Chemical tools to probe the proteasome

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

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3.1 Introduction

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] 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 chemoselective manner employing the copper (I) catalyzed Huisgen 1,3-dipolar cycloaddition ('click' reaction),[6] gaining potential activity-based profiling probes.

In this Chapter the synthesis of three alkyne functionalized BODIPY dyes with different optical properties is described. Using these newly developed dyes, a set of fluorescently labeled epoxomicin analogues is prepared. The natural product epoxomicin (Figure 1) 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 the catalytically active β-

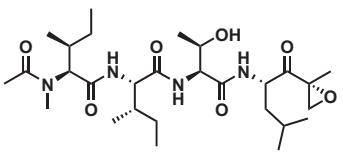


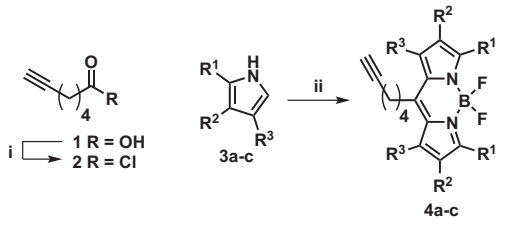
Figure 1. Epoxomicin

subunits of the proteasome, forms a very stable morpholine ring (see Chapter 1.4 for a more detailed description).[8] In Chapter 2 it is shown that the fluorescent proteasome probe MV151 is a powerful tool in proteasome research.[2] Because of the potency and selectivity of epoxomicin, fluorescently labeled analogues thereof could be very useful proteasome probes. Where the synthesis of the fluorophore BODIPY TMR, which is present in MV151, is rather laborious, the preparation of the acetylene functionalized BODIPY dyes described in this chapter is very straightforward.

3.2 Results and discussion

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 $BF_3{\cdot}OEt_2$, 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).

Scheme 1. Synthesis and spectroscopic data of acetylene functionalized BODIPY dyes.

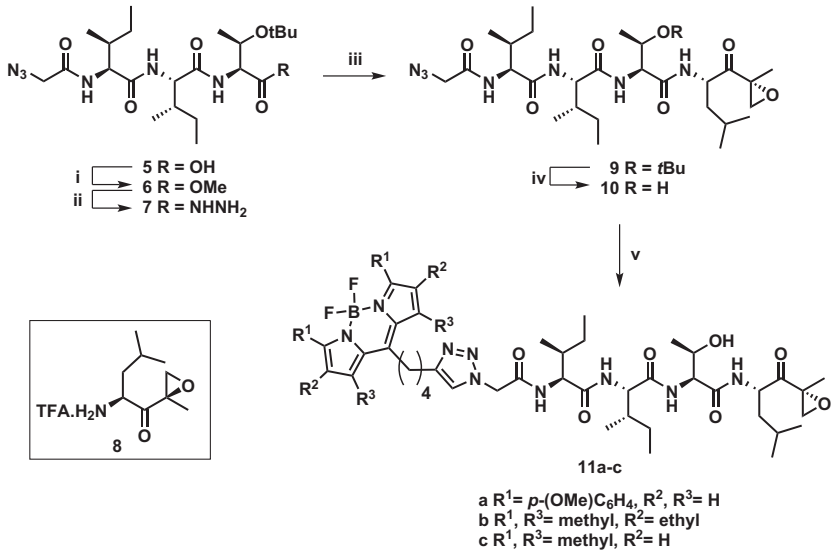


4	R^1	R^2	R^3	λ_{abs} (nm)	λ_{em} (nm)	stokes shift (nm)	ε (l/mol cm)
a	p-(OMe)C_6H_4	H	H	573	607	34	51 097
b	methyl	ethyl	methyl	519	529	10	66 807
c	methyl	H	methyl	496	503	7	84 161

Reagents and conditions: i) oxalyl chloride (1.5 equiv.), DMF (cat.), Tol., 3 hr. ii) (a) 1M **2** in DCE, **3a-c** (2.1 equiv.), 2 hr. 65 °C. (b) $BF_3{\cdot}OEt_2$ (5 equiv.), DiPEA (4 equiv.), **4a** 21%, **4b** 14%, **4c** 26%.

Next, the azido functionalized epoxomicin analogue "azidomicin" (**10**, Scheme 2) was synthesized. Tripeptide **5** capped with an *N*-terminal azidoacetyl moiety 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 excess hydrazine in refluxing methanol. *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

Scheme 2. Synthesis of fluorescent epoxomicin analogues **11a-c**.



Reagents and conditions: i) TMS-diazomethane (2 equiv.), MeOH/Tol. (1/1), 15 min., 97%. ii) hydrazine monohydrate (60 equiv.), MeOH, reflux, 37%. iii) (a) *t*BuONO, HCl, dioxane/DMF. (b) DiPEA, **8**, 11%. iv) TFA, 30 min., quant. v) **4a-c**, $CuSO_4$ (10 mol%), sodium ascorbate (15 mol%), *t*BuOH/Tol./H_2O (1/1/1), 80 °C, 12hr., **11a** 91%, **11b** 82%, **11c** 65% (2 steps from **9**).

diastereomer.[9] Acidic deprotection of the threonine γ-hydroxyl gave azidomicin **10**. Conjugation of azidomicin was executed 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 91%, 82% an 65% yield, respectively.

Being a potential two-step labeling activity-based probe itself, the proteasome inhibitory potential of azidomicin (**10**) was assessed in a competition experiment versus MV151[2] (Figure 2A). Lysates of the murine EL-4 cell line (expressing both the constitutive proteasome and the immunoproteasome) were incubated with increasing concentrations of **10** for 1 hour, before the residual proteasome activities were labeled with MV151. The proteins were denatured, separated on SDS-PAGE and the resulting gel was scanned on a fluorescence scanner. Azidomicin proved to be a very effective proteasome inhibitor, with potency in the same order of magnitude as its parent compound epoxomicin.[2] Having synthesized three fluorescently labeled epoxomicin analogues, their labeling profile was determined in the following experiment. EL-4 cell lysates were incubated with increasing concentrations of **11a-c** at 37 °C. After 1 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 (Figure 2B).[2] Although there are no significant differences in potency, **11a** seems to be the least powerful

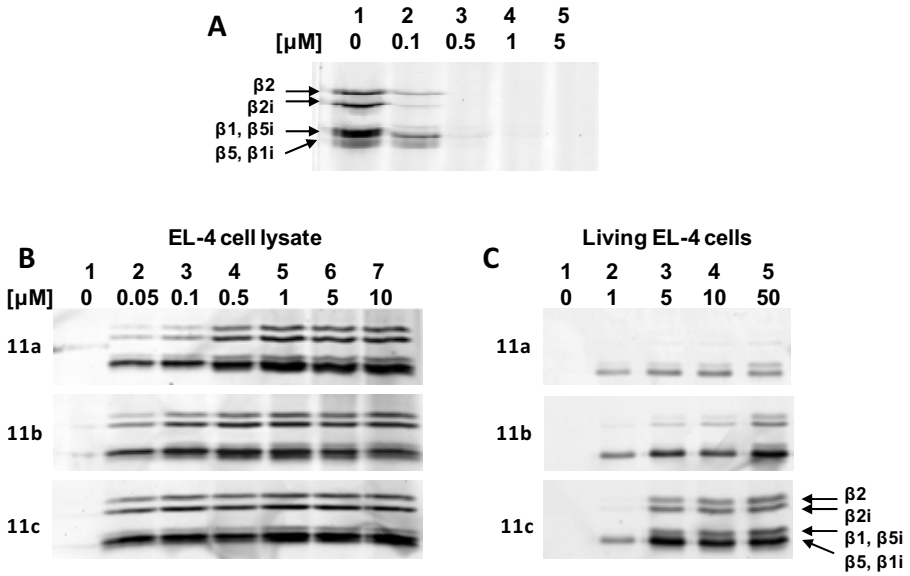


Figure 2. Competition study azidomicin and labeling profile of **11a-c.**
(**A**) EL-4 lysates (10 μg total protein) were incubated with the indicated concentrations of azidomicin (**10**) for 1 hr. at 37 °C. Residual proteasome activity was fluorescently labeled by incubation with 1 μM MV151 for 1 hr. at 37 °C. (**B** and **C**) In-gel readout of fluorescently labeled proteins by **11a-c** in (**B**) EL4 lysates and (**C**) living EL4 cells. (**B**) EL4 lysates (10μg total protein) were incubated with the indicated concentrations of **11a**, **11b** or **11c** for 1 hr. at 37 °C and resolved by SDS-PAGE. (**C**) Some $1{\cdot}10^6$ EL-4 cells were exposed to the indicated concentrations of **11a, 11b** or **11c** for 2 hr. at 37 °C. The cells were harvested, washed and lysed and the proteins were resolved by SDS-PAGE.

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 two hours. 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 (Figure 2C). Probe **11b** seems to be better cell permeable than **11a,** which is carrying the biggest and most hydrophobic BODIPY dye of the three. This characteristic leads to a greater affinity of the probe for the cell membrane, resulting in a slower distribution throughout the cytosol and the nucleus. The smallest and least hydrophobic epoxomicin analogue **11c** appears to be the most efficient in crossing the cell membrane and labeling the active proteasome subunits in living cells.

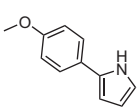
3.3 Conclusion

In conclusion, three acetylene functionalized BODIPY dyes **4a** (pink), **4b** (orange) and **4c** (green) have been synthesized and used in the synthesis of three fluorescently labeled epoxomicin analogues **11a-c**. The synthetic intermediate azidomicin (**10**) proved to be a very potent proteasome inhibitor, making this compound a potential activity-based

two-step labeling proteasome probe. This work results in the addition of three more fluorescently labeled proteasome probes to the proteasome profiling toolkit.[2,7,8,10,11] Apart from this, the alkyne functionalized BODIPY dyes can be conjugated any azide containing activity-based profiling probe and azido modified metabolite, potentially leading to valuable fluorescent biochemical tools.[12] 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.

Experimental section

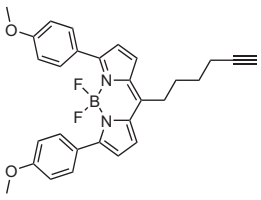
General: All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol.)(purum), ethyl acetate (EtOAc) (puriss.), diethyl ether (Et_2O) and light petroleum ether (PetEt) (puriss.) were obtained from Riedel-de Haën and distilled prior to use. Dichloroethane (DCE), dichloromethane (DCM), dimethyl formamide (DMF) and dioxane (Biosolve) were stored on 4Å molecular sieves. Methanol (MeOH) and *N*-methylpyrrolidone (NMP) were obtained from Biosolve. Tetrahydrofuran (THF) (Biosolve) was distilled from $LiAlH_4$ prior to use. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm), spraying with 20% H_2SO_4 in ethanol followed by charring at ~150 °C, by spraying with a solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 °C or spraying with an aqueous solution of $KMnO_4$ (20%) and K_2CO_3 (10%). Column chromatography was performed on Screening Devices silica gel (0.040 – 0.063 nm). LC/MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.6 mm column (Phenomenex, Torrance, CA, USA) (detection at 200-600 nm), coupled to a Thermo Finnigan LCQ Advantage Max mass spectrometer (Breda, The Netherlands) with electrospray ionization (ESI; system 1). The applied buffers were A: H_2O, B: MeCN and C: 1.0 % aq. TFA. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctylpthalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Jeol JNM-FX-200 (200/50), Bruker DPX-300 (300/75 MHz), Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory or a Bruker DMX-600 (600/150 MHz) with a cryoprobe. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. UV spectra were recorded on a Perkin Elmer, Lambda 800 UV/VIS spectrometer. Azidoacetic acid[13] and *tert*-butyl (S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylcarbamate[7b] were synthesised as described in literature.



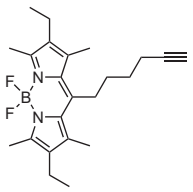
2-(4-methoxyphenyl)-1*H*-pyrrole (3a).[14] Pyrrole (3.50 ml, 50 mmol, 1.6 equiv.) was slowly added under an argon atmosphere to a cooled (0 °C) suspension of sodium hydride (2.0 g of a 60% dispersion in mineral oil, 50 mmol, 1.6 equiv.) in anhydrous THF (20 ml) and stirring was continued for 30 min. at room temperature. After cooling to 0 °C, a solution of $ZnCl_2$ (6.81 g, 50 mmol, 1.6 equiv.) in anhydrous THF (100 ml, 0.5M) was added *via canula*. After stirring for 10 min. at room temperature, di-*tert*-butyl-*o*-biphenylphosphine (75 mg, 0.25 mmol, 0.5 mol %) and $Pd(OAc)_2$ (56 mg, 0.25 mmol, 0.5 mol%) were added in one portion and the resulting mixture was degassed with Argon for 30 min. *p*-Bromoanisol

(3.50 ml, 31.25 mmol, 1 equiv.) was subsequently added dropwise to the reaction mixture and the resulting brown solution was stirred overnight at 65 °C. After cooling to room temperature, water (200 ml) and Et_2O (200 ml) were added and stirring was continued for 15 min., followed by filtration over celite. The filter cake was repeatedly washed with Et_2O (5 x 50 ml) and the filtrate was transferred to a separatory funnel. The organic layer was separated and the aqueous layer was extracted with Et_2O (2 x 50 ml). The combined organics was washed with brine (100 ml), dried over $MgSO_4$and concentrated under reduced pressure to afforded a yellow-brown solid which was purified by column chromatography (50 % PetEt in Tol → Tol) giving the title compound as a white solid (2.82 g, 16.3 mmol, 52%), which rapidly turned bluish upon exposure to air. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 8.57 (s, 1 H), 7.41 (d, *J* = 8.4 Hz, 2 H), 6.91 (d, *J* = 8.8 Hz, 2 H), 1.54 (s, 3 H); ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 156.76, 131.81, 125.96, 124.84, 118.16, 113.92, 108.95, 104.06, 54.94.

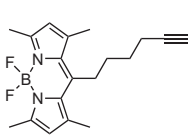
General procedure for the synthesis of BODIPY-alkyne (4a-c). Oxalylchloride (1.5 equiv.) was added dropwise to a solution of hept-6-ynoic acid (**1**, 1 equiv.) in anhydrous Tol. (10 ml). A catalytic amount of DMF (2 drops) was added and the solution was stirred at room temperature for 3 hr. After concentration *in vacuo*, the residue was co-evaporated with Tol. (2x) and the resulting crude hept-6-ynoyl chloride (**2**, yellow oil) was used without further purification. A 1M solution of hept-6-ynoyl chloride (**2**) in DCE was made and 1*H*-pyrrole (**3a-c**, 2.1 equiv.) was added. The resulting reaction mixture was stirred at 65 °C for 2 hr. After leaving to cool to room temperature, BF_3.OEt_2 (5 equiv.) was added over 5 min., followed by the dropwise addition of DiPEA (4 equiv.). Argon gas was then bubbled through the solution, and the reaction mixture was stirred for 12 hr. at ambient temperature, before being washed with H_2O and extracted with EtOAc. The organic layer was dried over Na_2SO_4 and the filtrate was immobilized on celite. Purification by flash chromatography afforded the crude product, which was then crystallized from Tol./heptane.



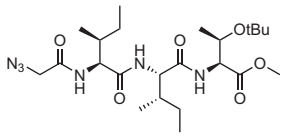
4,4-difluoro-8-(hept-6-yne)-3,5-di(2-(4-methoxyphenyl))-4-bora-3a,4a-diaza-s-indacene (4a). Following the general procedure, hept-6-ynoic acid (**1**, 0.69 ml, 5.48 mmol) and 2-(4-methoxyphenyl)-1*H*-pyrrole (**3a**, 2g, 4.8 mmol) were used. Purification by flash chromatography (PetEt → 10% EA in PetEt), followed by recrystallization afforded the title compound as golden cubes (0.48 g, 1.0 mmol, 21 %). ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 7.83 (d, *J* = 9.0 Hz, 4H), 7.23 (d, *J* = 4.3 Hz, 2H), 6.92 (d, *J* = 9.0 Hz, 4H), 6.58 (d, *J* = 4.4 Hz, 2H), 3.80 (s, 6H), 2.93-2.84 (m, 2H), 2.25 (dt, J_1 = 7.0, J_2 = 2.6 Hz, 2H), 1.99 (t, *J* = 2.6 Hz, 1H), 1.96-1.88 (m, 2H), 1.70-1.63 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ ppm 160.48, 157.49, 144.53, 136.03, 130.91, 126.66, 125.12, 119.90, 113.64, 83.60, 69.01, 55.17, 32.39, 29.99, 28.27, 17.95. λ_{abs} 573 nm, λ_{em} 607 nm. HRMS: calcd. for $[C_{29}H_{27}BF_2N_2O_2H]^+$ 485.22064, found 485.22038, calcd. for $[C_{29}H_{27}BFN_2O_2]^+$ 465.21441, found 465.21403.



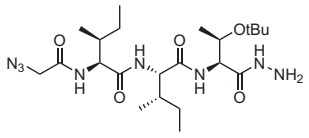
4,4-difluoro-8-(hept-6-yne)-1,3,5,7-tetramethyl-2,6-diethyl-4-bora-3a,4a-diaza-s-indacene (4b). Employing the general procedure, hept-6-ynoic acid (**1**, 2 ml, 15.85 mmol) and 2,4-dimethyl-3-ethyl-1*H*-pyrrole (**3b**, 4.5 ml, 33.29 mmol) were used. Purification by flash chromatography (PetEt → 1% acetone in PetEt) yielded the title compound (1.2 g, 3.1 mmol, 20% yield). Crystallisation afforded **4b** as green metallic cubes (0.8 g, 2.1 mmol, 14%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 2.94-2.85 (m, 2H), 2.48 (s, 6H), 2.37 (q, *J* = 7.5 Hz, 4H), 2.28 (s, 6H), 2.25-2.20 (m, 2H), 1.94 (t, *J* = 2.5 Hz, 1H), 1.78-1.62 (m, 4H), 1.03 (t, *J* = 7.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 151.83, 144.16, 135.55, 132.33, 130.73, 83.61, 68.78, 30.28, 28.66, 27.65, 17.94, 16.97, 14.64, 13.01, 12.17. λ_{abs} 519 nm, λ_{em} 529 nm. HRMS: calcd. for $[C_{23}H_{31}BF_2N_2H]^+$ 385.26211, found 385.26212, calcd. for $[C_{23}H_{31}BFN_2]^+$ 365.25588, found 365.25581.



4,4-difluoro-8-(hept-6-yne)-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (4c). Following the general procedures hept-6-ynoic acid (**1**, 1.24 ml, 9.83 mmol) and 2,4-dimethyl-1*H*-pyrrole (**3c**, 2.13 ml, 20.64 mmol) were used. Flash chromatography (PetEt → 10% acetone in PetEt) afforded the crude product, which was crystallised to yield the title compound **4c** as dark red-green crystals (811 mg, 2.5 mmol, 26%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 6.03 (s, 2H), 2.91-2.85 (m, 2H), 2.50 (s, 6H), 2.37 (s, 6H), 2.24 (dt, J_1 = 6.3, J_2 = 2.7 Hz, 2H), 1.95 (t, J = 2.6 Hz, 1H), 1.78-1.63 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 153.70, 145.86, 140.30, 131.30, 121.52, 83.60, 68.94, 30.45, 28.79, 27.71, 18.05, 16.15, 14.31. λ_{abs} 496 nm, λ_{em} 503 nm. HRMS: calcd. for $[C_{19}H_{23}BF_2N_2H]^+$ 329.19951, found 329.19961, calcd. for $[C_{19}H_{23}BFN_2]^+$ 309.19328, found 309.19333.

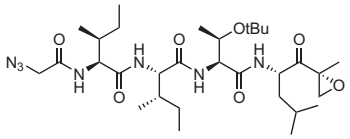


Azidoacetyl-Ile$_2$-Thr(*t*Bu)-OMe (6). MBHA functionalized polystyrene resin (3.57 g, 0.7 mmol/g, 2.5 mmol) was washed with NMP (3x) and DCM (3x) followed by addition of a preactivated mixture of HMPB linker (1.8 g, 7.5 mmol, 3 equiv.), BOP (3.3 g, 7.5 mmol, 3 equiv.) and DiPEA (2.48 ml, 15 mmol, 6 equiv.) in NMP. After 12 hr. of shaking, the resin was washed DCM (3x) and dried. The resin was transferred into a flask, coevaporated with DCE (2x), and condensed with Fmoc-Thr(*t*Bu)-OH (3.0 g, 7.5 mmol, 3 equiv.), under the influence of DIC (1.29 ml, 8.25 mmol, 3.3 equiv.) and DMAP (46 mg, 0.38 mmol, 5 mol%) in DCM for 2 hr. The resin was filtered and washed with DCM (2x), followed by a second condensation cycle. The loading of the resin was determined by spectrophotometric analysis (4.98 g, 0.44 mmol/g, 2.2 mmol, 88%). The obtained resin was submitted to two cycles of Fmoc solid-phase synthesis with Fmoc-Ile-OH as follows: a) deprotection with piperidine/NMP (1/4, v/v, 20 min.); b) washing with NMP (3×); c) coupling of Fmoc-amino acid (1.94 g, 5.5 mmol, 2.5 equiv.) in the presence of BOP (2.4 g, 5.5 mmol, 2.5 equiv.) and DiPEA (1.17 ml, 6.6 mmol, 3 equiv.) in NMP and shake for 2 hr.; d) wash with NMP (3×) and DCM (3×). Couplings were monitored for completion by the Kaiser test. After Fmoc-deprotection of the resin bound tripeptide (0.1 mmol), azidoacetic acid[13] (25 mg, 0.25 mmol, 2.5 equiv.), BOP (0.11 g, 0.25 mmol, 2.5 equiv.) and DiPEA (50 µl, 0.3 mmol, 3 equiv.) in NMP were added, and the resin was shaken for 12 hr. After washing with DCM (3×), the resin was subjected to mild acidic cleavage (TFA/DCM, 1/99, v/v, 10 min., 3x) and the collected fractions were coevaporated with Tol. (2x) to give the crude, partially protected tetrapeptide **5**, which was used without further purification. To a solution of peptide **5** in MeOH/Tol. (1/1, v/v), TMS-diazomethane (0.1 ml 2M in hexanes, 0.2 mmol, 2 equiv.) was added. The reaction mixture was stirred for 15 min., before being concentrated *in vacuo*. The crude title compound was crystallized from DCM/PetEt. Purification by flash chromatography (DCM → 2% MeOH in DCM) afforded Azidoacetyl-Ile$_2$-Thr(*t*Bu)-OMe as a white solid (28.5 mg, 97 µmol, 97%). ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 7.10 (d, J = 8.8 Hz, 1H), 6.92 (d, J = 8.7 Hz, 1H), 6.65 (d, J = 9.1 Hz, 1H), 4.53-4.44 (m, 3H), 4.23 (dq, J_1 = 6.1, J_2 = 1.6 Hz, 1H), 4.01 (q, J = 16.3, 2H), 3.71 (s, 3H), 1.88-1.80 (m, 2H), 1.61-1.46 (m, 2H), 1.28-1.17 (m, 2H), 1.16-1.07 (m, 13H), 0.96 (d, J = 6.8 Hz, 3H), 0.94-0.86 (m, 9H). ^{13}C NMR (125 MHz, $CDCl_3$): δ ppm 171.10, 170.75, 170.46, 166.54, 77.00, 74.14, 67.10, 57.75, 57.47, 57.46, 52.37, 52.09, 37.84, 37.80, 28.25, 24.94, 20.71, 15.21, 14.98, 11.40, 11.26. HRMS: calcd. for $[C_{23}H_{42}N_6O_6H]^+$ 499.32386, found 499.32380, calcd. for $[C_{23}H_{42}N_6O_6Na]^+$ 521.30580, found 521.30539.

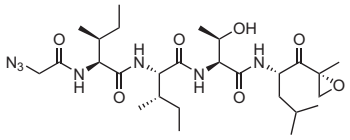


Azidoacetyl-Ile$_2$-Thr(*t*Bu)-hydrazide (7). Azidoacetyl-Ile$_2$-Thr(*t*Bu)-OMe (**6**, 46 mg, 92 µmol) was dissolved in MeOH and hydrazine monohydrate (0.27 ml, 5.5 mmol, 60 equiv.) was added. The reaction mixture was refluxed for 18 hr., before being concentrated in the presence of Tol. Purification by flash chromatography (DCM → 5% MeOH in DCM) afforded the title compound as a white solid (17.1 mg, 34 µmol, 37%). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD = 1/1, v/v): δ ppm 8.07 (d, J = 8.0 Hz, 2H), 7.81 (d, J = 8.7 Hz, 1H), 7.58 (d, J = 7.6 Hz, 1H), 4.40 (dd, J_1 = 7.9, J_2 = 3.3 Hz, 1H), 4.36-4.24 (m, 2H), 4.07 (dq, J_1 = 6.3, J_2 = 3.6 Hz, 1H), 3.96 (s,

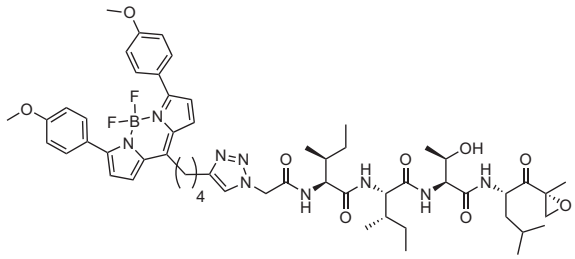
2H), 1.94-1.80 (m, 2H), 1.63-1.47 (m, 2H), 1.35-1.11 (m, 12H), 1.09 (d, *J* = 6.4 Hz, 3H), 0.96-0.88 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD = 1/1, v/v): δ ppm 174.89, 174.45, 172.85, 170.84, 77.70, 69.41, 61.09, 60.38, 60.34, 59.82, 54.56, 39.59, 39.09, 30.52, 27.55, 27.42, 21.10, 17.86, 17.76, 13.35, 13.26.



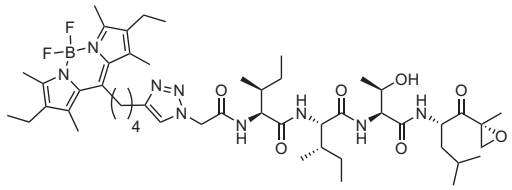
Azidoacetyl-Ile$_2$-Thr(*t*Bu)-leucinyl-2-methyloxiran (9). *Tert*-butyl (S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylcarbamate[7b] (9.5 mg, 35 µmol, 1.1 equiv.) was stirred in TFA, until TLC showed complete Boc deprotection. All volatiles were removed in the presence of Tol. (2×) yielding the crude leucine epoxyketone TFA salt (**8**), which was dissolved in DMF and neutralized with DiPEA (5.8 µl, 35 µmol, 1 equiv.). Azidoacetyl-Ile$_2$-Thr(*t*Bu)-hydrazide (**7**, 16 mg, 32 µmol) was dissolved in DMF/EtOAc (1/1, v/v), put under an argon atmosphere and cooled to -30 °C. HCl (22.5 µl 4M in dioxane, 90 µmol, 2.8 equiv.) and *tert*-butyl nitrite (4.2 µl, 35 µmol, 1.1 equiv.) were added and the reaction mixture was stirred for 3 hr. The leucine epoxyketone (**8**) solution and DiPEA (20.1 µl, 0.12 mmol, 3.8 equiv.) were added at -30 °C and the reaction mixture was stirred for 18 hr., before being concentrated *in vacuo*. Purification by flash chromatography (DCM → 1% MeOH in DCM) afforded a mixture of the title compound and a side product[15] as a white solid (14.7 mg). Column chromatography (*n*-hexane → 15% acetone in *n*-hexane) afforded azidoacetyl-Ile$_2$-Thr(*t*Bu)-leucinyl-2-methyloxiran (**9**) as a white solid (2.2 mg, 3.4 µmol, 11%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.66 (d, *J* = 7.5 Hz, 1H), 6.94-6.85 (m, 2H), 6.35 (d, *J* = 8.5 Hz, 1H), 4.46 (ddd, J_1 = 10.4, J_2 = 7.5, J_3 = 2.9 Hz, 1H), 4.37-4.26 (m, 3H), 4.13 (dq, J_1 = 6.4, J_2 = 4.3 Hz, 1H), 4.00 (q, *J* = 16.4 Hz, 2H), 3.34 (d, *J* = 5.1 Hz, 1H), 2.91 (d, *J* = 5.1 Hz, 1H), 1.94-1.76 (m, 2H), 1.75-1.54 (m, 3H), 1.52 (s, 3H), 1.29 (s, 9H), 1.27-1.24 (m, 2H), 1.19-1.09 (m, 2H), 1.07 (d, *J* = 6.5 Hz, 3H), 1.00-0.85 (m, 18H).



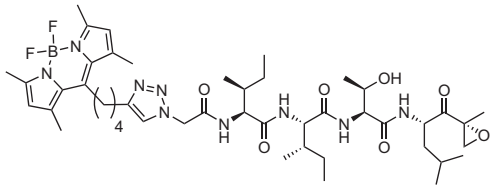
Azidoacetyl-Ile$_2$-Thr(*t*Bu)-leucinyl-2-methyloxiran (10). Azidoacetyl-Ile$_2$-Thr(*t*Bu)-leucinyl-2-methyloxiran (**9**, 10 mg, 15.6 µmol) was dissolved in TFA and stirred until TLC analysis revealed total consumption of **9**. The reaction mixture was coevaporated with Tol. (3×) resulting in azidomicin (**10**) as a white solid (9.1 mg, 15.6 µmol, quant.) and was used without purification.



Fluorescent epoxomicin analogue 11a. BODIPY-alkyne **4a** (7.6 mg, 15.6 µmol) and azidomicin (**10**, 9.1 mg, 15.6 µmol, 1 equiv.) were dissolved in a mixture of *t*BuOH and Tol. (1/1, 124.8 µl), before an aqueous solution of sodium ascorbate (31 µl 0.16 mM, 15 mol%) and an aqueous solution of $CuSO_4$ (31 µl 0.1 mM, 10 mol%) were added. The reaction mixture stirred at 60 °C for 12 hr., before being concentrated *in vacuo*. Purification by size-exclusion chromatography (Sephadex LH-20, eluens: MeOH) gave the title compound as a fine purple powder (19.4 mg, 14.2 µmol, 91%). ^{1}H NMR (400 MHz,$CDCl_3$ + drop MeOD): δ ppm 7.80 (d, *J* = 8.7 Hz, 4H), 7.52 (s, 1H), 7.23 (d, *J* = 3.7 Hz, 2H), 6.90 (d, *J* = 8.8 Hz, 4H), 6.54 (d, *J* = 4.0 Hz, 2H), 5.19-4.43 (m, 6H), 4.10-4.00 (m, 1H), 3.80 (s, 6H), 3.28 (d, *J* = 3.0 Hz, 1H), 2.93-2.83 (m, 3H), 2.68-2.49 (m, 2H), 1.90-1.15 (m, 15H), 1.14-0.99 (m, 4H), 0.94-0.69 (m, 18H). HRMS: calcd. for $[C_{56}H_{74}BF_2N_9O_9H]^+$ 1066.57434, found 1066.57512, calcd. for $[C_{56}H_{74}BF_2N_9O_9Na]^+$ 1088.55628, found 1088.55690.



Fluorescent epoxomicin analogue 11b. BODIPY-alkyne **4c** (6 mg, 15.6 µmol) and azidomicin (**10**, 9.1 mg, 15.6 µmol, 1 equiv.) were dissolved in a mixture of *t*BuOH and Tol. (1/1, 124.8 µl), before an aqueous solution of sodium ascorbate (31 µl 0.16 mM, 15 mol %) and an aqueous solution of $CuSO_4$ (31 µl 0.1 mM, 10 mol %) were added. The reaction mixture stirred at 60 °C for 12 hr., before being concentrated *in vacuo*. Column chromatography (DCM → 5% MeOH in DCM) yielded the title compound as an orange solid (12.4 mg, 12.8 µmol, 82%). ^{1}H NMR (400 MHz,$CDCl_3$ + drop MeOD): δ ppm 7.57 (s, 1H), 5.07 (dd, J_1 = 32.8, J_2 = 16.1 Hz, 2H), 4.54 (dd, J_1 = 10.5, J_2 = 2.8 Hz, 1H), 4.32 (d, J = 4.1 Hz, 1H), 4.25-4.19 (m, 2H), 4.14-4.06 (m, 1H), 3.30 (d, J = 4.9 Hz, 1H), 3.09-3.02 (m, 2H), 2.92 (d, J = 5.0 Hz, 1H), 2.83-2.77 (m, 2H), 7.57 (s, 1H), 2.48 (s, 6H), 2.41 (q, J = 7.7 Hz, 4H), 2.33 (s, 6H), 1.99-1.87 (m, 2H), 1.87-1.77 (m, 2H), 1.76-1.61 (m, 2H), 1.58-1.43 (m, 5H), 1.38-1.24 (m, 2H), 1.19-1.01 (m, 12H), 0.98-0.82 (m, 18H). HRMS: calcd. for $[C_{50}H_{78}BF_2N_9O_7H]^+$ 966.61581, found 966.61685, calcd. for $[C_{50}H_{78}BF_2N_9O_7Na]^+$ 988.59775, found 988.59809.



Fluorescent epoxomicin analogue 11c. BODIPY-alkyne **4b** (5.1 mg, 15.6 µmol) and azidomicin (**10**, 9.1 mg, 15.6 µmol, 1 equiv.) were dissolved in a mixture of *t*BuOH and Tol. (1/1, 124.8 µl), before an aqueous solution of sodium ascorbate (31 µl 0.16 mM, 15 mol %) and an aqueous solution of $CuSO_4$ (31 µl 0.1 mM, 10 mol %) were added. The reaction mixture stirred at 60 °C for 12 hr., before being concentrated *in vacuo*. Column chromatography (DCM → 5% MeOH in DCM) yielded the title compound as a fine orange powder (9.2 mg, 10.1 µmol, 65%). ^{1}H NMR (400 MHz,$CDCl_3$ + drop MeOD): δ ppm 7.57 (s, 1H), 6.08 (s, 2H), 5.08 (q, J = 16.1 Hz, 2H), 4.54 (dd, J_1 = 10.5, J_2 = 3.0 Hz, 1H), 4.31 (d, J = 4.1 Hz, 1H), 4.24-4.07 (m, 3H), 3.30 (d, J = 4.9 Hz, 1H), 3.06-2.98 (m, 2H), 2.91 (d, J = 5.0 Hz, 1H), 2.80 (t, J = 7.6 Hz, 2H), 2.50 (s, 6H), 2.41 (s, 6H), 1.97-1.88 (m, 2H), 1.87-1.78 (m, 2H), 1.77-1.64 (m, 2H), 1.51 (s, 3H), 1.50-1.28 (m, 6H), 1.13 (m, 4H), 0.98-0.83 (m, 18H). HRMS: calcd. for $[C_{46}H_{70}BF_2N_9O_7H]^+$ 910.55321, found 910.55425, calcd. for $[C_{46}H_{70}BF_2N_9O_7Na]^+$ 932.53515, found 932.53529.

***In vitro* proteasome labeling.** EL4 cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml penicillin and 10 µg/ml streptomycin in a 5% CO_2 humidified incubator at 37 °C. Cells were harvested and after flash freezing (N_2 (l)) the cells were lysed in digitonin containing lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl, 1 mM DTT, 0.025% digitonin) for 15 min. on ice and centrifuged at 20.000 g for 20 min. at 4 °C. The supernatant containing the cytosolic fraction was collected and the protein content was determined by Bradford assay. The resulting cell lysates (10µg total protein) were exposed to the probes **11a-c** with the indicated concentrations (10× solution in DMSO) for 1 hr. Reaction mixtures were boiled with Laemmli's buffer containing β-mercapto-ethanol for 3 min., before being resolved on 12.5% SDS-PAGE. Fluorescently labeled proteins were visualized by in-gel fluorescence scanning on a Typhoon variable mode imager (Amersham Biosciences, settings: **11a** λ_{ex} 532 nm, λ_{em} 610 nm; **11b** λ_{ex} 532 nm, λ_{em} 526 nm; **11c** λ_{ex} 488 nm, λ_{em} 520 nm).

Proteasome labeling in living cells. EL4 cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml penicillin and 10 µg/ml streptomycin in a 5% CO_2 humidified incubator at 37 °C. Some $1{\cdot}10^6$ cells were seeded in 6 cm Petri dishes and allowed to grow 12 hr. in 1 ml of medium. The cells were exposed to 0, 1, 5, 10 or 50 µM **11a-c** (100× solution in DMSO) for 2 hr., before being washed with PBS and harvested. After flash freezing (N_2 (l)) the cells were lysed in 60 µl digitonin containing lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl, 1 mM DTT, 0.025% digitonin) for 15 min. on ice and centrifuged at

20.000 g for 20 min. at 4 °C. The supernatant containing the cytosolic fraction was collected and the protein content was determined by Bradford assay. Some 20 µg protein was resolved by 12.5% SDS-PAGE. Fluorescently labelled proteins were visualised by in-gel fluorescence scanning on a Typhoon variable mode imager (Amersham Biosciences, settings: **11a** λ_{ex} 532 nm, λ_{em} 610 nm, **11b** λ_{ex} 532 nm, λ_{em} 526 nm, **11c** λ_{ex} 488 nm, λ_{em} 520 nm).

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15. LC/MS analysis of the reaction mixture before concentration *in vacuo* revealed a single product peak corresponding to **9** (R_f 10.17 min (linear gradient 10 → 90% B in 15 min), *m/z* 638.33 $[M+H]^+$). Purification by column chromatography (DCM → 1% MeOH in DCM) afforded a mixture of two compounds. LC/MS analysis (product 1: R_f 9.79 min (linear gradient 10 → 90% B in 15 min), *m/z* 674.40 $[M+H]^+$, showing the typical chlorine isotope pattern. **9**: R_f 10.17 min (linear gradient 10 → 90% B in 15 min), *m/z* 638.33 $[M+H]^+$) and ^1H NMR revealed partial opening of the epoxide by a chloride ion (probably originating from the DiPEA·HCl salt present in the reaction mixture), dramatically decreasing the yield.