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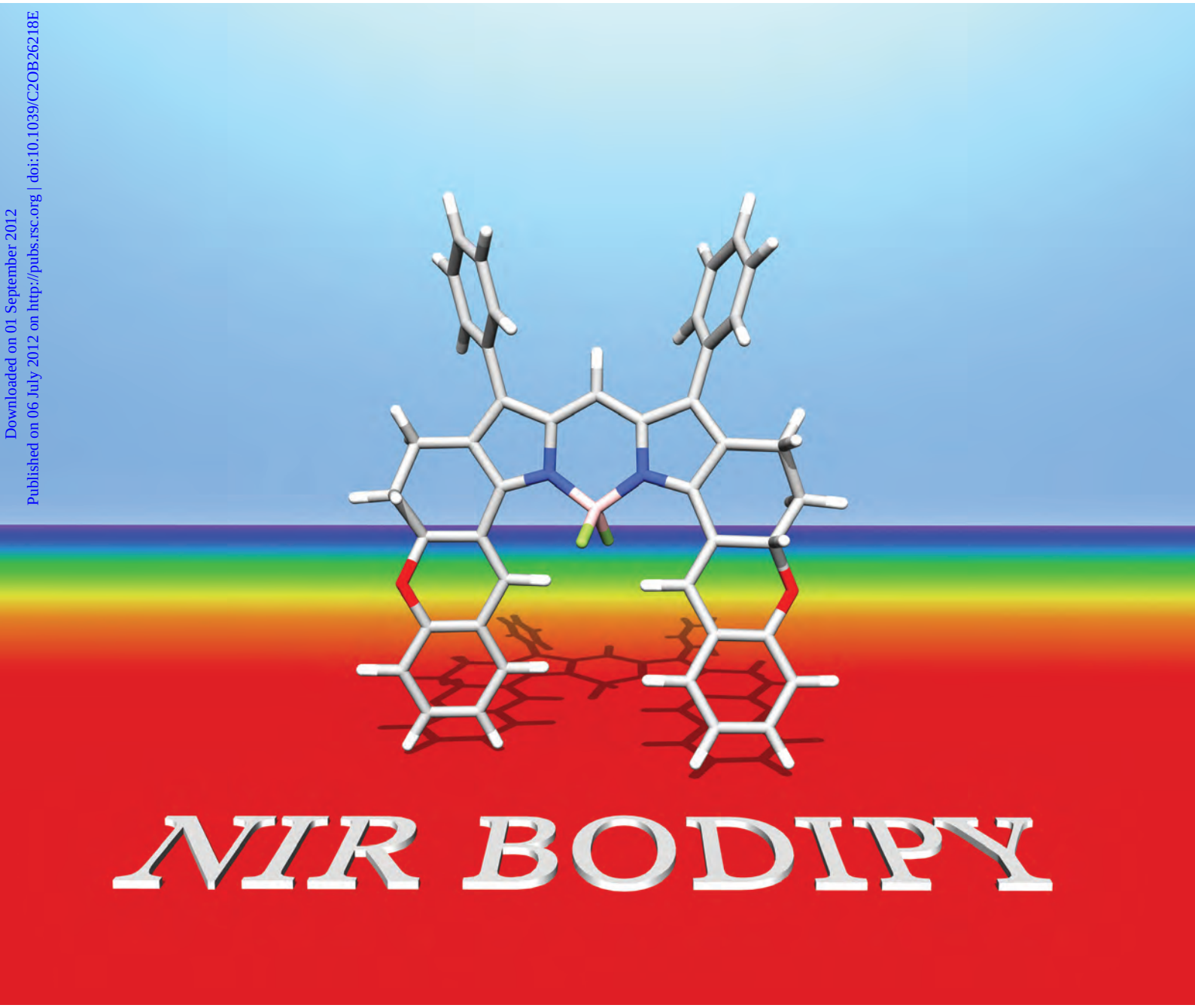

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FULL PAPER

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PAPER

A NIR BODIPY dye bearing 3,4,4*a*-trihydroxanthene moieties†

Xin-Dong Jiang,*[a] Ruina Gao,[a] Yi Yue,[a] Guo-Tao Sun[b] and Weili Zhao*[a,c]

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A novel 3,4,4*a*-trihydroxanthene-fused pyrrole **2** was synthesized by the reaction of 2,3,4,4*a*-tetrahydro-1*H*-xanthen-1-one with 3-phenyl-2*H*-azirine in the presence of LDA. Utilizing this pyrrole **2**, a NIR BODIPY **1** (λ_{abs} = 732 nm, λ_{em} = 747 nm) has been prepared. The new BODIPY **1** was stable, non-cytotoxic, and suited to labeling living cells for imaging assay in the NIR region.

Introduction

Near-infrared (NIR) fluorescent dyes can greatly reduce background absorption, fluorescence and light scattering, and improve the sensitivity and selectivity of fluorescent probes and sensors.[1] More importantly, NIR fluorescent dyes have deep penetration in tissues to allow *in vivo* imaging and photodynamic therapy (PDT).[2] The safe, non-invasive nature of NIR fluorescent imaging has attracted great interest in the design and synthesis of novel NIR fluorescent dyes.[3] NIR dyes such as azo dyes,[3a] cyanine dyes,[3b] quinone dyes,[3c,d] and phthalocyanine dyes[3e] have been developed so far; however, insufficient photo-stability and/or difficult modification obstructs their application. Most NIR fluorescent dyes possess highly conjugated systems which lead to significantly reduced fluorescence due to increased internal conversion.[3] BODIPY dyes are well-known to be highly fluorescent, very stable, and exceptionally insensitive to the polarity of solvents as well as to pH.[4] To develop NIR responsive BODIPY dyes, recent developments focused on the extension of π-conjugation, or rigidification of rotatable moieties in pyrroles (Fig. 1, **A**–**F**).[5,6] For BODIPY dyes, to extend the absorption/emission of the fluorescent dyes over 700 nm requires judicious design. Our previous work on rigidified pyrrole (**F**) derived restricted aza-BODIPYs paved the way to discover NIR fluorescent dyes absorbing and emitting over 700 nm.[7] Herein, we utilized the combination of extended conjugation and rigidification strategies and discovered a novel NIR BODIPY dye **1** (λ_{abs} = 732 nm) derived from 3,4,4*a*-trihydroxanthene-fused pyrrole **2** (Fig. 1).

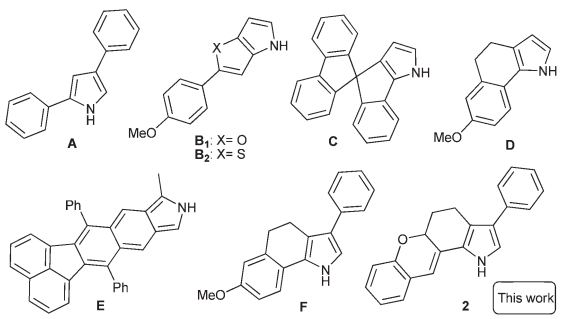

Fig. 1 Pyrrole skeletons used for NIR BODIPY (aza-BODIPY) dyes.

Results and discussion

Synthesis of the NIR compound 1

For the preparation of the novel 3,4,4*a*-trihydroxanthene-fused pyrrole **2**, 2,3,4,4*a*-tetrahydro-1*H*-xanthen-1-one[8] was employed to react with 3-phenyl-2*H*-azirine to give **2** in 14% yield in the presence of LDA (Scheme 1).[7] BODIPY **1** was successfully synthesized from **2** in 80% combined yield by condensation with triethyl orthoformate in the presence of 0.5 equiv. of TsOH, followed by complexation with $BF_3{\cdot}Et_2O$–Et_3N.[9]

Pale green crystals of **2** suitable for X-ray crystallography were obtained by recrystallization from *n*-hexane–CH_2Cl_2 (Fig. 2).[10] Due to the torsional rigidity, the angles of H12–C13–O1, H12–C13–C12, and H12–C13–C14 are all 107.1° which distorts from the ideal value of 109.2° in an sp^3 hybridized structure. Moreover, the dihedral angle of the C3–C11–C12–C13 restricted bond was 43.4°.

Photophysical properties of 1

To our delight, the combination of extended conjugation and rigidification resulted in the NIR absorption and emission of dye **1**. The absorption and emission spectra of **1** in $CHCl_3$ (λ_{abs} = 732 nm; λ_{em} = 747 nm) are illustrated in Fig. 3. Dye **1** exhibited

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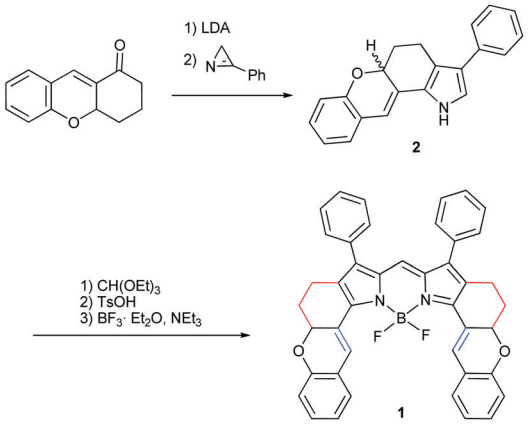
† Electronic supplementary information (ESI) available: HRMS-MALDI spectra, ^1H and ^{13}C NMR spectra, and crystallographic data. CCDC 878978 for **2**. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c2ob26218e



Scheme 1 Synthetic route to the novel pyrrole **2** and BODIPY **1**.

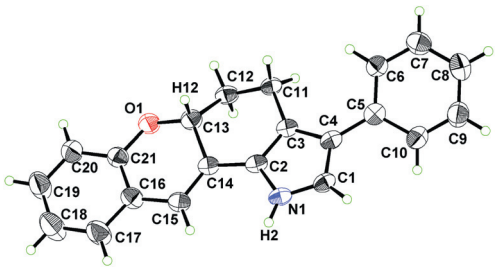


Fig. 2 Molecular structure and conformation for **2**. Carbon, nitrogen and oxygen atoms are depicted with thermal ellipsoids set at the 30% probability level. Selected bond lengths (Å) and angles (°) for *R*-**2**: C3–C11, 1.513(5); C11–C12, 1.480(6); C12–C13, 1.501(6); C13–O1, 1.438(5); O1–C21, 1.363(5); C3–C11–C12, 112.0(3); C11–C12–C13, 114.0(4); C12–C13–O1, 109.0(4); C13–O1–C21, 119.4(3).

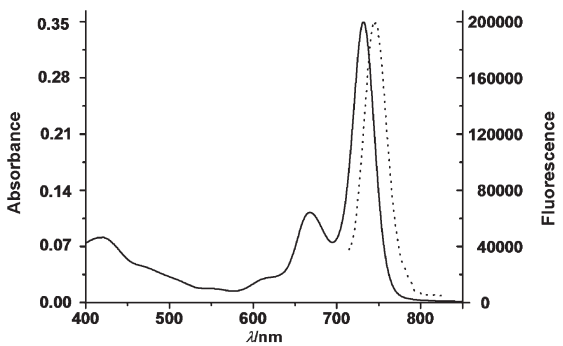


Fig. 3 Absorption (solid curve) and emission (dashed curve) spectra of **1** in $CHCl_3$ at 298 K.

moderate extinction coefficients (29 000 M^{-1} cm^{-1}) and a narrow full width at half maximum (32 nm).

The spectral characteristics of **1** were compared to those of the known dyes **3**–**5** (Table 1).[11] In comparison with the absorption maximum of **3** (λ_{abs} = 564 nm), merely rigidification (*cf.* **4**) or extended conjugation (*cf.* **5**) leads to about 70 nm of bathochromic shift. However, the combined effect of restricting the phenyl substituent and extending the π-conjugation in **1** is dramatic wherein 168 nm of bathochromic shift was accomplished in comparison with **3**. The fluorescence quantum yield (Φ_f) of the NIR BODIPY **1** was measured to be 0.06, which was bright enough for the staining experiments.

Stability and toxicity of 1

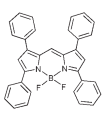
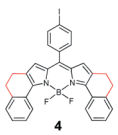
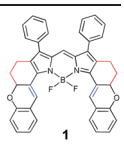
Stability and toxicity of a probe is very important for biological labeling. The stability measurements of BODIPY **1** in acidic and basic solutions[12] were carried out by monitoring the transmission at absorption maxima (Fig. 4). Dye **1** was found to be reasonably stable in a range of pH from 2 to 14 by monitoring the change of transmission in a solution of dye **1** in H_2O–DMSO (95 : 5, v/v) after 2 h. The light stability of **1** was evaluated by monitoring the intensity changes of the emission with time of irradiation in comparison with a typical visible fluorescent BODIPY dye, namely 1,3,5,7-tetramethyl-4-difluorobora-3*a*,4*a*-diaza-*s*-indacene (TM-BDP) (Fig. 5). As expected, the NIR BODIPY **1** was found to be slightly inferior to the visible TM-BDP.

As for the toxicity studies, MTT assays carried out on HepG2 cells indicated the non-cytotoxic nature of **1**, which is quite similar to that of TM-BDP (Fig. 6).

Cell-staining experiment of 1

For the cell-staining experiment, 2 × 10^{-5} M of dye **1** in PBS containing 0.5% (v/v) DMSO was incubated with HepG2 cells for 20 min at 37 °C and the excess dye was removed by washing with PBS prior to visualization. An ArrayScan® VTI HCS Reader inverted fluorescence microscope was employed for the

Table 1 Effect of extended conjugation and rigidification on photophysical properties

Dye	 3	 4	 5	 1
λ_{abs}(nm)	564	634	630	732
λ_{em}(nm)	593	647	639	747

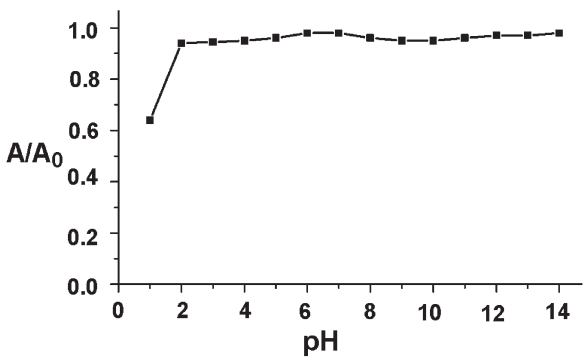


Fig. 4 The normalized transmission (A/A_0) of **1** in response to a pH range from 1–14 in H_2O–DMSO (95 : 5, v/v) after 2 h at room temperature.

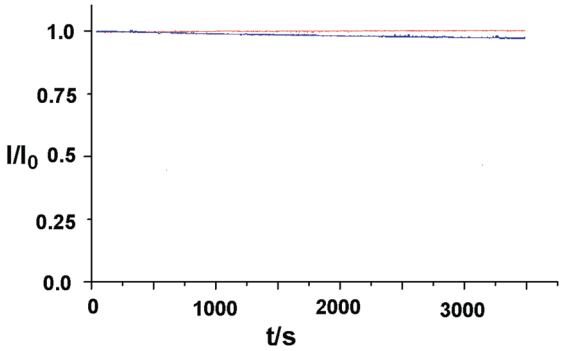


Fig. 5 Intensity variations of TM-DBP (red) and **1** (blue) under continuous irradiation with light (470 and 700 nm, respectively) in toluene.

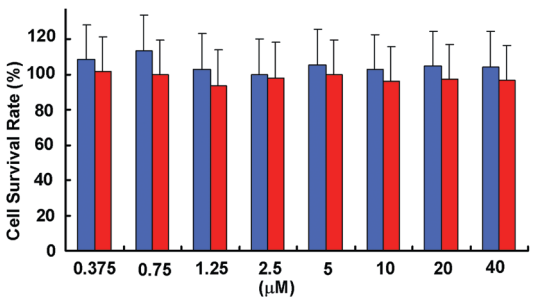


Fig. 6 Comparison of the cytotoxic effects of TM-BDP (blue) and **1** (red) on HepG2 cells.

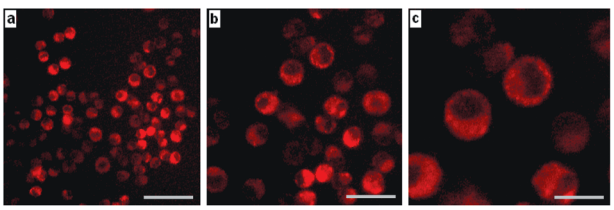


Fig. 7 Fluorescence microscope imaging of HepG2 cells following incubation with 2×10^{-5} M of **1** in PBS + 0.5% (v/v) DMSO (red color) for 20 min at 37 °C; dark area represents the cell nuclei. Scale bar: (a) 40 μm; (b) 20 μm; (c) 10 μm.

fluorescence image, with the excitation wavelength of the laser at 700 nm. The obtained images indicated that **1** was efficiently internalised by living cells. Dye **1** was membrane-permeable and was found to localize exclusively to the cytoplasm (red color) with nuclei intact (dark area) (Fig. 7).

Conclusions

A novel 3,4,4*a*-trihydroxanthene-fused pyrrole **2** was prepared. The structure of **2** was confirmed by single crystal X-ray analysis. BODIPY **1** (λ_{abs} = 732 nm, λ_{em} = 747 nm, Φ_f = 0.06 in $CHCl_3$) prepared from pyrrole **2** absorbed and emitted in the NIR region as a result of combined extension of conjugation and restriction. Dye **1** was fairly stable, non-cytotoxic, and suitable for labeling of living cells in the NIR region. Efforts are currently under way to develop nonsymmetrically substituted, water-soluble BODIPY/aza-BODIPY to allow conjugation for biosensing experiments.

Experimental

General

^{1}H NMR spectra were recorded on a VARIAN Mercury 300 MHz spectrometer. ^{1}H NMR chemical shifts (δ) are given in ppm downfield from Me_4Si, determined by residual chloroform (δ = 7.26 ppm). ^{13}C NMR spectra were recorded on a VARIAN Mercury 75 MHz spectrometer in $CDCl_3$, all signals are reported in ppm with the internal chloroform signal at δ 77.0 ppm as standard. Infrared spectra were recorded on an AVA TAR 360 using KBr pellets. Mass spectrometric measurements were performed by the mass spectrometry service of the ETHZ on a Bruker Reflex MALDI as matrix (20 kV).

Fluorescence spectra were recorded on FluoroSENS spectrophotometer and are reported as cm^{-1}. UV–vis spectra were recorded on Perkin-Elmer Lambda 35 UV–vis spectrophotometer at room temperature.

The fluorescence quantum yields (Φ_f) of the BODIPY systems were calculated using the following relationship (eqn (1)):

$$\Phi_f = \Phi_{ref}\, F_{sampl}\, A_{ref}\, n^2{}_{sampl} / F_{ref}\, A_{sampl}\, n^2{}_{ref} \qquad (1)$$

Here, F denotes the integral of the corrected fluorescence spectrum, A is the absorbance at the excitation wavelength, and n is the refractive index of the medium, ref and sampl denote parameters from the reference and unknown experimental samples, respectively. The reference system used was boronazadipyrromethene compound aza-BODIPY (Φ_f = 0.36 in chloroform).[6d]

The pH-dependence experiments were carried out by monitoring the change of absorbance at 732 nm of 5 μM of the dye **1** in DMSO–H_2O (95 : 5, v/v) in the pH range from 1–14 after 2 h at room temperature.

The photostability experiments were carried out using a 5 μM solution of the dye (TM-BDP or **1**) in toluene. After initial 10 min irradiation, the fluorescence change was recorded for 1 h at 540 or 750 nm under continuous excitation at 470 or 700 nm, respectively. The slit width was 8.0 nm for excitation, and 1 nm for emission.

Preparation of 3-phenyl-1,4,5,5*a*-tetrahydrochromeno[2,3-*g*]-indole 2

Under N_2, LDA (4.78 mmol) in THF (5 mL) was added to 2,3,4,4*a*-tetrahydro-1*H*-xanthen-1-one (0.902 g, 4.50 mmol) in THF (20 mL) at −78 °C. Then, 3-phenyl-2*H*-azirine (0.746 g, 0.636 mmol) in THF (2 mL) was added and the resulting mixture was stirred for 2 h at the same temperature. The reaction was allowed to warm up to room temperature slowly. It was quenched with water, neutralized with dilute HCl to a pH about 7. The mixture was extracted with CH_2Cl_2 (2 × 60 mL), and the organic layer was washed with brine (2 × 50 mL) and dried over anhydrous $MgSO_4$. After removal of the solvents by evaporation,

the resulting crude mixture was separated by column chromatography (*n*-hexane–CH_2Cl_2 = 1 : 1) to afford **2** as a pale green solid (190.6 mg, 0.636 mmol, 14%). Pale green crystals of **2** suitable for X-ray analysis were obtained by recrystallization from *n*-hexane–CH_2Cl_2. M.p.: 103–104 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (TMS, ppm): 8.27 (s, 1H), 7.44 (d, 2H, ^3J = 8.0 Hz), 7.37 (t, 2H, ^3J = 8.0 Hz), 7.24 (t, 2H, ^3J = 8.0 Hz), 7.07 (td, 1H, ^3J = 8.0 Hz, ^4J = 1.2 Hz), 7.02 (dd, 1H, ^3J = 8.0 Hz, ^4J = 1.2 Hz), 7.01 (s, 1H), 6.89 (td, 1H, ^3J = 8.0 Hz, ^4J = 1.2 Hz), 6.85 (d, 1H, ^3J = 8.0 Hz), 5.14–5.10 (m, 1H), 2.99–2.89(m, 2H), 2.55–2.51 (m, 1H), 2.22–2.11 (m, 1H). ^{13}C NMR (75 MHz, CDCl3), δ (TMS, ppm): 153.5, 135.2, 128.6, 127.6, 127.0, 126.3, 126.2, 125.9, 125.8, 124.3, 124.1, 121.7, 120.9, 118.4, 115.6, 108.2, 30.8, 21.0. IR (KBr) cm^{-1}: 2930, 2360, 2340, 1640, 1470, 1450, 1116, 1010, 760. HRMS-MALDI (*m*/*z*): $[M]^+$ Calcd For $C_{21}H_{17}NO$: 299.1305; found 299.1305.

Preparation of BODIPY dye 1

Under N_2, a solution of pyrrole **2** (46.1 mg, 0.153 mmol) and toluene-4-sulfonic acid (13.5 mg, 0.078 mmol) in triethyl orthoformate (2 mL) was stirred for 15 min at room temperature. The solution was diluted with ethyl acetate (10 mL), filtered, and the precipitated solid was washed with ethyl acetate and *n*-hexane. After removing the solvents by evaporation, the crude compound was obtained. Then, triethylamine (40 mg, 0.39 mmol) was added to this crude in $BF_3{\cdot}OEt_2$ (3 mL) and the reaction mixture was stirred for 15 min at room temperature. The mixture was extracted with CH_2Cl_2 (2 × 80 mL), and the organic layer was washed with brine (2 × 60 mL), then dried over anhydrous $MgSO_4$. The solvents evaporated to afford a dark green solid **1** (40.2 mg, 0.0612 mmol, 80%). M.p.: 270–271 °C (decomp.). λ_{abs} = 732 nm, λ_{em} = 747 nm of **1** in $CHCl_3$. The log *P* value of **1** is 4.110, when the solvent is DMSO. ^{1}H NMR (400 MHz, $CDCl_3$) δ (TMS, ppm): 8.26 (s, 1H), 7.54–7.27 (m, 14H), 7.25–7.20 (dd, 2H, ^3J = 8.0 Hz, ^4J = 1.6 Hz), 7.02 (td, 2H, ^3J = 8.0 Hz, ^4J = 1.6 Hz), 6.88 (d, 2H, ^3J = 8.0 Hz), 5.17–5.12 (m, 2H), 2.89–2.68 (m, 4H), 2.64–2.48 (m, 4H). Compound **1** was too insoluble to record a ^{13}C NMR spectrum. IR (KBr) cm^{-1}: 3400, 2930, 1590, 1525, 1407, 1226, 1205, 1116, 1072, 1045, 756. HRMS-MALDI (*m*/*z*): $[M]^+$ calcd for $C_{43}H_{31}BF_2N_2O_2$: 656.2441; found 656.2447.

In vitro cytotoxicity studies

HepG2 cells were used to compare the cytotoxicity/biocompatibility of the dye (**1** or TM-BDP) *via* a MTT test. HepG2 cells were incubated in DMEM with 10% (v/v) fetal bovine serum, 1% penicillin/streptomycin at 37 °C in 5% CO_2. HepG2 cells were seeded at a density of 5 × 10^4 cells per well (200 μL) in a 96-well plate. After 24 h, different concentrations including 0.375, 0.75, 1.25, 2.5, 5, 10, 20, 40 μM of the dye (**1** or TM-BDP) were added into the wells as 200 μL quantities (*n* = 8), respectively. Blank wells contained 200 μL of medium with the equivalent quantity of DMSO. After 24 h, a MTT solution (1 mg mL^{-1}, 20 μL) was added into each well and incubated for 4 h at 37 °C in 5% CO_2. Media was removed and the formazan was dissolved in 150 μL of DMSO. OD was measured at 570 nm using a microplate reader. The cytotoxicity of samples was investigated by relative cell survival rates (%). The absorbance of the blank wells was considered as that corresponding to 100% survival and the relative cell survival rates in the treated wells were calculated. The relative percent survival rates were statistically compared using the Student's *t*-test.

Labeling of living cells for imaging assay

ArrayScan® VTI HCS Reader inverted fluorescence microscope was used for the fluorescence images. The excitation wavelength of the laser was 700 nm. BODIPY **1** (2 × 10^{-5} M, 20 μL) was added to HepG2 cells in a six-compartment cell culture plate that contained 2.0 mL culture medium, and was incubated at 37 °C for 20 min. After removing the culture medium and washing with PBS twice, the fluorescence images of cells were taken, which confirmed the cell-membrane permeability of BODIPY **1**. Bright-field images in the cytoplasm confirmed the viability of the cells during the cell-staining experiment (Fig. 7).

X-ray crystal structure determination of compound 2

Crystals suitable for the X-ray structural determination were mounted on a Mac Science DIP2030 imaging plate diffractometer and irradiated with graphite monochromated Mo-Kα radiation (λ = 0.71073 Å) for the data collection. The unit cell parameters were determined by separately autoindexing several images in each data set using the DENZO program (MAC Science).[13] For each data set, the rotation images were collected in 3° increments with a total rotation of 180° about the ϕ axis. The data were processed using SCALEPACK. The structures were solved by a direct method with the SHELX-97 program.[14] Refinement on F^2 was carried out using the full-matrix least-squares by the SHELX-97 program.[14] All non-hydrogen atoms were refined using the anisotropic thermal parameters. The hydrogen atoms were included in the refinement along with the isotropic thermal parameters.

CCDC reference number 878978 for **2** contains the supplementary crystallographic data.

Acknowledgements

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10 Crystal data for **2**. $C_{21}H_{17}NO$, $M = 299.36$, orthorhombic, $a = 7.6581(1)$, $b = 8.0433(2)$, $c = 50.1404(7)$ Å, $\alpha = 90$, $\beta = 90$, $\gamma = 90°$, $V = 3088.47(10)$ Å^3, $T = 298(2)$ K, space group *Pbca*, $Z = 8$, Data/restraints/parameters = 2728/0/208, $R_1 = 0.0937$ ($I > 2\sigma(I)$), wR_2 (all data) = 0.2160, GOF = 1.152. CCDC reference number 878978 for **2**.

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