Photophysical Characteristics of Polarity-Sensitive and Lipid Droplet-Specific Phenylbenzothiadiazoles

Kilian Colas, Susanne Doloczki, Aikaterina Kesidou, Lourdes Sainero-Alcolado, Aida Rodriguez-Garcia, Marie Arsenian-Henriksson and Christine Dyrager

In this study, we present a series of solvatochromic phenylbenzothiadiazoles that display dual emission from the locally excited (LE) and intramolecular charge transfer (ICT) excited states. The donor-acceptor derivatives are highly sensitive to polarity changes, which can be monitored by differences in emission efficiency, spectroscopic shifts and variations of the LE/ICT ratio. One of the compounds in the series, containing a thiomethyl substituent, emerged as an excellent blue emitting stain for intracellular lipid droplets, a biomarker for various types of cancer. In addition, a non-emissive nitro derivative becomes fluorescent upon bioreduction in hypoxic cancer cells and accumulates in lipid droplets with a high signal-to-background ratio.

1. Introduction

Fluorescent probes are valuable molecular tools with a wide array of applications in fields such as materials, drug design and bioimaging. In the latter, fluorophores that are sensitive to changes in the environment (e.g., polarity, pH, oxygen level or redox potential) are particularly attractive since they can serve as sensors for the local surroundings in cellular systems. For instance, they can give off/on emission in cells with oxygen deficiency or color changes when accumulating in specific organelles or membranes. The most common class of environment-sensitive fluorophores are donor-acceptor (D–A) structures that exhibit different photophysical properties in environments of different polarity (e.g., solvatochromism). D–A fluorophores can undergo intramolecular charge transfer (ICT) in the excited state (Figure 1), a process that is facilitated by strong donors and promoted in polar solvents that stabilize the charge-separated excited state species. Emission from the ICT state is associated with increased non-radiative relaxation processes, which generally results in quenched fluorescence. For some fluorophores, charge transfer (ICT) can give rise to dual emission that originates from an equilibrium between the locally excited (LE) and the ICT states. ICT can also promote different conformations of the fluorophore in the excited state (i.e., going from $S_1$–LE to $S_1$–ICT). The most commonly described models are: planarization of the D and A units ($\varphi = 0^\circ$) to generate a planar intramolecular charge transfer state (PICT) or rotation ($\varphi = 90^\circ$) around the D–A single bond, forming a twisted intramolecular charge transfer (TICT) state. An additional feature of many fluorophores, in particular flat hydrophobic aromatics, is that they may form aggregates in solution, which can significantly affect the emission efficiency. Depending on the topology of the aggregates,
either of two phenomena can occur: formation of non-emissive clusters (aggregation-caused quenching, ACQ)\(^{[11a–d]}\) or formation of highly emissive aggregates (aggregation-induced emission, AIE)\(^{[11c,d]}\).

Here we present a new set of electronically diverse 2,1,3-benzothiadiazole (BTD)-based fluorophores. The aim of the study was to investigate the photophysical properties of the compounds in detail (focusing on substituent and solvent polarity effects), and also to explore their potential as imaging probes for fluorescent cell microcopy. We found that the D–A fluorophores displayed dual fluorescence, from the LE and ICT states, the predominance of which can be controlled by solvents of different polarity. Two compounds in the series were shown to specifically stain intracellular lipid droplets (LDs) in cancer cells. One of these emerged as a bright blue stain under normoxic conditions and the other as a bioreductive imaging agent that is activated in hypoxic cancer cells.

2. Results and Discussion

D–A fluorophores with a BTD unit exhibit favorable chemical and photophysical properties, e.g., strong electron-withdrawing capacity from the BTD unit that facilitates ICT, solid state fluorescence, large Stokes shifts, and red-shifted optical profiles.\(^{[1e,h,7b,12]}\) BTD has therefore been used extensively as an acceptor unit in polymeric D–A materials for various light technology applications.\(^{[1e,12c,d]}\) The emission properties of BTD-based fluorophores can be fine-tuned by linking their electron-deficient structure with electron-donating moieties to enable charge transfer within a D–A system.\(^{[7b,12c]}\) Accordingly, we synthesized a range of electronically diverse phenylbenzothiadiazole derivatives bearing electron-donating (EDG) or -withdrawing (EWG) groups (Scheme 1). Compounds 1–11 were easily prepared in a single step from 4-bromo-2,1,3-benzothiadiazole (Br–BTD) in moderate to excellent yields via Suzuki-Miyaura cross-coupling.\(^{[13]}\)

2.1. Photophysical Characterization

The photophysical properties of compounds 1–11 were investigated by UV-Vis absorption and emission spectroscopy in a broad variety of solvents of different polarity (Table 1 and SI Figures S1–11). Measurements in aqueous solution were performed in phosphate buffered saline (PBS, pH 7.4) with 5% DMSO in order to retain solubility while approximating physiological conditions.

2.1.1. Solvatochromism

The BTD derivatives containing strong EDGs 1–3 displayed absorption bands with maxima around 370–440 nm, while compounds containing weak donors (4 and 5) and the compounds with EWGs (6–11) showed absorption maxima centered at 330–360 nm. The absorption wavelengths (\(\lambda_{\text{Amax}}\)) for 1–11 are virtually independent of solvent polarity, indicating a non-polar nature of their respective ground states.\(^{[2,10b,14]}\) However, significant blue-shifts were observed in PBS solution for the D–A systems with the largest dipole moment (1, 2 and 3) which, together with observed poor aqueous solubility, suggest the presence of soluble nanoaggregates. The sulfonamide derivative 9 showed similar behavior, albeit in hexane, with a strong blue-shifted absorption band below 220 nm, also suggesting aggregation.

While dynamic light scattering measurements with 9 did not support this hypothesis, we could observe an inverted concentration dependence of the absorbance intensity. We also performed a simple filtration of the sample with a syringe filter, which led to a blank absorption spectrum, implying that some degree of non-ordered precipitation might occur. The emission wavelengths for 1–10 typically increased with solvent polarity, suggesting that the excited states are stabilized by polar solvents (e.g., positive solvatochromism).\(^{[2,14]}\) This effect was strongest for derivatives containing EDGs (1–5) and halides (6 and 7 – perhaps due to their positive mesomeric effect). The solvatochromic behavior was more modest with strong EWGs (8–10), except in aqueous environment. By contrast, 1–3 showed blue-shifted emission in PBS buffer, further supporting our aggregation hypothesis.\(^{[11a,15]}\) Compound 1 exhibits a...
unique behavior in the series: its fluorescence quantum yield gradually decreased with increased solvent polarity, and the emission efficiency was entirely quenched in DMSO, methanol and PBS solution (ΦF < 0.01). In polar protic solvents, this can be ascribed to hydrogen-bonding effects.\(^\text{[16]}\)

Compound 2, which also contains a strong hydrogen-bond acceptor, retains high quantum yield across most solvents before displaying suppressed quantum yields in MeOH and PBS solution (ΦF ≤ 0.01). In polar protic solvents, this can be ascribed to hydrogen-bonding effects.\(^\text{[16]}\)

Due to its relatively poor hydrogen-bonding capacity we expected that the thiomethyl derivative 3 would not readily quench in protic solvents. However, its quantum yield was completely suppressed in methanol (ΦF = 0.02) and relatively high in PBS (ΦF = 0.19), in which the formation of bright fluorescent aggregates was clearly observed. We did not see any trends in quantum yields when changing the polarity for the derivatives that incorporate strong EWGs (4, 6, and 7). Molar extinction coefficients (ε) were mostly unaffected by solvent or substituent changes, reaching up to 9800 M\(^{-1}\) cm\(^{-1}\) (3 in hexane). As expected, the nitro-substituted compound 11

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[a] 5% DMSO in phosphate buffered saline (PBS, 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4). [b] No emission detected. [c] Weak emission with low signal-to-noise ratio. [d] Not determined due to poor solubility. [e] Low wavelength absorption. [f] Emission due to aggregation.
showed no fluorescence in solution or in the solid state, in line with the documented tendency of nitro groups to quench emission through efficient non-radiative relaxation processes.\textsuperscript{[5b,17]} Nevertheless, 11 was included in the series as a promising hypoxia-activated fluorophore that upon enzymatic reduction in hypoxic cancer cells can be transformed into the corresponding emissive amine.\textsuperscript{[5b,17,22] All the other compounds (1–10) exhibit solid state fluorescence (SI, Figure S12), which implies that they could serve as valuable building blocks for material-based applications.\textsuperscript{[1b–e,11p]}

2.1.2. Substituent Effects

The different substituents on the phenyl unit and their impact on the photophysical properties were further compared in hexane (Figure 2), to minimize solvent effects. Traditionally, the electronic character of substituents is described with Hammett constants (\(\sigma_p\)) and in our case a clear correlation showed that the emission maxima decrease with increasing values of \(\sigma_p\) (i.e., with decreasing electron-donation capability). A minimum was reached with the phenyl derivative 5, and no further influence of the EWGs present in 6–8 and 10 was observed beyond this point. A similar trend emerged upon comparison of quantum yields. However, in both cases, the thiomethyl-substituted compound 3 clearly stood out. We believe this indicates that the Hammett constant does not properly reflect the properties of the sulfur heteroatom in this context, perhaps due to the interaction between its lone pairs and the conjugated \(\pi\) system. Alternatively, the substituents were compared in terms of polarizability using Taft’s parameters (Figure 2, bottom). This showed a clear correlation with the quantum yields for the most well-defined D–A compounds in the series (1–5), in which the thiomethyl derivative 3 seemed to perform the best, while the EWG substituents again showed no clear influence. Notably, Taft’s parameter did not correlate well with the emission maxima, suggesting that substituent effects in this context are better studied by a combination of descriptors rather than a single parameter.

2.1.3. Dual Emission

We found that, with the exception of 1, all emissive BTD derivatives (2–10) displayed dual emission, originating from the LE and ICT states (SI, Figures S2–10).\textsuperscript{[8a,9c,10,19]} The relative ratio of the two peaks is dependent on solvent polarity and the emission from the minor excited state generally appears as a shoulder with lower intensity. As illustrated in Figure 3A, the LE/ICT emission ratio decreased with increasing solvent polarity\textsuperscript{[20]} until the emission profile eventually derived predominantly from the ICT state. The electronic character of the substituents on the phenyl moiety also affected the LE/ICT ratios. A comparison of the Hammett \(\sigma\) constants\textsuperscript{[21]} showed that substituents with EDGs (\(-\text{NMe}_2, -\text{Me}, -\text{SMe} \text{ and } -\text{Me}\)) promote emission from the ICT state. The effect was most pronounced for 1, which incorporates the strongest donor substituent in the series (NMe\textsubscript{2}), giving rise to single emission profiles from the ICT state only, regardless of solvent. Similar behavior was displayed by the methoxy derivative 2, although dual emission appears in hexane. Compound 3 behaves similarly to 2, further suggesting that the Hammett constant alone does not fully reflect the properties of the thiomethyl group. The prospect of charge transfer was significantly smaller with weaker EDGs or with EWGs (higher \(\sigma_p\) constants). For example, phenyl-BTD 5 exhibited dual emission in all solvents, except PBS solution (Figure 3B). Its emission profile in hexane demonstrated a high LE/ICT ratio with LE as the highest emission peak and ICT as a red-shifted shoulder of significantly lower intensity (Figure 3C). In chloroform, emission peaks for 5 essentially reach the same magnitude, while the charge-separated ICT excited state was further stabilized in polar, protic methanol. The strong correlation between the inherent polarity of the compounds and the LE/ICT ratio suggests the ICT state is favored by increasing solvent polarity for systems with strong D–A character (1, 2, 3), whereas derivatives with
the ICT linearity indicates a significant difference between the dipole moment in the ground and excited states.\cite{10b} Instead, LE predominance leads to very small gradients and thus small changes in dipole moment, which is consistent with the expected increase in charge separation (ICT vs LE state). A few deviations from linearity in the L–M plots were observed. For compounds 4 and 9, these are likely due to similar intensities of the LE and ICT emission peaks in various solvents, which make their respective Stokes shift values less accurate. For compounds 8 and 10, these deviations may result from the apparent stabilization of the excited states by DMSO and PBS resulting in red-shifted emission bands and exceptionally large Stokes shifts.

2.1.5. Viscosity and Aggregation Experiments

D–A systems with TICT (as opposed to PICT) fluorescence are highly sensitive to the viscosity of their environment.\cite{9b,c,10} Viscous solvents are expected to restrict the intramolecular rotation around the D–A single bond (Figure 1), resulting in increased emission that originates from the LE state. This can be studied by measuring fluorescence emission intensity in MeOH/glycerol mixtures with increasing fractions of glycerol (containing a weak EDG without H-bonding ability, a neutral substituent and an EWG, respectively) (Figure 3D) shows a linear correlation between the solvents that favor emission from the LE state (i.e., PICT or TICT; Figure 1). Initially, the three strongest D–A systems in the series (1–3) were measured in MeOH/glycerol mixtures with increasing fractions of glycerol (\textit{f}g) (SI, Tables S1 and S2). Compounds 1 and 3, which display very weak or no emission in MeOH (Table 1), remained non-emissive up to 90 vol \% glycerol, while the methoxy derivative 2 showed a steady decrease of the emission with increasing the glycerol percentage (SI, Table S1 and S2). However, since the emission of these three compounds seems to be quenched through hydrogen bonding\cite{15a} (Table 1) and given that glycerol is a stronger hydrogen bond donor than MeOH, the observed steady decrease in fluorescence could not be attributed to either effect (PICT or TICT) with certainty.\cite{11b} Consequently, 4, 5 and 6 (containing a weak EDG without H-bonding ability, a neutral substituent and an EWG, respectively) were selected for analysis instead (SI, Table S1 and S2). These compounds displayed identical behavior; that of compound 5 is representative (Figure 4, top). The emission enhances with increasing fractions of glycerol up to a maximum at 40 vol \%, after which the emission intensity continuously drops. However, the bell-shaped response observed was inconclusive with regards to TICT vs PICT, and instead resembles aggregative behavior, as previously suggested for other fluorophores with similar characteristics.\cite{11c,d,10} Additional measurements were therefore performed in mixtures of THF/water with increasing fractions of water (\textit{fw}) in which the compounds have limited solubility (SI, Tables S3–6).\cite{11b} The results showed a spectroscopic behavior that is comparable to that observed in the viscosity experiments (Figure 4, bottom). Steady increase in fluorescence intensity (up to ca. 40 \% \textit{fw}) is characteristic of aggregation.
induced enhanced emission (AIEE)\(^{23}\) and subsequent decrease in intensity is consistent with polarity-induced quenching.\(^{11g,11,23a–e}\) From \(f_s = 60\%\), the latter effect overcomes the AIEE and suppresses the emission. Solubility factors may also be at play, with some degree of precipitation contributing to the decrease in fluorescence intensity. At high concentrations, bright aggregates are clearly visible while the solution remains strongly emissive (SI, Figure 21). For the compounds with the strongest D–A character (1–3), we could not conclude whether no aggregation occurs or the polarity-induced (and/or hydrogen-bonding-caused) quenching is simply too strong to observe any potential AIEE effect. However, we observed the precipitation of bright fluorescent solid particles from 3 in the PBS solution (containing 5\% DMSO), which likely explains its surprisingly high quantum yield (\(\Phi_F = 0.19\)).

2.2. Computational Studies

To further probe the nature of the ICT state (i.e., PICT or TICT, Figure 1), compounds 1–6 were investigated by DFT and TD-DFT calculations.\(^{26}\) Computed vertical excitation wavelengths in hexane and DMSO using the implicit solvation model based on density (SMD) largely coincide with the experimental data (SI, Table S7). The absorption energy of 1, however, is clearly underestimated in both solvents. The analysis of natural transition orbitals (NTO) of compounds 1–6 shows that the HOMO has electron delocalization over the entire system, while the LUMO is mainly localized on the BTD unit (SI, Table S9). This indicates charge transfer from the (substituted) phenyl moiety to the BTD acceptor for absorption into the first excited state. To explore the conformation in the ICT states, ground state and excited state geometries were optimized with both planar (\(\psi_s = 0^\circ\)) and twisted (\(\psi_s = 90^\circ\)) conformations of the aromatic rings (Figure 1). These calculations were performed with hexane as implicit solvent (SMD). Optimization of the ground state without geometry constraints resulted in dihedral angles around 35–38°, which are decreased in the optimized excited state geometries (11–17°) (SI, Table S8). This tendency towards planarization upon excitation is supported by energetic considerations. Calculating the energy difference between the twisted (T) and planar (P) first excited states (\(\Delta E_{S_1,\chi^\circ,\chi^\circ}\)) and comparing it with the corresponding ground state energy difference (\(\Delta E_{S_0,\chi^\circ,\chi^\circ}\)) should be indicative of whether the PICT or TICT state is favored.\(^{26}\) Each studied compound exhibits a very small positive \(\Delta E_{S_1,\chi^\circ,\chi^\circ}\), while the positive \(\Delta E_{S_0,\chi^\circ,\chi^\circ}\) is much larger, indicating that the two aromatic subunits tend to planarize in the excited state (PICT) rather than twist out of the plane (TICT).

2.3. Cell Studies

Beyond their traditional use in material sciences, BTD derivatives have gained interest as promising bioimaging agents for fluorescent cell microscopy.\(^{10,15}\) Recently, our group reported the use of compound 1\(^{[13]}\) as a selective stain for intracellular lipid droplets (LDs), an organelle that is overabundant in many types of cancer.\(^{24}\) Under malignant conditions, LDs serve as energy reservoirs for lipids that can be further mobilized within the cells to promote cell proliferation, tumor progression and cancer invasiveness.\(^{24,26}\) Thus, the development of fluorescent dyes that specifically stain LDs is highly valuable for the study of altered lipogenesis in cancer cell biology.\(^{24,25}\) Numerous LD-specific fluorophores have recently been reported in the literature and several of these are benzothiadiazole derivatives.\(^{23,26}\) We reasoned that structural analogues of 1 (compounds 2–11) also might accumulate in intracellular LDs, and this was further investigated using pediatric neuroblastoma cells (KCN-69n), one of the most severe and common form of cancer in children.\(^{27}\) The majority of the compounds (2 and 4–10) proved unsuitable for this purpose, as they were either unspecific, insufficiently bright or prone to crystallization in the cell media (SI, Figure S14 and S15). However, we were delighted to see that the thiomethyl-substituted derivative 3 once again stood out in the series. It provided a bright blue punctate pattern (Figure 5) identical to 1 (SI, Figure S14) - strongly suggesting LD accumulation. This derivative displayed an excellent signal-to-background ratio (SBR) in the blue channel (\(\text{SBR} = 14\)), while green and yellow channels revealed weak unspecific signals (SI, Figure S16). This is attributed to the solvatochromic properties of 3 while it does not quench in polar environment (as compound 1 does).\(^{24}\) The background signals arising from more polar environments are only observed in further excitation windows, thus allowing clean imaging in the blue channel. Colocalization experiments of compound 3 with Nile Red (a known and established LD stain) confirmed the LD specificity (Figure 6).\(^{28}\) To the best of our knowledge, this makes 3 one of the brightest blue LD-specific fluorescent probes reported to date (e.g., brightness in hexane, \(\varepsilon \times \Phi_F = 8330 \, \text{M}^{-1} \cdot \text{cm}^{-1}\)). Thus, it may be a useful alternative to Nile Red if LD-imaging explicitly is desirable in the blue channel. In addition, 3 showed to be highly LD-specific and did not seem
to stain other hydrophobic structures in the cell (Figure 6), which is a well-known feature of Nile Red.

We further sought to exploit the non-emissive nitro derivative 11 as a bioreductive imaging agent in hypoxic cancer cells. Oxygen deficiency (hypoxia) in tumor tissue derives from uncontrolled cell proliferation that outgrows the vascular supply. Substantial evidence indicates that hypoxia-induced proteomic and genomic changes regulate various pathological mechanisms that further promote cancer cell survival and tumor aggressiveness. These processes are mainly mediated by hypoxia-inducible factors (HIFs), which activate the transcription of genes coding for proteins involved in increased cell metabolism and metastatic progression. Some of these events create a harsh microenvironment that is characterized by overexpression of various reductive enzymes, such as oxygen sensitive nitroreductases (NTRs). An attractive approach for imaging hypoxic tumors is therefore to use optical chemosensors (off/on fluorophores) that are based on reducible nitroaromatic motifs.

We observed that 11 (BTD-phenyl-NO₂) is invisible when staining cells under normoxic conditions (20% O₂) but becomes visible in hypoxic cells (1% O₂) due to bioreductive transformation by NTRs that generate the corresponding emissive amine, BTD-phenyl-NH₂ (Figure 7). The result showed an intense green signal with high LD specificity (as evidenced by the identical pattern to 1, 3 and Nile red) and little to no background fluorescence (SBR = 7) - presumably due to hydrogen-bond quenching of the amino group in aqueous media. Two additional experiments were performed to further support the bioreduction of the nitro derivative 11 in hypoxic cells. Enzymatic reduction of 11 using NTR and NADH clearly showed transformation to the suggested emissive counterpart BTD-phenyl-NH₂ (SI, Figure S19 and S20). Compound 11 was also chemically reduced to BTD-phenyl-NH₂ and then used to stain neuroblastoma cells under normoxic conditions and Nile Red was not possible due to overlapping optical profiles. However,
the punctate staining pattern for 11 in hypoxic cancer cells (Figure 7) is clearly consistent with the observations for LD-specific 1 and 3 (SI Figure S14, and Figure 5). We foresee that 11 offers several important advantages as a hypoxia-activated fluorophore: a compact structure, straightforward one-step synthesis and high signal-to-background ratio. Notably, the cell viability after treatment with 1–11 (10 μM) was measured using the WST-1 assay (SI, Figure S13). No significant differences in viability could be observed after 48 hours incubation, suggesting non-cytotoxic profiles during the cell imaging experiments. This was also supported with observed unaltered cell morphology in comparison to controls.

3. Conclusion

We have described a series of donor-acceptor phenylbenothiazidazoles that display dual emission from the LE and ICT excited states. The LE/ICT ratio can be strictly controlled by changing the polarity of the solvent and/or by varying the electronic character of the donor motif in the structures. In addition, several of the compounds show an interesting aggregation-induced enhanced emission (AIEE) phenomenon when increasing the polarity of the environment. Two compounds in the series emerged as lipid droplet-specific probes for cancer cell imaging. Compound 3 is one of the rare blue LD-probes reported to date, while compound 11 becomes selectively activated in cancer cells under hypoxic conditions. Both compounds also display excellent signal-to-background ratios in cells, making them promising molecular tools for studying altered lipogenesis under normoxic and/or hypoxic conditions.

Experimental Section

Materials

All reagents were purchased from Sigma-Aldrich (Merck) or Fluorochem and used without further purification. Solvents used for synthesis or photophysical characterization were obtained from VWR Chemicals and used without further purification unless otherwise indicated. THF and toluene were distilled before use. Recombinant nitroreductase from Escherichia coli (≥90%) was purchased from Sigma-Aldrich (Merck). Phosphate buffered saline (PBS) tablets were obtained from VWR Chemicals and the PBS solutions were prepared with distilled water to obtain 10 mM phosphate, 137 mM NaCl, and 2.7 mM KCl at pH 7.4. Spectroscopic measurements in aqueous solution were performed in 5 % DMSO in PBS buffer to enhance compound solubility.

Chemical Characterization

NMR spectra were recorded at 298 K using an Agilent MR400-DD2 instrument at 400 MHz (1H), 101 MHz (13C) and 376 MHz (19F). Chemical shifts (δ) are reported in ppm using the residual solvent peak in CDCl3 (δCDCl3 = 7.26 and δCl3 = 77.2 ppm) or DMSO-d6 (δH = 2.50 and δD = 39.5 ppm) as internal reference.19F NMR spectra chemical shifts were calibrated to an external standard at 0.00 ppm (CFCl3). Coupling constants (J) are given in Hz and the apparent resonance multiplicity is reported as s (singlet), d (doublet), q (quartet) or m (multiplet). High-resolution mass spectrometry (HRMS) data (APCI) was determined at the Division of Mass Spectrometry, Department of Chemistry, Imperial College London, UK.

Photophysical Characterization

UV/Vis absorption spectra were acquired on a UV-1650PC Shimadzu instrument at room temperature using quartz cuvettes (10 mm). Absorption maxima (λmax) are reported in nm and the molar extinction coefficient (ε) in M⁻¹ cm⁻¹ with a margin of error of up to 6%. For each compound three data points with known different concentrations were acquired and the measured absorbances (≤1) were plotted against the concentrations. The molar extinction coefficients were then determined according to the Lambert-Beer law as the slope of the linear fit. Fluorescence measurements were carried out using a Spex Fluorolog 1680 0.22 m Double Spectrometer instrument. Fluorescence quantum yields (ΦF) were determined relative to fluorescein in 20 mMaq. NaOH (ΦF = 0.93)31 or quinine sulfate in 0.1 Maq. H2SO4 (ΦF = 0.55).32 Three fluorescence spectra were recorded per compound per solvent, and the areas under the curves were plotted against the absorbances at the excitation wavelength. The quantum yields were then calculated from the slope of the linear fit via the comparative method, with a margin of error of 0 to 10%. Reflective indices of solvents were adjusted for quantum yield calculations based on the excitation wavelength used.33 The excitation wavelength (360 or 430 nm) and concentrations for the quantum yield measurements were selected so that the absorbance was below 0.1 to prevent self-absorption effects. Obtained raw data was processed using Origin-Pro 8 software. Emission spectra illustrated herein were normalized to the peak maximum of interest and smoothed using an FFT filter function with 5 point window. Spectra with weak emissions and low signal-to-noise ratio (compound 1, 8 and 10 in DMSO; compound 3 in MeOH; compound 1 and 2 in PBS buffer with 5% DMSO) were smoothed using an FFT filter function with 30 point window.

Viscosity Experiments

Samples for viscosity experiments were prepared as 10⁻³ M solutions in volume fractions of 0%, 10%, 20%, 40%, 60%, 80% and 90% of glycerol in methanol. Emission spectra were measured with excitation at the maximum absorption wavelength of the corresponding compound in methanol. Peak areas of emission spectra were integrated and plotted against the glycerol fraction.

Aggregation Fluorescence Experiments

Samples for aggregation experiments were prepared as 10⁻³ M solutions in volume fractions of 0%, 10%, 20%, 40%, 60%, 80% and 90% of distilled water in THF. Emission spectra were measured with excitation at the maximum absorption wavelength of the corresponding compound in THF. Peak areas of emission spectra were integrated, and quantum yields were estimated from single-point measurements and plotted against the water fraction, respectively.

Computational Details

All calculations were performed using Gaussian 0924 with density functional theory (DFT) and time-dependent density functional theory (TD-DFT) for ground states and excited states, respectively. The CAM-B3LYP/6-31+G** level of theory gave results in good agreement with experimental data. The solvation model based on
density (SMD) was used to consider implicit solvation effects. For vertical excitation calculations, state-specific solvation was taken into account. Geometry optimizations were followed by vibrational frequency calculations to confirm the nature of the obtained structure as energetic minimum. Natural transition orbitals (NTO) were calculated in gas phase and visualized using Avogadro (1.2.0).

The energy difference between PICT (P) and TICT state (T) in the ground state (n = 0) and in the excited state (n = 1) were determined respectively as follows [Equation (1)]:

\[ \Delta E_{n}^{T-P} = E_{n}^{T} - E_{n}^{P} \]

(1)

\( E_{n}^{T} \) was calculated using geometry constraints freezing the dihedral angle between the two aromatic rings at 90°. Accordingly, \( E_{n}^{P} \) was calculated using geometry constraints freezing the dihedral angle at 0° during optimization.

**Cell Culture**

The human neuroblastoma cell line KCN-69n (ATCC, USA) was grown in MEME:Nutrient Mixture F-12 (1:1) medium containing 10% fetal bovine serum (HyClone), 1% non-essential amino acids (NEAA) (Sigma-Aldrich), 1% GlutaMAX (Gibco) and 1% penicillin-streptomycin (Sigma-Aldrich). Cells were cultured in a humidified environment at 37°C and 5% CO₂.

**Cell Viability Assay**

The cell viability of neuroblastoma cells (KCN-69n) was investigated in parallel. After 48 h in culture, 10 μL of WST-1 (Roche) were added per well and the cells were further incubated for 1 h (at 37°C). Subsequently, the absorbance was measured at 480 nm whose characterization data was in accordance with reported literature.

**Synthesis**

**General procedure for the Pd-mediated Suzuki coupling:**

The synthesis was adapted from a previously reported procedure. According to a solution of 4-bromo-2,1,3-benzothiadiazole (100 mg, 0.47 mmol, 1.0 equiv.) and K₂CO₃ (190 mg, 1.38 mmol, 3.2 equiv.) in toluene/methanol (1:1, 13 mL), the relevant arylboronic acid or boronate ester (0.98 mmol, 2.1 equiv.) and PEPPSI-IPr (6.3 mg, 2 mol%) were added. The reaction mixture was stirred at 80°C for 2 h, then allowed to cool down to r.t. and further stirred overnight unless otherwise specified. The reaction mixture was poured into water (50 mL) and extracted with EtOAc (3 × 30 mL). The combined organic layers were washed with water (2 × 30 mL), dried over Na₂SO₄ and concentrated in vacuo. The crude product was purified by column chromatography.

**4-(4-(N,N-Dimethylamino)phenyl)-2,1,3-benzothiadiazole (1)**

The compound was synthesized according to the general procedure using 4-(N,N-dimethylamino)phenylboronic acid pinacol ester (241 mg, 0.98 mmol, 2.1 equiv.). Purification by column chromatography (hexane/CH₂Cl₂ : 1, Rᵣ = 0.25) gave 1 as an orange solid (103 mg, 87%) whose characterization data was in accordance with reported literature. **¹H NMR** (400 MHz, CDCl₃): δ (ppm) = 7.91–7.87 (m, 3H), 7.66–7.61 (m, 2H), 6.80 (d, J = 8.4 Hz, 2H), 3.05 (s, 6H); **¹C NMR** (101 MHz, CDCl₃): δ (ppm) = 155.7, 153.7, 150.5, 134.6, 130.0, 129.8, 125.8, 125.1, 118.8, 112.2, 40.4.

**4-(4-Methylphenyl)-2,1,3-benzothiadiazole (2)**

The compound was synthesized according to the general procedure using 4-methoxyphenylboronic acid (148 mg, 0.98 mmol, 2.1 equiv.). Purification by column chromatography (hexane/CH₂Cl₂ : 1, Rᵣ = 0.37) gave 2 as a bright green solid (104 mg, 93%) whose characterization data was in accordance with reported literature. **¹H NMR** (400 MHz, CDCl₃): δ (ppm) = 7.95 (d, J = 7.4, 2.4 Hz, 1H), 7.91–7.88 (m, 2H), 7.67–7.63 (m, 2H), 7.09–7.05 (m, 2H), 3.89 (s, 3H); **¹C NMR** (101 MHz, CDCl₃): δ (ppm) = 159.8, 155.6, 153.6, 134.2, 130.4, 129.8, 129.7, 126.9, 119.8, 114.1, 55.4.

**4-(4-Methylphenyl)-2,1,3-benzothiadiazole (3)**

The compound was synthesized according to the general procedure using 4-methylphenylboronic acid (116 mg, 0.69 mmol, 1.3 equiv.). Purification by recrystallization from CH₂Cl₂/hexane gave 3 as dark green crystalline solid (93 mg, 94%). **¹H NMR** (400 MHz, CDCl₃): δ (ppm) = 7.96 (m, 2H), 7.86 (m, 2H), 7.70–7.65 (m, 2H), 7.40 (m, 2H), 2.56 (s, 3H); **¹C NMR** (101 MHz, CDCl₃): δ (ppm) = 155.7, 153.7, 138.3, 134.0, 133.9, 129.6, 129.5, 127.2, 126.4, 120.4, 15.7. HRMS (APCI): calcd for [C₁₆H₁₆N₂S⁺ + H⁺] = 259.0358; found 259.0352.

**4-(4-Methylphenyl)-2,1,3-benzothiadiazole (4)**

The compound was synthesized according to the general procedure using p-tolyboronic acid (133 mg, 0.98 mmol, 2.1 equiv.). Purification by column chromatography (hexane/toluene 10:1, Rᵣ = 0.30) gave 4 as a pale green solid (86 mg, 82%). **¹H NMR** (400 MHz, CDCl₃): δ (ppm) = 7.99–7.95 (m, 1H), 7.83 (d, J = 7.9 Hz, 2H), 7.67–7.66 (m, 2H), 7.35 (d, J = 7.9 Hz, 2H), 2.54 (s, 3H); **¹C NMR** (101 MHz, CDCl₃): δ (ppm) = 155.6, 153.6, 138.3, 134.6, 134.5, 129.6, 129.3, 129.1, 127.3, 120.2, 21.3. HRMS (APCI): calcd for [C₁₆H₁₆N₂S⁺ + H⁺] = 227.0637; found 227.0631.

**Staining of Cells and Fluorescence Microscopy**

Stock solutions of the compounds in DMSO were prepared at 10% fetal bovine serum (HyClone), 1% non-essential amino acids (NEAA) (Sigma-Aldrich), 1% GlutaMAX (Gibco) and 1% penicillin-streptomycin (Sigma-Aldrich). Cells were cultured in a humidified environment at 37°C and 5% CO₂.

**Control and experimental pictures were processed identically.**
4-(4-Chlorophenyl)-2,1,3-benzothiadiazole (8)

The compound was synthesized according to the general procedure using 4-chlorophenylboronic acid (153 mg, 0.98 mmol, 2.1 equiv.). Purification by column chromatography (hexane/CH₂Cl₂ 10:1, Rᵣ=0.29) gave 7 as a colourless solid (55 mg, 48%) whose characterization data was in accordance with reported literature.¹⁰¹

¹¹ H NMR (400 MHz, CDCl₃): δ (ppm) = 8.08–8.00 (m, 1H), 7.90–7.87 (m, 2H), 7.71–7.67 (m, 2H). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) = 155.3, 153.3, 153.7, 134.5, 133.3, 130.5, 129.5, 128.8, 127.6, 126.9. HRMS (APCI): calcd for [C₁₀H₇ClN₂S⁺]+ 247.0391; found 247.0387.

4-(4-Fluorophenyl)-2,1,3-benzothiadiazole (6).

The compound was synthesized according to the general procedure using 4-fluorophenylboronic acid (137 mg, 0.98 mmol, 2.1 equiv.). Purification by column chromatography (hexane/CH₂Cl₂ 10:1, Rᵣ=0.29) gave 6 as a pale yellow solid (100 mg, 74%) whose characterization data was in accordance with reported literature.²⁹

¹¹ H NMR (400 MHz, CDCl₃): δ (ppm) = 8.31–8.28 (m, 1H), 8.16–8.12 (m, 2H), 8.11 (dd, J = 8.5, 1.3 Hz, 1H), 7.79 (dd, J = 7.0, 1.3 Hz, 1H), 7.74 (dd, J = 8.5, 7.0 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) = 155.5, 152.9, 147.5, 143.6, 132.0, 130.1, 129.4, 128.7, 128.3, 127.7, 126.7, 63.2. HRMS (APCI): calcd for [C₁₁H₈F₂N₂O⁺]+ 258.0332; found 258.0327.

Chemical reduction of compound 11

A round-bottom flask was charged with 11 (26 mg, 0.11 mmol) and Pd/C (5 mg, 15 wt %). The flask was evacuated and backfilled with H₂ (three times). CH₂Cl₂ was added and the resulting solution was stirred at rt. overnight under a H₂ atmosphere (balloon). The crude mixture was filtered over Celite and the filtrate concentrated in vacuo. The resulting crude was purified by column chromatography (pentane/EtOAc 3:2, Rᵣ=0.50) to yield the desired compound as a yellow fluorescent solid (19 mg, 86%) whose characterization data was in accordance with reported literature.²⁹ H NMR (400 MHz, DMSO-d₆): δ (ppm) = 7.94–7.89 (m, 1H), 7.79–7.75 (m, 2H), 7.73–7.66 (m, 2H), 6.76–6.71 (m, 2H), NH₂ not observed.

NTR reduction of compound 11(32)

A 1 mM stock solution of compound 11 in DMSO was used for the experiment. Compound 11 (50 µM final concentration), NADH (500 µM final concentration) and NTR (1 µg/µl final concentration) were dissolved in PBS buffer (pH = 7.4) to a total volume of 10 mL in which the final DMSO concentration was 5%. The solution was stirred at 37°C for 45 min and then directly transferred to a quartz cuvette to measure the fluorescence. A parallel blank experiment without NTR was performed under the same conditions.

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