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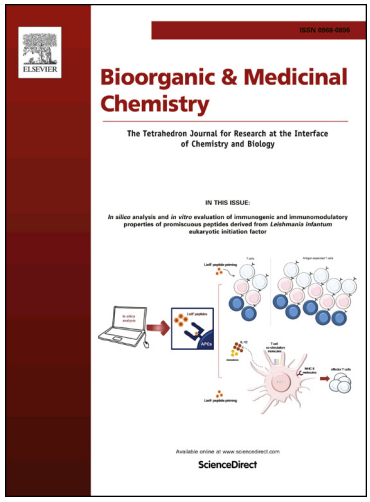

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Graphical Abstract

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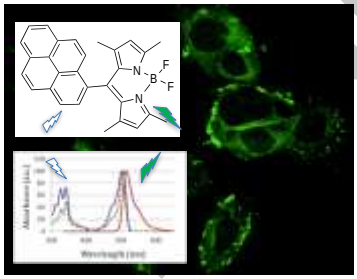

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Cellular imaging using BODIPY-, pyrene- and phthalocyanine- based conjugates

Faustine Bizet,[1] Martin Ipuy,[1] Yann Bernhard,[1] Vivian Lioret,[1] Pascale Winckler,[2] Christine Goze,[1] Jean-Marie Perrier-Cornet,[2] Richard A. Decréau[1*]

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ABSTRACT

Fluorescent Probes aimed at absorbing in the blue/green region of the spectrum and emitting in the green/red have been synthesized (as the form of dyads-pentads), studied by spectrofluorimetry, and used for cellular imaging. The **synthesis** of phthalocyanine-pyrene **1** was achieved by cyclotetramerization of pyrenyldicyanobenzene, whereas phthalocyanine-BODIPY **2c** was synthesized by Sonogashira coupling between tetraiodophthalocyanine and meso-alkynylBODIPY. The standard four-steps BODIPY synthesis was applied to the BODIPY-pyrene dyad **3** starting from pyrenecarbaldehyde and dimethylpyrrole. ^{1}H, ^{13}C, ^{19}F, ^{11}B-NMR, ICP, MS, and UV/Vis spectroscopic analyses demonstrated that **2c** is a mixture of BODIPY-Pc conjugates corresponding to an average ratio of 2.5 BODIPY per Pc unit, where its bis, tris, tetrakis components could not be separated. **Fluorescence emission studies** (μM concentration in THF) showed that the design of the probes allowed excitation of their antenna (pyrene, BODIPY) in the blue/green region of the spectrum, and subsequent transfer to the acceptor platform (BODIPY, phthalocyanine) followed by its emission in the green/red (with up to 140-350 nm overall Stokes shifts). The fluorescent probes were used for **cellular imaging** of B16F10 melanoma cells upon solubilization in 1% DMSO containing RPMI or upon encapsulation in liposomes (injection method). Probes were used at 1-10 μM concentrations, cells were fixed with methanol and imaged by biphoton and/or confocal microscopy, showing that probes could achieve the staining of cells membranes and not the nucleus.

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1. Introduction

In biphoton imaging, deeper penetration of light in tissues is possible compared to monophoton excitation [1]. Conjugating a blue-absorbing dye to another NIR-emitting-dye will make emission in the NIR possible provided that either FRET or through bond energy transfers (TBET) occur. Hence, such a strategy is a two-fold approach that will minimize the autofluorescence on the way in (biphoton) and way out (TBET followed by emission in the NIR). The rational of our study was to develop dyads/pentads of fluorophores in order to harvest light on a large window in biphoton microscopy. We selected three well-known classes of fluorophores that span the optical window: Pyrene (380 nm, blue), BODIPY (500 nm, green), and Phthalocyanine (Pc, 680 nm, near-IR) [2] (which are also known as robust and efficient photosensitizers in photodynamic therapy [3ab]). Hence, we were interested in fluorophore conjugates that could be used in cellular imaging upon transferring light from 300 nm to 700 nm (as in Pyrene-Pc **1a** reported by Ozcesmeci *et al.* [4]), 500 nm to 700 nm (as in BODIPY-Pc **2c**) and 300 nm to 500 nm (as in Pyrene-BODIPY **3a** reported by Goze *et al.* [5]). Some of these fluorescent molecular assemblies have been previously reported but have not been used as probes for cellular imaging. However, the design of BODIPY-Pc conjugates **2a** and **2b** reported by Göl *et al.* [6a] and Osati *et al.* [6b], respectively, are slightly different than **2c** depicted in Figure 1. Göl et al. BODIPY-Pc conjugate **2b** has either one phenyl ring or a -CH_2O- between the BODIPY and the alkyne, whereas our system **2c** provides a shorter connection between Pc and BODIPY that are separated with only one alkynyl group. The rationale for such an optimized design was to hope for a better transfer between the two antennas within the dyad by increasing the conjugation between the two antennas. At this stage of the study the yields of transfers were not measured. [7]

2. Materials and Methods

2.1. Materials: Chemicals were used as provided.

2.2. Measurements and Instrumentations

LC-MS mass spectrometry analyses, Analytical and reverse phase HPLC were performed on a Ultimate 3000 Thermo DIONEX apparatus. MALDI-TOF mass spectrometry analyses were performed on an Ultraflex II LRF 2000 apparatus, using dithranol and DHB as a matrix. ICP analyses were performed on a ICP-AES iCAP 7400 double visée Thermo apparatus. Low-resolution ESI analyses were carried out on an Amazon SL (BRUKER) mass spectrometer. High-resolution ESI analyses were performed on a LTQ Orbitrap XL (THERMO) apparatus. ^{1}H RMN , ^{19}F NMR and ^{11}B NMR analyses were performed on a 300 MHz Bruker Avance III NanoBay NMR apparatus. UV/Vis absorption spectra were measured using a UV-Visible AGILENT CARY 50 spectrophotometer. Fluorescence measurements,

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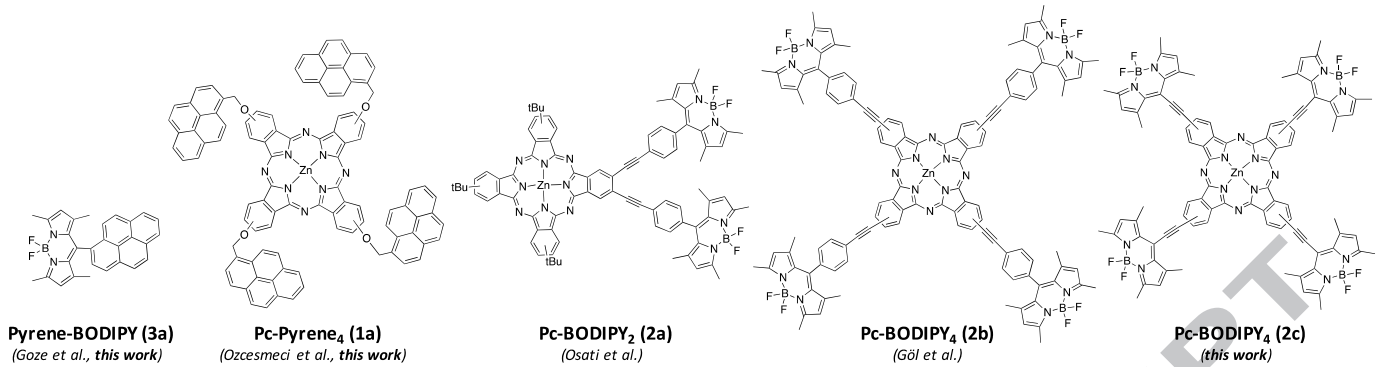

Fig.1. Synthetic dyads/triads/pentads described in the literature (1, 2a, 3) and reported in this work (2c), that span the 300-700 nm spectrum region.

excitation and emission spectra were recorded on a Fluorolog Jobin Yvon Horiba Xenon Lamp spectrometer. Biphotonic images were collected on a Nikon A1-MP scanning microscope (Nikon, Japan). Imaging was carried out with a ×25 Apo LWD objective (NA: 1.1, Water Immersion, Nikon, Japan) at a scanning speed of 0.5 frame per second. An IR laser (Chameleon, Coherent) was used to provide a 780nm excitation. Fluorescence emission was collected on four detection channels (channel 1 (blue) : FF01-492/SP-25 filter [400-492 nm] ; channel 2 (green) : FF03-525/50-25 filter [500-550 nm] ; channel 3 (orange) : FF01-575/25-25 filter [563-588 nm] ; channel 4 (red) FF01-629/56-25 filter [601-657 nm], Semrock). Confocal images were collected using a Nikon C1Si Eclipse TE 2000 confocal microscope (Nikon, Japan). Imaging was carried out with a ×100 PlanApo objective (NA: 1.4, oil, Nikon, Japan). Two laser diodes were used as light sources, which delivered 488 and 561 nm wavelength light. Fluorescence emission was collected by a spectral detector, using collection bands of [489-648]nm with 488nm excitation and [566-721]nm for 561nm excitation.

2.3. Methods

2.3.1. Syntheses of compounds 1-9

[2,9(10),16(17),23(24)-tetramethoxypyrenylphthalocyanina to]zinc(II) (1):

4-(Pyren-1-ylmethoxy)-phthalonitrile (**5**) (70 mg, 0.19 mmol), zinc acetate (10 mg, 0.048 mmol), 1,8-diazabicyclo{5.4.0}undec-7-ene (21 mg, 0.19 mmol) and pentanol (10 mL) were placed in a round bottom flask. The mixture was heated to reflux at 140°C for 22 h. The reaction mixture was subsequently allowed to come to room temperature, and then an ice bath was added to precipitate the product down. The precipitate was filtrated on a Büchner, rinsed with water, methanol, ethanol and acetone. Yield = 17%.

^{1}H NMR (300 MHz, DMSO-d_6, 300 K): δ (ppm)= 6.2 (s, 8H); 7.8 – 8.7 (m, 48H). MS (MALDI-TOF): m/z= 1497.89 $[M+H]^+$, 1283.83 $[M-(CH_2\text{-pyr})+H]^+$, 1069.78 $[M-(CH_2\text{-pyr})_2+2H]^+$, 855.53 $[M-(CH_2\text{-pyr})_3+3H]^+$, 641.13 $[M-(CH_2\text{-pyr})_4+4H]^+$. (Calcd for $C_{100}H_{57}N_8O_4Zn$: 1497.37 (exact mass); 1498.95). UV-Vis (THF): λ_{max} (nm)= 329, 344, 611, 680.

Phthalocyanine-Bodipy conjugates (2c) (Sonogashira coupling):

Pc-I_4 (**7**) (100 mg, 0.092 mmol), the alkyne-BODIPY (**6**) (100 mg, 0.36 mmol) and copper iodide (35 mg, 0.18 mmol) were put in a two-necked round bottom flask. Dry tetrahydrofuran (10 mL) and triethylamine (5 mL) were added, and the resulting mixture was degased upon three series of freeze-pump-thaw-nitrogen refill cycles. Tetrakis-(triphenylphosphine)-palladium (35 mg, 0.03 mmol) was added, and the mixture was stirred for 18 h. Ammonium chloride was added to quench the reaction, the organic phase was extracted with dichloromethane, washed with water and with a saturated solution of ammonium chloride, and the solvents were removed under reduced pressure. The resulting powder was washed with methanol and dichloromethane. Yield = 60%

^{1}H NMR (300 MHz, DMSO-d_6, 300 K): δ (ppm)= 2.5 - 3 (broad s, H from BODIPY methyl) ; 6.04 (broad s, H from BODIPY core) (hard to define the exact number of proton, no well-define peak), 7.0 – 8.5 (broad signal, H from Pc) ^{19}F NMR (280 MHz, d-DMSO, 300 K): δ (ppm)= -146 ppm (not well-defined peak).^{11}B NMR (128 MHz, d-DMSO, 300 K): δ (ppm)= 0.524 (not well-defined peak). MALDI-TOF: m/z= 1224.85 $[M_{mono}+H]^+$; 1214.82 $[M_{mono}-9]^+$, 1369.06 $[M_{bis}+H]^+$; 1349.06 $[M-F]^+$; 1359.03 $[M_{bis}-9]^+$; 1513.26 $[M_{tris}-9]^+$; 1493.26 $[M-F]^+$; 1503.23 $[M_{tris}-9]^+$; 1647.42 $[Mtetra-9]^+$(calcd for $C_{92}H_{68}B_4F_8N_{16}Zn$: 1656.53 (exact mass); Mono 1223.88 ; Bis 1368.10 ; Tris 1512.32 ; Tetra 1656.23). ICP-AES: Zinc $_{theorical}$ = 3.94%, Zinc $_{exp}$= 3.83%, Boron $_{theorical}$ = 2.61%, Boron $_{exp}$= 1.63%. UV-Vis (DCM) : λ_{max} (nm)= 374, 567, 697.

Pyrenyl-BODIPY(3):

Pyrenecarboxaldehyde (230 mg, 1 mmol) and dimethylpyrrole (0.215 mL, 2 mmol) were put in a round bottom flask then 30 mL of dry dichloromethane was added under nitrogen atmosphere, followed by one drop of trifluoroacetic acid. The solution became red, and the mixture was stirred for 16h under nitrogen atmosphere. p-Chloranil (250 mg, 1 mmol) was added and the solution was stirred for 30 min under nitrogen atmosphere. Triethylamine (3 mL, 22 mmol) and boron trifluoride diethyletherate (3 mL, 24 mmol) were added in the mixture that was previously cooled in an ice bath. The mixture was stirred for 3 h at room temperature. Then the organic phase was washed with water and dried with magnesium sulphate, and solvents were removed under reduced pressure. The resulting crude product was filtered on a plug of silica gel (dichloromethane). The filtrates were collected, the solvent was removed, and the resulting solid was subjected to silica gel column chromatography (silica gel, eluent ethyl acetate : petroleum ether, gradient from 6 :94 to 12 : 88 vol.). Yield = 22%.

^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm)= 0.87 (s, 6H) ; 2.6 (s, 6H) ; 5.93 (s, 2H) ; 7.8 – 8.4 (m, 9H). ^{19}F NMR (280 MHz, $CDCl_3$, 300 K): δ (ppm)= -146 (q, $^1J_{F\text{-}B}$= 32 Hz, 2F). ^{11}B NMR (128 MHz, $CDCl_3$, 300 K): δ (ppm)= 0.995 (t, $^1J_{B\text{-}F}$= 32 Hz, 1B). ESI-MS: m/z= 471.17 $[M+Na]^+$, 447.18 $[M-H]^-$ (calcd for $C_{29}H_{23}BF_2N_2$: 448.19 (exact mass); 448.31 (FW)). UV-Vis (DCM) : λ_{max} (nm)= 327, 342, 504.

Pyrenylmethanol (4):

Pyrene-aldehyde (1 g, 4.3 mmol) and dry tetrahydrofuran (20 mL) were placed in a round bottom flask. Sodium borohydride (165 mg, 4.3 mmol) was added in small portions,

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together with small portions of methanol to help the solubilisation (total volume of added methanol = 10 mL). An orange solution was obtained. The reaction was quenched with a 2% concentrated hydrochloric acid solution. The solvent was removed under reduced pressure. The white powder obtained was dissolved in dichloromethane, washed with water and the organic phase was dried with magnesium sulphate. The solvent was removed under reduce pressure. The resulting solid was subjected to silica gel column chromatography (eluent = dichloromethane). Yield = 88%

^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm)= 1.87 (s, 1H) ; 5.39 (s, 2H) ; 7.9 – 8.4 (m, 9H). ^{13}C NMR {^{1}H} (75 MHz, $CDCl_3$, 300 K): δ (ppm)= 64,03; 123.15; 124.87; 124.93; 125.13; 125.42; 125.45; 126.14; 126.19; 127.54; 127.63; 128.07; 128.97; 130.95; 131.42; 131.44; 133.94. ESI-MS: m/z= 254.97 $[M+Na]^+$, 214.99 $[M-OH]^+$(Calcd. for $C_{17}H_{12}O$: : 232.09 (exact mass); 232.28 (FW)). UV-Vis (DCM) : λ_{max} (nm)= 314 ; 327 ; 344.

4-(pyren-1-ylmethoxy) phthalonitrile (5):

4-Nitrophthalonitrile (87 mg, 0.5 mmol) and 1-pyrenylmethanol (**4**) (116 mg, 0.5 mmol) were put in a round bottom flask. Dimethylsulfoxyde (10 mL) was added and the mixture was stirred until the powder was completely dissolved. Potassium carbonate (250 mg, 1.75 mmol) was added in small portions over a period of 2 h. The reaction mixture was stirred for 22h at room temperature. Then it was poured in 50 mL of water that results in the precipitation of a white solid. The solid was isolated by filtration on a Büchner, then rinsed with water, heptane and diethyl ether. Yield = 59%.

^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm)= 5.80 (s, 2H) ; 7.24 (s, 1H) ; 7.34 (d, ^{3}J= 8.7 Hz, 1H) ; 7.68 (d, ^{3}J= 8.7 Hz, 1H) ; 8 – 8.25 (m, 9H). **ESI-MS:** m/z= 356.98 $[M-H]^-$, 381.08 $[M+Na]^+$ (Calcd for $C_{25}H_{14}N_2O$: 358.11 (exact mass); 358.39). UV-Vis (DCM) : λ_{max} (nm)= 315 ; 329 ; 346.

TMS-deprotected BODIPY alkyne (6):

TMS-protected BODIPY-alkyne (**8**) (400 mg, 0.999 mmol) was dissolved in 80 mL of methanol. Potassium fluoride (290 mg, 4.995 mmol) was added and the solution was stirred for 90 min. The solvent was removed under reduced pressure and the powder was dissolved in dichloromethane. The organic phase was extracted with dichloromethane, washed with water and dried over magnesium sulphate. The solvent was removed under reduced pressure to afford a brown powder. Yield = 70%

^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm)= 2.43 (s, 6H) ; 2.5 (s, 6H) ; 3.89 (s, 1H) ; 6.04 (s, 2H). ^{13}C NMR {^{1}H} (75 MHz, $CDCl_3$, 300 K): δ (ppm)= 14.77; 15.60; 79.37; 94.52; 119.06; 121.19; 133.46; 142.75; 155.23. ^{19}F NMR (280 MHz, $CDCl_3$, 300 K): -146 ppm (q, $^1J_{F-B}$= 65.5, 32.7 Hz, 2F). ESI-MS: m/z= 273.04 $[M+H]^+$, 295.01 $[M+Na]^{+\cdot}$ (calcd for $C_{15}H_{15}BF_2N_2$: 272.13 (exact mass); 272.10 (FW)). UV-Vis (DCM) : λ_{max} (nm)= 338, 509, 544.

[2,9(10),16(17),23(24)-tetraiodophthalocyaninato]zinc (Pc-I$_4$) (7):

4-Iodo-phthalonitrile (**9**) (1 g, 3.95 mmol), 1,8-diazabicyclo{5.4.0}undec-7-ene (602 mg, 3.95 mmol) and zinc acetate (180 mg, 0.98 mmol) were put in a round bottom flask and 10 mL of pentanol were added. The mixture was heated at 130°C and turned blue. After 23 h of reflux, the solution was allowed to come to room temperature affording a dark precipitate. Pentanol was removed, and the precipitate was resuspended in methanol and filtrated. It was subsequently rinsed with a 1M hydrochloric acid solution until the filtrate turned colourless, then it was rinsed with water. Yield = 75%.

^{1}H NMR (300 MHz, d-DMSO, 300 K): δ (ppm)= 8,38 (d, ^{3}J= 7.7 Hz, 4H) ; 8.63 (m, 4H) ; 9.13 (m, 4H). MALDI-TOF: m/z= 1080.79 $[M+H]^+$ (Calcd for $C_{32}H_{12}I_4N_8Zn$: 1079.67 (exact mass); 1081.49 (FW)). UV-Vis (DMSO) : λ_{max} (nm)= 357 ; 615 ; 682.

TMS-protected BODIPY-alkyne (8):

Dimethylpyrrole (1 g, 10 mmol) was put in a round bottom flask under nitrogen atmosphere, and 22 mL of dried dichloromethane were added. The resulting mixture was sparged with nitrogen bubbling for 90 mins. Then 3-(trimethylsilyl)-2-propynal (0.733 mL, 5 mmol) was added drop wise at -70°C over a period of 30 min. The solution became purple. After the addition, stirring was carried on for 15 more minutes and removed. The solution was allowed to come to room temperature during 3 h. The solution became orange. First, p-Chloranil (1.23 g, 10 mmol) was added, the reaction mixture was stirred for 1 h until it turned purple. Then, triethylamine (6.74 mL, 50 mmol) was added, the reaction was stirred for 30 min at room temperature. **Finally, boron trifluoride diethyl etherate** was added drop wise and the reaction was stirred for 16 h. Dichloromethane was added to the mixture and the organic phase was extracted with dichloromethane, washed with water and dried over magnesium sulphate. The solvent was removed under reduced pressure. Acetone was added until there was no more smoke emission, and was removed under reduced pressure. The solid obtained was subjected to column chromatography (silica gel, using a petroleum ether: dichloromethane mixture (70 : 30 vol.) as the eluent). Yield = 70%.

^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm)= 0,27 (s, 9H) ; 2.5 (s, 6H) ; 2.4 (s, 6H) ; 6.04 (s, 2H). ^{13}C NMR {^{1}H} (75 MHz, $CDCl_3$, 300 K): δ (ppm)= 0.56; 14.73; 15.65; 100.62; 115.11; 120.20; 120.91; 133.21; 142.37; 154.55. ESI-MS: m/z= 345 $[M+H]^+$; 325 $[M-F]^+$; 367 $[M+Na]^{+\cdot}$ (calcd for $C_{18}H_{23}BF_2N_2Si$: 344.17 (exact mass); 344.28 (FW))

4-Iodo-phthalonitrile (9):

4-amino-phthalonitrile was placed in a three-necked round bottom flask. 50 g of crushed ice and 80 mL of hydrochloric acid (37%) were added. Then a solution of sodium nitrite (3 mg, 43 mmol) in water (30 mL) was slowly added to the reaction mixture at 5°C over 30 min. The reaction mixture was stirred at 5°C for 1h. The precipitate formed was filtrated and washed with water (50 mL). The filtrate was added dropwise on a potassium iodide solution (7 mg, 42 mmol) in 30 mL of water at 5°C over a period of 1 h. The reaction mixture was warmed to room temperature during 14 h. An orange powder was filtrated on Büchner, and dissolved in toluene (affording an orange filtrate). The organic phase was washed with saturated bicarbonate sodium solution, saturated sodium thionite solution, brine, then dried over magnesium sulphate. After concentration under reduced pressure, the resulting solid was subjected to column chromatography (silica gel, eluent = dichloromethane: toluene, 50: 50 vol.). Yield= 51%.

^{1}H NMR (300 MHz, d-DMSO, 300 K): δ (ppm)= 7.85 (d, ^{3}J= 7.8 Hz, 1H); 8.31 (dd, ^{3}J= 7.8, 1.6 Hz, 1H) ; 8.58 (d, ^{3}J= 1.6 Hz, 1H). ^{13}C NMR {^{1}H} (75 MHz, $CDCl_3$, 300 K): δ (ppm)= 99.78; 113.95; 115.05; 115.22; 117.16; 134.12; 142.18; 142.59. ESI-

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MS: m/z= 276.81 $[M+Na]^+$; 252.83 $[M-H]^-$; (calcd for $C_8H_3IN_2$: 253.93 (exact mass); 254.03 (FW)).

BODIPY-dicyanobenzene conjugate (14)

4-Iodo-phthalonitrile (**9**) (55.6 mg, 0.22 mmol), the alkyne-BODIPY (**6**) (60 mg, 0.22 mmol) and copper iodide (6,9 mg, 0.036 mmol) were put in a two-necked round bottom flask. Dry tetrahydrofuran (10 mL) and triethylamine (4 mL) were added, and the resulting mixture was degased upon three series of freeze-pump-thaw-nitrogen refill cycles. Tetrakis-(triphenylphosphine)-palladium (11,5 mg, 0.01 mmol) was added, and the mixture was stirred for 22 h. Ammonium chloride was added to quench the reaction, the organic phase was extracted with dichloromethane, washed with water and with a saturated solution of ammonium chloride, dried over $MgSO_4$, filtered, and the solvents were removed under reduced pressure. The resulting residue was subjected to column chromatography on silica gel using a dichloromethane-petroleum ether mixture (4:1 vol.) as the eluent. Yield = 60%

^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm)= 2.49 (s, 6H) ; 2.54 (s, 6H) ; 6.10 (s, 2H), 7.84 (m, 3H). ^{19}F NMR (280 MHz, $CDCl_3$, 300 K): -145.9 ppm (q, $^1J_{F-B}$= 65.5, 32.7 Hz, 2F). ESI-MS: m/z= 273.04 $[M+H]^+$, 295.01 $[M+Na]^+$. (calcd for $C_{23}H_{17}BF_2N_4$: (exact mass); 397.02 [M-H], 380.13 $[M-F]^+$ (FW)).

2.3.2. Fluorescence studies

Fluorescence measurements were performed on a Jasco FP-8500 spectrofluorometer equipped with a Xe source. Excitation was performed at 329 nm (**1a**), 551 nm (**2c**), and 328 nm (**3a**). Emission spectra were recorded for an absorbance at 329 nm (pyrene), 551 nm (BODIPY) comprised between 0.03 and 0.07.

2.3.3. Cell Culture and Fixation

B16F10 melanoma cells were grown in RPMI supplemented with 10% fetal calf serum and 1% streptomycin and penicillin. Two days before imaging studies, cells were harvested and placed in 8-well microscopy chambers (8 chambers polystyrene vessel (with tissue culture treated glass slides; BD Falcon Culture slides Ref 3541 18)). Cells were incubated with non-supplemented RPMI mixed with probes **1**,**2** in solution in DMSO (the % of which, upon addition in the well, does not exceed 1,5%) or probe **3** as a liposomal suspension. Upon 1 h incubation, the medium was removed and cells were rinsed with PBS. Then cold methanol (stored at -30°C) was added in each well, and the plates chambers were stored for 5 min at 4°C in the fridge. Methanol was then carefully removed from the well, and replaced with cold PBS (4°C) and the plate chambers were stored on ice up to the biphoton microscope. Then the walls were removed, PBS was added on the cells, and a microscopy glass was added on the cells prior to microscopy study.

2.3.4. Preparation of Liposomes

Liposomes were prepared by the injection method following earlier procedures [3b,]. Two solutions have been prepared : 1,2-dipalmitoyl-*sn*- glycero-3-phosphocholine (14.6 mg) in ethanol (1 mL) (solution 1), and the probe (5 µmol) in chloroform (10 mL) (solution 2). 100 µmol of each solution were mixed and quickly injected using an Hamilton seringe into a vigorously stirred PBS (10 mL) at 60°C. Stirring at 60°C went on for 2 min, then heating was stopped and the solution was gradually cooled down to room temperature under stirring. The liposome suspension was used immediately.

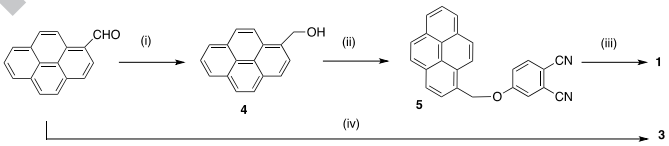
2.3.5. Imaging studies

Upon fixation (using methanol) cells were imaged using a biphoton microscope and then a confocal microscope.

3. Results and Discussions

3.1. Synthesis and Characterization of probes

The syntheses of pyrene-containing probes **1** and **3** were the most straightforward to carry out. Pyrene-Pc **1** was synthesized by reduction of pyrene carboxaldehyde with $NaBH_4$ to afford pyrenyl carbinol **4** that undergoes S_NAr with nitrodicyanobenzene to afford pyrenyloxydicyanonobenzene 5. The latter was subsequently cyclotetramerized in the presence of zinc salt and DBU to afford target **1**, because of solubility issues it was only characterized by HRMS and UV/Vis. Moreover, ^{1}H-NMR analysis allowed to identify the presence of key signals (albeit very broad). The Pyrene-BODIPY conjugate **3** was synthesized in a three-steps one-pot procedure starting from a TFA-catalyzed condensation of pyrenyl-carbinol and dimethylpyrrole, followed by *p*-chloranil oxidation, deprotonation with triethylamine followed by borylation with BF_3-etherate. Target **3** was characterized by ^{1}H-NMR, ^{13}C-NMR, ^{11}B-NMR (doublet at ca. 1 ppm), ^{19}F-NMR (doublet of triplet at -146 ppm) and UV/Vis (characteristic absorption band at 327, nm and 342nm and 504).



Scheme 1. Synthesis of pyrene-containing targets (**1**) and (**3**) (i) $NaBH_4$, THF, MeOH, yield: 88% ; (ii) K_2CO_3, DMSO, yield: 59% ; (iii) $Zn(OAc)_2$, DBU, pentanol, yield: 17% ; (iv) a) dimethylpyrrole, TFA, b) *p*-chloranil, c) NEt_3, d) $BF_3.Et_2O$, yield: 22%.

Pc-BODIPY **2c** was synthesized from alkynyl-BODIPY **6** (scheme 2A) and tetraiodophthalocyanine $ZnPCI_4$ **7** (scheme 2B). The condensation of dimethylpyrrole and trimethylsilylpropynal led to the formation of dipyrromethane that was oxidized, deprotonated and borylated to afford **8** in an overall three-steps one pot procedure. It should be noted that no acid catalyst was necessary in the synthesis of BODIPY **6**, unlike that of BODIPY **3** (scheme 1 and 2). The protective group in **8** was removed to afford unprotected alkynyl-BODIPY **6**. Tetraiodophthalocyanine synthon **7** was synthesized from aminodicyanobenzene as follows: the latter was reacted with sodium nitrite to afford intermediate diazonium salt (none isolated), which reacts with iodide to afford iododicyanobenzene **9**. DBU-catalyzed cyclotetramerization of the latter in refluxing pentanol afforded $ZnPcI_4$ synthon **7** (Scheme 2B). This synthon was obtained as a mixture of regioisomers that cannot be separated. Subsequent Sonogashira coupling (Cu(I) / Pd(0) catalyzed) between iodinated phthalocyanine **7** and alkynyl-BODIPY **6** afforded BODIPY-Pc conjugate **2c**. It was obtained as a mixture with various degree of fonctionnalisation (from 1 to 4 BODIPY unit per Pc as determined by MALDI-TOF MS) because of incomplete Sonogashira coupling. The mixture was purified by a series of washings with methanol to remove impurities. It was eventually subjected to Sonogashira coupling again, which increased the conjugation rate in BODIPY. Several attempts to separate the different BODIPY-Pc conjugates by standard chromatography on silica failed.

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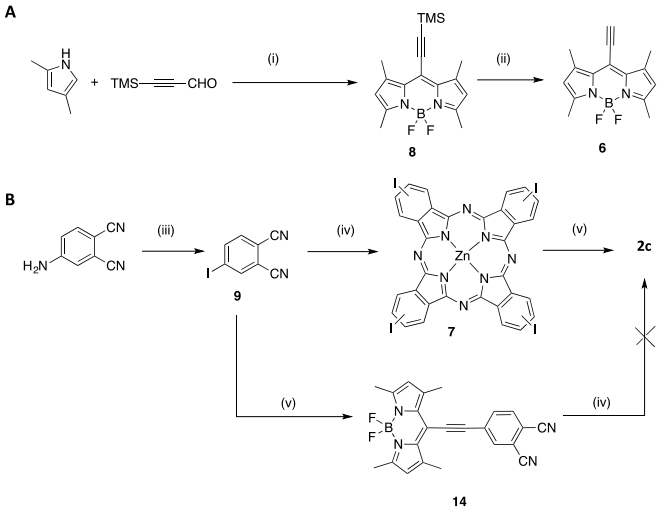

Scheme 2. Synthesis of synthons, such as BODIPY **6** and $ZnPcI_4$ **7** and subsequent Sonogashira coupling between **6** and **7** leading to target **2c**. (i) *p*-chloranil, NEt_3, $BF_3.Et_2O$, yield: 70%; (ii) KF, MeOH, yield: 70 % (iii) $NaNO_2$, KI, CH_3OH, yield: 51%; (iv) DBU, $Zn(OAc)_2$, pentanol, yield in **7**: 75%; (v) **6**, $Pd(PPh_3)_4$, CuI, Et_3N, THF, yield in **2c**: 60%; yield in **14** : 40%.

The characterization of the statistical mixture in **2c** was carefully achieved in light of the spectroscopic signatures of its two components (BODIPY **6** and Pc **7**). It was achieved using an array of spectroscopic tools such as HRMS, UV-Vis, ^1H, ^{11}B and ^{19}F NMR and also ICP (Figure 2 A-E). They offered a series of spectroscopic handles that allowed to conclude about the nature of **2c**. *(a)* First, upon extensive extractions and washings, TLC of **2c** shows only one purple spot on TLC (with a short tail), indicative of the removal of non-phthalocyanine and non-BODIPY materials. *(b)* MALDI-TOF mass spectrometry shows the molecular peaks of species that correspond to phthalocyanine bearing two to four BODIPY moieties. The corresponding peaks with loss of fluorine atoms were also observed. *(c)* ICP analysis gave an Zn/B ratio corresponding to about 2.5 Boron atoms (from the BODIPY) per atom of Zinc (from the Pc). *(d)* ^1H NMR of **2c** is not as resolved as that of phthalocyanine precursor **7**. However, it clearly shows the protons of the phthalocyanine isoindolic moieties (7 - 8 ppm) and the protons of the BODIPY moieties, such as pyrrolic protons (6 - 6,5 ppm), and methyl protons (3 ppm). The integration of these two signals roughly concurs with the BODIPY/Pc ratio determined by ICP. Note that the broad signals may be the result from stacking and the presence of multiple regioisomers. *(e)* The proof of the presence of the BODIPY moiety was also shown by ^{11}B NMR and ^{19}F NMR signals at δ (ppm)= 0.524 et δ (ppm)= -146 ppm, respectively. *(f)* UV-Vis spectroscopy performed in dichloromethane shows three bands that correspond to the Pc moiety at 379 nm (Soret) and 689 nm (Q bands), and that of the BODIPY moiety at 379 nm and 555 nm. Not only all bands appear to have a broad shape, but also the Q bands appear flat. Upon addition of a coordinating solvent in the solution, such as pyridine (in red) or tetrahydrofuran, the intensity of Q bands increased significantly with respect to that of other bands. Moreover, a bathochromic shift was observed. These data are the signature of a natural aggregation phenomenon that is inhibited upon addition of coordinating molecules preventing the molecular stacking. Altogether, these spectroscopic data indicate that **2c** is a statistical mixture that corresponds to the grafting of about 2.5 BODIPY per Pc, where the final molecular construct indicates a strong tendency to aggregate in non-coordinating solvents.

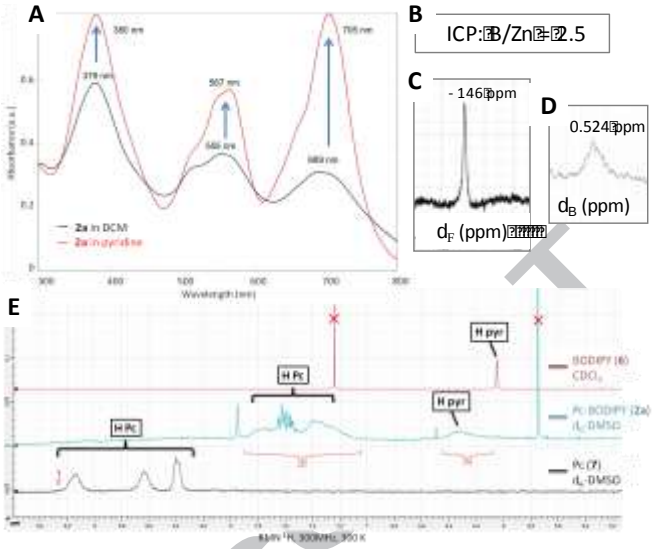

Fig. 2. Characterization of **2c**: A. UV/Vis in non-coordinating solvent (dichloromethane, black) and in the presence of coordinating pyridine (red); B: ICP-AES; B: Mass Spectrometry; C: ^{19}F-NMR; D: ^{11}B-NMR; E: ^1H-NMR.

Attempts to couple up to four BODIPY were made : (i) the isolated statistical mixture was subjected again to Sonogashira coupling up to three times. Upon removal of non-phthalocyanine components by washings, ICP and ^1H-NMR measurement indicated a slight improvement, with a ratio close to 1:4 (i.e. four BODIPY per Pc). (ii) Finally, another synthetic approach consisted in coupling alkynyl-BODIPY **6** with the iodophthalonitrile precursor **9** first to afford BODIPY-phthalonitrile **14**. Subsequent cyclotetramerization of **14** did not work in our hands, unlike what Durmus reported with an analogous system (6).

3.2. Absorption and Fluorescence emission studies

The overlay of the absorbance spectra of Pc-pyrene **1** and its precursor pyrenyl-methanol **4** and Pc **7** shows a good match between their two maximum absorption bands : bands at 329 nm and 345 nm correspond to the pyrene, whereas bands at 611 nm and 678 nm correspond to the Pc (Fig. 3A). Fluorescence emission spectra obtained upon excitation at 329 nm show that the bands corresponding to pyrene are no longer present, whereas that corresponding to Pc are present (Fig. 3B). This indicates that the energy transfer occuring from pyrene to Pc occurs, as previously described by Ozcesmeci *et al*.

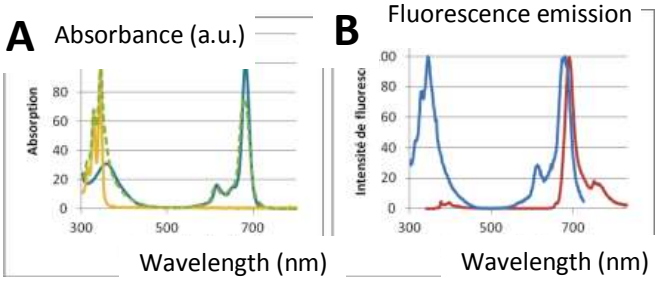

Fig. 3. Pc-pyrene **1**. A : Absorption spectra of 1-pyrenylmethanol **(4)** (yellow) in DCM, Pc-I_4 **(7)** (blue) in DMSO and **(1)** (dotted green) in DMSO. B : Pc-pyrene **1** emission spectrum (red) and excitation spectrum (purple) measured in DMSO (λ ex = 329 nm; λ em = nm)

The overlay of the absorbance spectra of Pc-BODIPY **2c** and that of its two components BODIPY **6** and Pc **7** shows a good match between their maximas, which appear at 373 and 551 nm with

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BODIPY **(6)**, and 373 and 694 nm with Pc **7** (Fig. 4A). Upon excitation at 551 nm (BODIPY), subsequent fluorescence emission spectra show that the transfer from the BODIPY antenna towards the Pc platform occurred (Fig. 4B). Indeed, emission peak corresponding to the maximum emission of Pc is observed (700 nm). Moreover, when the excitation was achieved at 373 nm subsequent fluorescence emission also occured leading to a similar emission spectrum.

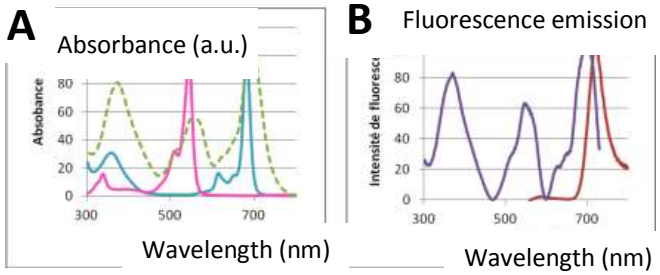

Fig 4. BODIPY-Pc **2c**. A : Absorption spectra of BODIPY-alkyne **6** (pink, measured in DCM), phthalocyanine precursor Pc-I_4 **7** (blue, measured in DMSO) and phthalocyanine-BODIPY conjugate **2c** (dotted green, measured in THF). B : BODIPY-Pc **2c** emission spectrum (red, λ_{ex}= 551 nm) and excitation spectrum (purple) measured in THF (λ em = nm) .

The overlay of the absorbance spectra of pyrene-BODIPY **3** with that of its components, pyrene and BODIPY, shows two absorption maxima at 328 and 343 nm for the pyrenyl component, and a maximum at 504 nm that corresponds to the BODIPY. Fluorescence emission spectrum of **3** shows a single band at 515 nm that corresponds to the emission of BODIPY. It also indicates that there is a complete transfer from the pyrene moiety to the BODIPY as no band was found below 500 nm (Fig. 5). Hence, there is no more fluorescence signal corresponding to pyrene.

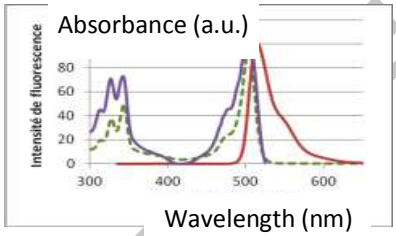

Fig. 5. BODIPY-pyrene **3**. Absorption spectrum (dotted green), emission spectrum (red) and excitation spectrum (purple) measured in DCM (λ_{ex} = 328 nm; λ ex = 515 nm)

Table 1. Absorption and emission data.

Compound	Solvent	Abs. max. (nm)	Em. Max. (nm)
Pyrene-OH (**4**)	DCM	329, 345	355
PcI_4 (**7**)	DMSO	373, 611, 678	690
Pc-$Pyrene_4$ (**1a**)	DMSO	373, 694	700
BODIPY-CCH (**6**)	DCM	360, 551	515
BODIPY-Pyrene (**3a**)	DMSO	345, 551	515
Pc-$BODIPY_4$ (**2a**)	DCM	373, 551, 694	700

3.3. Cellular Imaging studies

Cell imaging studies were carried out in light of previous reports using BODIPY, Pc, pyrene monomeric probes [2b, 11]. Here, prior entrapment of fluorescent probes **1-3** in SUV liposomes was attempted following our earlier procedure [3b,10]. It was successful with BODIPY-pyrene conjugate **3** (Fig. 6) but we failed to reach a successful incorporation rate with Pc-containing species **1-2** (for lack of solubility reasons in solvent classically used for liposomes, Supp. Info Fig S9). Hence, the latter were used as a solution in DMSO and subsequently injected in RPMI such as DMSO content does not reach more than 1%. Controls, i.e. untreated cells that do not contain any probe (raw 1, Figure 6) barely fluoresce upon two-photon excitation at various wavelengths and whatever filter was used (blue, green, red channels). In these images, it should be noted that the background remained dark whereas stained cells are well defined. Staining of membranes and cytoplasm occurred, whereas the nucleus remains unstained.

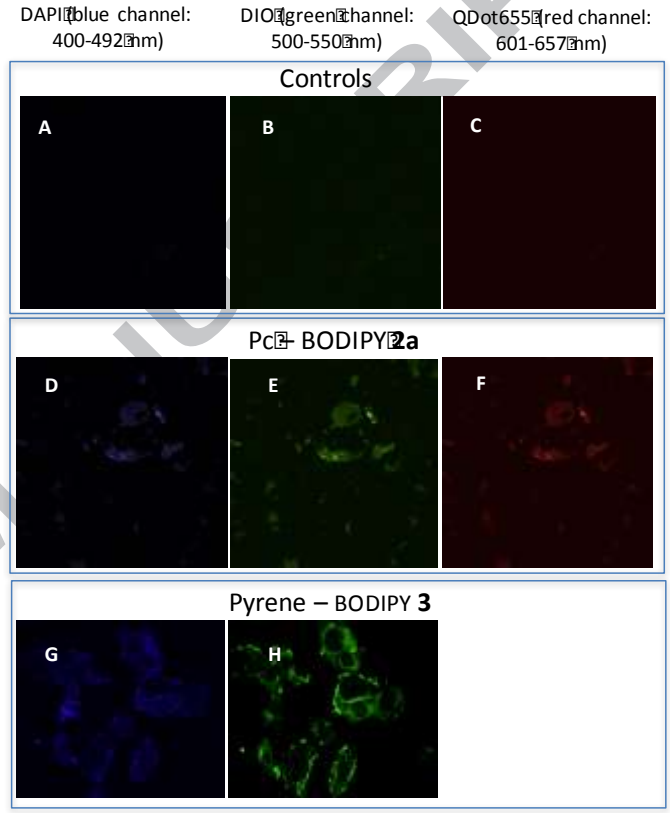

Fig 6. Fluorescence biphoton microscopy images (exc. 750 nm; channels: blue (A,D,G), green (B, E, H) and red (C, F)) of B16-F10 melanoma cells upon treatment and fixation with methanol: untreated cells (controls, images A-C), and cells incubated with 1 µM of probes: probe Pc-BODIPY **2c** (solution in RPMI with 1.5% DMSO, images D-F) and Pyrene-BODIPY **3** (liposome suspension, images G-H).

This is unlike cells treated with Pc-BODIPY conjugate **2c** (raw 2, Figure 6) in solution in RPMI with 1% DMSO: strong fluorescence emission was observed mostly in the green canal (E, BODIPY emission) but also in the blue (D) and red canal (F). Cells treated with Pc-Pyrene conjugate **1** (in solution with 1% DMSO) are fluorescent with fluorescence emission mostly in the blue (pyrene) and red (Pc) windows (data not shown, see Fig S9 in the SI). Interestingly, cells treated with a liposomal solution of BODIPY-pyrene conjugate **3** emit in the blue (pyrene, G) and green (BODIPY, H) windows and barely in the red. These results were revisited using confocal microscopy (Figure 7): it also indicates that cells were stained with fluorescent probes **2c**, results with different canals also indicate that transfers from one antenna to the other occurred and may be reminiscent of the spectrofluorimetry studies (fluorescence emission spectra Figures 3-5). Altogether microscopy and spectrofluorimetry studies seem to indicate that transfers from the donor to the acceptor moiety within a fluorophore conjugate do occur. However, these two studies do not seem to be a mirror image of each other: upon

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excitation at (750-800 nm) subsequent cell imaging remain possible in the green canal indicating emission of the BODIPY emission, which spectrofluorimetry studies showed does not occur. Such differences may possibly be explained from various environnement: DMSO (spectrofluorimetry) vs cellular environnement (microscopy studies).

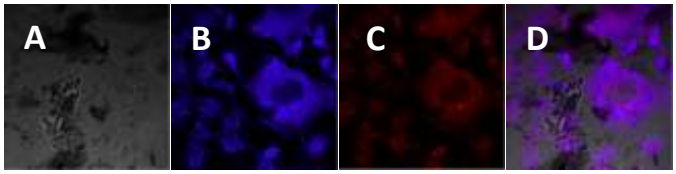

Fig 7. Confocal images of B16F10 cells incubated with a solution of **2c** (1 μM) in solution (excitation at 405 nm) : transmission (A), fluorescence with collection at 400-500 nm (B) or 668 – 678 nm (C), and superposition (D).

4. Conclusion

Herein we reported the syntheses of several fluorophores conjugates and subsequent B16F10 cellular imaging (biphoton, confocal) upon irradiation in the 300-500 nm window with a resulting emission beyond 500 nm and up to 720 nm. The probes that were developed are pyrene-phthalocyanine **1** (ca. 300-700 nm), pyrene-BODIPY **3** (ca. 300-500 nm), and BODIPY-phthalocyanine **2c** (ca. 500-700 nm). The design and syntheses of these conjugates was reminiscent of other reported structures. From a synthetic standpoint, the conjugation of up to four BODIPY per Pc platform was difficult to achieve: a mixture of BODIPY-Pc conjugates, consisting in 2.5 BODIPY per Pc platform was obtained instead. The probes are not soluble in water, hence they were formulated in DMSO (1% vol. in RPMI) or in liposomes. The proof-of-concept of the study was met, all probes **1-3** led to efficient staining of cells, with a good contrast and dark background, with staining of membranes and cytoplasm. However, a complete correlation between microscopy and spectrofluorimetry studies could not be noticed: spectrofluorimetry studies showed that upon excitation of the BODIPY antenna (in the BODIPY-Pc conjugate), the cascade energy transfer to the Pc acceptor appear to be complete because no subsequent emission of the BODIPY antenna occured, whereas a strong emission of the Pc moiety was observed. However, biphoton microscopy still shows intense BODIPY emission in the green canal upon excitation at 750-800 nm (ca. 400-450 nm).

Acknowledgments

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Supplementary data

NMR and mass spectra of **4-6, 8, 14** are listed as supplementary material

Acronyms

FRET: Förster Resonance Energy Transfer
TBET: Through-Bond Energy Transfer
DBU: 1,8-DiazaBicyclo[5.4.0]Undec-7-ene
Pc: Phthalocyanine
BODIPY : BOron DIPYromethene
NMR: Nuclear Magnetic Resonance
UV-Vis: UltraViolet-Visible
DMSO: Dimethyl sulfoxyde
DCM: Dichloromethane
$CDCl_3$: deuterated chloroform
THF: Tetrahydrofuran
PBS: Phosphate Buffered Saline
RPMI: « Roswell Park Memorial Institute » medium
TMS: TriMethylSilyl
ICP-AES: Inductively Coupled Plasma - Atomic Emission Spectrometry
LC-MS: Liquid Chromatography – Mass Spectrometry
HPLC: High Performance Liquid Chromatography
MALDI-TOF: Matrix-Assisted Laser Desorption/Ionisation - Time of Flight
ESI-MS: Electrospray Ionization Mass Spectrum

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