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# Water-Soluble Triarylborane Chromophores for One- and Two-Photon Excited Fluorescence Imaging of Mitochondria in Cells

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**Abstract:** Three water-soluble tetracationic quadrupolar chromophores comprising two three-coordinate boron  $\pi$ -acceptor groups bridged by thiophene-containing moieties were synthesised for biological imaging applications. The derivative **3** containing the bulkier 5-(3,5-Me<sub>2</sub>C<sub>6</sub>H<sub>2</sub>)-2,2'-(C<sub>4</sub>H<sub>2</sub>S)<sub>2</sub>-5'-(3,5-Me<sub>2</sub>C<sub>6</sub>H<sub>2</sub>) bridge is stable over a long period of time, exhibits a high fluorescence quantum yield and strong one- (OPA) and two-photon absorption (TPA), with a TPA cross-section of 268 GM at 800 nm in water. Confocal laser scanning fluorescence microscopy studies in live cells indicate localisation of the chromophore at the mitochondria; moreover, cytotoxicity measurements prove biocompatibility. Thus, chromophore **3** has excellent potential for one- and two-photon excited fluorescence imaging of mitochondrial function in cells.

## Introduction

Three-coordinate organoboron compounds have aroused much interest for various optical and electronic applications.<sup>[1]</sup> Due to its vacant p<sub>z</sub>-orbital, three-coordinate boron is a strong  $\pi$ -electron acceptor when connected to a conjugated  $\pi$ -system. The trigonal planar geometry and Lewis acidity of the boron atom facilitate

attack by nucleophiles, resulting in bond cleavage or the formation of a four-coordinate boron species, which inhibits the boron atom from being part of the delocalised  $\pi$ -system. To prevent the attack of nucleophiles such as water, kinetic stabilisation can be achieved by introducing sterically demanding substituents, such as mesityl (Mes) or 2,4,6-(CF<sub>3</sub>)<sub>3</sub>C<sub>6</sub>H<sub>2</sub> (FMes),<sup>[2]</sup> to the boron atom, or by incorporation of the boron atom in a rigid, planar structure.<sup>[3]</sup> Only small anions, such as fluoride or cyanide, are able to overcome the steric bulk and attack the boron centre, which is exploited for anion-selective sensing.<sup>[4]</sup> Triarylboranes are also used in organic light-emitting diodes (OLEDs) as electron-transporting, emitting and hole-blocking materials.<sup>[5]</sup>

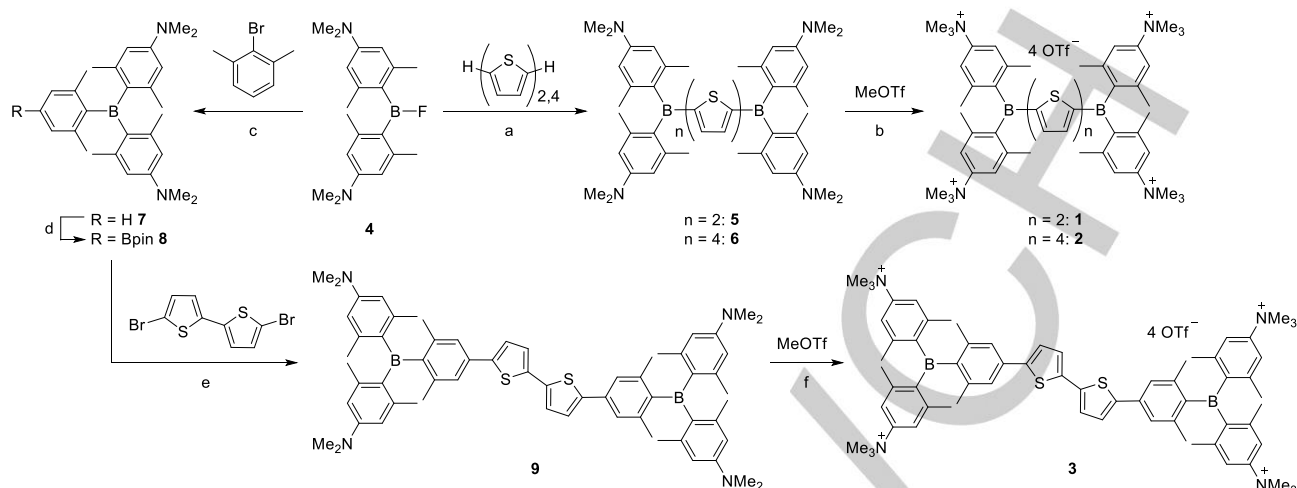
The large change in dipole moment upon excitation of compounds including a triarylborane moiety as an electron acceptor results in large first and second-order molecular hyperpolarisabilities  $\beta$  and  $\gamma$ .<sup>[6]</sup> These interesting 2<sup>nd</sup> and 3<sup>rd</sup> order non-linear optical properties indicate that triarylboranes should be excellent components of chromophores that undergo two-photon absorption (TPA). Several octupolar and quadrupolar compounds using this boron acceptor were reported and their TPA cross-sections ( $\sigma_2$ ) and fluorescence quantum yields ( $\Phi_f$ ) were measured to develop structure-TPA relationships.<sup>[7]</sup> In previous work, we reported that the insertion of thiophene rings into the  $\pi$ -bridge of A- $\pi$ -A chromophores (A = boryl acceptor; here, B(Mes)<sub>2</sub>) results in a remarkable increase of the TPA cross-section, and synthesised a quadrupolar compound with a TPA cross-section of 1930 GM at 770 nm that is, as far as we know, the highest  $\sigma_2$ /m.w. of all compounds containing B(Mes)<sub>2</sub> and thiophene groups reported to date.<sup>[8]</sup> Because the TPA maximum of each of these chromophores is located in the near-infrared (NIR) biological transparent window, the reported chromophores are potentially good candidates for two-photon excited fluorescence (TPEF) microscopy of living cells and tissues. However, these prototype compounds were not designed to be water-soluble, posing formidable challenges for *in vitro* or *in vivo* application, and it is this important aspect that we develop in this study while maintaining their aqueous stability and favourable optical properties.

Only a few examples of water-soluble triarylboranes have been reported to date.<sup>[9]</sup> Gabbai and co-workers achieved water-solubility by successively replacing the *para*-methyl groups of trimesitylborane with cationic ammonium substituents, and used two such compounds as efficient cyanide sensors in water.<sup>[10]</sup> They and two other groups made use of a similar method for the preparation of water-soluble triarylboranes with phosphonium substituents for anion sensing.<sup>[11]</sup> Recently, a water-soluble, nonionic triarylborane, containing polyethylene glycol chains as the hydrophilic groups, was reported by Yang and co-workers as an efficient fluorescence indicator for ATP in the cytoplasm and cell membrane.<sup>[12]</sup> Furthermore, while our work was in progress,

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**Scheme 1.** Synthesis of the compounds **1-3**. a) *n*-BuLi, THF,  $-78^{\circ}\text{C}$  to r.t.; b)  $\text{CH}_2\text{Cl}_2$ , r.t.; c) *t*-BuLi, THF,  $-78^{\circ}\text{C}$  to r.t.; d)  $\text{B}_2\text{pin}_2$ ,  $[\text{Ir}(\mu\text{-OMe})(\text{COD})_2]$ , dtbpy, THF,  $80^{\circ}\text{C}$ ; e)  $\text{Pd}_2(\text{dba})_3$ , SPhos, KOH, toluene,  $\text{H}_2\text{O}$ ,  $85^{\circ}\text{C}$ ; f) MeOTf,  $\text{CH}_2\text{Cl}_2/\text{Et}_2\text{O}$  1:3, r.t.

the same authors reported a water-soluble triarylborane containing Cu(II)-cyclen. While non-fluorescent itself, it can serve as a one- and two-photon excited fluorescence turn-on probe for  $\text{H}_2\text{S}$  at mitochondria.<sup>[13]</sup>

For TPEF imaging in biological systems, we prepared analogues of our previous quadrupolar compounds with trimethylammonium substituents for enhanced water-solubility. These substituents are not just promising due to their hydrophilic character, but are also expected to enhance the accumulation in the mitochondria.<sup>[14]</sup> These features profile the molecules as potential sensors for the mitochondrial membrane potential, providing direct information about the status of a cell's power plants.<sup>[15]</sup> Importantly, the use of such dyes, if amenable for TPA, is potentially beyond *in vitro* use on single cells, populations of cells, or united cell structures, but may very well expand into *in vivo* applications by virtue of the aforementioned accessibility of deeper cell layers and tissues for NIR light. Measurements of time-lapse acute mitochondrial responses to, e.g., drug exposure, inducible gene knock-in/-out or exposure to other challenges could provide immediate information on secondary respiratory challenges to mitochondria, thereby providing on-the-fly read-out of cell damage. Other potential applications include live cell imaging of diseased vs. healthy tissue, e.g., to understand the underlying mechanisms of dynamic transport in neurodegenerative diseases such as glaucoma<sup>[16]</sup> or Alzheimer's disease.<sup>[17]</sup>

In this paper, we present three quadrupolar chromophores, **1-3**, containing cationic triarylborane acceptors (Scheme 1). The  $\pi$ -bridge has been modified by the number of thiophene spacers and the nature of the aryl substituent adjacent to the boron atom. Their linear photophysical properties were examined experimentally and theoretically. With the water-stable derivative **3** we demonstrate herein one- and two-photon excited fluorescence imaging of the mitochondria in cells, due to its remarkable fluorescence quantum yield and high two-photon

cross section in water. Co-localisation and cytotoxicity studies show that dye **3** is an excellent candidate for the use as a new mitochondrial imaging agent.

## Results and Discussion

### Thiophene-boron directly connected chromophores **1** and **2**

**Synthesis.** The synthesis of the compounds **1** and **2** is summarised in Scheme 1. Compounds **5** and **6** were prepared *via* reaction of bis[4-(*N,N*-dimethylamino)-2,6-xylyl]fluoroborane **4**<sup>[18]</sup> with dilithiated bithiophene or quaterthiophene. Neutral compounds **5** and **6** were methylated with methyl triflate in  $\text{CH}_2\text{Cl}_2$ , and the products **1** and **2** precipitated in quantitative yield. Both compounds **1** and **2** were found to be water-soluble at concentrations suitable for fluorescence microscopy (Table 1), especially noting that commercially available chromophores for mitochondrial imaging are generally dissolved in dimethyl sulfoxide (DMSO).

**Linear optical properties.** The absorption and emission spectra of the methylated dyes **1** and **2** were measured in water (Fig. 1A) and MeCN (Figs. S30 and S31, see ESI). The absorption spectra recorded in water exhibit a broad band at 426 nm for compound **1**, whereas an elongated quaterthiophene  $\pi$ -system shifts the absorption by *ca.* 30 nm to the red for chromophore **2**. The extinction coefficients, measured in MeCN, due to enhanced stability (*vide infra*) and solubility, range from 28 000 to 48 000  $\text{M}^{-1}\text{cm}^{-1}$  (Table 1). The emission spectra are broad, with maxima spread over a *ca.* 150 nm range for the different compounds. The smaller quadrupolar compound, **1**, has an emission maximum in water at 451 nm, with a small Stokes shift of 1 300  $\text{cm}^{-1}$ . By insertion of two more thiophene rings into the bridging unit, the emission of **2** is bathochromically



(Fig. 2A and Table S2). A progressive blue-shift of the emission is observed on going from water to (polar aprotic or protic) organic solvents of decreasing polarity. UV-vis measurements over 72 h clearly demonstrate that the increased steric protection provided by the additional methyl groups in **3** dramatically enhances stability in water (Fig. 2B). Furthermore, **3** is more stable in water than the commercially available imaging chromophore MitoTracker Red CMXRos (Figs. S46-S47).

### DFT calculations

Density Functional Theory (DFT) calculations were carried out in order to examine the effects of the linker groups on the dihedral angles around the boron centre and its influence on conjugation. The geometry of each of the compounds **1-3** was optimised using DFT (B3LYP/6-31G\*, gas phase). The structures all show the expected propeller arrangement of the aryl groups about the rigorously trigonal planar boron centre. The exchange of the thiendiyl linkers at the boron atom in **1** and **2** for xylylene groups in **3**, leads to an increased twist of the aryl group with respect to the  $BC_3$  plane (**1**: 16.2°; **2**: 13.7°; and **3**: 43.2°). This reduced conjugation with the boron atom leads to the LUMO of **3** being 0.37 eV higher in energy than that of **2**. The  $\pi$ -bridge backbone remains relatively planar in all cases (inter-ring dihedral angles **1**: 14.3°; **2**: 1.5 and 10.3°; and **3**: 18.9 and 12.4°).

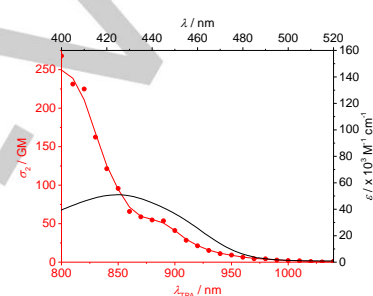
Time Dependent-DFT (TD-DFT) calculations were carried out in order to obtain information on the nature of the optical transitions, and to compute the expected UV-vis absorption spectra of the compounds and compare this with the experimental spectra. TD-DFT calculations excellently reproduced the experimental absorption maxima of the lowest energy bands, the deviation in energy being within 0.02-0.15 eV in simulated MeCN solution. Full details of these results and those in the gas phase are presented in the ESI (Figs. S53-S55). Introduction of the xylylene groups in **3** leads to a hypsochromically shifted absorption spectrum relative to **2**, in line with the experimental spectra. As seen in the natural transition orbitals (NTOs) (Figs. S53-S55) the  $S_1 \leftarrow S_0$  transitions of all three compounds contain a significant contribution from population of the empty boron  $p_z$ -orbital, albeit that the transitions are predominantly  $\pi-\pi^*$ . The boron acceptor thus increases the conjugation length of the  $\pi$ -system. For TPA and TPEF applications, we also considered the  $S_2 \leftarrow S_0$  transition, as this is the lowest energy allowed TPA transition in a quadrupolar chromophore; thus, the NTOs for these transitions are plotted in Figs. S53-S55.

### Two-photon absorption

Table 1 summarises the results of TPA measurements of chromophore **3** by using a two-photon excited fluorescence method.<sup>[21]</sup> Due to the quantum selection rules for centrosymmetric molecules, the TPA maximum does not occur at the doubled wavelength of the one-photon absorption (OPA) maximum, but is located at a shorter wavelength. Indeed, as observed in Fig. 3, in which the TPA and rescaled OPA are compared, the maximum TPA is observed at higher energy, corresponding to an excited state which is not one-photon allowed. This is in agreement with the typical behavior of quadrupolar

molecules.<sup>[22]</sup> The lowest-excited state is, however, slightly OPA allowed (as indicated by the shoulder between 850 and 900 nm), most probably due to conformational freedom responsible for slight deviations from ideal centrosymmetry.

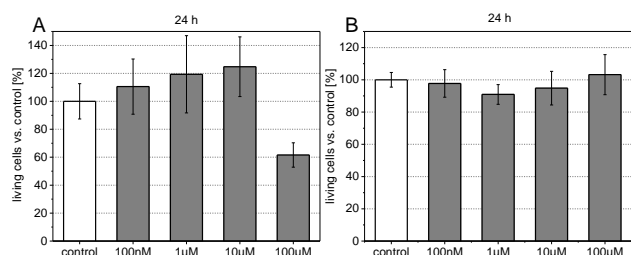
It was not possible to determine the actual maximum of the TPA (Fig. 3), which is calculated to be at 792 nm (Table S8), as we have not measured beyond 800 nm, but at 800 nm, we observe a very large TPA cross section of 268 GM in  $H_2O$ , which is increased to 693 GM in MeCN (Fig. S49). This value is more than five times higher than that reported recently for the only other example of a triarylborane mitochondrial imaging chromophore (120 GM at 765 nm in DMSO).<sup>[13]</sup> Due to the sizeable fluorescence quantum yields, relatively large values of the two-photon brightness (27 and 285 GM in water and MeCN, respectively) have been measured, making dye **3** of much promise for two-photon imaging in tissues.



**Figure 3.** One-photon absorption (OPA) (black) and two-photon absorption (TPA) spectra (red) of **3** in  $H_2O$ .

### Cell cytotoxicity and live cell imaging

In light of the above photophysical properties and water-stability of **3**, and thus its potential as a chromophore for live cell imaging, we next examined its possible cytotoxicity in cells. Therefore, we exposed three different cell lines - murine-fibroblasts (NIH 3T3), human embryonal kidney (HEK 293T), and human-hepatic origin (HepG2-16) - to serial dilutions of **3** and also LiOTf and studied the cell metabolic activity with a colorimetric (WST-1) assay (Figs. 4 and S50-S52). These experiments confirmed that compound **3** did not influence the cell viability at concentrations as high as 10  $\mu M$ . We have also checked the triflate counterion, as its lithium salt, for cytotoxicity and found that it showed no toxicity up to 100  $\mu M$  (for cytotoxicity results of **1** and **2**, see ESI Figs. S50-S52). We therefore suggest that compound **3** can be safely used in live cell imaging applications and that this class of compounds shows potential for the development of *in vivo* diagnostics to probe mitochondrial function.

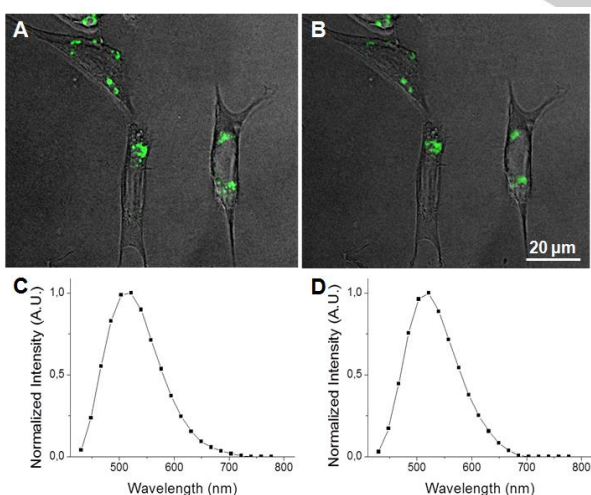


**Figure 4.** The metabolic activity of NIH 3T3 cells was measured after 24 h with serial dilution of **3** (A) and LiOTf (B) using a colorimetric assay. The results show the relative cell viability as a percentage of the untreated control (white bars) + the standard deviation.

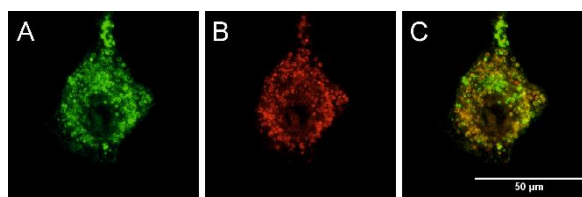
Thus, living mouse embryo fibroblast cells (NIH 3T3) were treated with a 10  $\mu\text{M}$  concentration of chromophore **3**. Visualisation by confocal laser scanning fluorescence microscopy showed that **3** penetrated the cell membrane of living cells and localised at the mitochondria as confirmed by co-localisation experiments with the commercial mitochondrial imaging agent MitoTracker Red CMXRos (Fig. 5).

**Figure 5.** Confocal microscope live cell image of murine NIH 3T3 fibroblast cells with (A) 10  $\mu\text{M}$  of dye **3** and (B) 125 nM of MitoTracker Red CMXRos. (C) Merged image indicating the co-localisation of compound **3** with mitochondria.

**Figure 6.** Confocal microscope image of POS 1 cells after 8 h of incubation with dye **3** (300 nM in the media): merge of transmission image (in grey) and fluorescence image (green) showing the internalisation of the dye within the cells: (A) one-photon excitation ( $\lambda_{\text{ex}} = 405\text{nm}$ ;  $\lambda_{\text{em}} = 500\text{-}600\text{ nm}$ ) and (B) two-photon excitation ( $\lambda_{\text{ex}} = 800\text{ nm}$ ;  $\lambda_{\text{em}} = 500\text{-}600\text{ nm}$ ); (C) emission spectrum upon one-photon excitation ( $\lambda_{\text{ex}} = 405\text{nm}$ ; 20 nm step) of the dye within the cell; (D) emission spectrum upon two-photon excitation ( $\lambda_{\text{ex}} = 800\text{ nm}$ ; 20 nm step) of the dye within the cell.



Based on the sizeable two-photon brightness of dye **3** in water, we also tested **3** as a two-photon dye to stain POS 1 cells - a cell line derived from an osteosarcoma tumour. The two-photon imaging experiments (and parallel confocal imaging under one-photon excitation) were performed using a 300 nM concentration.



As clearly shown in Fig. 6, fluorescence images under standard one-photon and two-photon excitation show the same localisation of the dye. Furthermore, emission spectra of the uptaken dyes were acquired demonstrating that the dye structure is retained upon internalisation in the cells. Hence, the steric protection around the empty  $p_z$ -orbital at the boron atom not only confers stability of dye **3** in pure water, but is also sufficient to make it stable in a cellular environment. The blue-shifted emission compared to that observed in pure water can be related to environmental effects, suggesting that the dye is located in a less polar environment.

## Conclusions

In conclusion, three water-soluble quadrupolar chromophores with triarylborane acceptors were synthesised. The two compounds **1** and **2** are bright emitters in water, but were shown to decompose, due to hydrolysis at the boron centre. In contrast, **3** has enough steric protection around the empty  $p_z$ -orbital at the boron atom that it is sufficiently stable in water. We have proved as well that **3** localises in mitochondria by co-localisation experiments, and that our new chromophore is not toxic at concentrations suitable for imaging purposes. We have shown that **3** is more stable than the commercial available MitoTracker Red CMXRos, while the solubility in water remains the same. We report here the first TPA cross-section measurement of a triarylborane in water, being 268 GM at 800 nm, which is very large; hence, **3** is suitable for two-photon live cell microscopy. We have reported herein a three-coordinate boron-containing chromophore for mitochondrial TPEF imaging, profiling this compound as a water-soluble, biocompatible mitochondrial tracker for *in vitro* live cell imaging applications. Future application as a diagnostic tool for clinical use should, in spite of the promising data set obtained with respect to (cellular) biocompatibility, be re-assessed in light of the outcome from robust (pre-)clinical safety studies. Optimisation of such chromophores to enhance quantum yields and TPA cross-sections and to tune emission wavelengths is currently in progress.

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**Keywords:** borane • luminescence • two-photon excited fluorescence • mitochondrion • cell imaging

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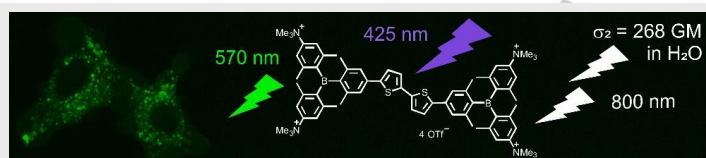
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