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Trihydroxytrioxatriangulene—An Extended Fluorescein and a Ratiometric pH Sensor

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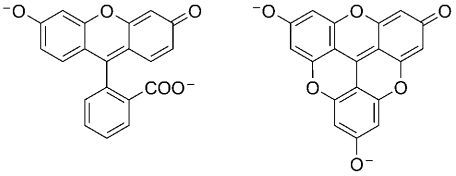
Dedicated to Professor Klaus Bechgaard

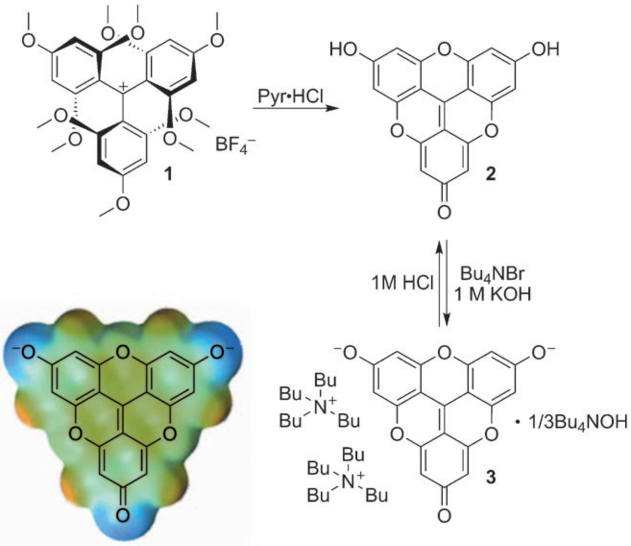
The high fluorescence quantum yield, non-toxicity and ease of synthesis have made fluorescein (Scheme 1) the dye of choice in medicine and science for more than 100 years.[1] It was first synthesised by A. Baeyer in 1871[2] and is extensively used in fluorescent sensors[3] and bioimaging,[1a,4] but has also found more exotic uses: for example the colouring of rivers.[1c] Fluorescein is still in focus of the development of new dyes and several variations of fluorescein, for instance the Tokyo Greens, have recently been synthesised.[5] In these dyes, modifications of the appending benzoic acid residue has allowed for tailoring of both the photophysical properties and their applicability in biologically relevant systems.[3a,6]



Scheme 1. The fully deprotonated structure of fluorescein (left) and the dianion of trihydroxytrioxatriangulenium (H-TOTA^{2-}, right).

In this communication we report the synthesis and properties of trihydroxytrioxatriangulenium (H-TOTA) (Scheme 1), a new[7] planar D_{3h} symmetric extension of fluorescein with intense absorption and high fluorescence quantum yield. H-TOTA displays pH sensitive absorption and fluorescence properties, and we demonstrate its use as a ratiometric pH sensor with high sensitivity at physiological pH.

The synthesis of the H-TOTA ring system is straight forward (Scheme 2) and H-TOTA is obtained in one step from the readily available tris(2,6-dimethoxyphenyl)methylium·BF_4 salt (**1**).[8] A combination of ether cleavage and ring



Scheme 2. Synthetic procedure to H-TOTA and the calculated electron density of the dianion, H-TOTA^{2-} (bottom left). Blue indicates high electron density and red low.

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closure reactions leads to formation of H-TOTA by heating **1** in molten pyridinium hydrochloride at 170 °C for 150 min.[9] The cooled acidic reaction mixture is poured into water and neutralised by titration with NaOH to precipitate the neutral dye, H-TOTA (**2**). The crude product **2** is obtained in 90 % yield, but the compound is difficult to purify and characterise due to its low solubility in all common solvents. Improved solubility and further purification is achieved by dissolving **2** in alkaline water, in which it dissolves as the dianion H-TOTA^{2-}, and precipitating it as the tetrabutylammonium salt **3**. After recrystallisation pure **3** was obtained in 30 % overall yield. The moderate yield can be explained by incomplete precipitation due to the high solubility of H-TOTA^{2-} in alkaline water. ^{1}H NMR shows that **3** contains a constant excess of tetrabutylammonium ions corresponding to inclusion of a 1/3 equivalent of tetrabutylammonium hydroxide. The tetrabutylammonium salt **3** is soluble in water and most common organic solvents. Pure **2** can be obtained by neutralisation of a concentrated aqueous solution of **3** with hydrochloric acid, whereupon **2** precipitates.

Based on simple resonance arguments, the dianion of H-TOTA is expected to exist as a completely planar D_{3h} symmetric π-system, with the two negative charges equally distributed between the three phenolate/quinoide oxygens at the corners of the trioxatriangulene skeleton. This is confirmed by the calculated electron density shown in Scheme 2. We consider this a unique structural motif that, in conjunction with the photophysical properties of the system, makes it suitable for supramolecular assemblies of charged planar aromatic molecules.[10] Similar symmetrical charge delocalisation is found in the triangulenium cations, allowing for close packing of similarly charged molecules and suggesting that the H-TOTA^{2-} ion may form similar columnar aggregates.[8b, 9b, 10a, 11]

The effective chromophore unit in fluorescein is of the oxonol type, in which two oxygen atoms are connected through an odd number of sp^2-hybridised carbon atoms, a motif also known from other famous colourants such as phenolphthalein and naturally occurring anthocyanins.[12] In H-TOTA this linear chromophore is extended into two dimensions by attaching three oxygen atoms to the periphery of a trioxatriangulene core, which has recently been applied in a number of planar and highly stabilised cationic dyes.[8b, 9, 11b, 13]

H-TOTA, fluorescein and all other oxonol type dyes are intrinsically pH sensitive, since protonation/deprotonation of the terminal oxygen atoms results in significant changes in the chromophore.[12] This is the basis for the pH indicator action of phenolphthalein, some fluorescein derivatives, such as SNARF (seminaphthorhodafluor), and other fluorescent pH probes.[14] The extensive interest in and use of fluorescein preceded complete mapping of the pH response by several decades.[1c, 4a, 15] The pH response and behaviour of the excited states of fluorescein is exceedingly complex with seven differently protonated forms possible. This is due to the carboxylic acid on the phenyl group that adds the possibility of additional protonation and lactone formation. The full complex set of equilibria, together with their photophysical properties, was resolved by the groups of Kubista and Sawyer in the mid 1990s.[16] The pH responsive groups of the H-TOTA system are the three phenolic groups; thus H-TOTA is expected to have four interconvertible states: H-TOTA$^+$, H-TOTA^0, H-TOTA$^-$ and H-TOTA^{2-}. This was found to be the case and Figure 1 shows the four different states and their absorption and emission spectra. The spectra were recorded in DMSO with 20 mol % water (6 % v/v) and the acidity of the solution was controlled by the addition of acid or base in order to obtain the different protonation states (see Supporting Information for details). The fun-

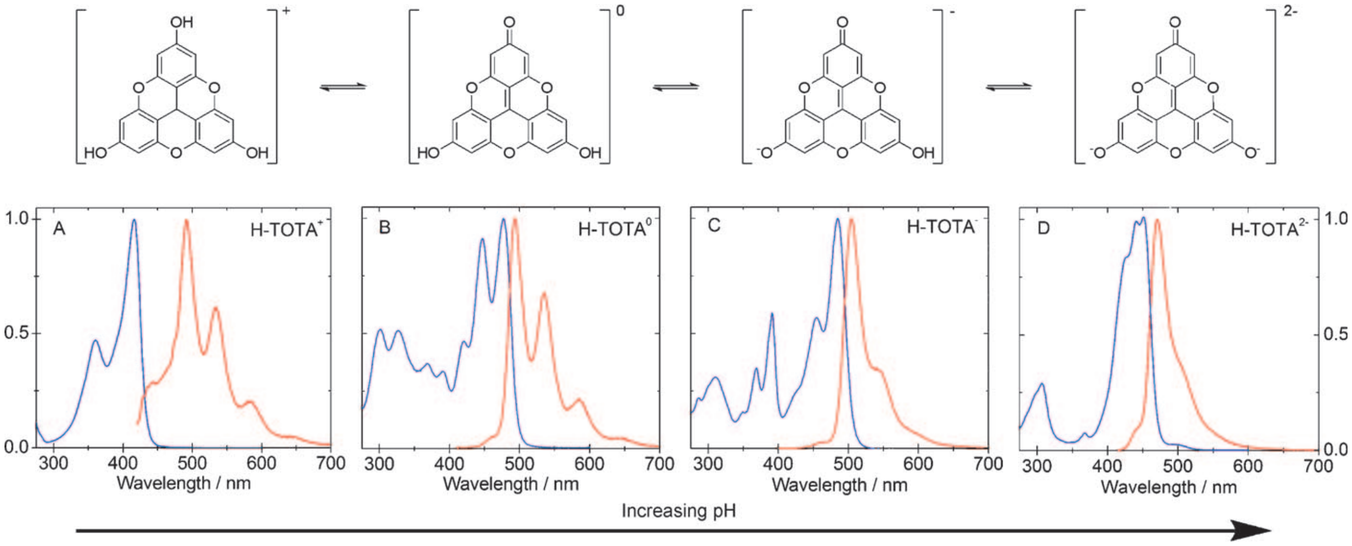

Figure 1. The four different protonation states of H-TOTA with their corresponding absorption (blue) and emission (red) spectra in 80:20 mol % DMSO:H_2O (6 % v/v H_2O) mixture.

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damental photophysical data for the four forms of H-TOTA in DMSO and in water are compiled in Table 1.

Table 1. Photophysical properties of the four different protonation states of H-TOTA in DMSO and water.

	Species	ε (λ_{max}) [$\text{M}^{-1}\text{cm}^{-1}$ ([nm])]	λ_{fl} [nm]	ϕ_{fl}[c]	τ [ns]
DMSO[a]	H-TOTA^{2-}	76000 (451)	471	0.65	3.3
	H-TOTA^{-}	55000 (485)	505	0.87	4.2
	H-TOTA^{0}	35000 (477)	493	0.62	4.3
	H-TOTA^{+}	57000 (416)	491	<0.01	3.2
H_2O[b]	H-TOTA^{2-}	63000 (425)	459	0.80	3.2
	H-TOTA^{0}	23000 (429)	474	0.05	3.8
	H-TOTA^{+}	38000 (413)	477	0.30	2.9

[a] 80:20 mol% DMSO/H_2O (6% v/v H_2O). [b] 95:5 mol% H_2O/DMSO (17% v/v DMSO). [c] Measured against quinine in 0.1 M H_2SO_4.

The fully protonated H-TOTA^{+} ion shows an intense absorption at 416 nm and a weak, red-shifted emission (ϕ_{fl} <0.01) at 491 nm in acidic DMSO/water solvent mixture (Figure 1 A). The emission spectrum of the cation is found to be almost identical to that of the neutral form (H-TOTA^{0}), suggesting that the emission originates from the same species. This can be assigned to an excited state deprotonation of H-TOTA^{+}, that is, a lowering of the pK_a of the excited state; this is a phenomenon known for several hydroxyl containing dyes[17] including fluorescein.[16a] This assumption is supported by the observation of a mirror image relationship between the absorption and emission spectra of H-TOTA^{0} (Figure 1 B) and the finding that the excitation spectrum of H-TOTA^{+} matches its absorption spectrum, despite the fact that its emission spectrum is identical to that of H-TOTA^{0}. Thus, we assign the low fluorescence quantum yield of H-TOTA^{+} in DMSO to an efficient excited state deprotonation, which in most cases is accompanied by nonradiative deactivation. A low yield of the formation of excited H-TOTA^{0} from H-TOTA^{+} is consistent with the observed excited state lifetime of 3.2 ns, which otherwise would be much too long for the low quantum yield (<0.01). The absorption spectrum of H-TOTA^{0} (Figure 1 B) is red-shifted to 477 nm and is more structured compared to the cation. The absorption of the monoanion H-TOTA^{-} is further red-shifted with a maximum at 485 nm and an emission following the mirror image rule (emission maximum at 505 nm, Figure 1 C). The dianion H-TOTA^{2-} (Figure 1 D) exhibits a significant blue-shift when compared to the monoanion. H-TOTA^{2-} shows a strong absorption at 451 nm and an emission spectrum with a maximum at 471 nm. Except for the cation, which undergoes an excited state photoreaction, all forms of the H-TOTA system are highly fluorescent in DMSO with quantum yields from 0.62 to 0.85.

While all four predicted protonation states of H-TOTA could be identified in DMSO (6% v/v H_2O), pH-titrations in water lead to a different result. As shown in Figure 2, only three different species can be detected in aqueous solution. At high pH (>9.5) the dianionic H-TOTA^{2-} is the only species present. In water H-TOTA^{2-} displays a strong ab-

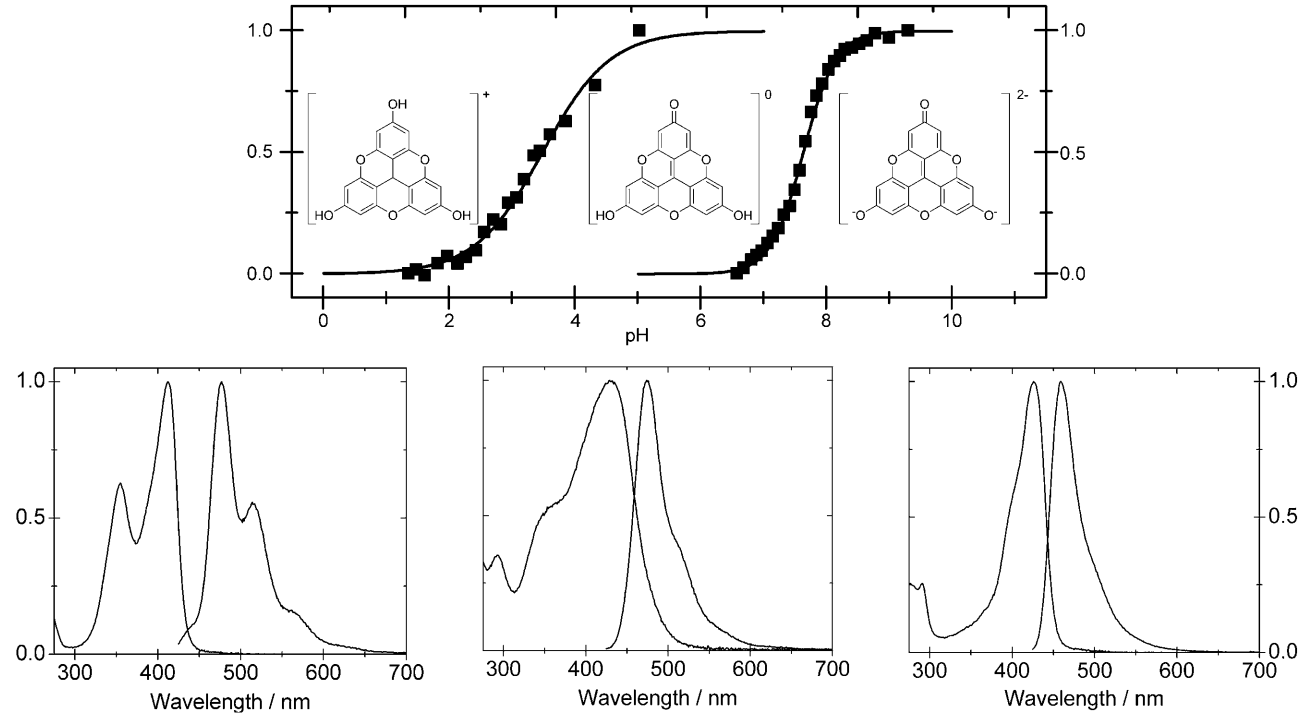

Figure 2. pH titration in a 95:5 mol% H_2O:DMSO mixture (top) and the normalised absorption and emission spectra for the three different protonation states detected (bottom). The lines in the top panel are fitted sigmoidal curves. The titration is performed with 5 mol% DMSO (17% v/v) in the water to avoid artefacts due to the low solubility of the neutral species.

sorption at 425 nm and emission at 459 nm with a quantum yield of 0.80. At lower pH H-TOTA^{2-} is in equilibrium with its acid form, with a p$K_a = 7.6$. The spectral properties of this species imply that it is the neutral species H-TOTA^0, leaving the missing protonation state to be the monoanion H-TOTA$^-$ (see Supporting Information for details). The reason for this unusual two-proton process is believed to be specific solvation of the hydrophobic core of the neutral form, in agreement with the low water solubility. This is manifested in the absorption spectrum by a broad absorption peak with only weak vibrational fine structure. We speculate that the energy gain on going from the neutral form over the partially hydrophilic monoanion to the dianion is so great that the monoanion is not stable in water. Further lowering of the pH yields the acid form, H-TOTA$^+$, which has a p$K_a = 3.7$. By comparing the spectra it can be seen that, as in DMSO, H-TOTA$^+$ acts as an excited state acid and the fluorescence can be assigned to photogenerated H-TOTA^0. However, the excited state deprotonation is less prone to non-radiative deactivation in water and the overall emission quantum yield rises to 0.30 without any significant change in fluorescence lifetime (Table 1).

The fact that the pK_a for the H-TOTA^{2-}/H-TOTA^0 equilibrium in water (7.6) is close to physiological pH (7.4) suggests that this dye could be used as a fluorescent pH sensor in biologically relevant conditions.[18] Figure 3 demonstrates H-TOTA as a ratiometric pH sensor at nanomolar concentrations in phosphate-buffered saline (PBS).[14,19] The dye is excited at two different wavelengths, at which the absorption is dominated by one of the species (here 350 and 425 nm, inset in Figure 3), and the ratio between the two emission intensities at 470 nm is collected. The main advantage of a ratiometric probe is that the pH is determined from an emission ratio and thus is insensitive to exact dye loading. The individual data points in Figure 3 were collected for random dye concentrations between 10 and 100 nM. The data was collected with a short integration time (0.1 s) allowing for improved sensitivity. Experiments at different emission wavelengths show that any emission wavelength between 470 and 530 nm can be used to determine the pH with equal sensitivity. Thus, a long-pass filter is sufficient when collecting the emission ratio, implying that H-TOTA can be used to measure pH by fluorescence microscopy. It should be noted that the physiological buffer shifts the pK_a by nearly one pH unit from 7.6 to 6.7 and, while H-TOTA^0 precipitates at high concentrations, it is completely soluble in the concentration range relevant for the pH measurements.

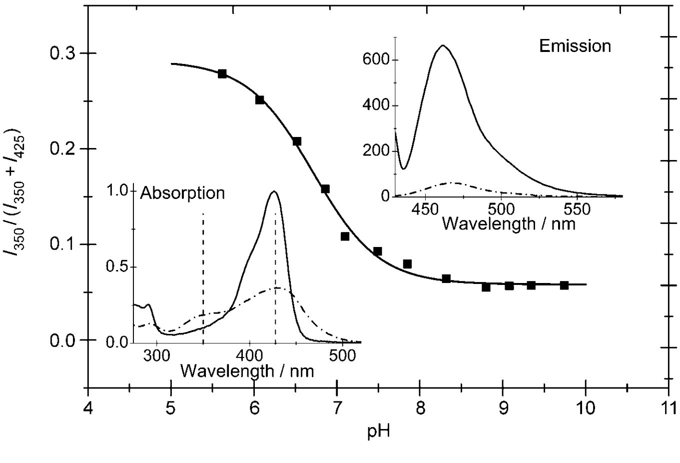

Figure 3. Ratiometric pH titration of the fluorescence of H-TOTA in PBS buffer. Emission spectra are measured at two different excitation wavelengths indicated in the absorption spectra (bottom left) by dashed lines. The ratio of the emission intensities at 470 nm, $I_{350}/(I_{350}+I_{425})$, is plotted versus the pH of the solution. The emission spectra at pH 7.5 are shown (top right) with excitation at 350 (dashed line) and at 425 nm (full line). Measurements were performed in PBS buffer with 130 mM NaCl, with an integration time of 0.1 s and a dye concentration of 10–100 nM.

To conclude: a new dye system, H-TOTA, has been synthesised in one step from readily available starting materials. H-TOTA is a symmetrically extended fluorescein analogue with an absorption coefficient and quantum yield comparable to that of fluorescein; the main difference is a blue shift in absorption and emission of approximately 60 nm for H-TOTA^{2-}.[1a] H-TOTA displays solvent and pH-dependent photophysical properties and can be used as a fluorescent ratiometric pH probe with high sensitivity at physiological pH. We also expect the planar and threefold symmetric ionic H-TOTA^{2-} fluorophore to be an excellent building block in ionic supramolecular self-assemblies.

Experimental Section

The materials were used as received. Emission measurements were done using a Fluorolog-3 from Horiba. The fluorescence lifetimes were measured by using time-correlated single-photon counting (TC-SPC), exciting the samples at 405 nm and detecting through a 425 nm long-pass filter. Absorption spectra were recorded on a Varian Cary 100. The ratiometric pH titration was performed on a Perkin–Elmer LS50 fluorimeter. Measurements were done in either 95:5 mol% (17% v/v DMSO) or 20:80 mol% (6% v/v H_2O) H_2O/DMSO mixtures, excepting the ratiometric titration, which was performed in PBS buffer. The pH was adjusted by using KOH and acetic acid, always keeping the DMSO/H_2O ratio constant. Tris(2,4,6-trimethoxyphenyl)methylium tetrafluoroborate (**1**) was synthesised as reported in reference [8b].

Synthesis of bis(tetrabutylammonium)-2,6,10-trioxo-4,8,12-trioxatriangulenium (3): Compound **1**·BF_4 (1.00 g, 1.67 mmol) and pyridinium hydrochloride (10 g, 87 mmol) were heated to 170 °C for 2.5 h, and the reaction was monitored by MALDI-TOF mass spectrometry. After completion, the hot reaction mixture was poured into H_2O (250 mL) and aqueous KOH (1 M, 60 mL) was added, resulting in pH ≈6. The crude product (**2**) precipitated and was collected by filtration and washed repeatedly with water; isolated yield: 90% (500 mg) The crude product was dissolved in aqueous KOH (1 M, 10 mL) and tetrabutylammonium bromide (3.22 g, 10 mmol) was added. The mixture was left to precipitate for 12 h at room temperature and the crude tetrabutylammonium salt was collected by filtration. The solid was dissolved in acetonitrile (50 mL), the mixture was filtered and CCl_4 (300 mL) was added to precipitate the product as a yellow-brown powder in a yield of 30% (475 mg). ^{1}H NMR (500 MHz, DMSO): $\delta = 5.69$ (s, 6H), 3.20 (m, 19H), 1.57–1.49 (m, 19H), 1.37–1.19 (m, 19H), 0.90 ppm (t, $J = 7.9$ Hz, 28H); ^{13}C NMR (126 MHz, D_2O): $\delta = 178.58$, 153.43, 130.59, 102.25, 102.19, 92.68, 58.10, 23.17, 19.22, 12.94 ppm; UV/Vis (DMSO): $\lambda_{max}(\log \varepsilon) = 451$ (4.9), 441 (4.9), 426 (sh; 4.8), 368(3.8), 307 nm (4.3); MS (MALDI-TOF): m/z: 332.8 $[M]^+$; HR ESI-TOF: m/z calcd for $C_{19}H_9O_6{}^+$: 333.0394; found: 333.0382; elemental analysis calcd (%) for $C_{169}H_{277}K_6N_7O_{25}$ (3×**3**·6 KOH·Bu_4NOH): C 66.7, H 9.2, N 3.2; found: C 66.7, H 9.3, N 3.0.

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Keywords: dyes/pigments · fluorescence · photophysics · sensors · triangulenium

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