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Anthony Nina-Diogo, Janek Hyzewicz, Marie-paule Hamon, Jérémy Forté, Serge Thorimbert, et al.. Synthesis of New Bodipy Hydrazone Fluorescent Probes for the Detection of Carbonylated Proteins Generated by Oxidative Stress. *ChemBioChem*, 2024, 25 (19), pp.e202400093. 10.1002/cbic.202400093 . hal-04627813

HAL Id: hal-04627813

<https://hal.science/hal-04627813v1>

Submitted on 27 Aug 2024

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Synthesis of New Bodipy Hydrazide Fluorescent Probes for the Detection of Carbonylated Proteins Generated by Oxidative Stress

Anthony Nina-Diogo,^[a] Janek Hyzewicz,^[b] Marie-Paule Hamon,^[b] Jeremy Forté,^[a] Serge Thorimbert,^[a] Bertrand Friguet,^{*[b]} and Candice Botuha^{*[a]}

Oxidative stress is a cellular disorder implicated in various severe diseases and redox biology and represents an important field of research for the last decades. One of the major consequences of oxidative stress is the carbonylation of proteins, which is also a reliable marker to assess protein oxidative modifications. Accumulation of carbonylated proteins has been associated with aging and age-related diseases and can ultimately causes cell death. Detection of these oxidative modifications is essential to understand and discover new treatments against oxidative stress. We describe the design and

the synthetic pathway of new BODIPY fluorescent probes functionalized with hydrazide function for protein carbonyl labeling to improve existing methodologies such as 2D-Oxi electrophoresis. Hydrazide BODIPY analogues show very good fluorescent properties such as NIR emission up to 633 nm and quantum yield up to 0.88. These new probes were validated for the detection and quantification of carbonylated proteins with 2D-Oxi electrophoresis using mouse muscle protein extracts, as well as both flow cytometry and microscopy using oxidant stressed C2 C12 cells.

Introduction

Oxidative stress is defined as an imbalance between the generation of reactive oxygen species (ROS) from oxygen metabolism and the capacity of the organism to reduce them, favoring excessive ROS production. In non-pathological conditions, ROS contribute to cell signaling and defense against pathogens. However, pathological conditions, such as cancer, neurodegeneration or atherosclerosis, can be linked with excessive ROS production leading to oxidative stress.^[1–4] Oxidative stress also acts in Alzheimer's disease and diabetes.^[5–7] Its effects can be significant as it is increasingly considered as a cause and not only a consequence of cancers, mutagenesis or inflammation. Excessive ROS production, due to endogenous (e. g: mitochondrial respiratory chain oxidation) and exogenous (e. g: exposure to oxidants and UV light) factors, causes irreversible damage to proteins and generates carbonylated proteins.^[8–10] The accumulation of these oxidatively modified proteins has

been associated with aging and age-related diseases and can induce cell death. Analysis of this protein modification may provide access to new information or treatments for these diseases.

The 2D-Oxi electrophoresis and its variation the 2D-Oxi DIGE are technologies for detecting of carbonylated proteins with fluorescent cyanine hydrazides dyes.^[11–12] Cyanine hydrazides (Cy–Hz) have two important advantages: high sensitivity and good stability at different pH. However, their relatively low solubility in a biological environment can lead to drawbacks for covalent labeling. In addition, commercial Cy–Hz have relatively small Stokes shifts which limit their use in reduced photoluminescence self-absorption application.

We thus decided to design and synthesize new hydrazide dyes based on a BODIPY core suitable for 2D-Oxi-DIGE technologies able to overcome Cy–Hz drawbacks and provide better solubilities.

BODIPY (4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene) are fluorescent compounds discovered in 1968.^[13] They are considered as an important scaffold for designing new fluorescent probes due to a neutral global charge, high quantum yield and high molar extinction coefficients.^[14–15] BODIPY are generally stable at different pH, and possess a backbone easy to modify, which allowed tuning the absorption and/or emission properties. These characteristics are the strengths of BODIPY to be used as probes for optical imaging of biological samples^[16] and also *in cellulo* labeling due to their lipophilic structure.^[17–19]

The design of BODIPY as fluorescent probes for the 2D-Oxi DIGE technique seems a good alternative to cyanine-based probes. Indeed, the use of a commercial BODIPY-hydrazide (BODIPY–Hz) in the presence of Cy-3 and Cy-5 hydrazides has been shown to be compatible with this technique without unfavorable interactions with the gel or carbonylated

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Supporting information for this article is available on the WWW under
<https://doi.org/10.1002/cbic.202400093>

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proteins.^[20] Moreover, there are few existing BODIPYs possessing a hydrazide function for conjugation with carbonyl compounds which limits the possibilities of using them as a variety of probes to analyze samples.^[21–23] Most of them are commercially available and contain the hydrazide function linked directly to one of the pyrrole rings which leaves little scope for modulating fluorescence properties elsewhere. In addition, most of them emit in the same area that is the orange-red (fluorescence emission wavelength (590–620 nm) and few in the red wavelength region (> 620 nm), highly desirable for biological applications.

Based on the optical properties of BODIPY and to improve the 2D-Oxi electrophoresis technology, we herein propose to design and synthesize new red emitting BODIPY probes possessing a hydrazide function for protein carbonyls labeling. Unlike previously reported BODIPY-Hz,^[20–23] we wanted to possess BODIPY-Hz for which both the absorption and emission wavelengths are easy to tune. We thus introduced either electro-donating or electro-withdrawing groups (EDG and EWG) onto the pyrrole backbone and connected the hydrazide function at the meso position of the BODIPY. We considered the mono- or di-substitutions of the pyrrole rings with aryl or heteroaryl groups in order to keep the molecular weight close to the cyanine hydrazide one. In addition, we also desired to introduce (hetero)aromatic ring to reinforce planarity and thus increase π - π overlap of the polycyclic BODIPY backbone to reduce optical gaps and shift to the red area. Especially thienyl rings are known as a donor scaffold in the fields of organic electronics to reduce homo-lumo gap by a donor acceptor approach. We have developed a 5-steps synthesis allowing a simple and general access to BODIPY functionalized with ester groups to the final BODIPY-Hz.

Photophysical properties of both BODIPY-ester and BODIPY-Hz derivatives were studied.

Selected fluorophores have been tested for the 2D-Oxi electrophoresis technology and their protein labelling compared with usual Cy-Hz. To go further, we used BODIPY-Hz to label carbonylated proteins directly on C2 C12 cells both for fluorescence microscopy and flow cytometry.

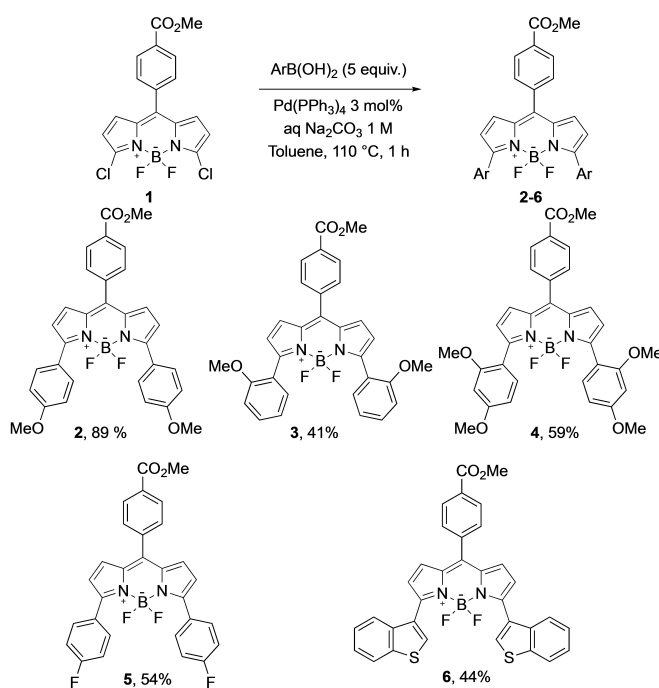
Results

Synthesis of BODIPY Derivatives: BODIPY Esters and Hydrazides

The synthesis of BODIPY often starts with the preparation of substituted pyrroles which are transformed in few steps into the desired BODIPY. Other approaches consist in preparing a halogen-substituted BODIPY as common platform and take advantage of the possible divergent metal catalyzed reaction. It appeared that pallado-catalyzed coupling reactions such as Suzuki-Miyaura or Stille reactions, were efficient methods to functionalize BODIPYs.^[24–26] We considered this pathway to be more suitable for our aims and selected that approach to obtain an easy road to the target BODIPY-Hz.

According to a described procedure, we obtained the 3,5-dichloro-BODIPY **1** in 3-steps (see experimental part) starting from commercial pyrrole and 4-formyl benzoate.^[27] To install para-methoxyphenyl groups on 3,5-dichloro-BODIPY **1**, we initially tried a Suzuki coupling under microwave irradiation, following a described procedure (1,2-DME as solvent, 3 mol% of Pd(PPh₃)₄ as catalyst 150 °C).^[28] Unfortunately, these conditions were not optimal since only 50% conversion of the starting substrate **1** into the expected product **2** has been observed. We thus tested a conventional heating in toluene in the presence of Na₂CO₃ and were pleased to get the expected BODIPY **2** bearing two 4-methoxyphenyl groups, in 89% isolated yield in less than 30 min (Scheme 1). We applied these reaction conditions to other substituted arylboronic acids and isolated corresponding BODIPY **2–5** bearing either electron-donating or electron-withdrawing groups, in moderate to good yields (Scheme 1).

The presence of strong electron-donating substituted aryls on positions 3 and 5 of BODIPY generally red-shift their emission wavelengths. For this purpose, we also introduced electron-rich heteroaryls such as benzothiophenyl or 2- and 3-thienyl groups at both C3 and C5 positions of the BODIPY scaffold.^[29] 3-benzothiophenyl boronic acid delivered the desired BODIPY **6** with moderate yield (Scheme 1). As expected, the use of 2-thienyl boronic acid which is considered not stable enough in classical Suzuki's coupling conditions did not allow to get the expected 2-thienyl substituted BODIPY **8** even after 24 h.^[30] We thus run Stille reaction instead, with 10 equivalent of 2-(tributylstannyl)thiophene **7**, 3% mol of Pd(PPh₃)₄ as catalyst, in refluxing toluene.^[31] After 5 h of reaction, the

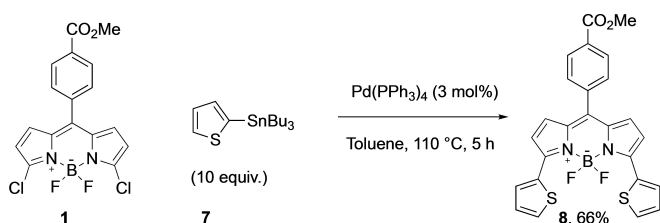


Scheme 1. Synthesis of functionalized BODIPY esters via Suzuki-Miyaura cross coupling reaction.

disubstituted BODIPY **8** was successfully obtained in 66% yield (Scheme 2).

However, the synthesis of 3-thienyl substituted BODIPY **11** proved tricky. The Stille reaction between 10 equiv. of 3-(tributyltin)thiophene **9** and BODIPY **1** didn't occur as well as the one with its 2-thienyl analogue. The conversion was sluggish and even after 15 hours only the mono substituted-BODIPY **10** was isolated in 18% yield (Table 1 entry 1). Overloading in palladium catalyst and longer reaction time pushed the reaction to the completion and the expected dithiophenyl BODIPY **11** could be isolated in 52% yield (Table 1 entry 2). Finally, to facilitate the conversion of **1** into **11**, while keeping the loading of palladium down to 3 mol%, we introduced 10 equivalents of LiCl to improve the efficiency of the catalyst,^[32] which proved beneficial since the disubstituted BODIPY **11** was isolated to 59% after 1 hour at reflux.

We next took advantage of reactivity of 2- and 3-(tributyltin)thiophene respectively **7** and **9** to prepare the corresponding dissymmetric disubstituted BODIPY **12** using a

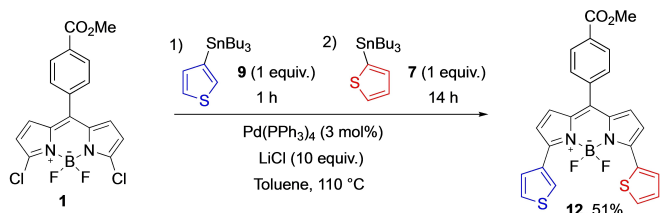


Scheme 2. Synthesis of 2-thienyl substituted BODIPY esters via Stille cross coupling reaction.

Table 1. Optimization of the synthesis of the 3-thienyl disubstituted BODIPY **11**.

Entry	X mol%	Y equiv.	Additive	Time	Yield (%) ^[a]
1	3	10	–	15	18 (10)
2	9	10	–	48	52 (11)
3	3	5	LiCl (10 equiv.)	1	59 (11)

[a] Isolated yield.



Scheme 3. Synthesis of dissymmetric BODIPY esters bearing 2- and 3-thienyl substituents.

two-steps one pot procedure. Using first 1 equivalent of 3-(tributyltin)thiophene **9** in the presence of excess LiCl, followed by the addition of 2-(tributyltin)thiophene **7**, the BODIPY **12** bearing two different thienyl groups was obtained in a reasonable 51% yield (Scheme 3). An X-Ray analysis of the crystals allowed to secure the final double differentiated substitution^[33] (see supporting information, Figure S1).

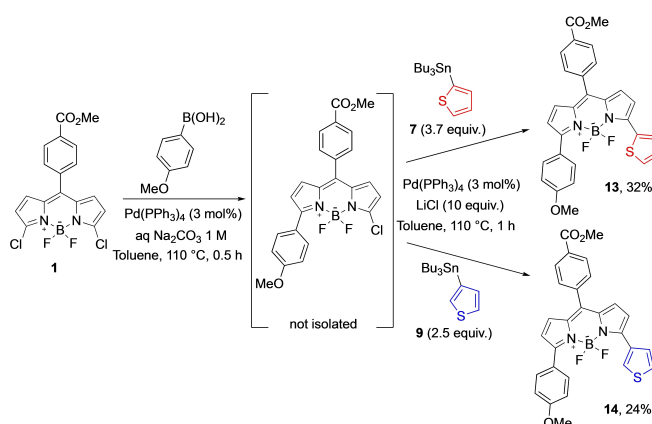
In order to extend our panel of electron-rich BODIPYs, we prepared other dissymmetric compounds using one-pot sequential Suzuki-Miyaura/Stille coupling reactions. In the presence of Pd(PPh₃)₄ as catalyst and a base, 1.1 equivalent of 4-methoxyphenyl boronic acid was reacted first with 3,5-dichloro BODIPY **1** followed by 3.7 equivalents of 2-(tributyltin)thiophene **7** or 2.5 equivalents of 3-(tributyltin)thiophene **9** and an excess of LiCl. The mono-substitution reaction was carefully followed by TLC to avoid the formation of undesired diaryl substituted BODIPY **2**. Addition of an excess of tin derivatives was thus required to get the expected dissymmetric BODIPY **13** or **14** with traces of dithienyl BODIPY **8** or **11** as byproducts. Following this original approach, the expected compounds BODIPY **13** and BODIPY **14** were isolated in 32 and 24% yield respectively (Scheme 4).

Finally, all the corresponding BODIPY–Hz were obtained by mixing the BODIPY esters with an excess of 1 M hydrazine solution in THF during 12 h. The BODIPY–Hz **15–21** diversely substituted were obtained in good quantitative yields (Scheme 5).

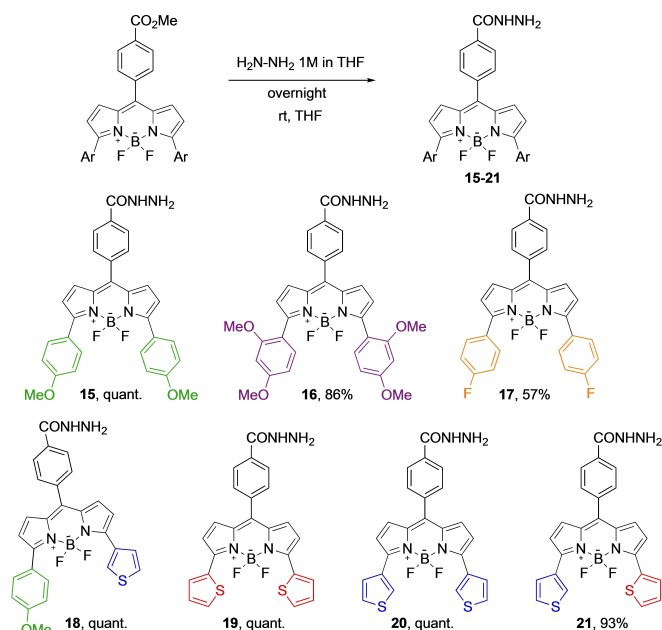
UV-vis Absorption and Fluorescence Properties Evaluation

Next, the photophysical properties of BODIPY **1** to **5** were studied. Figure 1 and Table 2 display the normalized absorption spectra of BODIPY **1** to **5** in methanol at a concentration of 10⁻⁵ M. The maxima of absorption are observed from 555–620 nm ($\epsilon = 1448$ to 30837 m⁻¹cm⁻¹) whereas the emission bands appear from 585 to 636 nm.

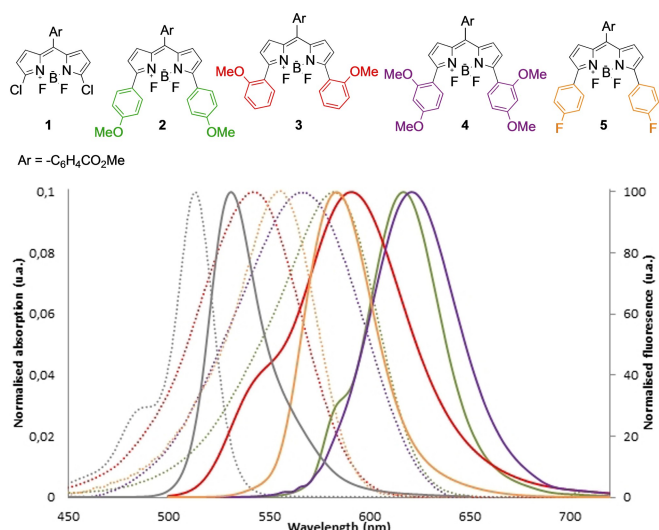
As expected, a bathochromic shift of 40 to 50 nm in the UV-vis absorption and 55 to 91 nm for the emission wavelengths are observed for disubstituted BODIPYs **2–5** as compared to



Scheme 4. Synthesis of dissymmetric BODIPY esters bearing thienyl and 4-methoxyphenyl substituents.



Scheme 5. Synthesis of diversely functionalized BODIPY-Hz.

Figure 1. UV-vis absorption spectra (dotted line) and emission spectra (full) in methanol at 10^{-5} M for BODIPY ester 1–5 after excitation at the maximum absorption wavelength.

BODIPY 1 with a dramatic increase for electron-rich BODIPY 2 and 4 substituted by 4-methoxyphenyl and 2,4-dimethoxyphenyl respectively.

Indeed, as shown in Figure 1, the bis (2,4-dimethoxyphenyl)-BODIPY 4 affords the longest emission wavelength up to 621 nm and a maximum absorption peak centered at 567 nm resulting in a Stokes shift of 54 nm. In comparison, bis (4-methoxyphenyl) BODIPY 2 and bis (2-methoxyphenyl) BODIPY 3 with only two methoxy groups have Stokes shifts of 35 nm and 49 nm respectively. Interestingly, the emission wavelength of BODIPY 4 (λ_{em} = 621 nm) is close to the one of BODIPY 2 (λ_{em} = 617 nm) while its absorption peak (λ_{abs} = 567 nm) is located

Table 2. UV-vis and fluorescence properties of BODIPY esters 2–6, 8, 11–14.

BODIPY ^[a]	λ_{abs} ^[b]	ϵ ^[c]	λ_{em} ^[d]	Stokes Shift ^[e]	Φ (F) ^[f]
2	582	27754	617	35	0.10
3	542	32903	591	49	0.04
4	567	37912	621	54	0.03
5	555	31352	585	30	0.16
6	584	40319	623	39	0.29
8	620	30377	636	16	0.45
11	589	36278	609	20	0.85
12	603	31578	623	20	0.69
13	601	32026	628	27	0.36
14	585	28372	615	30	0.39

[a] All compounds were pre-solubilized in DMSO at 10^{-3} M and used it as mother solution to prepare 10^{-5} M diluted solutions in methanol. [b] Absorption maxima measured in solution (10^{-5} M) at room temperature in nm. [c] Extinction coefficient at absorption maxima in 10^3 M⁻¹ cm⁻¹. [d] Fluorescence wavelengths at room temperature at a concentration of 10^{-5} M. [e] Stokes shifts in nm. [f] Fluorescence quantum yields were measured using a cresyl violet (λ_{exc} = 500 nm, quantum yield = 0.54 as a reference).

between absorption bands of BODIPY 2 (λ_{abs} = 582 nm) and 3 (λ_{abs} = 542 nm). In addition, the red-shifted emission wavelength observed for BODIPY 2 is more important than for BODIPY 3. This difference comes from the presence of a higher electronic delocalization in BODIPY 2 between the electron-acceptor BODIPY core and the two electron-donor groups i.e. the 4-methoxy phenyl groups which allows an efficient charge transfer in the system Whereas, for BODIPY 3, the ortho position of the methoxy groups influences the overall conformation forcing the phenyl group to turn out of the plane of the molecule, thus reducing the efficiency of charge transfer. Meanwhile, BODIPY 5 possessing 4-fluorophenyl groups, absorbs at 555 nm and emits at 585 nm for a Stokes shift of 30 nm. The small bathochromic effect could be explained by the presence of strong electron-withdrawing fluoride atom in the phenyl ring which decreases overall electronic delocalization. BODIPYs 2–5 showed low to modest fluorescence quantum yields efficiency depending of the substitution in the range of 0.03–0.04 for BODIPYs 3 and 4 and 0.1 for BODIPYs 2 and 5. Low quantum yields can be explained by conformational changes of the structure or π -stacking.^[34]

We next studied the optical properties of diverse thienyl substituted BODIPY esters 8, 11 and 12. BODIPY 8 possessing 2-thienyl groups emits at the highest wavelength up to 636 nm albeit with the lowest Stokes shift of 16 nm (Figure 2). The 3-thienyl substituted BODIPY 11 presents a small bathochromic shift with a maximum absorption at 589 nm and maximum of emission at 609 nm compared to the others 2-thienyl or 2-benzothieryl substituted BODIPY which possess an extended π -conjugation. Interestingly BODIPY 12 possessing both 2-thienyl and 3-thienyl substituents is found to be an “average” of compounds 8 and 11 with its maxima (λ_{abs} = 603 nm, λ_{em} = 623 nm). In addition, the Stokes shift of benzothieryl substituted BODIPY (6) is two times higher from 20 to 39 nm than

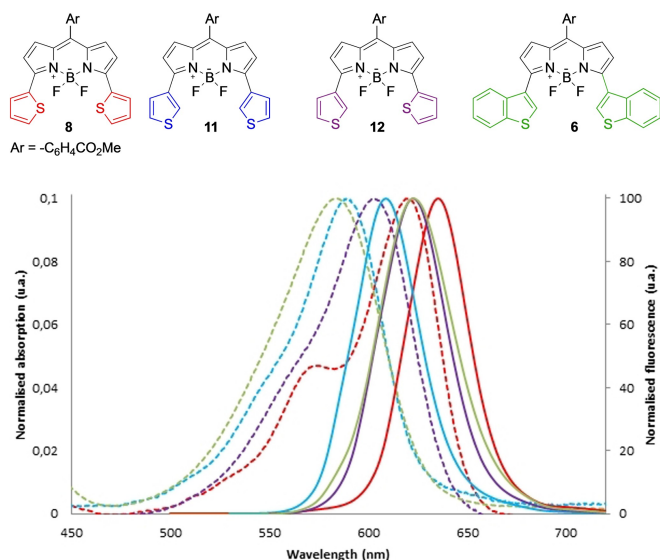


Figure 2. UV-vis absorption spectra (dotted line) and emission spectra (full) in methanol at 10^{-5} M for BODIPY esters **8**, **11**, **12** and **6** after excitation at the maximum absorption wavelength.

the one with 3-thienyl groups (BODIPY **11**), with a longer emission maximum from 609 to 623 nm and a smaller absorption wavelength from 589 to 584 nm. The presence of thienyl groups induces better fluorescence quantum yield efficiency from very good for 3-thienyl BODIPY **11** (0.85) to moderate for 2-thienyl BODIPY **12** (0.69) compared of those obtained with benzothienyl substituents. These results demonstrate that the position of the sulphur atom on the thienyl ring influences both the absorption and emission spectra of BODIPYs as well as the fluorescence quantum yield which also depends on the presence or absence of extended π -conjugation. This is clearly the case for π -extended benzothienyl substituted BODIPY **6** in which the quantum yield (0.29) is much lower than for thienyl analogues **8**, **11** and **12** (0.45, 0.85 and 0.65 respectively).

Finally, spectra of BODIPYs **13** and **14** possessing both 4-methoxyphenyl and 2-thienyl or 3-thienyl substituent were recorded and are shown in supporting information (Table 2, Figure S2 and S3). For BODIPY **13**, the absorption and emission maxima are located at 601 nm and 628 nm respectively. These results are similar to the data recorded for asymmetrical BODIPY **12** albeit with a lower quantum yield of 0.36. Compound **14** follows the same behavior as symmetrical compounds **2** (4-methoxyphenyl) and **11** (3-thienyl) with absorption and emission maxima, respectively at 585 nm and 615 nm. In addition, likewise in the case of BODIPY **13**, lower quantum yield (0.39) than the symmetrical analogues, was observed for BODIPY **14**. (Table 2)

The photophysical measurements of BODIPY–Hz **15–21** have also been achieved in methanol and the resulting optical data are summarized in Table 3 and the spectra are in Figure S4 and Figure S5.

Absorption and fluorescence spectra are quite similar to those of their ester analogues with a difference of 1–2 nm. This

Table 3. UV-vis and fluorescence properties of BODIPY–Hz **15–21**.

BODIPY–Hz ^[a]	λ_{abs} ^[b]	ϵ ^[c]	λ_{em} ^[d]	Stokes Shift ^[e]	Φ (F) ^[f]
15	583	34530	616	33	0.18
16	566	14549	619	53	0.06
17	554	19264	580	26	0.20
18	586	10429	612	26	0.49
19	619	26314	633	14	0.47
20	588	24567	606	18	0.88
21	602	20608	619	17	0.66

[a] All compounds were pre-solubilized in DMSO at 10^{-3} M and used it as mother solution to prepare 10^{-5} M diluted solutions in methanol. [b] Absorption maxima measured in solution (10^{-5} M) at room temperature in nm. [c] Extinction coefficient at absorption maxima in $10^3 \text{ M}^{-1} \text{ cm}^{-1}$. [d] Fluorescence wavelengths at room temperature at a concentration of 10^{-5} M. [e] Stokes shift in nm [f] Fluorescence quantum yields measured using a cresyl violet ($\lambda_{\text{exc}} = 500 \text{ nm}$, quantum yield = 0.54 as a reference).

is not surprising since the overall electronic structure of the substituted BODIPY remains unchanged. Indeed, only the electron acceptor function at the 8-position of the BODIPY core has been modified, the methoxy group having been replaced by the hydrazine one.

In summary, among all the BODIPY–Hz evaluated in this study, symmetrical BODIPY **19** bearing 2-thienyl groups and BODIPY **20** bearing 3-thienyl groups appear to get the best photophysical properties i.e. good fluorescence quantum yields and specific fluorescence windows. More precisely, for the biological evaluation as fluorescent probes in 2D-Oxi electrophoresis, the BODIPY–Hz need to have similar profile to cyanine–Hz used to label the carbonylated proteins. BODIPY **19** and **20** both possess emission wavelength with high quantum yield ($\lambda_{\text{abs}} = 619$ and $\lambda_{\text{em}} = 633 \text{ nm}$, $\Phi = 0.47$) and ($\lambda_{\text{abs}} = 588$ and $\lambda_{\text{em}} = 606 \text{ nm}$, $\Phi = 0.88$) which are distinct enough to be compared to cyanine3–Hz ($\lambda_{\text{abs}} = 555$ and $\lambda_{\text{em}} = 570 \text{ nm}$, $\Phi = 0.31$) and cyanine5–Hz ($\lambda_{\text{abs}} = 646$ and $\lambda_{\text{em}} = 662 \text{ nm}$, $\Phi = 0.2$) respectively.

Evaluation of BODIPY Hydrazide for the Detection of Carbonylated Proteins

Materials and Methods

Cell Culture

C2 C12 myoblasts were cultivated in 42,5% Dulbecco's Modified Eagle's Medium and 42,5% Ham's F12 medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum, in presence of 2 g/L glucose or 2 g/L galactose.

Mouse Muscle Samples Preparation

Eight-week-old C57BL/6 male mice were used in this study. All procedures were performed in accordance with national and

European legislations, in conformity with the Public Health Service Policy on Human Care and Use of Laboratory Animals under the license 75-1102. All animal studies were approved by our institutional Ethics Committee "Charles Darwin" (Permit number: 01361.03) and conducted according to the French and European laws, directives, and regulations on animal care (European Commission Directive 86/609/EEC). Mice were sacrificed by cervical dislocation prior sampling of *latissimus dorsi* skeletal muscle. Samples were prepared according to the previously described protocol of Wumaier et al.^[35] Briefly, mouse skeletal muscles were homogenized in solubilization buffer A (50 mM NaCl, 2 mM 6-aminohexanoic acid, 1 mM EDTA, 50 mM imidazole/HCl, pH 7.0) using an UltraTurax. After measurement of protein concentration using the Bradford method, 2 μ L of 20% digitonin were added to 100 μ g of proteins to set a digitonin/protein ratio of 4 g/g.^[36] After 10 min incubation on ice, samples were then centrifuged for 30 min at 20,000 g to remove non solubilized material.

1D-Gel Electrophoresis

Proteins of *latissimus dorsi* muscle from a wild type C57BL/6 J mouse were extracted in an UTC Buffer (7 M urea; 2 M thiourea, 2% CHAPS, 10 mM DTT). After a quantification using a Bradford assay, four samples containing 10 μ g of total proteins were labeled with a fluorescent hydrazide dye, either a Cy3-Hz, a Cy5-Hz, a BODIPY-Hz 19 or a BODIPY-Hz 20. Labeled samples were resolved by SDS-PAGE in Any-KD precast gels (Bio-Rad). After three washes in 7% acetic acid/10% ethanol, gels were scanned with a ChemiDoc MP imaging system (Biorad, California, USA) for monitoring the fluorescence of the cyanine and BODIPY dyes, and then incubated on Instant Blue Ultrafast Protein Stain (Sigma Aldrich Chimie S.a.r.l, St Quentin Fallavier, France) for coloration of total proteins.

Sample Preparation for 2D-Oxi Electrophoresis

50 μ g of total proteins of *latissimus dorsi* muscle from a wild type C57BL/6J mouse were labeled either with both fluorescent hydrazide dyes Cy3-Hz and BODIPY-Hz 19, or with both Cy5-Hz and BODIPY-Hz 20. Briefly, proteins were incubated 30 minutes on ice in presence of fluorescent dyes. Then unfixed fluorescent dyes were removed by using a 2D Clean up kit from GE Healthcare (GE Healthcare Europe, Velizy-Villacoublay, France).

Migration in First Dimension

Samples were loaded on Drystrips gel (pH 3–11 NL), previously rehydrated with UTC buffer containing 1.2% DeStreak and 0.5% of pharmalytes. Then the electrofocalisation of proteins was done on Ettan IPGphor 3 devices from GE Healthcare (GE Healthcare Europe, Velizy-Villacoublay, France) and migrated during 7 hours under a gradient voltage of 500 V for 1 h, 1000 V

for 1 h and 8000 V for 2.5 h. Then gels stayed for 3 hours at 8000 V.

Migration in Second Dimension

Following the first dimension, and after equilibration as previously described,^[37] strips were applied on the top of Criterion TGX precast gels 4–20% polyacrylamide IGP + 1 well, 11 cm (Biorad, California, USA) for protein separation by electrophoresis. Gels were then scanned with a ChemiDoc MP imaging system (Biorad, California, USA) for monitoring the fluorescence of the cyanine and BODIPY dyes, and then incubated on Instant Blue Ultrafast Protein Stain (Sigma Aldrich Chimie S.a.r.l, St Quentin Fallavier, France) for coloration of total proteins.

Immunofluorescence

We prepared 6 samples of C2 C12 cells: 2 control plates without or with H₂O₂ treatment (30 minutes in the presence of H₂O₂ 0.5 mM), 2 plates without or with H₂O₂ treatment labeled with BODIPY-Hz 19 probes and 2 plates without or with H₂O₂ treatment labeled with BODIPY-Hz 20 probes. All samples were labeled with DAPI (4',6-diamidino-2-phénylindole) to detect cell nuclei. Then, C2 C12 cells were fixed in acetone during 15 minutes, permeabilized with 1% Bovine Serum Albumin containing 0.1% saponin during 60 minutes and rinsed three times with PBS before fluorescence detection using a Leica DMI8 microscope.

Flow Cytometry

C2 C12 cells were incubated in hydrogen peroxide 500 μ M during one hour and permeabilized in acetone or not. A control sample without labeling was prepared. 10 μ L of a 10 mM fluorophore solution (BODIPY-Hz 19 or Cy5-Hz) was added on the other cells. Labeling was carried out at 4 °C for 3 hours. Then, the samples were subjected to three washes (centrifugation, supernatant removal, pellet dispersion in 200 μ L of PBS) to remove the excess fluorescent marker. Fluorescent signal was detected by flow cytometry using MACSQuant X. The same excitation and emission wavelengths were used for both probes. Data were analyzed with the FlowJo software.

Evaluation of BODIPY Hydrazide for the Detection of Carbonylated Proteins by 1D-gel Electrophoresis

The monitoring of carbonyl labeling was performed on proteins of *latissimus dorsi* muscle from a wild type C57BL/6J mouse incubated in the presence of the different BODIPY-Hz 15 to 21. After a protein separation by 1D-gel electrophoresis, as expected we found that only the signal from BODIPY-Hz 19 and 20 was detectable (data not shown). Then, we decided to

focus on these molecules that are referred as probes **19** and **20** in the rest of the study.

We labeled 50 μg of proteins from the *latissimus dorsi* muscle with the commercial Cy3-Hz or Cy5-Hz dyes and the

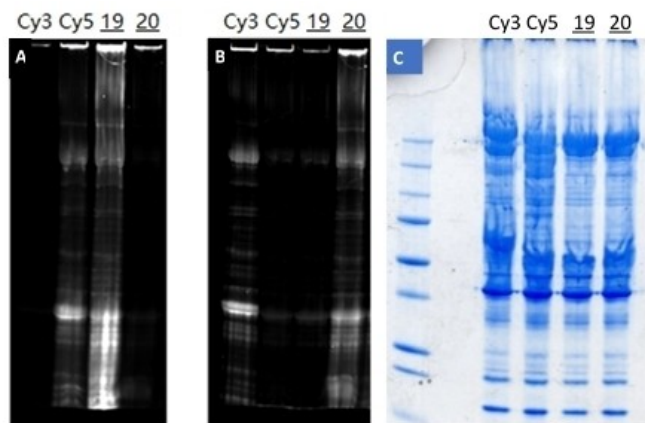


Figure 3. Detection of carbonylated proteins by Cy3-Hz, Cy5-Hz, **19** or **20** probes after a protein separation by 1D-gel electrophoresis using excitation and emission wavelengths of A) Cy5-Hz and **19** probes or B) Cy3-Hz and **20** probes. C) Coomassie blue staining.

same amount of proteins with the probes **19** and **20**. Labeled proteins were separated in 4–20% polyacrylamide gel. Fluorescence was revealed with the ChemiDoc MP imaging system (Figure 3A) and charge control was performed by Coomassie blue staining. We found that excitation and emission signals of the probe **19** correspond to Cy5-Hz dye (Figure 3A). Similarly signals of the probe **20** correspond to Cy3-Hz dye (Figure 3B).

Evaluation of BODIPY-Hz probes **19** and **20** for the Detection of Carbonylated Proteins in 2D-Oxi Electrophoresis

We then performed the detection of carbonylated proteins separated according to isoelectric point and molecular weight according a 2D-Oxi electrophoresis protocol (Figure 4). As for the 1D-gel electrophoresis, proteins of *latissimus dorsi* muscle were labeled either with both the commercial Cy3-Hz and **19** or with both Cy5-Hz and **20**. Labeled proteins were electrofocused in first dimension on a 3–11 NL strip, separated in second dimension on a 4–20% polyacrylamide gel and revealed using ChemiDoc MP imaging system. Fluorescent signal of carbonylated proteins labeled with the commercial Cy5-Hz (Figure 4A)

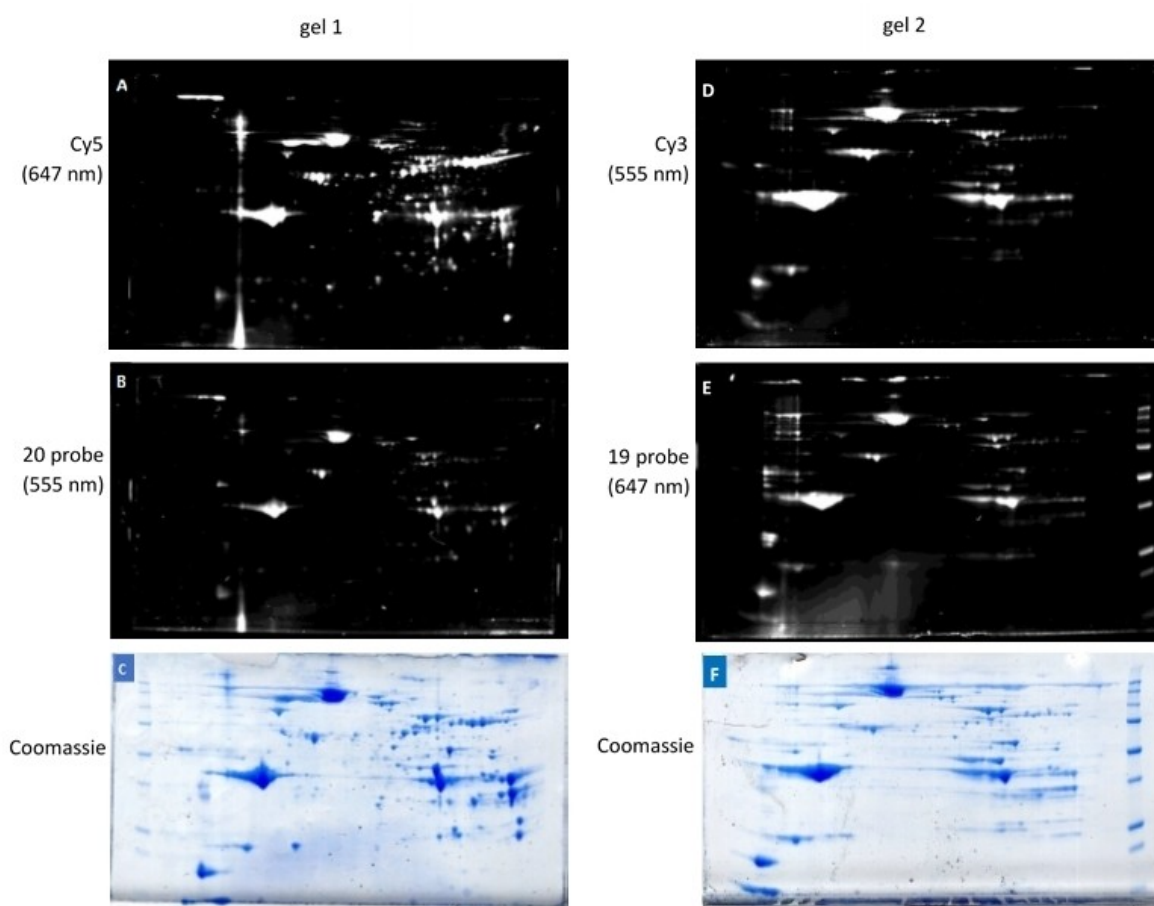


Figure 4. Detection of carbonylated proteins with the 2D-Oxi electrophoresis method. The gel 1 was scanned using excitation and emission wavelengths of A) Cy5-Hz or B) **20** probes. C) Coomassie blue staining of gel 1. Gel 2 was scanned using excitation and emission wavelengths of D) Cy3-Hz or E) **19** probes. F) Coomassie blue staining of gel 2.

or **20** (Figure 4B) was strongly similar. We revealed almost the same proteome, with only few differences but more spots for proteins labeled with **20** between 50 kDa and 37 kDa. Protein staining with Coomassie blue for charge control (Figure 4C) confirms the presence of the proteins. This difference suggests a better labeling rate with BODIPY–Hz compared to Cy5–Hz.

In contrast to the labeling with the Cy5–Hz and the probe **20** (Figure 4A and 4B), the comparison between Cy3–Hz (Figure 4D) and the probe **19** labeling (Figure 4E) was not conclusive. Although both dyes detected similar protein spots, the Cy3–Hz labeling was more intense.

Comparison between fluorescence signal and staining of total proteins (Figure 4F) showed that proteome is detected, without labeling or migration problems. Differences can be attributed to a technical variation during the precipitation step, but also to a lower sensitivity of probe **19**. Hence, we proceeded to further experiments to test if the hydrazide BODIPY fluorophores **19** and **20** could be used in replacement of cyanines Cy3–Hz and Cy5–Hz.

Evaluation of BODIPY–Hz Probes **19** and **20** for the Detection of Carbonylated Proteins in Cell Culture

We ran experiments to determine if the new BODIPY–Hz probes **19** and **20** could label carbonylated proteins directly in C2 C12 cells in culture. Fluorescent signal was not detected in wells containing C2 C12 cells labeled with probes **19** and **20**, but not exposed to oxidative stress (Figures 5B and C). On the other hand, we observed a fluorescent signal in wells housing C2 C12 cells incubated with H₂O₂ prior incubation with **19** and **20** probes (Figures 5D and E). We noted that fluorescent signal was slightly weaker with probe **19** than with probe **20**. In all cases,

both probes are specific of oxidatively damaged molecules since absence of oxidative damage also means absence of fluorescent signal.

Evaluation of BODIPY–Hz Probes **19** and **20** for the Detection of Carbonylated Proteins by Flow Cytometry

Flow cytometry is a cellular biology analysis technology used to characterize sample populations. Flow cytometry can, for example, be used to determine the type of population present in the blood for various pathological conditions (endothelial dysfunction, leukemia allergies, etc.).^[38,39] In addition, this tool provides the means to quantify the intensity of fluorescence signal emitted by agents targeting molecules expressed on or in cells and from that an assessment of the extent of the process under study.

This is why, in order to precise the results reported in the Figure 4, we decided to measure protein carbonylation levels using flow cytometry and limiting our experiment to probe **19** only.

Indeed, we have a doubt concerning the specificity of the probe **20**: in spite of intensive labeling in cell culture, **20** probe could not label the same proteins that Cy3–Hz. In contrary, probe **19** and Cy5–Hz dye labeled the same proteins. Thus, we decided to keep **19** probe and we performed flow cytometry experiment for further validation (Figure 6).

Non permeabilized cells labeled with Cy5–Hz dye were sorted in two populations P1 and P2 (Figure 6A). Population P1 (37, 4% of cells) showed a fluorescence intensity 100 times lower than population P2 (58,7% of cells). Non permeabilized cells labeled with **19** probe were grouped in a single P2 population comprising 97.9% of the C2 C12 population (Fig-

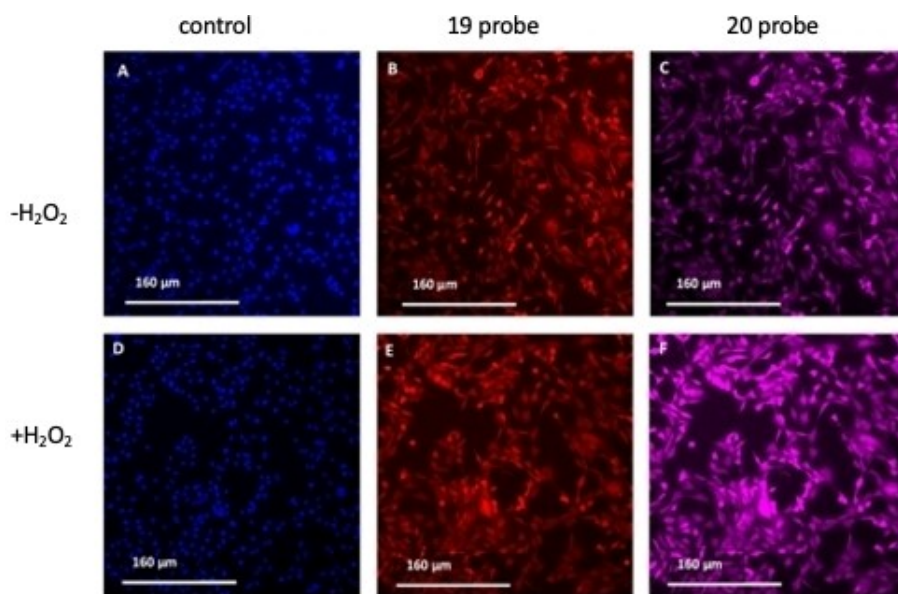


Figure 5. Immunofluorescent staining of C2 C12 immortalized myoblasts. A) control C2 C12 cells, B) C2 C12 cells labeled with probe **19**, C) C2 C12 labeled with probe **20**, D) control C2 C12 cells with H₂O₂ treatment, E) C2 C12 cells with H₂O₂ treatment labeled with probe **19**, F) C2 C12 cells with H₂O₂ treatment labeled with probe **20**.

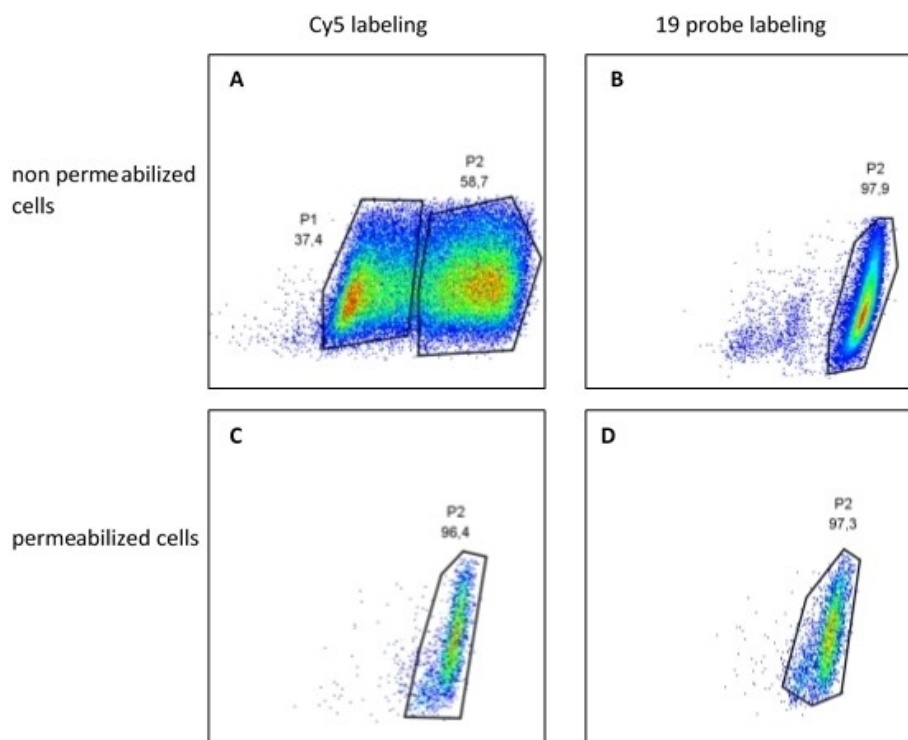


Figure 6. Flow cytometry analysis using laser red 635 nm and band pass filter 655–730 nm of non permeabilized C2 C12 cells labeled with A) Cy5-Hz or B) 19 probes, and permeabilized C2 C12 cells labeled with C) Cy5-Hz or D) 19 probes.

ure 6B). The existence of two populations of non permeabilized cells labeled with Cy5-Hz can be explained by a difference in labeling. The gate P1 gathers up cells labeled on the surface, on their plasma membrane. In the case of P2 cells, the fluorophore Cy5 hydrazide was internalized, which allowed carbonylated proteins labeling in the cytosol.

This hypothesis has been confirmed by a second experiment in which the C2 C12 cells were permeabilized before labeling (Figures 6C and D). Indeed, permeabilized cells with Cy5-Hz labeling were found in only one P2 population similar to the P2 population with 19 probe labeling, whether cells are permeabilized or not. Given the fact that BODIPY are known to internalize into cells, we deduced that 19 probe can target carbonylated proteins in the cytosol of intact cells.

Discussion

Protein carbonylation is a post-translational modification that consist in formation of a reactive aldehyde ($-\text{CH}=\text{O}$) or ketone ($>\text{C}=\text{O}$) residues on proteins. Amino acids susceptible to oxidation-mediated carbonylation are lysine (Lys), arginine (Arg), proline (Pro), and threonine (Thr) residues.^[40] There are two types of protein carbonylation: “primary protein carbonylation” which is due to oxidation of the four cited residues, initiated by reactive oxygen species (such as hydrogen peroxide) and often catalyzed by metals. “Secondary protein carbonylation” is caused by the addition of aldehyde moieties to such amino acids as lysine (Lys) and arginine (Arg) side

chains resulting in their carbonylation. These aldehyde bodies are formed during the process of lipid peroxidation (such as malondialdehyde or 4-hydroxynonenal) or during the oxidation of carbohydrates (for example methylglyoxal).

Aldehyde or ketone residues on proteins can react with 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones. Indeed, oxidized proteins are detected with anti-2,4-dinitrophenyl hydrazone antibodies.^[41] These probes have been progressively replaced by fluorescent hydrazide able to link covalently to carbonylated residues.^[20] This progress made the detection and quantification of carbonylated proteins by 2D Oxi electrophoresis and 2D-Oxi DIGE techniques much easier. Still, cyanine dyes such as Cy3-Hz and Cy5-Hz exhibit relatively low solubility in a biological environment that can lead to drawbacks for covalent labeling and the development of new fluorescent probes could be of valuable interest.

In this study we designed and synthesized seven different BODIPY-Hz probes. Two of them, 19 and 20 displayed expected fluorescent signal and were chosen for the detection of carbonylated proteins. This selection step, based on the emission of fluorescence after binding biological samples, was easy to perform. However, since we had two possible candidates for detection of carbonylated proteins, BODIPY-Hz probes 19 and 20, we did not investigate the other BODIPY-Hz as they produced weak amount of fluorescence. The second step of selection was based on the accuracy of the detection of carbonylated proteins in biological samples using different technical approaches.

We performed 2D-Oxi electrophoresis, fluorescence microscopy and flow cytometry experiments. Since 2D Oxi electrophoresis experiment evidenced a not as good sensitivity of the probe **20** we decided to focus on probe **19**. On the other hand, fluorescence microscopy experiment showed that both probes **19** and **20** emitted fluorescence only after exposure of C2 C12 cells to hydrogen peroxide. The flow cytometry experiments demonstrated that probe **19** showed the same efficiency on permeabilized and non permeabilized cells.

Other experiments may be performed to further validate the **19** and **20** probes, such as labeling of proteins submitted to metal-catalyzed oxidation *in vitro* in addition to our *ex vivo* experiments.^[20] Indeed, we already found that cells exposed by hydrogen peroxide were successfully labeled by our probes **19** and **20** by fluorescence microscopy and by flow cytometry for probe **19** but it is important to ensure that these probes recognize only carbonylated residues.

The Translational Study on Protein Carbonylation

This study on the development of new fluorescent BODIPY–Hz probes has raised interesting questions about the characteristics of the probes that need to be further investigated. For example, is the labeling capacity of the probes depending on the nature of the carbonylated residue? Does labeling occur similarly with a product of primary carbonylation and a product of secondary carbonylation? Does the probe have a similar affinity with ketone and aldehyde moieties? Does the fixation on biological samples affect the emission of fluorescence? And, do the preparation steps of the biological samples affect the binding ability and fluorescent capacities of the probes?

Another topic to be further investigated is related to the significance of protein carbonylation. Carbonylation is a marker of oxidative stress, that is a pathological condition defined by an imbalance between production of reactive oxygen or nitrogen species and their elimination by the reductive capacity of the cell. Labeling carbonylated proteins *in vitro* or *ex vivo* gives information on an already fixed state of oxidative damage, but labeling them in living cells in real time could inform on their appearance and clearance of these protein oxidative modifications. Recent researches showed the possibility to modulate the fluorescence of a BODIPY in function of his state of oxidation.^[42] Next step could be to design a turn-on BODIPY hydrazide probe that would emit no fluorescence in absence of carbonylated residue and become fluorescent as soon as a residue is labeled. This molecule could not only monitor in real time the appearance and clearance of oxidative damage, but also inform about the location and kinetics of clearance mechanisms.

Conclusions

In conclusion, we described the design and the synthesis of new BODIPY-based probes possessing a hydrazide function for carbonylated proteins labeling. Through a multi-step synthesis, we have prepared and characterized a library of diversely

substituted BODIPY esters and hydrazides with aryles and heteroaryles functions allowing an easy modulation of their optical properties. Aryles and heteroaryles substituents were introduced to the BODIPY platform by Suzuki-Miyaura or Stille coupling reactions. We have obtained new fluorescent probes excitable at 554 nm–619 nm, and emitting at wavelengths up to 633 nm with quantum efficiencies between 10% and 88%. These new BODIPY–Hz were successfully used as fluorescent probes for carbonylated proteins labeling in 2D-Oxi electrophoresis and *ex vivo* experiments such as fluorescence microscopy and flow cytometry. We have shown that BODIPY is compatible with the 2D-Oxi electrophoresis technology, and that it can be used directly for proteins labeling in cells monitored by fluorescence microscopy. The results in flow cytometry show also that the BODIPY–Hz **19** probe can enter the cell for labeling of cytosolic proteins and not only of membrane proteins as previously observed with Cy–Hz.

Experimental Section

Synthesis and Analytical Data

General Information

All reagents and solvents were purchased from commercial suppliers, Sigma-Aldrich, TCI, Alfa-Aesar or fluorochem. All reactions were monitored by TLC on silica gel 60 F254 plates and revealed using UV lamp (λ 254 nm). Flash chromatography was carried out on a Merck silica gel 60 F 254. Reactions involving air or moisture sensitive reagents were carried out under an inert atmosphere (argon) with oven-dried glassware. THF was distilled over sodium/benzophenone. All final compounds were analyzed by high-resolution ESI mass spectrometry (HRMS) in MeOH using a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with an electrospray ion source. NMR spectra were recorded on a Bruker Avance spectrometer at 300 or 400 MHz for ¹H and 101 MHz or 75.5 MHz for ¹³C. Chemical shifts (δ) are given in ppm, referenced to the residual solvent, coupling constant (*J*) are measured in hertz (Hz). Multiplicity is indicated as follows: s, singlet; bs broad signal; d, doublet; t, triplet; q, quadruplet; dd, doublet of doublet; m, multiplet. The melting point were performed on electrothermal IA 91000. UV/Vis absorption spectra were measured using a Cary 50 (Varian) spectrophotometer at 20 °C. Emission spectra were performed on JASCO J-815 CD spectrofluorometer at 20 °C.

The methyl 4-(3,7-dichloro-5,5-difluoro-5H-4 λ⁴,5 λ⁴-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzoate BODIPY **1** compound was synthesized according to the published procedure.^[43]

Synthesis of BODIPY Esters

General procedure A: Under argon, BODIPY **1** (1 equiv.), Pd(PPh₃)₄ (3% mol) and dry toluene (0.01 M) were introduced in a sealed tube. An aqueous solution of Na₂CO₃ (1 M) were added to the suspension followed by the corresponding boronic acid (5 or 10 equiv.) portion wise. The mixture was stirred at reflux for 30 min to 5 h. The organic layer was recovered and the aqueous layer was extracted three times with dichloromethane. The combine organic layers were washed with water then brine. The organic layer was dried over MgSO₄ and concentrated under vacuum. The crude

product was purified either by recrystallization or by silica gel chromatography to afford the title compound.

Methyl 4-(5,5-difluoro-3,7-bis(4-methoxyphenyl)-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzoate. BODIPY 2. The compound is obtained according to general procedure A from BODIPY 1 (150 mg, 0.379 mmol), Pd(PPh₃)₄ (14 mg), 4-methoxybenzeneboronic acid (575 mg, 3.79 mmol) and an aqueous solution of Na₂CO₃ (7.5 mL, 1 M) in toluene (40 mL) for 30 min at reflux. The crude product was recrystallized in methanol to afford the title product as a blue solid. M = 180 mg, 89% yield. ¹H NMR (300 MHz, CDCl₃) δ 8.22 (d, *J* = 8.3 Hz, 2H), 7.91 (d, *J* = 9.0 Hz, 2H); 7.68 (d, *J* = 8.3 Hz, 2H) 6.99 (d, *J* = 9.0 Hz, 2H), 6.80 (d, *J* = 4.4 Hz, 2H), 6.65 (d, *J* = 4.3 Hz, 2H), 4.02 (s, 3H), 3.88 (2 s, 6H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 166.6, 161.0 (2 C), 159.0 (2 C), 140.8, 139.1 (2 C), 136.1, 131.6 (2 C), 131.3 (2 C), 130.7 (2 C), 130.2 (2 C), 129.5 (2 C), 125.1, 120.9 (4 C), 113.9 (4 C), 55.4 (2 C), 52.6. The experimental data are in accordance with those reported in the literature.^[28]

Methyl 4-(5,5-difluoro-3,7-bis(2-methoxyphenyl)-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzoate. BODIPY 3. The compound is obtained according to general procedure A from BODIPY 1 (50 mg, 0.127 mmol), Pd(PPh₃)₄ (4.7 mg), 2-methoxybenzeneboronic acid (199 mg, 1.27 mmol) and an aqueous solution of Na₂CO₃ (2.5 mL, 1 M) in toluene (13 mL) for 4 h at reflux. The crude product was by silica gel chromatography (eluent dichloromethane) to afford the title product as a pink-purple solid. M = 28 mg, 41% yield. ¹H NMR (300 MHz, CDCl₃) δ 8.20 (d, *J* = 8.2 Hz, 2H), 7.79 – 7.65 (m, 4H), 7.40 – 7.28 (m, 2H), 7.00 (t, *J* = 7.5 Hz, 2H), 6.92 (d, *J* = 8.3 Hz, 2H), 6.78 (d, *J* = 4.3 Hz, 2H), 6.60 (d, *J* = 4.3 Hz, 2H), 4.00 (s, 3H), 3.78 (s, 6H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.7, 157.3 (2 C), 155.5, 141.8, 137.9 (2 C), 134.2 (2 C), 131.1 (2 C), 131.0 (2 C), 130.9 (2 C), 130.8 (2 C), 129.8 (2 C), 129.2 (2 C), 122.8, 121.1 (2 C), 119.7 (2 C), 111.4 (2 C), 55.6 (2 C), 52.4; ¹⁹F NMR (282 MHz, DMSO-*d*₆) δ –132.56 (dd, *J* = 61.8, 31.3 Hz, 2F); IR (film, cm⁻¹) 2926, 1722, 1548, 1272, 1121, 1071; mp 121–123 °C; HRMS (ESI⁺): calcd for C₃₁H₂₅BF₂N₂O₄Na: 561.1773; found: 561.1793 [M + Na].

Methyl 4-(3,7-bis(2,4-dimethoxyphenyl)-5,5-difluoro-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzoate. BODIPY 4. The compound is obtained according to general procedure A from BODIPY 1 (50 mg, 0.127 mmol), Pd(PPh₃)₄ (4.4 mg, 0.0038 mmol), 4-dimethoxybenzeneboronic acid (120 mg, 0.64 mmol) and an aqueous solution of Na₂CO₃ (2.5 mL, 1 M in H₂O) in toluene (10 mL) for 30 min at reflux. The crude product was purified by silica gel chromatography (eluent dichloromethane/cyclohexane 7/3) to afford the title product as a dark blue solid. M = 43 mg, 59% yield. ¹H NMR (300 MHz, CD₂Cl₂) δ 8.18 (d, *J* = 8.3 Hz, 2H), 7.70 (d, *J* = 8.3 Hz, 2H), 7.61 (d, *J* = 8.2 Hz, 2H), 6.80 (d, *J* = 4.1 Hz, 2H), 6.62 (d, *J* = 4.2 Hz, 2H), 6.59 – 6.51 (m, 4H), 3.96 (s, 3H), 3.84 (s, 6H), 3.78 (s, 6H); ¹³C NMR (75 MHz, CDCl₃) δ 166.6, 162.1 (2 C), 159.1 (2 C), 155.7, 140.4, 139.4 (2 C), 135.2 (2 C), 133.0 (2 C), 131.4 (2 C), 130.7 (2 C), 129.4 (2 C), 129.0 (2 C), 122.8, 114.8 (2 C), 104.5 (2 C), 98.9 (2 C), 55.9 (2 C), 55.5 (2 C), 52.5; ¹⁹F NMR (282 MHz, CD₂Cl₂) δ –134.14 (dd, *J* = 62.8, 31.7 Hz, 2F); IR (film, cm⁻¹) 2930, 1722, 1572, 1466, 1253, 1129; mp 212–213 °C; HRMS (ESI⁺): calcd for C₃₃H₂₉BF₂N₂O₆Na: 621.1984; found: 621.2005 [M + Na].

Methyl 4-(5,5-difluoro-3,7-bis(4-fluorophenyl)-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzoate. BODIPY 5. The compound is obtained according to general procedure A from BODIPY 1 (50 mg, 0.127 mmol), Pd(PPh₃)₄ (4.4 mg, 0.0038 mmol), 4-fluorobenzeneboronic acid (76 mg, 0.54 mmol) and an aqueous solution of Na₂CO₃ (2 mL, 1 M in H₂O) in toluene (10 mL) for 30 min at reflux. The crude product was purified by silica gel chromatography (eluent dichloromethane/cyclohexane 6/4) to afford the title compound as a purple solid. M = 33 mg, 51%

yield. ¹H NMR (300 MHz, CD₂Cl₂) δ 8.20 (d, *J* = 8.6 Hz, 2H), 7.92–7.80 (m, 4H), 7.69 (d, *J* = 8.6 Hz, 2H), 7.22–7.09 (m, 4H), 6.90 (d, *J* = 4.4 Hz, 2H), 6.66 (d, *J* = 4.3 Hz, 2H), 3.97 (s, 3H); ¹³C NMR (75 MHz, CD₂Cl₂) δ 166.8, 164.2 (d, *J* = 255 Hz, 2 C), 158.7 (2 C), 143.6, 138.9 (2 C), 136.7 (2 C), 132.5 (2 C), 132.2 (2 C), 131.5 (2 C), 130.6 (d, *J* = 98 Hz, 4 C), 129.3; 129.2, 121.7 (d, *J* = 2 Hz,), 115.8 (d, *J* = 22 Hz, 4 C), 52.9; ¹⁹F NMR (282 MHz, CD₂Cl₂) δ –111.41 (m, 2F), –132.42 (dd, *J* = 64.2, 32.1 Hz, 2F); IR (film, cm⁻¹) 1706, 1601, 1545, 1164, 1070; HRMS (ESI⁺): calcd for C₂₉H₁₉BF₄N₂O₂Na: 537.1368; found: 537.1368 [M + Na]; mp 211–212 °C.

Methyl 4-(3,7-bis(benzo[*b*]thiophen-3-yl)-5,5-difluoro-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzoate. BODIPY 6. The title product was synthesized according to general procedure A from BODIPY 1 (50 mg, 0.127 mmol) and 1-benzothien-3-ylboronic acid (113 mg, 0.63 mmol). The crude was purified over silica gel (eluent DCM/Cyclohexane 7/3) to afford the title compound as a dark blue solid. M = 33 mg, 44% yield. ¹H NMR (300 MHz, CDCl₃) δ 8.34 (s, 2H), 8.25 (d, *J* = 8.2 Hz, 2H), 7.94–7.84 (m, 4H), 7.73 (d, *J* = 8.2 Hz, 2H), 7.47–7.30 (m, 4H), 6.95 (d, *J* = 4.4 Hz, 2H), 6.85 (d, *J* = 4.3 Hz, 2H), 4.02 (s, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 166.5, 152.5, 141.59, 140.1 (2 C), 138.8 (2 C), 138.3 (2 C), 135.8, 131.9 (2 C), 131.4, 131.3, 131.2, 130.7 (2 C), 130.5 (2 C), 129.7 (2 C), 127.4 (2 C), 124.8 (2 C), 124.7 (2 C), 123.1 (2 C), 123.0 (2 C), 121.40, 52.6; ¹⁹F NMR (282 MHz, CDCl₃) δ –133.36 (dd, *J* = 62.8, 31.4 Hz, 2F); IR (film, cm⁻¹) 2922, 1720, 1569, 1531, 1148; HRMS (ESI⁺): calcd for C₃₃H₂₁BF₂N₂O₂S₂Na: 613.1105; found: 613.0385 [M + Na]; mp 272–273 °C.

Methyl 4-(5,5-difluoro-3,7-di(thiophen-2-yl)-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzoate. BODIPY 8. In a sealed tube under argon, BODIPY 1 (90 mg, 0.23 mmol, 1 eq), Pd(PPh₃)₄ (8.1 mg, 3 mol%) and dry toluene (23 mL) were mixed. 2-(tributylstannyl)thiophene (753 μ L, 2.3 mmol, 10 eq) was added and the reaction mixture was heated at reflux for 5 h. The reaction was cooled at rt and 10 mL of an aqueous solution of KF (10% in H₂O) were added. The organic layer was recovered and the aqueous layer was extracted three times with dichloromethane. The combine organic layers were washed with water then brine. The organic layer was dried over MgSO₄ and concentrated under vacuum. The crude product was recrystallized in MeOH to afford the title compound as a dark blue solid. M = 35 mg, Yield 66%. ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.15 (d, *J* = 8.2 Hz, 2H), 8.09 (dd, *J* = 3.8, 1.1 Hz, 2H), 7.88 (dd, *J* = 5.1, 1.1 Hz, 2H), 7.77 (d, *J* = 8.2 Hz, 2H), 7.29 (dd, *J* = 5.0, 3.8 Hz, 2H), 7.04 (d, *J* = 4.4 Hz, 2H), 6.88 (d, *J* = 4.5 Hz, 2H), 3.94 (s, 3H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 165.4, 149.7 (2 C), 138.8 (2 C), 137.6 (2 C), 135.5 (2 C), 133.0 (2 C), 131.2 (2 C), 131.0 (2 C), 130.9, 130.4 (2 C), 129.9, 128.8 (2 C), 128.5 (2 C), 120.9, 51.9; ¹⁹F NMR (282 MHz, CD₂Cl₂) δ –139.56 (dd, *J* = 65.8, 32.8 Hz, 2F); IR (film, cm⁻¹) 1709, 1544, 1269, 1131, 1033; HRMS (ESI⁺): calcd for C₂₅H₁₇BF₂N₂O₂S₂Na: 513.0689; found: 513.0686 [M + Na]; mp > 300 °C.

Methyl 4-(3-chloro-5,5-difluoro-7-(thiophen-3-yl)-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzoate. BODIPY 10. In a sealed tube under argon, BODIPY 1 (50 mg, 0.127 mmol), Pd(PPh₃)₄ (4.4 mg, 3 mol%) and dry toluene (10 mL) were mixed. A solution of 3-(tributylstannyl)thiophene in toluene (2.54 mL, 1.27 mmol, 0.5 M) was added and the reaction mixture was heated at reflux for 15 h. The reaction was cooled at rt and 5 mL of an aqueous solution of KF (10% in H₂O) were added. The organic layer was recovered and the aqueous layer was extracted three times with dichloromethane. The combine organic layers were washed with water then brine. The organic layer was dried over MgSO₄ and concentrated under vacuum. The crude product was purified by silica gel chromatography (eluent: dichloromethane/Cyclohexane 9/1) to afford the title compound as a dark pink solid. M = 10 mg, Yield 18%. ¹H NMR (300 MHz, CDCl₃) δ 8.44 (dd, *J* = 3.0, 1.3 Hz, 1H),

8.22–8.09 (m, 2H), 7.70 (dd, $J=5.1, 1.3$ Hz, 1H), 7.65–7.54 (m, 2H), 7.41 (dd, $J=5.1, 2.9$ Hz, 1H), 6.90–6.79 (m, 2H), 6.74–6.64 (m, 1H), 6.40 (dd, $J=4.3, 1.2$ Hz, 1H), 3.99 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 166.2, 155.4, 137.8, 132.9, 132.8, 132.4, 131.9, 130.5 (2 C), 130.3, 129.6 (2 C), 128.8, 128.5, 125.9, 121.9, 117.8, 114.2, 52.5. ^{19}F NMR (282 MHz, CDCl_3) δ –143.15 (dd, $J=61.2, 30.5$ Hz). IR (film, cm^{-1}) 2922, 1722, 1572, 1546, 1084; HRMS (ESI⁺): calcd for $\text{C}_{21}\text{H}_{14}\text{BClF}_2\text{N}_2\text{O}_2\text{SNa}$: 465.0422; found: 465.0432 [M + Na]

methyl 4-(5,5-difluoro-3,7-di(thiophen-3-yl)-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzoate. BODIPY 11. In a sealed tube under argon, BODIPY 1 (50 mg, 0.127 mmol), $\text{Pd}(\text{PPh}_3)_4$ (4.4 mg, 3 mol%) and dry toluene (10 mL) were mixed. A solution of 3-(tributylstannyl)thiophene in toluene (1.27 mL, 0.633 mmol, 0.5 M) and LiCl (54 mg, 1.27 mmol) were added and the reaction mixture was heated at reflux for 1 h. The reaction was cooled to rt and 10 mL of an aqueous solution of KF (10% in H_2O) were added. The organic layer was recovered and the aqueous layer was extracted three times with dichloromethane. The combine organic layers were washed with water then brine. The organic layer was dried over MgSO_4 and concentrated under vacuum. The crude product was recrystallized in methanol to afford the title compound as a dark blue solid $M=37$ mg, Yield 59%. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 8.48 (dd, $J=2.9, 1.3$ Hz, 2H), 8.15 (d, $J=8.3$ Hz, 2H), 7.85 (dd, $J=5.2, 1.3$ Hz, 2H), 7.80 (d, $J=8.3$ Hz, 2H), 7.72 (dd, $J=5.1, 2.9$ Hz, 2H), 7.15 (d, $J=4.5$ Hz, 2H), 6.92 (d, $J=4.5$ Hz, 2H), 3.93 (s, 3H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 165.7, 151.9 (2 C), 140.0 (2 C), 138.0 (2 C), 135.3 (2 C), 132.1 (2 C), 131.0, (2 C) 130.9 (2 C), 130.5 (2 C), 129.4, 129.2, 128.9 (2 C), 126.7 (2 C), 121.5, 52.5; ^{19}F NMR (376 MHz, $\text{DMSO}-d_6$) δ –136.31 (dd, $J=67.2, 33.4$ Hz, 2F); IR (film, cm^{-1}) 1713, 1525, 1273, 1138, 1058; HRMS (ESI⁺): calcd for $\text{C}_{25}\text{H}_{17}\text{BF}_2\text{N}_2\text{O}_2\text{S}_2\text{Na}$: 513.0689; found: 513.0687 [M + Na]; mp > 300 °C.

Methyl 4-(5,5-difluoro-3-(thiophen-2-yl)-7-(thiophen-3-yl)-5H-5 λ^4 ,6 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzoate. BODIPY 12. In a sealed tube under argon, BODIPY 1 (75 mg, 0.19 mmol), $\text{Pd}(\text{PPh}_3)_4$ (6.6 mg, 0.0057 mmol) and dry toluene (10 mL) were mixed. A solution of 2-(tributyltin)thiophene in toluene (380 μL , 0.19 mmol, 0.5 M) and LiCl (80 mg, 1.9 mmol) were added. The reaction was degassed for 10 min with argon. The reaction was heated at reflux for 1 h30 (monitored by TLC (eluent methylene chloride) until the appearance of the double coupling product. The mixture was cooled to rt and 3-(tributyltin)thiophene (73 mg, 0.19 mmol) was added to the mixture. The reaction was stirred at reflux 15 h. After cooling to room temperature, an aqueous solution of KF (15 mL, 10% in H_2O) was added to the mixture under stirring. The aqueous layer was extracted with methylene chloride three times. The combined organic layers were washed with brine, dried over MgSO_4 and the solvent was removed in vacuo to give the title product as a crude product. Purification of the crude product by silica gel chromatography (eluent methylene chloride /cyclohexane 7/3) afforded a solid which was triturated with cyclohexane to give the title compound as a dark blue solid. $M=47$ mg. Yield 51%. ^1H NMR (300 MHz, CD_2Cl_2) δ 8.40–8.29 (m, 1H), 8.23–8.11 (m, 3H), 7.75–7.62 (m, 3H), 7.60–7.55 (m, 1H), 7.49–7.39 (m, 1H), 7.28–7.17 (m, 1H), 6.83 (d, $J=8.1$ Hz, 4H), 3.96 (s, 3H). ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 166.2, 152.9, 150.6, 140.2, 138.5, 136.4, 136.2, 133.9, 132.8, 132.0, 131.8, 131.6, 131.2 (2 C), 130.9, 130.7, 130.6, 129.6 (2 C), 129.3, 129.2, 126.9, 122.0, 121.7, 52.7; ^{19}F NMR (282 MHz, CD_2Cl_2) δ –138.65 (dd, $J=66.1, 33.0$ Hz, 2F); IR (film, cm^{-1}) 1711, 1571, 1536, 1526, 1469, 1270, 1043; HRMS (ESI⁺): calcd for $\text{C}_{25}\text{H}_{17}\text{BF}_2\text{N}_2\text{O}_2\text{S}_2\text{Na}$: 513.0690; found: 513.0691 [M + Na]; mp 293–294 °C.

Methyl 4-(5,5-difluoro-3-(4-methoxyphenyl)-7-(thiophen-2-yl)-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzoate. BODIPY 13. In a sealed tube under argon, BODIPY 1 (50 mg,

0.12 mmol), $\text{Pd}(\text{PPh}_3)_4$ (5.9 mg, 0.0051) and dry toluene (15 mL) were introduced. An aqueous solution of Na_2CO_3 (2 mL, 1 M in H_2O) and 4-methoxybenzeneboronic acid (22 mg, 0.14 mmol) were added. The reaction was degassed for 10 min with argon. The reaction was heated at reflux for 30 min (monitored by TLC (eluent DCM) until the appearance of the double coupling product). The mixture was cooled to rt, then 2-(tributyltin)thiophene (150 μL , 0.47 mmol) and LiCl (54 mg, 1.27 mmol) were added to the mixture. The reaction was stirred at reflux for 3 h. After cooling to room temperature, 10 mL of an aqueous solution of KF (10% in H_2O) were added to the reaction mixture. The aqueous layer was extracted with methylene chloride three times. The combined organic layers were washed with brine, dried over MgSO_4 and the solvent was removed in vacuo to give the title product as a crude product. Purification of the crude product by silica gel chromatography (eluent methylene chloride /cyclohexane 6/4) afforded the title compound as a purple solid. $M=21$ mg. Yield 32%. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 8.15 (dd, $J=8.4, 1.6$ Hz, 2H), 7.98 (m, 3H), 7.81 (dd, $J=4.8, 1.2$ Hz, 1H), 7.77 (dd, $J=8, 2$ Hz, 2H), 7.23 (dd, $J=4.8, 1.2$ Hz, 1H), 7.09 (dd, $J=6.8, 2.4$ Hz, 2H), 6.98 (d, $J=3.2$ Hz, 1H), 6.90 (s, 2H), 6.86 (d, $J=3.2$ Hz, 1H), 3.94 (s, 3H), 3.88 (s, 3H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 165.4, 160.6, 158.1, 149.3, 139.5, 137.6, 135.5, 135.1, 133.0, 130.8, 130.8, 130.7, 130.4, 130.3 (2 C), 130.2, 129.5, 128.7 (2 C), 128.2 (2 C), 123.9, 121.2, 120.5, 113.7 (2 C), 55.0, 51.8; ^{19}F NMR (282 MHz, CD_2Cl_2) δ –139.57 (dd, $J=65.8, 32.8$ Hz, 2F); IR (film, cm^{-1}) 2922, 1703, 1572, 1542, 1467, 1039; HRMS (ESI⁺): calcd for $\text{C}_{28}\text{H}_{22}\text{BF}_2\text{N}_2\text{O}_3\text{S}$: 515.1407; found: 515.1403 [M + H]; mp 232–233 °C

Methyl 4-(5,5-difluoro-3-(4-methoxyphenyl)-7-(thiophen-3-yl)-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzoate. BODIPY 14. In a sealed tube under argon, BODIPY 1 (100 mg,

0.25 mmol), $\text{Pd}(\text{PPh}_3)_4$ (8.8 mg, 0.007 mmol) and dry toluene (20 mL) were introduced. An aqueous solution of Na_2CO_3 (5 mL, 1 M in H_2O) and 4-methoxybenzeneboronic acid (40 mg, 0.26 mmol) were added. The reaction was degassed for 10 min with argon. The reaction was heated at reflux for 40 min (monitored by TLC (eluent DCM) until the appearance of the double coupling product). The mixture was cooled to rt, then a solution of 3-(tributyltin)thiophene in toluene (1.5 mL, 0.75 mmol) and LiCl (100 mg, 2.38 mmol) were added to the mixture. The reaction was stirred at reflux for 15 h. After cooling to room temperature, 10 mL of an aqueous solution of KF (10% in H_2O) were added to the reaction mixture. The aqueous layer was extracted with methylene chloride three times. The combined organic layers were washed with brine, dried over MgSO_4 and the solvent was removed in vacuo to give the title product as a crude product. Purification of the crude product by silica gel chromatography (eluent methylene chloride /cyclohexane 7/3) afforded the title compound as a dark blue solid. $M=30$ mg. Yield 23%. ^1H NMR (300 MHz, CDCl_3) δ 8.26 (dd, $J=2.9, 1.3$ Hz, 1H), 8.19 (d, $J=8.0$ Hz, 2H), 7.95 (d, $J=8.8$ Hz, 1H), 7.69–7.59 (m, 3H), 7.34 (dd, $J=5.1, 3.0$ Hz, 1H), 7.01 (d, $J=8.8$ Hz, 2H), 6.82–6.71 (m, 3H), 6.65 (d, $J=4.4$ Hz, 1H), 3.99 (s, 3H), 3.88 (s, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 166.5, 161.1, 159.3, 152.7, 140.6, 139.1, 136.3, 135.9, 132.6, 131.6, 131.4, 131.4, 130.7 (2 C), 130.4, 130.0, 129.6 (2 C), 129.0, 128.8, 125.5, 125.1, 121.2, 120.7, 114.1 (2 C), 55.5, 52.6; ^{19}F NMR (282 MHz, CDCl_3) δ –135.22 (dd, $J=65.4, 32.7$ Hz, 2F); IR (film, cm^{-1}) 2925, 1716, 1543, 1469, 1274, 1057; HRMS (ESI⁺): calcd for $\text{C}_{28}\text{H}_{21}\text{BF}_2\text{N}_2\text{O}_3\text{SNa}$: 537.1231; found: 537.1251 [M + Na]; mp 189–190 °C.

Synthesis of BODIPY Hydrazides

General procedure B: In a round bottom flask under argon, a solution of hydrazine in THF (57 equiv, 1 M) and the corresponding BODIPY (1 equiv.) in dry THF (0.7 mL) were introduced. The reaction was stirred at rt for 15 h. The mixture was concentrated to remove

excess of hydrazine and the resulting solid was triturated with Et₂O to afford the title compound.

4-(5,5-difluoro-3,7-bis(4-methoxyphenyl)-5H-4λ⁴,5λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzohydrazide.

BODIPY 15. The title compound was obtained as a dark red solid according to the procedure B from BODIPY 2 (53 mg, 0.098 mmol) and hydrazine in THF (5.7 mL, 1 M). M = 33 mg. Yield 62%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 10.00 (s, 1H), 8.03 (d, *J* = 8.3 Hz, 2H), 7.86 (d, *J* = 9.0 Hz, 4H), 7.74 (d, *J* = 8.4 Hz, 2H), 7.05 (d, *J* = 8.9 Hz, 4H), 6.94 – 6.82 (m, 4H), 4.63 (s, 2H), 3.83 (s, 6H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 165.0, 160.6 (2 C), 157.7 (2 C), 141.1, 136.0 (2 C), 135.4, 134.8 (2 C), 131.0 (2 C), 130.5 (2 C), 127.0 (2 C), 124.3 (2 C), 121.2 (4 C), 113.9 (4 C), 55.3 (2 C); ¹⁹F NMR (376 MHz, CD₂Cl₂) δ –131.94 (dd, *J* = 64.6, 32.0 Hz, 2F); IR (film, cm⁻¹) 3327, 1566, 1466, 1140, 1056; HRMS (ESI⁺): calcd for C₃₀H₂₅BF₂N₄O₃K: 577.1625; found: 577.1637 [M + K]; mp 160–161 °C.

4-(3,7-bis(2,4-dimethoxyphenyl)-5,5-difluoro-5H-4λ⁴,5λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzohydrazide.

BODIPY 16. The title compound was obtained as a dark blue solid, according to the general procedure B from BODIPY 4 (44 mg, 0.074 mmol) and hydrazine in THF (4.2 mL, 1 M). The crude product was triturated with cyclohexane. M = 38 mg. Yield 86%. ¹H NMR (300 MHz, CD₂Cl₂) δ 7.95 (d, *J* = 7.9 Hz, 2H), 7.73 (d, *J* = 7.9 Hz, 2H), 7.63 (d, *J* = 8.5 Hz, 2H), 6.83 (d, *J* = 4.3 Hz, 2H), 6.65 (d, *J* = 3.4 Hz, 2H), 6.57 (m, 4H), 5.35 (dd, *J* = 1.1; 1.0 Hz, 2H), 3.87 (s, 6H), 3.81 (s, 6H), 2.70 (bs, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 162.3, 159.4 (2 C), 155.7 (2 C), 141.6, 136.7, 135.4, 135.0 (2 C), 132.6 (2 C), 130.8 (2 C), 130.7 (2 C), 129.6 (2 C), 127.5 (2 C), 123.1 (2 C), 114.8 (2 C), 105.6 (2 C), 99.4 (2 C), 56.4 (2 C), 55.9 (2 C). ¹⁹F NMR (282 MHz, CD₂Cl₂) δ –134.15 (dd, *J* = 62.8, 31.5 Hz, 2F); IR (film, cm⁻¹) 2941, 1719, 1609, 1253, 1070, 1029; HRMS (ESI⁺): calcd for C₃₂H₂₉BF₂N₄O₅: 599.2272; found: 599.2270 [M + H]; mp 204–205 °C.

4-(5,5-difluoro-3,7-bis(4-fluorophenyl)-5H-4λ⁴,5λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzohydrazide. **BODIPY 17.** The title compound was obtained as a dark purple solid according to the general procedure B from BODIPY 5 (30 mg, 0.058 mmol) and hydrazine in THF (3.1 mL, 1 M). The crude product was purified by silica gel chromatography (eluent : methylene chloride /MeOH 98/2). M = 17 mg. 57% Yield. ¹H NMR (300 MHz, CD₂Cl₂) δ 7.92 (d, *J* = 7.9 Hz, 2H), 7.89 – 7.81 (m, 4H), 7.70 (d, *J* = 7.8 Hz, 2H), 7.58 (bs, 1H), 7.20 – 7.09 (m, 4H), 6.90 (d, *J* = 4.4 Hz, 2H), 6.66 (d, *J* = 4.3 Hz, 2H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 164.6, 162.6 (d, *J* = 255 Hz, 2 C), 156.9 (2 C), 142.9, 135.3 (2 C), 134.8, 134.1, 131.2 (2 C), 130.8 (2 C), 130.0 (2 C), 128.2 (2 C), 126.7 (2 C), 121.1 (2 C), 115.9, 115.8, 114.8 (d, *J* = 22 Hz, 4 C); ¹⁹F NMR (282 MHz, CD₂Cl₂) δ –111.31–111.51 (m, 2F), –132.43 (dd, *J* = 64.3, 32.2 Hz, 2F); IR (film, cm⁻¹) 3325, 1602, 1567, 1433, 1137, 1058; HRMS (ESI⁺): calcd for C₂₈H₁₉BF₄N₄ONa: 537.1480; found: 537.1479 [M + Na]; mp 188–189 °C.

4-(5,5-difluoro-3-(4-methoxyphenyl)-7-(thiophen-3-yl)-5H-5λ⁴,6λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzohydrazide.

BODIPY 18. The title compound was obtained as a dark purple solid according to the general procedure B from BODIPY 14 (30 mg, 0.06 mmol) and hydrazine in THF (3.3 mL, 1 M). The crude product was used without further purification. M = 30 mg. Quantitative yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.77 (bs, 1H), 8.30 (s, 1H), 8.04 (d, *J* = 7.7 Hz, 2H), 7.94 (d, *J* = 8.5 Hz, 2H), 7.81–7.67 (m, 3H), 7.63 (s, 1H), 7.15–6.99 (m, 3H), 6.99–6.76 (m, 3H), 4.79 (bs, 2H), 3.87 (s, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.0, 160.6, 160.0, 134.8, 132.2 (2 C), 130.9 (2 C), 130.8 (2 C), 130.2, 130.2, 130.1, 128.6, 127.9, 126.8 (2 C), 126.2, 126.0 (2 C), 125.4, 121.0, 113.9 (2 C), 113.2 (2 C), 55.2; ¹⁹F NMR (282 MHz, DMSO-*d*₆) δ –133.36 (dd, *J* = 66.3, 33.3 Hz, 2F); IR (film, cm⁻¹) 3346, 1657, 1605, 1544, 1074, 1059; HRMS (ESI⁺): calcd

for C₂₇H₂₁BF₂N₄O₂S: 515.1519; found: 515.1506 [M + H]; mp 272–273 °C.

4-(5,5-difluoro-3,7-di(thiophen-2-yl)-5H-4λ⁴,5λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzohydrazide. **BODIPY 19.** The title compound was obtained as a dark purple solid according to the general procedure B from BODIPY 8 (30 mg, 0.06 mmol) and hydrazine in THF (3.5 mL, 1 M). The crude product was used without further purification. M = 30 mg. Quantitative yield. ¹H NMR (600 MHz, DMSO-*d*₆) δ 9.99 (bs, 1H), 8.11 (dd, *J* = 3.8, 1.1 Hz, 2H), 8.02 (d, *J* = 8.2 Hz, 2H), 7.92 (dd, *J* = 5.0, 1.1 Hz, 2H), 7.72 (d, *J* = 8.2 Hz, 2H), 7.31 (dd, *J* = 5.1, 3.8 Hz, 2H), 7.08 (d, *J* = 4.5 Hz, 2H), 6.90 (d, *J* = 4.5 Hz, 2H), 4.75 (bs, 2H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 165.0, 149.7 (2 C), 139.6 (2 C), 135.8 (2 C), 135.8 (2 C), 134.7, 133.2, 131.6 (2 C), 131.5 (2 C), 130.5 (2 C), 130.4 (2 C), 129.0 (2 C), 127.0 (2 C), 121.1; ¹⁹F NMR (282 MHz, DMSO-*d*₆) δ –137.40 (dd, *J* = 66.8, 33.3 Hz, 2F); IR (film, cm⁻¹) 3348, 1569, 1469, 1134, 710; HRMS (ESI⁺): calcd for C₂₄H₁₇BF₂N₄O₁S₂Na: 513.0797; found 513.0801: [M + Na]; mp 229–230 °C.

4-(5,5-difluoro-3,7-di(thiophen-3-yl)-5H-4λ⁴,5λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzohydrazide. **BODIPY 20.** The title compound was obtained as a dark purple solid, according to the general procedure B from BODIPY 11 (32 mg, 0.06 mmol) and hydrazine in THF (3.7 mL, 1 M). The crude product was dissolved in dichloromethane (10 mL) and the organic layer washed with H₂O (10 mL) and brine (10 mL). The organic layer was dried over MgSO₄ and concentrated under vacuum. M = 32 mg, 90% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.00 (bs, 1H), 8.47 (dd, *J* = 2.9, 1.3 Hz, 2H), 8.03 (d, *J* = 8.4 Hz, 2H), 7.85 (dd, *J* = 5.1, 1.3 Hz, 2H), 7.76–7.69 (m, 4H), 7.14 (d, *J* = 4.5 Hz, 2H), 6.91 (d, *J* = 4.5 Hz, 2H), 4.66 (bs, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.0, 151.7 (2 C), 140.6 (2 C), 136.0 (2 C), 135.4 (2 C), 134.7 (2 C), 132.1 (2 C), 130.5 (2 + 1 C), 129.3, 128.9 (2 C), 127.0 (2 C), 126.7 (2 C), 121.3; ¹⁹F NMR (282 MHz, DMSO-*d*₆) δ –136.26 (dd, *J* = 67.1, 33.4 Hz, 2F); IR (film, cm⁻¹) 2921, 1610, 1567, 1140, 1058; HRMS (ESI⁺): calcd for C₂₄H₁₇BF₂N₄O₁S₂Na: 513.0797; found: 513.0809 [M + Na]; mp. 220–221 °C.

4-(5,5-difluoro-3-(thiophen-2-yl)-7-(thiophen-3-yl)-5H-5λ⁴,6λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzohydrazide.

BODIPY 21. The title compound was obtained as a dark purple solid according to the general procedure B from BODIPY 12 (30 mg, 0.06 mmol) and hydrazine in THF (3.5 mL, 1 M). The crude product was triturated with cyclohexane. M = 28 mg. 93% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.77 (bs, 1H), 8.49 – 8.33 (m, 1H), 8.04 (dd, *J* = 11.8, 5.8 Hz, 3H), 7.84 (d, *J* = 5.3 Hz, 2H), 7.74–7.62 (m, 3H), 7.31–7.25 (m, 1H), 7.12–7.04 (m, 1H), 7.04–6.97 (m, 1H), 6.97–6.80 (m, 2H), 4.62 (bs, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 164.7, 151.8, 151.6, 149.3, 139.9, 135.5, 135.3, 134.7, 133.0, 131.9, 130.9, 130.5, 130.0, 129.9 (2 C), 128.7, 128.4, 128.3, 128.3, 126.6 (2 C), 126.0, 121.0, 120.5; ¹⁹F NMR (282 MHz, DMSO-*d*₆) δ –136.73 (dd, *J* = 66.5, 33.2 Hz, 2F); IR (film, cm⁻¹) 3310, 1658, 1566, 1470, 1134, 1042; HRMS (ESI⁺): calcd for C₂₄H₁₇BF₂N₄O₂Na: 513.0797; found: 513.0800 [M + Na]; mp 220–221 °C.

Supporting Information

X-Ray diffraction structure of BODIPY 12. ¹H NMR and ¹³C NMR spectra. UV-Vis and fluorescence spectra of BODIPY 13 and 14 and BODIPY–Hz 15–21.

Acknowledgements

We thank Sorbonne Université and CNRS for funding, the Fédération de Recherche de Chimie Moléculaire de Paris Centre (FR2769) for providing technical access to analytical instruments. We thank the "Interface Pour le Vivant" doctoral program from Sorbonne Université for a PhD grant to A. Nina-Diogo. We thank AFM postdoc fellowship for Janek Hyzewic.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: carbonylated proteins · BODIPY · fluorescent probes · 2D-Oxi electrophoresis · flow cytometry · microscopy

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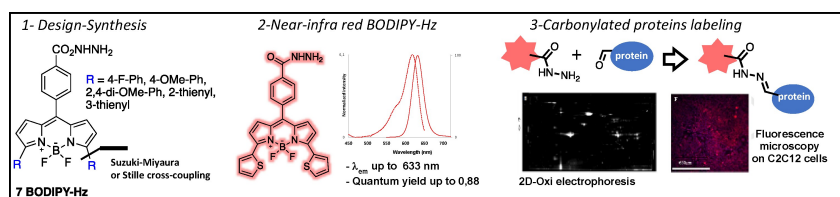
Manuscript received: January 31, 2024

Revised manuscript received: March 28, 2024

Accepted manuscript online: May 2, 2024

Version of record online: ■■, ■■

RESEARCH ARTICLE



Insert text for Table of Contents here. We report the design and the synthesis of new BODIPY possessing a hydrazide function (BODIPY-Hz), an emission in the near-infra red area and high quantum yield. We show the use of these new BODIPY-Hz as fluorescent probes for carbonylated

proteins labeling. We demonstrate that the new BODIPY-Hz are compatible with the 2D-Oxi electrophoresis technology, and that it can be used directly for proteins labeling in cells monitored by fluorescence microscopy.

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1 – 15

Synthesis of New Bodipy Hydrazide Fluorescent Probes for the Detection of Carbonylated Proteins Generated by Oxidative Stress

