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Isothiocyanato Boron Dipyrromethenes—The First BODIPY Analogues of Fluorescein Isothiocyanate

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ABSTRACT

Two boron complexes of 5-phenyldipyrromethenes bearing isothiocyanate groups on the phenyl ring have been synthesized for the first time. The utility of these new fluorescence probes for labeling biologically relevant proteins is demonstrated on two monoclonal antibodies that bind to antigens overexpressed on cancer cells. Spectral comparison of the two structures reveals significant photophysical differences, including bathochromically shifted excitation and emission bands, increased molar absorptivity and a large increase in fluorescence quantum yield of approximately 10 times. Differences in photophysical parameters are linked to hindered rotation of the phenyl ring in one of the probes.

INTRODUCTION

The boron complexes of dipyrromethene (BODIPY, a registered trademark of Molecular Probes Inc., Eugene, OR) have received considerable interest in the last decade as chromophoric units for incorporation into light harvesting arrays (1); as components of sensors for zinc (2), nitric oxide (3), and pH (4) and as fluorescent probes for use in biological systems (5,6). Many different derivatives of these photoactive complexes have been synthesized, and recently some inventive methods for direct functionalization of the BODIPY fluorophore have been published (7,8); however, curiously, no BODIPY analogues of fluorescein isothiocyanate (FITC) have been reported. FITC is enormously useful for labeling biomolecules bearing amino groups (9) including proteins, peptides and suitably functionalized oligonucleotides, which can then be tracked by fluorescence techniques such as fluorescence microscopy and flow cytometry. The BODIPY fluorophore offers many advantages compared to fluorescein, including a narrow emission bandwidth, spectra that are less sensitive to polarity and pH, longer excited state lifetimes, and a large two-photon cross-section for multiphoton excitation.

Recently, we have reported on use of the isothiocyanate group as a convenient conjugation "handle" for the coupling of photoactive molecules such as porphyrins, chlorins, bacteriochlorins and phthalocyanines to biomolecules with free amino groups (10–13). We now wish to report that this strategy can be successfully adapted to generate BODIPY isothiocyanates, and that these molecules can be used to fluorescently label monoclonal antibodies.

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MATERIALS AND METHODS

General. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-LA-400 spectrometer (at 400 MHz) and are referenced downfield to tetramethylsilane. UV and visible spectra were recorded on an Agilent 8453 UV-visible spectrophotometer. Fluorescence spectra were recorded on an Aminco-Bowman Series 2 Luminescence Spectrometer. Accurate masses were obtained from EPSRC Mass Spectrometry Service, Swansea, Wales. All commercial chemicals and solvents were of reagent grade or higher and were used as received, unless otherwise specified. All experiments with moisture- or air-sensitive compounds were performed in anhydrous solvents under a nitrogen atmosphere. Thin-layer chromatography (TLC) analyses were performed on Merck silica gel 60 plates (F_{254}, 0.2 mm thick). Flash column chromatography was performed with MP silica gel 60 (32–63) with all of the crude reaction mixtures being preadsorbed onto silica gel prior to separation, unless otherwise stated. All purified compounds were found to contain only one component by TLC analysis. HPLC analyses were performed using a Jasco HPLC system with diode array detector on a Phenomenex Luna (C18, 50 × 4.6 mm) column (flow rate, 1 mL min^{-1}; eluent A, H_2O containing 0.1% trifluoroacetic acid; eluent B, methanol; gradient 0 min, 0% B; 10 min 75% B; 13 min, 75% B; 17 min, 0% B; 18 min, 0% B).

Synthesis of N,N'-difluoroboryl-5-(4-isothiocyanatophenyl)-dipyrromethene (3). 5-(4-Nitrophenyl) dipyrromethane (**1**) was synthesized as previously described (11). **1** (1.5 g, 5.6 mmol) was dissolved in dry tetrahydrofuran (100 mL) and 10% palladium/carbon (0.5 g) was added. The mixture was saturated with hydrogen gas and stirred for 16 h. The Pd/C was removed by filtration and the solvent evaporated *in vacuo*. The residue was crystallized from dichloromethane and washed with pentane to give 5-(4-aminophenyl)-dipyrromethane (1.27 g, 96%). 5-(4-Aminophenyl)-dipyrromethane (0.85 g, 3.5 mmol) was dissolved in 1,4-dioxane (20 mL) and stirred with sodium hydrogen carbonate (1.6 g, 19.2 mmol) under nitrogen. 9-Fluorenylmethyl-chloroformate (1.66 g, 6.4 mmol) was dissolved in 1,4-dioxane (46 mL) and added to the stirring mixture. The reaction was then stirred for a further 2 h. Solvent was removed and the product partitioned in to dichloromethane, which was washed with water (100 mL) then brine (100 mL) and finally dried over anhydrous magnesium sulphate. Solvent was removed and the product was purified by column chromatography (silica, DCM) to give 5-(4-(*N*-fluorenylmethoxycarbonyl)amino)phenyl)-dipyrromethane (1.2 g, 75%). 5-(4-(N-fluorenylmethoxycarbonyl)amino)phenyl)-dipyrromethane (200 mg, 0.425 mmol) was dissolved in a mixture of dichloromethane (10 mL) and methanol (3 mL) and added to a stirred solution of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (108 mg, 0.48 mmol) in dichloromethane (DCM). The mixture was stirred for 1 h at room temperature under a nitrogen atmosphere. Solvent was removed and the product was purified by column chromatography (silica, DCM/MeOH 49:1) to give 5-(4-(N-fluorenylmethoxycarbonyl)amino)phenyl)-dipyrromethene (120 mg, 61%). 5-(4-(N-fluorenylmethoxycarbonyl)amino)phenyl)-dipyrromethene (100 mg, 0.21 mmol) was dissolved in dichloromethane under nitrogen. To this stirred solution was added triethylamine (23 μL, 5 mmol) then boron trifluoride etherate (35 μL, 0.26 mmol). The mixture was

stirred for 45 min and the solvent was removed *in vacuo* to give crude *N,N'*-difluoroboryl-5-(4-(N-(fluorenylmethoxycarbonyl)amino)phenyl)-dipyrromethene (**2**) which was purified by column chromatography to give **2** (77 mg, 72%). **2** (50 mg, 0.1 mmol) was dissolved in dichloromethane (10 mL) and piperidine (10 drops) was added. The solution was stirred for 20 min and the solvent removed *in vacuo*. Purification of the crude product by column chromatography (silica, EtOAc/DCM 1:3) gave *N,N'*-difluoroboryl-5-(4-aminophenyl)-dipyrromethene (26 mg, 96%). *N,N'*-Difluoroboryl-5-(4-aminophenyl)-dipyrromethene (10 mg, 35 μmol) was dissolved in dry dichloromethane under nitrogen. To this solution was added 1,1'-thiocarbonyldi-2(1H)-pyridone (40.6 mg, 175 μmol) and the mixture was stirred for 30 min. Solvent was removed *in vacuo* and the crude product purified by column chromatography (silica, DCM) to give **3** (8 mg, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.58 (d, J = 4.0 Hz, 2H), 6.90 (d, J = 4.0 Hz, 2H), 7.40 (d, J = 8.4 Hz, 2H), 7.59 (d, J = 8.4 Hz, 2H), 7.96 (s, 2H); ES-HRMS Anal. Calcd. For $C_{14}H_{10}N_3F_2SB$ ([M+NH$_4$]) 343.0995 found 343.0998; TLC (Silica, DCM) R_f = 0.85; UV-visible (DCM) λ_{max} = 505 nm (ε = 57,000 $M^{-1}cm^{-1}$); fluorescence (DCM) λ_{emm} = 515 nm.

Synthesis of N,N'-*difluoroboryl-2,8-diethyl-1,3,7,9-tetramethyl-5-(4-isothiocyanatophenyl)-dipyrromethene (**6**).* Acetamidobenzaldehyde (0.457 g, 2.8 mmol) and kryptopyrrole (0.756 mL, 5.6 mmol) were dissolved in anhydrous dichloromethane (125 mL) under nitrogen. TFA (0.02 mL) was added and the mixture was left stirring under nitrogen, at room temperature for 16 h. DDQ (640 mg, 2.8 mmol) was added and the mixture was stirred for 1 h. The reaction mixture was washed with water, and then dried over Na_2SO_4. After removal of the solvent *in vacuo*, the crude mixture was purified by column chromatography (silica, DCM:MeOH 9:1) to give 2,8-diethyl-1,3,7,9-tetramethyl-5-(4-acetamidophenyl)-dipyrromethane (**4**) (371 mg, 34%). **4** (300 mg, 0.77 mmol) was dissolved in 20 mL of MeOH, then 20 mL of 1 *N* HCl was added and the mixture was heated at reflux at 100°C for 1 h (protected from light). The reaction mixture was neutralized with 2 *N* NaOH (1.73 g of NaOH dissolved in 21.6 mL H_2O). The aqueous solution was extracted with DCM, and the combined organic extracts were dried over Na_2SO_4. The solvent was then removed *in vacuo* to obtain crude 2,8-diethyl-1,3,7,9-tetramethyl-5-(4-aminophenyl) dipyrromethene. The crude product was dissolved in 50 mL of anhydrous DCM; 1 mL of dry triethylamine was added under nitrogen, followed by 1 mL of $BF_3 \cdot OEt_2$. The reaction mixture was stirred under nitrogen, at room temperature, protected from light, for 3–4 h. The reaction mixture was washed with water and brine, and then dried over Na_2SO_4. After removing the solvent, crude product was purified by column chromatography on silica with DCM to give *N,N'*-difluoroboryl-2,8-diethyl-1,3,7,9-tetramethyl-5-(4-aminophenyl)-dipyrromethene (**5**) (101 mg, 33%). **5** (97 mg, 0.245 mmol) was dissolved in anhydrous DCM (45 mL) and then 1,1'-thiocarbonyldi-2(1H)-pyridone (114 mg, 0.49 mmol) was added under nitrogen. The reaction mixture was stirred under nitrogen, at room temperature, protected from light for 2 h. The solvent was removed and crude product was purified by column chromatography (silica, *n*-hexane:DCM 4:3) to give **6** (104 mg; 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.98 (t, J = 7.6 Hz, 6H), 1.30 (s, 6H), 2.30 (q, J = 7.6 Hz, 4H), 2.53 (s, 6H), 7.29 (d, J = 8.7 Hz, 2H), 7.36 (d, J = 8.7 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) 11.95, 12.56, 14.61, 17.08, 126.43, 129.91, 132.11, 133.15, 134.96, 138.00, 154.35; EI-HRMS Anal. Calcd. For $C_{22}H_{26}N_3F_2SB$ ([M^+]) 437.1903 found 437.1902; HPLC (C_{18}) R_t = 10.8 min (100%); UV-visible (DCM) λ_{max} = 528 nm (ε = 89 500 $M^{-1}cm^{-1}$); fluorescence (DCM) λ_{emm} = 540 nm.

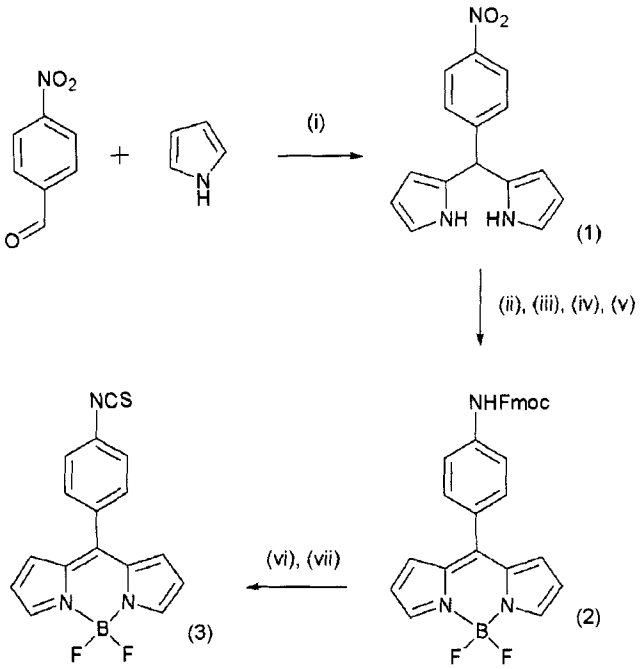
Conjugation to monoclonal antibodies. The murine antibodies anti-EpCAM and anti-CD146 were purchased from Serotec. Anti-EpCAM recognizes a 40 kDa cell–cell adhesion molecule up-regulated on some carcinomas, particularly colorectal, whereas anti-CD146 binds to a cell adhesion molecule up-regulated in melanoma cells.

Conjugation was carried out in a 1-mL reaction volume containing 1 mg mL^{-1} antibody in 0.5 *M* bicarbonate buffer (pH 9.2) and either a 10× or 20× molar excess of compound **6**. The reaction vessels were agitated gently at room temperature for 1 h and protected from light. The conjugates were purified using Sephadex G25 columns (Amersham) and eluted with PBS. The degree of labeling (the number of moles of **6** conjugated per mole of antibody) was calculated using spectroscopic methods.

Polyacrylamide gel electrophoresis. SDS-PAGE was carried out on the XCell SureLock mini cell apparatus (Invitrogen, Paisley, UK). Ten percent polyacrylamide resolving gel mixture (10 mL: 2.5 mL 40% acrylamide/bisacrylamide, 2.5 mL 1.5 *M* Tris-HCl pH 8.8, 0.1 mL 10% SDS, 50 μL 10% ammonium persulphate, 10 μL TEMED, 4.85 mL H_2O) was prepared and poured into a cassette. Once the gel had polymerized, 4% stacking gel mixture (10 mL: 1 mL 40% acrylamide/bisacrylamide, 1.25 mL 1 *M* Tris-HCl pH 6.8, 0.1 mL 10% SDS, 50 μL 10% ammonium persulphate, 20 μL TEMED, 7.6 mL H_2O) was poured into the cassette with a comb inserted to form wells. After polymerization, the gel was placed into the electrophoresis tank and covered in running buffer (25 m*M* Tris, 200 m*M* glycine, 0.1% SDS). The samples were mixed (1:1) with loading buffer (0.5 *M* Tris-HCl pH 6.8, 5% SDS, 20% glycerol, 0.05% bromophenol blue) and boiled for 5 min. A 20 μL sample was loaded onto the gel along with a protein ladder (between 13–205 KDa; 10 μL; Invitrogen). The gel was developed at a voltage of 100 V until the dye front had reached the bottom of the resolving gel.

The gel was illuminated with UV light before staining with coomassie blue stain overnight, followed by several changes of coomassie blue destain.

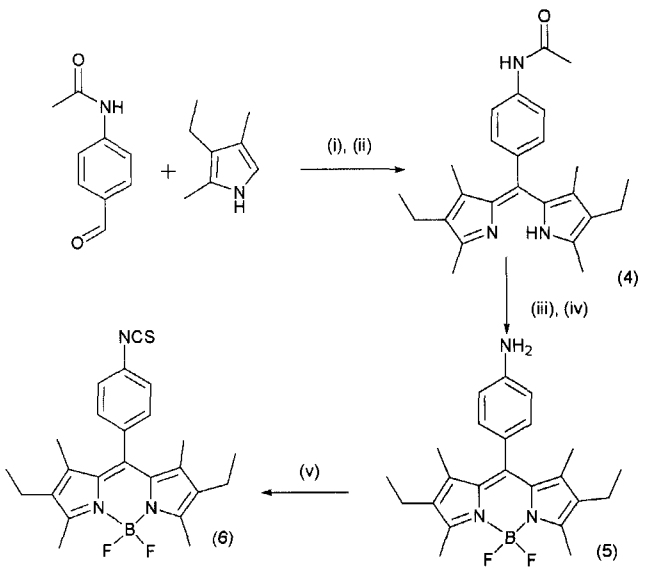
Fluorescence microscopy. LoVo (Human colon adenocarcinoma) cells were seeded 2 days in advance at 5×10^4 cells mL^{-1} in 35 mm Iwaki glass-bottomed petri dishes. On the day of imaging, the cells were washed twice in PBS and incubated for 30 min at 37°C and in 5% CO_2 atmosphere with the appropriate antibody-conjugated porphyrin diluted in Ham-12 medium supplemented with 1% L-glutamin and 10% fetal calf serum. The petri dishes were then washed twice again with PBS and 2 mL of Hank's Balanced Salt Solution was added to each. Light for imaging was delivered using a Xenon short-arc lamp and a Cairn Optoscan Monochromator tuned to 488 nm excitation λ and a filter cube without an excitation filter but with a 505 nm dichroic mirror and a 535 nm band pass emission filter with 50 nm half band width. The images were acquired with an Orca CCD camera mounted on a triocular Leica DM IRB microscope.



Scheme 1. Reagents and conditions: (i) TFA, (ii) H_2/Pd/C, THF, (iii) FmocCl, $NaHCO_3$, dioxane, (iv) DDQ, toluene, (v) $BF_3 \cdot OEt_2$, DCM, (vi) piperidine, DCM, (vii) TDP, DCM.

RESULTS AND DISCUSSION

Initially, we synthesized 5-(4-nitrophenyl)dipyrromethane (**1**) which we had previously used as a porphyrin building block (11). Reduction of the nitro group by catalytic hydrogenation gave the corresponding 5-(4-aminophenyl)dipyrromethane which was amino-protected with Fmoc (fluorenylmethoxycarbonyl), thus allowing DDQ oxidation of the dipyrromethane core to the conjugated dipyrromethene and subsequent boron complexation (**2**). Finally, deprotection of the amine and treatment with 1,1'-thiocarbonyldi-2(1H)-pyridone (TDP) gave the required *N, N'*-



Scheme 2. Reagents and conditions: (i) TFA, DCM, (ii) DDQ, DCM, (iii) HCl_{aq}, MeOH, (iv) $BF_3 \cdot OEt_2$, Et_3N, DCM, (v) TDP, DCM.

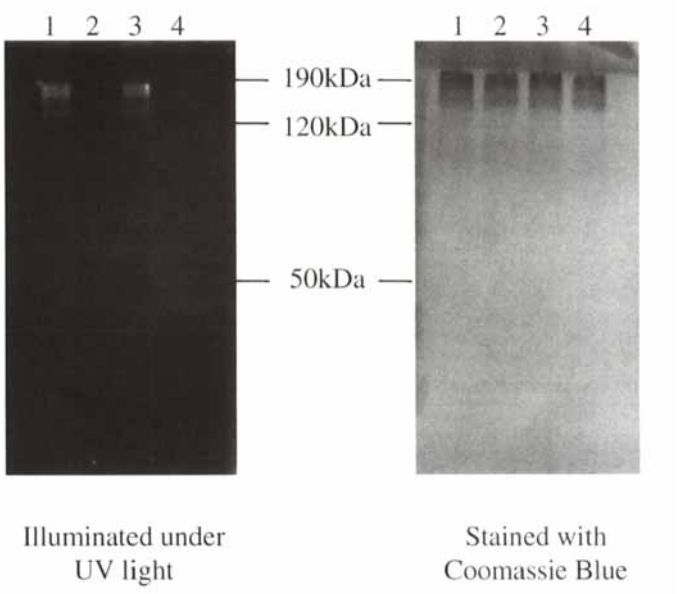


Figure 1. Polyacrylamide gel electrophoresis of **6**-conjugated antibodies. 4.5 μg of conjugated (lane 1) and unconjugated (lane 2) anti-EpCAM and conjugated (lane 3) and unconjugated (lane 4) anti-CD146 separated on a 10% gel, illuminated under UV light (left panel) and stained with coomassie blue (right panel).

difluoroboryl-5-(4-isothiocyanatophenyl)dipyrromethene (**3**) in 16% overall yield (Scheme 1). The final product was characterized by NMR, HRMS, TLC, UV-visible and fluorescence spectroscopy, the latter techniques confirming the characteristic excitation and emission bands for BODIPY-type fluorophores centered at 505 and 515 nm respectively. In order to quantify the fluorescence of **3** the fluorescence quantum yield was determined (14); this revealed that the fluorescence of **3**, although clearly detectable, was quite weak, with a value of 0.05. Low fluorescence quantum yields (0.007) have previously been reported for dipyrromethene complexes of zinc substituted with aryl rings at the 5 position when the aryl ring is free to rotate into coplanarity with the dipyrromethene π system (15). The fluorescence of **3** is sufficient to allow its use as a fluorescent probe; however it was of interest to explore if the quantum yield could be increased in the NCS–BODIPY system by hindering rotation of the 5-phenyl group.

In order to restrict rotation of the 5-phenyl ring, 4-acetamido benzaldehyde was reacted with 3-ethyl-2,4-dimethylpyrrole (kryptopyrrole) to give 2,8-diethyl-1,3,7,9-tetramethyl-5-(4-acetamidophenyl) dipyrromethane (Scheme 2). DDQ oxidation gave the corresponding dipyrromethene (**4**), which upon acid hydrolysis of the acetamido group and complexation with boron gave the 5-(4-aminophenyl) BODIPY analogue (**5**). Finally, treatment with TDP gave the required NCS–BODIPY (**6**) in 11% overall yield (Scheme 2). NMR, HRMS, HPLC, UV-visible and fluorescence spectroscopy all confirmed the identity and purity of **6**. Determination of the quantum yield for **6** indicated a 10-fold increase in fluorescence efficiency to 0.5. Interestingly, in addition to the increase in fluorescence quantum yield the structural modifications in moving from **3** to **6** also resulted in bathochromic shifts of both the excitation and emission maxima to 528 and 540 nm respectively, and an increase in molar absorptivity, from 56 666 $M^{-1}cm^{-1}$ to 89 550 $M^{-1}cm^{-1}$.

It was decided to investigate the applicability of **6** for labeling monoclonal antibodies, as FITC is commonly used to fluorescently tag this class of protein.

The murine antibodies anti-EpCAM and anti-CD146 were selected for labeling. Anti-EpCAM recognizes a 40 kDa cell–cell adhesion molecule up-regulated on some carcinomas, particularly colorectal, whereas anti-CD146 binds to a cell adhesion molecule up-regulated in melanoma cells.

Conjugation was carried out in a 1-mL reaction volume containing 1 mg mL^{-1} antibody in 0.5 *M* bicarbonate buffer (pH 9.2)

Table 1. Degree of labeling of monoclonal antibodies (MAb) with **6**

	Degree of labeling (moles **6**:moles Mab)	
MAb	10× Loading ratio	20× Loading ratio
Anti-EpCAM	3.9	7.4
Anti-CD146	6.1	9.1

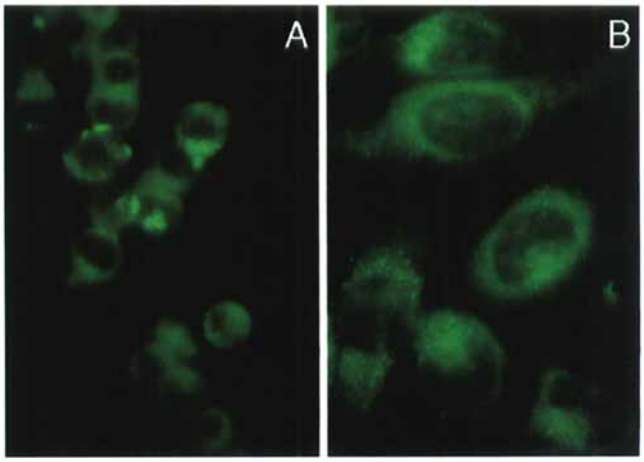


Figure 2. Fluorescence microscopy (excitation = 488 nm; emission = 535-nm bandpass filter) images (40× magnification) of (A) cells expressing EpCAM after incubation with anti-EpCAM antibody labeled with **6** (loading ratio 20×), and (B) cells expressing CD146 after incubation with anti-CD146 antibody labeled with **6** (loading ratio 10×). Cells were washed to remove unbound material before imaging.

using 10× and 20× molar excesses of **6** respectively. The reaction vessels were agitated gently at room temperature for 1 h, while protected from light. Conjugates were then purified by gel permeation chromatography. The degree of labeling (moles of **6** per mole of antibody) was calculated spectroscopically (Table 1).

In order to ensure covalent conjugation of **6** to the antibodies had indeed occurred, as opposed to noncovalent binding often observed with hydrophobic fluorophores (11), polyacrylamide gel electrophoresis was performed. As such, 5 μg of conjugated and unconjugated antibody was loaded onto a 10% nonreducing polyacrylamide gel. After separation the gel was illuminated under UV light to visualize the antibodies labeled with **6**. This was followed by coomassie blue staining to visualize the total protein present (Fig. 1).

Finally, in order to confirm that antibody functionality was retained after labeling with **6**, cells expressing EpCAM and CD146 respectively were incubated with the corresponding labeled antibody, washed to remove unbound antibody, and visualized by fluorescence microscopy (Fig. 2).

CONCLUSION

In conclusion we have synthesized, for the first time, BODIPY analogues bearing isothiocyanate groups and demonstrated their applicability for fluorescence labeling of proteins bearing primary amino groups. Because of inherent photophysical advantages of the BODIPY fluorophore, these molecules have great potential advantages in the areas of multiphoton fluorescence microscopy and fluorescence polarization assays.

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