An Electrophilic, Activation-free Fluorogenic Reagent for Labeling Bioactive Amines

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ABSTRACT: Herein we report the preparation of BODIPY mesoionic acid fluorides through a short sequence involving an isocyanide multicomponent reaction as the key synthetic step. These novel BODIPY acid fluorides are water-stable electrophilic reagents that can be used for the fluorescent derivatization of amine-containing biomolecules using mild and activation-free reaction conditions. As a proof of principle, we have labelled the antifungal natamycin and generated a novel fluorogenic probe for imaging a variety of human and plant fungal pathogens, with excellent selectivity over bacterial cells.

INTRODUCTION
Electrophilic tags have enabled the functionalization of nucleophilic metabolites and biomolecules for in vitro profiling and/or imaging studies.[1] Some of the first examples of electrophilic labels include dansyl chloride,[2] Sanger[3] and Edman reagents.[4] Since then, the reactivity and application of different electrophilic groups for tagging purposes has been reported. For instance, the group of Cravatt pioneered the use of electrophiles in activity-based protein profiling.[5,6] Likewise, the HaloTag technology has become a broad and powerful tool to fluorescently label proteins.[7] In the context of small molecule labeling, most electrophilic tagging protocols rely on a variety of reactions, including conjugate additions and SoAR, as well as the conventional formation of amides from carboxylic acids and amines with participation of a variety of coupling reagents.[9-20] These conjugation reactions proceed with high yields but render side products (e.g. NHS in the case of succinimidyl esters) and, to some extent, are sensitive to small amounts of water. In order to overcome these limitations, we have developed a new reagent to fluorescently label amine-containing bioactive compounds using a simple, one-step transformation with an electrophilic BODIPY derivative.

Our groups have recently described the synthesis of dipolar acid fluorides through a multicomponent reaction (MCR)[21-23] involving isoquinolines, isocyanides and trifluoroacetic acid anhydride (TFAA).[24] This process provides an efficient path to structures otherwise inaccessible through classical synthesis. Interestingly, dipolar acid fluorides display remarkable resistance to water hydrolysis while maintaining high reactivity against amine nucleophiles. We have employed isoquinoline dipolar acid fluorides (1) for imaging intracellular histamine[25] as well as for tagging oligonucleotides (Scheme 1),[26] but their short excitation and emission wavelength hamper their application for cell imaging studies. The derivatization of the BODIPY structure using MCR chemistry has proven an excellent strategy for the generation of unconventional architectures with unique spectral properties.[27-28] We envisaged that the conjugation of dipolar acid fluorides to the BODIPY fluorescent scaffold, which has excellent photophysical and permeability properties,[29-34] would render useful electrophilic BODIPY dyes for rapid and activation-free labeling of amine-containing bioactive compounds.

RESULTS AND DISCUSSION
We designed the synthesis of BODIPY dipolar acid fluorides (2, Scheme 2) in two consecutive steps. First, the conjugation of 4-isoquinoline boronic acid to BODIPY aryl iodides via Suzuki coupling to render suitable fluorescent isoquinolines (3, Scheme 2). Secondly, BODIPY-isoquinoline conjugates were used in MCRs with TFAA and isocyanides to yield the corresponding BODIPY dipolar acid fluorides (Scheme 2).
Suzuki employed two nucleophilic isocyanides (i.e. cyclohexyl and benzyl) to obtain the corresponding dipolar acid fluorides. Quinolines 3 was only observed when benzyl isocyanide was used. The adducts 1:2:3 and yielding a series of BODIPY dipolar acid fluorides under conventional Suzuki cross-coupling conditions (Scheme 3). The adducts 3a (para, 83%) and 3b (meta, 90%) were obtained in excellent yields, and were subjected to MCR to obtain the corresponding dipolar acid fluorides (2). We employed two nucleophilic isocyanides (i.e. cyclohexyl and benzyl) to explore the effect of different groups in the final acid fluorides. Quinolines 3a and 3b reacted with the different isocyanides and TFAA in DCM, using relative stoichiometry 1:2:3 and yielding a series of BODIPY dipolar acid fluorides (2a-d, Scheme 3) in good yields (for synthetic details and characterization, see Electronic Supporting Information (ESI)). Notably, the formation of Arndtsen-dipoles (5a and 5b, Scheme 3) was only observed when benzyl isocyanide was used.

Since m- and p-iodinated BODIPY dyes have been reported to perform differently in some conjugation reactions, we performed MCRs using the two regioisomers. We isolated both p- and m-BODIPY derivatives (4a and 4b, respectively), and successfully coupled them to 4-isoquinoline boronic acid under conventional Suzuki cross-coupling conditions (Scheme 3). The adducts 3a (para, 83%) and 3b (meta, 90%) were obtained in excellent yields, and were subjected to MCR to obtain the corresponding dipolar acid fluorides (2). We employed two nucleophilic isocyanides (i.e. cyclohexyl and benzyl) to explore the effect of different groups in the final acid fluorides. Quinolines 3a and 3b reacted with the different isocyanides and TFAA in DCM, using relative stoichiometry 1:2:3 and yielding a series of BODIPY dipolar acid fluorides (2a-d, Scheme 3) in good yields (for synthetic details and characterization, see Electronic Supporting Information (ESI)). Notably, the formation of Arndtsen-dipoles (5a and 5b, Scheme 3) was only observed when benzyl isocyanide was used.

Scheme 2. Retrosynthetic analysis for BODIPY dipolar acid fluorides (2).

Scheme 3. Synthesis of a collection of BODIPY dipolar acid fluorides (2a-d).

A major limitation of many fluorescent labeling reagents is their potential hydrolysis in aqueous media, which is particularly relevant when tagging bioactive molecules. Previous mesionic acid fluorides showed excellent resistance to hydrolysis; hence we assessed the chemical integrity of the BODIPY dipolar acid fluorides in aqueous media. HPLC-MS analysis confirmed their high stability in aqueous environments, even under basic conditions (Figure S1 in ESI). This unique feature represents an advantage over other carbonyl-containing labelling reagents (e.g. succinimidyl esters) for reduced fluorescence background and purification steps.

We then evaluated the fluorescence properties of the different BODIPY acid fluorides. All derivatives displayed typical excitation and emission wavelengths of the BODIPY structure (Table 1 and Figure S4 in ESI), with higher extinction coefficients observed for m- over p-derivatives. No significant differences in the wavelength maxima or the fluorescence quantum yields were detected between p- and m-derivatives (i.e., 2a vs. 2b, 2c vs. 2d), only with slightly higher quantum yields in cyclohexyl over benzyl derivatives (i.e., 2a vs. 2c, 2b vs. 2d). These results correlate with our analysis of the structural and energetic arrangements of the molecular orbitals for these compounds (Figure S2 in ESI). In these studies, we observed that both p- and m-cyclohexyl derivatives 2a and 2b showed a preferential parallel disposition of the BODIPY and isoquinoline cores linked through the perpendicular phenyl spacer, with energy diagrams of the frontier orbitals showing minimal differences between regioisomers (Figure S3 in ESI). All BODIPY dipolar fluorides exhibited high fluorescence quantum yields in the green region of the spectrum, making them useful molecules for the fluorescence labeling of amine-containing compounds.

Table 1. Spectral properties of BODIPY dipolar acid fluorides in EtOH.

<table>
<thead>
<tr>
<th>Compound</th>
<th>λabs. (nm)</th>
<th>λem. (nm)</th>
<th>ε (M⁻¹cm⁻¹)</th>
<th>QY*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>502</td>
<td>514</td>
<td>16,100</td>
<td>0.56</td>
</tr>
<tr>
<td>2b</td>
<td>502</td>
<td>514</td>
<td>85,500</td>
<td>0.59</td>
</tr>
<tr>
<td>2c</td>
<td>501</td>
<td>512</td>
<td>11,400</td>
<td>0.45</td>
</tr>
<tr>
<td>2d</td>
<td>502</td>
<td>514</td>
<td>66,100</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* Determined using fluorescein in basic EtOH as standard (QY: 0.92).

In view of the high extinction coefficient and quantum yield of the BODIPY dipolar acid fluoride 2b, we decided to examine its application as a labeling reagent for natamycin, a naturally-occurring antifungal agent with high affinity and selectivity for fungal cells. Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane where it inhibits vacuole fusion at the priming phase and interferes with membrane protein functions. As it has been reported for other antimicrobial drugs, we envisaged that mesionic analogues of natamycin could be used as probes for selective imaging of fungal infection sites. The complex polyene macrolide structure of natamycin includes several functional groups (e.g. epoxides, alcohols, carboxylic acids, amines), which demands highly specific labeling reagents for its derivatization (Figure 1). Notably, the exclusive reactivity of the acid fluoride 2b against amine groups enabled the preparation of the fluorescent analogue of natamycin 6 (Figure 1) by simply mixing the acid fluoride 2b with natamycin in DMF at 40 °C, without any prior activation or additional reagent. The conjugation reaction was completed in several hours, and we isolated the conjugate 6 in high purity (>95%) and synthetic yield (80%). Furthermore, the conjugation reactions between natamycin and other BODIPY acid fluorides proceeded with similarly good results (for details and characterization data, see derivatives 7 and 8 in ESI), which validates our approach as a general methodology for labeling amine-containing biomolecules using activation-free reaction conditions. The efficient derivatization of natamycin with different acid fluorides opens the possibility of extending the...
chemical diversity of this set of labelling reagents by using various isocyanides to tune their selectivity without compromising their performance. To the best of our knowledge, these are the first fluorescent derivatives of natamycin described to date.

![Chemical structure](image)

**Figure 1.** Synthesis of a fluorescent analogue of natamycin under mild and activation-free reaction conditions. Inset) Pictograms of solutions of 6 (50 µM) in 1,4-dioxane (left) and PBS (right) upon irradiation with a 365 nm UV-lamp.

The spectral characterization of compound 6 corroborated that the fluorescent conjugate retained the excitation and emission maxima wavelengths of the BODIPY acid fluoride 2b (Figure S5 in the SI). Because electron-rich BODIPY derivatives have been reported as environmentally-sensitive dyes,[35,40,41] we assessed the fluorescence emission of compound 6 in different environments. Natamycin strongly binds to ergosterol, which is a key component of the membranes of fungal cells, thus we determined the fluorescence quantum yields of compound 6 in mixtures of 1,4-dioxane and PBS (phosphate buffer saline) as an indication of its behaviour in lipid membranes and intracellular environments, respectively.[42,43] Notably, compound 6 displayed around 70-fold higher fluorescence emission in 1,4-dioxane, which mimics the dielectric constant of hydrophobic environments found in lipid bilayers (Figure 1 and Figure S6 in ESI).[44] This feature suggests that BODIPY acid fluorides might be used as reagents for the fluorogenic labeling of biomolecules that preferentially localize in hydrophobic environments, such as cellular membranes.

In order to examine the application of compound 6 as a fluorescent probe for imaging fungal cells, we incubated the natamycin analogue 6 with several filamentous human and plant fungal pathogens - namely *Fusarium solani*, *F. oxysporum* and *Aspergillus flavus* - and acquired fluorescence images of the different species using live cell confocal microscopy. As shown in Figure 2, compound 6 brightly stained the three fungal species since ergosterol, the binding target of natamycin, is present in the plasma membranes of all fungal pathogens, except the highly co-evolved lung pathogen *Pneumocystis* spp.. Furthermore, we analysed the staining of compound 6 in the different species over time (Figure 2d). In this kinetic study, we observed that the binding of compound 6 at fungal cells increased sharply in the first minutes and reached saturation after 20 minutes, which asserts the potential of compound 6 for the rapid identification of fungal cells using fluorescence readouts.

![Fluorescence images](image)

**Figure 2.** Fluorescence images of fungal cells after incubation with the natamycin analogue 6 (5 µM) for 20 min. a) *F. solani*, b) *F. oxysporum*, c) *A. flavus*. Scale bar: 20 µm. d) Time-course analysis of the fluorescence emission of compound 6 in the different strains (*F. oxysporum* (red), *A. flavus* (green), *F. solani* (blue)).

![Fluorescence images](image)

**Figure 3.** Compound 6 brightly stains fungal cells with high selectivity over bacterial cells. Brightfield and fluorescence images of *F. oxysporum* (a, b) and *Pseudomonas aeruginosa* (c, d) after incubation with compound 6 (5 µM) for 20 min. Scale bar: 10 µm.
Finally, we assessed the selectivity of compound 6 as an imaging probe for fungal cells over other microbes (e.g., bacteria) which do not contain ergosterol in their membrane structures. We incubated compound 6 in both fungi (F. oxysporum) and bacteria (Pseudomonas aeruginosa) under the same experimental conditions, and acquired fluorescence images by confocal microscopy. As shown in Figure 3, compound 6 brightly stained fungal cells (F. oxysporum) whereas no staining was observed in bacterial cells. These results confirm that the derivatization of natamycin with the BODIPY acid fluoride 2b did not impair the binding of natamycin for ergosterol in fungal cells, and assert the potential of the BODIPY analogue 6 as a fluorescent agent for selective imaging of fungal infection sites.

CONCLUSIONS

In conclusion, we have developed a new class of mesoionic BODIPY acid fluorides as highly reactive fluorescent labeling reagents for amine-containing biomolecules. These new reagents display excellent fluorescence properties in the green region of the spectrum as well as high stability in aqueous media and basic conditions. As a proof-of-concept, we have modified the complex polyene structure of the antifungal drug natamycin to produce the first fluorescent analogues of natamycin described to date. Notably, the conjugation between the BODIPY acid fluorides and natamycin was performed in very mild conditions, without the need for protective groups or activation reagents, and without producing any side products. The resulting BODIPY-natamycin conjugates have been used for selective imaging of fungal cells using fluorescence microscopy, and showed broad applicability to a variety of fungal species and very high selectivity over bacterial cells. Altogether, these mesoionic BODIPY acid fluorides represent a new methodology for the non-invasive labeling of biomolecules and will open many opportunities in the development of optimal fluorescence imaging probes.

ASSOCIATED CONTENT

Supporting Information
Experimental procedures, spectral and characterization data (NMR, HRMS) and additional biological assays. The Supporting Information is available free of charge on the ACS Publications website.

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGEMENTS

D. C.-L., D. W. D and N. D. R acknowledge the support of the Global Action Fund for Fungal Infection (104684). M.V. acknowledges the support of the Medical Research Council, the Marie Curie Integration Grant (333847), and the Biotechnology and Biological Sciences Research Council (BB/M025160/1). R.L. acknowledges the support of DGICYT-Spain (CTQ2015-67870-P), Generalitat de Catalunya (2014SGR137).

REFERENCES


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Activation-free
Stable to hydrolysis
Selective imaging of fungal cells

R’NC
TFAA

MCR

NH-natamycin

Selective imaging of fungal cells