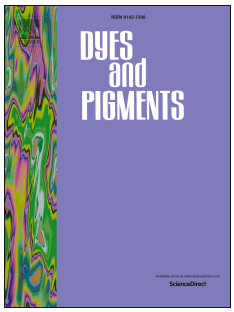

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Synthesis and characterization of two novel red-shifted isothiocyanate BODIPYs and their application in protein conjugation

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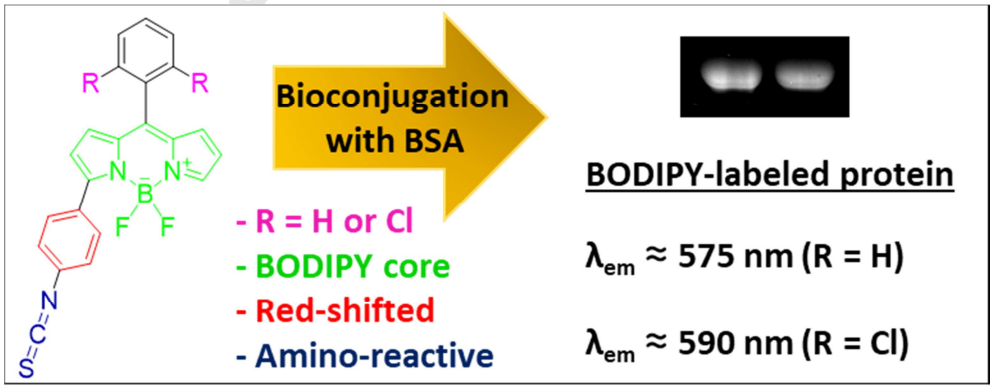
Abstract

Fluorescent organic small molecules are very important tools for researchers in the fields of biochemistry and biotechnology, and boron-dipyrromethene (BODIPY) class of organic fluorophores gained much attention in recent years due to their remarkable photophysical properties and chemical versatility. Their application often involves conjugation with biomolecules, which can be accomplished by inserting a reactive functionality to the fluorescent dye. In this paper we present the preparation and characterization of two unpublished BODIPYs bearing the electrophilic isothiocyanate group, widely used for conjugation with proteins and biological amines. These dyes were prepared by inserting a nitrobenzene group to the BODIPY core via direct arylation, which was later converted to the isothiocyanate functionality. The arylated BODIPYs retained their fluorescence and showed red-shifted absorption and fluorescence spectra, when compared to previously published isothiocyanate-BODIPYs. Their application in bioconjugation was tested with bovine serum albumin (BSA) and was confirmed from spectroscopic and electrophoretic analysis.

Graphical abstract



Keywords: BODIPY; Bioconjugation; Isothiocyanate; Protein; Fluorescence, Arylation

Highlights

- Preparation of two unpublished amino-reactive BODIPYs
- A red-shifted and highly fluorescent isothiocyanate-BODIPY is presented
- Bioconjugation with a model protein was accomplished
- BODIPY labelled proteins with fluorophore:protein (F:P) ratio between 0.5 and 5.5

1. Introduction

In recent decades boron-dipyrromethene derivatives, more commonly known by the acronym "BODIPY", found widespread application as fluorescent dyes in several fields of technology [1-4]. The BODIPY core is a pyrrolic dye characterized by a tricyclic structure in which the C-2 of two pyrroles are connected by a methine bridge, and the pyrrolic nitrogen atoms are complexed with a boron atom, usually as a part of a difluoroboryl group (Figure 1). The BODIPY core is a highly emitting fluorophore, a consequence of their typical high fluorescence quantum yields and molar absorptivity. The fluorescence spectra of the BODIPY core is centred close to 500 nm and is weakly Stokes shifted, however their usual fluorescence properties can be manipulated *via* chemical modifications [5,6].

Since their first report, more than 40 years ago [7], several methods for the preparation and chemical modification of BODIPYs have been reported in the literature, rendering the BODIPY core an excellent choice for the development of tailored fluorescent dyes [6,8]. Red-shifted BODIPYs, for example, can be achieved from substitution reactions at positions 3 and 5, while a very well-established method to prepare blue-shifted BODIPYs involves the insertion of amines at position 8 [8-10].

The application of fluorescent dyes in biochemistry commonly involves bioconjugation with biomolecules, such as antibodies and proteins in general. In this context, fluorescent dyes are commonly functionalized with the isothiocyanate amino-reactive group, aiming to prepare fluorophores for bioconjugation [11]. Fluorescein isothiocyanate (FITC) is the classic example of an isothiocyanate-functionalized fluorophore and some BODIPY derivatives have also been synthesized, however their peak fluorescence is usually around 500 nm [12,13]. For this paper we designed two isothiocyanate-functionalized BODIPYs, with an aryl group at position 3 for a slightly red-shifted emission (Figure 1).

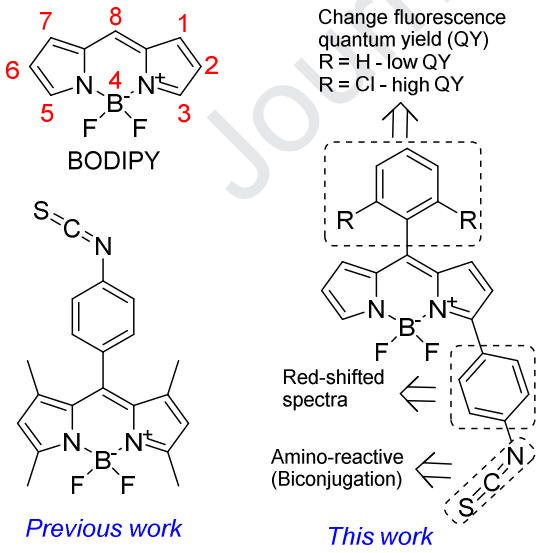
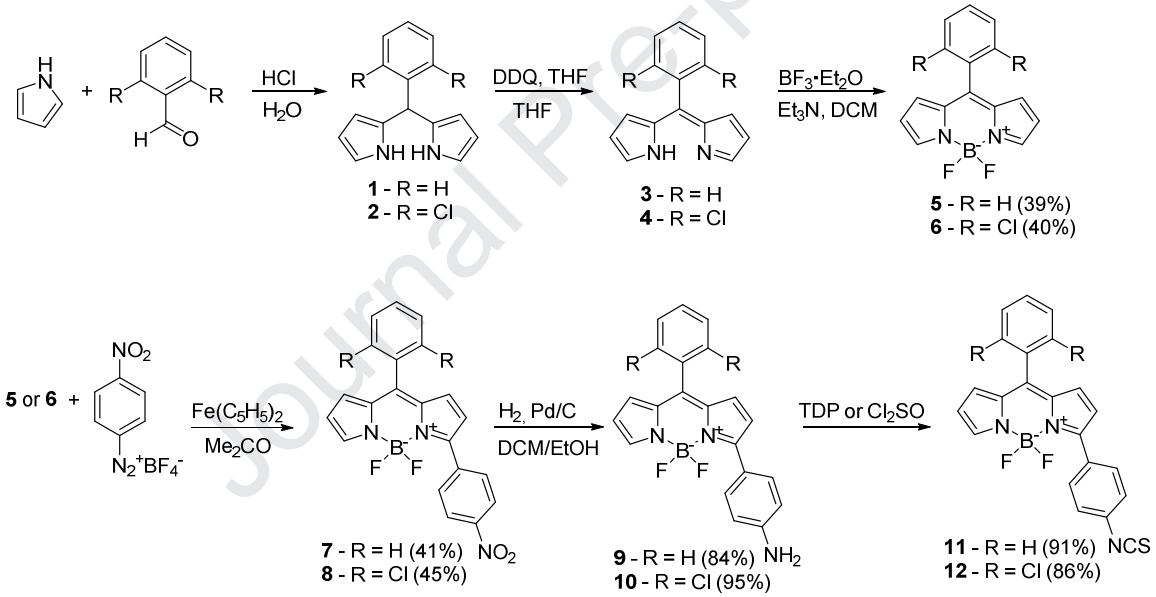


Figure 1. Top left: General structure and numbering system of the BODIPY core. Bottom left: Chemical structure of a previously published isothiocyanate-BODIPY. Right: Design of the red-shifted BODIPYs presented in this paper.

2. Results and discussion

2.1 Synthesis

The synthesis of isothiocyanate BODIPYs (Scheme 1) started with the preparation of dipyrromethanes **1** and **2**, from the aqueous reaction of pyrrole with benzaldehyde (for compound **1**) or 2,6-dichlorobenzaldehyde (for compound **2**). Based on our previous experience the dipyrromethanes **1** and **2** were not isolated, instead, the crude products were readily oxidized with DDQ to yield dipyrrins **3** and **4**, respectively [14]. The complexation of compounds **3** and **4** with $BF_3{\cdot}Et_2O$ in organic basic medium yielded, respectively, BODIPYs **5** and **6** in 39% and 40%, for the three reaction steps. We used a direct arylation procedure based on a radical reaction with an aryl diazonium salt to prepare compounds **7** and **8** (41% and 45% yield) [15]. The objectives with the arylation reaction were to shift the absorption and emission spectra and to introduce the nitro functionality as a precursor of the isothiocyanate group. The formation of diarylated BODIPYs seemed as an unavoidable side reaction, however the two products could be easily separated *via* silica gel column chromatography. The last two steps were functional group conversions, starting with the reduction of nitro groups of **7** and **8** to the aromatic amines in **9** (84%) and **10** (95%) via catalytic hydrogenation [12]. Finally, the conversion of amines **9** and **10** to the isothiocyanate derivatives **11** and **12** were accomplished using different methods. Compound **9** was converted to compound **11** in 91% yield using 1,1'-Thio-carbonyldi-2,2'-pyridone (TDP), while the preparation of **12** from **10** was done with thiophosgene, in a reaction with 86% yield [12,13].



Scheme 1. Synthetic route applied for the preparation of isothiocyanato-BODIPYs **11** an **12**.

The chemical structure of compounds **7** – **12** were confirmed by mass spectrometry and nuclear magnetic resonance spectroscopy, and the characterization data for compounds **7** - **12** can be found as supplemental file. The two functional group conversions leading to the isothiocyanate functionality (compounds **11** and **12**) from the nitrophenyl substituted BODIPY (compounds **7** and **8**) can be easily confirmed *via* ^{1}H-NMR spectroscopy. In figure 2 a low field section of the ^{1}H-NMR containing the signals of the aromatic hydrogens of compounds **8**, **10** and **12** are shown.

The hydrogens at the substituting aromatic ring, indicated as H^a and H^b, can be unambiguously correlated with their respective signals from their multiplicity and integration. Hydrogens H^a and H^b in compound **8** are strongly deshielded, due to the electron withdrawing effect of the

nitro group and the BODIPY core, which is reflected on their chemical shifts (8.33 ppm and 8.14 ppm, respectively). As expected, an upfield shift was observed for H^a and H^b in compound **10** (7.97 ppm for H^b and 6.75 ppm for H^a), and its conversion to the isothiocyanate derivative **12** resulted in an downfield shift of H^a (7.33 ppm) while the chemical shift of H^b was unaltered (8.01 ppm). A similar trend was observed for the chemical shifts of hydrogen atoms directly linked to the BODIPY core. In general, the reduction of the nitro group of compound **8** to the amino group in compound **10** resulted in an upfield shift, which was partially reverted when the amino group was converted to isothiocyanate in compound **12**. This observation confirms the existence of a resonance interaction between the BODIPY core and the phenyl group at position 3 and can be exemplified by the chemical shift of H^c, which was 7.95 ppm for compound **8**, 7.75 ppm for compound **10** and 7.88 ppm for compound **12**. Moreover, the absence of resonance interaction between the BODIPY core and the meso-aromatic substituent is well reported, [16,17] and it explains the absence of any relevant upfield or downfield shift for the hydrogens in the 2,6-dichlorophenyl substituent, indicated as H^d.

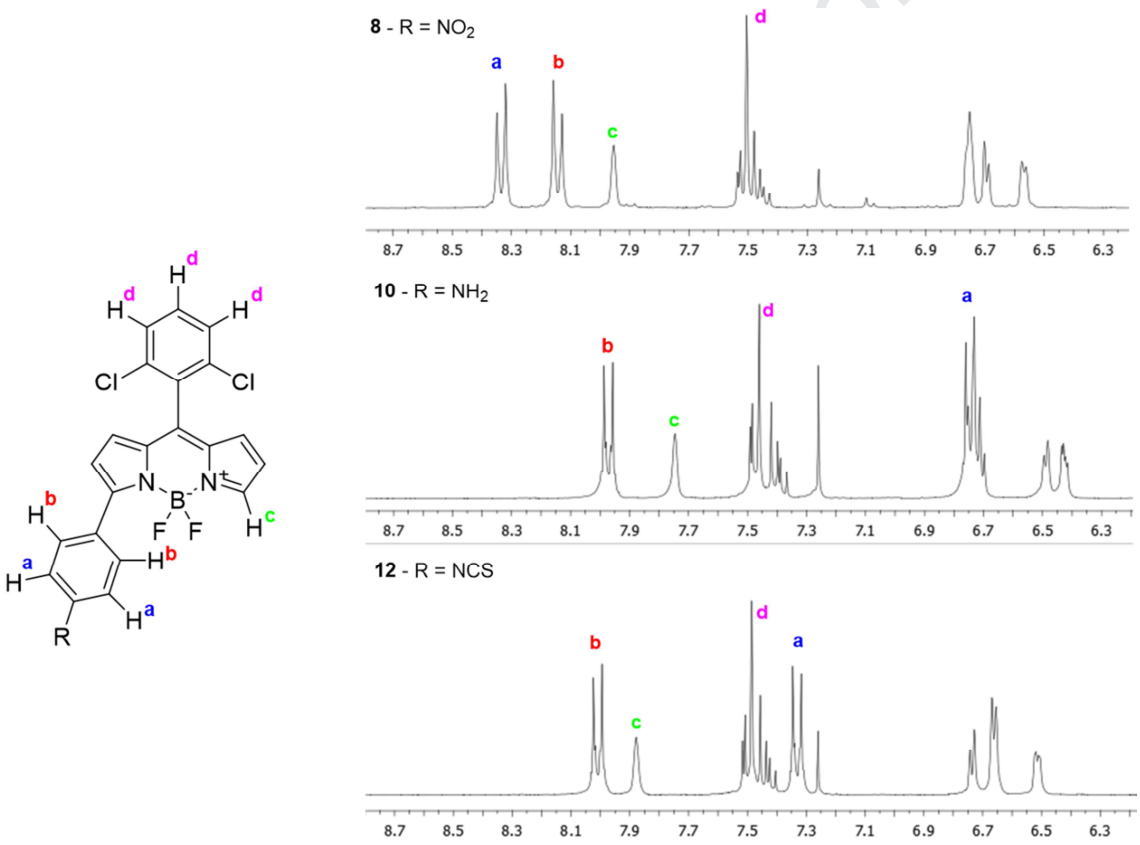


Figure 2. Low field section of the ^{1}H-NMR of compounds **8**, **10** and **12**, confirming the functional group conversions.

2.2 Photophysical properties

The UV-Vis absorption and the fluorescence emission of compounds **11** and **12** were characterized in selected solvents, to study their solvatochromic behaviour. Pentane (C_5H_{12}), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), ethyl acetate (EtOAc), acetone (Me_2CO), methanol (MeOH) and dimethylsulfoxide (DMSO) were selected for this characterization. A solution in aqueous buffer (PBS pH 7.4) could not be tested, once both compounds were completely insoluble in this solvent system. This was not surprising, as BODIPY dyes are often water-insoluble and chemical modifications are commonly applied to enhance their solubility

[18]. For biochemical applications, DMF and DMSO are often applied as co-solvents to work around the low solubility of organic fluorescent dyes.

The results of the photophysical characterization are presented in table 1 as the wavelength of maximum absorption and emission (λ_{max}), the full width at half maximum for absorption and emission spectra (FHWM), the Stokes shift and the fluorescence quantum yield (ϕ_{fl}). As expected, a red-shifted λ_{max} was observed for the absorption and emission of both compounds, when compared to the isothiocyanate-BODIPYs previously described in the literature [12,13]. Absorption and fluorescence maxima of *meso*-(2,6-dichlorophenyl)-BODIPYs are known to be slightly red-shifted when compared to meso phenyl-BODIPYs, [15,19] which was also observed for compound **12** whose absorption and fluorescence spectra were consistently more red-shifted (10 to 15 nm) when compared to compound **11**. The FWHM parameter is shown as an estimate for peak width, and a slightly narrower peaks were observed for compound **12** when compared to **11**. The effect of solvent polarity over absorption and emission spectra was very weak and no clear trend was observed, as can be visualized in their spectra (supplemental file, Figures S1 – S4).

Regarding fluorescence quantum yields, previous reports indicates a stronger fluorescence emission for *meso*-(2,6-dichlorophenyl)-BODIPYs when compared to meso-phenyl BODIPYs [15,19]. This observation is explained by nonradiative decay pathways related to conformational changes of the S1 excited state in meso-phenyl BODIPYs, which is dramatically diminished in sterically hindered substituents, such as 2,6-dichlorophenyl, that prevents aryl rotation [16,17]. Our results are in accordance with these observations, as fluorescence quantum yields of **11** ranged from 0.05 to 0.13, while for compound **12** the results were closer to unity, ranging from 0.85 to 0.99.

Stokes shifts are presented in wavenumber and reflects the energy dissipation due to vibrational relaxation and the reorganization of solvents around the excited state [20]. Our results indicated slightly reduced Stokes shift for compound **12**, which is probably due to their red-shifted absorption and emission peaks, as the shifts are very similar if expressed in wavelength (between 23 and 29 nm for BODIPY **11**, and between 22 and 26 nm for BODIPY **12**). A slightly shorter Stokes shift was observed in pentane, a hydrocarbon solvent. We have previously reported a similar trend for other BODIPYs and it might reflect the absence of solvent dipole reorientation due to its low polarity [20,21].

Table 1. Photophysical properties of compounds **11** and **12** in seven organic solvents

Compound and solvent	Absorption		Emission		Stokes shift[b]	ϕ_{fl}
	λ_{max}[a]	FWHM[b]	λ_{max}[a]	FWHM[b]		
11						
C_5H_{12}	538	1562	561	1027	762	0.09
CH_2Cl_2	539	1661	566	1094	885	0.09
$CHCl_3$	540	1665	567	1060	882	0.14
EtOAc	537	1646	562	1062	828	0.07
CH_3COCH_3	536	1666	561	1079	831	0.05
MeOH	534	1695	561	1075	901	0.05
DMSO	543	1717	572	1084	934	0.10
12						
C_5H_{12}	551	1434	573	898	697	0.99
CH_2Cl_2	552	1530	578	935	815	0.99
$CHCl_3$	554	1551	578	944	749	0.95

EtOAc	550	1559	573	922	730	0.85
CH_3COCH_3	550	1517	576	886	821	0.90
MeOH	547	1606	571	932	768	0.94
DMSO	557	1567	583	881	801	0.97

[a]data shown in nm [b]data shown in cm^{-1}

2.3 Bioconjugation

BODIPYs **11** and **12** were designed as fluorescent probes for protein bioconjugation, and their application was tested using BSA as a model protein. Each compound was used in independent bioconjugation reactions with two different fluorophore:BSA molar ratio (10:1 and 25:1). Aiming to eliminate non-conjugated fluorophore, the products were submitted to a centrifugal dialysis against distilled water using a Vivaspin® Turbo 15 centrifugal concentrators (30 KDa MWCO), which we will refer as protocol A. The purification *via* protocol A seemed inefficient as the non-conjugated dye precipitated and was not visually observed in the dialysate, probably due to the low water-solubility of BODIPY dyes. To test another purification protocol (which we will refer to as protocol B), new batches of conjugation products were prepared using the same procedure. For purification protocol B we added three cycles of centrifugal dialysis against aqueous ethanol 70%, which better solubilizes the non-conjugated dye, followed by three cycles of dialysis against water. In protocol B the elimination of non-conjugated dyes could be visually observed by the colour and fluorescence appearing in ethanolic dialysate. Additionally, no precipitation was observed during this protocol, and the conjugated protein could be retrieved as a slightly coloured solid after lyophilization (supplemental file, Figures S5 and S6).

Figure 3 shows the absorption and emission spectra recorded for the BSA-BODIPY conjugates prepared with 25 eq of each dye and purified using Protocol B. UV-Vis absorption spectroscopy revealed the typical protein band at 280 nm, and the BODIPY band at 547 nm for BODIPY **11** and at 560 nm for BODIPY **12**. Fluorescence spectroscopy indicated that the BODIPY dyes retained their fluorescence after conjugation, with fluorescence emission peaks slightly shifted to 575 nm and 590 nm for BODIPYs **11** and BODIPY **12**, respectively. Normalized absorption and fluorescence spectra of each BSA-BODIPY conjugate can be found in the supplemental file (Figiures S7 – S10).

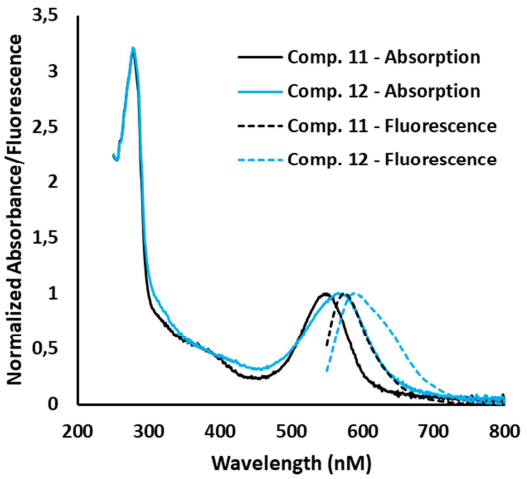


Figure 3. Normalized absorption (full line) and fluorescence (dotted line) spectra of compounds **11** (black) and **12** (blue) after conjugation with BSA.

Even though fluorescence quantum yield was not calculated for the BSA-BODIPY, their fluorescence intensity was seemingly less intense when compared to the non-conjugated dyes. Larger slit opening was necessary to record the fluorescence spectra of BSA-BODIPY conjugates when compared to solutions of the non-conjugated dyes with similar absorbance. This reduction of fluorescence intensity does not prevent their application in bioconjugation protocols and could be related to interactions with the protein/aqueous environment [24,25], or result from the formation of thiourea functionality. To better understand this observation, small-scale test reactions between the isothiocyanato-BODIPYs and an excess of diethylamine were performed (Figure S11). TLC control indicated an instantaneous full conversion of BODIPYs **11** and **12** to a more polar and less bright thiourea-BODIPY. Photophysical characterization of these thiourea-BODIPYs in methanol (Figure S12) indicated a slightly red-shifted absorption (λ_{max} = 543 nm, for compound **11**; λ_{max} = 555 nm for compound **12**) and emission spectra (λ_{max} = 572 nm, for compound **11**; λ_{max} = 581 nm for compound **12**, and reduced fluorescence quantum yields (ϕ_{fl} = 0.03 for compound **11**; ϕ_{fl} = 0.11 com compound **12**). This data suggests that the formation of thiourea functionality could explain the changes in the photophysical properties observed after protein conjugation.

The ratio between the absorbance of protein and that of the dye was used to estimate the degree of protein labelling (also known as F/P ratio), which estimates the number of fluorophore molecules per molecule of protein [24]. As expected, a slightly higher degree of protein labelling was observed when 25 equivalents of the fluorophore were used in the bioconjugation reaction (Table 2). Additionally, a lower F:P ratio was observed when the bioconjugation product was purified in the dialysis with ethanol, reinforcing the conclusion that this a better purification method.

Table 2. Application of **11** and **12** in bioconjugation with BSA.

Comp.	ε^a	CF[b]	Dye excess	Purification	F:P ratio
11	36000	0.36	10 eq	Protocol A	3.24
			10 eq	Protocol B	0.56
			25 eq	Protocol A	5.31
			25 eq	Protocol B	0.67
12	28000	0.44	10 eq	Protocol A	2.19
			10 eq	Protocol B	0.64
			25 eq	Protocol A	2.54
			25 eq	Protocol B	0.81

[a]Molar extinction coefficient in DMSO (M^{-1} cm^{-1}) [b]Correction factor calculated from the absorption spectra of the unconjugated dyes in aqueous solution (DMSO as co-solvent).

To further confirm conjugation of the BODIPY dyes SDS-PAGE experiments were performed to analyse the bioconjugation products. These experiments revealed strong fluorescent protein bands, regardless of the molar excess used in the bioconjugation step or the purification procedure. Figure 4 shows the SDS-PAGE of BSA conjugated with BODIPY **11** (10 eq in lane 1 and 25 eq. in lane 2) and BODIPY **12** (10 eq, in lane 3 and 25 eq. in lane 4), all of them purified by purification protocol A. Non-conjugated BSA was loaded to the gel as a control and, as expected, was only observed in the gel after comassie blue stain (lane 5). Similar results were

observed for the BSA-BODIPY conjugates purified *via* protocol B, despite their lower F:P ratio (supplemental file, Figure S11).

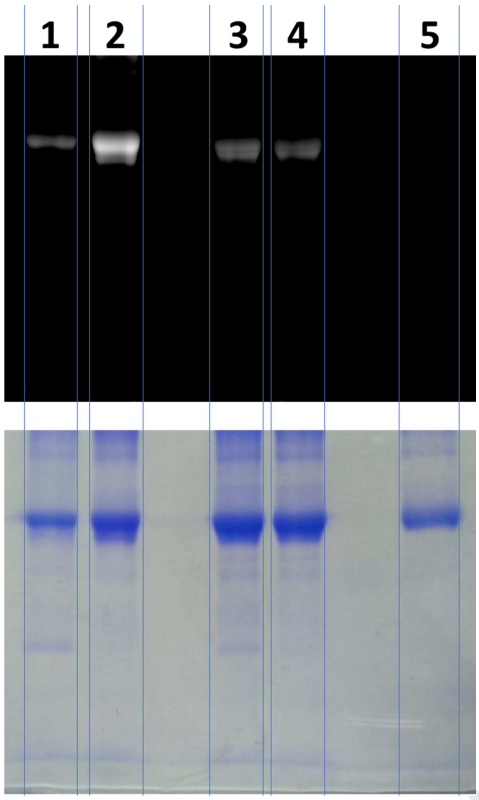


Figure 4. SDS-PAGE gel of BSA-BODIPY conjugates after purification via protocol A. Top: Visualised for fluorescence emission before staining. Bottom: Visualised after comassie blue staining. Lane 1: Product of the conjugation of BSA with 10 eq of BODIPY **11**. Lane 2: Product of the conjugation of BSA with 25 eq of BODIPY **11.** Lane 3: Product of the conjugation of BSA with 10 eq of BODIPY **12**. Lane 4: Product of the conjugation of BSA with 25 eq of BODIPY **12**.

3. Concluding Remarks

Herein we showed the synthesis and photophysical characterization of two isothiocyanate-functionalized BODIPY dyes, designed for application in bioconjugation reactions. As planned, the fluorescence of both compounds was red-shifted when compared to previously published isothiocyanate-BODIPYs, and the fluorescence quantum yield strongly depends on the aromatic substituent at position 8. Both compounds were completely non-water soluble, which makes the use of co-solvents necessary. Nevertheless, both were successfully applied in bioconjugation reactions with a model protein and their application in the conjugation with antibodies will be further studied by our research group. Additionally, enhancing their solubility in water could result in simpler bioconjugation and purification protocols, and is a current goal our research group working on.

4. Experimental

4.1 Syntheses

The syntheses were performed in appropriate round-bottom glass flasks with magnetic stirring. The reactions were monitored using silica gel thin layer chromatography (TLC) in aluminium foils with fluorescent indicator. Mixtures of hexane, ethyl acetate and

dichloromethane were used as mobile phase for TLC analyses, which were visualized under UV light. Purification was performed via column chromatography on silica gel (0.060–0.200 mm, 60 Å), and organic solvents were evaporated using a rotary evaporator at 40–50 °C. For novel compounds the chemical structures were determined by ^{13}C NMR (75 or 101 MHz), ^{1}H NMR (300, 400 or 600 MHz), low-resolution mass spectrometry (electrospray ionization) and high-resolution mass spectrometry (electrospray ionization with time-of-flight). The preparation of compounds **5** and **6** is well stablished in our group and were performed according to previously published procedures [25-27].

*Preparation of BODIPYs **7** and **8** by arylation*: Approximately 0,9 mmol of the starting BODIPYs **5** (300 mg) and **6** (241 mg) were dissolved in acetone (5 mL) in different round-bottom flasks at room temperature under magnetic stirring. Approximately 1 equivalent (0,9 mmol) of 4-nitrobenzenediazonium tetrafluorborate (212 mg) was added to the stirring solution. A strict stoichiometric relation should be followed, as the formation of diarylated BODIPY is an important side reaction. Following, 20 mol% of Ferrocene (33 mg, 0,18 mmol) dissolved in acetone (5 mL) was added dropwise for 10 minutes. After the ferrocene addition was completed the reaction was left stirring at room temperature for 15 minutes, after which TLC control indicated partial conversion to the desired product and the formation of a small amount of the diarylated BODIPY. Each reaction was separately poured into diethyl ether (100 mL), extracted three times with distilled water and dried over magnesium sulphate. Each solution was filtered, evaporated in a rotary evaporator, and the residue was purified chromatographically (hexane/ethyl acetate) to yield the desired products **7** (144 mg, 0,368 mmol, 41% yield) and **8** (185 mg, 0,405 mmol, 45% yield).

*Preparation of BODIPYs **9** and **10** by catalytic hydrogenation:* Approximately 0,36 mmol of the starting BODIPYs **7** (140 mg) and **8** (165 mg) were dissolved in a mixture of dichloromethane (9 mL) and ethanol (1 mL) in different round-bottom flasks at room temperature under magnetic stirring. Palladium on activated charcoal (20 mol%) was added to the solution as a heterogenous catalyst. The reaction flask was purged with hydrogen gas, sealed with appropriated silicon septum and left under magnetic stirring for 15 min at room temperature and H_2 atmosphere (1 atm). TLC control indicated full conversion of the starting material and the formation of new products, which were purified chromatographically (hexane/ethyl acetate) to yield the desired products **9** (109 mg, 0,304 mmol, 84% yield) and **10** (148 mg, 0,345 mmol, 95% yield).

*Preparation of BODIPY **11**:* To a stirring solution of BODIPY **9** (42 mg, 0,117 mmol) in chloroform (5 mL) at room temperature 1,1'-Thio-carbonyldi-2,2'-pyridone (46 mg, 0,194 mmol) was added. After 1.5 hour under magnetic stirring at room temperature TLC control indicated the full conversion of the starting material to a new compound, which was purified chromatographically (hexane/ethyl dichloromethane) to yield the desired product **11** (43 mg, 0,106 mmol, 91% yield).

*Preparation of BODIPY **12**:* To a stirring solution of BODIPY **10** (80 mg, 0,187 mmol) in dimethylformamide (5 mL) under cooling a bath ($0^{o}C$) and an inert atmosphere, Na_2CO_3 was added (95 mg, 0,9 mmol). After 15 minutes under magnetic stirring, phosgene (67 uL, 101 mg, 0,9 mmol) was added to the solution, which was kept stirring at cooling bath and inert atmosphere. After 1 hour, TLC control showed full conversion of the starting material to a new compound. The reaction was poured into ethyl acetate, extracted three times with distilled water and dried over magnesium sulphate. The solution was filtered, solvent was evaporated

in a rotary evaporator and the residue was purified chromatographically (hexane/ethyl acetate) to yield the desired product **12** (76 mg, 0,161 mmol, 86% yield).

4.2 Photophysical characterization

UV-Vis absorption spectra of the fluorescent dyes in seven organic solvents were recorded in a Genesys™ 10S spectrophotometer (Thermo Scientific). Fluorescence spectra of the fluorescent dyes in the same seven solvents were recorded in a Cary Eclipse fluorescence spectrophotometer (Agilent). UV-Vis absorption spectra of the conjugated protein were recorded in Evolution® 300 spectrophotometer (Thermo-Scientific). Fluorescence quantum yields were calculated by a comparative method using Rhodamine-101 in acidified methanol as a reference (φ = 0.96, excitation at 535 nm for compound **11** and at 550 nm para for compound **12**). In this experiment, the absorbance of five diluted solutions of the fluorescent compound was recorded at 535 nm for compound **11** and at 550 nm for compound **12**, so that the absorbance of each solution was no higher than 0.1 and no lower than 0.01. These same solutions were analysed in a fluorescence spectrophotometer, and the area under the emission band was calculated using the equipment's software. Following, absorbance and integrated fluorescence are plotted and the angular coefficient of the linear regression is used in the following equation to calculate the fluorescence quantum yield. Where ϕ_x and ϕ_{st} are the fluorescence quantum yield of the studied dye and the reference, m_x and m_{st} are the angular coefficient calculated for the studied dye and the reference, and n_x and n_{st} are the refractive index of the solvents used to solubilize the studied dye and the reference.

$$\phi_x = \phi_{st}\left[\frac{m_x}{m_{st}}\right]\left[\frac{n_x}{n_{st}}\right]^2$$

4.3 Bioconjugation

The bioconjugation reactions were performed in 2 mL of aqueous bicarbonate buffer (0,5 M; pH = 9,2) containing DMSO (5 % v/v) as co-solvent. The final concentration of BSA was 1 mg/mL (approximately 15 µM) and the fluorescent dye was added in a final concentration of 150 µM (for 10 eq experiments) or 375 µM (for 25 eq experiments). After 1 hour reacting at 38°C in a heating bath, the solution was submitted to a centrifugal dialysis using Vivaspin turbo 15 (Sartorius), with a 30 kDa molecular weight cut-off. Two distinct dialysis protocols were tested in different bioconjugation reactions: a single dialysis against distilled water (4000 RPM, 10 minutes – Protocol A); a triple dialysis against aqueous ethanol 70% v/v (4000 RPM, 20 minutes – Protocol B). Purification procedures using ethanol 70% were followed by a solvent exchange step in which the solution was dialysed against distilled water for three times (4000 RPM, 10 minutes). After the dialysis the aqueous solutions were evaporated in a centrifugal evaporator CentriVap (LABCONCO).

Degree of protein labelling: UV-Vis absorption spectra of the conjugated protein were recorded in Evolution® 300 spectrophotometer (Thermo-Scientific) and used to estimate the dye:protein molar ratio (F/P ratio). The molar extinction coefficient of each fluorescent dye (ε) was calculated from the absorbance at maximum wavelength of each compound in DMSO solutions of known concentration. Absorbance was plotted against molar concentration and a linear regression curve was plotted for the dataset with a linear relation. ε was calculated from the angular coefficient of the linear regression. The correction factor (CF) equals the absorbance at 280 nm (A_{280}) of the dye divided by the absorbance at peak maxima (A_{max}) of the

dye in aqueous solution containing 30% of DMSO as co-solvent. Finally, F/P ratio could be calculated from the following equation [22].

$$\frac{A_{max} \times \varepsilon_{prot}}{(A_{280} - A_{max}CF) \times \varepsilon_{fluor}}$$

Polyacrylamide gel electrophoresis: SDS-PAGE was carried out on the Mini-Protean Tetra Cell apparatus (BioRad, Hercules, USA). Polyacrylamide resolving gel mixture (5 mL) was prepared using polyacrylamide 12%, bisacrylamide 0.3%, SDS 0.1% in Tris-HCl buffer (0.375 mM, pH 8.8) and polymerized with ammonium persulfate 0.1% and tetramethylethylenediamine 20 µL. This gel mixture was poured into a cassette and after complete polymerization the polyacrylamide stacking gel was prepared and poured over. Polyacrylamide stacking gel mixture (3 mL) was prepared using polyacrylamide 3%, bisacrylamide 0.3%, SDS 0.1%, in Tris-HCl buffer (0.125 mM, pH 6.8) and polymerized with ammonium persulfate 0.1% and tetramethylethylenediamine 20 µL. After polymerization, the gel was placed into the electrophoresis tank and covered in running buffer (0.025 mM Tris, 0.192 mM glycine, 0.1% SDS). The samples were prepared by diluting the protein samples in distilled water in a final concentration of 4 mg/mL. This solution was mixed with loading buffer (0.31 M Tris- HCl pH 6.8, 0,2% SDS, 50% glycerol, 0.01% bromophenol blue), and a 20 µL sample was loaded onto the gel. The gel was developed at a voltage of 120 V for two hours. The gel was illuminated with UV light and registered photographically, before staining overnight in staining solution (0.025% "Coomassie Brilliant Blue" R250, 15% methanol, 7% acetic acid, in distilled water), followed by several changes of destain solution (50% methanol, 10% acetic acid, in distilled water).

Acknowledgements

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Supplementary Data

Synthesis and characterization of two novel red-shifted isothiocyanate BODIPYs and their application in protein conjugation.

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Spectroscopical characterization data

BODIPY **7**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (d, *J* = 9.0Hz, 2H), 7,52 (s, 1H), 7,52 – 7.40 (m, 5H), 7,02 (d, *J* = 9.0 Hz, 2H), 6,97 (d, *J* = 4.5 Hz, 1H), 6.80 (d, *J* = 4.3 Hz, 1H), 6.70 (d, *J* = 4.5 Hz, 1H), 6.49 (dd, *J* = 4.3, 2.0 Hz, 1H), 3.81 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.5, 160.9, 1445, 141.2, 137.6, 134.4, 134.0, 133.2, 131.5, 130.6, 130.4, 128.7, 128.5, 124.6, 121.2, 117.7, 114.1, 55.5. LRMS (ESI) 375 $(M + H)^+$, 397 $(M + Na)^+$, 413 $(M + K)^+$.

BODIPY **8**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (d, *J* = 8.7 Hz, 1H), 8.14 (d, *J* = 8.7 Hz, 1H), 7.95 (s, 1H), 7.56 – 7.40 (m, 3H), 6.75 (s, 1H), 6.69 (d, *J* = 4.3 Hz, 1H), 6.57 (d, *J* = 4.0 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.6, 148.2, 146.3, 140.3, 138.1, 136.9, 135.2, 134.9, 131.5, 131.2, 130.4, 130.3, 130.3, 128.3, 123.5, 120.9, 120.1. LRMS (EI): 457, 438 (M - F), 422 (M - Cl).

BODIPY **9**: ^{1}H NMR (400 MHz, DMSO-d6) δ 7.97 (d, *J* = 8.9 Hz, 2H), 7.73 (s, 1H), 7.65 – 7.54 (m, 5H), 7.12 (d, *J* = 4.8 Hz, 1H), 6.99 (d, *J* = 4.8 Hz, 1H), 6.67 (d, *J* = 8.9 Hz, 2H), 6.64 (d, *J* = 3.9 Hz, 1H), 6.51 (dd, *J* = 3.9, 2.1 Hz, 1H), 6.24 (br, 2H). ^{13}C NMR (101 MHz, DMSO-d6) δ 162.0, 152.4, 139.8, 137.5, 137.4, 133.8, 133.5, 132.3, 132.2, 130.3, 130.1, 128.5, 124.9, 122.4, 117.1, 116.4, 113.3. HRMS (ESI): calculated for $C_{21}H_{16}BF_2N_3$, $[M + H]^+$: 360.1484, found: 360.1459, $[M + Na]^+$: 382.1303, found: 382.1285.

BODIPY **10**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.97 (d, *J* = 8.8 Hz, 2H), 7.75 (s, 1H), 7.54 – 7.34 (m, 3H), 6.82 – 6.66 (m, 4H), 6.49 (d, *J* = 3.8 Hz, 1H), 6.43 (dd, *J* = 3.8, 1.9 Hz, 1H), 4.11 (br, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.1, 149.5, 140.1, 137.9, 135.7, 132.9, 132.2, 132.1, 132.0, 131.6, 131.0, 128.3, 125.3, 122.2, 121.2, 117.2, 114.6. HRMS (ESI): calculated for $C_{21}H_{14}BCl_2F_2N_3$, $[M + H]^+$: 428.0699, found: 428.0686, $[M + Na]^+$: 450.0524, found: 450.0505, $[M + K]^+$: 466.0263, found: 466.0290.

BODIPY **11**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (d, *J* = 8.8 Hz, 2H), 7.81 (s, 1H), 7.44 – 7.54 (m, 5H), 7.26 (d, *J* = 8.8 Hz, 2H), 6.90 (d, *J* = 4.4 Hz, 1H), 6.83 (d, *J* = 4.1 Hz, 1H), 6.60 (d, *J* = 4.3 Hz, 1H), 6.48 (dd, *J* = 4,1, 1,6 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.9, 146.0, 143.3, 137.3, 136.7, 134.5, 134.0, 132.6, 131.1, 130.8, 130.7, 130.6, 130.5, 128.5, 125.7, 120.5, 118.6.

BODIPY **12**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.01 (d, *J* = 8.8 Hz, 2H), 7.88 (s, 1H), 7.40 – 7.52 (m, 3H), 7.33 (d, *J* = 8.8 Hz, 2H), 6.74 (d, *J* = 4.4 Hz, 1H), 6.66 (d, *J* = 4.4 Hz, 2H), 6.51 (d, *J* = 2.8 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ^{13}C NMR (75 MHz, $CDCl_3$) δ 159.1, 144.4, 139.2, 137.2, 135.5, 134.2, 132.9, 131.6, 131.4, 131.0, 130.9, 130.8, 128.9, 128.4, 125.9, 121.3, 119.2. HRMS (ESI): calculated for $C_{22}H_{12}BCl_2F_2N_3S$, $[M + H]^+$: 470.0268, found: 470.0268.

UV-Vis absorption spectra

BODIPY **11**

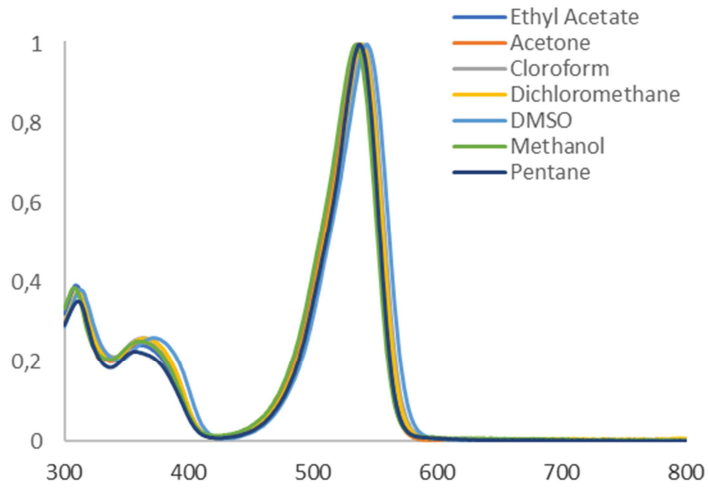

Figure S1. Normalized UV-Vis absorption spectra of BODIPY **11** in seven organic solvents.

BODIPY **12**

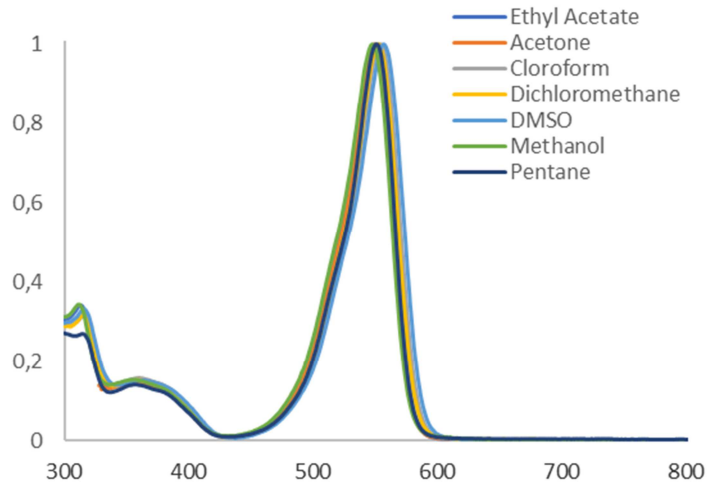

Figure S2. Normalized UV-Vis absorption spectra of BODIPY **12** in seven organic solvents.

Fluorescence emission spectra

BODIPY **11**

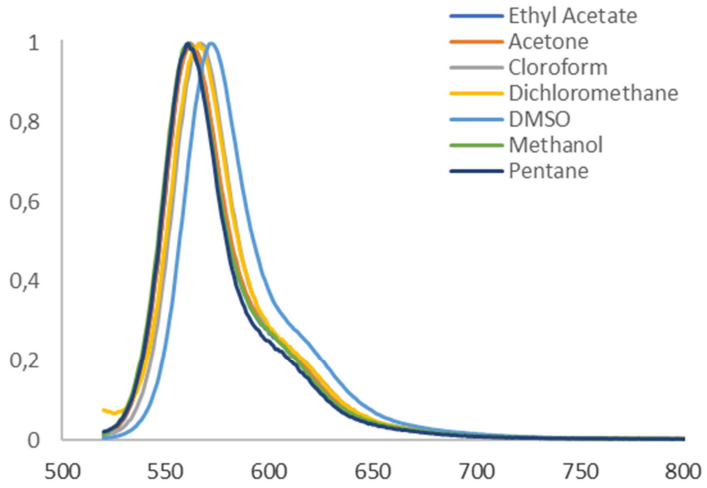

Figure S3. Normalized fluorescence spectra of BODIPY **11** in seven organic solvents.

BODIPY **12**

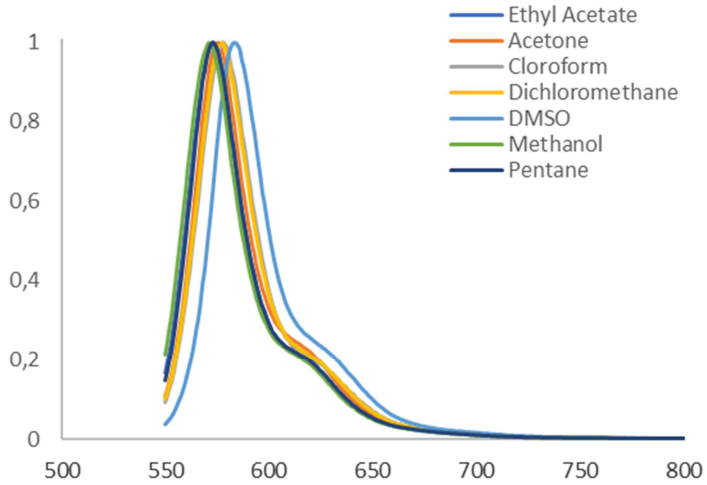

Figure S4. Normalized fluorescence spectra of BODIPY **12** in seven organic solvents.

Bioconjugation Purification Protocol

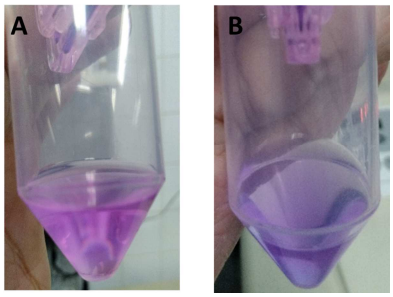

Figure S5. Appearance of dialysis solution of two BSA-BODIPY conjugates purified with Protocol B after a dialysis cycle. **A**: Compound **11**, 25 eq. **B**: Compound 12, 25 eq. No colour was observed when dialysis was performed in water (Protocol A).

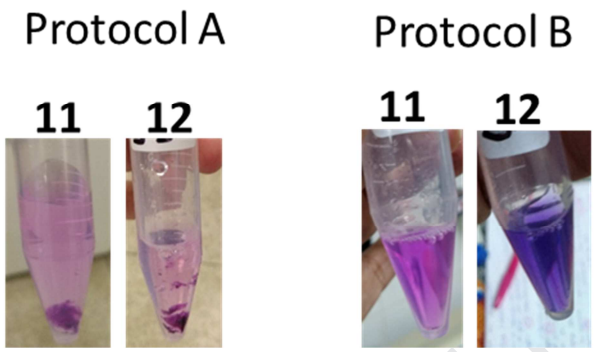

Figure S6. Appearance of aqueous solutions of BSA-BODIPY conjugates prepared with compounds **11** or **12** and purified with Protocol A or Protocol B. Solubilization of BSA-BODIPY conjugates purified *via* Protocol A was less efficient, probably due to the lower solubility of non-conjugated dyes in water, hindering the dialysis in this purification protocol. No solubilization issues were observed for the BSA-BODIPY conjugates purified *via* Protocol B.

Spectra of BSA-BODIPY conjugates

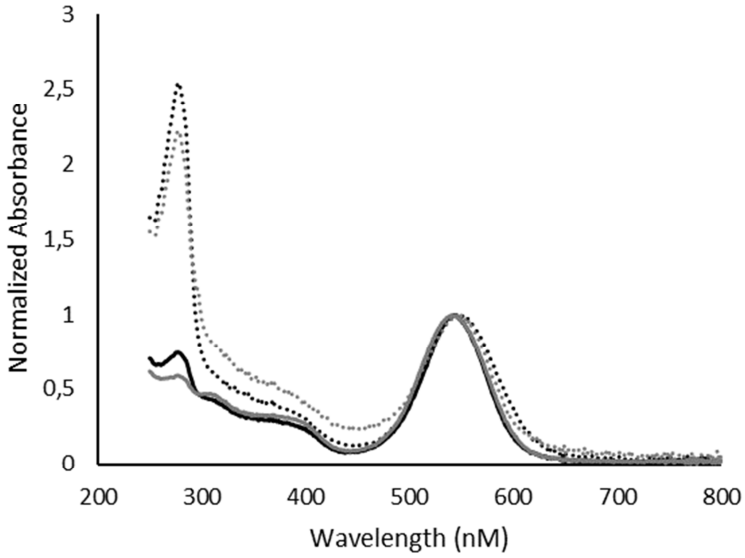

Figure S7. UV-Vis absorption spectra of BSA-BODIPY conjugates prepared using compound **11** in different molar ratios (10 eq or 25 eq) and purified with different protocols (Protocol A or Protocol B). Spectra were normalized for the maximum wavelength of BODIPY absorption. **Full black line:** 10 eq, Protocol A. **Dotted black line:** 10 eq, Protocol B. **Full grey line:** 25 eq, Protocol A. **Dotted grey line:** 25 eq, Protocol B.

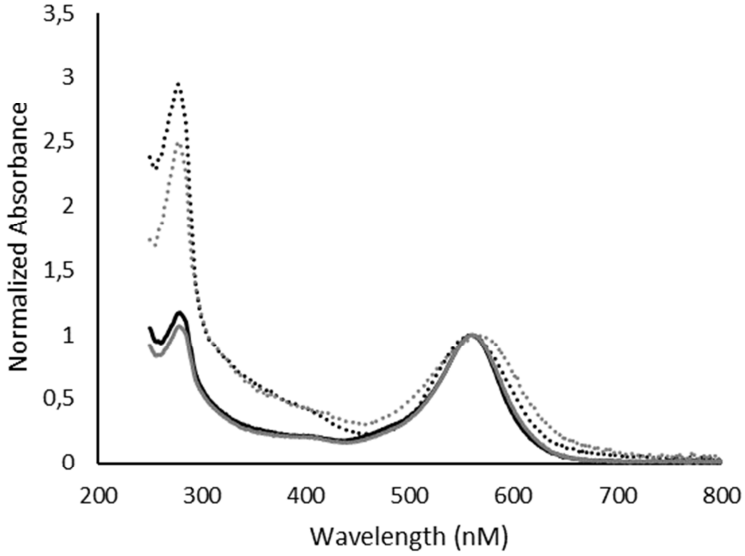

Figure S8. UV-Vis absorption spectra of BSA-BODIPY conjugates prepared using compound **12** in different molar ratios (10 eq or 25 eq) and purified with different protocols (Protocol A or Protocol B). Spectra were normalized for the maximum wavelength of BODIPY absorption. **Full black line:** 10 eq, Protocol A. **Dotted black line:** 10 eq, Protocol B. **Full grey line:** 25 eq, Protocol A. **Dotted grey line:** 25 eq, Protocol B.

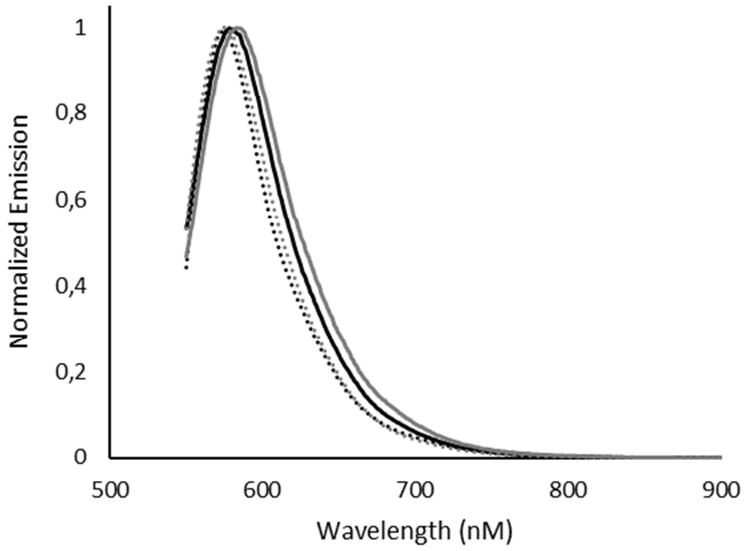

Figure S9. Normalized fluorescence emission spectra of BSA-BODIPY conjugates prepared using compound **11** in different molar ratios (10 eq or 25 eq) and purified with different protocols (Protocol A or Protocol B). **Full black line:** 10 eq, Protocol A. **Dotted black line:** 10 eq, Protocol B. **Full grey line:** 25 eq, Protocol A. **Dotted grey line:** 25 eq, Protocol B.

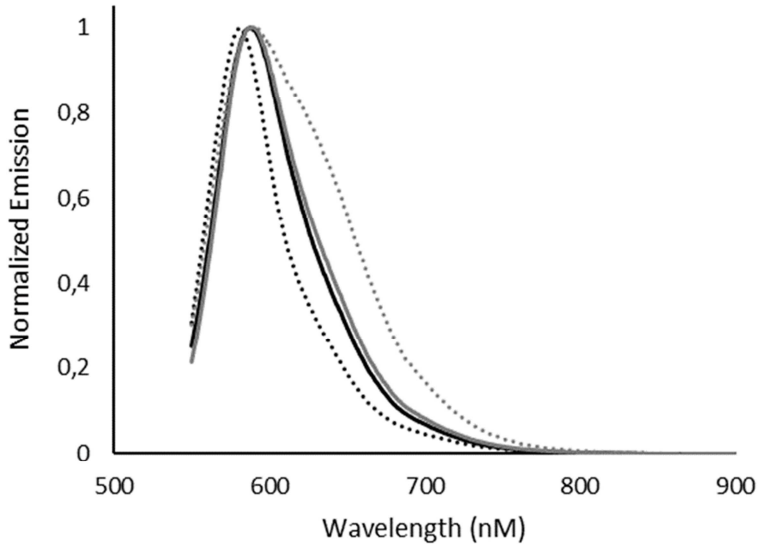

Figure S10. Normalized fluorescence emission spectra of BSA-BODIPY conjugates prepared using compound **12** in different molar ratios (10 eq or 25 eq) and purified with different protocols (Protocol A or Protocol B). **Full black line:** 10 eq, Protocol A. **Dotted black line:** 10 eq, Protocol B. **Full grey line:** 25 eq, Protocol A. **Dotted grey line:** 25 eq, Protocol B.

Reaction of isothiocyanato-BODIPYs with diethylamine

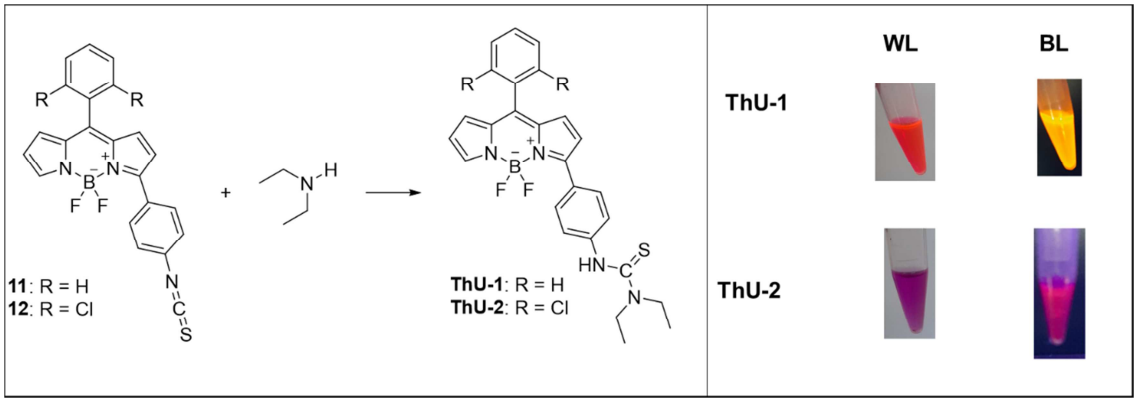

Figure S11. Left panel: Test reactions between compounds **11** and **12** with diethylamine for the formation of thiourea-BODIPYs **ThU-1** and **ThU-2**. Right panel: Solutions of thiourea BODIPYs **ThU-1** and **ThU-2** in acetone visualized under white light (WL) and black light (BL).

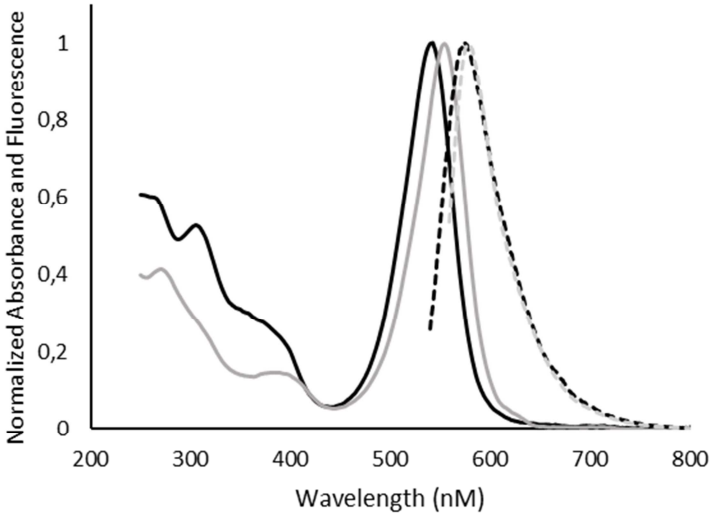

Figure S12. Absorption and emission spectra of thiourea-BODIPYs **ThU-1** and **ThU-2** in Methanol. **Full black line:** Absorption spectra of **ThU-1**. **Full grey line:** Absorption spectra of **ThU-2**. **Dotted black line:** Emission spectra of **ThU-1**. **Dotted grey line:** Emission spectra of **ThU-2**.

SDS-PAGE

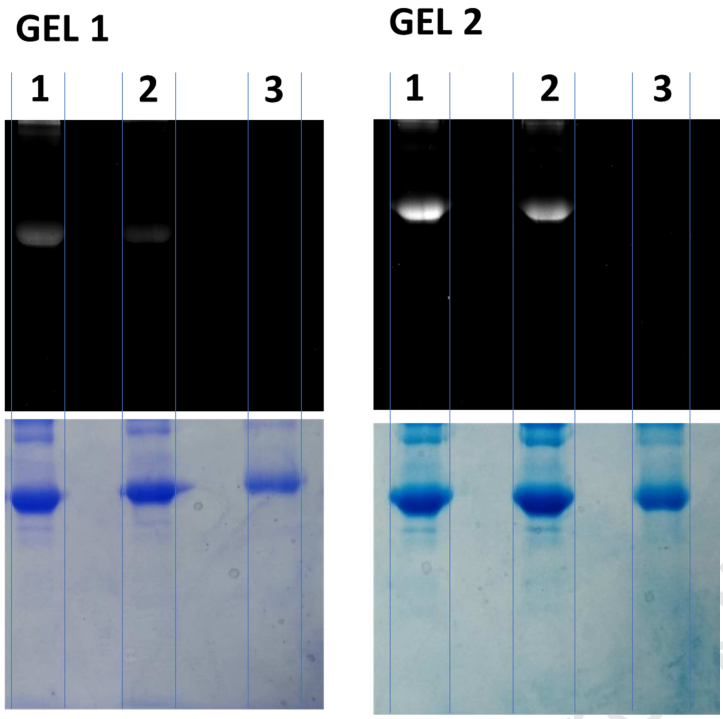

Figure S13. SDS page of BSA-BODIPY conjugates purified via protocol B. **Gel 1, lane 1:** compound **11**, 10 eq; **Gel 1, lane 2:** L compound **12**, 10 eq; **Gel 1, lane 3:** non-conjugated BSA; **Gel 2, lane 1:** compound **11**, 25 eq; **Gel 2, lane 2:** compound **12**, 25 eq; **Gel 2, lane 3:** non-conjugated BSA.

Highlights

- Synthesis of two amino-reactive red-shifted BODIPYs with isothiocyanate group
- NMR and photophysical characterization
- Amino-reactive BODIPYs successfully conjugated with BSA as a model protein
- BODIPY-BSA conjugates retained fluorescence and were visualized via SDS-PAGE

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: