Chemical tools to probe the proteasome

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7 Diels-Alder two-step activity-based proteasome labeling

7.1 Introduction

As discussed in Chapter 1.5, two general strategies for two-step activity-based protein profiling have been described. These are the so called "click" ligation, based on the Huisgen [2+3]-cycloaddition[1] and the Staudinger-Bertozzi ligation.[2] Both these strategies rely on azido functionalized probes or reporter groups. To gain flexibility and the opportunity to examine multiple proteins in a single experiment, activity-based protein profiling research would greatly benefit from the availability of a larger panel of bioorthogonal two-step labeling techniques.[3] An optimal two-step ligation reaction should be chemoselective, bioorthogonal, efficient under mild reaction conditions and proceed in aqueous media. One of the most classical organic chemical reactions, the Diels-Alder [4+2] cycloaddition,[4] fits most of the aforementioned qualifications. This reaction of a conjugated diene and an electronically matched dienophile proceeds via a concerted mechanism and is highly chemoselective. The physiological conditions of a two-step labeling approach seem to fit the Diels-Alder reaction, since the reaction is accelerated in aqueous media by the hydrophobic effect.[5] The Diels-Alder reaction has successfully been applied in the bioconjugation of carbohydrates to proteins[6] and the tagging[7] and surface immobilization of oligonucleotides.[8] Waldmann and co-workers recently developed a Diels-Alder based site-specific chemoselective labeling, ligation and surface immobilization strategy for peptides and proteins.[9]

This Chapter describes the development of chemical tools to perform a Diels-Alder based two-step labeling strategy as depicted in Figure 1. The synthesis of four diene

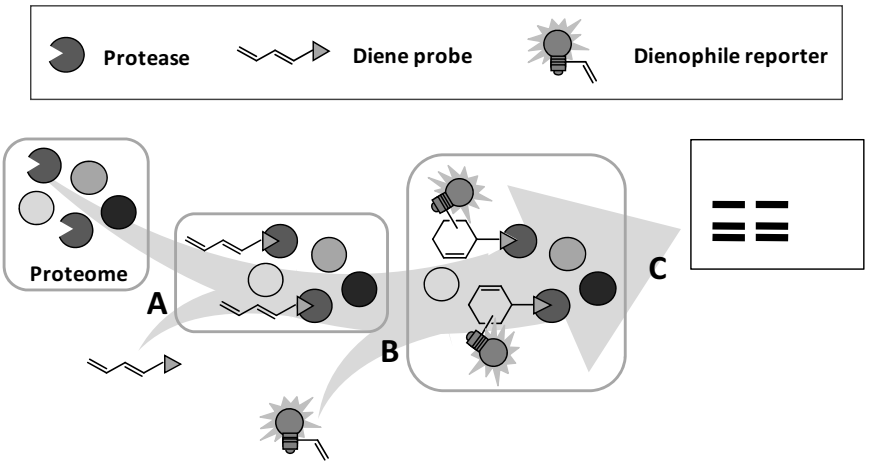


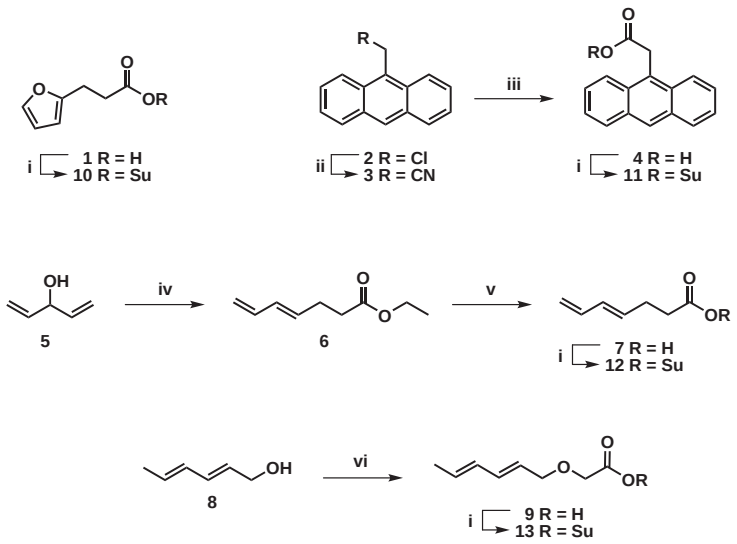
Figure 1. Schematic representation of a Diels-Alder based two-step protein profiling strategy.
(**A**) A protease is targeted by a diene functionalized probe. (**B**) Treatment of the diene tagged protease with a reporter group equipped dienophile results in the labeling of the protease. (**C**) SDS-PAGE, followed by detection results in the labeling profile of the diene functionalized probe.

functionalized epoxomicin analogues is described. The influence of the *N*-terminal diene modification on the capacity to inhibit the proteasome is determined in a competition assay versus MV151 (Chapter 2). A two-step labeling strategy was applied on purified 20S proteasomes spiked in RAW cell lysate, in which a BODIPY TMR tagged maleimide derivative was used as the fluorescent dienophile in the Diels-Alder ligation.

7.2 Results and discussion

The cyclic dienes furylpropionic acid **1** and 9-anthracenylacetic acid **4**, and the linear dienes hepta-4,6-dienoic acid **7** and (hexa-2,4-dienyloxy)acetic acid **9** were chosen for the synthesis of a panel of *N*-terminally diene modified epoxomicin analogues. Since some of these dienes are acid labile, it was reasoned that these functionalities are best introduced in the final step of the synthesis, after full deprotection of the epoxomicin synthon **14**. The syntheses commenced with the preparation of the diene functionalized carboxylic acids (Scheme 1). Apart from the commercially available 3-(2-furyl)propionic acid (**1**), the diene functionalized carboxylic acids were synthesized as follows. 2-(Anthracen-9-yl)acetic acid (**4**) was obtained from 9-chloromethyl anthracene (**2**) by reaction with KCN, followed by hydrolysis of the resulting nitrile **3** with NaOH in refluxing 2-methoxyethanol. (*E*)-hepta-4,6-dienoic acid (**7**) was synthesized in two steps from penta-1,4-dien-3-ol (**5**). Treatment with triethyl orthoacetate in refluxing toluene under mildly acidic conditions gave ethyl ester **6** via an orthoester Claisen rearrangement.[10] Hydrolysis of ethyl hepta-4,6-dienoate (**6**) with NaOH in methanol/dioxane mixture gave **7**. The final diene functionalized carboxylic acid, 2-((2*E*,4*E*)-hexa-2,4-dienyloxy)acetic acid (**9**), was obtained from

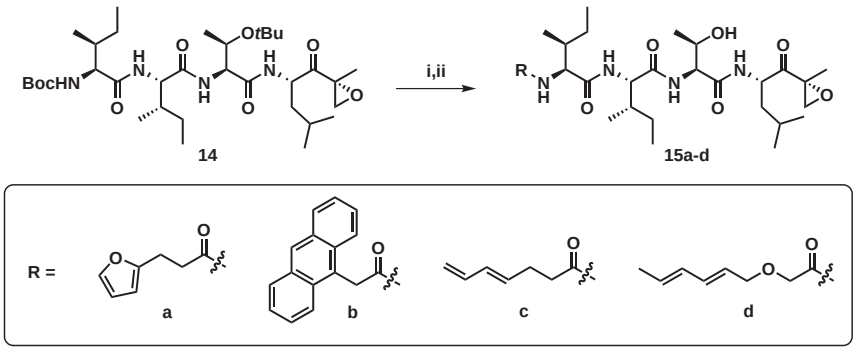
Scheme 1. Synthesis of diene succinimidyl esters.



Reagents and conditions: i) HOSu (4 equiv.), EDC·HCl (4 equiv.), DCE/DMF, rt, 16 hr., **10** 71%, **11** 81%, **12** 78%, **13** 80%. ii) KCN (1.5 equiv.), DMSO/H_2O, 60 °C, 1 hr. then rt, 16 hr., 94%. iii) (a) NaOH (2.5 equiv.), 2-methoxyethanol, reflux, 3 hr. (b) 1M HCl, rt, 16 hr., 56%. iv) triethyl orthoacetate (5 equiv.), propionic acid (cat.), Tol., reflux, 3 hr., 94%. v) 1M NaOH/MeOH/1,4-dioxane (1/1/1, v/v/v), rt, 1 hr., 68%. vi) (a) NaH (2 equiv.), THF, rt, 30 min. (b) 2-bromoacetic acid (1 equiv.), rt, 16 hr., 73%.

(2*E*,4*E*)-hexa-2,4-dien-1-ol (**8**) after deprotonation with NaH and reaction with bromoacetic acid. The four diene functionalized carboxylic acids **1, 4, 7** and **9** were converted into their corresponding succinimidyl ester by condensation with *N*-hydroxysuccinimide under the influence of EDC·HCl. The protected tetrapeptide epoxyketone Boc-Ile-Ile-Thr(*t*Bu)-LeuEK (**14**)[11] was fully deprotected, and condensed with the diene succinimidyl esters **10-13** to afford the four *N*-terminally diene modified epoxomicin analogues **15a-d** (Scheme 2).

Scheme 2. Synthesis of diene functionalized epoxomicin analogues.



Reagents and conditions: i) TFA/DCM (1/1, v/v), rt, 15 - 45 min, ii) R-OSu (**10-13**), DiPEA, DCE/DMF, rt, 1.5 - 18 hr., **15a** 82%, **15b** 39%, **15c** 64%, **15d** quant.

The proteasome inhibitory potential of the diene equipped epoxomicin analogues **15a-d** was assessed in a competition experiment versus the fluorescent broad-spectrum proteasome probe MV151 (Chapter 2).[12] Lysates of the murine EL-4 cell line (expressing both the constitutive proteasome and the immunoproteasome) were incubated with increasing concentrations of **15a-d** for 1 hr., before labeling of the residual proteasome activities with MV151. The proteins were denatured, separated on SDS-PAGE and the resulting gels were scanned on a fluorescence scanner (Figure 2). All four *N*-terminally

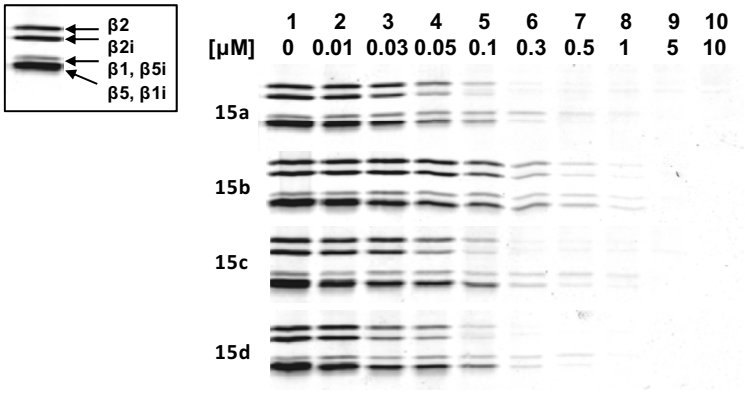
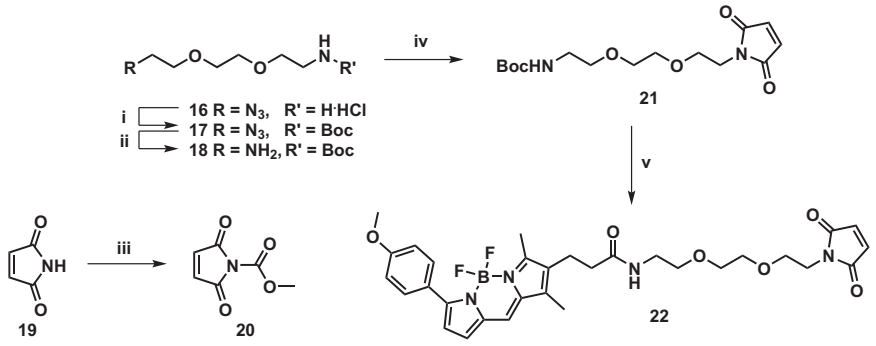


Figure 2. Competition assay of diene functionalized epoxomicin analogues **15a-d** versus MV151.
EL-4 lysates (10 μg total protein) were incubated with the indicated concentrations **15a-d** for 1 hr. at 37 °C. Residual proteasome activity was fluorescently labeled by incubation with 1 μM MV151 for 1 hr. at 37 °C.

diene modified epoxomicin analogues **15a-d** proved to be inhibitors of the proteasome with similar inhibitory profiles and potencies in the same order of magnitude as the parent inhibitor epoxomicin.[13] Although the differences in potency are small, the most bulky derivative, anthracenyl epoxomicin **15b** proved the least potent inhibitor of the four.

Scheme 3. Synthesis of fluorescent dienophile.



Reagents and conditions: i) Boc_2O (1.1 equiv.), Et_3N (2 equiv.), DCM, rt, 50 min., 90%. ii) (a) Ph_3P (1.2 equiv.), THF, rt, 5 hr., (b) H_2O (few drops), rt, 2 hr. iii) methyl chloroformate (1 equiv.), NMM (1 equiv.), EtOAc, 0 °C, 2 hr., 52%. iv) **20** (1.3 equiv.), sat. aq. $NaHCO_3$, 0 °C, 30 min., then rt, 45 min., 31% (2 steps). v) (a) TFA/DCM (1/1, v/v), rt, 10 min. (b) BODIPY TMR-OSu (1 equiv.), DiPEA (6 equiv.), DCE, rt, 16 hr., 34%.

Having synthesized a panel of diene functionalized proteasome inhibitors, their applicability in a Diels-Alder based two-step proteasome labeling approach was investigated. To this end, a reporter group equipped with a dienophile was designed and synthesized. Inspired by the results published by Waldmann and co-workers,[9] maleimide was chosen as the dienophile. A BODIPY TMR fluorophore tethered by a hydrophilic linker to the maleimide was synthesized as depicted in Scheme 3. After Boc-protection of the free amine in **16**,[11] the azide was reduced by a Staudinger reduction to give mono-protected di-amine **18**. Introduction of the maleimide moiety was carried out by treatment with Nefkens reagent analog **20**.[14] The synthesis was finalized by acidic deprotection of **21** and subsequent acylation with BODIPY TMR-OSu (Chapter 2) to give the fluorescently tagged dienophile **22**.

Initial Diels-Alder two-step proteasome labeling experiments were performed on purified 20S proteasomes, employing diene functionalized epoxomicin analogue **15d** as the potential probe as follows. Purified 20S proteasome was exposed to 5 µM **15d** or 1 µM MV151 as a positive control for 1 hr. at 37 °C, before the proteins were denatured with urea and DTT. To prevent Michael addition of exposed cysteine residues on the fluorescently tagged maleimide, the samples were treated with Ellman's reagent[15] (5,5'-dithio-bis(2-nitrobenzoic acid), DTNB) to cap the free thiols. The same denaturation and capping procedure was performed on RAW cell lysate, which was added to prevent loss of diene functionalized proteasome subunits during workup.[16] The samples were subjected to chloroform/methanol precipitation to separate the proteins from the excess reagents. The precipitate was taken up in Diels-Alder buffer (5 mM NaH_2PO_4, 20 mM NaCl, 0.2 mM $MgCl_2$, pH 6.0)[9] containing 2 mM urea and 10 mM $Cu(NO_3)_2$ and the samples were incubated with 25 µM fluorescently tagged dienophile **22** for 16 hr. at 37 °C. Copper nitrate was added to

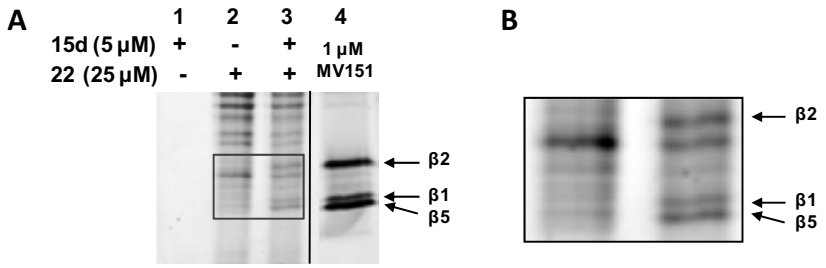


Figure 3. Diels-Alder two-step labeling of spiked purified 20S proteasome.
(**A**) Purified 20S proteasome (100 ng) was incubated with 5 µM **15d** for 1 hr. at 37 °C, followed by denaturation with 8M urea and 5 mM DTT for 30 min. at 55 °C and treatment with 30 mM DTNB for 3.5 hr. at rt. After addition of denatured and DTNB-treated RAW cell lysate, the samples were subjected to chloroform/methanol precipitation and redissolved in Diels-Alder buffer (pH 6.0) containing 2 mM urea and 10 mM $Cu(NO_3)_2$. Where indicated, the samples were exposed to 25 µM **22** for 16 hr. at 37 °C. The reactions were terminated by chloroform/methanol precipitation. Proteins were separated by SDS-PAGE, followed by in-gel fluorescence readout. (**B**) Magnification of the box indicated in Figure 3A.

the ligation reactions, since copper(II) salts are known to catalyze Diels-Alder cycloadditions in aqueous media.[17,18] The ligation reactions were terminated by precipitation of the proteins. The precipitated proteins were resolved by SDS-PAGE and fluorescently labeled proteins were visualized by in-gel fluorescence readout (Figure 3). The gel revealed a considerable amount of labeling in the **15d** naïve sample (Figure 3A, lane 2), indicating aspecific fluorescent labeling by dienophile **22**. However, the sample treated with both the diene functionalized proteasome inhibitor **15d** and the fluorescently tagged dienophile **22** revealed three additional bands, not present in the **15d** naïve sample (Figure 3A, lane 3. Magnified in Figure 3B). This finding, and the fact that the labeling closely resembles that of the fluorescent proteasome probe MV151 indicates the Diels-Alder two-step labeling of proteolytically active proteasome β subunits.

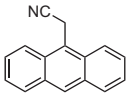
7.3 Conclusion

The synthesis of four *N*-terminally diene modified epoxomicin analogues **15a-d** is described. The introduction of the dienes did not influence the proteasome inhibitory potential as judged by the competition experiment versus MV151, which showed potencies in the same order of magnitude as for the parent inhibitor epoxomicin. Furthermore, the fluorescently tagged dienophile **22** was synthesized. Initial Diels-Alder two-step proteasome labeling experiments performed on purified 20S proteasome showed the potential of the Diels-Alder as an alternative ligation method in activity-based protein profiling, although considerable optimizations are needed to extrapolate this methodology to labeling of endogenously expressed proteasomes.

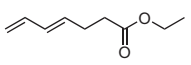
Experimental section

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 1,4-dioxane (Biosolve) were stored on 4Å molecular sieves. Dimethyl sulfoxide (DSMO), 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), by 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 by spraying with an aqueous solution of $KMnO_4$ (20%) and K_2CO_3 (10%). Column chromatography was performed on silica gel (0.040-0.063 nm) obtained from Screening Devices. LC/MS analysis was performed on a LCQ Adventage Max (Thermo Finnigan) equipped with a Gemini C18 column (Phenomenex). The applied buffers were A: H_2O, B: MeCN and C: 1.0 % aq. TFA. ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Jeol JNM-FX-200 (200/50) or Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory. Chemical shifts (δ) are given relative to tetramethylsilane as internal standard. All presented ^{13}C-APT spectra are proton decoupled.

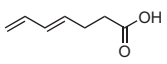
Coupling constants are given in Hz. Boc-Ile-Ile-Thr(*t*Bu)-Leu-ek **14** and 2-(2-(2-azidoethoxy)ethoxy)ethanamine·HCl **16** were synthesized as described in Chapter 4.



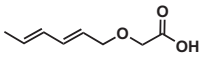
2-(anthracen-9-yl)acetonitrile (3). A solution of 9-(chloromethyl)anthracene (**2**) (2.27 g, 10 mmol) in DMSO (15 ml) was heated to 60 °C, before addition of a solution of KCN (0.98 g, 15 mmol, 1.5 equiv.) in H_2O (3 ml). The reaction mixture was stirred at 60 °C for 1 hr., then allowed to cool down to rt and stirred overnight. After addition of H_2O (40 ml), the precipitate was filtrated, washed with H_2O and dried *in vacuo* to yield **3** as yellow crystals (2.04 g, 9.4 mmol, 94%). ^{1}H NMR (200 MHz, $CDCL_3$): δ ppm 8.53 (s, 1H), 8.13 (ddd, J_1 = 20.7, J_2 = 8.4, J_3 = 0.4 Hz, 4H), 7.59 (dddd, J_1 = 14.8, J_2 =7.7, J_3 =6.6, J_4 =1.1 Hz, 4H), 4.61 (s, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 131.21, 129.33, 128.66, 127.07, 125.13, 122.77, 117.65, 77.62, 77.00, 76.35, 15.96.



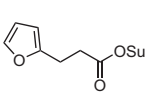
(*E*)-ethyl hepta-4,6-dienoate (6). To a solution of penta-1,4-dien-3-ol (**5**) (0.29 ml, 3.0 mmol) in toluene was added triethyl orthoacetate (2.8 ml, 15 mmol, 5 equiv.) and one drop of propionic acid. The reaction mixture was refluxed for 3 hr., before being concentrated *in vacuo*. Purification by column chromatography (PetEt → 5% EtOAc in PetEt) yielded compound **6** as a colorless oil (0.43 g, 2.8 mmol, 94%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 6.44-5.99 (m, 2H), 5.81-5.60 (m, 1H), 5.05 (ddd, J_1 = 13.3, J_2 = 11.5, J_3 = 1.6 Hz, 2H), 4.14 (q, J = 7.1 Hz, 2H), 2.37 (s, 4H), 1.25 (t, J = 7.1, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 172.12, 136.46, 132.18, 131.55, 115.05, 59.72, 33.31, 27.37, 13.75.



(*E*)-hepta-4,6-dienoic acid (7). (*E*)-ethyl hepta-4,6-dienoate (**6**) (0.43 g, 2.8 mmol) was dissolved in a mixture of 1,4-dioxane/MeOH/1M NaOH (1/1/1, v/v/v) and stirred for 1 hr. After adding DCM, the mixture was washed with sat. aq. $NaHCO_3$ (1x). The aqueous layer was acidified with 1M HCl, followed by extraction with DCM (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 25% EtOAc in PetEt) gave hepta-4,6-dienoic acid (**7**) (0.24 g, 1.9 mmol, 68%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 6.43-6.00 (m, 2H), 5.78-5.61 (m, 1H), 5.26-4.87 (m, 2H), 2.56-2.33 (m, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 179.26, 136.67, 132.04, 115.74, 33.47, 27.26.

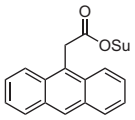


2-((2*E*,4*E*)-hexa-2,4-dienyloxy)acetic acid (9). (2*E*,4*E*)-hexa-2,4-dien-1-ol (**8**) (3.0 g, 30 mmol) was added to a suspension of NaH (60% in mineral oil, 2.5 g, 61 mmol, 2 equiv.) in freshly distilled THF under argon atmosphere. The reaction mixture was stirred for 30 min., after which bromoacetic acid (4.2 g, 30 mmol, 1 equiv.) was added. The reaction mixture was stirred overnight, before being quenched with a 3M KOH solution. The aqueous layer was then washed with Et_2O (2x), acidified with 6M HCl and extracted with $CHCl_3$ (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (DCM + 1% AcOH → 5% MeOH in DCM + 1% AcOH) yielded acid **9** (3.4 g, 22 mmol, 73%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 11.58 (s, 1H), 6.20 (dd, J_1 = 15.2, J_2 = 10.5 Hz, 1H), 6.04 (ddd, J_1 = 14.7, J_2 = 10.5, J_3 = 1.3 Hz, 1H), 5.71 (dq, J_1 = 13.6, J_2 = 6.7 Hz, 1H), 5.62-5.53 (m, 1H), 4.08 (t, J = 3.2 Hz, 4H), 1.73 (d, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 175.58, 134.55, 130.60, 130.27, 124.61, 71.51, 65.80, 17.75.

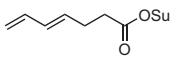


3-(2-furyl)propanoyl-OSu (10). A solution of 3-(2-furyl)propanoic acid (**1**) (0.98 g, 7.0 mmol) in DCE/DMF was put under argon atmosphere, before HOSu (3.2 g, 28 mmol, 4 equiv.) and EDC·HCl (5.3 g, 28 mmol, 4 equiv.) were added. The reaction mixture was stirred overnight, after which EtOAc was added and the mixture was washed with 1M HCl (2x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (PetEt

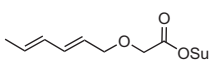
→ 40% EtOAc in PetEt), yielding OSu-ester **10** (1.2 g, 4.9 mmol, 71%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.38-7.28 (m, 1H), 6.29 (dd, J_1 = 3.2, J_2 = 1.9 Hz, 1H), 6.11 (dd, J_1 = 3.2, J_2 = 0.8 Hz, 1H), 3.14-2.90 (m, 4H), 2.84 (s, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 169.04, 167.46, 152.32, 141.50, 109.99, 105.60, 29.09, 25.14, 22.51.



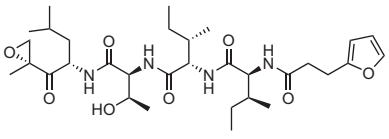
2-(anthracen-9-yl)acetyl-OSu (11). 2-(anthracen-9-yl)acetonitrile (**3**) (2.04 g, 9.4 mmol) was dissolved in 2-methoxyethanol (15 ml), before NaOH (0.94 g, 23.5 mmol, 2.5 equiv.) was added. The reaction mixture was refluxed for 3 hr., before addition of H_2O (60 ml). The mixture was extracted with Et_2O (2x) and the aqueous layer was acidified to pH 2 using 1M HCl, and left to stand overnight. The precipitate was filtered, washed with H_2O, dried *in vacuo* to give the acid **4** (1.24 g, 5.2 mmol, 56%), which was used without further purification. 2-(anthracen-9-yl)acetic acid (**4,** 0.86 g, 3.6 mmol) was coevaporated with Tol., dissolved in DCE/DMF and put under argon atmosphere, before HOSu (1.6 g, 14 mmol, 4 equiv.) and EDC·HCl (2.8 g, 14 mmol, 4 equiv.) were added. The reaction mixture was stirred overnight, after which EtOAc was added. The mixture was washed with 1M HCl (2x) and the organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (PetEt → 40% EtOAc in PetEt), yielding OSu-ester **11** (0.98 g, 2.9 mmol, 81%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 8.48 (s, 1H), 8.22 (d, J = 9.1 Hz, 2H), 8.03 (d, J = 8.4 Hz, 2H), 7.64-7.45 (m, 4H), 4.92 (s, 2H), 2.76 (s, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 168.89, 166.58, 131.25, 130.43, 129.13, 128.16, 126.67, 125.01, 123.55, 122.85, 30.30, 25.27.



(*E*)-hepta-4,6-dienoyl-OSu (12). A solution of acid **7** (0.17 g, 1.3 mmol) in DCE/DMF was put under argon atmosphere, before addition of HOSu (0.60 g, 5.2 mmol, 4 equiv.) and EDC.HCl (1.0 g, 5.2 mmol, 4 equiv.). The reaction mixture was stirred overnight. EtOAc was added and the mixture was washed with 1M HCl (2x). The combined organics were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 40% EtOAc in PetEt) gave OSu-ester **12** (0.23 g, 1.0 mmol, 78%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 6.30 (td, J_1 = 16.9, J_2 = 10.2 Hz, 1H), 6.14 (dd, J_1 = 15.1, J_2 = 10.5 Hz, 1H), 5.76-5.65 (m, 1H), 5.09 (dd, J_1 = 51.1, J_2 = 13.5 Hz, 2H), 2.80 (s, 4H), 2.71 (t, J = 7.4 Hz, 2H), 2.54-2.47 (m, 2H).

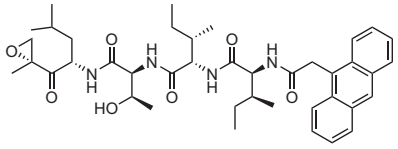


2-((2*E*,4*E*)-hexa-2,4-dienyloxy)acetyl-OSu (13). 2-((2*E*,4*E*)-hexa-2,4-dienyloxy)acetic acid (**9**, 3.42 g, 21.9 mmol) was dissolved in DCE/DMF and put under argon atmosphere, before HOSu (4.70 g, 40.5 mmol, 1.8 equiv.) and EDC·HCl (7.80 g, 40.5 mmol, 1.8 equiv.) were added. The reaction mixture was stirred overnight, before EtOAc was added and the mixture was washed with 1M HCl. The organics were separated and dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 50% EtOAc in PetEt) gave OSu-ester **13** (4.41 g, 17.4 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 6.26 (dd, J_1 = 15.2, J_2 = 10.4 Hz, 1H), 6.07 (dd, J_1 = 14.1, J_2 = 11.5 Hz, 1H), 5.81-5.71 (m, 1H), 5.66-5.53 (m, 1H), 4.41 (s, 2H), 4.16 (d, J = 6.6 Hz, 2H), 2.85 (s, 4H), 1.77 (d, J = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 168.72, 165.91, 135.31, 131.28, 130.35, 124.34, 72.06, 64.36, 25.52, 18.08.

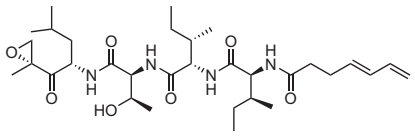


3-(2-furyl)propanoyl-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyloxirane (15a). Fully protected tetrapeptide epoxyketone **14** (0.16 g, 0.25 mmol) was treated with TFA/DCM (1/1, v/v) for 15 min., before being concentrated in the presence of toluene. The resulting TFA salt was dissolved in DCE/DMF and neutralized with DiPEA (0.17 ml, 1.0 mmol, 4 equiv.), followed by addition of a solution of OSu-ester **10** (0.18 g, 0.75 mmol, 3 equiv.) in DCE/DMF. The reaction mixture was stirred under argon atmosphere for 1.5 hr. Next, DCM was added and the mixture was washed with H_2O (1x). The aqueous layer was extracted with EtOAc (1x), the organic layers were combined and MeOH was added until the solution became clear. The organics were dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*.

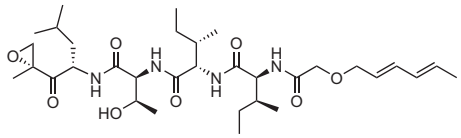
Purification by column chromatography (DCM → 4% MeOH in DCM) afforded title compound **15a** (0.13 g, 0.20 mmol, 82%) as a white solid. LC/MS analysis: R_t 8.50 min (linear gradient 10 → 90% B in 15 min), *m/z* 621.3 $[M+H]^+$, 1241.3 $[2M+H]^+$. ^1H NMR (200 MHz, $CDCl_3$/MeOD): δ ppm 8.34 (d, *J* = 9.4 Hz, 1H), 7.87 (d, *J* = 7.4 Hz, 1H), 7.79 (d, *J* = 7.9 Hz, 1H), 7.26 (s, 1H), 7.15 (d, *J* = 8.8 Hz, 1H), 6.24 (dd, J_1 = 3.1, J_1 = 1.9 Hz, 1H), 6.00 (dd, J_1 = 3.2, J_1 = 0.7 Hz, 1H), 4.80-4.50 (m, 3H), 4.28-4.01 (m, 2H), 3.29 (d, *J* = 4.8 Hz, 1H), 3.03-2.90 (m, 2H), 2.88 (d, *J* = 4.9 Hz, 1H), 2.75-2.46 (m, 3H), 1.87-1.54 (m, 7H), 1.52 (s, 3H), 1.49-1.34 (m, 1H), 1.10 (d, *J* = 6.2 Hz, 3H), 0.93-0.71 (m, 18H).



2-(anthracen-9-yl)acetyl-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyl-oxirane (15b). Fully protected tetrapeptide epoxyketone **14** (0.16 g, 0.25 mmol) was treated with TFA/DCM (1/1, v/v) for 15 min, before being concentrated in the presence of toluene. The resulting TFA salt was dissolved in DCE/DMF, followed by the addition of DiPEA (0.09 ml, 0.50 mmol, 2 equiv.) and a solution of OSu-ester **11** (0.25 g, 0.75 mmol, 3 equiv.) in DCE/DMF. The reaction mixture was stirred under argon atmosphere for 18hr., before DCM was added and the mixture was washed with H_2O (1x). The aqueous layer was extracted with EtOAc (1x), the organic layers were combined and MeOH was added until a clear solution had formed. The organics were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (DCM → 2% MeOH in DCM) afforded title compound **15b** (70 mg, 98 µmol, 39%). LC/MS analysis: R_t 10.17 min (linear gradient 10 → 90% B in 15 min), *m/z* 717.3 $[M+H]^+$, 1455.4 $[2M+Na]^+$. ^1H NMR (200 MHz, $CDCl_3$/MeOD): δ ppm 8.455 (s, 1H), 8.18 (d, *J* = 8.8 Hz, 2H), 8.03 (d, *J* = 7.7 Hz, 2H), 7.61-7.41 (m., 5H), 6.74 (d, *J* = 8.0 Hz, 1H), 4.65 (s, 2H), 4.61-4.48 (m, 1H), 4.29-4.01 (m, 4H), 3.29 (d, *J* = 4.9 Hz, 1H), 2.90 (d, *J* = 4.9 Hz, 1H), 1.80-1.24 (m, 8H), 1.50 (s, 3H), 1.00-0.64 (m, 22 H).

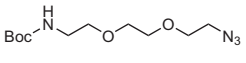


(*E*)-hepta-4,6-dienoyl-Ile-Ile-Thr(tBu)-leucinyl-(*R*)-2-methyloxirane (15c). Tetrapeptide epoxyketone **14** (0.13 g, 0.22 mmol) was treated with TFA/DCM (1/1, v/v) for 15 min, before being concentrated in the presence of toluene. The resulting TFA salt was dissolved in DCE/DMF and neutralized with DiPEA (0.14 ml, 0.80 mmol, 4 equiv.), followed by addition of a solution of OSu-ester **12** (0.13 g, 0.60 mmol, 3 equiv.) in DCE. The reaction mixture was stirred under argon atmosphere for 2 hr., before DCM was added and the mixture was washed with H_2O (1x). The aqueous layer was extracted with EtOAc (1x), the organic layers were combined and MeOH was added until a clear solution was obtained. The organics were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (DCM → 4% MeOH in DCM) afforded title compound **15c** (78 mg, 0.13 mmol, 64%). LC/MS analysis: R_t 8.94 min (linear gradient 10 → 90% B in 15 min), *m/z* 607.3 $[M+H]^+$, 1213.3 $[2M+H]^+$, 1819.1 $[3M+H]^+$. ^1H NMR (200 MHz, $CDCl_3$): δ ppm 8.27 (d, *J* = 8.1 Hz, 1H), 7.85 (d, *J* = 7.9 Hz, 1H), 7.63 (d, *J* = 6.9 Hz, 1H), 7.01 (d, *J* = 8.9 Hz, 1H), 6.37-6.00 (m, 2H), 5.66 (td, J_1 = 14.5, J_2 = 6.2 Hz, 1H), 5.03 (ddd, J_1 = 13.3, J_2 = 11.1, J_3 = 1.6 Hz, 2H), 4.79-4.49 (m, 3H), 4.28-4.08 (m, 2H), 3.30 (d, *J* = 4.9 Hz, 1H), 2.90 (d, *J* = 4.9 Hz, 1H), 2.86-2.82 (m, 1H), 2.52-2.28 (m, 4H), 1.85-1.56 (m, 6H), 1.53 (s, 3H), 1.48-1.33 (m, 2H), 1.11 (d, *J* = 6.5 Hz, 3H), 0.96-0.76 (m, 18H).

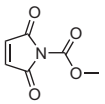


2-(hexa-2,4-dienyloxy)acetamido-Ile-Ile-Thr-leucinyl-2-methyloxirane (15d). The fully protected epoxketone **14** (0.16 g, 0.25 mmol) was deprotected in TFA/DCM (1/1 v/v) for 15 min, before being concentrated in the presence of toluene. The crude TFA salt was dissolved in DCE/DMF and neutralized with DiPEA (0.18 ml, 1.0 mmol, 4 equiv.), before a solution of OSu ester **13** (0.21 g, 0.81 mmol, 3.2 equiv.) in DCE/DMF was added. The reaction mixture was stirred under argon atmosphere for 2.5 hr., before DCM was added. The mixture was washed with H_2O and the aqueous

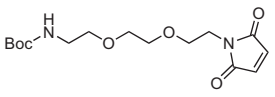
layer was extracted with EtOAc. MeOH was added to the combined organics until a clear solution was obtained. The solution was dried over $MgSO_4$, filtered and evaporated. Purification by column chromatography (10% acetone in hexane → 30% acetone in hexane) yielded the title compound **15d** (0.16 g, 0.25 mmol, quant.) as a white solid. ^{1}H NMR (400 MHz, $CDCL_3$): δ ppm 8.43 (d, *J* = 9.0 Hz, 1H), 7.94 (d, *J* = 7.4 Hz, 1H), 7.45 (d, *J* = 9.1 Hz, 1H), 7.30 (s, 1H), 6.19 (dd, J_1 = 15.2, J_2 = 10.4 Hz, 1H), 6.05 (dd, J_1 = 14.0, J_2 = 11.4 Hz, 1H), 5.79-5.66 (m, 1H), 5.63-5.54 (m, 1H), 4.86-4.69 (m, 1H), 4.55 (td, J_1 = 9.8, J_2 = 5.7 Hz, 3H), 4.18-3.99 (m, 1H), 3.97 (s, 2H), 3.89 (d, *J* = 15.4 Hz, 2H), 3.35 (d, *J* = 4.9 Hz, 1H), 2.89 (d, *J* = 4.9 Hz, 1H), 1.76 (d, *J* = 6.7 Hz, 2H), 1.71-1.58 (m, 2H), 1.52 (s, 3H), 1.43-1.17 (m, 2H), 1.11 (d, *J* = 6.3 Hz, 3H), 1.08-0.94 (m, 2H), 0.90 (d, *J* = 6.5 Hz, 6H), 0.87-0.75 (m, 12H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 208.20, 171.81, 171.21, 170.53, 169.80, 134.45, 130.80, 130.40, 125.02, 71.78, 68.65, 67.12, 59.17, 57.52, 56.86, 56.69, 52.35, 50.73, 39.13, 38.01, 37.92, 25.13, 25.05, 24.91, 23.21, 21.09, 17.98, 17.26, 16.74, 15.22, 15.20, 11.33, 11.25.



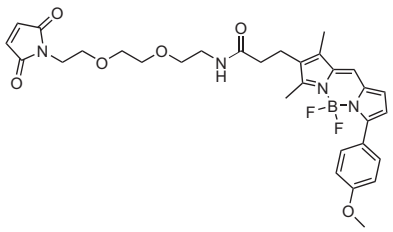
***t*Butyl 2-(2-(2-azidoethoxy)ethoxy)ethylcarbamate (17).** To a solution of 2-(2-(2-azidoethoxy)ethoxy)ethanamine·HCl (**16**) (1.5 g, 5.4 mol) in DCM was added triethylamine (1.5 ml, 11 mmol, 2 equiv.) and Boc_2O (1.3 g, 5.9 mmol, 1.1 equiv.). The reaction mixture was stirred for 50 min., before being concentrated *in vacuo*. Purification of the crude product by column chromatography (DCM → 3% MeOH in DCM) gave the title compound as a colorless oil (1.3 g, 4.9 mmol, 90%). LC/MS analysis: R_t 7.00 min (linear gradient 10 → 90% B in 15 min), *m/z* 274.8 $[M+H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 5.07 (s, 1H), 3.73-3.59 (m, 6H), 3.58-3.50 (m, 2H), 3.45-3.24 (m, 6H), 1.45 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 154.91, 77.46, 69.36, 69.12, 68.96, 49.50, 39.28, 27.27.



Nefkens reagent analog (20). A solution of maleimide (**19**) (0.99 g, 10 mmol) and *N*-methyl morpholine (1.1 ml, 10 mmol, 1 equiv.) in EtOAc was cooled to 0 °C. Methylchloroformate (0.80 ml, 10 mmol, 1 equiv.) was added and the reaction mixture was stirred 2 hr. The reaction mixture was washed sat. aq. $NaHCO_3$ (3x) and the aqueous layer was extracted with EtOAc (1x). The combined organics were dried over $MgSO_4$, filtered and concentrated *in vacuo*, to yield the crude product **20** (0.80 g, 5.2 mmol, 52%) which was used without any further purification. LC/MS analysis: R_t 1.29 min (linear gradient 10 → 90% B in 15 min), *m/z* 156.0 $[M+H]^+$. ^{1}H NMR (200 MHz, acetone-d_6): δ ppm 7.05 (s, 2H), 3.89 (s, 3H). ^{13}C NMR (50 MHz, acetone-d_6): δ ppm 166.93, 148.80, 136.27, 54.12.



***t*Butyl 2-(2-(2-(2,5-dioxo-pyrrol-1-yl)ethoxy)ethoxy)ethylcarbamate (21).** Boc-protected azide **17** (1.0 g, 3.6 mmol) was dissolved in THF, before triphenylphosphine (1.2 g, 4.4 mmol, 1.2 equiv.) was added. The reaction mixture was stirred for 5 hr., after which a few drops of H_2O were added. After stirring for an additional 2 hr., toluene was added and the mixture was extracted with 1M HCl (4x). The combined aqueous layers were alkalized using sat. aq. $NaHCO_3$ and 3M KOH, and extracted with DCM (3x) and EtOAc (3x). The combined organics were dried over $MgSO_4$, filtered and concentrated *in vacuo* to give amine **18** (0.65 g, 2.6 mmol, 72%). LC/MS analysis: R_t 3.86 min (linear gradient 10 → 90% B in 15 min), *m/z* 249.0 $[M+H]^+$, 496.9 $[2M+H]^+$. The crude compound **18** was dissolved in sat. aq. $NaHCO_3$ solution, cooled to 0 °C, before Nefkens reagent analog **20** (0.52 g, ~3.4 mmol, 1.3 equiv.) was added. The reaction mixture was stirred at 0 °C for 30 min., before being allowed to warm up to rt for 45 min. The mixture was extracted with chloroform (3x) and the combined organics were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 70% EtOAc in PetEt) yielded maleimide **21** (0.36 g, 1.1 mmol, 31% over two steps). LC/MS analysis: R_t 6.37 min (linear gradient 10 → 90% B in 15 min.), *m/z* 328.9 $[M+H]^+$, 351.1 $[M+Na]^+$. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 6.72 (s, 2H), 5.04 (s, 1H), 3.85-3.41 (m, 10H), 3.39-3.20 (m, 2H), 1.45 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 170.37, 155.66, 133.86, 78.70, 69.81, 69.51, 67.42, 40.00, 36.67, 28.06.



(*N*)-BODIPY TMR-2-(2-(2-(2,5-dioxo-pyrrol-1-yl)ethoxy)ethoxy)ethyl-amine (22). Compound **21** (65 mg, 0.20 mmol) was treated with TFA/DCM (1/1, v/v) for 10 min, before being concentrated in the presence of toluene. The resulting TFA salt was dissolved in DCE and neutralized with DiPEA (0.20 ml, 1.2 mmol, 6 equiv.), followed by addition of BODIPY TMR-OSu (99 mg, 0.20 mmol, 1 equiv.). The reaction mixture was stirred under argon atmosphere overnight. DCE was added and the mixture was washed with H_2O (1x). The aqueous layer was extracted with EtOAc (2x), the organic layers were combined and MeOH was added until a clear solution was obtained. The organics were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (toluene → 30% acetone in toluene) afforded title compound **22** as a purple solid (41 mg, 68 µmol, 34%). LC/MS analysis: R_t 8.55 min (linear gradient 10 → 90% B in 15 min.), *m/z* 589.4 $[M-F]^+$, 1216.8 $[2M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$): δ ppm 7.89-7.83 (m, 2H), 7.07 (s, 1H), 6.99-6.95 (m, 2H), 6.94 (d, *J* = 4.1 Hz, 1H), 6.61 (s, 2H), 6.53 (d, *J* = 4.0 Hz, 1H), 6.35 (t, *J* = 4.9 Hz, 1H), 3.85 (s, 3H), 3.68 (t, *J* = 5.5 Hz, 2H), 3.58 (t, *J* = 5.4 Hz, 2H), 3.52-3.41 (m, 6H), 3.41-3.35 (m, 2H), 2.77 (t, *J* = 7.4 Hz, 2H), 2.53 (s, 3H), 2.36 (t, *J* = 7.5 Hz, 2H), 2.21 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 171.82, 170.72, 160.28, 159.69, 139.88, 134.80, 134.07, 130.77, 130.62, 127.67, 125.45, 122.63, 118.16, 113.66, 70.07, 69.76, 69.74, 67.74, 55.21, 39.17, 37.09, 36.16, 20.05, 13.12, 9.54.

***In vitro* competition assay in EL-4 cell lysates**

EL-4 cell lysates (10 µg total protein) were incubated for 1 hr. at 37 °C with the indicated concentrations of the inhibitor (10x solution in DMSO) in a total reaction volume of 10 µL (H_2O/DMSO 9/1, v/v), prior to incubation with MV151 (1 µM) for 1 hr. at 37 °C in a total reaction volume of 11 µL (H_2O/DMSO 9/2, v/v). The reaction mixtures were boiled for 3 minutes with 4 µL 4x Laemmli's sample buffer containing β-mercapto-ethanol and resolved on 12.5% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly on a Typhoon Variable Mode Imager (Amersham Biosciences) using Cy3/Tamra settings (λ_{ex} 532, λ_{em} 560).

Diels-Alder ligation on denatured purified 20S proteasome

Purified 20S proteasome (100 ng per reaction) was exposed to 5 µM **15d** or 1 µM MV151 for 1 hr. at 37 °C in a total reaction volume of 10 µl (H_2O/DMSO 9/1, v/v). The samples were then denaturated with 8 M urea for 15 min. at rt followed by addition of DTT (5 mM final concentration) for 30 min. at 55 °C, and capping with 30 mM DTNB for 3.5 hr. at rt. After addition of RAW cell lysates (denatured and capped with 30 mM DTNB according to the same method as described above), the proteins were crashed out by chloroform/methanol precipitation. The proteins were taken up in Diels-Alder buffer (pH 6.0) containing 6M urea, followed by addition of 2 volumes of Diels-Alder buffer (5 mM NaH_2PO_4, 20 mM NaCl, 0.2 mM $MgCl_2$, pH 6.0) containing 10 mM $Cu(NO_3)_2$. The denatured proteins were incubated with 25 µM **22** for 16 hr. at 37 °C in a total reaction volume of 16.5 µL (H_2O/DMSO 10/1, v/v). The reaction was terminated by chloroform/methanol precipitation. The proteins were taken up in Laemmli's sample buffer containing β-mercapto-ethanol, heated at 55 °C for 15 min. and resolved on 12.5% SDS-PAGE, before in-gel fluorescence readout of the labeled proteins using the Cy3/Tamra settings (λ_{ex} 532, λ_{em} 560) on the Typhoon Variable Mode Imager (Amersham Biosciences).

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