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Acetylene functionalized BODIPY dyes and their application in the synthesis of activity based proteasome probes

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Abstract—The synthesis of three acetylene functionalized BODIPY dyes is described. These dyes are used to fluorescently modify an azido functionalized epoxomicin analogue employing the Huisgen 1,3-dipolar cycloaddition, resulting in a panel of fluorescent epoxomicin derived proteasome probes.

The ease of detection by direct in-gel readout and visualization in living cells make fluorophores powerful tools in activity based proteomics. Tagging a probe with a fluorescent dye enables rapid assessment of the labeling profile, and in certain examples allows monitoring of intracellular localization of the target proteins. Tagging can be achieved by connecting the dye to the activity based probe directly[1,2] or in a two-step labeling fashion.[3] Boradiazaindacenes (BODIPY dyes)[4] are fluorescent dyes with attractive properties, such as their photochemical stability, high molar absorptivity, high fluorescence quantum yield, and the fact that their fluorescence properties can be altered by varying the substitution pattern on the core and the flanking pyrroles. Symmetrical BODIPY dyes are relatively easy to access by condensation of an acid chloride with two equivalents of any given 1*H*-pyrrole.[5] We envisaged that condensing a variety of pyrroles with 6-heptynoic acid chloride would lead to a panel of optical diverse fluorescent dyes bearing a terminal alkyne. These dyes can be used to fluorescently label an azido functionalized inhibitor in a chemo selective manner employing the copper (I) catalyzed Huisgen 1,3-dipolar cycloaddition ('click' reaction),[6] gaining potential activity based profiling probes.

The natural product epoxomicin is a potent and selective proteasome inhibitor.[7] The selectivity of epoxomicin for the proteasome is governed by the α′,β′-epoxyketone warhead, which, upon reaction with the N-terminal threonine of one of the three catalytically active β-subunits of the proteasome, forms a morpholine ring.[8] Because of the potency and selectivity of epoxomicin, fluorescently labeled analogues thereof could be very useful proteasome probes. Herein we describe the synthesis of three alkyne functionalized BODIPY dyes and the application of these in the synthesis of three fluorescently labeled epoxomicin analogues.

The synthesis of the alkyne functionalized BODIPY dyes commenced with the treatment of 6-heptynoic acid (**1**) with oxalyl chloride (Scheme 1). Crude acid chloride **2** was condensed with either 2-(4-methoxyphenyl)-1*H*-pyrrole (**3a**), 3-ethyl-2,4-dimethyl-1*H*-pyrrole (**3b**) or 2,4-dimethyl-1*H*-pyrrole (**3c**), followed by treatment with DiPEA and BF_3 etherate, giving BODIPY dyes **4a** (pink), **4b** (orange), and **4c** (green). The extinction coefficients ε of the three new dyes were determined to be in the range of that reported for commercial BODIPY-TMR (around 60,000, see Scheme 1).

Next, we synthesized azido functionalized epoxomicin analogue **10** (Scheme 2). Tripeptide **5** bearing an N-terminal azido acetyl cap was prepared by standard Fmoc-based solid phase peptide synthesis. Treatment of **5** with TMS-diazomethane gave methyl ester **6**, which in turn was transformed into hydrazide **7** upon treatment with

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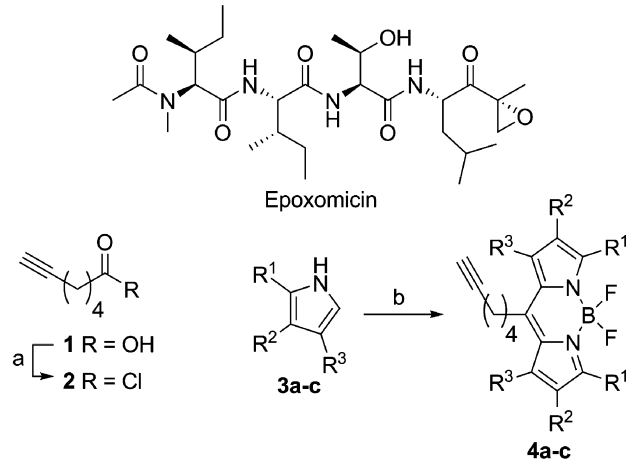
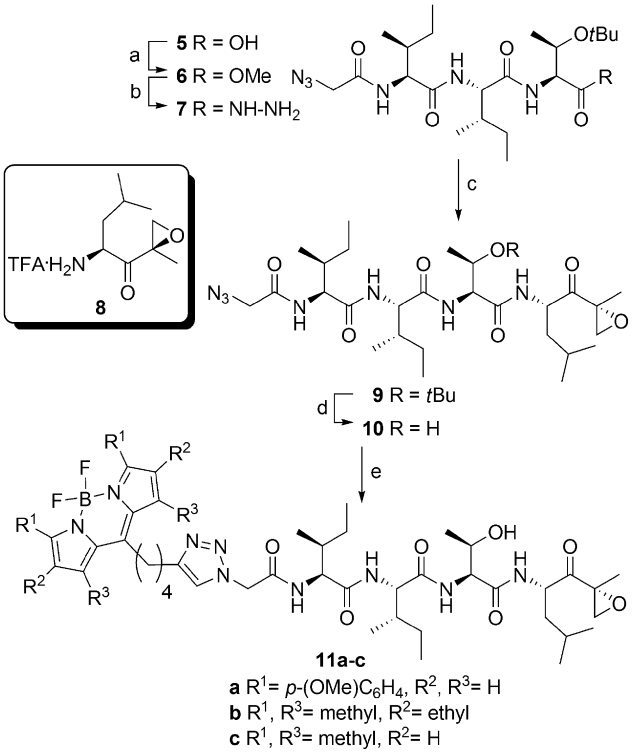


4	R^1	R^2	R^3	λ_{abs} (nm)	λ_{em} (nm)	stokes shift (nm)	ε (l/mol cm)
a	*p*-(OMe)C_6H_4	H	H	573	607	34	51 097
b	methyl	ethyl	methyl	519	529	10	66 807
c	methyl	H	methyl	496	503	7	84 161

Scheme 1. Synthesis and spectroscopical data of acetylene functionalized BODIPY dyes. Reagents and conditions: (a) oxalylchloride (1.5 equiv), DMF (cat.), Tol., 3 h; (b) i—1 M **2** in DCE, **3a–c** (2.1 equiv), 2 h 65 °C; ii—$BF_3{\cdot}OEt_2$ (5 equiv), DiPEA (4 equiv), **4a** 21%, **4b** 14%, **4c** 26%.



Scheme 2. Synthesis of fluorescent epoxomicin analogues **11a–c**. Reagents and conditions: (a) TMS-diazomethane (2 equiv), MeOH/Tol. (1:1), 15 min, 97%; (b) hydrazine monohydrate (60 equiv), MeOH, reflux, 37%; (c) i—*t*-BuONO, HCl, dioxane/DMF; ii—DiPEA, **8**, 11%; (d) TFA, 30 min; (e) **4a–c**, $CuSO_4$ (10 mol %), sodium ascorbate (15 mol %), *t*-BuOH/Tol./H_2O (1:1:1), 80 °C, 12 h, **11a** 91%, **11b** 82%, **11c** 65%, two steps from **9**.

excess hydrazine. In situ generation of the acyl azide using *t*-BuONO under acidic conditions and subsequent neutralization with DiPEA and treatment with leucine epoxyketone **8** gave protected epoxomicin analogue **9** as a single diastereomer (block-coupling of the leucine epoxyketone **8** with peptide **5** using BOP as a condensating agent resulted in a mixture of diastereomers due to epimerization at the threonine α-position). After deprotection, azidomicin **10** was reacted with the alkyne functionalized BODIPY dyes under the influence of $CuSO_4$ and sodium ascorbate resulting in the three fluorescent peptide epoxyketones **11a**, **11b**, and **11c** in good to excellent yields.

Having three fluorescently labeled epoxomicin analogues in hand, we set out to establish their labeling pattern. Lysates of the murine EL4 cell line (expressing both the constitutive proteasome and the immunoproteasome) were incubated with increasing concentrations of **11a–c** at 37 °C. After one hour the proteins were denatured and separated on SDS–PAGE, and the wet gel slabs were scanned on a fluorescence scanner. All three fluorescent epoxomicin analogues revealed bands of labeled proteins, the molecular weight of which correspond to the proteolytically active proteasomal β-subunits (Fig. 1A–C).[2] Although there are no big differences in potency, **11a** seems to be the least potent probe of the three. It should be noted that the efficiency of proteasome labeling is the combined result of binding affinity and the extinction coefficient of the probes, and proteasome probe **11a** contains the BODIPY dye that has the lowest ε value.

The cell permeability of the three probes was scrutinized by exposing living EL4 cells to increasing concentrations of **11a–c** for 2 h. After washing, the cells were lysed, the proteins denatured, and separated on SDS–PAGE. The scans revealed a marked difference in cell permeability between the three probes (Fig. 1D–F). Probe **11b** seems to be better cell permeable than **11a**, bearing the biggest and most hydrophobic BODIPY dye of the three. The smallest and the least hydrophobic epoxomicin analogue (**11c**) appears to be best capable of crossing the cell membrane and labeling the proteasome.

In conclusion, we have synthesized three acetylene functionalized BODIPY dyes **4a** (pink), **4b** (orange), and **4c** (green), and used these in the synthesis of fluorescently labeled epoxomicin analogues **11a–c**. Our work results in the addition of three more fluorescently labeled proteasome probes to the proteasome profiling toolkit.[2,7–10] Apart from this, the alkyne functionalized BODIPY dyes can be used to conjugate to any azide containing activity based profiling probe and azido modified metabolite, potentially leading to valuable fluorescent biochemical tools.[11] Varying the substitution pattern on the core and the flanking pyrroles of the BODIPY not only changes the fluorescence properties of the dye, but also has a dramatic effect on the bioavailability of the fluorophore. Currently, our lab is focussing on the two-step labeling of azido modified target proteins in the proteome using the acetylene functionalized BODIPY dyes.

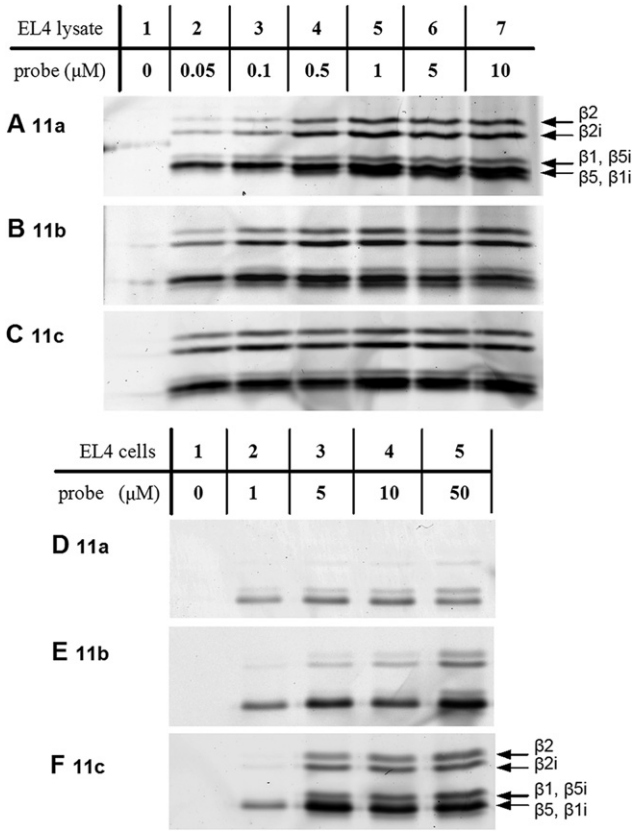


Figure 1. In-gel readout of fluorescently labeled proteasome subunits in EL4 lysates (A–C) and living EL4 cells (D–F). (A–C) EL4 lysates (10 μg total protein) were incubated with the indicated concentrations of **11a** (A), **11b** (B), or **11c** (C) for 1 h at 37 °C and resolved by SDS–PAGE. (D–F) EL4 cells (1×10^6 cells) were exposed to the indicated concentrations of **11a** (D), **11b** (E), or **11c** (F) for 2 h at 37 °C. The cells were harvested and lysed and the proteins were resolved by SDS–PAGE.

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Supplementary data

Experimental procedures and analytical data for all compounds are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.09.025.

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