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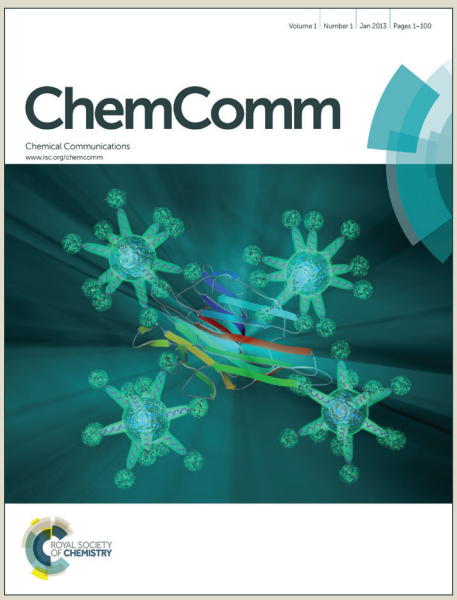

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Highly emissive water-soluble Tetraazaperopyrenes as fluorescence markers

Cite this: DOI: 10.1039/xoxxoooooox

Lena Hahn, [a] Simin Öz[b], Hubert Wadepohl[a], and Lutz H. Gade*[a]

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

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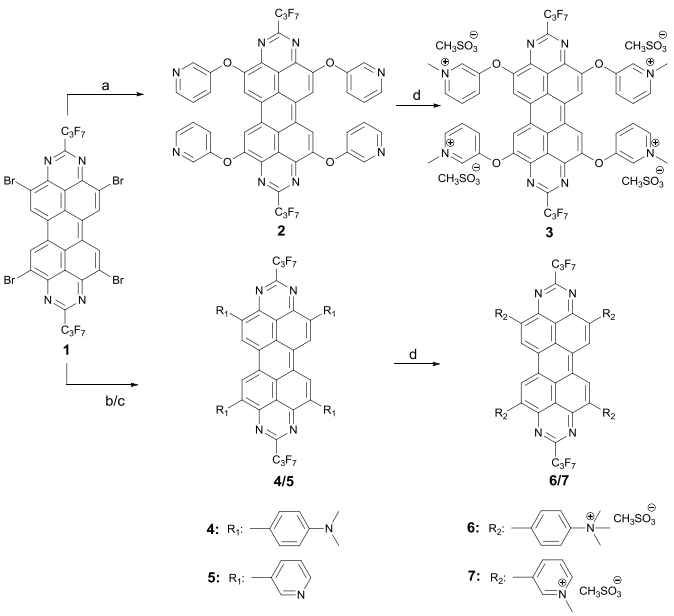
Water-soluble tetraazaperopyrene derivatives (TAPPs) have been synthesized, which show both high photostability and high fluorescence quantum yields (> 80 %) in water. Furthermore, delivery of the dye into cells demonstrated selective staining of the nucleus.

The application of fluorescence microscopy has become a powerful tool for the investigation of biological systems as well as in clinical diagnosis and therapy.[1,2] As a consequence, the use of fluorescent markers for cellular compartments[3] or biomolecules such as DNA[4–6], RNA[7–9] or proteins[10] has expanded rapidly in recent years.[11,12] However, these markers have to meet certain requirements such as high water solubility, high molecular extinction coefficients and fluorescence quantum yields in an aqueous medium. This has to be matched by high chemical and photophysical stability, as well as low cytotoxicity. Even though there is a large number of water-soluble dyes commercially available today most of them possess low photochemical stability and/or low fluorescence quantum yields.[1,13] Hence, considerable efforts have been devoted towards the synthesis of water-soluble chromophores[14–21] which combine the above mentioned features.[22–27] Many of these systems are functionalized perylenetetracarboxydiimides (PDIs) which bear charged substituents or polar dendritic sidechains.

In recent years, we developed a synthesis of a new class of polyheterocyclic aromatics, 1,3,8,10-tetraazaperopyrenes (TAPPs) possessing properties which are complementary to those of PDIs.[28] While the unsubstituted TAPPs are sparingly soluble, core functionalization of TAPPs demonstrated a drastic increase of their solubility in common organic solvents and resulted in chromophores possessing remarkable absorption and fluorescence properties.[29–31] Owing to these photochemical characteristics water-soluble TAPPs were thought to be suitable candidates for the application as biomarkers. In this work we present a first account of the synthesis and photophysical properties of such water soluble systems as well as their application as cellular fluorescent markers.

One strategy to render organic dyes water soluble is the incorporation of charged functional groups into the molecular structure.[18,24] Based on this concept, the strategy for the synthesis of water-soluble TAPP derivatives was the introduction of *N*-heterocycles or amino groups to the 4,7,11,14-positions of the TAPP core and the subsequent quaternization of the nitrogen atoms. The synthesis of the resulting tetracationic TAPP derivatives **3, 6, 7** was accomplished in three steps (Scheme 1).



Scheme 1 a) 3-Pyridinol, N-methylpyrrolidone (NMP), Cs_2CO_3, 100 °C, 4 d, 71 %; b/c) 4-dimethylaminophenylboronic acid b)/ pyridine-3-boronic ester c), [$PdCl_2$(dppf)], toluene, K_2CO_3/H_2O (1 M), 105 °C, 3 d, 79 % (4), 75 % (5); d) 1. methyl iodide, methanol, 80 °C, 24 h 2. silver methanesulfonate, methanol, rt, 12 h, 90-94 %.

The first step of the synthesis of **3** was phenoxylation of 2,9-bisperfluorobutyryl-4,7,11,14-tetrabromo-1,3,8,10-tetraazaperopyren (**1**), analogous to the approach chosen by Müllen et al. for PDI derivatives,[18] by treatment with 3-pyridinol in 1-methyl-2-pyrrolidone (NMP) resulting in the formation of **2** in moderate yields. Direct aryl-aryl coupling employed in the synthesis **4** and **5** was accomplished by Suzuki coupling of **1** with either pyridine-3-boronic ester or 4-dimethylaminophenylboronic acid, respectively.

The next step, the quaternization of the nitrogen atoms, was achieved by reaction of **2**, **4** and **5** with an excess of methyliodide, followed by a salt metathesis with silver methanesulfonate to give the desired water-soluble compounds **3**, **6** and **7** in quantitative yields. Each of the TAPP derivatives was structurally characterized by NMR spectroscopy as well as mass spectrometry. Their photophysical properties in water were investigated by UV/VIS and fluorescence spectroscopy. An overview of the optical properties of compounds **3**, **6** and **7** is given in Table 1**.**

Table 1. Photophysical properties of **3a**,**5a** and **7a** recorded in water

	λ_{max} [nm] (log ε)	λ_{em} [nm] (Stokes-shift)	ϕ_{em}	τ [ns]
3	472 (4.56)	504 (1344 cm^{-1})	0.72	3.42
6	484 (4.31)	512 (1130 cm^{-1})	0.80	2.80
7	474 (4.63)	492 (772 cm^{-1})	0.80	3.03

All of the newly synthesized TAPP derivatives are red in the solid state and display green fluorescence in solution. The UV/VIS absorption spectra of compound **3**, **6** and **7** recorded in water display characteristic visible $\pi^* \leftarrow \pi$ absorption bands with a maximum between 472 and 484 nm and high extinction coefficients in water (between log 4.56 and log 4.82). As expected, the emission spectra of these compounds image the mirror symmetry of absorbance spectra with Stokes shifts in the range of 950-1350 cm^{-1} (Figure 1).

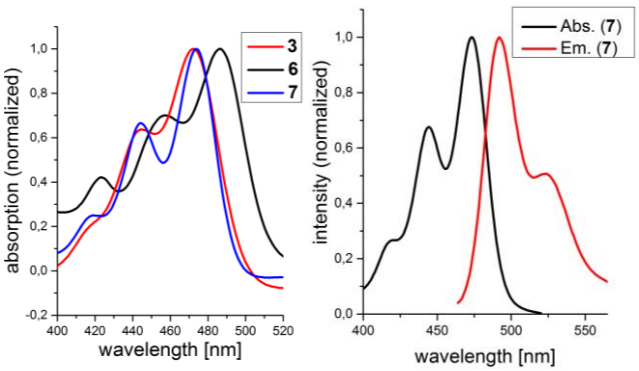


Figure 1: Normalized absorption spectra of **3**,**6** and **7** in water (10^{-7} M) (left), normalized absorption and emission spectra (excitation wavelength: 460 nm) of **7** in water (right).

Notably, these TAPP salts were found to be characterized by remarkably high fluorescence quantum yields (FQY) of up to 80 % in water, with compound **3** possessing the lowest FQY of 72 %. We attribute the latter to the flexible diarylether linkages in compound **3** which cause partial fluorescence quenching.

As a next step the effect of the counter ion on the FQY and the water solubility of this class of compounds was investigated. Upon reaction of the neutral precursor **2** with either dimethyl sulfate or methyl trifluoromethansulfonate we obtained the methylsulfate (**7a**) and trifluoromethylsulfonate salts (**7b**), respectively. The photophysical properties of **7a** and **7b** remained essentially unchanged compared to **7** with an absorption maximum at 474 and an emission maximum at 492 (Table 2).

Table 2. Influence of the counter ion on water solubility and FQY.

	Counter ion	Water solubility	ϕ_{em}	log ε
7	$CH_3SO_3^-$	2.0 x 10^{-3} mol L^{-1}	80%	4.63
7a	$CH_3SO_4^-$	2.1 x 10^{-3} mol L^{-1}	82%	4.82
7b	$CF_3SO_3^-$	2.2 x 10^{-6} mol L^{-1}	80%	4.63

The FQY of the three salts are in the range of 80-82 %, demonstrating that the counter ion seems to have no significant effect on the FQY. However the water solubility is strongly dependent on the counter ion. Whereas compounds **7** and **7a** we found to be highly soluble in water, the solubility of trifluoromethylsulfonate salt **7b** was lower by a factor of 1000.

Crystals of compound **7b**, which were suitable for X-ray diffraction, were grown from methanol and its molecular structure is displayed in Figure 2. As observed for most TAPP derivatives[28–31] tetraazaperopyrene core of the dye is almost planar (RMSD 0.025 Å) with the two perfluoroalkyl substituents pointing in opposite directions above and below the TAPP core. The planes of the N-methyl pyridinium substituents are at 35.8(1)° and 46.43(8)° to the TAPP core.

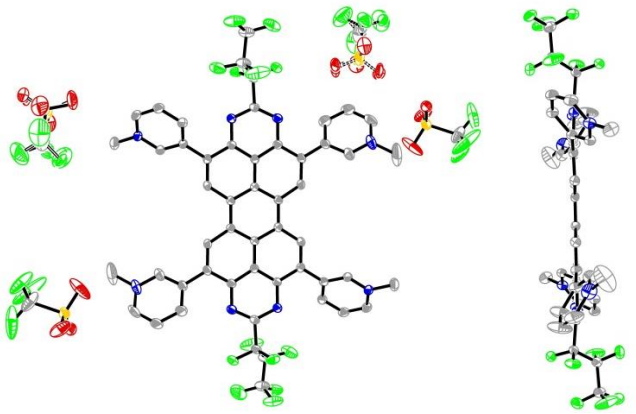


Figure 2: Top view (left) and side view (right) of compound **7a**, displaying the structure of the TAPP dye. Thermal ellipsoids are drawn at 50% probability level, hydrogen atoms were omitted for clarity.

The water-solubility of the synthesized dyes raised the question of their applicability as fluorescent markers in biological samples. In an initial step towards characterizing the effects of the dye in living cells a cervical carcinoma cell line (HeLa) was used. We treated HeLa cells with various concentrations (0 to 100 µM) of **3**,**6** and **7a** respectively for 24 h at 37°C and determined the metabolic activity of cells by measuring the intracellular ATP levels.[32] The results demonstrated IC_{50} values in micro molar ranges suggesting a weak cellular response (Figure 3). Furthermore the cytotoxicity of the methylsulfate salt **7a** remained low even at relatively high concentrations of the dye. Therefore, all subsequent studies were performed with this compound.

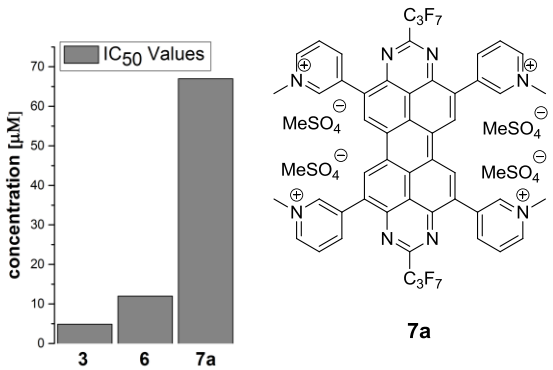


Figure 3: IC_{50} values of compound **3**, **6** and **7a** after treatment of HeLa cells for 24h.

Next, we investigated the accumulation of the fluorescent marker in HeLa cells. We incubated PFA-fixed and permeabilized cells with 20 µM of **7a** for 1h at ambient temperature which were subsequently

investigated by confocal microscopy. The results displayed in Figure 4 show that significant amounts of the fluorescent marker were accumulated within the nuclei of the cells. In contrast almost no dye was detected within the cytoplasm reflecting the specificity of the compound for chromatine. Figure 4b shows an enlarged image of a cell fixed during mitosis which clearly demonstrates the preferential accumulation of the dye at the chromatides. Additionally, staining of HeLa cells with both DAPI (4',6-diamidino-2-phenylindol), an established DNA marker, and **7a** desplayed colocalization of both compounds within the nucleus, further confirming the selective staining behaviour of **7a** (Figure in ESI) .

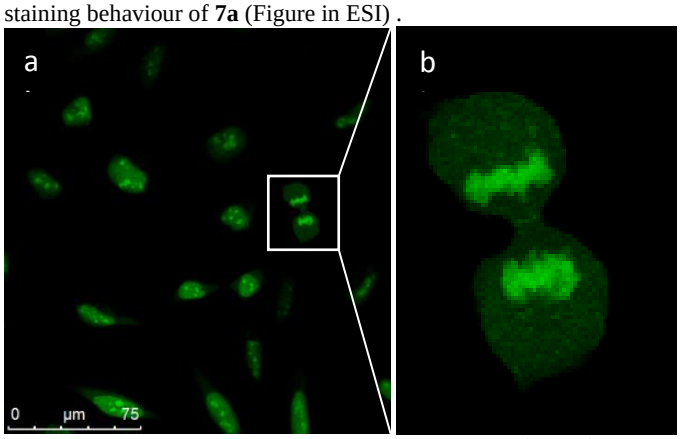


Figure 4: a) HeLa cells incubated with **6a**; b) enlargement of a cell fixed during mitosis.

In conclusion, we have developed a synthesis for the highly water soluble TAPP derivatives that possess remarkable absorption and emission properties with fluorescence quantum yields of up to 82 % in water. We note that the chosen approach does not involve the addition of bioconjugation moieties.[19-21] First studies into their interaction with living cells show that the compounds exhibit relatively low cytotoxicity with IC_{50} values of up to 67 µM after treatment for 24 hours. Incubation of fixed cells with **7a** demonstrated selective staining of the cell nuclei. More specifically the dye was localized especially at DNA rich regions of the chromatides, as demonstrated by a cell in the stage of mitosis. This suggests a direct interaction with DNA, and the investigation of the specific interaction of the fluorescent TAPP salts with DNA is the objective of current and future studies in our group.

Notes and references

[a] L. Hahn, Prof. Dr. H. Wadepohl, Prof. Dr. L. H. Gade
Anorganisch-Chemisches-Institut, Universität Heidelberg
Im Neuenheimer Feld 270, 69120 Heidelberg (Germany)
Fax: (+49) 6221545609
E-mail: lutz.gade@uni-hd.de
[b] S. Öz
Division of Epigenetics, DKFZ-ZMBH Alliance, German Cancer Research Center, Heidelberg, Germany
E-mail: s.oez@dkfz.de

† Financal support from the University of Heidelberg as well as the doctoral college "Verknüpfung molekularer π-Systeme zu Funktionsmaterialien" funded by the Landesgraduiertenförderung of Baden-Württemberg is gratefully acknowledged. The authors thank Thimon Schwaebel for determining the fluorescence lifetimes and Nevcin Sentürk for imaging. Electronic Supplementary Information (ESI) available: General experimental methods; compound characterization and X-ray crystallographic data of compound **5**. See DOI: 10.1039/c000000x/

[#] *Crystal data:* $C_{56}H_{32}F_{26}N_8O_{12}S_4$, M = 1631.13, monoclinic, a = 10.5849(6), b = 9.8070(4), c = 29.9716(13) Å, β = 99.183(5) °, V = 3071.3(3) Å^3, T = 110(1) K, space group $P2_1/n$, Z = 2, Cu K_α X-radiation, λ = 1.5418 Å, 44255 reflections measured, 5881 unique (R_{int} = 0.0639) , $wR(F^2)$ [all unique data] = 0.1584, $R(F)$ [$F_o > 4\sigma(F_o)$] = 0.0609.

[#2] CCDC 986570 and 986571 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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