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Cu^{II}-induced fluorescence quenching of a BODIPY fluorophore

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Dedicated to Professor Peter Comba on the Occasion of his 65th Birthday.

Abstract: The ongoing developments in fluorescence microscopy have pushed the interest in new fluorescent probes with specific properties. Aside of controlling emissive states by light irradiation, defined control by changing the chemical environment has come into focus. In this context, we designed a fluorescent probe by conjugation of bipyridine, acting as sensor for Cu^{II}, to a BODIPY derivative at the shortest possible distance between sensor and chromophore. Here, we present the synthesis of the BODIPY-probe along with its crystal structure. We found a strong dependence of absorption and emission upon Cu^{II}-complex formation decreasing the fluorescence quantum yield to ca. 1% in respect to the pure probe. We hypothesize that further functionalization with a linker could make this compound and interesting probe for fluorescence microscopy.

Introduction

In recent years, the interest in novel fluorescent probes increased due to improved labelling approaches for cell experiments,[1] and promising applications in microscopy.[2,3] What makes them interesting is that the chemical environment can be used to gain control of the spectroscopic states.

Fluorescent probes can be realized as turn-off or turn-on probes reacting with an emission decrease or increase, respectively. Aside of collisional quenching,[4] fluorescence can be controlled by a variety of different mechanisms, like Förster resonance energy transfer (FRET),[5] photo-induced electron transfer (PET),[3,6] (twisted) internal charge transfer ((T)ICT),[7] or through bond energy transfer (TBET).[8] The field gained interest as isomerisation, [9] long-lived dark states,[10] or adduct formation[11] supported the advancements in super-resolution fluorescence microscopy. Also, chemical transformations leading to changed fluorescence emission have been widely studied, e.g. intramolecular cyclization of rhodamine derivatives,[12] DNA hybridization,[13] protonation,[14] imine tautomerization in photo-acids,[15] reversible redox reactions,[16] enzymatic cleavage,[17] and several more.[18]

To become effective, fluorescent probes should emit as much photons as possible upon excitation which points to the importance of choosing the fluorescent dye. Here, the important parameters are molecular brightness and photo-stability where the molecular brightness is the product of extinction coefficient and quantum yield. Good fluorophores usually show extinction coefficients of more than 100,000 $M^{-1}cm^{-1}$ and a quantum yield of 90% or higher. It is commonly known that high quantum yields are achieved when non-radiative transitions e.g. by internal conversion (IC) or intersystem crossing (ISC) are suppressed. Rigid structures lacking flexibility, like conjugated aromatic rings, or substituents, like Fluorine, are therefore favoured for the design of fluorescent probes, while substituents, like Bromine or Iodine, lead to a significant reduction of the quantum yield due to the heavy atom effect.[19] Photo-stability is governed by chemical reactions occurring in the excited state. While many of these reactions may occur from the triplet state, i.e. by spin exchange and subsequent reaction with triplet oxygen, it is also known that excitation at short wavelength (UV) can lead to photo-induced decomposition of fluorophores.

Beyond this, also a high Stokes shift and a narrow emission band is of advantage as it simplifies optical separation of the emitted light from scattered excitation. For designing fluorescent probes, the sensor unit switching the emission, is of equal importance as the fluorophore. When driven by metal ion coordination, not only the quenching mechanisms play an important role but also the kinetics of metal ion coordination. Additional requirements are imposed by the envisioned application, e.g. for single-molecule based super-resolution microscopy only a small fraction of molecules must be in the on-state such that non-overlapping point-spread functions can be imaged in each frame. The achievable labelling density is also limited by residual fluorescence emission of molecules in their off-state. Both effects are directly governed by the properties of the ligand, i.e. its switching kinetics and the quenching mechanism controlling the fluorophore emission. For instance, in single-molecule localization microscopy based on reversible metal ion complexation the dissociation reaction plays an important role because the on-state duration must match the integration times of the image detector to maximise localisation precision whilst minimizing the total image acquisition time to achieve a full reconstruction. In this sense, Boron-dipyrromethene (BODIPY) dyes have high extinction coefficients with relatively narrow absorption bands in the visible range and are thereby interesting candidates for metal ion sensors. [20] Due to their high quantum yields and good photo-stabilities they are even used in single-molecule fluorescence microscopy experiments. [21]

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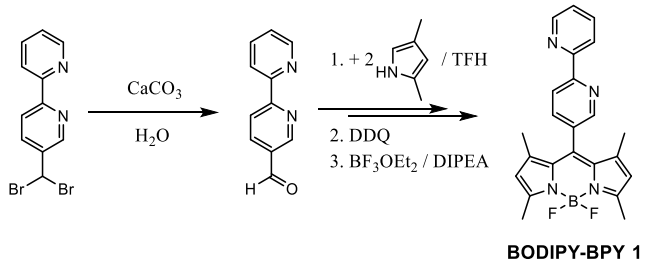

Scheme 1. Syntheses of BODIPY-BPY **1**.

In previous studies, the bidentate bipyridine was found a suitable ligand for designed Cu^{II}-dependent switchable fluorescent probes.[2,22–25] Therefore, we designed a bipyridine functionalized turn-off probe with the shortest possible distance to the chromophore to maximize quenching upon coordination of Cu^{II}. We synthesized a bipyridine/BODIPY conjugate BODIPY-BPY **1** (Scheme 1) and characterized its spectroscopic properties in presence of Cu^{II}.

Results and Discussion

Synthesis and molecular structure

The well-known synthesis of symmetric BODIPY dyes starts with condensation of substituted pyrroles with an aldehyde.[26–28] The intermediate dipyrromethane is then oxidized, e.g. by a quinone, and reacted with borontrifluoroetherate. Frequently, the pyrroles are substituted with two alkyl groups in position 2 and 4 to sterically prevent rotation of the residue introduced by the aldehyde which otherwise leads to a reduced fluorescence quantum yield. [27] Based on this route we synthesized a probe in which the ligand bipyridine is directly coupled to the BODIPY chromophore such that metal ion binding can influence their spectroscopic properties.

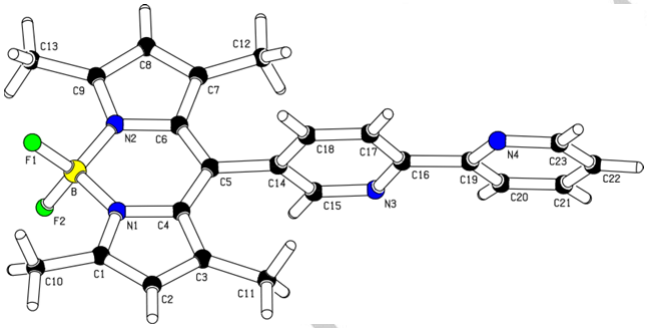

Figure 1. Crystal structure of BODIPY-BPY **1**.

Synthesis of BODIPY-BPY **1** starts with hydrolysis of 5-(dibromomethyl)-2,2'-bipyridine to form the corresponding aldehyde, followed by BODIPY synthesis according to standard procedures (Scheme 1). Purification and recrystallization yields red crystals suited for crystallographic analysis (fig. 1). In BODIPY-BPY **1** the bipyridine moiety is directly coupled to the chromophore suggesting a strong influence on spectroscopic states. On the other hand, the planes of bipyridine and chromophore are orthogonally oriented due to steric hindrance by the two methyl groups in 3 and 7 position preventing direct π-π-interaction. The nitrogen atoms in the two pyridine rings of the bipyridine moiety are pointing in opposite directions and must be reorganized upon coordination to metal ions, like Cu^{II}.

Spectroscopic characterization

The absorption spectrum of BODIPY-BPY **1** (fig. 2) shows the BODIPY absorption band at 502 nm [29] along with aromatic side bands (236 nm) and the typical bipyridine band at 285 nm.[26] In methanol we found a molar extinction of 100,000 $M^{-1}cm^{-1}$ that is reduced in MOPS buffered aqueous solution to ca. 79,000 $M^{-1}cm^{-1}$.

Upon addition of increasing Cu^{II} amounts absorbance is further reduced by ca. 20% when reaching saturation also showing a slight red shift by ca. 5 nm (fig. 2). At the same time the bipyridine band at 285 nm shows a pronounced bathochromic shift by ca. 33 nm. Additionally, isosbestic points can be observed indicating a second species, i.e. the Cu^{II} complex with the bipyridine moiety, influencing the spectroscopic properties of BODIPY-BPY **1**. Cu^{II} coordination is reversible as the BODIPY absorbance (dashed line) is fully recovered upon addition of excess EDTA.

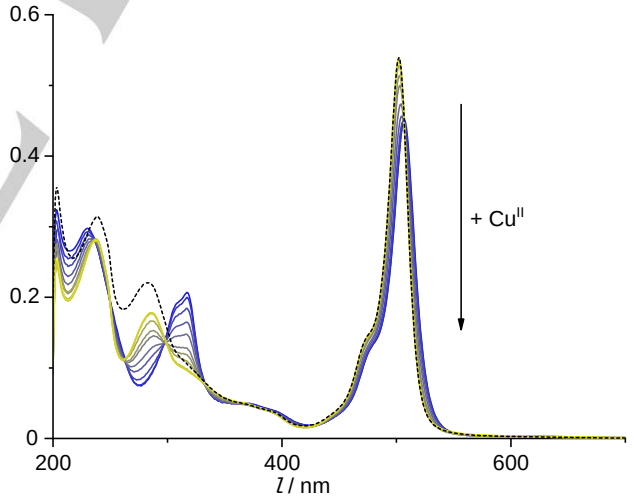

Figure 2. Absorption spectra of BODIPY-BPY **1** (23 µM; yellow) in 10 mM MOPS buffered aqueous solution (λ_{max} = 502 nm) and at increasing Cu^{II} concentrations as indicated by colour shading from yellow to blue (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 14, 19 µM of $CuSO_4$). While the BODIPY absorbance is only weakly shifted to 507 nm, a strong bathochromic shift is visible for the bipyridine band from 285 to 317 nm. Addition of excess EDTA fully recovers the BODIPY absorption band (dashed line).

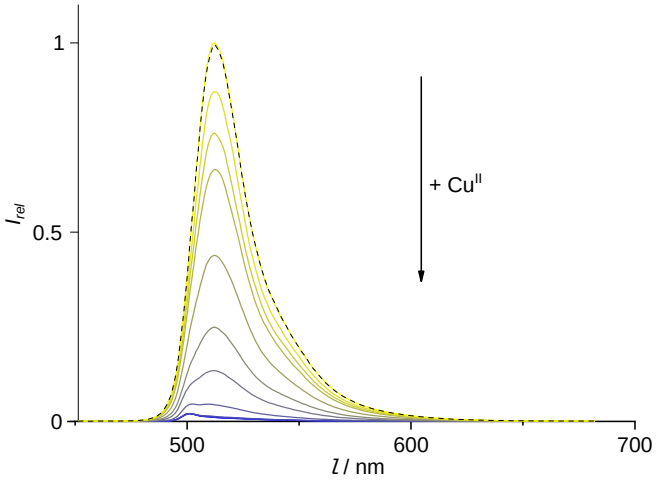

Figure 3. Emission spectra of BODIPY-BPY **1** (λ_{exc} = 458 nm) in 10 mM MOPS buffered aqueous solution and at increasing Cu^{II} concentrations as indicated by colour shading from yellow to blue (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 14, 19 µM of $CuSO_4$). Excess of EDTA fully recovers the BODIPY emission (dashed line).

BODIPY-BPY **1** itself is strongly fluorescent showing an emission band at 517 nm typical for BODIPY derivatives. The relative quantum yield in methanol was determined to be 24% using fluorescein as standard.

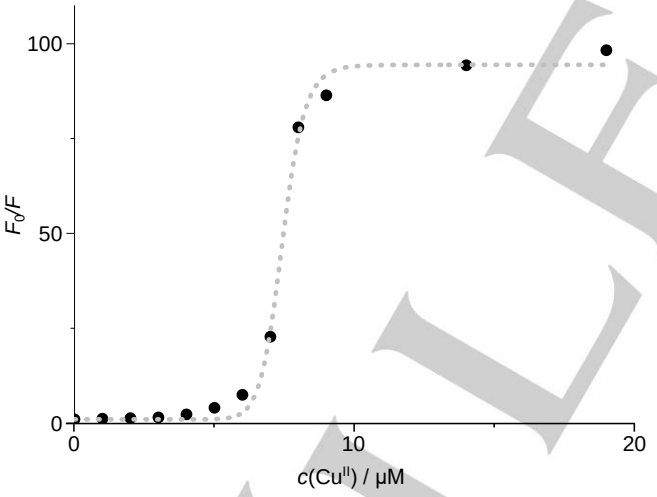

Figure 4. Stern-Volmer plot of the titration of BODIPY-BPY **1** (7 µM) in 10mM MOPS buffered aqueous solution with $CuSO_4$. The titration yields a sigmoidal shape levelling off at ca. 94.4 reflecting a residual fluorescence intensity of about 1% (dashed line shows the logistic function to guide the eye).

In contrast to the rather weak changes in absorbance, strong quenching of fluorescence emission can be observed upon Cu^{II} coordination (fig. 3). Quenching of the fluorescence happens instantaneously upon addition of a $CuSO_4$ accompanied by a bathochromic shift of ca. 10 nm. Fluorescence emission is fully recovered along with absorption upon addition of an excess of EDTA supporting reversible coordination of Cu^{II}. Figure 4 shows a Stern-Volmer plot of the titration with Cu^{II}. Here, F_0/F is plotted over the Cu^{II} concentration c(Cu^{II}), where F_0 is the fluorescence intensity of a pure BODIPY-BPY **1** solution and F is the fluorescence intensity measured at a given c(Cu^{II}). The data shows a sigmoidal shape levelling off close to F_0/F = 94.4 reflecting a residual fluorescence emission of the Cu^{II} complex with BODIPY-BPY **1** of ca. 1%. Similar observations have been made when using methanol as solvent. Obviously, simple complex formation cannot account for the observed sigmoidal shape. One might speculate that two BODIPY-BPY **1** could be coordinated to a single metal cation at low Cu^{II} concentrations but this effect is not reflected in the absorbance data. More extensive spectroscopic studies might shed light on this issue.

Conclusions

Overall, we have synthesized and characterized a new fluorescent probe sensitive to Cu^{II}. The probe BODIPY-BPY **1** combines the bright emission of the known BODIPY fluorophore with sensory activity of bipyridine. Since chromophore and coordination site are in close proximity, we observed strong spectroscopic effects by coordination of Cu^{II} on absorbance and emission of the BODIPY moiety. We found that the quenching by Cu^{II} is based on the coordination equilibrium and can be reverted by addition of EDTA.

Although absorbance and emission are showing the same trend upon addition of Cu^{II}, the strong quenching of fluorescence emission cannot be justified by the change in absorbance alone. It is well known that bipyridine itself doesn't show fluorescence emission because free rotation of the pyridine rings in solution promote alternative decay mechanisms. In contrast to redox inert metal cations, the Cu^{II} complex with bipyridine becomes not fluorescent, although coordination by Cu^{II} stabilizes the two rings in plane as indicated by the red shift of the bipyridine band.[30] This observation points towards additional quenching effects by interaction metal-ligand orbitals influencing the electron distribution as well as mesomeric effects.

In previous studies of similar probes, we have observed comparable effects and could exclude energy transfer as possible quenching mechanism. We found that the overlap between dye emission and ligand/complex absorption was too small to enable energy transfer.[2,23–25,31] However, as complex and chromophoric centre were always in close proximity, as is the case for BODIPY-BPY **1**, photo-induced electron transfer (PET) remains a possible quenching mechanism. Further studies and an eventual proof of PET in such systems could bring up interesting possibilities of such probes for light-controlled redox states of Cu^{II} complexes, e.g. in catalytic reactions.

Beyond this, functionalization of fluorescent metal indicators have an interesting perspective in the field of microscopy, e.g. as probes for super-resolutions microscopy or as additional approach for multiplexing in microscopy to increase the number of simultaneously observable targets.[2,24] Moreover, functionalized probes could be used for implementing sensory

layers in nanostructured materials adding function to certain regions.

Experimental Section

Synthesis

General Methods. The BODIPY-BPY probe was synthesized starting with alkaline hydrolysis of 5-(dibromomethyl)-2,2'-bipyridine to [2,2'-bipyridine]-5-carbaldehyde as precursor in the well described BODIPY synthesis.[27,28]

Preparation of 9-(2,2'-bipyridine-5-yl)-1,3,5,7-tetramethyl boron dipyrromethene (BODIPY-BPY) 1: 2,4-Dimethylpyrrol (97%, 1.38 g, 1.5 ml, 14.4 mmol) and (2,2'-bipyridine)-5-carbaldehyd **2** (1.34 g, 7.3 mmol) are successively dissolved in 150 mL DCM (abs.) under inert-gas atmosphere (Ar) at room temperature. After addition of catalytic amounts of TFA the mixture is stirred for approx. 3 h. When the reaction is finished (residual substrate checked by TLC), 2,3-Dichloro-5,6-dicyano-pbenzochinon (98%, 1.65 g, 7.3 mmol in 50 ml DCM) is added and stirred for another 10 min before addition of an excess of DIPEA (30.0 ml, 176.4 mmol) and BF_3OEt_2 (10.0 ml, 79.0 mmol). The reaction mixture is stirred for another 2 h and then purified by extraction with water. After separation of the organic phase it is dried over $NaSO_4$ and the solvent is removed in a rotary evaporator. The product is further purified by column chromatography (SiO_2; DCM/EtOAc 1:3) and recrystallization in DCM/EtOAc (1:8) yielding red crystals. The yield was 24% based on **(2)**. $C_{23}H_{21}BF_2N_4$ (402.25581). Elemental analysis didn't yield satisfying results ($C_{23}H_{21}BF_2N_4$ (402.26); C 67.17 (calc. 68.68); H 5.53 (calc.5.26); N 13.04 (calc. 13.93) and has been reported being difficult for BODIPY derivatives due to the presence of boronnitride.[32]. Therefore, purity of the compound was proven by 1H-NMR and MS ESI (see supporting information) and low amounts of the compound were further purified by HPLC for the spectroscopic experiments. **^{1}H-NMR** (300 MHz, $CDCl_3$) δ (ppm) = 8.72 (ddd, J=4.8, 1.9, 0.9, 1H, 2), 8.65 – 8.55 (m, 2H, 5, 12), 8.53 – 8.43 (m, 1H, 9), 7.93 – 7.82 (m, 1H, 6), 7.78 (dd, J=8.1, 2.3, 1H, 11), 7.37 (ddd, J=7.6, 4.8, 1.2, 1H, 1), 6.02 (s, 2H, 20, 23), 2.57 (s, 6H, 28, 30), 1.46 (s, 6H, 27, 29). **^{13}C-NMR** (75 MHz, $CDCl_3$) δ (ppm) = 156.76 (10), 156.45 (19, 24), 155.32 (4), 149.50 (2), 148.04 (12), 142.98 (14, 18), 137.67 (13), 137.27 (6), 137.20 (11), 131.65 (21, 22), 131.35 (7), 124.45 (1), 121.85 (9), 121.46 (5), 121.11 (20, 23), 77.58, 77.16, 76.74, 15.36 (27, 29), 14.80 (28, 30). **MS** (ESI pos): m/z = 403.30 ([M+H]+). **UV/VIS** (H_2O): λ_{max} (ε): 502 (0.53), 236 (0.28), 285 (0.18) nm.

Preparation of (2,2'-bipyridine)-5-carbaldehyde 2: A mixture of 5-(dibromomethyl)-2,2'-bipyridine (3 mg, 9.2 nmol) and $CaCO_3$ (2.0 mg, 20.1 nmol) in 100 ml H_2O is boiled for 6 h under reflux. After cooling to room temperature the mixture is extracted three times with 100 ml DCM each. The united organic phase is dried over $NaSO_4$ before the solvent is removed in a rotary evaporator. The product is further purified by column chromatography (SiO_2, PE/EtoAC 5:1). The yield was 85% based on **(4)**. $C_{11}H_8N_2O$ (184.19). **^{1}H-NMR** (600 MHz, $CDCl_3$): δ (ppm) = 7 38 (t, ^{1}J(H) = 12 Hz, 1 H), 7.86 (t, ^{1}J(H) = 7 Hz, 1 H), 8.27 (d, ^{1}J(H) = 8 Hz, 1 H), 8.49 (d, ^{1}J(H) = 8 Hz, 1 H), 8.60 (d, ^{1}J(H) = 8 Hz, 1 H), 8.71 (d, ^{1}J(H) = 4 Hz, 1 H), 9.11 (s, 1 H), 10.15 (s, 1 H). **^{13}C-NMR** (150 MHz, $CDCl_3$): δ (ppm) = 121.35, 122.28, 124.85, 131.11, 136.97, 137.25, 149.47, 151.69, 154.71, 160.61, 190.64. **MS** (ESI pos): m/z = 184.10 ([M+H]+).

X-Ray Crystal structure determination

Crystal data: BODIPY-BPY 1: $C_{23}H_{21}BF_2N_4$, M_r = 402.25, triclinic, space group *P*-1, *a* = 9.3868(2), *b* = 9.7303(3), *c* = 10.8644(3) Å, α = 92.910(2), β = 96.618(2), γ = 103.217(3) °, *V* = 956.54(5) Å^3, *Z* = 2, d_{calc} = 1.397 Mg·m^{-3}, μ = 0.797 mm^{-1}, F_{000} = 420. *T* = 120(1) K, θ range 4.1 to 70.6 °. Index ranges *h*, *k*, *l* ±11, ±11, ±13. Reflections measd.: 76817, indep.: 3580 [R_{int} = 0.0456], obsvd. [$I > 2\sigma(I)$]: 3472. Final *R* indices [$F_o > 4\sigma(F_o)$]: $R(F)$ = 0.0460, $wR(F^2)$ = 0.1195, *GooF* = 1.098. Difference density: max, min 0.267, -0.296 e·Å^{-3}.

Data collection: Agilent Technologies Supernova-E CCD diffractometer, Cu-K_α radiation, microfocus X-Ray tube, multilayer mirror optics, λ = 1.5418 Å. Lorentz, polarization and numerical absorption correction (max., min. transmission factors: 0.919, 0.862).[33] Structure solution: *ab initio* charge flipping.[34] Refinement: full-matrix least squares methods based on F^2,[35] all non-hydrogen atoms anisotropic, most hydrogen atoms located and refined (except those of the methyl groups, which were input at calculated positions and refined as variable metric rigid groups).

CCDC 1838079 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via https://www.ccdc.cam.ac.uk/data_request/cif.

^{1}H- and ^{13}C-NMR spectra: NMR spectra were recorded on a Varian Mercury Plus 300 MHz spectrometer or a Varian 500 MHz NMR System. Peak shifts were reported relative to solvent peaks according to Fulmer et al.[36] The chemical shift δ is stated in parts per million (ppm), the coupling constants J in Hertz (Hz) in respect to ^{1}H-^{1}H-coupling. Signal multiplicities are indicated by: s = Singulett, d = Dublett, dd = Dublett of Dubletts, t = Triplett, q = Quartett, m = Multiplett.

Mass Spectrometry: Bruker ApexQe hybrid 9.4 T FT-ICR. For MALDI spectra DCTB was used as matrix.

Optical Spectroscopy: All measurements were carried out in ultra-micro-quarzglass cuvettes (105.251-QS, Hellma Analytics, Müllheim) with an optical path length of 3mm and a minimum volume of 45 µl. UV/VIS absorption was measured on a "Cary 500 Scan" (Varian/Agilent Technologies) against air. Spectra were corrected for absorption of the cuvette and the pure solvent. Fluorescence spectra were taken on a "Cary Eclipse 500" (Varian/Agilent Technologies) at an excitation wavelength just below the absorption maximum to minimize light scattering. Fluorescence spectroscopy was carried out at concentrations well below 10^{-3} M (usually at ca. 10^{-6} M) using 100 µl sample to avoid inner filter effects.

Titrations: Titration with Cu^{II} was performed by stepwise addition of 1 µl $CuSO_4$ stock solution and careful mixing by loading the pipette multiple times. Fluorescence spectra were recorded after equilibration. Concentrations were corrected for the respective dilution in each titration step. Fluorescence intensities were measured by integrating the emission spectra over ± 10 nm around maximum emission to minimize noise fluctuations.

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Keywords: fluorescent switches • metal ion sensing • fluorescence spectroscopy

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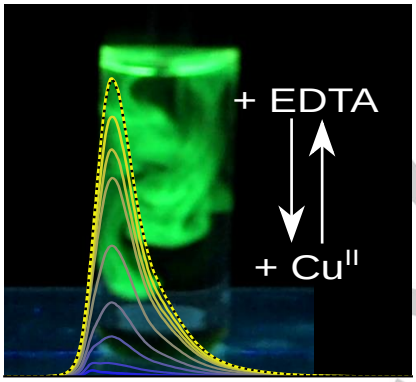

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Cu^{II}-induced fluorescence quenching of a BODIPY fluorophore

Dirk-Peter Herten, Andreas Haderspeck, Felix Braun, Hubert Wadepohl

Figure S1: ^{1}H-NMR of Compound **1**

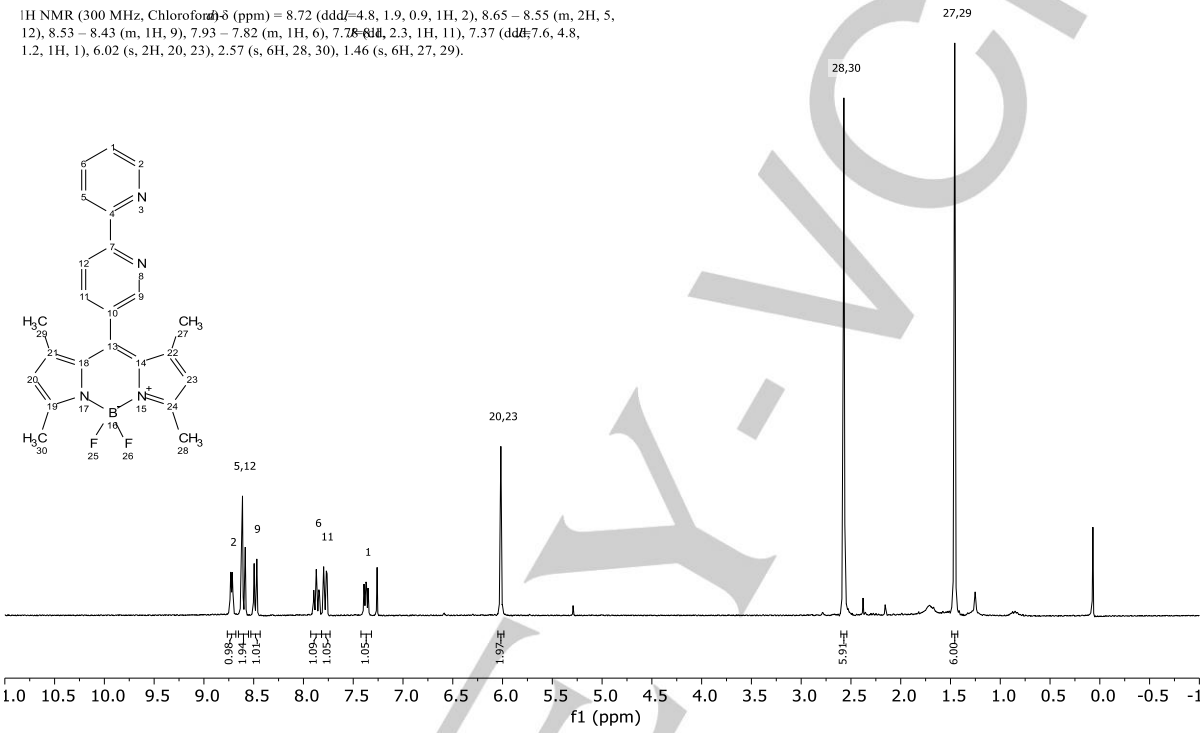

Figure S2: ^{13}C-NMR of Compound **1**