{ ns} \).69 Note that BSA is not a perfect spherical rotor, and the value determined will be influenced by the position and relative orientation of the dye in the bioconjugate.

While emission from either of the azaoxa-triangulenium dyes can be used to follow the rotational motion of large biomolecules for more than 100 ns,27 there is a clear difference in the behaviour of 4a-BSA and 5c-BSA. Where ADOTA fluorescence is quenched in the conjugates (4a-BSA) when compared to the non-bounded dyes (4a), the DAOTA fluorescence appears to be enhanced upon conjugation, as seen by the significantly increased fluorescence lifetime (Fig. 3). As the fluorescence lifetime only report on the emitting population of dyes, the actual emission intensity of each dye was determined. Fig. 4 shows the results for 4a-BSA and 5c-BSA. The total emission intensity of either dye is decreased upon conjugation to BSA, but the effect is much less pronounced for the DAOTA derivative. We rationalised the quenching of the ADOTA fluorescence as a result of reductive PET quenching by tryptophan,50 a process the increased cation stability of DAOTA makes less favoured (see ESI for details†).51 Thus, we see less quenching of the DAOTA fluorescence in the 5c-BSA conjugates. The significant increase in lifetime upon conjugation of 5c to BSA must be due to a reduction in the non-specific solvent induced quenching of a population of DAOTA dyes that is partially shielded by the protein surface, while the reduction in overall intensity must be due to an almost fully quenched population of 5c-BSA. The quenched population does not contribute to the time-resolved emission decay profile, and will not influence the fluorescence anisotropy. The net result is that the fluorescence quantum yield of the 5c-BSA conjugate at \( \phi_0 = 0.34 \) is very close to that of the free dye 5c in PBS at \( \phi_0 = 0.35 \), although with a more complicated time-resolved fluorescence decay profile (see ESI†).

Conclusions

The syntheses of six new derivatives of diazoxatriangulenium (DAOTA) salts were reported, and it was shown that these dyes can be accessed via two synthetic routes. One set of substituents may favour one route over the other.

The photophysical properties of the DAOTA fluorophore were investigated in view of using the red emitting, long fluorescence lifetime dye in fluorescence polarisation assays. We showed that the DAOTA fluorophore undergoes unspecific solvent fluorescence quenching in aqueous buffer, reducing the fluorescence lifetime from 19 ns in acetonitrile solution to 14 ns in PBS solution. 5c was conjugated to BSA, and we found that 5c-BSA had a significantly increased longest fluorescence lifetime component at 21.2 ns (as well as intensity weighted average fluorescence lifetime of 19.2 ns). Furthermore, the overall emission intensity of the conjugates (5c-BSA) was found to be equal to that of the free dye 5c) measured in PBS solution, as 5c is less quenched by tryptophan. Thus the DAOTA fluorophore, with emission further in the red and a longer fluorescence lifetime in biomolecule conjugates, was found to be superior to the ADOTA fluorophore as a probe for developing fluorescence polarisation assays for large biomolecules.

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Notes and references