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PAPER

Fluorescent properties and resonance energy transfer of 3,4-bis(2,4-difluorophenyl)-maleimide†

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A fluorescent compound 3,4-bis(2,4-difluorophenyl)-maleimide **1** from the 3,4-diaryl-substituted maleimides was synthesized and determined to have a Stokes shift of 140 nm (λ_{abs} 341 nm, λ_{em} 481 nm), a high fluorescent quantum yield (Φ_{fl} 0.61) and an extinction coefficient $\varepsilon_{(340)}$ of 48 400 M^{-1} cm^{-1} in dichloromethane. For the first time we demonstrated the successful implementation of a 3,4-diaryl-substituted maleimide molecule as a donor component in FRET experiments.

Introduction

Fluorescent molecules find important applications as sensor elements, biomolecular probes, and substrates in material and life sciences. Despite the large variety of available fluorescent dyes, new fluorophoric systems possessing a large Stokes shift, a high quantum yield, and a high photostability are of continued interest.[1] Well known fluorophores such as cyanines, tetramethyl rhodamines (TMR), and fluoresceins are characterized by broad excitation and emission ranges and small Stokes shifts (less than 30 nm in some cases), which limit their application in Förster resonance energy transfer (FRET) systems due to partial absorption of the incident light by the FRET acceptor partner.[2a] At the same time while Alexa 350 and Alexa 430 dye are characterized with a high Stokes shift, greater than 70 nm, they exhibit lower extinction coefficient.[2b] In addition, fluorescein conjugates appended to biological molecules are quite often photolabile and their fluorescence is significantly quenched prior to photodissociation. In contrast, the use of the diaryl-substituted maleimides (DMs) as fluorescence probes in bioscience applications have not been extensively explored. Despite their promising photophysical characteristics such as high fluorescent quantum yields ($\Phi_{fl} > 0.10$), good extinction coefficients ($\varepsilon_{\lambda} > 5000$ M^{-1} cm^{-1}), and large Stokes shifts ($\Delta > 90$ nm), the DM molecules have been investigated primarily for material science applications.[3] Recent reports demonstrated the design and the application of fluorescent metal sensors based on monoindole-substituted maleimides and the development of an efficient organic light-emitting diode (OLED) based upon a naphthylphenylamino substituted *N*-methyl-3,4-diphenyl-maleimide.[4] The ongoing effort to generate small-molecule fluorescent probes with high fluorescent quantum yield, a large Stokes shift and high photostability, prompted us to investigate the photophysical characteristics of a new fluorescent dye from the DM family, 3,4-bis(2,4-difluorophenyl)-maleimide **1**, and its functionalized derivatives. The simple and efficient synthetic route to yield **1** and the convenient way to functionalize that moiety with appropriately installed chemical handles, makes this fluorophore easily accessible. Herein we present the identification and spectroscopic characterization of 3,4-bis(2,4-difluorophenyl)-maleimide **1** and its first reported use as a donor component in FRET systems.

Results and discussion

Photophysical properties

The analysis of the photophysical properties of **1** revealed a high extinction coefficient $\varepsilon_{(340)}$ 48 400 M^{-1} cm^{-1}, a large Stokes shift (Δ) of 140 nm in MeOH (λ_{abs} 341 nm; λ_{em} 481 nm), and a very good fluorescence quantum yield in DCM (Φ_{fl} 0.61) (Fig. 1). It is important to note that when compared to the reported DM molecules ($\varepsilon_{(\lambda)}$ 2180 to 14 000 M^{-1} cm^{-1} and Φ_{fl} 0.02 to 0.52), **1** exhibits a higher extinction coefficient ($\varepsilon_{(\lambda)}$ 13 490 to 48 400 M^{-1} cm^{-1}) and excellent quantum yield in a variety of solvents (Φ_{fl} 0.08 to 0.61) in addition to the unusually large Stokes shift.

These advantageous spectral characteristics render **1** as a suitable donor component in a FRET system, exemplified in the large Stokes shift which is essential for an efficient energy transfer between the donor and acceptor with a minimal direct excitation of the donor. Therefore, having a donor with an excitation maxima being sufficiently apart from the excitation acceptor,

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results in a more accurate measurement of the energy transfer phenomena.

An efficient one step synthesis of **1** was achieved by allowing (2,4-difluoro-phenyl)-acetonitrile to react with iodine and sodium methoxide at −85 °C (Scheme 1).[3] The exact structure of **1** was confirmed by ^{1}H-, ^{13}C-, ^{19}F-NMR spectroscopy, and by high resolution mass spectrometry. An X-ray diffraction structure was also obtained, demonstrating that **1** has noncrystallographic C_2-symmetry with the two difluorophenyl rings not being co-planar to the maleimide ring plane (Fig. 2).

In an attempt to implement the fluorescent probe into a FRET system, we generated derivatives **1a**, **1b**, and **1c** containing chemical handles, enabling the linkage to a FRET partner of interest.[1] Conceivably, such types of derivatives were easily obtained by substituting the DM fluorophore with various functionalities tethered to the nitrogen. Much like the parent compound **1**, the analogs **1a**, **1b**, and **1c** display high fluorescence quantum yields, high molar extinctions ($\varepsilon_{(\lambda)}$ 5990 to 23 000), and a large Stokes shifts (Δ 115 to 143 nm depending on solvent).

Bathochromic shifts were observed in the emission spectra for **1** and its derivatives when moving from nonpolar to polar solvents (Table 1). The largest bathochromic shift in the emission spectra, as well as the largest values for the Stokes shift for all compounds was recorded in the presence of the protic polar solvent methanol (Δ 135 nm for **1c** to Δ 143 nm for **1b**) while the lowest values were observed for the more polar aprotic solvent CH_3CN (Δ 122 nm for **1b** to Δ 130 nm for **1**). In contrast, the absorption maxima of **1** and **1a–1c** display only small differences between solvents. This observation suggests that hydrogen bonding between **1** and the methanol solvent stabilizes the singlet excited state.

Seliskar and McGlynn[5a] characterized the lowest lying singlet state of the unsubstituted maleimide as a $1^1A_1 \rightarrow 1^1B_2$ transition of primarily n → π* character. However, it is well known that transitions to n → π* states are strongly dependent upon the nature of the solvent while transitions to π → π* states are not as strongly influenced.[5b] Thus, the fact that the absorption maxima of **1**, **1a–1c** do not exhibit a significant solvent dependence would suggest that the excitation is largely of π → π* origin while the lowest energy emitting state is of n → π* character thus accounting for the significant solvent dependence of the Stokes shift. The re-ordering of the excitation state may be a

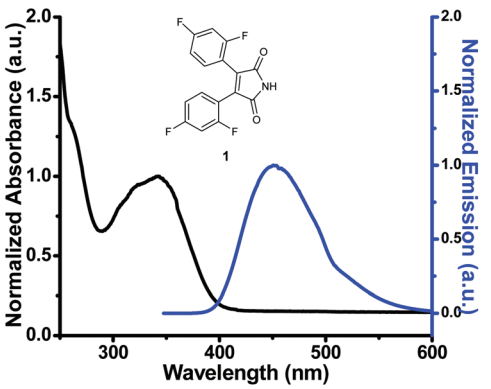
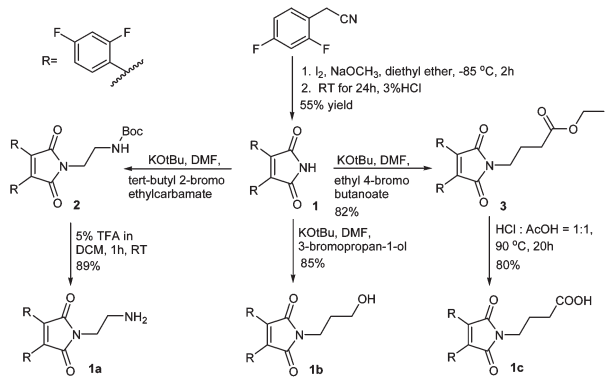


Fig. 1 Normalized absorption and emission of **1** in DCM.



Scheme 1 Synthesis of 3,4-bis(2,4-difluorophenyl)-maleimide **1** and the analogs **1a**, **1b**, and **1c**.

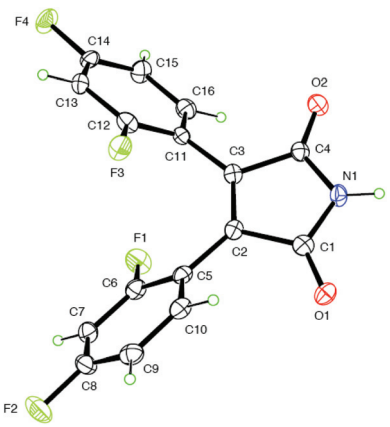


Fig. 2 X-ray crystal structure of **1** with 50% ellipsoids.

Table 1 Solvent dependent absorption and emission maxima of compounds **1**, **1a–1c**

	ε	**1** $\lambda_{abs}/\lambda_{fl}$ [a] (Stokes)	**1a** $\lambda_{abs}/\lambda_{fl}$ [a] (Stokes)	**1b** $\lambda_{abs}/\lambda_{fl}$ [a] (Stokes)	**1c** $\lambda_{abs}/\lambda_{fl}$ [a] (Stokes)	Φ_{fl} [b]
Hexanes	1.89	345/460 (115)	334/445 (111)	346/465 (119)	342/461 (119)	0.083
Toluene	2.38	345/463 (118)	331/448 (117)	346/469 (123)	346/464 (118)	0.06
DCM	8.93	345/468 (123)	332/454 (122)	348/467 (119)	350/469 (119)	0.61
CH_3OH	32.7	341/481 (140)	332/469 (137)	340/483 (143)	346/481 (135)	0.313
CH_3CN	37.5	338/468 (130)	325/452 (127)	342/464 (122)	344/469 (125)	0.296

[a] Absorption and emission spectra were recorded in 5×10^{-6} M solutions, λ_{ex} 340 nm. [b] Quantum yield of **1** was measured against perylene.

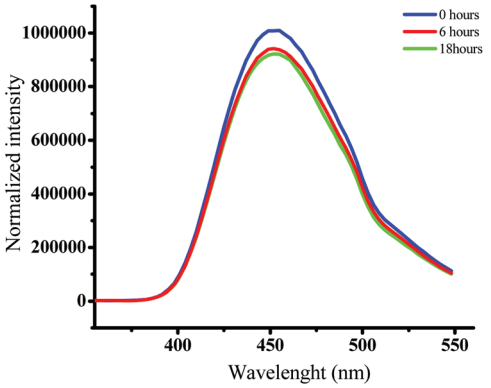


Fig. 3 Normalized emission spectra of **1** (DCM, 1×10^{-5} M) after irradiation with Xe source for 6–18 hours, (λ_{exc} = 340 nm).

result of the phenyl substitution on the parent maleimide ring and this is currently under investigation.

pH sensitivity and photobleaching stability

The pH sensitivity and stability towards photobleaching of **1** were explored as such preconditions are important when selecting a fluorophore for general biological or FRET applications. First, the photobleaching test was performed by monitoring the intensity of the absorption and fluorescence spectra of **1** in DCM irradiated by a broad spectrum Xe source (150 W) for an extended period of time (6–18 hours). This experiment indicated that **1** has a very good photo-stability with a negligible loss of fluorescence (~2%) after 18 hours of illumination (Fig. 3).

Maleimides are known to undergo intersystem crossing leading to formation of an excited triplet state.[5,6] If the energy of the triplet of **1** is estimated to be close to that of the parent maleimide structure, the quantum yield of triplet formation of **1** in CH_3CN is ~0.37 and increases with less polar solvents.[5] The low photobleaching susceptibility of **1**, despite the substantial efficiency of triplet formation, may be an intrinsic property related to the polyfluorination of the aromatic rings. It has been reported that substitution by heavy atoms, such as fluorine in the case of **1** and its derivatives, significantly improves the photobleaching stability of cyanine dyes substituted with sulfonyl groups despite the sulfonyl group's propensity to increase triplet yields.[7]

Due to the low aqueous solubility of **1**, the pH dependence of the fluorescence could not be accurately recorded in aqueous media. Therefore, experiments to determine a potential pH dependence were carried out in organic solvents by analyzing the photophysical properties of **1** under acidic (TFA) and basic (2,6-lutidine) environments. It was observed that the spectral profile of **1** was not influenced by the acidic media (Fig. 4A), while a steady decrease of the fluorescence intensity was observed when **1** was titrated with 2,6-lutidine (Fig. 4B). Rehm–Weller analysis using E_{ox}(lutidine) of 1.89 V (SCE) and E_{red}-(maleimide) = −1.03 V (SCE) and 2.65 V for the energy of the singlet excited state of **1** in DCM (λ_{max} = 468 nm) results in a positive potential ~0.3 V indicating the mechanism of quenching is not electron transfer between **1** and 2,6 lutidine.[8]

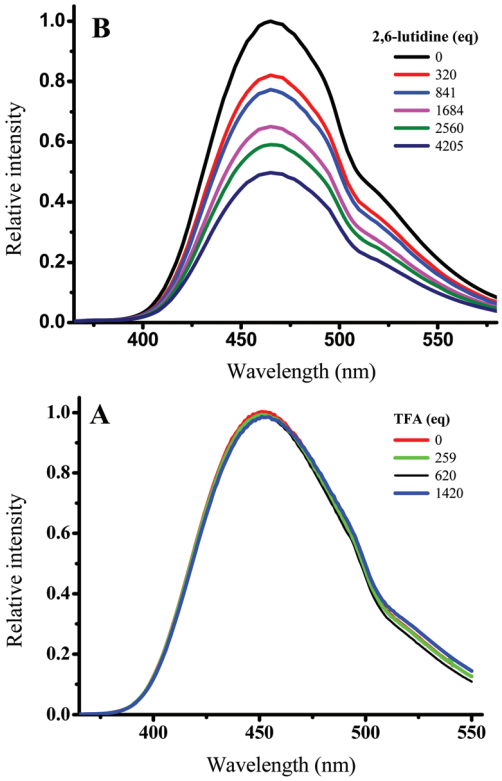


Fig. 4 Fluorescence pH dependence of **1** under (**A**) acidic conditions, TFA (**1** in CH_3CN, 5×10^{-6} M) and (**B**) basic conditions, 2,6-lutidine (**1** in CH_3CN, 5×10^{-6} M), λ_{exc} = 340 nm.

The quenching effect was even more pronounced when a stronger base such as triethylamine was titrated to **1**. The decrease in the fluorescence intensity of **1** at higher pH is attributed to the deprotonation of the bisamido group and the generation of a non-fluorescent monoanion. In addition, the fact that the alkylated analog **1b** displayed higher stability to basic media confirmed the above mechanism of a base-induced reduction in fluorescence.† The fluorescence reduction was found to be reversible upon treatment of the anion with acid.† This suggests that the n → π* transition of the excited singlet state to the ground state primarily involves the lone pair on the nitrogen while also confirming the role of hydrogen bonding in stabilizing the excited state.

FRET system with quencher moiety as an acceptor

Considering the advantageous photophysical properties of DMs **1** and **1a–1c**, we evaluated **1c** as a donor component in a FRET peptide substrate of β-secretase, a key enzyme responsible for Alzheimer's disease.[9] The sequence of the peptide was derived from the β-secretase cleavage site of the Swedish APP mutation (previously reported by Anna Spec). The envisioned FRET peptide contains DM donor moiety and an acceptor (quencher) motif allowing for depopulation of the donor excited state *via* an intramolecular FRET mechanism. The peptide synthesis was performed by using standard Fmoc chemistry on solid phase with subsequent coupling of the donor–acceptor pair.† The fluorophore donor **1c** was appended to the N-terminus, and the

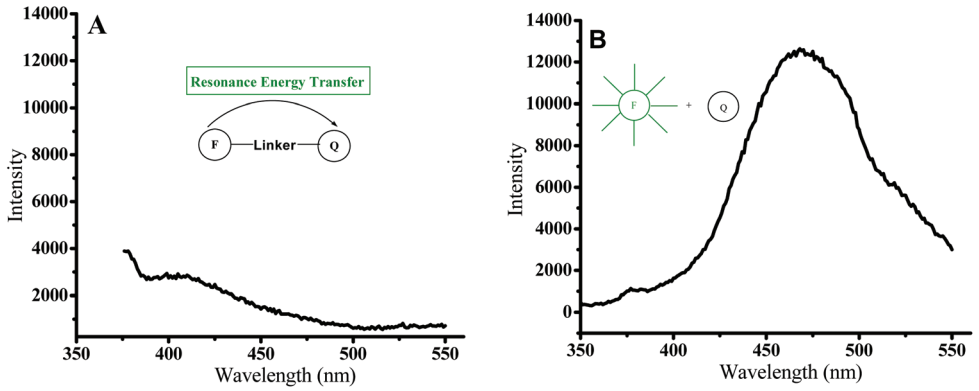


Fig. 5 Emission spectra of **A**. The tethered donor–acceptor FRET peptides and **B**. FRET peptide after enzymatic cleavage, (DMSO 1×10^{-5} M), λ_{exc} = 340 nm.

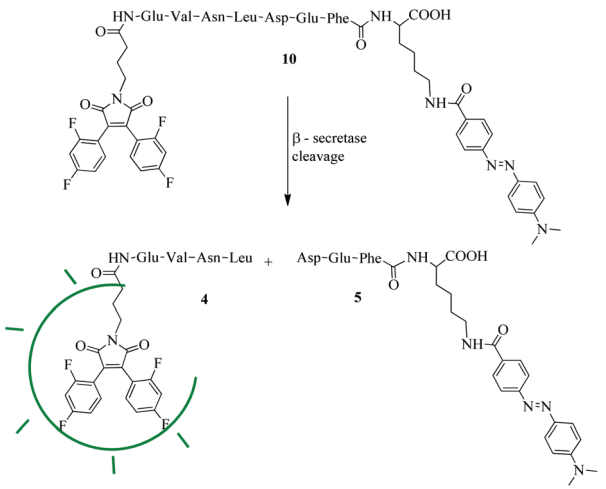


Fig. 6 FRET peptide cleaved by β-secretase.

fluorescence quenching energy acceptor, dimethylaminoazobenzene-2′-carboxylic acid (DABCYL) was attached at the C-terminus (Fig. 6).

The donor emission which displays a maxima centered at λ_{em} 455 nm suitably overlaps with the absorption of the quencher centered at λ_{abs} 440 nm, fulfilling one of the major requirements for an efficient intramolecular FRET system.† The Förster-Radius (R_0) calculated for this FRET system is 36 Å.† The proper quencher selection was confirmed in a preliminary experiment in which the two molecules, **1b** and the acceptor (Dabcyl) were covalently linked through a coupling reaction (compound **9** in the ESI†) and the FRET-based quenching mechanism was observed due to the close proximity between the FRET partners (λ_{ex} 340 nm). The absorption and the emission profile of **9** is presented in the ESI† and clearly indicates that fluorescence quenching occurs.† These results were consistent with the spectral profile obtained from the actual FRET peptide system (Fig. 5A and 5B). The FRET substrate entails a "fluorescence dequenching" motif in which the quenched fluorophore participating in the intramolecular FRET becomes fluorescent once the substrate/acceptor is cleaved by the enzyme, β-secretase. Incubation of the FRET peptide with β-secretase (for 1 hour at room temperature) results in the cleavage of the Leu–Asp bond and the formation of two fragments 4 and 5 that separate the fluorophore from the quencher (Fig. 6).

Therefore, the fluorescence from the DM containing fragment is recovered as the two pairs are no longer in the proximity required for the intramolecular FRET to occur. The emission profile of the FRET system before and after the enzymatic cleavage are presented on Fig. 5A and 5B and clearly indicate a change in the fluorescence signal attributed to the efficient energy transfer between the compatible donor–acceptor partners. Diffusional quenching between the two FRET components in solution was found to be negligible based upon titration experiments in which the quencher was gradually introduced to a solution containing the donor. No significant quenching was observed even at 1.5 equivalents of the quencher.†

A kinetic study of the enzymatic cleavage of the peptide system revealed that the intensity of the restored fluorescence (in RFU) is enhanced in a concentration-dependent fashion with a K_m 0.29 μM and a V_{max} 28.9 μM min^{-1}.† These results confirmed our expectation that the DM derivatives can be successfully incorporated into FRET experiments.

FRET system with bright fluorophore moiety as an acceptor

Encouraged by these results, the application of **1** was expanded by the design of a second FRET system using another chromophore as an acceptor-free-base tetraphenylporphyrin (TPP).[10] This molecule was chosen as a suitable acceptor due to the TPPs high molar extinction coefficient $\varepsilon_{(417)}$ 470 300 M^{-1} cm^{-1} (in THF), sufficient spectral overlap with the emission band of the donor molecule centered at λ_{abs} 450 nm and to the large Stokes shift of the donor. The low energy emission (λ_{max} ~640 nm) of the TPP avoids any undesired overlap between the donor and the acceptor emissions and the primary absorption band of TPP (Soret band ~416 nm) is red shifted relative to the absorption band of **1** which also minimizes the partial absorption of the excitation energy by the porphyrin (primary inner-filter effect). The synthetic pathway to the final FRET complex was done as a result of a spontaneous reaction between **1a** and the pre-activated succinimidyl ester of TPP **8** (Fig. 7).†

When compound **7** was excited at 270 nm or 340 nm, significant fluorescence enhancement typical for the acceptor emission

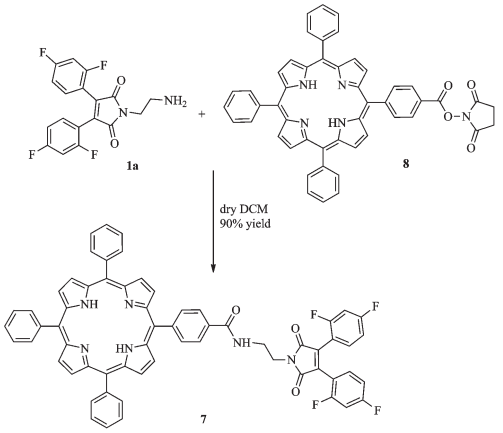


Fig. 7 Synthesis of the FRET paired system.

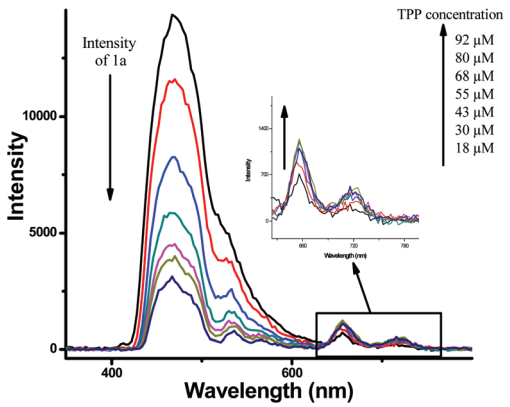


Fig. 8 Diffusional FRET between the non-covalently bound donor–acceptor pair. Emission spectra were obtained by gradual titration of the acceptor into a donor containing sample (λ_{exc} = 340 nm, 2.5 × 10^{-5} M of **1a** in toluene).

was observed (λ_{max} ~640 nm). This is particularly important since in a control experiment it was confirmed that the acceptor unit alone does not emit under these conditions.† The Förster-Radius (R_0) calculated for this FRET system is 26 Å. The FRET efficiency was qualitatively estimated by quantifying the emission ratio change (RRC). This value was measured by taking the product between the intensity of the donor and the acceptor's emissions at their respective maxima for the covalently attached entity **7** and dividing that by the same product for an equimolar sample of the free components in solution (see ref. 11 for description of method). The experimental data revealed that there was a 4.5-fold signal increase in the case of the covalently linked complex† which is in a similar range as SFP/YFP (RRC = 3), Cerulean/YFP (RRC = 2), and BFP/GFP (RRC = 5) FRET pairs.[11] In addition, diffusional FRET was explored in a concentration dependent manner by two parallel titration experiments in which one of the chromophores was kept at a constant concentration, and the other was titrated, monitoring the emission signal after each titration step. In both experiments, regardless of which component was kept at a constant concentration, an increase in the fluorescence intensity of the acceptor emission and a simultaneous decrease in the emission from the donor were observed due to diffusional quenching/FRET (Fig. 8).

Conclusions

In summary, we have presented new fluorophore 3,4-bis(2,4-difluorophenyl)-maleimide **1** and its derivatives that possess favorable fluorescence properties suitable for biological and biomedical applications. In addition we demonstrated that **1** can easily be decorated with functional handles that enhance its conjugation to the biological target of interest, without altering its fluorescent properties. Furthermore, for the first time we demonstrated the implementation of the DM moiety as an acceptor unit in two FRET systems.

Experimental section

General information

All of the reagents and solvents are commercially available and were used as purchased, without further purification. Column chromatography was carried out using Merck Kieselgel 60 H silicagel. ^{1}H NMR, ^{13}C NMR were recorded on a Bruker 250 MHz and Varian 400 MHz NMR spectrometer. All ^{1}H NMR experiments were reported in δ units, parts per million (ppm) downfield of TMS and were measured relative to the signals for chloroform (7.26 ppm) and deuterated methanol (3.35, 4.78 ppm). All ^{13}C NMR spectra were reported in ppm relative to the signals for chloroform (77 ppm) and dimethylsulfoxide (49.3 ppm) with 1H decoupled observation. HRMS were taken on an Agilent G1969A LC/MSD TOF. Fluorescence measurements were conducted using a Photon Counting Spectrophotometer ISS (PC1) and the absorption measurements were recorded on Shimadzu UV-2401-PC. Absorption and fluorescence spectra were recorded in 1 cm path length quartz cuvette. All solvents used for spectroscopy experiments were spectrophotometric grade.

3,4-Bis(2,4-difluorophenyl)-maleimide 1. A suspension of (2,4-difluoro-phenyl)-acetonitrile (153 mg, 1 mmol) and iodine (254 mg, 1 eq) in diethyl ether was reacted with freshly prepared sodium methoxide in methanol solution (113 mg, 2.1 mmol in 10 mL dry methanol) at −85 °C. The reaction mixture was brought to room temperature and after two hours it was treated with 3% HCl until reaching pH 7. The reaction was completed after stirring at room temperature for 24 hours and the crude mixture was washed with water (20 mL), saturated solution of sodium chloride (15 mL), and extracted with diethyl ether (30 mL × 3). After extraction with diethyl ether, the combined organic phases were dried over anhydrous sodium sulfate and concentrated. The product **1** (177 mg, 55%) was obtained by flash chromatography (DCM : methanol = 10 : 2). R_f = 0.55 (DCM : methanol = 9 : 2). ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.44 (s, 1H), 7.50 (td, J = 8.3, 6.4 Hz, 2H), 6.97 (td, J = 8.0, 2.4 Hz, 2H), 6.85–6.77 (m, 2H). ^{13}C NMR (63 MHz, $CDCl_3$) δ 169.49 (s), 166.37–158.64 (m), 134.39 (s), 132.48 (dd, J = 9.9, 4.3), 113.34 (ddd, J = 15.2, 4.0), 112.10 (dd, J = 21.6, 3.6 Hz, 7H), 104.84 (t, J = 25.4 Hz). DEPT 90 ^{13}C NMR (63 MHz, $CDCl_3$) δ 132.37 (dd, J = 10.0, 4.3 Hz), 111.99 (dd, J = 21.6, 3.7 Hz), 104.74 (t, J = 25.4 Hz). HRMS (ESI) calcd for $C_{16}H_7F_4NO_2$ $[M + H]^+$: 322.0412, found: 322.0485.

1-(2-Aminoethyl)-3,4-bis(2,4-difluorophenyl)-1*H*-pyrrole-2,5-dione 1a. The synthesis of (2-bromo-ethyl)-carbamic acid *tert*-butyl ester were performed according to a reported procedure.[1] The suspension of 3,4-bis-(2,4-difluorophenyl)-maleimide **1** (321 mg, 1 mmol) in 10 mL dry DMF was cooled to 0 °C and reacted with (2-bromo-ethyl)-carbamic acid *tert*-butyl ester (336 mg, 1.5 eq) and potassium *tert*-butoxide (2 eq, 225 mg) under inert atmosphere. The reaction was completed after 36 hours and the crude mixture was washed with saturated solution of sodium bicarbonate and extracted with ethyl acetate (3 × 20 mL). After extraction with ethyl acetate the combined organic phases were dried over anhydrous sodium sulfate and concentrated. The protected crude {2-[3,4-bis-(2,4-difluoro-phenyl)-2,5-dioxo-2,5-dihydro-pyrrol-1-yl]-ethyl}-carbamic acid *tert*-butyl ester **2** was not purified but further reacted with 5% TFA solution in DCM and stirred at room temperature for 3 hours in order to remove the Boc group. The crude mixture was washed with water (20 mL) and extracted with DCM (3 × 20 mL). The combined organic phases were dried over anhydrous sodium sulfate and concentrated. The product (323 mg, 89%) was obtained by chromatography (DCM : methanol = 7 : 3). R_f = 0.55 (DCM : methanol = 8 : 2). ^{1}H NMR (400 MHz, CD_3OD) δ 7.57 (td, J = 8.4, 6.4 Hz, 2H), 7.09–7.03 (m, 2H), 7.03–6.95 (m, 2H), 3.99–3.94 (m, 2H), 3.27–3.23 (m, 2H), 1.26 (s, 2H). ^{13}C NMR (63 MHz, $CDCl_3$) δ 169.55 (s), 166.22–158.62 (m), 133.29 (s), 132.47 (dd, J = 9.9, 4.4 Hz), 113.67 (ddd, J = 15.1, 3.9, 2.2 Hz), 111.94 (dd, J = 21.6, 3.6 Hz), 104.70 (t, J = 25.6 Hz), 38.31 (s), 18.03 (s). HRMS (ESI) calcd for $C_{18}H_{12}F_4N_2O_2$ $[M + H]^+$: 365.0835, found: 365.0896.

3,4-Bis(2,4-difluorophenyl)-1-(3-hydroxypropyl)-1*H*-pyrrole-2,5-dione 1b. The suspension of 3,4-bis(2,4-difluorophenyl)-maleimide **1** (321 mg, 1 mmol) in dry 10 mL DMF was cooled to 0 °C and was reacted with potassium *tert*-butoxide (2 eq, 225 mg) and 3-bromopropan-1-ol (1.5 eq, 207 mg) under inert atmosphere. The reaction was completed after 24 hours at room temperature and the crude mixture was washed with saturated solution of sodium bicarbonate (15 mL) and extracted with DCM (3 × 30 mL). After extraction with ethyl acetate the combined organic phases were dried over anhydrous sodium sulfate and concentrated. The desired product **1b** (322 mg, 85%) was obtained by chromatography (DCM : methanol = 10 : 3). R_f = 0.45 (DCM : methanol = 8 : 2). ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.49 (td, J = 8.3, 6.4 Hz, 2H), 6.96 (ddd, J = 8.0, 2.5, 1.3 Hz, 2H), 6.86–6.74 (m, 2H), 3.82 (t, J = 6.4 Hz, 2H), 3.66 (t, J = 5.7 Hz, 2H), 1.93–1.82 (m, 2H). ^{13}C NMR (63 MHz, $CDCl_3$ δ 169.93 (s), 166.22–158.57 (m), 133.39 (s), 132.41 (dd, J = 9.9, 4.3 Hz), 113.33 (ddd, J = 15.2, 4.0), 111.94 (dd, J = 21.6, 3.6 Hz), 104.69 (t, J = 25.4 Hz), 59.33 (s), 35.36 (s), 31.33 (s). HRMS (ESI) calcd for $C_{19}H_{13}F_4NO_3$, $[M + H]^+$: 380.0832, found: 380.0812.

4-(3,4-Bis-(2,4-difluorophenyl)-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)butanoate 3. The suspension of 3,4-bis(2,4-difluorophenyl)-maleimide **1** (321 mg, 1 mmol) in 10 mL dry DMF was cooled to 0 °C and was reacted with potassium *tert*-butoxide (2 eq, 225 mg) and with 4-bromo-butyric acid (293 mg, 1.5 eq) under inert atmosphere. The reaction was stirred for 24 hours at room temperature and the crude mixture was washed with saturated solution of sodium chloride (15 mL) and extracted with ethyl acetate (3 × 30 mL). After extraction with ethyl acetate the combined organic phases were dried over anhydrous sodium sulfate and concentrated. The desired product **3** was (357 mg, 82%) was obtained by chromatography (hexane : ethyl acetate = 6 : 4). R_f = 0.45 (hexane : ethyl acetate = 7 : 3). ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.43 (dd, J = 8.2, 6.4 Hz, 2H), 6.94 (td, J = 8.0, 2.4 Hz, 2H), 6.78–6.65 (m, 2H), 4.14–3.95 (m, 2H), 3.65 (t, J = 6.7 Hz, 2H), 2.31 (t, J = 7.3 Hz, 2H), 2.04–1.86 (m, 2H), 1.15 (dd, J = 10.4, 3.8 Hz, 3H). ^{13}C NMR (63 MHz, $CDCl_3$) δ 172.63 (s), 169.47 (s), 162.40–158.51 (m), 133.29 (s), 132.48 (dd, J = 10.0, 4.2 Hz), 113.62 (ddd, J = 15.0, 3.8, 2.2 Hz), 111.91 (dd, J = 21.6, 3.6 Hz), 104.65 (t, J = 25.5 Hz), 60.59 (s), 38.08 (s), 31.64 (s), 23.87 (s), 14.21 (s). HRMS (ESI) calcd for $C_{22}H_{17}F_4NO_4$, $[M + H]^+$: 436.1094, found: 436.1078.

4-[3,4-Bis-(2,4-difluoro-phenyl)-2,5-dioxo-2,5-dihydro-pyrrol-1-yl]-butyric acid 1c. The suspension of 4-[3,4-bis-(2,4-difluoro-phenyl)-2,5-dioxo-2,5-dihydro-pyrrol-1-yl]-butyric acid ethyl ester **3** (435 mg, 1 mmol) in 15 mL HCl : AcOH = 1 : 1 was refluxed at 90 °C for 20 hours. The crude product was extracted with DCM (20 mL × 3) and the combined organic phases were dried over anhydrous sodium sulfate and concentrated. The desired product **1c** (326 mg, 80%) was obtained by chromatography (DCM : methanol = 7 : 3). R_f = 0.52 (DCM : methanol = 8 : 2). ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.43 (td, J = 8.3, 6.5 Hz, 2H), 6.97 (td, J = 8.0, 2.4 Hz, 2H), 6.80–6.69 (m, 2H), 3.68 (t, J = 6.8 Hz, 2H), 2.39 (t, J = 7.3 Hz, 2H), 1.98 (dd, J = 14.2, 7.2 Hz, 2H). ^{13}C NMR (63 MHz, $CDCl_3$) δ 178.91 (s), 169.60 (s), 167.25–156.95 (m, 2H), 133.38 (s), 132.50 (dd, J = 10.0, 4.1 Hz), 113.58 (ddd, J = 15.3, 3.9, 2.2 Hz), 112.03 (dd, J = 21.6, 3.5 Hz), 104.77 (t, J = 25.6 Hz), 38.01 (s), 31.40 (s), 23.49 (s). HRMS (ESI) calcd for $C_{20}H_{13}F_3NO_4$, $[M + H]^+$: 408.0781, found: 408.0742.

***N*-{2-[3,4-Bis-(2,4-difluoro-phenyl)-2,5-dioxo-2,5-dihydro-pyrrol-1-yl]-ethyl}-4-(10,15,20-triphenyl-porphyrin-5-yl)-benzamide 7.** The synthesis of 4-(10,15,20-triphenyl-porphyrin-5-yl)-benzoic acid 2,5-dioxo-pyrrolidin-1-yl ester **8** was performed by following a reported procedure[2] and the precursor of **8**, 4-(10,15,20-triphenyl-porphyrin-5-yl)-benzoic acid was obtained through hydrolysis following a previously reported procedure.[3] The suspension of 4-(10,15,20-triphenyl-porphyrin-5-yl)-benzoic acid 2,5-dioxo-pyrrolidin-1-yl ester **8** (755 mg, 1 mmol) in 10 mL dry DCM was reacted with 1-(2-amino-ethyl)-3,4-bis-(2,4-difluoro-phenyl)-pyrrole-2,5-dione (**1a**) (364 mg, 1 eq). The reaction mixture was stirred for 24 hours at room temperature and the product formation was monitored by TLC and MALDI-TOF. The desired product **7** (904 mg, 90%) was obtained by chromatography (DCM : methanol = 9 : 1). R_f = 0.50 (100% DCM). MALDI-TOF, calculated for: 1006.309, found: 1006.310. ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.89 (d, J = 5.5 Hz, 5H), 8.81 (d, J = 4.8 Hz, 2H), 8.46 (d, J = 8.2 Hz, 2H), 8.33 (d, J = 8.1 Hz, 2H), 8.29–8.18 (m, 6H), 7.77 (td, J = 4.7, 2.3 Hz, 9H), 7.51 (td, J = 8.3, 6.5 Hz, 2H), 6.99 (td, J = 8.2, 2.1 Hz, 2H), 6.82 (ddd, J = 11.0, 8.9, 2.4 Hz, 2H), 3.78 (t, 2H), 2.46 (t, J = 7.3 Hz, 2H), −2.73 (s, 1H). ^{13}C NMR (63 MHz, $CDCl_3$) δ 169.99, 169.24, 167.47–158.54 (m), 147.16 (s), 142.12 (s),

134.64 (s), 133.40 (s) 132.44 (dd, J = 11.0, 5.1 Hz), 129.62 (s), 129.55 (s) 128.05 (s), 128.87 (s) 126.81 (s), 120.70 (s), 120.51 (s), 118.68 (s), 113.55 (m), 112.01 (dd, J = 21.6, 3.6 Hz), 104.77 (t, J = 25.6 Hz), 52.52 (s,), 31.33 (s). MALDI-TOF, calculated 1004.31, found 1005.33.

4-(4-Dimethylamino-phenylazo)-benzoic acid 3-[3,4-bis-(2,4-difluoro-phenyl)-2,5-dioxo-2,5-dihydro-pyrrol-1-yl]-propyl ester 9. A suspension of **1b**, 3,4-bis-(2,4-difluoro-phenyl)-1-prop-2-ynyl-pyrrole-2,5-dione, (379 mg, 1 mmol) in dry 10 mL DCM was reacted with 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (287 mg, 1.5 eq), 4-dimethylaminopyridine (61 mg, 0.5 eq) and 4-(4-dimethylamino-phenylazo)-benzoic acid (DABCYL) (269 mg, 1 eq). The reaction mixture was stirred for 24 hours at room temperature and the product formation was monitored by TLC and LC/MS. The cruder material was washed with water and extracted with DCM (20 mL × 3). The combined organic phases were dried over anhydrous sodium sulfate and concentrated. The desired product **9** (560 mg, 80%) was obtained by chromatography (DCM : methanol = 8 : 2). R_f = 0.52 (DCM : methanol = 8 : 2). ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.13 (d, J = 8.6 Hz, 2H), 7.86 (dd, J = 18.7, 8.8 Hz, 4H), 7.44 (td, J = 8.2, 6.5 Hz, 2H), 6.75 (td, J = 8.0, 2.4 Hz, 2H), 6.77 (ddd, J = 9.2, 3.5, 1.8 Hz, 4H), 4.43 (t, J = 5.9 Hz, 2H), 3.90 (t, J = 6.7 Hz, 2H), 2.30–2.14 (m, 2H). ^{13}C NMR (63 MHz, $CDCl_3$) δ 169.47 (s), 166.53 (s), 162.46–156.14 (m), 153.02 (s), 143.81 (s), 133.40 (s), 132.49 (dd, J = 10.0, 4.1 Hz), 130.62 (s), 130.06 (s), 125.63 (s), 122.14 (s), 113.77 (ddd, J = 15.1, 3.9, 2.2 Hz), 112.19 (dd, J = 21.6, 3.6 Hz), 104.74 (t, J = 25.6 Hz), 104.34 (s), 62.70 (s), 40.38 (s), 36.29 (s), 27.81. HRMS (ESI) calcd for $C_{34}H_{26}F_4N_4O_4$, $[M + H]^+$: 631.1890, found: 631.1855.

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