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Water-Soluble Red-Emitting Distyryl-Borondipyrromethene (BODIPY) Dyes for Biolabeling

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Abstract: A series of water-soluble red-emitting distyryl-borondipyrromethene (BODIPY) dyes were designed and synthesized by using three complementary approaches aimed at introducing water-solubilizing groups on opposite faces of the fluorescent core to reduce or completely suppress self-aggregation. An additional carboxylic acid functional group was introduced at the pseudo-*meso* position of the BODIPY scaffold for conjugation to amine-containing biomolecules/biopolymers. The optical properties of these dyes were evaluated under simulated physiological conditions (i.e., phosphate-buffered saline (PBS), pH 7.5) or in pure water. The emission wavelength (λ_{max}) of these labels was found in the 640–660 nm range with quantum yields from modest to unprecedentedly high values (4 to 38%). The bioconjugation of these distyryl-BODIPY dyes with bovine serum albumin (BSA) and the monoclonal antibody (mAb) 12A5 was successfully performed under mild aqueous conditions.

Keywords: biolabeling · BODIPYs · fluorescence · sulfonated linker · water soluble

Introduction

The red to near-IR absorbing window of biological tissues is typically in the 600–900 nm range. In this optical window, the scattering and absorption of light by biological samples and the background signal from cellular autofluorescence (300–550 nm) are dramatically reduced.[1] Thus, red-absorbing and emitting fluorophores tend to offer the best resolution and greatest contrast in biological labeling experiments and the detection of various (bio)analytes either in vitro or in vivo. In this context, the engineering and study of red-emitting dyes are of great interest and applicability.[2] Several criteria, such as high fluorescence quantum yields (ϕ) under physiological conditions, high absorption coefficients (ε), relatively long lifetimes (1–5 ns), high photostability, good water-solubility, and the presence of an additional functional group for bioconjugation, have, however, to be met.[3] This is a formidable challenge and only a limited number of such labels belonging to the family of cyanine or rhodamine dyes are currently commercially available.[4,5,6] Recently, a new class of water-soluble red-emitting carbopyronines displaying very attractive spectroscopic features has also been studied.[6d]

Unlike that of such fluorescent markers, the synthesis of water-soluble and bioconjugable red-emitting borondipyrromethene dye (BODIPY) derivatives has been little studied, despite their promising photophysical properties.[7] In 2006, Atilgan and co-workers reported the first synthesis of a water-soluble NIR fluorescent BODIPY dye by introduction of several PEG-type linkers both onto the 8-*meso*-phenyl and 3,5-distyryl moieties of the BODIPY core.[8] Further functionalization with a dipicolylamine-derived binding unit provided a sensitive and selective ratiometric chemosensor for Zn^{II} ions.[9] However, the lack of an additional functional group for bioconjugation excluded the use of such water-soluble dyes as biolabeling reagents. More recently, anionic and cationic substituted BF_2-chelated tetraarylazadipyrromethene derivatives (aza-BODIPY), bearing sulfonic acid, carboxylic acid, or quaternary amine moieties have been synthesized.[10] However, the location of two water-solubilizing groups on the same side of the aza-BODIPY scaffold led to amphiphile-like species fluorescent only in aqueous solutions containing additives that disrupt aggregates.

As an alternative, we developed two synthetic strategies to introduce ionizable sulfonate groups onto BODIPY scaffolds and thereby improve their solubility in water and aqueous buffers. The first protocol relied on the introduc-

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tion of polysulfonated pseudopeptidyl linkers derived from α-sulfo-β-alanine onto the pseudo *meso*-position (functionalized with a carboxylic acid) by a post-synthetic Schotten–Baumann amidification reaction.[11,12] The second approach was based on the formation of polar zwitterions (sulfobetaine moieties) by quaternization of bis(dimethylpropargylamine) BODIPY derivatives with 1,3-propanesultone.[13] Both methods could be efficiently applied to green-emitting BODIPY derivatives, causing them to become highly soluble in water without affecting their photophysical properties. However, our first attempts to introduce (α-sulfo-β-alanine)-type linkers onto the pseudo *meso*-8-position of a red-emitting distyryl-BODIPY scaffold led to fluorescence-quenching of the resulting water-soluble dyes in aqueous buffers due to the formation of aggregates.[14]

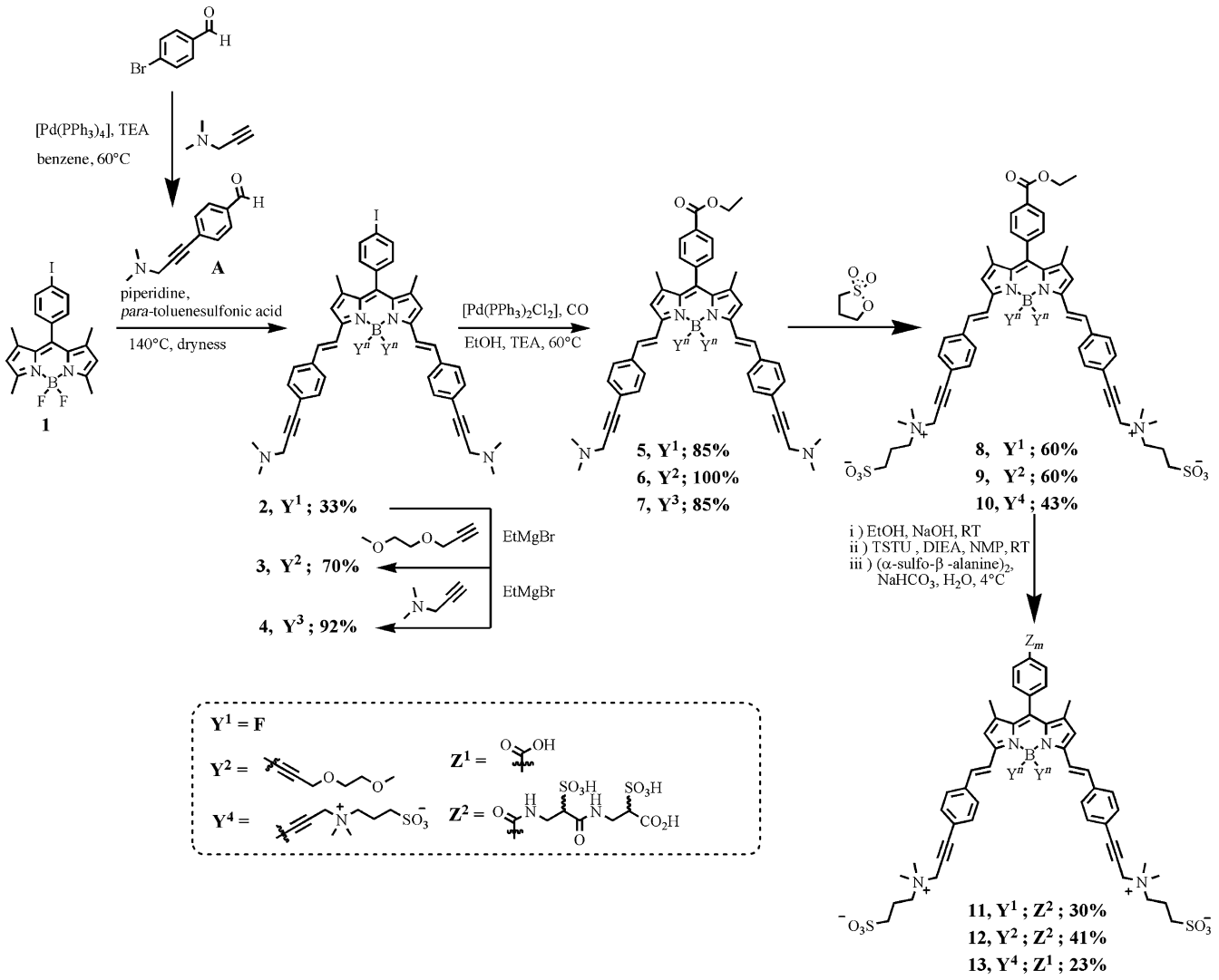
Herein, we wish to report an alternative and more sophisticated synthetic method to produce series of water-soluble BODIPY derivatives that retain their strong red emission in aqueous environments and are suitable for biolabeling applications. The basic concept was the introduction of several water-solubilizing groups on opposite faces of the distyryl-BODIPY scaffold through three complementary synthetic approaches.[15] The first involves the formation of the sulfobetaine moieties through quaternization of dimethylamino groups previously introduced on the styryl arms. The second approach consists of the substitution of the fluorine atoms of BODIPY with the appropriate ethynyl Grignard reagent. In this approach, short PEG-type linkers or sulfobetaine moieties are introduced onto the boron atom as the water-solubilizing groups. Such functionalization of the distyryl-BODIPY involves not only improving the dye's water-solubility, but introduces an additional functional group for conjugation to biomolecules. Thus, an ethyl ester group was introduced by a Pd-catalyzed carboalkoxylation reaction with an aromatic halide group in the pseudo *meso*-position of the BODIPY dye, and subsequent saponification gave the carboxylic acid derivative. The carboxylic acid can be converted into the corresponding active *N*-hydroxysuccinimidyl (NHS) ester for bioconjugation to amine-containing biomolecules/biopolymers.[3] Interestingly, this NHS ester derivative also offers a third anchoring point for a polysulfonated pseudopeptidyl linker designed to improve the dye's water-solubility and resistance to self-aggregation. In addition to the practical implementation of these different water-solubilizing methodologies, the application of these new dyes in biolabeling has been demonstrated through direct conjugation to a model protein bovine serum albumin (BSA) and a monoclonal antibody (12A5).

Abstract in French: *De nouveaux colorants fluorescents à architecture distyrylborondipyrromethene (BODIPY), émettant dans le rouge et solubles dans l'eau, ont été préparés en utilisant trois méthodes efficaces de fonctionnalisation chimique qui permettent l'introduction de groupements hydrophiles (motifs pseudo-PEG et sulfonate) sur les parties nord et sud de leur squelette conjugué, et ce afin de réduire ou de supprimer totalement l'agrégation en solution aqueuse. La solubilité dans l'eau des fluorophores ainsi obtenus est élevée et leurs propriétés optiques en milieu physiologique remarquables et comparables à celles déterminées dans les solvants organiques pour les composés hydrophobes parents. De plus, la présence d'une fonction acide carboxylique en position* méso *de ces composés distyryl-BODIPY a permis le marquage covalent de protéines d'intérêt (albumine de sérum bovin et anticorps monoclonaux 12A5) via la formation de liens amide dans des conditions biocompatibles.*

Results and Discussion

Synthesis of water-soluble distyryl-BODIPY dyes: First, the benzaldehyde derivative **A** was prepared in quantitative yield from 4-bromobenzaldehyde and dimethylaminopropyne by using a Pd^0-promoted cross-coupling reaction under mild conditions (Scheme 1). Then, a Knoevenagel condensation reaction between iodophenyl tetramethyl-BODIPY[16] **1** and 4-[3-(dimethylamino)-1-propyn-1-yl]benzaldehyde (**A**) in a mixture of dry toluene and piperidine gave the key blue distyryl derivative **2** in 33% yield after careful column chromatography. Then, the fluorine atoms on the boron center were substituted by reaction with the Grignard reagent of an ethyleneglycol (EG) chain or dimethylamino propyne, leading to the corresponding compounds **3** and **4** in yields of 70 and 92%, respectively. Carboalkoxylation[17] promoted by Pd^0 efficiently converted the *meso*-iodine to the corresponding ethyl ester group and gave the functionalized distyryl-BODIPY derivatives **5**–**7**.

Subsequently, quaternization of the dimethylpropargylamine moieties with 1,3-propanesultone was performed in an anhydrous organic solvent (DMF or 1,2-dichloroethane) at 60 °C to afford sulfobetaine derivatives **8**–**10**. NMR spectroscopy and mass spectral data are in full agreement with the assigned molecular structures. For example, the well-resolved ^{1}H NMR spectrum of **10** is shown in Figure 1. It clearly exhibits all the characteristic signals of this sulfonated distyryl-BODIPY compound. The integration of eight protons at $\delta = 7.84$ ppm corresponds to the phenyl subunits on the distyryl arms; the four protons observed at $\delta = 8.31$ and 7.64 ppm belong to the AB system of the phenyl at the pseudo-*meso* position. The quaternary dimethylammonium groups give signals at $\delta = 3.35$ and 3.04 ppm integrating for 12 H in total. The four protons at $\delta = 2.39$ and 2.14 ppm are assigned to the CH_2 groups of the sulfobetaine residues on the styryl arms and on the boron center, respectively. In addition, the observed 16 Hz proton–proton coupling constant is in agreement with the *E* configuration for the double bonds, already reported for this type of Knoevenagel condensation on BODIPY dyes.[18] Since compound **10** with four sulfobetaine groups was found to be very soluble in water, no further sulfonation of its *meso*-phenyl substituent was required. Thus, this compound was only subjected to an alkaline hydrolysis to generate the bioconjugatable carbox-



Scheme 1. Synthesis of water-soluble distyryl-BODIPY dyes **11**–**13** (TEA = triethylamine).

ylic acid **13** in a 23% isolated yield after flash column chromatography on a RP-C_{18} silica gel column.

In contrast, the bis(sulfobetaine) derivatives **8** and **9** were found to be poorly water-soluble and a further Schotten–Baumman amidification reaction with dipeptidyl linker (α-sulfo-β-alanine)$_2$ was therefore applied. After saponification of the ethyl ester, these compounds were quantitatively converted into the corresponding NHS ester by treatment with the peptide coupling uronium reagent *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyl uronium tetrafluoroborate (TSTU) and *N*,*N*-diisopropylethylamine (DIEA) in dry *N*-methyl-2-pyrrolidone (NMP).[19] Thereafter, the crude NHS ester was subjected to aminolysis with the dipeptide (α-sulfo-β-alanine)$_2$ in aqueous $NaHCO_3$ (pH 8.5) to give the water-soluble distyryl-BODIPY dyes **11** and **12**. RP-HPLC purification by using aqueous triethylammonim bicarbonate (TEAB, pH 7.5) buffer and acetonitrile as eluents, followed by desalting on Dowex H^+ ion-exchange resin provided **11** and **12** in pure forms with an overall yield of 30–40% for the last three steps. Their structures were confirmed by ESI mass spectrometry measurements and their purity was checked by RP-HPLC.

We assumed that the low overall yield for the synthesis of **13** was due to the chemical instability of the ethynylaryl substituents. Thus, we decided to construct the styryl arms of the BODIPY core by using the 4-[(dimethylamino)methyl]-benzaldehyde (**B**) instead of the 4-[3-(dimethylamino)-1-propyn-1-yl]benzaldehyde derivative (**A**) as the starting material. An original three-step synthetic protocol aimed at preparing this unusual aldehyde was developed as described in Scheme 2. First, the radical bromination of 4-iodotoluene gave the 4-iodobenzyl bromide in 46% yield. Subsequent nucleophilic substitution with dimethylamine in THF afforded the *N*-(4-iodobenzyl)-*N*,*N*-dimethylamine in 93% yield. Finally, a Pd^0-promoted formylation reaction provided the targeted 4-[(dimethylamino)methyl]benzaldehyde (**B**) in a good 73% yield.

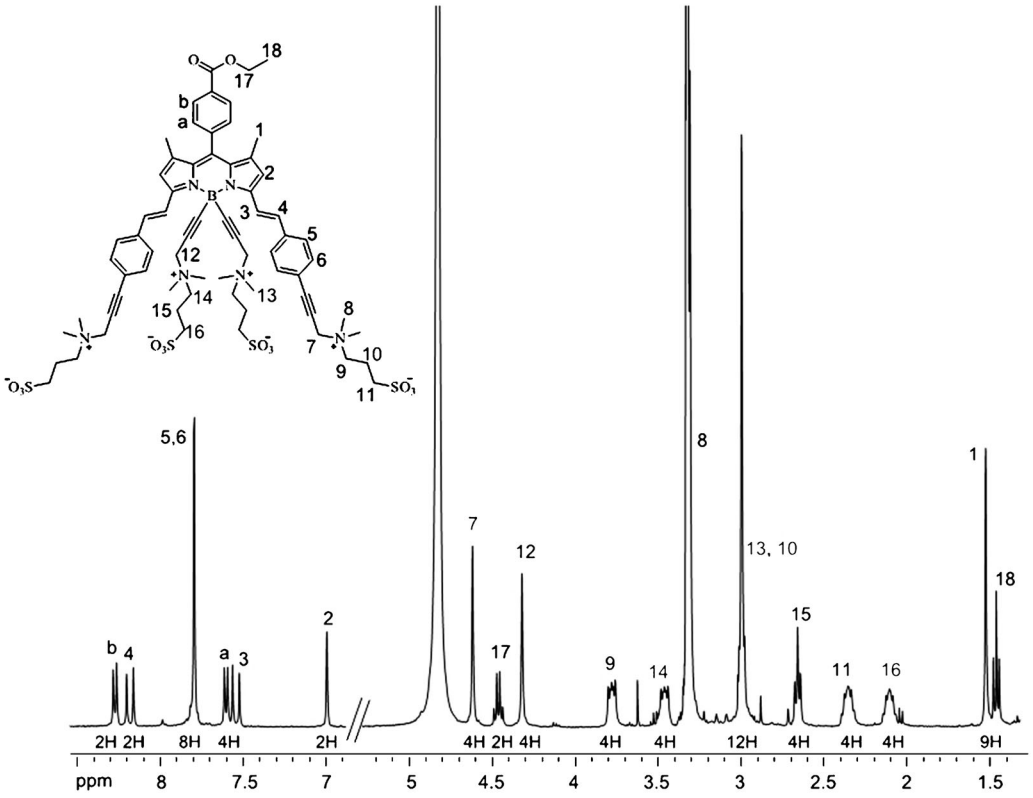
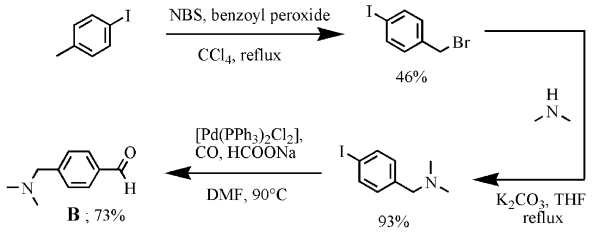


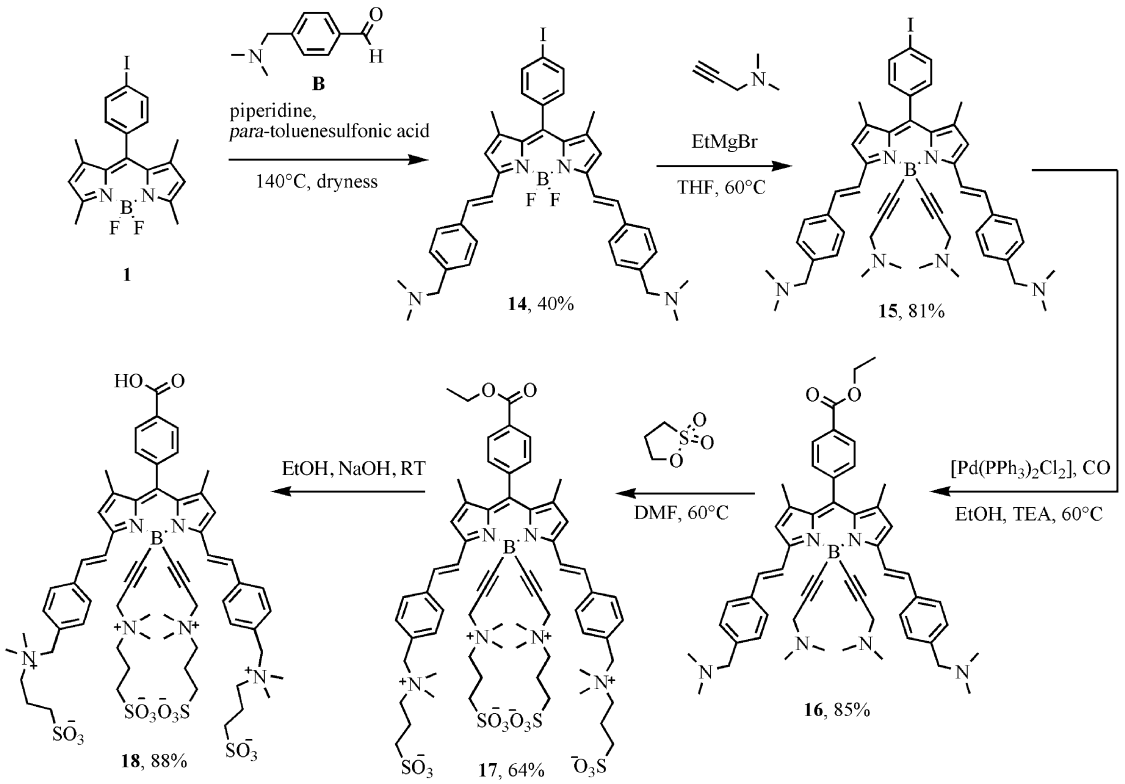
Figure 1. ^{1}H NMR spectrum (400 MHz) of sulfonated distyryl-BODIPY dye **10** recorded in a CD_3OD/D_2O mixture. Despite the solubility of this compound in water, CD_3OD was added to get a better resolved spectrum.



Scheme 2. Synthesis of 4-[(dimethylamino)methyl]benzaldehyde (**B**).

This novel benzaldehyde derivative was used in the well-established Knoevenagel condensation reaction involving **1**, to give the distyryl-BODIPY **14** (see Scheme 3 for the synthesis of BODIPY dye **14**) in a 40% yield. Subsequent substitution of the fluorine atoms by the alkynyl-Grignard reagent from *N*,*N*-dimethylaminopropyne gave dye **15** in 81% yield. Thereafter, a Pd-catalyzed carboalkoxylation reaction provided the ethyl ester **16** and quaternization of the tertiary amino groups by treatment with an excess of 1,3-propanesultone in dry DMF provided the tetra-sulfonated derivative **17**, which was purified by flash column chromatography on a RP-C_{18} silica gel column. Finally, saponification of the ethyl ester was performed under standard conditions to give the water-soluble and bioconjugatable distyryl-BODIPY dye **18**. Thus, a significantly higher overall yield was obtained for this five-step synthesis relative to that in the preparation of the first tetrakis(sulfobetaine) BODIPY dye **18** (overall yield: 16% against 3% for dye **13**). The ^{1}H NMR spectrum of **18** is shown in Figure 2 and supports the molecular structure of this free acid distyryl-BODIPY dye.

Photophysical properties: The red-emitting distyryl-BODIPY dyes **11**–**13** and **18** were found to be readily soluble in water and related aqueous buffers in the concentration range (1 μM to 10 mM) largely suitable for biolabeling applications. Their optical properties were evaluated under simulated physiological conditions (i.e., phosphate buffered saline (PBS), pH 7.5) or pure water and the corresponding data are collected in Table 1. In aqueous solution, dye **11**, despite the presence of a disulfonated tail and two sulfobetaine residues on the distyryl-BODIPY scaffold, displays unusual features, with a dual absorption band, a lowered intensity of the main π–π* absorption, and a broadening of all absorption bands (Figure 3). Dye **11** shows a low absorption coefficient ($\approx 20000\,M^{-1}cm^{-1}$) and a low fluorescence quantum yield (4%; Table 1, entry 6). The dual absorption, the breadth of the absorption bands, and the hypsochromic shift of one to 601 nm is in keeping with the formation of non-emissive aggregates.[14,20] Interestingly, the addition of BSA protein (5% (*w*/*v*) in PBS) led to an increase of the quantum yield to 20% (Figure 4). This protein is known to enhance the emission of many fluorophores due to a combination of rigidization, reduction in the polarity of the dye's mi-



Scheme 3. Synthesis of the water-soluble distyryl-BODIPY dye **18**.

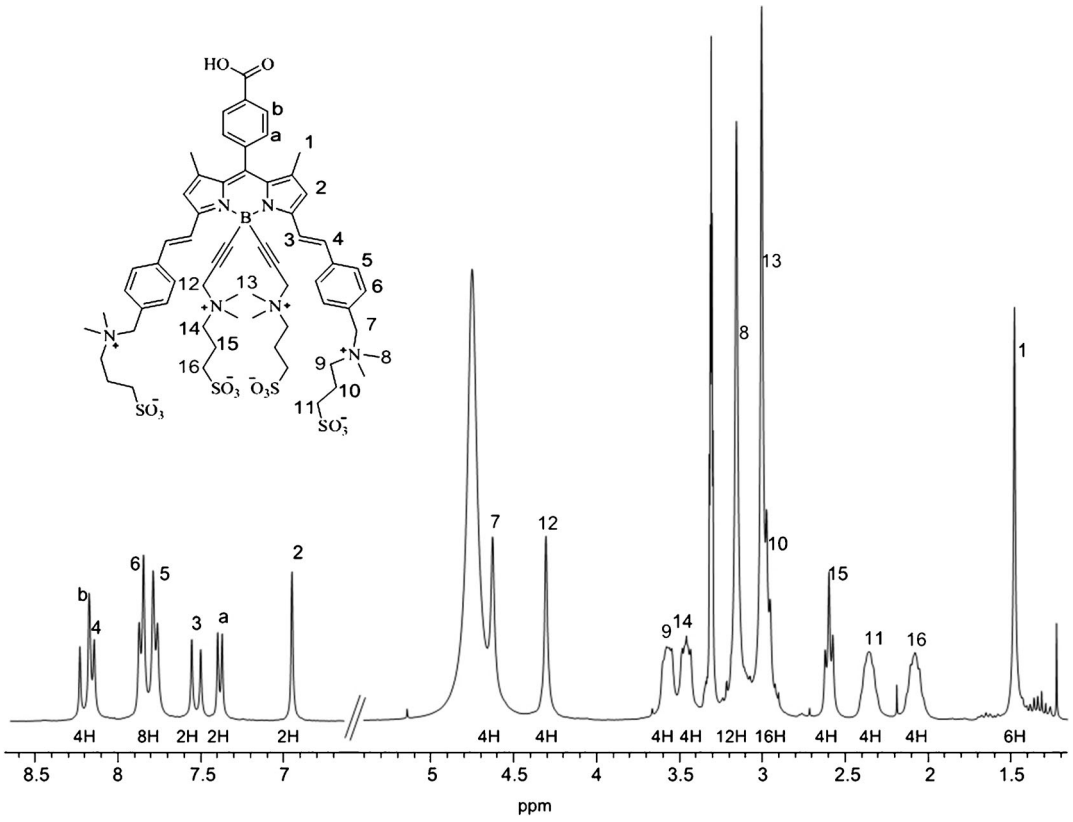


Figure 2. ^{1}H NMR spectrum of sulfonated distyryl-BODIPY dye **18** recorded in CD_3OD/D_2O (300 MHz). Despite the solubility of this compound in water, CD_3OD was added to get a better resolved spectrum.

Table 1. Spectroscopic properties of BODIPY dyes studied in this work. Data for the less energetic absorption band.

Compound	λ_{abs} [nm]	ε [$M^{-1}cm^{-1}$]	λ_{em} [nm]	ϕ [%][a]	τ [ns][b]	k_r [$10^7 s^{-1}$][c]	k_{nr} [$10^7 s^{-1}$][d]
2 (CH_2Cl_2)	646	110600	661	46	5.2	8.8	10.4
3 (CH_2Cl_2)	647	108500	659	47	4.8	9.8	11.0
4 (CH_2Cl_2)	648	92200	661	26	5.2	5.0	14.2
5 (CH_2Cl_2)	646	122200	659	43	4.6	9.3	12.4
7 (CH_2Cl_2)	647	83500	663	30	4.8	6.3	14.6
11 (PBS)	601, 649	17900, 19300	655	4	–	–	–
12 (PBS)	642	55100	657	22	–	–	–
13 (PBS)	641	58000	657	25	2.7	9.3	27.8
14 (CH_2Cl_2)	632	64500	640	40	6.5	6.2	9.2
15 (1,4-dioxane)	634	81300	645	25	6.3	4.0	11.9
16 (1,4-dioxane)	634	84200	649	36	5.3	6.8	12.1
17 (H_2O)	630	76600	643	30	3.4	8.8	20.6
17 (EtOH)	636	92000	646	35	4.5	7.8	14.4
18 (H_2O)	628	71000	641	38	3.9	9.7	15.9

[a] Standard used for quantum yield measurements: cresyl violet in EtOH ($\phi=51\%$), $\lambda_{exc}=578$ nm[32] for **2**–**7** and **14**–**18**, and sulfoindocyanine dye Cy 5.0 in PBS ($\phi=20\%$), $\lambda_{exc}=600$ nm[5] for **11**–**13**. All ϕ are corrected for changes in refractive index. [b] Lifetime. [c] $k_r=\phi/\tau$. [d] $k_{nr}=(1-\phi)/\tau$, assuming that the emitting state is produced with unit quantum efficiency.

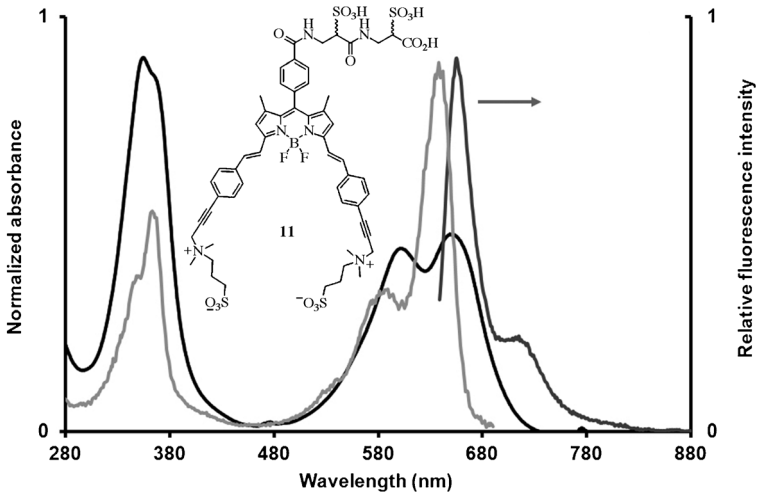


Figure 3. Absorption and emission ($\lambda_{exc.}=600$ nm) spectra of dye **11** in PBS at 25°C. ——: Absorption; ——: emission; ——: excitation.

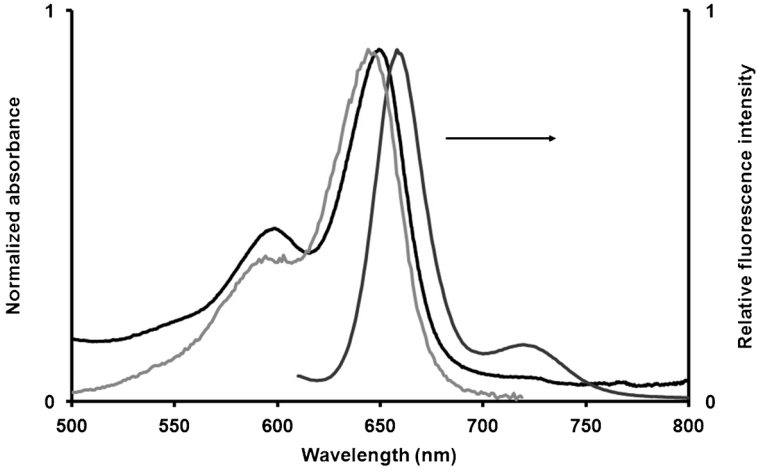


Figure 4. Normalized absorption and fluorescence emission ($\lambda_{exc}=600$ nm) spectra of **11** in PBS+5% (*w*/*v*) BSA at 25°C. ——: Absorption; ——: emission; ——: excitation.

croenvironment (binding in the hydrophobic BSA pocket), and deaggregation.[21a] Moreover, PBS solutions containing 5% BSA are often used as a model for body fluids.

The substitution of both fluorine atoms at the boron center by EG chains dramatically improves the spectroscopic properties of the distyryl-BODIPY dye under physiological conditions. Thus, in aqueous solution, the optical properties of **12** in the red region are quite similar to those of standard distyryl-BODIPY derivatives in organic solvents (Table 1).[22] The absorption spectrum of the dye has its lowest energy absorption maximum centered at 642 nm, corresponding to the $S_0 \rightarrow S_1$ (0–0) transition of the BODIPY core, with an absorption coefficient of $55100\,M^{-1}cm^{-1}$ (Figure 5). The shoulder centered at about 590 nm can be assigned to the 0–1 vibrational band of the same transition.[23] The strong absorption band with an absorption coefficient of $76800\,M^{-1}cm^{-1}$ centered at 366 nm can probably be assigned to the $\pi \rightarrow \pi^*$ transition of the styryl moieties.[24]

In emission, in contrast to the BODIPY dye **11**, the boron-EG-substituted BODIPY dye **12** exhibits a strong fluorescence emission centered at 657 nm, with a fluorescence quantum yield of 22%. The absence of aggregates was confirmed by recording the excitation spectrum, which perfectly matches the absorption spectrum. In comparison with **11**, the better quantum yield and the absence of aggregation of **12** indicate that the substitution of fluorine atoms at the boron center by bulky polar functional groups can efficiently prevent the aggregation of the distyryl-BODIPY dye in aqueous media. This fact was also confirmed with tetrakis(sulfobetaine) BODIPY dyes **13** and **18** (Table 1 and Figures 6 and 7).

Indeed, the introduction of four sulfobetaine residues onto one face of the BODIPY scaffold totally overcomes the water-solubility issues with the distyryl-BODIPY dyes, removing the need for the introduction of a further polysulfonated tail onto the pseudo *meso*-position. The optical properties of

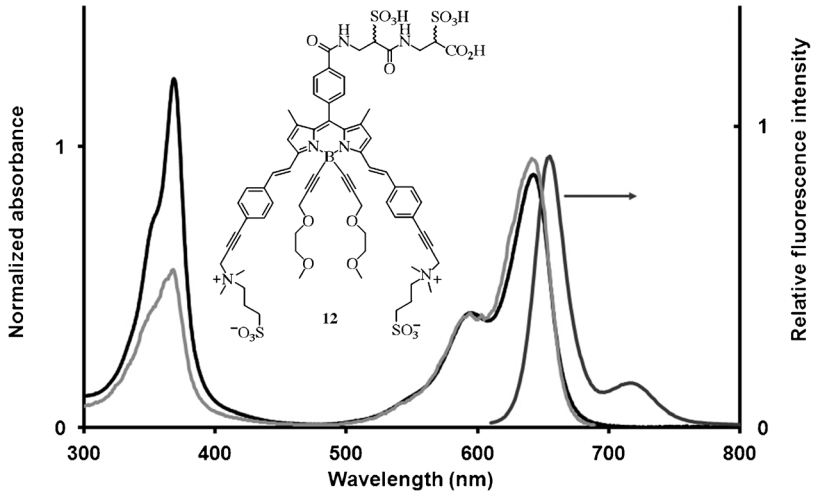


Figure 5. Absorption and emission ($\lambda_{exc}=600$ nm) spectra of water-soluble BODIPY dye **12** in PBS at 25 °C. ——: Absorption; ——: emission; ——: excitation.

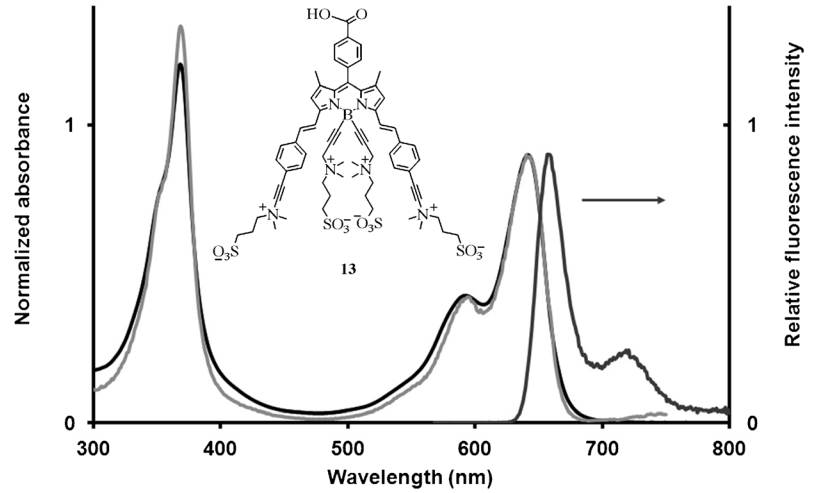


Figure 6. Absorption, excitation ($\lambda_{em}=680$ nm), and emission ($\lambda_{exc}=600$ nm) spectra of dye **13** in PBS at 25 °C. ——: emission; ——: Absorption; ——: excitation.

13 are quite similar to those of the dye **12** (Figure 6). In the UV/Vis absorption spectrum, the lowest energy absorption maxima is centered at 641 nm, with an absorption coefficient of 58000 $M^{-1}cm^{-1}$. The strong absorption centered at 369 nm can probably be assigned to the $\pi\rightarrow\pi^*$ transitions of the styryl groups. In fluorescence, the dye exhibits a strong emission maximum centered at 657 nm with a fluorescence quantum yield of 25%. The absorption, emission, and excitation spectra of the dye confirm the absence of aggregation in aqueous solution.

The optical properties of **18** were also evaluated in pure water (Figure 7 and Table 1). In aqueous solution, the carboxylic acid **18** shows similar optical properties to those of **12** and **13**. In the UV/Vis absorption spectrum, the lowest energy absorption maximum is centered at 628 nm with an absorption coefficient of 71000 $M^{-1}cm^{-1}$. The strong absorption of the $\pi\rightarrow\pi^*$ transitions of the styryl groups appears at 352 nm. In fluorescence, the dye exhibits an emission maximum at 641 nm with a good fluorescence quantum yield of 38%. Once again, the absorption, emission, and excitation spectra confirm the absence of aggregation in water. In addition, slight hypsochromic shifts of about 14 nm were observed in both the absorption and emission maxima of dye **18** relative to those of the structural analogue **13**, due to the reduced π-conjugation of the BODIPY core.

Bioconjugation: Since the most emissive water-soluble BODIPY dyes, **12** and **13**, are functionalized with a free carboxylic acid group, their ability to label proteins and antibodies through reactions of their active ester with the NH_2 groups of ε-lysine residues present in these biopolymers was assessed. BSA and the monoclonal antibody (mAb) 12A5 that recognizes the influenza hemagglutinin (HA) epitope tag[25] were chosen as the protein and the antibody, respectively, for these bioconjugation experiments.

To compare the labeling performances of these novel red-fluorescent markers with those of classic CyDye reagents, similar reactions were performed with sulfoindocyanine dye Cy 5.0[5] under the same experimental conditions. To avoid possible harmful effects of organic solvents and additives towards the mAb (or protein) structure and functional activity, a carboxylic acid activation protocol performed in deionized water and involving the use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and sulfo-NHS instead of TSTU/DIEA in a polar aprotic solvent, such as DMF or DMSO, was preferred.[26] In this case, the one-pot procedure without isolating the corresponding sulfo-NHS ester of the BODIPY dye was preferred because these labeling reactions were typically performed on a small scale (<1 mg of dye). However, isolation of the active ester of the BODIPY dye can be performed by RP-HPLC if necessary.

BSA and anti-HA mAb were labeled through overnight incubation with a 13- and 31-fold molar excess of BODIPY derivatives **12** and **13**, respectively, in PBS (pH 7.5). The re-

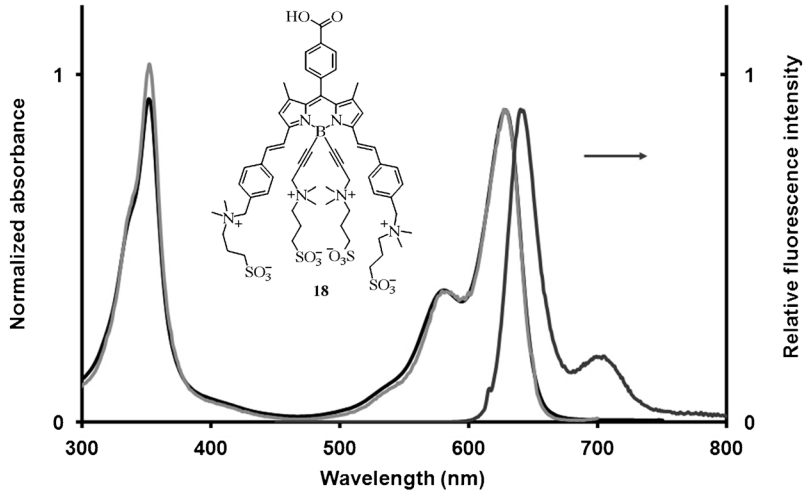


Figure 7. Absorption, excitation ($\lambda_{em}=680$ nm), and emission ($\lambda_{exc}=600$ nm) spectra of water-soluble BODIPY dye **18** in deionized water at 25 °C. ——: Absorption; ——: emission; ——: excitation.

sulting protein fluorescent conjugates were purified by size-exclusion chromatography on a Sephadex G-25 column.

Table 2 contains the absorption/emission wavelengths and quantum yields of fluorescent proteins in PBS, together with the attached fluorophore/protein molar ratios (*F*/*P*). The *F*/*P* ratio was calculated from the absorbance of the fluorescent protein conjugate at 280 nm (corrected for BODIPY/cyanine dye contribution) and the integral absorption of the dye's longest wavelength absorption band. Use of the integral absorption instead of the commonly employed molar absorption coefficient at the dye's absorption maximum is more appropriate for the calculation of *F*/*P* ratios of conjugates showing conjugation-induced changes in the shape of the fluorophore's absorption band.[21]

The *F*/*P* values achieved with the *meso*-substituted BODIPY dye **12** were significantly higher than those achieved with the dye **13** and Cy 5.0, which suggests a greater reactivity of its sulfo-NHS ester. Indeed, the added (α-sulfo-β-alanine)dipeptidyl spacer enables projection of the bioconjugable CO_2H group further away from the sterically hindered distyryl-BODIPY core than in **13**, thus ensuring a better capability for the acylation of primary amines within proteins, especially under pH-neutral conditions.

When compared to the noncovalently bound water-soluble distyryl-BODIPY dyes **12** and **13**, the absorption maxima of the fluorescent protein conjugates are slightly redshifted by about 8 nm, whereas emission maxima remain unchanged (Figures 8 and 9). In addition, the conjugated dye **13** shows a more pronounced tendency to form aggregates, as revealed by the enlargement of these absorption bands and the blueshift of one of these bands to 588 nm. The lesser tendency of the conjugated dye **12** to form nonemissive aggregates is probably a further positive effect of the disulfonated tail grafted onto the *meso*-phenyl substituent. The observed conjugation-induced diminution in fluorescence efficiency is not surprising and is attributed to a combination of effects, with the main contributions arising from the formation of non-fluorescent aggregates (H-type homodimers) and from fluorescence energy resonance transfer (FRET) from the fluorescent monomeric dyes to the increasing number of non-emissive dimers on the biopolymers.[21a] Nonetheless, these

Table 2. Spectral properties in PBS of the proteins labeled with water-soluble red-emitting dyes **12**, **13**, or Cy 5.0 and fluorophore to protein molar ratios (*F*/*P*).

Conjugated fluorescent dye	λ_{abs} [nm][a]	λ_{em} [nm]	ϕ [%][b]	*F*/*P*
mAb-**12**	370, 603, 650	657	7	6.6
BSA-**12**	372, 603, 651	657	6	1.5
mAb-**13**	370, 588, 650	659	5	4.5
BSA-**13**	371, 588, 650	658	5	0.8
mAb-Cy 5.0	610, 650	667	12	3.1
BSA-Cy 5.0	609, 651	663	3	1.0

[a] The absorption signal arising from the protein at about 280 nm is omitted. [b] Determined at 25 °C by using sulfoindocyanine dye Cy 5.0 ($\Phi_F=20$ % in PBS at 25 °C) as a standard.[5]

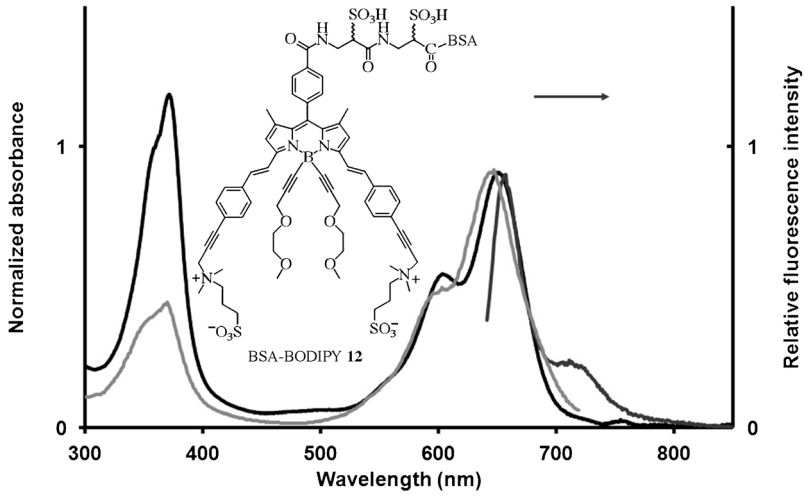


Figure 8. Normalized absorption and emission ($\lambda_{exc}=600$ nm) spectra of conjugate BSA–**12** in PBS at 25 °C. ——: Absorption; ——: emission; ——: excitation.

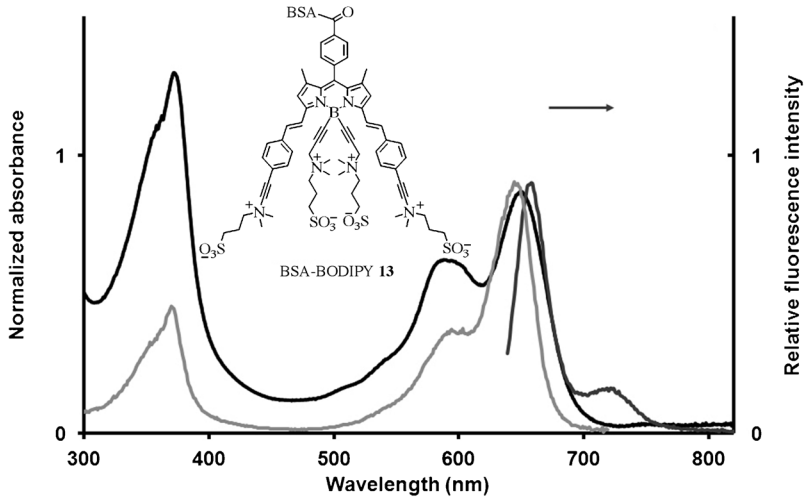


Figure 9. Normalized absorption and emission (λ_{exc} = 600 nm) spectra of conjugate BSA–**13** in PBS at 25 °C. ——: Absorption; ——: emission; ——: excitation.

red labels maintain moderate fluorescence quantum yields upon bioconjugation and are expected to have utility in fluorescent biosensing applications, especially those involving the design of activatable fluorescent probes.[27]

Conclusion

In this contribution, four novel water-soluble and bioconjugable red fluorophores based on a distyryl-BODIPY scaffold were synthesized and studied. Our synthetic approach is based on the introduction of water-solubilizing groups (sulfonic acid and sulfobetaine moieties) at the final stages of the BODIPY synthesis, through efficient alkylation and/or acylation of preintroduced functional reactive groups (tertiary amine and carboxylic acid). Thus, the time-consuming handling and purification (by RP-HPLC or RP-C_{18} flash column chromatography) of highly polar fluorescent materials is advantageously limited to the final product. We found that the fluorescence quantum yield of these distyryl-BODIPY dyes in aqueous solution is primarily dependent on the substitution pattern of their boron atom, which also determines the aggregation behavior. Sterically demanding subsitutents on the boron center and negatively charged sulfonate groups dramatically reduce the tendency to aggregate, thereby leading to bright fluorophores with quantum yields in the 22–38% range. To our knowledge, these are the highest values reported to date for red-emitting BODIPY dyes under physiological conditions. These new dyes were also tested for protein labeling and the conjugates obtained display satisfactory fluorescence properties. With compounds **12**, **13**, and **18**, we have established attractive new diagnostic tools for in vitro and in vivo fluorescence applications.

Experimental Section

General remarks: Column chromatography purifications were performed either on silica gel 60 M (40–63 μm) from Macherey–Nagel or on aluminum oxide 90 standardized from Merck. Reversed-phase flash column chromatography was performed on octadecyl-functionalized silica gel (mean pore size 60 Å, 37–74 μm) from Aldrich. TLC analyses were carried out on Macherey–Nagel Alugram Sil G/UV_{254} aluminum sheets. All reactions were performed under a dry atmosphere of argon by using standard Schlenk tube techniques. All chemicals were used as received from commercial sources without further purification unless otherwise stated. All solvents were dried by following standard procedures (CH_2Cl_2: distillation over P_2O_5, $C_2H_4Cl_2$: distillation over P_2O_5, diethyl ether: distillation over Na^0/benzophenone, DMF: distillation over BaO, THF: distillation over Na^0/benzophenone, toluene: disillation over Na^0). NMP (peptide synthesis grade) was purchased from Iris Biotech GmbH. The HPLC-gradient grade acetonitrile (CH_3CN) and methanol (CH_3OH) were obtained from VWR. PBS (100 mM phosphate + 150 mM NaCl, pH 7.5) and aqueous mobile phases for HPLC were prepared by using water purified with a Milli-Q system (purified to 18.2 MΩ cm). 4-Iodobenzyl bromide,[28] *N*-(4-iodobenzyl)-*N*,*N*-dimethylamine,[29] $[PdCl_2(PPh_3)_2]$,[30] and $[Pd(PPh_3)_4]$[31] were prepared according to literature procedures. Disulfonated linker α-sulfo-β-alaninyl-α-sulfo-β-alanine was prepared from β-alanine by using synthetic procedures recently reported by us.[12] Sulfoindocyanine dye Cy 5.0 (also named Cy 5.29) was prepared by using a literature procedure.[5]

Instruments and methods: Ion-exchange chromatography (for desalting water-soluble BODIPY dyes) was performed with an Econo-Pac Disposable chromatography column (Bio-Rad, #732–1010) filled with an aqueous solution of Dowex 50WX8–400 (Alfa Aesar, ≈7 g for 10 mg of dye, 15 × 50 mm bed), regenerated by using a 10% HCl aqueous solution and equilibrated with deionized water. Size-exclusion chromatography (for purification of fluorescently labeled proteins) was performed with an Econo-Pac Disposable chromatography column (Bio-Rad, #732–1010) filled with an aqueous solution of Sephadex G-25 Fine (Amersham Biosciences AB, 15 × 40 mm bed), equilibrated with PBS (0.01 M phosphate, 0.015 M NaCl, pH 7.5). ^{1}H- and ^{13}C NMR spectra were recorded on Bruker AC 200, Avance 300, and Avance 400 spectrometers working at 200, 300, or 400 MHz, respectively. Chemical shifts are expressed in parts per million (ppm) from the residual nondeuterated solvent signal. *J* values are expressed in Hz. Analytical HPLC was performed on a Thermo Scientific Surveyor Plus instrument equipped with a PDA detector. Semi-preparative HPLC was performed on a Thermo Scientific SPECTRASYSTEM liquid chromatography system (P4000) equipped with a UV/Vis 2000 detector. MS (ESI) were obtained either with a Finnigan LCQ Advantage MAX (ion trap) apparatus or a JEOL JMS-T100 LO Acc TOF. Elemental analysis was conducted by an Elementar vario MICRO Cube.

HPLC separations: Four chromatographic systems were used for the analytical experiments and purification steps.

System A: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 100 mm) with CH_3CN and trifluoroacetic acid (aqueous TFA, 0.1%, pH 2.2) as eluents (20% CH_3CN (5 min), followed by a linear gradient from 20 to 100% (32 min) of CH_3CN) at a flow rate of 1.0 mL min^{-1}. Triple UV/Vis detection was achieved at 254, 530, and 640 nm.

System B: Semi-preparative RP-HPLC (Varian Kromasil C_{18} column, 10 μm, 21.2 × 250 mm) with CH_3CN and 0.1% aqueous TFA as eluents

(10% CH_3CN (10 min), followed by a linear gradient from 10 to 100% (45 min) of CH_3CN) at a flow rate of 16.0 mL min^{-1}. Dual visible detection was achieved at 366 and 635 nm.

System C: System A with aqueous triethylammonium acetate (TEAA, 100 mM, pH 7.0) instead of 0.1% aqueous TFA.

System D: Semi-preparative RP-HPLC (Varian Kromasil C_{18} column, 10 μm, 21.2×250 mm) with CH_3CN and aqueous triethylammonium bicarbonate (TEAB 50 mM, pH 7.5) as eluents (5% CH_3CN (10 min), followed by a linear gradient from 5 to 100% (47 min) of CH_3CN) at a flow rate of 16.0 mL min^{-1}. Dual visible detection was achieved at 366 and 640 nm.

Spectroscopic measurements: UV/Vis spectra were recorded by using a Shimadzu UV-3600 dual-beam grating spectrophotometer with a 1 cm quartz cell. Fluorescence spectra were recorded on a HORIBA Jobin–Yvon fluoromax 4P spectrofluorimeter. All fluorescence spectra were corrected. The following equation was used to determine the relative fluorescence quantum yield was determined by using Equation (1) in which A is the absorbance at the excitation wavelength (in the range 0.01–0.1 A.U.), F is the area under the corrected emission curve, n is the refractive index of the solvents (at 25°C) used in measurements, and the subscripts S and X represent standard and unknown, respectively. The following standards were used: Cresyl violet in ethanol (ϕ=50%, λ_{exc}=546 nm or ϕ=51%, λ_{exc}=578 nm) and Cy 5.0 in PBS (ϕ=20%, λ_{exc}=600 nm).[5,32] Luminescence lifetimes were measured on an Edinburgh Instruments spectrofluorimeter equipped with an R928 photomultiplier and a PicoQuant PDL 800-D pulsed diode connected to a G^{w}Instect GFG-8015G delay generator. No filter was used for the excitation. Emission wavelengths were selected by a monochromator. Lifetimes were deconvoluted with FS-900 software by using a light-scattering solution (LUDOX) for instrument response.

$$\phi(X) = (A_S/A_X)(F_X/F_S)(n_X/n_S)^2\phi(S) \quad (1)$$

4-[3-(Dimethylamino)-1-propyn-1-yl]benzaldehyde (A): $[Pd(PPh_3)_4]$ (0.36 g, 0.32 mmol) and 1-dimethylamino-2-propyne (0.67 g, 8.10 mmol) were sequentially added to a degassed solution of 4-bromobenzaldehyde (1.0 g, 5.40 mmol) in benzene (3 mL) and triethylamine (3 mL) under an argon atmosphere. The resulting reaction mixture was stirred at 60°C for 10 h until the complete consumption of the starting material was observed by TLC analysis. The mixture was then evaporated and purified by chromatography on a silica gel column with the following eluents (CH_2Cl_2 100%, and then CH_2Cl_2/CH_3OH 97:3) to afford the desired compound (1.13 g, quant. yield). ^{1}H NMR ($CDCl_3$, 200 MHz): δ=10.00 (s, 1H), 7.82 (d, J_{HH}=8.3 Hz, 2H), 7.58 (d, J_{HH}=8.3 Hz, 2H), 3.54 (s, 2H), 2.42 ppm (s, 6H); MS (ESI, positive mode): m/z (%): calcd for $C_{12}H_{13}NO$: 187.1; found: 188.1 $[M+H]^+$ (100); elemental analysis calcd (%) for $C_{12}H_{13}NO$: C 76.98, H 7.00, N 7.48; found: C 76.64, H 6.82, N 7.17.

Compound 2: A mixture of 8-(4-iodophenyl)-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (**1**; 500 mg, 1.11 mmol) and benzaldehyde derivative **A** (415 mg, 2.22 mmol) was dissolved in dry toluene (20 mL) and piperidine (0.5 mL). The resulting reaction mixture was heated at 140°C in a Dean–Stark apparatus for 2 h. Thereafter, the Dean–Stark receiver was removed and the mixture was heated with argon bubbling until the solvent was completely removed. The resulting crude product was treated with saturated aqueous $NaHCO_3$ and deionized water, and finally extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$ and evaporated to dryness. The crude residue was purified by chromatography on a silica gel column with a step gradient of CH_3OH (3–7%) in CH_2Cl_2 as the mobile phase giving the distyryl-BODIPY **2** (193 mg, 22%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ=7.86 (d, J_{HH}=7.7 Hz, 2H), 7.71 (d, J_{HH}=16.3 Hz, 2H), 7.56 (d, J_{HH}=7.9 Hz, 4H), 7.46 (d, J_{HH}=7.8 Hz, 4H), 7.22 (d, J_{HH}=16.4 Hz, 2H), 7.08 (d, J_{HH}=7.7 Hz, 2H), 6.65 (s, 2H), 3.52 (s, 4H), 2.40 (s, 12H), 1.48 ppm (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ=152.9, 142.2, 138.6, 136.4, 135.9, 134.8, 133.5, 130.6, 127.6, 123.8, 119.9, 118.4, 95.1, 86.7, 85.7, 48.9, 44.5, 15.2 ppm; MS (ESI, positive mode): m/z (%): calcd for $C_{43}H_{40}BF_2IN_4$: 787.2; found: 788.2 $[M+H]^+$ (100); elemental analysis calcd (%) for $C_{43}H_{40}BF_2IN_4$: C 65.50, H 5.11, N 7.11; found: C 65.22, H 4.64, N 6.82.

Compound 3: A solution of 3-(2-methoxyethoxy)-1-propyne (68 mg, 0.60 mmol) in dry THF (5 mL) was placed in a Schlenk tube under an argon atmosphere. A 1.0 M solution of EtMgBr in THF (0.52 mL) was then added and the reaction mixture was stirred at 60°C for 1 h. The resulting anion was then transferred through a cannula to a solution of distyryl-BODIPY **2** (120 mg, 0.15 mmol) in dry THF (3 mL) previously placed in a Schlenk tube under an argon atmosphere. The resulting reaction mixture was stirred at 60°C for about 15 min, until the complete consumption of the starting BODIPY was observed by TLC analysis. Then, deionized water (3 mL) was added. The mixture was then dissolved in CH_2Cl_2 and sequentially washed with deionized water and brine. The organic layer was dried over anhydrous $MgSO_4$ and evaporated to dryness. The crude product was purified by chromatography on a silica gel column with the following eluent (CH_2Cl_2/CH_3OH 93:7) to afford bis-(ethynyl) BODIPY **3** (104 mg, 70%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ=8.21 (d, J_{HH}=16.5 Hz, 2H), 7.86 (d, J_{HH}=7.7 Hz, 2H), 7.58 (d, J_{HH}=8.0 Hz, 4H), 7.48 (d, J_{HH}=8.1 Hz, 4H), 7.13 (m, 4H), 6.66 (s, 2H), 4.15 (s, 4H), 3.56 (s, 4H), 3.49 (m, 4H), 3.20 (s, 6H), 3.16 (m, 4H), 2.43 (s, 12H), 1.47 ppm (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ=152.0, 140.7, 138.5, 137.0, 135.1, 133.8, 132.5, 130.7, 127.4, 123.4, 121.8, 118.7, 92.1, 86.0, 68.5, 59.0, 48.7, 44.2, 29.9, 15.4 ppm; MS (ESI, positive mode): m/z (%): calcd for $C_{55}H_{58}BIN_4O_4$: 975.4; found: 977.2 $[M+H]^+$ (100); elemental analysis calcd (%) for $C_{55}H_{58}BIN_4O_4 \cdot H_2O$: C 66.40, H 6.08, N 5.63; found: C 66.12, H 5.74, N 5.44.

Compound 4: A solution of 1-dimethylamino-2-propyne (52 mg, 0.62 mmol) in dry THF (3 mL) was placed in a Schlenk tube under an argon atmosphere. A 1.0 M solution of EtMgBr in THF (0.435 mL) was added and the reaction mixture was and stirred at 60°C for 1 h. The resulting anion was then transferred through a cannula to a solution of distryryl-BODIPY **2** (100 mg, 0.124 mmol) in dry THF (3 mL) previously placed in a Schlenk tube under an argon atmosphere. The resulting reaction mixture was stirred at 60°C for 15 min until the complete consumption of the starting BODIPY was observed by TLC analysis. Then, the mixture was sequentially treated with deionized water and brine and extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$ and evaporated to dryness. The crude product was purified by chromatography on a Al_2O_3 column with the following eluent (CH_2Cl_2/CH_3OH 97:3) to give bis(ethynyl) BODIPY **4** (114 mg, quant. yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ=8.39 (d, J_{HH}=16.4 Hz, 2H), 7.86 (d, J_{HH}=8.2 Hz, 2H), 7.61 (d, J_{HH}=8.2 Hz, 4H), 7.47 (d, J_{HH}=8.0 Hz, 4H), 7.15 (d, J_{HH}=16.6 Hz, 2H), 7.11 (d, J_{HH}=8.1 Hz, 2H), 6.67 (s, 2H), 3.53 (s, 4H), 3.20 (s, 4H), 2.42 (s, 12H), 2.20 (s, 12H), 1.47 ppm (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ=151.8, 140.3, 138.2, 137.8, 136.8, 135.1, 133.4, 132.1, 131.8, 130.6, 127.2, 123.3, 122.0, 118.5, 94.8, 90.8, 86.5, 85.4, 48.9, 48.7, 44.3, 34.1, 29.7, 22.4, 15.2, 14.1 ppm; MS (ESI, positive mode): m/z (%): calcd for $C_{53}H_{56}BIN_6$: 913.4; found: 915.2 $[M+H]^+$ (100); elemental analysis calcd (%) for $C_{53}H_{56}BIN_6 \cdot H_2O$: C 66.95, H 6.36, N 8.84; found: C 66.78, H 6.21, N, 8.64.

Compound 5: Distyryl-BODIPY **2** (100 mg, 0.12 mmol) was dissolved in a mixture of absolute EtOH (4 mL) and triethylamine (3 mL). $[PdCl_2(PPh_3)_2]$ (9 mg, 0.012 mmol) was added and the reaction mixture was kept under a CO atmosphere for 4 h and heated at 60°C. Thereafter, the mixture was treated with saturated aqueous $NaHCO_3$ and deionized water and extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$. The solvent was then removed and the crude product was purified by chromatography on silica gel column with the following eluent (CH_2Cl_2/CH_3OH 93:7) to afford the ethyl ester **5** in a pure form (78 mg, 85%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ=8.18 (d, J_{HH}=8.1 Hz, 2H), 7.70 (d, J_{HH}=16.4 Hz, 2H), 7.54 (d, J_{HH}=8.1 Hz, 4H), 7.43 (d, J_{HH}=8.1 Hz, 4H), 7.42 (d, J_{HH}=7.7 Hz, 2H), 7.20 (d, J_{HH}=16.2 Hz, 2H), 6.62 (s, 2H), 4.41 (q, J_{HH}=7.2 Hz, 2H), 3.51 (s, 4H), 2.39 (s, 12H), 1.42 (t, J_{HH}=7.3 Hz, 3H), 1.40 ppm (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ=166.2, 152.9, 142.2, 139.9, 137.9, 136.4, 133.4, 132.3, 131.4, 130.5, 128.9, 127.6, 123.7, 120.0, 118.5, 86.3, 85.9, 61.6, 48.9, 46.4, 44.4, 15.0, 14.5 ppm; MS (ESI, positive mode): m/z (%): calcd for $C_{46}H_{45}BF_2N_4O_2$: 733.4;

found: 735.4 $[M+H]^+$ (100); elemental analysis calcd (%) for $C_{46}H_{45}BF_2N_4O_2$: C 75.20, H 6.17, N 7.62; found: C 74.89, H 5.92, N 7.45.

Compound 6: Distyryl-BODIPY **3** (104 mg, 0.10 mmol) was dissolved in a mixture of absolute EtOH (4 mL) and triethylamine (3 mL). $[PdCl_2(PPh_3)_2]$ (8 mg, 0.01 mmol) was added and the reaction mixture was kept under a CO atmosphere for 5 h and heated at 60 °C. Thereafter, volatiles were removed and the resulting residue was purified by chromatography on a silica gel column with the following eluent (CH_2Cl_2/CH_3OH 93:7) to afford the ethyl ester **6** in a pure form (116 mg, quantitative yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.24 (d, J_{HH} = 16.3 Hz, 2H), 8.19 (d, J_{HH} = 8.5 Hz, 2H), 7.58 (d, J_{HH} = 8,3 Hz, 4H), 7.48 (d, J_{HH} = 8.3 Hz, 4H), 7.46 (d, J_{HH} = 7.9 Hz, 2H), 7.13 (d, J_{HH} = 16.5 Hz, 2H), 6.65 (s, 2H), 4.43 (q, J_{HH} = 7.2 Hz, 2H), 4.15 (s, 4H), 3.59 (s, 4H), 3.49 (m, 4H), 3.20 (s, 6H), 3.16 (m, 4H), 2.46 (s, 12H), 1.45 (t, J_{HH} = 7.3 Hz, 3H), 1.41 ppm (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 166.2, 152.1, 140.7, 140.3, 138.3, 132.5, 131.3, 130.4, 129.1, 127.4, 123.2, 121.9, 118.8, 92.1, 86.4, 85.4, 61.6, 59.6, 59.0, 48.7, 46.0, 44.1, 29.9, 15.2, 14.5, 8.8 ppm; MS (ESI, positive mode): *m/z* (%): calcd for $C_{58}H_{63}BN_4O_6$: 921.5; found: 922.4 $[M+H]^+$ (100); elemental analysis calcd (%) for $C_{58}H_{63}BN_4O_6{\cdot}H_2O$: C 74.03, H 6.96, N 5.95; found: C 73.78, H 6.70, N 5.66.

Compound 7: The same procedure as described above was applied to distyryl-BODIPY **4** (100 mg, 0.11 mmol). Purification of the crude product by chromatography on an Al_2O_3 column with the following eluent (CH_2Cl_2/CH_3OH 93:7) afforded the ethyl ester **7** in a pure form (80 mg, 85%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.35 (d, J_{HH} = 16.3 Hz, 2H), 8.17 (d, J_{HH} = 8.1 Hz, 2H), 7.57 (d, J_{HH} = 8.4 Hz, 4H), 7.43 (dd, 6H), 7.06 (d, J_{HH} = 16.3 Hz, 2H), 6.64 (s, 2H), 4.41 (q, J_{HH} = 7.4 Hz, 2H), 3.49 (s, 4H), 3.17 (s, 4H), 2.37(s, 12H), 2.17 (s, 12H), 1.42 (t, J_{HH} = 7.2 Hz, 3H), 1.39 ppm (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 166.0, 151.9, 140.3, 138.0, 136.8, 133.4, 132.1, 131.5, 128.9, 127.2, 123.3, 122.0, 118.5, 90.7, 86.6, 85.4, 61.4, 48.9, 44.3, 29.7, 15.0, 14.3 ppm; MS (ESI, positive mode): *m/z* (%): calcd for $C_{56}H_{61}BN_6O_2$: 859.5; found: 860.3 $[M+H]^+$ (100); elemental analysis calcd (%) for $C_{56}H_{61}BN_6O_2{\cdot}H_2O$: C 76.52, H 7.22, N 9.56; found: C 76.22, H 6.89, N 9.37.

Compound 8: Distyryl-BODIPY **5** (62 mg, 0.084 mmol) was dissolved in dry DMF (3 mL). 1,3-Propanesultone (103 mg, 0.85 mmol) was added and the resulting reaction mixture was stirred at 60 °C overnight, until the complete consumption of the starting material was observed by TLC analysis (EtOH/H_2O 8:2). Thereafter, the reaction mixture was centrifuged. The recovered precipitated product was purified by chromatography on a silica gel column with the following eluant (EtOH/H_2O 7:3) and finally recrystallized with a mixture of methanol and EtOAc to afford the bis(sulfobetain) **8** in a pure form (36 mg, 60%). ^{1}H NMR (CD_3OD/$CDCl_3$, 300 MHz): δ = 8.03 (d, J_{HH} = 8.2 Hz, 2H), 7.56 (d, J_{HH} = 16.3 Hz, 4H), 7.44 (d, J_{HH} = 8.1 Hz, 4H), 7.36 (d, J_{HH} = 8.3 Hz, 4H), 7.29 (d, J_{HH} = 8.2 Hz, 2H), 7.08 (d, J_{HH} = 16.2 Hz, 4H), 6.52 (s, 2H), 4.31 (s, 4H), 4.27 (m, 2H), 3.05 (s, 12H), 2.75 (m, 4H), 2.09 (m, 4H), 1.27 ppm (m, 9H); ^{13}C NMR (CD_3OD/$CDCl_3$, 75 MHz): δ = 166.4, 152.6, 142.7, 139.6, 137.7, 136.7, 133.7, 132.8, 131.8, 131.0, 129.3, 127.5, 123.9, 121.0, 117.9, 86.7, 85.5, 61.6, 61.4, 53.4, 51.9, 51.8, 18.3, 15.2, 14.3 ppm; MS (ESI, positive mode): *m/z* (%): calcd for $C_{52}H_{57}BF_2N_4O_8S_2$: 977.4; found: 979.4 $[M+H]^+$ (100); the compound was too hygroscopic for suitable elemental analysis.

Compound 9: Distyryl-BODIPY **6** (50 mg, 0.054 mmol) was dissolved in dry $C_2H_4Cl_2$ (3 mL). 1,3-Propanesultone (66 mg, 0.54 mmol) was added and the resulting reaction mixture was stirred at 60 °C overnight, until the complete consumption of the starting material was observed by TLC analysis (EtOH/H_2O 8:2). Thereafter, the reaction mixture was centrifuged. The recovered precipitated product was purified by chromatography on a silica gel column by using the following eluent (EtOH/H_2O 7:3) and finally recrystallized with a mixture of methanol and EtOAc to afford the bis(sulfobetain) **9** in a pure form (38 mg, 60%). ^{1}H NMR (CD_3OD/$CDCl_3$, 300 MHz): δ = 8.24 (d, J_{HH} = 16.4 Hz, 2H), 8.16 (d, J_{HH} = 8.1 Hz, 2H), 7.61 (d, J_{HH} = 8.2 Hz, 4H), 7.55 (d, J_{HH} = 8.2 Hz, 4H), 7.44 (d, J_{HH} = 8.3 Hz, 2H), 7.14 (d, J_{HH} = 16.2 Hz, 2H), 6.67 (s, 2H), 4.49 (s, 4H), 4.39 (q, J_{HH} = 7.3 Hz, 2H), 4.09 (s, 4H), 3.69 (m, 4H), 3.28 (m, 4H), 3.21 (s, 12H), 3.14 (m, 10H), 2.90 (m, 4H), 2.24 (m, 4H), 1.39 ppm (m, 9H); ^{13}C NMR (CD_3OD/$CDCl_3$, 75 MHz): δ = 166.2, 151.5, 140.8, 139.8, 138.5, 132.9, 131.5, 130.9, 130.1, 128.7, 127.2, 122.5, 119.9, 118.6, 91.8, 67.9, 63.1, 61.4, 59.1, 58.2, 55.3, 41.9, 18.7, 14.7, 13.9 ppm; MS (ESI, positive mode): *m/z* (%): calcd for $C_{64}H_{75}BN_4O_{12}S_2$: 1165.5; found: 1167.5 $[M+H]^+$ (100); the compound was too hygroscopic for suitable elemental analysis.

Compound 10: Distyryl-BODIPY **7** (88 mg, 0.102 mmol) was dissolved in dry DMF (2 mL). 1,3-Propanesultone (250 mg, 2.05 mmol) was added and the resulting reaction mixture was stirred at 60 °C for 10 h. Then, addition of EtOAc led to precipitation of the crude product, which was recovered by centrifugation. Purification by flash chromatography on a RP-C_{18} silica gel column by using the following eluent (H_2O/THF 85:15) afforded **10** in a pure form (60 mg, 43%). ^{1}H NMR (CD_3OD/D_2O, 400 MHz): δ = 8.31 (d, J_{HH} = 8.4 Hz, 2H), 8.22 (d, J_{HH} = 16.2 Hz, 2H), 7.84 (s, 8H), 7.58 (d, J_{HH} = 16.2 Hz, 2H), 7.04 (s, 2H), 4.66 (s, 4H), 4.50 (q, J_{HH} = 7.2 Hz, 2H), 4.36 (s, 4H), 3.82 (m, 4H), 3.50 (m, 4H), 3.35 (s, 12H), 3.04 (s, 16H), 2.70 (m, 4H), 2.39 (m, 4H), 2.14 (m, 4H), 1.56 (s, 6H), 1.50 ppm (t, J_{HH} = 7.20 Hz, 3H); ^{13}C NMR (CD_3OD/D_2O, 100 MHz): δ = 153.0, 143.5, 139.1, 136.3, 134.5, 132.9, 131.6, 130.2, 128.6, 122.7, 120.5, 92.6, 79.2, 64.2, 63.8, 62.8, 56.6, 20.1, 15.2, 14.6 ppm; MS (ESI, positive mode): *m/z* (%): calcd for $C_{68}H_{85}BN_6O_{14}S_4$: 1347.5; found: 1349.6 $[M+H]^+$ (100); the compound was too hygroscopic for suitable elemental analysis.

Compound 11

Saponification: Ethyl ester **8** (21.4 mg, 0.02 mmol) was dissolved in a mixture of EtOH (1.5 mL) and deionized water (0.75 mL), and then aqueous 1.0 M NaOH (0.33 mL, 0.33 mmol) was added. The resulting reaction mixture was stirred at RT until the complete consumption of the staring material was observed by RP-HPLC (system A) and MS (ESI). The reaction was quenched by adding concentrated HCl (30 μL, 0.36 mmol) and purified by RP-HPLC (system B, 1 injection, t_R = 22.7–25.0 min). The product-containing fractions were lyophilized to give the carboxylic acid intermediate as a blue amorphous powder (10.4 mg, 50%). HPLC (system A): t_R = 15.9 min (compared to t_R = 19.9 min for ethyl ester **8**); MS (ESI, positive mode): *m/z* calcd for $C_{50}H_{53}BF_2N_4O_8S_2$: 950.33; found: 951.20 $[M+H]^+$.

Conversion into N-*hydroxysuccinimidyl ester*: BODIPY carboxylic acid (10.4 mg, 8.9 μmol, 1 equiv) was dissolved in dry NMP (410 μL). A solution of TSTU (507 μL) in dry NMP (35.6 mg, 13.3 equiv) and a 2.0 M solution of DIEA (35 μL) in dry NMP (70 μmol, 8 equiv) were sequentially added and the resulting mixture was protected from light and stirred at RT overnight. The reaction was checked for completion by RP-HPLC (system A). The resulting crude NHS ester was used without further purification. HPLC (system A): t_R = 17.1 min (compared to t_R = 15.9 min for the free carboxylic acid).

Coupling reaction: α-Sulfo-β-alaninyl-α-sulfo-β-alanine (80 mg, 0.15 mmol) was dissolved in aqueous 0.25 M $NaHCO_3$ buffer (pH 8.2, 1.2 mL) and the resulting solution was cooled to 4 °C. The crude solution of NHS ester in NMP (see above) was added dropwise to this stirred solution and the resulting reaction mixture was stirred at RT. The reaction was checked for completion by RP-HPLC (system C). Thereafter, the mixture was diluted with aqueous 50 mM TEAB and purified by RP-HPLC (system D). The product-containing fractions were lyophilized to give the TEA salt of water-soluble BODIPY. Desalting by ion-exchange chromatography followed by lyophilization afforded the acid form of **11** as a blue amorphous powder (0.75 mg, yield 6%, mixture of two racemic diastereomers). HPLC (system C): t_R = 11.9 min, purity 96%; MS (ESI, negative mode): *m/z* (%): calcd for $C_{56}H_{63}BF_2N_6O_{16}S_4$: 1252.3; found: 1251.0 $[M-H]^-$ (100); this compound was obtained in too small amounts to be well-characterized by NMR spectroscopy.

Compound 13: Ethyl ester **10** (60 mg, 0.04 mmol) was dissolved in a mixture of EtOH (2 mL) and deionized water (0.5 mL), and then NaOH (18 mg, 0.4 mmol) was added. The resulting reaction mixture was stirred at RT for 10 h until the complete consumption of the starting ester was observed by TLC analysis (EtOH/H_2O 7:3). The reaction was quenched by adding aqueous 2% HCl solution until pH ≈7 was obtained, and subsequent addition of EtOAc led to precipitation of the crude product, which was isolated by centrifugation. Purification by flash chromatography on a RP-C_{18} silica gel column by using the following eluent (H_2O/

THF 95:5) afforded **13** in a pure form (15 mg, 25%). ^{1}H NMR (CD_3OD/D_2O, 200 MHz): δ=8.26 (d, J_{HH}=16.2 Hz, 2H), 8.20 (d, J_{HH}=7.9 Hz, 2H), 7.98 (s, 8H), 7.61 (d, J_{HH}=16.2 Hz, 2H), 7.41 (d, J_{HH}=8.3 Hz, 2H), 6.99 (s, 2H), 4.67 (s, 4H), 4.37 (s, 4H), 3.80 (m, 4H), 3.51 (m, 4H), 3.46 (s, 12H), 3.04 (s, 16H), 2.72 (m, 4H), 2.39 (m, 4H), 1.54 ppm (s, 6H); ^{13}C NMR (CD_3OD/D_2O, 50 MHz): δ=153.2, 144.1, 142.3, 139.6, 138.0, 136.5, 135.0, 133.6, 131.8, 129.5, 123.1, 120.8, 93.0, 79.7, 64.6, 64.3, 57.1, 20.6, 15.6 ppm; HPLC (system C): t_R=4.3 min, purity 97%; MS (ESI, positive mode): *m*/*z* (%): calcd for $C_{66}H_{81}BN_6O_{14}S_4$: 1319.5; found: 1321.3 $[M+H]^+$ (100), 660.6 $[(M+2H)/2]^{2+}$ (50); elemental analysis calcd (%) for $C_{66}H_{81}BN_6O_{14}S_4{\cdot}H_2O$: C 59.18, H 6.25, N 6.27; found: C 58.99, H 5.92, N 5.99.

4-[(Dimethylamino)methyl]benzaldehyde (B): *N,N*-Dimethyl-*N*-(4-iodobenzyl)amine (500 mg, 1.92 mmol), anhydrous sodium formate (156 mg, 2.30 mmol), and $[PdCl_2(PPh_3)_2]$ (69 mg, 0.096 mmol) were dissolved in dry DMF (10 mL). The resulting reaction mixture was stirred at 100°C for 4 h with bubbling CO gas. Thereafter, the reaction mixture was diluted with EtOAc, washed with deionized water (3×70 mL), and dried over anhydrous $MgSO_4$. After evaporation, the resulting residue was purified by chromatography on an Al_2O_3 column by using CH_2Cl_2 as the eluent to afford **B** in a pure form (283 mg, 91%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ= 9.93 (s, 1H), 7.77 (d, J_{HH}=8.0 Hz, 2H), 7.42 (d, J_{HH}=8.0 Hz, 2H), 3.43 (s, 2H), 2.20 ppm (s, 6H); other spectroscopic data are identical to those already reported in the literature.[33]

Compound 14: BODIPY **1** (450 mg, 0.70 mmol) and 4-[(dimethylamino)-methyl]benzaldehyde **B** (227 mg, 1.39 mmol) were dissolved in dry toluene (5 mL) and piperidine (0.5 mL). The resulting reaction mixture was heated at 140°C in a Dean–Stark apparatus for 2 h. Thereafter, the Dean–Stark receiver was removed and the mixture was heated with argon bubbling until the solvent was completely removed. The resulting crude product was treated with saturated aqueous $NaHCO_3$ and deionized water, and finally extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$ and evaporated to dryness. The resulting residue was purified by chromatography on an Al_2O_3 column by using CH_2Cl_2 as the eluent to afford the distyryl-BODIPY **14** (210 mg, 40%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ=7.84 (d, J_{HH}=8.3 Hz, 2H), 7.70 (d, J_{HH}=16.6 Hz, 2H), 7.57 (d, J_{HH}=8.3 Hz, 4H), 7.33 (d, J_{HH}=8.2 Hz, 4H), 7.24 (d, J_{HH}=16.4 Hz, 2H), 7.08 (d, J_{HH}=8.2 Hz, 2H), 6.63 (s, 2H), 3.49 (s, 4H), 2.27 (s, 12H), 1.47 ppm (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ= 142.1, 138.5, 136.5, 135.8, 134.9, 130.7, 127.8, 119.2, 118.3, 64.0, 45.3, 29.9, 22.9, 15.2, 14.3 ppm; MS (ESI, positive mode): *m*/*z* (%): calcd for $C_{39}H_{40}BF_2IN_4$: 739.2; found: 740.2 $[M+H]^+$ (100); elemental analysis calcd (%) for $C_{39}H_{40}BF_2IN_4$: C 63.26, H 5.44, N 7.57; found: C 63.03, H 5.13, N 7.34.

Compound 15: A solution of 1-dimethylamino-2-propyne (121 mg, 1.46 mmol) in dry THF (3 mL) was placed in a Schlenk tube under an argon atmosphere. A 1.0 M solution of EtMgBr in THF (1.28 mL) was added and the reaction mixture was stirred at 60°C for 1 h. The resulting anion was then transferred through a cannula to a solution of distryryl-BODIPY **14** (260 mg, 0.35 mmol) in dry THF (3 mL) previously placed in a Schlenk tube under an argon atmosphere. The resulting reaction mixture was stirred at 60°C for 15 min until the complete consumption of the starting BODIPY was observed by TLC analysis. Then, the mixture was sequentially treated with deionized water and brine and then extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$ and evaporated to dryness. The crude product was purified by chromatography on an Al_2O_3 column with a step gradient of CH_3OH (0–1%) in CH_2Cl_2 as the mobile phase to give the bis(ethynyl) BODIPY **15** (247 mg, 81%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ=8.34 (d, J_{HH}=16.3 Hz, 2H), 7.80 (d, J_{HH}=8.3 Hz, 2H), 7.57 (d, J_{HH}=8.0 Hz, 4H), 7.29 (d, J_{HH}= 8.3 Hz, 4H), 7.13 (d, J_{HH}=16.4 Hz, 2H), 7.06 (d, J_{HH}=8.4 Hz, 2H), 6.61 (s, 2H), 3.42 (s, 4H), 3.17 (s, 4H), 2.23 (s, 12H), 2.15 (s, 12H), 1.43 ppm (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ=152.2, 140.2, 139.8, 138.3, 137.6, 136.2, 135.4, 134.1, 131.6, 130.8, 129.6, 127.5, 121.4, 118.4, 94.7, 90.7, 64.3, 49.0, 45.5, 44.3, 15.3 ppm; MS (ESI, positive mode): *m*/*z* (%): calcd for $C_{49}H_{56}BIN_6$: 865.4; found: 866.2 $[M+H]^+$ (100); elemental analysis calcd (%) for $C_{49}H_{56}BIN_6{\cdot}2H_2O$: C 65.19, H 6.70, N 9.31; found: C 64.84, H 6.62, N 9.04.

Compound 16: Distyryl-BODIPY **15** (100 mg, 0.11 mmol) was dissolved in a mixture of absolute EtOH (4 mL) and triethylamine (3 mL). $[PdCl_2(PPh_3)_2]$ (8 mg, 0.01 mmol) was added and the reaction mixture was kept under a CO atmosphere for 5 h and heated at 60°C. Thereafter, volatiles were removed and the resulting residue was dissolved in CH_2Cl_2. The organic layer was washed with saturated aqueous $NaHCO_3$, deionized water, dried over anhydrous $MgSO_4$, and then evaporated to dryness. The resulting residue was purified by chromatography on an Al_2O_3 column by using a step gradient of CH_3OH (0–1%) in CH_2Cl_2 as the mobile phase to afford the ethyl ester **16** in a pure form (80 mg, 85%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ=8.34 (d, J_{HH}=16.4 Hz, 2H), 8.16 (d, J_{HH}=8.2 Hz, 2H), 7.58 (d, J_{HH}=8.1 Hz, 4H), 7.43 (d, J_{HH}=8.4 Hz, 2H), 7.30 (d, J_{HH}=8.4 Hz, 4H), 7.14 (d, J_{HH}=16.5 Hz, 2H), 6.62 (s, 2H), 4.41 (q, J_{HH}=7.1 Hz, 2H), 3.43 (s, 4H), 3.18 (s, 4H), 2.24 (s, 12H), 2.16 (s, 12H), 1.41 (t, J_{HH}=7.2 Hz, 3H), 1.38 ppm (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ=166.3, 152.3, 140.6, 139.8, 137.8, 136.2, 134.2, 131.4, 130.3, 129.6, 127.5, 121.4, 118.5, 90.6, 64.3, 61.5, 53.6, 49.0, 45.6, 44.1, 15.2, 14.5 ppm; MS (ESI, positive mode): *m*/*z* (%): calcd for $C_{52}H_{61}BN_6O_2$: 811.5; found: 813.5 $[M+H]^+$ (100); elemental analysis calcd (%) for $C_{52}H_{61}BN_6O_2$: C 76.83, H 7.56, N 10.34; found: C 76.53, H 7.32, N 10.11.

Compound 17: BODIPY **16** (80 mg, 0.10 mmol) was dissolved in dry DMF (2 mL). 1,3-Propanesultone (240 mg, 1.97 mmol) was added and the resulting reaction mixture was stirred at 60°C for 12 h. Then, addition of EtOAc led to precipitation of the crude product, which was recovered by centrifugation. Purification by flash chromatography on a RP-C_{18} silica gel column by using the following eluent (H_2O/THF 85:15) afforded **17** in a pure form (82 mg, 63%). ^{1}H NMR (CD_3OD/D_2O, 300 MHz): δ=8.31 (d, J_{HH}=7.2 Hz, 2H), 8.28 (d, J_{HH}=16.1 Hz, 2H), 7.93 (d, J_{HH}= 7.3 Hz, 4H), 7.84 (d, J_{HH}=7.3 Hz, 4H), 7.61 (d, J_{HH}=16.4 Hz, 2H), 7.05 (s, 2H), 4.69 (s, 4H), 4.50 (q, J_{HH}=6.9 Hz, 2H), 4.38 (s, 4H), 3.62 (m, 4H), 3.54 (m, 4H), 3.21 (s, 12H), 3.07 (s, 12H), 3.02 (m, 4H), 2.64 (m, 4H), 2.41 (m, 4H), 2.15 (m, 4H), 1.56 (s, 6H), 1.50 ppm (t, J_{HH}=6.6 Hz, 3H); ^{13}C NMR (CD_3OD/D_2O, 75 MHz): δ=167.9, 153.2, 143.7, 140.8, 136.3, 135.6, 132.9, 131.7, 130.3, 129.8, 122.3, 120.6, 85.4, 69.4, 63.8, 56.8, 51.2, 50.0, 49.7, 20.1, 15.3, 14.8 ppm; MS (ESI, positive mode): *m*/*z* (%): calcd for $C_{64}H_{85}BN_6O_{14}S_4$: 1299.5; found: 1301.3 $[M+H]^+$ (100), 651.2 $[(M+2H)/2]^{2+}$ (55); elemental analysis calcd (%) for $C_{64}H_{85}BN_6O_{14}S_4{\cdot}1.5H_2O$: C 57.86, H 6.68, N 6.33; found: C 57.52, H 6.53, N 6.69.

Compound 18: Ethyl ester **17** (70 mg, 0.05 mmol) was dissolved in a mixture of EtOH (2 mL) and deionized water (0.5 mL), and then NaOH (21 mg, 0.5 mmol) was added. The resulting reaction mixture was stirred at RT for 10 h until complete consumption of the starting ester was observed by TLC analysis (EtOH/H_2O 7:3). The reaction was quenched by adding aqueous 2% HCl solution until pH ≈7 was obtained, and subsequent addition of EtOAc led to precipitation of the crude product, which was isolated by centrifugation. Purification by flash chromatography on a RP-C_{18} silica gel column by using a step gradient of THF (5–10%) in deionized water as the mobile phase afforded **18** in a pure form (60 mg, 88%). ^{1}H NMR (CD_3OD/D_2O, 300 MHz): δ=8.26 (d, J_{HH}=16.3 Hz, 2H), 8.21 (d, J_{HH}=8.3 Hz, 2H), 7.92 (d, J_{HH}=8.2 Hz, 4H), 7.83 (d, J_{HH}= 8.2 Hz, 4H), 7.58 (d, J_{HH}=16.2 Hz, 2H), 7.44 (d, J_{HH}=8.3 Hz, 2H), 7.01 (s, 2H), 4.66 (s, 4H), 4.36 (s, 4H), 3.64 (m, 4H), 3.51 (m, 4H), 3.21 (s, 12H), 3.06 (s, 12H), 3.03 (m, 4H), 2.65 (t, J_{HH}=6.8 Hz, 4H), 2.42 (m, 4H), 2.14 (m, 4H), 1.53 ppm (s, 6H); ^{13}C NMR (CD_3OD/D_2O, 75 MHz): δ=152.9, 143.8, 139.9, 135.5, 133.0, 131.6, 129.7, 122.2, 120.5, 85.3, 80.2, 69.3, 67.1, 63.7, 56.8, 51.1, 20.0, 15.3 ppm; MS (ESI, positive mode): *m*/*z* (%): calcd for $C_{62}H_{81}BN_6O_{14}S_4$: 1271.5; found: 1273.4 $[M+H]^+$ (100), 637.2 $[(M+2H)/2]^{2+}$ (35); elemental analysis calcd (%) for $C_{62}H_{81}BN_6O_{14}S_4{\cdot}2.5H_2O$: C 56.48, H 6.57, N 6.37; found: C 56.18, H 6.29, N 6.76.

Preparation of BODIPY/cyanine–protein conjugates

General procedure for the conversion into N*-hydroxysulfosuccinimidyl ester*: The water-soluble BODIPY dye carboxylic acid (0.37 μmol, weighed in a 0.5 mL Eppendorf microtutube) was dissolved in deionized water (50 μL). An aqueous solution of water-soluble carbodiimide (30 μL, EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, 12–15 equiv) and an aqueous solution of sulfo-NHS (10 μL, 0.17 mg,

0.78 μmol, 2 equiv) were sequentially added and the resulting reaction mixture was protected from light and periodically stirred by use of a vortex mixer. The reaction was checked for completion by RP-HPLC (system A or C). The resulting *N*-hydroxysulfosuccinimidyl ester was used in the next labeling step without purification.

Sulfo-NHS ester from ***12***: HPLC (system C): $t_R = 10.3$ min (compared to $t_R = 9.7$ min for water-soluble BODIPY carboxylic acid **12**).

Sulfo-NHS ester from ***13***: HPLC (system A): $t_R = 12.1$ min (compared to $t_R = 13.1$ min for water-soluble BODIPY carboxylic acid **13**).

Fluorescent labeling of proteins: The solution of *N*-hydroxysulfosuccinimidyl ester (see above, 45 μL, 185 nmol, 14- or 31-fold excess according to protein) was added to a solution of BSA (500 μL, 1.8 mg mL^{-1}, 13 nmol) or anti-HA mAb (1.8 mg mL^{-1}, 6 nmol) in phosphate buffer (pH 7.4). The resulting mixture was protected from the light and periodically stirred by use of a vortex mixer. The reaction was left at 4 °C overnight. Thereafter, the BODIPY–BSA or BODIPY–mAb conjugate was purified by size-exclusion chromatography with an Econo-Pac Disposable chromatography column (Bio-Rad, #732–1010) filled with an aqueous solution of Sephadex G-25 Fine (Amersham Biosciences AB, 15 × 40 mm bed), equilibrated with PBS (0.01 M phosphate, 0.015 M NaCl, pH 7.5). The dye-to-protein ratio (*F*/*P*) of these conjugates was determined spectrophotometrically by measuring their absorbance at 280 and 642 nm and inserting the measured values into Equation (2) in which A_{280} is the absorbance of the protein at 280 nm, ${}^{P}\varepsilon_{280}$ is the extinction coefficient of the protein at 280 nm, A_{max} is the absorbance of the BODIPY label as its absorption maximum, ${}^{F}\varepsilon_{max}$ is the extinction coefficient of the fluorophore at the absorption maximum, and ${}^{F}\varepsilon_{280}$ is the extinction coefficient of the fluorophore at 280 nm (10767 for **12**, 12618 for **13**, and 25464 M^{-1} cm^{-1} for Cy 5.0). We used a correction procedure for the visible part of the spectra at high *F*/*P* ratios. Specifically, we considered the integrated area under the visible part of the spectrum (and not the height of the dye peak) for quantization of the dye in solution. We assumed that the quantity of the dye is proportional to the integrated visible absorption spectrum or area under the curve (AUC) from 475 to 750 nm, and calculated a correction factor for each conjugate equal to the ratio of the AUC for the conjugate to the AUC for the free nonbound label (normalized spectra). Anti-HA mAb and BSA have an extinction coefficient at 280 nm of 295000 and 43824 M^{-1} cm^{-1} respectively.

$$F/P = A_{max}{}^{P}\varepsilon_{280}/(A_{280}{}^{F}\varepsilon_{max} + A_{max}{}^{F}\varepsilon_{280}) \quad (2)$$

Further fluorescent labeling experiments were performed with sulfoindocyanine dye Cy 5.0 (${}^{F}\varepsilon_{max} = 250000$ M^{-1} cm^{-1}) under the same conditions.

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Go bio! Water-soluble red-emitting borondipyrromethene (BODIPY) derivatives have been prepared by using mild sulfonation procedures (see figure). The sterically demanding substituents on boron and sulfonate groups on the top/bottom parts of the distyryl-BODIPY scaffold effectively suppress their aggregation in aqueous solution. These bright fluorophores show promise as biomolecule labels.

Biolabeling

S.-l. Niu, C. Massif, G. Ulrich, P.-Y. Renard, A. Romieu, R. Ziessel** ..... ▮▮▮▮–▮▮▮▮

Water-Soluble Red-Emitting Distyryl-Borondipyrromethene (BODIPY) Dyes for Biolabeling