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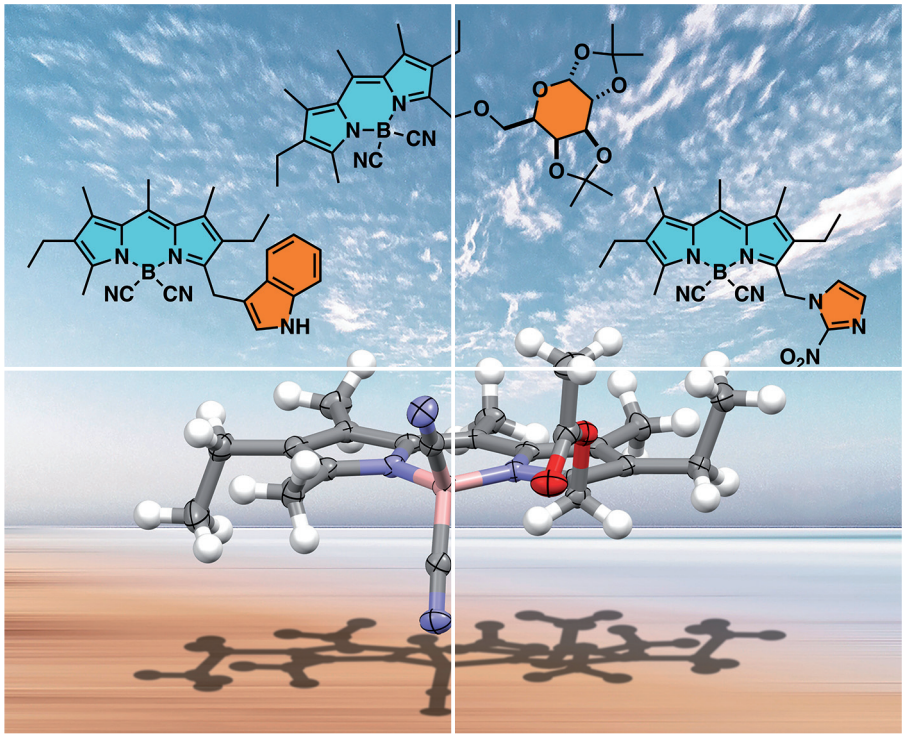


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# Acetoxymethyl-BODIPY dyes: a universal platform for the fluorescent labeling of nucleophiles†

Alberto Blázquez-Moraleja, [a] Larissa Maierhofer, ‡[a] Enrique Mann, ‡[a] Ruth Prieto-Montero, ‡[b] Ainhoa Oliden-Sánchez, ‡[b] Lucía Celada, [c] Virginia Martínez-Martínez, *[b] María-Dolores Chiara *[c] and Jose Luis Chiara *[a]

Current methods for the preparation of functional small-molecule fluorophores generally require labor-intensive, multi-step synthetic routes for all the major chromophoric groups. In spite of recent significant contributions from numerous laboratories, the paucity of rapid, straightforward and wide-scope synthetic strategies in this field is limiting the development of advanced probes for bioimaging, sensing and therapeutic applications. We describe herein a general and robust methodology for the one-step fluorescent labeling of a wide variety of molecules having *C*-, *N*-, *P*-, *O*-, *S*-, or halide-nucleophilic centers, using stable and readily available acetoxymethyl-BODIPYs as reagents in the presence of an acid catalyst. This modular methodology allows a very facile preparation of mono- and di-functional probes incorporating a broad assortment of biomolecules, enzyme cofactors, natural products, and other chromophores, as well as chemical functionalities for a wide range of applications including bioorthogonal conjugation, polymerization, and supramolecular chemistry, among others. The photophysical properties and preliminary applications of the new probes in live-cell imaging were also studied. The described strategy enables the high-throughput engineering of novel BODIPY dyes with diverse functionalities for basic and applied science with potential for innovative technological applications.

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## 1. Introduction

Genetically encoded fluorescent proteins[1] and small-molecule fluorescent probes[2] have evolved as essential facilitating tools for the study of biology at the single cell and subcellular levels with high spatiotemporal resolution. Although small-molecule fluorophores can hardly surpass the great specificity of fluorescent proteins, their physicochemical properties and photostabilities can be readily modulated through chemical modification, further allowing the introduction of appropriate functionalities for applications in biolabeling, biotargeting, and sensing that are often beyond the reach of genetic approaches.[2,3] In general, current methods for the preparation of functional small-molecule fluorophores require labor-intensive, multi-step synthetic routes for all the major chromophoric groups.[2] Among these, BODIPYs have become prominent in recent years due to their high photostability, excellent photophysical properties, and high chemical versatility.[4–6] The development of functionalization methods that are general, efficient, selective, and highly versatile is currently one of the main challenges in fluorophores' chemistry in general and in BODIPYs in particular, which display a richer chemistry than other fluorophores in current use. Although different methodologies have been described for the introduction of new functionalities at every position of the BODIPY chromophore, the vast majority involve the use of multistage routes that need to be individually tailored to each final target dye, which entails greater synthetic effort, overall loss of chemical yield and increased economic costs. For these reasons, there is great interest in the development of direct functionalization methods of pre-formed BODIPYs,[7,8] which ideally allow the introduction of the new functionality in a single and final reaction step.

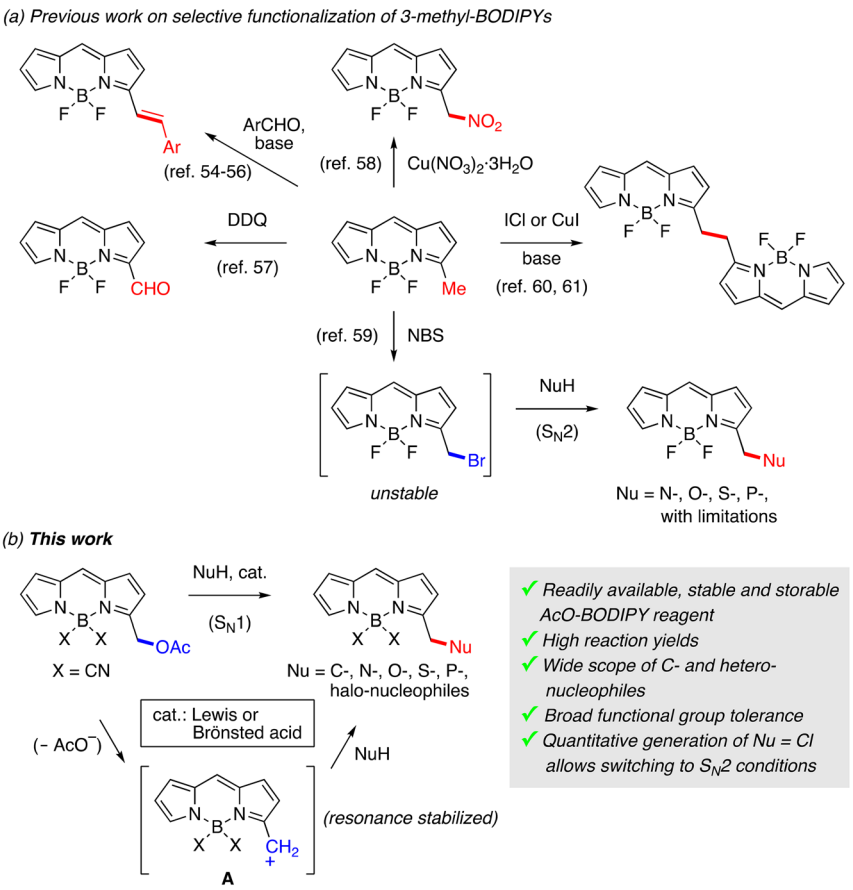
[a]*Instituto de Química Orgánica General (IQOG-CSIC), Juan de la Cierva 3, 28006 Madrid, Spain. E-mail: jl.chiara@csic.es*
[b]*Departamento de Química Física, Universidad del País Vasco-EHU, Facultad de Ciencia y Tecnología, Apartado 644, 48080 Bilbao, Spain. E-mail: virginia.martinez@ehu.eus*
[c]*Instituto de Investigación Sanitaria del Principado de Asturias, Instituto Universitario de Oncología del Principado de Asturias (IUOPA), CIBERONC, Universidad de Oviedo, Hospital Universitario Central de Asturias, 33011 Oviedo, Spain. E-mail: mdchiara.uo@uniovi.es*

†Electronic supplementary information (ESI) available. Supplementary tables and figures, complete experimental procedures, X-ray diffraction data of **9**, computational details, live-cell microscopy images, and copies of $^{1}$H and $^{13}$C NMR spectra of all new compounds (PDF). CCDC 2177585 contains the supplementary crystallographic data for compound **9**. For the corresponding 1D- and 2D-NMR datasets in the standard JCAMP-DX format and other electronic formats see DOI: https://doi.org/10.1039/d2qo01099b

‡These authors have contributed equally to this work.

In general, most post-functionalization strategies described for BODIPYs require the presence or prior introduction of a reactive atom or group (*e.g.* halogen,[9–19] formyl,[20–24] carboxyl,[25–31] thioether,[32–36] alkyne,[37–44] azide,[45–48] or tetrazine[49–53]) that enables the final functionalization reaction. These reactive groups can be directly attached either to the chromophore backbone (at boron or carbon) or to peripheral positions separated by one or several bonds from the chromophoric nucleus. However, all previously described methods present limitations in terms of: (1) the variety of substituents that can be introduced under the same reaction conditions (generally, carbon and heteroatomic substituents require different conditions); (2) the type of reactive functionality that should be present in the new substituents, which affects their price, availability and structural diversity; (3) the chemical compatibility between the pre-existing functional groups on the BODIPY and on the new substituent under the reaction conditions; and (4) the yield of the post-functionalization reaction.

The objective of this work was to develop a general strategy for the post-functionalization of BODIPYs that allowed the direct and straightforward incorporation of a wide variety of molecules under similar reaction conditions. Most widely used BODIPYs have alkyl-substituted pyrrole rings, which stabilize cations, radicals and anions at the benzylic-type carbon, particularly those at C3 and C5, offering an interesting opportunity for directly incorporating new functionality at these reactive positions. Not surprisingly, this reactivity has already been exploited in the functionalization of 3/5-methyl-substituted BODIPYs (Scheme 1a) using: (a) Knoevenagel reaction with aromatic aldehydes,[54–56] (b) oxidation to aldehyde with DDQ,[57] (c) nitration,[58] (d) electrophilic bromination followed by *in situ* nucleophilic substitution,[59] and (e) oxidative dimerization promoted by ICl or CuI under basic conditions.[60,61] Interestingly, Ulrich and Ziessel's[59] two-step/one-pot protocol has been successfully used for the introduction of *N*-, *O*-, *P*- and *S*-nucleophiles. However, this approach shows some drawbacks: (i) the bromo-methylated intermediate is a very unstable compound that cannot be isolated and, consequently, the bromination and substitution steps must be carried out "in a single flask" for each transformation; (ii) reaction times are highly variable, from a few minutes to several days, depending on the nucleophile; (iii) it is limited in relation to the type of nucleophiles that can participate in the reaction. Thus, in the case of *O*-nucleophiles, only alcohols have been described; in the case of *N*-nucleophiles, only azide and secondary amines render the substitution product, while primary amines generate complex reaction mixtures; and (iv) there are no examples




**Scheme 1** Post-functionalization strategies of 3-methyl-BODIPYs (substituents at other positions were removed for clarity).

of introduction of *C*-nucleophiles, which is possibly its most important limitation.

Our new method (Scheme 1b) was inspired by these former functionalization strategies. A key prerequisite of our approach was the incorporation of a stable reactive group that required prior activation under catalytic conditions to participate in the functionalization reaction. Thus, the reactive but non-activated BODIPY can be easily isolated and stored for later use in the substitution reaction. A functional group that fulfills these requirements is the acetoxymethyl group, widely used in the synthesis of porphyrins,[62–65] but, somehow surprisingly, not yet in the chemistry of BODIPYs despite the structural similarities between both families of dyes. Acid-catalyzed activation of this group can readily generate a resonance-stabilized carbocation intermediate (**A**), which will considerably expand the diversity of nucleophilic groups that can participate in the $S_N1$-type process including also different types of *C*-nucleophiles, in contrast to previous methods.

In this work, we describe the preparation of acetoxymethyl-BODIPYs by direct acyloxylation of the corresponding 3/5-methyl-BODIPYs and their efficient acid-catalyzed reaction with hetero- and carbon-nucleophiles to afford a wide variety of new functional BODIPYs for multiple applications. The photophysical properties of the new probes as well as some preliminary applications in live-cell microscopy will also be described.

## 2. Results and discussion

### 2.1. Synthesis of 3/5-acetoxymethyl-BODIPYs

When we started this work, there was already an example of a BODIPY dye with acetoxymethyl groups at C3 and C5 reported by Boyer *et al.* in 1994 in an article that is better known for first describing the regioselective oxidation of the methyl groups at C3/C5 to formyl groups with DDQ.[57] The authors prepared the 3,5-di(acetoxymethyl)-BODIPY **1** by a classical method of acetoxylation using lead tetraacetate, which was originally reported for alkyl-substituted pyrroles[62] and other heteroaromatic and aromatic compounds (Scheme 2a). However, attempts to hydrolyze the acetate groups under acidic or basic conditions always led to complex reaction mixtures and the compound did not awake further synthetic interest.

Although F-BODIPYs show great chemical robustness under diverse reaction conditions, strong acids and bases cause decomplexation of the $BF_2$ group.[66–73] Substitution of the fluorine atoms at boron with other substituents barely affects the positions of the spectroscopic bands (absorption and emission) of the chromophore, but allows to modulate its photo- and chemo-stability. Among the substituents that have been introduced on the boron atom of BODIPYs instead of fluorine (halo-, *C*-, *O*-, and *N*-derivatives),[7,8,68,74] the cyano group provides the greatest stability improvement in acid medium, while also enhancing the fluorescence of the chromophore.[75–78] For our planned strategy, the introduction of cyano groups on the boron atom was a conceivable requirement for our acetoxymethyl-BODIPY reagent to withstand the acidic conditions of the substitution reaction. Thus, we prepared 4,4′-dicyano-BODIPYs (CN-BODIPYs) **5–7** in quantitative yield from the corresponding F-BODIPYs **2–4**, respectively, following a described protocol (Scheme 2a),[75,79] without any need for chromatographic purification.

We first tested the reaction of F-BODIPYs **2** and **3** with Pb$(OAc)_4$ in acetic acid using the reported procedure,[57] with minor modifications (Scheme 2b; see the ESI† for details). In the case of the peralkylated BODIPY **2**, the reaction led to the regioselective formation of 3,5-bis(acetoxymethyl)-BODIPY **1**, as described.[57] In contrast, the reaction of BODIPY **3**, with an aryl group at C8, was slower and gave exclusively the mono-acetoxy derivative **8** under the same conditions. The acyloxylation of CN-BODIPY **5** (Scheme 2b) with $Pb(OAc)_4$ (2.4 equiv.) at room temperature exclusively yielded the mono-acetoxy product **9** in good yield, in contrast to its parent F-BODIPY **2**, which generated the diacetoxy-F-BODIPY **1** under these conditions. Using a higher temperature (40–50 °C) and a higher excess of $Pb(OAc)_4$ (3–4 equiv.) gave the diacetoxy-CN-BODIPY **10** in very good yield. Compound **10** was not stable on prolonged contact with silica gel, but it could be easily purified by recrystallization from hexane/$CH_2Cl_2$. The acyloxylation of CN-BODIPY **7** proceeded under the same thermal conditions to afford a mixture of mono- and di-acetoxy-CN-BODIPY (**11** and **12**, respectively), together with minor amounts of triacetoxy-CN-BODIPY **13**. Interestingly, these acetoxy-CN-BODIPYs could be purified on silica gel column chromatography without decomposition.

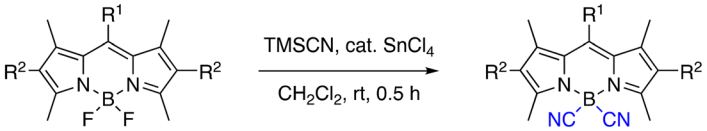
The structure of **9** was confirmed by X-ray diffraction (Fig. 1). As previously described[57] for **1**, the acetyl group adopts an orthogonal orientation with respect to the median plane of the chromophore, which is the most suitable electronic prealignment for the heterolytic cleavage of the C–OAc bond after Lewis acid complexation, with generation of intermediate carbocation **A** (see Scheme 1). As observed for other CN-BODIPYs,[76] the B–N bond lengths (1.531 and 1.538 Å) are slightly shorter than the mean value of 1.540 Å determined for its F-BODIPY precursor **2**.[80] The boron atom has an almost ideal tetrahedral geometry with bond angles for N–B–N, C–B–N and C–B–C of 108.4, 109.6 (mean value), and 110.0°, respectively.

A plausible mechanistic proposal for this reaction is discussed in the ESI† to explain the lower reactivity observed for compounds with better electron-withdraing groups (CN- *versus* F-BODIPYs: **5** and **2**, respectively; and 8-phenyl- *versus* 8-methyl-BODIPYs: **4** and **2**, respectively).

With a view to subsequent chemical transformations that would require a non-acetoxy leaving group, we tried to deacetylate **8** to generate the hydroxymethyl-derivative **14**, which can also be a suitable leaving group in the $S_N1$ reaction. As observed by Boyer *et al.*,[57] the transesterification reaction of **2** in MeOH under different basic conditions (NaOMe, $K_2CO_3$ or $Et_3N$) always led to complex reaction mixtures. We finally found very mild deprotection conditions[81] using metallic mag-

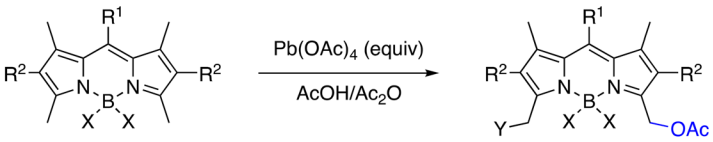
 

a) Synthesis of starting CN-BODIPYs

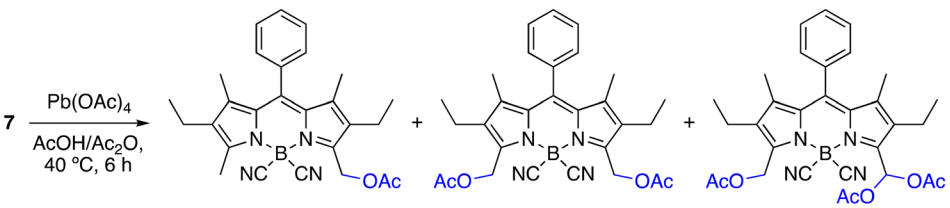



**2**, $R^1$ = Me, $R^2$ = Et — **5**, quant.
**3**, $R^1$ = 2,4,6-$Me_3C_6H_2$ (Mes), $R^2$ = H — **6**, 96%
**4**, $R^1$ = Ph, $R^2$ = Et — **7**, 99%

b) Acyloxylation reactions

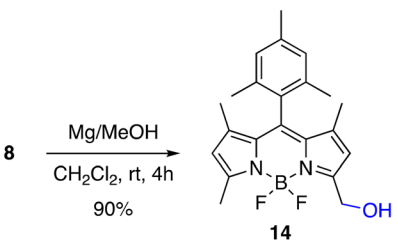



| Starting BODIPY | $R^1$ | $R^2$ | X | $Pb(OAc)_4$ (equiv) | Temp. (°C) | Time (h) | Product | Y | Yield (%) |
|---|---|---|---|---|---|---|---|---|---|
| **2** | Me | Et | F | 2.1 | 0-25 | 4.5 | **1** | AcO | 66 |
| **3** | Mes | H | F | 2.1 | 0-25 | 3.0 | **8** | H | 62 |
| **5** | Me | Et | CN | 2.4 | 0-25 | 2.5 | **9** | H | 72 |
| **5** | Me | Et | CN | 3.0 | 50 | 5.5 | **10** | AcO | 87 |




| $Pb(OAc)_4$ (equiv) | **11** | **12** | **13** |
|---|---|---|---|
| 2.6 | 49% | 32% | 2% |
| 4.0 | 24% | 44% | 5% |

c) Deacetylation reaction




**14**

**Scheme 2** Synthesis of starting CN-BODIPYs and acetoxymethyl-BODIPYs, and deacetylation of the latter.

nesium in MeOH at room temperature, which afforded **14** in very good yield (Scheme 2c). However, due to the success of our initial approach for directly activating the acetate group (see below), this alternative route was not further explored.

### 2.2. Reaction of 3-acetoxymethyl-BODIPYs with carbon and heteroatom nucleophiles

Preliminary studies of the substitution reaction of acetoxymethyl-F-BODIPYs **1** with isobutanol as model nucleophile in $CH_2Cl_2$ or MeCN at room temperature using either Brønsted (*p*-TsOH) or Lewis ($Et_2O{\cdot}BF_3$, TMSOTf, $Sc(OTf)_3$, $Cu(OTf)_2$) acid catalysts (2.5 mol%) afforded complex reaction mixtures with generation of very polar non-fluorescent products in all cases. This result points to a probably acid-promoted decomplexation of the $BF_2$ group generating the corresponding dipyrromethene.[68–73] We next assayed the CN-BODIPY derivative **9**, with an expected significantly enhanced stability under acidic conditions, as commented above. A qualitative parallel

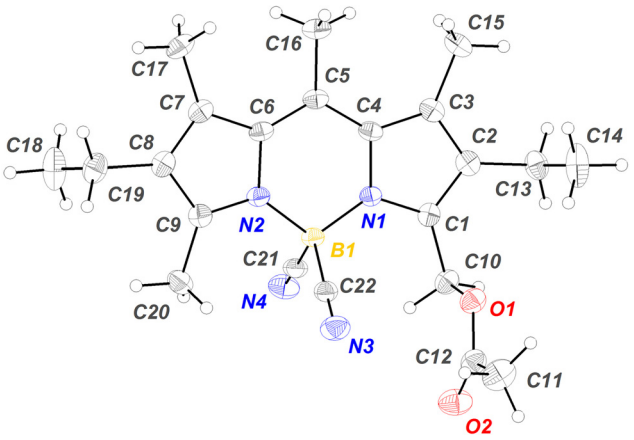


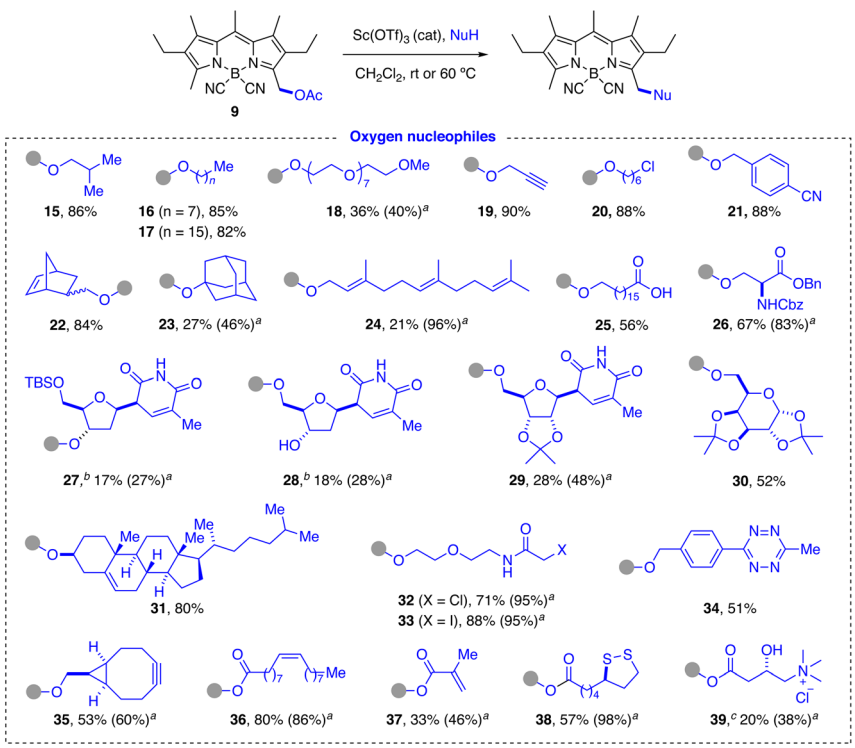

**Fig. 1** Molecular structure of compound **9** from X-ray crystal structure analysis.

screening with a larger group of acid catalysts using the same model test reaction with **9** (see ESI† for details) showed that Sc $(OTf)_3$ was the most efficient and general acid catalyst and was therefore selected for most of the substitution reactions described below.

**2.2.1. Synthesis of mono-substituted BODIPYs.** The initial optimization study with a simple alcohol, allowed us to generalize this new methodology for the efficient incorporation of a wide array of hetero- and *C*-nucleophiles. The general procedure consisted of treating an acetoxymethyl-CN-BODIPY with a small excess of the nucleophile (1.2–2.0 equiv.) in anhydrous solvent ($CH_2Cl_2$ or MeCN) at room temperature in the presence of 2.5–15.0 mol% of the acid catalyst (generally Sc $(OTf)_3$), without the need to use an inert atmosphere.

*O-Nucleophiles.* A wide variety of *O*-nucleophiles were incorporated at the methylene group of 3-acetoxymethyl-CN-BODIPY **9** using the general reaction conditions (Scheme 3). Primary (**15–18**, **20**, **22**, **25**, **26**, **28–30**, **32**, **33**, **35**), secondary (**27**, **31**) and tertiary (**23**) alkanols, as well as benzylic (**21**, **34**), allylic (**24**) and propargylic alcohols (**19**) were successfully labeled in moderate to very good yields. The reaction is compatible with the presence of ester (**26**), nitrile (**21**), carbamoyl (**26**), carboxamido (**32**, **33**), imido (**27–29**), trialkylsilyl ether (**27**), isopropylidene acetal (**29**, **30**), 1,2,4,5-tetrazino (**34**), disulfide (**38**), and chloro-/iodo-alkyl groups (**20**, **32**, **33**). Carboxylic acids were also good substrates, although a larger excess of the nucleophile (up to 5 equiv.) was generally required to obtain good product yields (**36–39**). In this case, the reaction is actually in thermodynamic equilibrium with the acetic acid released in the substitution reaction. This equilibrium can also explain the chemoselectivity observed in the reaction with simple hydroxy acids (**25**). However, in the case of more complex hydroxy acids (**39**) with a secondary hydroxyl group, the corresponding ester was obtained, although in moderate yield.

The method allowed us to fluorescently label natural products (farnesol: **24**), as well as a wide variety of biomolecules, including lipids (cholesterol: **31**, oleic acid: **36**), carbohydrates

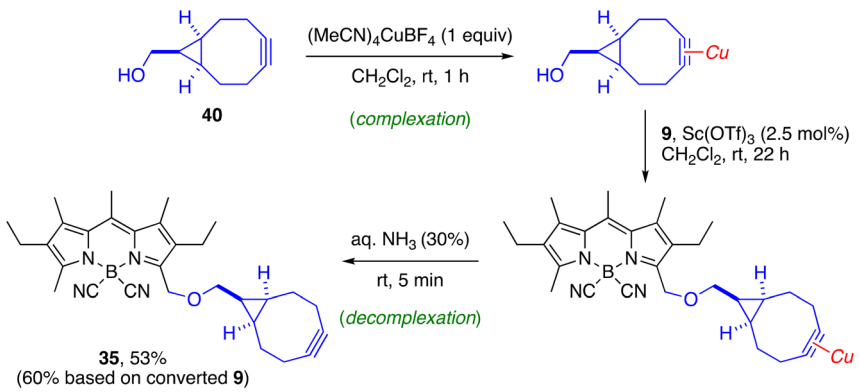



**Scheme 3** Reaction with *O*-nucleophiles. Reaction conditions: Sc$(OTf)_3$ (2.5–10 mol%), NuH (1.2–5.0 equiv.), 0.5–30 h (see ESI† for further details). [a]Yield based on converted **9**. [b]Products formed in the same reaction. [c]In MeCN.

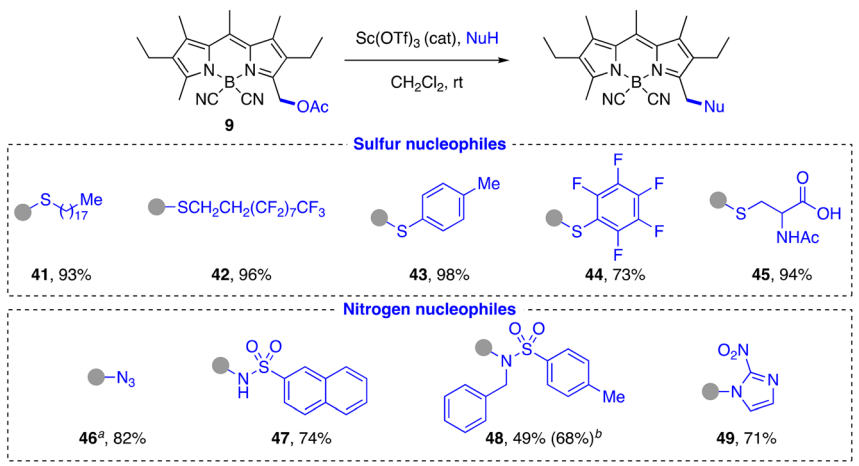
(D-galactose: **30**), nucleosides (partially protected derivatives of D-deoxyribose: **27**, **28**, and D-ribose: **29**), amino acids (L-serine: **26**), hydroxy acids with a tetraalkylammonium group (carnitine: **39**), or enzyme cofactors (lipoic acid: **38**). In addition, we were able to introduce functionalities for complexation with cyclodextrins (adamantyl group: **23**), for polymerization (methacrylate group for radical polymerization: **37**; norbornene group for ring-opening alkene metathesis-polymerization (ROMP): **22**), for bioorthogonal conjugation[82–84] (propargyl group for Cu(I)-catalyzed "click" cycloaddition reaction: **19**; cyclooctyne group for strain-promoted alkyne–azide cycloaddition (SPAAC): **35**; chloro-/iodo-acetamido group for selective labeling of cysteines: **32**, **33**; 1,2,4,5-tetrazine ring for inverse-electron-demand Diels–Alder cycloadditions:[85] **34**), and for selective fluorescent labeling of HaloTag fusion proteins (6-chlorohexyl chain: **20**).[86–88]

The diversity of O-substituents included in this study allowed us to identify some limitations of the method. The first, as already mentioned, is the need to use an excess of nucleophile in the case of carboxylic acids, due to the reversibility of the substitution reaction under acidic conditions. As expected, yields decreased in the case of molecules with acid-sensitive groups, such as acetals (**29**, **30**) or trialkylisilyl ethers (**27**). In the latter case, the reaction afforded the expected product **27** together with the corresponding desilylated derivative **28**. Not surprisingly, polyoxygenated substrates (**18**, **26–30**, **32**, **33**, **39**) slowed down the reaction due to their ability to complex the metal catalyst in competition with the activation of the acetate group, which required the use of a greater amount of catalyst and/or a higher reaction temperature, including microwave irradiation in some cases (**39**) (see ESI† for experimental details). Other strong metal chelating groups, such as the cyclooctyne group, sequestered the scandium catalyst very effectively. The incorporation of the cyclooctyne unit **40** could only be achieved following a described strategy for transient protection of the cyclooctyne by **1 : 1** complexation with a cationic copper(I) salt, which allowed the preparation of **35** in moderate overall yield (Scheme 4).[89,90]

*S-Nucleophiles.* As expected from their higher nucleophilicity, the substitution reaction with thiols was faster and more efficient than with alcohols (Scheme 5). Aliphatic (**41**, **42**, **45**) and aromatic (**43**, **44**) thiols were readily incorporated in high




**Scheme 4** Synthesis of cyclooctyne-functionalized probe **35** from acetoxymethyl-BODIPY **9** by a transient protection strategy of the cyclooctyne.




**Scheme 5** Reaction with *S*- and *N*-nucleophiles. [a]NuH: $TMSN_3$. [b]Based on converted **9**.

yield (70–90%), including polyfluorinated systems (**42**, **44**) and partially protected biomolecules such as *N*-acetyl-L-cysteine (**45**). In the latter case, the observed chemoselectivity is both of kinetic origin, due to the higher nucleophilicity of the thiol group in comparison with the carboxylic acid group, and of thermodynamic origin, due to the reversibility of the reaction with the carboxylate group.

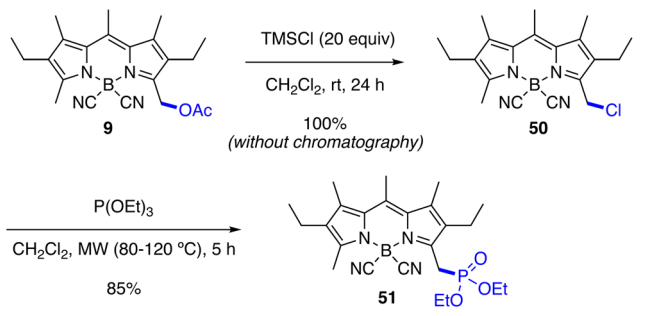
*N-Nucleophiles.* As we already verified in the study of *O*- and *S*-nucleophiles, amides and carbamates are not good substrates in this substitution reaction, nor would amines be expected to react due to their incompatibility with the acid catalysis conditions. Thus, we decided to test other types of *N*-nucleophiles that did not present these problems, either because of their greater acidity, as in the case of sulfonamides or 2-nitroimidazole, or because of their higher nucleophilicity, as occurs with the azide anion. Thus, primary and secondary sulfonamides (**47**, **48**, respectively), which can generate a transient nucleophilic anion, enabled the reaction in moderate to good yield, the former being more reactive due to the lower steric hindrance (Scheme 5). Trimethylsilyl azide yielded the azidomethyl-CN-BODIPY **46** in high yield and with appreciably faster kinetics than in the case of sulfonamides. Compound **46** is an interesting fluorescent reagent for bioorthogonal conjugation and sensing.[46,91,92] (NH)-Heterocyles, such as 2-nitroimidazole, were efficiently alkylated under the standard conditions, affording in this case a potential nitroreductase probe (**49**).[93]

*Cl- and P-Nucleophiles.* The reaction of **9** with an excess of TMSCl (20 equiv.) in $CH_2Cl_2$ at room temperature quantitatively furnished the chloromethyl-derivative **50** in the absence of a metal catalyst, where the reagent acted as acid catalyst as well as nucleophile precursor (Scheme 6). Although **50** was unstable on silica gel, it could be isolated in quantitative yield and high purity by simple evaporation of the reaction mixture.[59] This chlorinated compound can be directly used for $S_N2$-type substitution transformations, as described for its bromo-analogue.[59] Thus, the Arbuzov reaction with triethyl phosphite under microwave irradiation yielded the phosphonate **51** in good yield (Scheme 6).

*C-Nucleophiles.* In contrast to previously described approaches,[59] the same reaction conditions that were efficient for the functionalization of heteronucleophiles proved to be equally effective for the introduction of *C*-nucleophiles. Thus, electron-rich aromatic (phenols: **56**, **57**, **58**; azulene: **59**) and heteroaromatic (pyrrole: **60**; indole: **61**) compounds efficiently yielded the BODIPY-labeled product in a Friedel–Crafts-type process (Scheme 7). The reaction with phenols was highly chemo- and regioselective, exclusively affording the 2-hydroxyphenyl-derivative in very good yields. No trace of the alternative *O*-alkylated derivative or the 4-hydroxyphenyl-regioisomer were detected. The method allowed us to fluorescently label partially protected L-tyrosine (**57**) and a polyfunctional natural product such as resveratrol (**58**). The reaction with the tyrosine derivative proceeded with high chemo- and regioselectivity and was fully compatible with the presence of the carbamate and the methyl ester groups. The reaction with resveratrol required the use of a greater amount of catalyst, longer reaction time and a higher temperature, but proceeded regioselectively to give the *C*-derivative **58** in moderate yield as major product, with the BODIPY chromophore covalently bonded to the most electron-rich aromatic ring at the most electron-rich carbon. The reaction with guaiazulene, a natural derivative of azulene, was also highly regioselective, affording exclusively the product alkylated at the electron-rich 5-membered ring. The structures of these compounds were fully confirmed by careful one- and two-dimensional $^1H$ and $^{13}C$ NMR studies. Indole and pyrrole also gave the substitution reaction with good or very good yields under the standard conditions, with the expected regioselectivity for electrophilic attack on this type of heterocycles. The good reactivity observed for pyrroles led us to also test BODIPYs with a free position on the pyrrolic rings as nucleophiles. To avoid decomposition in the presence of the acid catalyst, we used a 4,4′-dicyano-BODIPY (**6**) as acceptor (Scheme 2a). Despite the presence of alkyl substituents flanking the reactive CH carbon of the pyrrole rings of **6**, the reaction of an equimolecular mixture of **6** and **9** under the standard conditions led to formation of the expected statistical mixture of mono- and di-substitution products **62** and **63**, respectively, in close to the expected theoretical yields (50% and 25%, respectively).

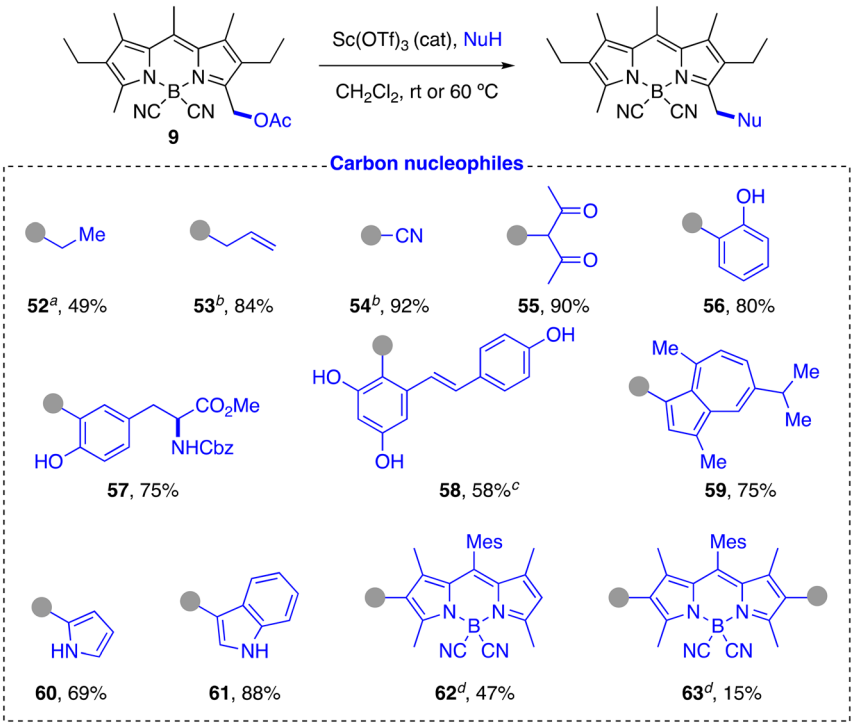
In addition to carbocyclic and heterocyclic systems, acyclic *C*-nucleophiles can also be incorporated. Thus, *C*-trimethylsilyl derivatives, including allyltrimethylsilane and trimethylsilyl cyanide, as well as compounds with an active methylene group such as acetylacetone, reacted very efficiently to yield the expected alkylation products **53**, **54**, and **55**, respectively, in high yield. Compound **55** presented the characteristic keto–enol equilibrium in solution, with a practically equimolecular ratio of both species in $CDCl_3$ at room temperature, as observed by $^1H$ NMR (see ESI†).[94,95] Simple organometallic compounds such as diethylzinc were also successfully tested, although in this case it was necessary to use a stoichiometric amount of trimethylsilyl triflate as promoter to afford the alkylated product **52**, which indicates the probable participation of a triflate intermediate in the substitution reaction.[96]

Unlike other related methods, the great versatility of the described post-functionalization strategy allowed not only to incorporate a wide diversity of heteronucleophiles in BODIPYs,




**Scheme 6** Reaction with halogen and phosphorous nucleophiles.




**Scheme 7** Reaction with carbon nucleophiles. [a]NuH: $Et_2Zn$, using $Me_3SiOTf$ as catalyst. [b]NuH: $Me_3SiNu$. [c]Yield of the mixture of regioisomers. Major regioisomer is shown, isolated by recrystallization. [d]Products formed in the same reaction (Mes: mesityl).

but also to extend the carbon chain of the chromophore with a broad variety of cyclic and acyclic *C*-nucleophiles, including biomolecules (tyrosine: **57**), natural products (resveratrol: **58**, guaiazulene: **59**), and other chromophores (**62** and **63**) in a direct and very efficient way using similar reaction conditions.

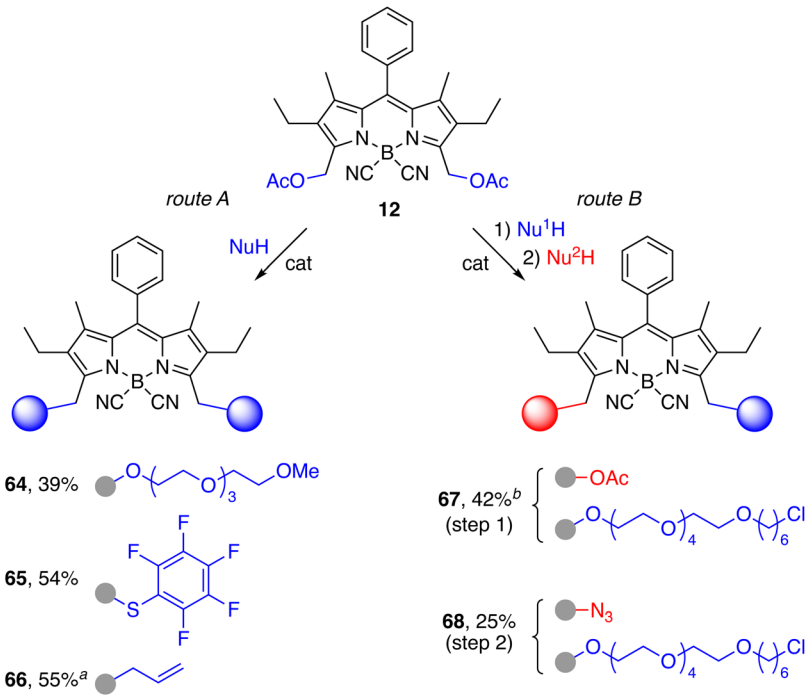
**2.2.2. Synthesis of bifunctional BODIPYs.** Our methodology can also be applied to the preparation of homo- and hetero-bifunctional BODIPYs, starting from the corresponding 3,5-bis(acetoxymethyl)-CN-BODIPY **12** (Scheme 8). Unlike monoacetoxy-BODIPYs, the efficient activation of the diacetoxylated derivative required mild thermal activation (50–60 °C). Under these conditions, the reaction with an excess of *O*-, *S*- or *C*-nucleophiles (>2 equiv.), afforded the expected homodisubstituted-BODIPYs **64–66**, respectively, in moderate yields. Compound **64** contains two tetraethylene glycol monomethyl ether chains to improve its solubility in water, while compounds **65** and **66** are interesting fluorescent cross-linkers for bioconjugation or peptide clamping[97] and for radical or ring-opening metathesis polymerization in the preparation of photonic materials, respectively.

Heterobifunctional BODIPYs can also be obtained in a two-step procedure employing two different nucleophiles, as exemplified by fluorescent probe **68** displaying a chlorohexyl chain as a selective ligand for halotagged fusion proteins,[86–88,98] and an azido group for biorthogonal conjugation to a second functional molecule of interest[99] (Scheme 8). The chlorohexyl substituent was first connected to a tetraethylene glycol chain, following a described method,[100] in order to improve the water solubility of the probe and also to increase the distance between the chromophore and the protein to boost the efficiency of the bioconjugation reaction. A two-fold excess of diacetoxy-BODIPY **12** was used with respect to the alcohol in order to optimize the ratio of the monofunctionalization product **67**. As already observed for other polyoxygenated alcohols, the reaction required the use of a larger amount of Sc $(OTf)_3$ and a higher temperature than the standard procedure. Mono-substituted product **67** was obtained in moderate yield (42%; 70% based on total conversion of **12**), along with a small amount of homodisubstituted derivative **69** (6%) and unreacted **12** (40%) (see ESI†). The azide group was introduced next under similar conditions using $TMSN_3$ as the nucleophile to afford the heterobifunctional BODIPY **68** in a modest 25% yield.

### 2.3. Photophysical study of some representative compounds

With a view to their possible applications in live-cell microscopy, we carried out a primary study of the photophysical properties of some of the most representative fluorescent probes obtained. The new BODIPYs were studied in three different solvents to determine the influence of the polarity of the medium: AcOEt, as a lipophilic aprotic polar organic solvent; MeOH, as a protic polar organic solvent; and phosphate-buffered saline (PBS), as the typical aqueous medium used in live-cell microscopy experiments.

Table S1† lists the photophysical properties determined in the three solvents for the most representative compounds




**Scheme 8** Synthesis of homo- and hetero-bifunctional probes. Reaction conditions: NuH (5 equiv.), $Tf_2NH$ (15–32 mol%), MeCN, 60 °C, 1.5–7.0 h (route A); $Nu^1H$ (0.5 equiv.), $Sc(OTf)_3$ (14 mol%), $CH_2Cl_2$, 50 °C, 24 h (route B, step 1); $Nu^2H$: $TMSN_3$ (1.5 equiv.), $Sc(OTf)_3$ (10 mol%), 50 °C, 24 h (route B, step 2). [a]$Sc(OTf)_3$ (10 mol%) was used as catalyst. [b]Homo-disubstituted product with two chloroalkyl chains (**69**, 6% yield; see ESI† for details) and unreacted **12** (40%) were also isolated.

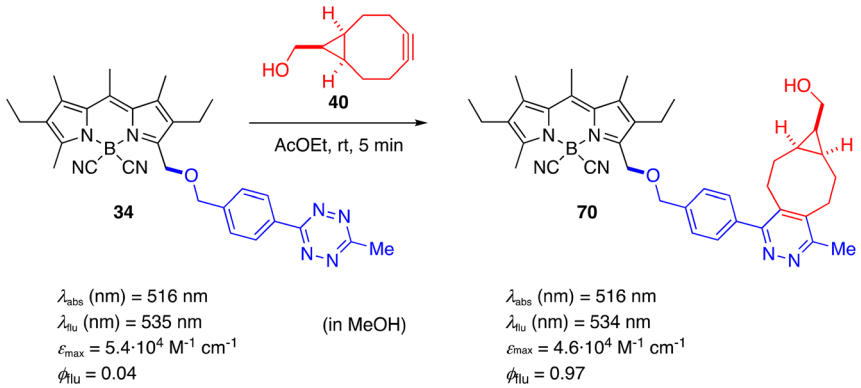
derived from *O*-nucleophiles (**15–39**), including the starting acetoxymethyl-derivative **9** (Fig. S1†). Compound **9** shows a similar photophysical behavior as previously reported for its parent CN-BODIPY **5**, without the acetoxy group.[79] In aqueous medium (PBS), a considerable drop in the molar absorption coefficient and fluorescent emission accompanied by band broadening was also observed together with the appearance of a shorter lifetime (3 ns) than that typical of BODIPYs (6–7 ns). These observations can be explained by the formation of dye aggregates in this very polar medium. The same tendency was observed for other compounds in this series, with some exceptions that will be discussed below (Fig. S2†). In general, quantum yields were maintained or only slightly decreased when going from AcOEt to MeOH, but significantly drop in PBS for the compounds with the most lipophilic substituents, as observed for **9**. In contrast, compounds **18**, **28**, **29**, and **32**, with more hydrophilic substituents that improve water solubility, maintained a high fluorescense in the three solvents studied. Compound **34**, with a 6-phenyl-1,2,4,5-tetrazine ring, showed very poor or almost no fluorescent emission, likely assigned to a FRET process (the absorption of tetrazine overlap with the emission of BODIPY),[49,101–104] but possibly also to a PET process (from BODIPY to tretazine, which is a strong electron withdrawer).[105] Like other previously described tetrazine-BODIPYs, compound **34** is an excellent reagent in inverse electron demand Diels–Alder cycloadditions (IEDDA)[86,106] with a wide variety of dienophiles, particularly strained alkenes and alkynes. The cycloaddition reaction, which occurs with loss of $N_2$, deactivates the energy transfer process and restores the fluorescent emission. Thus, addition of strained cyclooctyne **40** to a solution of **34** at room temperature very rapidly (<2 min) yielded the cycloaddition product **70** with a 24-fold increase in fluorescence emission (Scheme 9). Compound **34** is an interesting fluorogenic probe for bioorthogonal conjugation in no-wash live-cell microscopy.

In general, the *S*-derivatives have a higher solubility in aqueous medium and better fluorescent emission than similar *O*-derivatives (Table S2 and Fig. S3†). In this group, compounds with a polyfluorinated substituent (**42** and **44**) showed higher molar absorption coefficient and higher fluorescent emission in AcOEt than similar non-fluorinated analogs (**41** and **43**, respectively). The more hydrophilic *N*-acetyl-L-cysteine derivative **45** maintained a moderately high fluorescent efficiency in PBS medium.

The derivatives of the *N*-nucleophiles (**46–49**), showed high fluorescence quantum yields ($\phi \geq 0.84$) in both AcOEt and MeOH, with the exception of the 2-nitroimidazolyl-BODIPY **49** (Table S2†). However, these compounds were not fluorescent in PBS, unlike their *O*- and *S*-derivative analogs, possibly due to their lower solubility in water (Fig. S3†). Although it is known that nitro-derivatives are usually non-fluorescent, mainly by activation of an intersystem crossing process,[107,108] compound **49** showed moderate fluorescence efficiency in AcOEt and MeOH.

The presence of chlorine (**50**) or a phosphonate group (**51**) (Table S2 and Fig. S3†) barely affected the absorption and




**Scheme 9** Inverse electron demand Diels–Alder cycloaddition reaction of tetrazine **34** with cyclooctyne **40**.

emission bands, but significantly improved fluorescent emission in aqueous medium (PBS), probably due to the lower lipophilic nature of these derivatives.

The photophysical properties of the *C*-derivatives were highly dependent on the nature of the newly introduced carbon moiety (Table S3†). Thus, compounds with acyclic alkyl systems (**52–55**) and the phenol derivative **56** have very similar properties to their parent **9**, while compounds with electron-rich aromatic systems, including guaiazulene (**59**) (Fig. S4†), pyrrole (**60**), and indole (**61**) derivatives, showed very low fluorescence in the three solvents studied. These results could be interpreted in terms of a photoinduced electron transfer (PET) process taking place between the newly introduced electron-rich ring and the BODIPY chromophore, which quenches fluorescence. The contribution of this PET process was theoretically modeled by DFT calculations in the case of compounds **59–61**, where the guaiazulene, pyrrole and indole rings act as electron donors (reductive PET). The calculations indicate that the lowest energy electronic transition occurs from the HOMO−1 to the LUMO, both located mainly on the BODIPY chromophoric system (Fig. 2 and Fig. S5†). The thermodynamically feasible electron transfer from HOMO (mainly located on the guaiazulene and indole system) to the semi-vacant HOMO−1 after excitation prevents the excited electron from returning to its own ground state, thus causing a non-radiative decay that is responsible for the dramatic quenching of fluorescence observed for these compounds in all solvents studied (Table S3†). A similar effect on fluorescence has been described in other BODIPY derivatives with azulene[109] or indole[110] substituents. The multichromophoric systems **62** and **63** showed an intense absorption capacity (high molar absorption coefficient) due to the presence of two and three BODIPY units, respectively, and kept an efficient emission in AcOEt. These photophysical features are impaired in MeOH and PBS due to the inherent organophilic nature of these compounds (Table S3†).

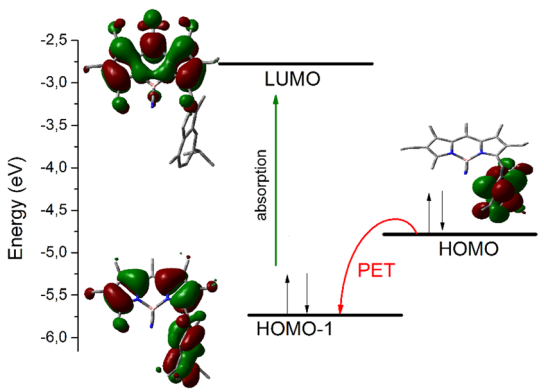



**Fig. 2** Deactivation of the fluorescence of compound **59** by photo-induced electron transfer (PET), modeled by DFT calculations (B3LYP/6-311+G*).

Table S4† lists the photophysical properties of the di-substituted derivatives **64** and **66**, together with their parent diacetoxy-compound **12** (Fig. S6†). As with the monosubstituted analogs, the nature of the new substituents had a negligible effect on the absorption and emission frequencies, but their lipophilic/hydrophilic character had a substantial impact on the molar absorption coefficient and fluorescent emission. Thus, a considerable drop in the magnitude of both properties was observed in PBS for the more lipophilic and less water-soluble **12** and, in particular, for **66**, attributed to dye aggregation. In fact, their fluorescent decay curves became biexponential in this solvent with the appearance of a very short lifetime (<0.1 ns), than that typical for BODIPYs. In contrast, the introduction of the two hydrophilic tetraethylene glycol substituents in compound **64** endowed it with a better water solubility that aided to maintain a moderately good fluorescence quantum yield in PBS leading to a monoexponential decay curve.

### 2.4. Live-cell microscopy studies

We have carried out a preliminary screening of the new compounds as fluorescent probes in live-cell microscopy, using two different cancer cell lines: HeLa and SCC38 (from human cervix and larynx squamous cell carcinoma, respectively). The compounds were incubated with live cells at three different concentrations (50 nM, 100 nM and 500 nM) for 30 minutes, before washing with PBS to remove excess dye. The most suit-

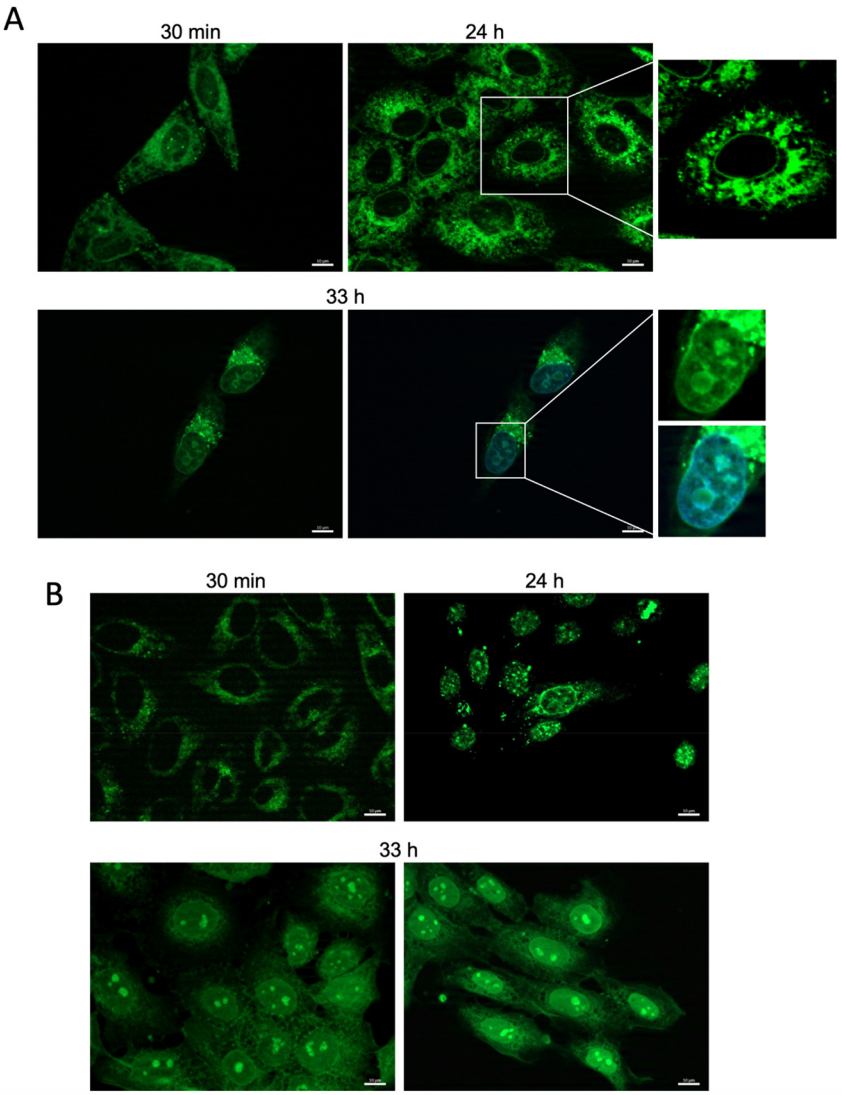



**Fig. 3** Representative images of HeLa (A) and SCC38 (B) cells stained with compound **28** (500 nM) for 30 minutes, 24 h or 33 h, as indicated. In panel A (33 h), cells were finally fixed and stained with DAPI (4′,6-diamidino-2-fenilindol), a fluorescent dye that binds to adenine and thymine enriched regions in the DNA. Insets in A show magnified images to better visualize the staining of the nuclear envelope (picture above) and the nucleoli (pictures below) in HeLa cells. Images clearly show time-dependent migration of the fluorescence from the cytoplasm (30 min) to the nuclear envelope (24 h) and the nucleoli (33 h) in the two cell lines. Scale bar: 10 μm.

able concentrations for visualization were 50 nM and 100 nM. In general, this screening showed that most compounds were able to cross the cell membrane and gave a specific subcellular staining without showing any cytotoxicity, with the exception of the following (the new substituent is indicated in parentheses): **9** (acetoxymethyl), **17** (hexadecanol), **18** (octaethylene glycol), **37** (methacrylic ester), **41** (octadecanethiol), **42** (heptadecafluorodecanethiol), **44** (pentafluorophenyl ring), **47** (naphthalenesulfonamide), and **55** (acetylacetone), which were found to be cytotoxic at the concentrations tested. Some compounds showed only weak cell staining due to their low water solubility and/or low permeability through the cell membrane: **36** (oleic ester), **38** (lipoic ester), **46** (azide), **51** (diethyl phosphonate), and **54** (cyano). As mentioned, some of the new probes showed specific subcellular staining in this preliminary screening (Fig. S7 and S8†). Fig. 3 shows live-cell microscopy images of compound **28** (thymidine derivative), as an illustrative example of those compounds with strong and specific subcellular staining. An interesting time-dependent staining involving the nuclear membrane and nucleoli was observed in this case, while maintaining a good cell viability over a 33 h-incubation time. This dynamic phenomenon is under investigation in our group.

## 3. Conclusions

We have developed a general and robust methodology for the direct incorporation of a wide variety of *C*-, *N*-, *P*-, *O*-, *S*-, and

halo-nucleophiles into functional BODIPY conjugates in a single reaction step. The new method employs stable 3-acetoxymethyl- or 3,5-diacetoxymethyl-4,4-dicyano-BODIPYs as reagents, which are readily available in two high yielding steps from the corresponding 3,5-dimethyl-4,4-difluoro-derivatives using previously described procedures. The nucleophilic substitution reaction takes place under mild acid-catalyzed conditions with remarkably broad substrate scope and functional group tolerance. Alcohols of all kinds, carboxylic acids, hydroxyacids, alkylic and aromatic thiols, primary and secondary sulfonamides, 1H-N-heterocycles, azide, chloride, and trialkylphosphites can all be easily attached to the BODIPY reagent in good yields using the same procedure. Unlike previously described approaches,[59] our strategy also allows the incorporation of a broad diversity of *C*-nucleophiles, including electron-rich aromatic and heteroaromatic compounds, trimethylsilyl carbon nucleophiles, active methylene compounds, and organometallic reagents. Our approach provided a direct access to mono- and di-functional probes incorporating biomolecules (lipids, carbohydrates, nucleosides, amino acids, L-carnitine), enzyme cofactors (lipoic acid), and natural products (farnesol, guaiazulene), as well as chemical functionalities for a wide range of applications including bioorthogonal conjugation, polymerization, and supramolecular chemistry, among others. In addition, this strategy also proved successful in the one-step synthesis of new multichromophoric systems, including BODIPY oligomers and a BODIPY-azulene diad. We believe that this novel and simple approach will significantly expand the synthetic toolbox for the preparation of functional BODIPY-based probes and could be easily implemented by specialists and non-specialists alike to rapidly tailor and deploy advanced probes for innovative applications in bioimaging, sensing and the preparation of new photonic materials.

## Author contributions

J.L.C. conceived and designed the study. V.M.-M., M.-D.C. and J.L.C. supervised the research project. A.B.-M., L.M. and E.M. performed the synthesis and structural characterization of probes. R.P.-M., A.O.-S. and V.M.-M. performed the photophysical studies. L.C., M.-D.C. and A.B.-M. performed the live-cell microscopy studies. V.M.-M. and J.L.C. performed the DFT theoretical calculations. V.M.-M., M.-D.C. and J.L.C. co-wrote the paper. All authors contributed to the final interpretation of the experimental results and critically revised the manuscript. All authors have read and approved the final version of the manuscript.

## Data availability

Experimental procedures, X-ray diffraction data of **9**, DFT computational details, photophysical characterization data, live-cell microscopy images, and copies of $^{1}H$ and $^{13}C$ NMR spectra of compounds (PDF) can be found in the ESI.† The datasets of 1D- and 2D-NMR spectra supporting this article are available from: **https://doi.org/10.20350/digitalCSIC/14707**.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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