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Two-Step Labeling of Endogenous Enzymatic Activities by Diels–Alder Ligation

Lianne I. Willems, Martijn Verdoes, Bogdan I. Florea, Gijsbert A. van der Marel, and Herman S. Overkleeft*[a]

A ligation strategy based on the Diels–Alder [4+2] cycloaddition for the two-step activity-based labeling of endogenously expressed enzymes in complex biological samples has been developed. A panel of four diene-derivatized proteasome probes was synthesized, along with a dienophile-functionalized BODIPY(TMR) tag. These probes were applied in a Diels–Alder labeling procedure that enabled us to label active proteasome β-subunits selectively in cellular extracts and in living cells. We were also able to label the activity of cysteine proteases in cell extracts by utilizing a diene-derivatized cathepsin probe. Importantly, the Diels–Alder strategy described here is fully orthogonal with respect to the Staudinger–Bertozzi ligation, as demonstrated by the independent labeling of different proteolytic activities by the two methods in a single experiment.

Introduction

The introduction of bioorthogonal chemistry has given a great impetus to chemical biology research. A bioorthogonal reaction can be defined as a chemical transformation between two functional groups that takes place selectively within complex biological samples such as cell extracts, tissues, or even living organisms.[1] By allowing the temporal separation of a molecular probe and a reporter group or affinity tag, bioorthogonal chemistry maximizes the potential for selective modification of a specific biomolecule in a desired experimental setting. The power of this strategy was initially demonstrated by Bertozzi and co-workers in the cell-surface labeling of glycoproteins.[2–4] In a seminal experiment, *N*-azidoacetylmannosamine was added to the medium of cultured Jurkat cells, resulting in the presence of azides on the cell-surface sialic acid residues present in *N*-glycans. These were then selectively modified with a biotin entity by treatment with a phosphine reagent, a strategy known as the Staudinger–Bertozzi ligation, which allowed the monitoring of cell surface glycoconjugates. A second important orthogonal bioconjugation method involves the reaction between an azide and an alkyne—the Huisgen [2+3] cycloaddition or click reaction.[5,6] Nowadays, both copper(I)-catalyzed click reactions[5,6] and copper-free strain-promoted click strategies[7–11] are used. Bioorthogonal ligation reactions are applied to study a variety of biological processes, in particular those involving post-translational modifications.[12–15]

Another area of research that has benefited from bioorthogonal chemistry is two-step activity-based protein profiling (ABPP). The direct introduction of a reporter group into an active-site-directed chemical probe could affect the cell permeability of the probe, affinity for a target enzyme, and/or selectivity over other enzymes. These problems can be avoided by introducing the tag in a later stage, after binding of the probe to a target enzyme. We demonstrated the feasibility of the Staudinger–Bertozzi ligation for two-step labeling of the proteasome, employing an azide-functionalized activity-based proteasome probe and a biotin-derivatized phosphine reagent for bioorthogonal ligation, and demonstrated its use in the direct identification of a proteasome subunit-specific inhibitor.[16,17] Cravatt and co-workers were the first to report the use of click chemistry for activity-based profiling of serine hydrolases.[18]

Whereas the examples described above make use of azides or alkynes as ligation handles and rely either on Staudinger–Bertozzi ligation or on click chemistry, other small groups also warrant consideration for use in bioorthogonal ligation reactions. In view of its selectivity and efficiency under mild, aqueous conditions, we considered the Diels–Alder [4+2] cycloaddition as a potential alternative. Several reports have described the use of Diels–Alder ligation strategies for bioconjugation, including the modification of oligonucleotides and DNA strands,[19–26] surface immobilization of oligonucleotides,[27,28] interior surface modification of viral capsids,[29] microarray development,[30–35] conjugation of carbohydrates and proteins,[36,37] and the surface immobilization and site-specific ligation of peptides and proteins.[38,39] A few related strategies that use the inverse-electron-demand Diels–Alder reaction for protein labeling have recently been reported.[40–42] By using this approach, live cells could be labeled in vitro by first targeting cell surface receptors with a dienophile-bearing monoclonal antibody, and then by adding a tetrazine conjugated to a fluorescent tag in order to label the pretargeted antibody on the cell surface.[41,42] Here we describe the development of a Diels–

[a] *L. I. Willems, Dr. M. Verdoes, Dr. B. I. Florea, Prof. G. A. van der Marel, Prof. H. S. Overkleeft*
Leiden Institute of Chemistry and
Netherlands Proteomics Centre, Gorlaeus Laboratories
Einsteinweg 55, 2333 CC Leiden (The Netherlands)
Fax: (+31) 71-5274307
E-mail: h.s.overkleeft@chem.leidenuniv.nl

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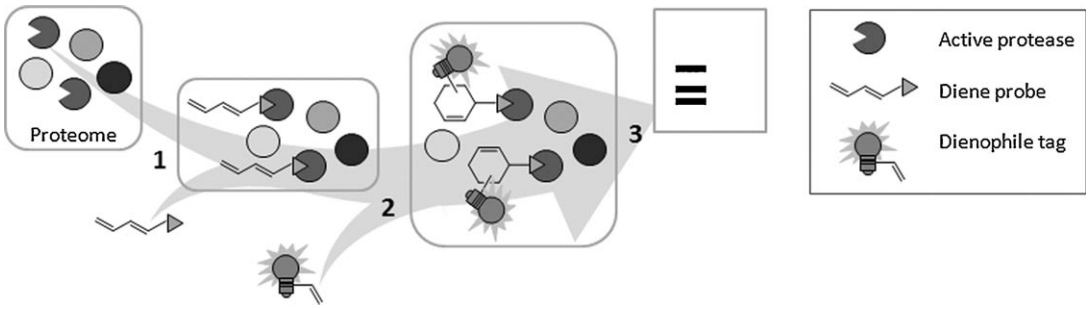


Figure 1. The Diels–Alder ligation in two-step ABPP. Schematic representation of the Diels–Alder-based labeling strategy, entailing: 1) targeting of active proteases by a diene-derivatized probe, 2) ligation of the protease-bound diene with a dienophile-functionalized tag, and 3) visualization of labeled proteases by SDS-PAGE analysis and fluorescence detection.

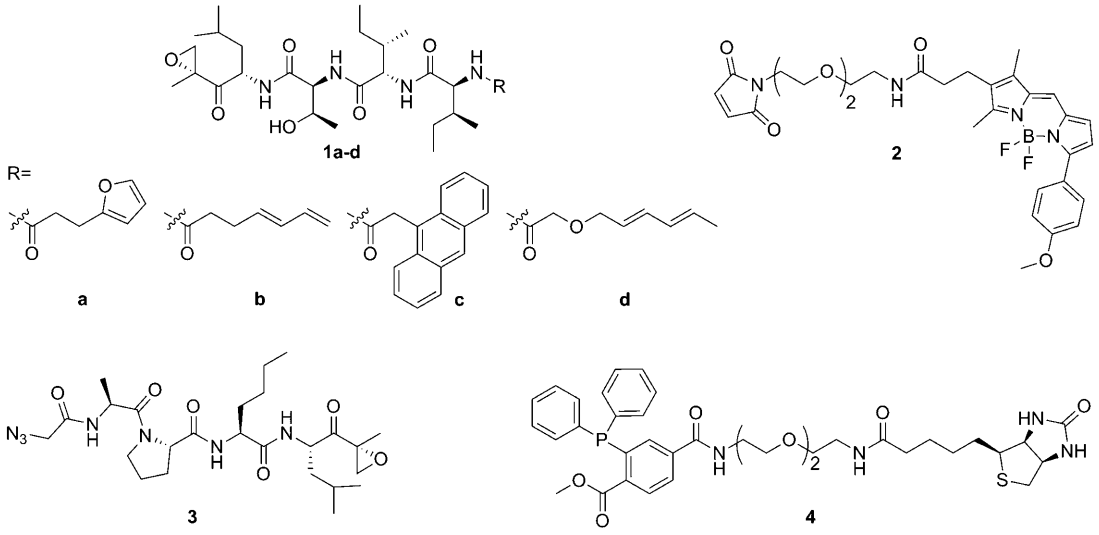
Alder ligation procedure for the profiling of endogenous enzymatic activities and explore its suitability for labeling of endogenously expressed enzymes in complex biological samples. Our approach is applicable to the labeling of various classes of enzymes and is orthogonal with respect to the Staudinger–Bertozzi ligation, allowing both strategies to be used in a single experiment for the independent labeling of different biomolecules.

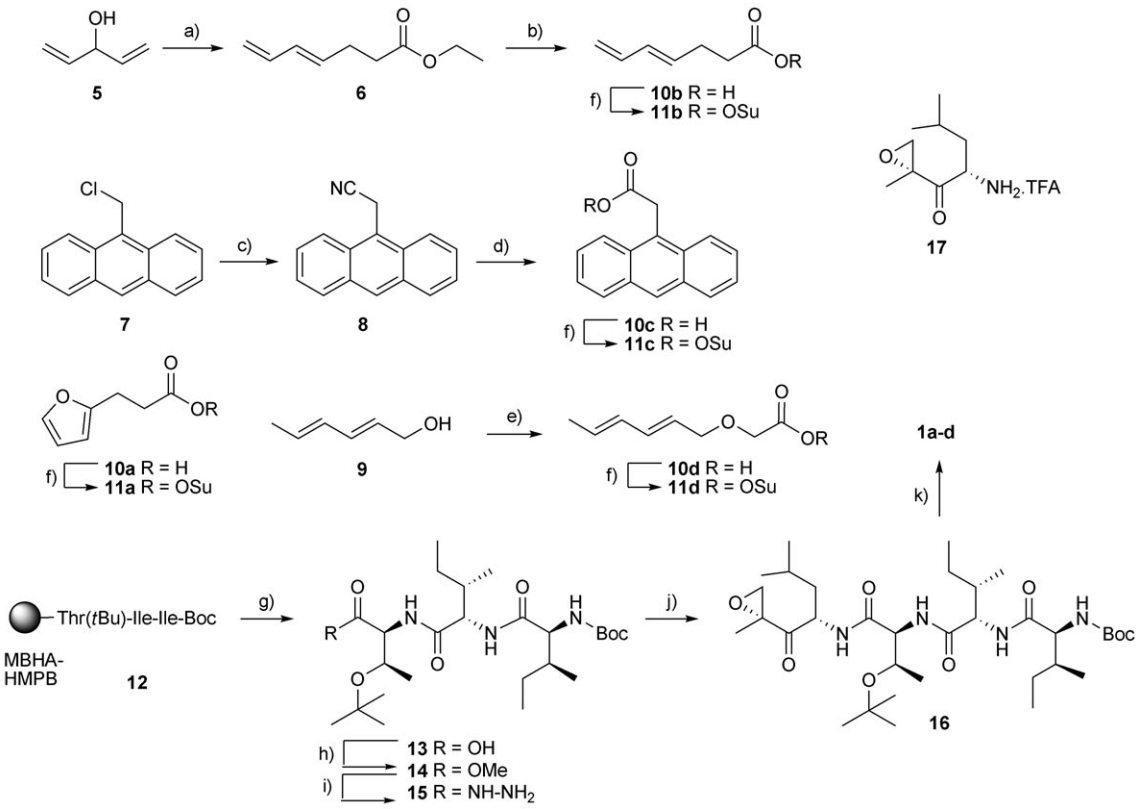
Results and Discussion

Our Diels–Alder-based ABPP strategy, as outlined in Figure 1, involves the labeling of active enzymes with a diene-derivatized active-site-directed probe followed by reaction with a dienophile modified with a fluorescent tag in order to visualize the labeled enzymes. In the first instance, we evaluated the validity of this approach for the two-step labeling of catalytically active subunits of the 20S proteasome, a highly conserved protease complex that is responsible for degrading the majority of cellular proteins.[43] For this purpose, a panel of four diene-functionalized proteasome probes (**1a–d**) was synthesized along with a dienophile-derivatized BODIPY(TMR)-tag (**2**). The maleimide functionality conjugated to the fluorescent BODIPY moiety in **2** has been reported to be an excellent dienophile for use in Diels–Alder-based ligation procedures on proteins.[38,39] The design of probes **1a–d** was based on the proteasome inhibitor epoxomicin,[44,45] extended at its N terminus with one of four different conjugated dienes.

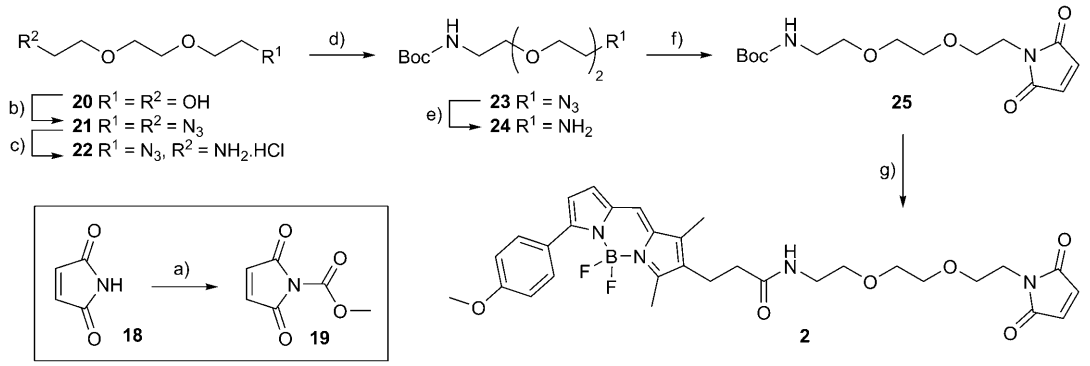
The probes **1a–d** were synthesized as depicted in Scheme 1. The tripeptide recognition element was obtained by standard solid-phase peptide chemistry (**12**) followed by mild acidic cleavage from the resin (**13**). The partially protected tripeptide **13** was then coupled through an azide-coupling procedure to the leucine-derived epoxyketone warhead amine **17**, which was synthesized essentially as described previously.[45] After deprotection of the N-terminal amine in **16**, treatment with the *N*-hydroxysuccinimidyl esters of four different diene-containing acids (**11a–d**) afforded the probes **1a–d**.

The dienophile-modified tag **2** was generated by starting from triethylene glycol (**20**; Scheme 2). The alcohol moieties were transformed into the tosylates, which were both substituted with NaN_3 to afford compound **21**, after which one of the azides could be selectively reduced and protected, yielding compound **23**. The free amine **24** resulting from reduction of the remaining azide was coupled to the activated maleimide **19** to give the maleimide-functionalized poly(ethylene





Scheme 1. Synthesis of the diene-functionalized proteasome probes. Reagents and conditions: a) triethyl orthoacetate, propionic acid, toluene, reflux, 3 h, 94%; b) 1 M NaOH/MeOH/1,4-dioxane, RT, 1 h, 68%; c) KCN, DMSO, 60 °C, 1 h, then RT, overnight (o/n), 94%; d) i: NaOH, 2-methoxyethanol, reflux, 3 h, ii: HCl, H_2O, RT, o/n, 56%; e) i: NaH, THF, RT, 30 min, ii: 2-bromoacetic acid, RT, o/n, 73%; f) HOSu, EDC·HCl, DCE/DMF, RT, o/n, **11 a** 71%, **11 b** 78%, **11 c** 81%, **11 d** 80%; g) TFA/DCM (1:99, *v/v*), RT, 6×10 min; h) TMS diazomethane, MeOH/toluene, RT, 3.5 h, 64% from MBHA-HMPB resin; i) H_2NNH_2·H_2O, MeOH, reflux, o/n, 76%; j) *t*BuONO, HCl, DMF/EtOAc, −30 °C, 1 h, ii: **17**, DiPEA, DMF/EtOAc, −30 °C to RT, o/n, 89%; k) i: TFA/DCM (1:1, *v/v*), RT, 15–45 min, ii: R-OSu (**11 a**–**d**), DiPEA, DCE/DMF, RT, 1.5–18 h, **1 a** 82%, **1 b** 64%, **1 c** 39%, **1 d** 100%.



Scheme 2. Synthesis of the dienophile-functionalized fluorescent tag. Reagents and conditions: a) methyl chloroformate, *N*-methylmorpholine, EtOAc, 0 °C, 2 h, 52%; b) i: tosyl chloride, Et_3N, DMAP, DCM, RT, 16 h, ii: NaN_3, TBAI, DMF, 80 °C, 16 h, 78%; c) Ph_3P, HCl (5%), toluene, 0 °C, 16 h, 79%; d) Et_3N, Boc_2O, DCM, RT, 50 min, 90%; e) i: Ph_3P, THF, RT, 5 h, ii: H_2O, RT, 2 h, 72% crude); f) **19**, saturated aqueous $NaHCO_3$, 0 °C, 30 min, then RT, 45 min, 31% over 2 steps; g) i: TFA/DCM (1:1, *v/v*), RT, 10 min, ii: BODIPY(TMR)-OSu, DiPEA, DCE, RT, o/n, 34%.

glycol) spacer **25**. Deprotection and condensation with BODIPY(TMR)-OSu[46] then afforded the BODIPY(TMR)-maleimide **2**.

We then set out to establish whether the four diene-containing probes **1 a**–**d** had the ability to inhibit the activity of the proteasome. This was tested by performing a competition experiment against the fluorescent activity-based proteasome probe MV151 (Figure 2).[46] EL-4 cell extracts were exposed to increasing concentrations of the probes **1 a**–**d** for 2 hours, followed by exposure to MV151 for 1 hour to label residual proteasome activity. After SDS-PAGE analysis, in-gel fluorescence detection of the labeled proteins revealed that probes **1 a**–**d** inhibited MV151 labeling of the active proteasome β-subunits

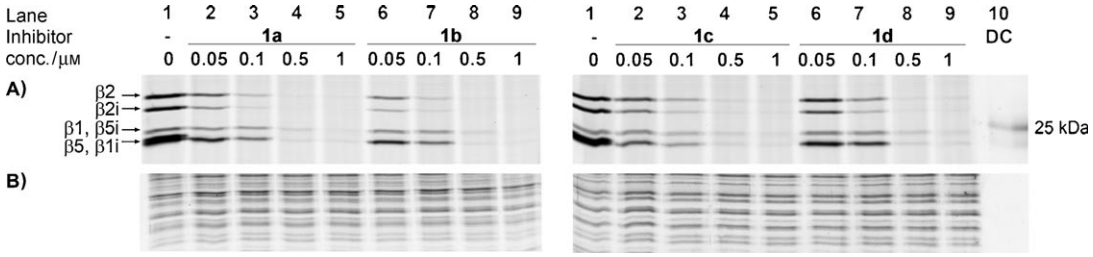


Figure 2. Diene-containing epoxomicin derivatives inhibit proteasome activity in vitro. EL-4 cell lysates (10 μg total protein per reaction) were exposed to increasing concentrations of the diene-derivatized probes **1a–d** for 2 h at 37 °C, followed by exposure to MV151 (1 μM) for 1 h at 37 °C. Labeled proteins were resolved by SDS-PAGE and detected by fluorescence readout (A) followed by Coomassie staining as a loading control (B). Proteasome β-subunits are designated on the basis of reported labeling by MV151.[46] DC: Dual Color protein standard.

with similar potencies. Complete inhibition was observed at inhibitor concentrations of 0.5–1 μM and above.

We next turned our attention to the two-step Diels–Alder labeling procedure. In the first instance, the maleimide-derivatized tag **2** was shown to react with the diene-containing probes **1a–d** in the absence of proteins to give the corresponding Diels–Alder adducts (see the Supporting Information). Two-step Diels–Alder ligation experiments were then performed in EL-4 cell extracts. After exposure to inhibitors **1a–d** (1 μM) for 1 hour at 37 °C, the proteins were denaturated and cysteine residues were masked with Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid), DTNB]. This is necessary in order to avoid labeling resulting from Michael addition of (cysteine) thiol groups to maleimide **2**. Remaining thiol-containing reagents were removed by chloroform/methanol (c/m) precipitation,[47] after which the proteins were exposed to the BODIPY-(TMR)-maleimide **2** (25 μM) at 37 °C overnight at pH 6.0. After excess maleimide-tag **2** had been washed away in a second precipitation step, the fluorescently labeled proteins were resolved by SDS-PAGE and visualized directly with the aid of a fluorescence scanner (Figure 3).

Although there is a considerable degree of background fluorescence, three fluorescent bands corresponding to proteasome β-subunits are clearly visible after labeling with the diene-derivatized epoxomicin analogues **1d**, **1a**, and **1b** (lanes 1, 5, and 7, respectively). These signals were completely blocked by addition of an excess of epoxomicin (lanes 2, 6, and 8), demonstrating that the observed bands indeed reflect labeling of proteasome active sites. The large differences in labeling intensity between the diene-derivatized probes indicate that the nature of the diene is of crucial importance for the ligation efficiency with maleimide **2**. The complete absence of specific labeling in lane 3 suggests that anthracenyl-containing inhibitor **1c** is unable to undergo Diels–Alder ligation with the maleimide **2** efficiently when bound to the proteasome. The ligation step proceeds most efficiently for the noncyclic dienes in **1d** (lane 1) and, especially, **1b** (lane 7), which is somewhat surprising because these dienes are not fixed in the *cis* configuration that is required for Diels–Alder cycloaddition.

In an effort to improve selectivity and to reduce background labeling, the concentration of the diene-derivatized probe **1b** and the conditions for cysteine masking were varied (Figure 4 and Figure S1 in the Supporting Information, respectively). Proteasome labeling became stronger with increasing concentra-

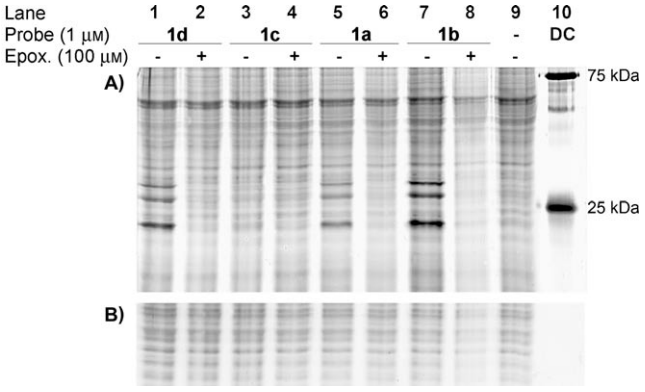


Figure 3. Labeling of proteasome activity in cell extracts by using the Diels–Alder ligation. EL-4 cell lysates were exposed to **1a–d** (1 μM) in the presence or in the absence of epoxomicin (100 μM) for 1 h at 37 °C, prior to denaturation, masking of cysteine residues with DTNB, and c/m precipitation. The proteins were then exposed to BODIPY(TMR)-maleimide **2** (25 μM) in Diels–Alder buffer (pH 6.0) overnight at 37 °C; 50 μg total protein/reaction was resolved by SDS-PAGE and labeled proteins were detected by fluorescence readout (A) followed by Coomassie staining as a loading control (B).

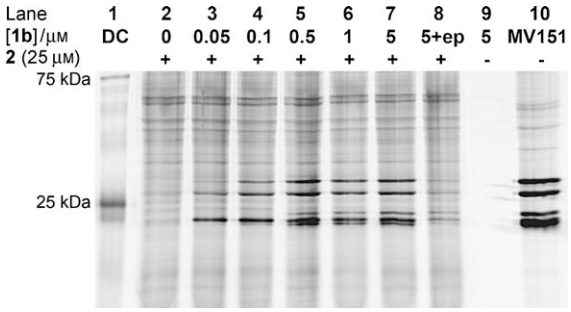


Figure 4. Effect of diene-functionalized probe concentration on Diels–Alder-based proteasome labeling. EL-4 cell lysates were exposed to increasing concentrations of the probe **1b** for 1 h at 37 °C prior to denaturation, masking of cysteine residues, and c/m precipitation. As a control, lysates were exposed either to **1b** in the presence of epoxomicin (100 μM, +ep) or to MV151 (1 μM). The proteins were then exposed to BODIPY(TMR)-maleimide **2** (25 μM) overnight at 37 °C in Diels–Alder buffer (pH 6.0); 50 μg total protein per reaction was resolved by SDS-PAGE and labeled proteins were detected by fluorescence readout.

tions of **1 b** and reached saturation at around 0.5 μM of the inhibitor (Figure 4), which is consistent with the competition assay shown in Figure 2. The concentration of diene-containing probe did not affect the amount of background fluorescence, so we assume that the background fluorescence is not caused by nonspecific reactions of the inhibitor **1 b** but rather by the maleimide-functionalized tag **2**, which might react with unprotected cysteine residues.

Consistently with this observation, the concentration of capping reagent (DTNB) proved to be of crucial importance for the amount of background labeling (Figure S1). The concentration of the capping reagent needs to be sufficiently high in order to reduce background fluorescence. A concentration that is too high, on the other hand, reduces not only background fluorescence but also specific labeling; this might be due to the fact that the large amount of reagent is not removed completely by a single c/m precipitation step and can therefore interfere with the subsequent ligation reaction.

Subsequently, efforts were made to improve the efficiency of the Diels–Alder ligation by varying the concentration of the BODIPY(TMR)-maleimide **2** (Figure 5 A) and the reaction time of the ligation step (Figure 5 B). From Figure 5 A it is apparent that both background fluorescence and specific labeling are enhanced at increasing concentrations of maleimide **2**. A dienophile concentration between 10 μM (lane 5) and 25 μM (lane 6) gives the best results in terms of signal intensity and background fluorescence. Figure 5 B reveals similar increases both in specific and in nonspecific labeling with increasing reaction times. The best results were obtained after Diels–Alder ligation for 4 or 20 hours (lane 5 and lane 6, respectively). At a reaction time of 20 hours, proteasome labeling reaches levels comparable to that of MV151 (lane 9), when used at the same concentration as the diene-derivatized probe. We chose a reaction time of 20 hours and a dienophile concentration of 25 μM for subsequent Diels–Alder ligation procedures on the proteasome.

Having shown that the Diels–Alder ligation strategy can successfully be applied to the profiling of active proteasomes in vitro, we also tested the utility of the approach for in situ labeling. The diene-functionalized probe **1 b** was first demonstrated to be cell-permeable by treatment of cultured EL-4 cells with the probe for 2 hours, after which the cells were lysed and residual proteasome activity was labeled with MV151 (Figure 6 A). The probe **1 b** was able to inhibit the activity of all proteasome β-subunits in situ, with an inhibitory potency approximately ten-fold lower in living cells than in cell extracts (see Figure 2).

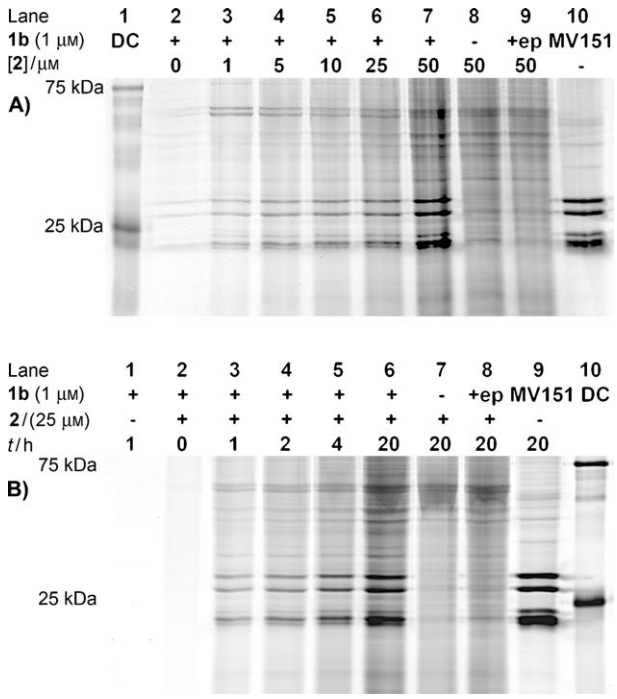


Figure 5. Effects of dienophile concentration and reaction time on Diels–Alder ligation efficiency. EL-4 cell lysates were exposed to the probe **1 b** (1 μM) for 1 h at 37 °C prior to denaturation, masking of cysteine residues, and c/m precipitation. As a control, the lysates were exposed either to **1 b** in the presence of epoxomicin (+ep, 100 μM) or to MV151 (1 μM). The proteins were then exposed either A) to increasing concentrations of the BODIPY-TMR-maleimide **2** overnight, or B) to compound **2** (25 μM) for the indicated time at 37 °C (pH 6.0); 50 μg total protein/reaction was resolved by SDS-PAGE and labeled proteins were detected by fluorescence readout.

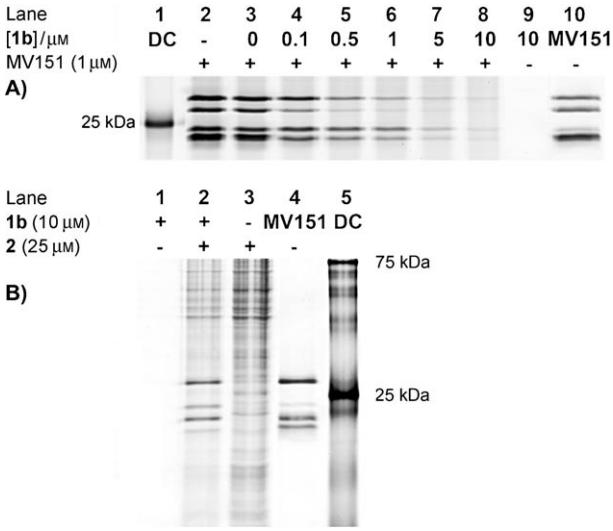
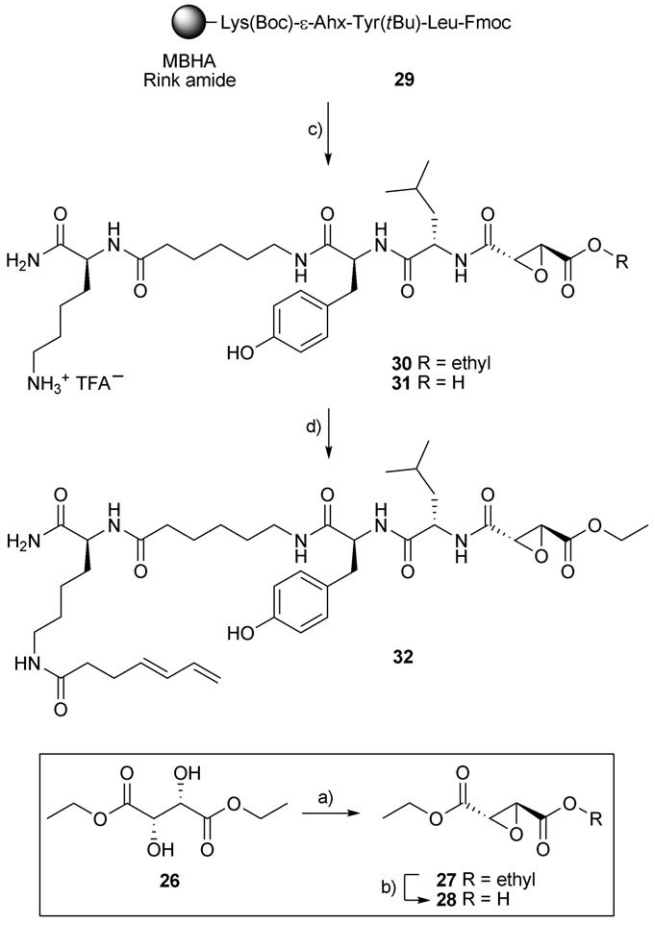


Figure 6. In situ labeling of proteasome activity with the diene-derivatized probe **1 b**. Living EL-4 cells were exposed for 2 h at 37 °C either to the indicated concentrations of the probe **1 b** or to MV151 (1 μM) as a control, before being harvested and lysed. A) The lysates (10 μg total protein per reaction) were exposed to MV151 (1 μM) for 1 h at 37 °C. B) The lysates (20 μg total protein per reaction) were subjected to denaturation, masking of cysteine residues, and c/m precipitation before being exposed to the BODIPY-(TMR)-maleimide **2** (25 μM) overnight at 37 °C (pH 6.0). Labeled proteins were resolved on SDS-PAGE and detected by fluorescence readout.

The activity of the proteasome β-subunits was next labeled by use of the following Diels–Alder ligation strategy. Viable EL-4 cells were treated with probe **1 b** (10 μM) for 2 hours, after which the cells were harvested and lysed. The cell lysates were subjected to the Diels–Alder protocol with the maleimide **2** as before (Figure 6 B). In lysates from cells treated with **1 b** (lane 2), fluorescent bands can be distinguished that are not

present in lysates from untreated cells (lane 3) and correspond to the active proteasome β-subunits labeled by MV151 (lane 4). These results reveal specific labeling of proteasome activity in situ. In comparison with the procedures in which cell extracts were exposed to the diene-functionalized probe (Figures 3–5), exposure of cells prior to cell lysis seems to give a small decrease in background labeling.

In order to demonstrate the applicability of the Diels–Alder strategy in ABPP further, we also employed the procedure for labeling of cysteine proteases of the papain family. We therefore synthesized the diene-functionalized probe **32**, derived from the activity-based cysteine cathepsin probe DCG-04[48] (Scheme 3). The synthesis of probe **32** involved the generation of peptidic recognition element **29** and coupling of epoxysuccinate warhead **28** by solid-phase peptide chemistry, cleavage from the resin and deprotection to give compound **30**, and finally coupling of the lysine ε-amine to the diene-functionalized activated ester **11 b** to afford the diene-derivatized DCG-04 analogue **32**.



Scheme 3. Synthesis of the diene-functionalized cathepsin probe. Reagents and conditions: a) i: HBr in AcOH (33%), 0 °C, 15 min, then RT, o/n, ii: AcCl, EtOH, reflux, 3.5 h, iii: DBU, Et_2O, 0 °C, 4.5 h, 56%; b) KOH, EtOH, 0 °C, 3 h, then RT, 2 h, 86%; c) i: piperidine/NMP (1:4, *v/v*), RT, 30 min, ii: **28**, DiPEA, BOP, NMP, RT, o/n, iii: TFA/TIS/H_2O (95:2.5:2.5, *v/v/v*), RT, 2 h, **30** 67%, **31** 33%; d) **11 b**, DiPEA, DCE/DMF, RT, o/n, 76%.

The inhibitory potency of the probe **32** was evaluated in a competition assay against the fluorescent activity-based cysteine cathepsin probe N_3-BODIPY(TMR)-DCG-04 (Figure 7 A).[49]

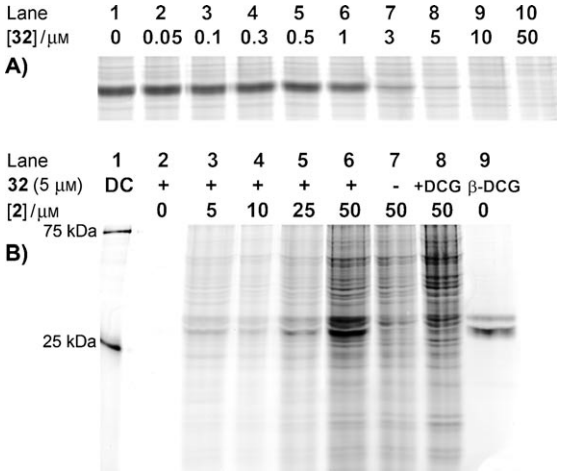


Figure 7. Labeling of cathepsin activity with the aid of the diene-derivatized DCG-04 analogue **32**. RAW cell lysates (50 μg total protein per reaction) were exposed to the indicated concentrations of the probe **32** for 1 h at 37 °C. As a control, lysates were exposed either to **32** in the presence of DCG-04 (+DCG, 25 μM) or to N_3-BODIPY(TMR)-DCG-04 (β-DCG, 0.5 μM). The lysates were next either: A) exposed to N_3-BODIPY(TMR)-DCG-04 (0.5 μM) for 1 h at 37 °C to label residual cathepsin activity, or B) subjected to denaturation, masking of cysteine residues, and Diels–Alder ligation with the indicated concentrations of the BODIPY(TMR)-maleimide **2** overnight at 37 °C (pH 6.0). Labeled proteins were resolved on SDS-PAGE and detected by fluorescence readout.

RAW cell extracts were exposed to increasing concentrations of compound **32** and then to N_3-BODIPY(TMR)-DCG-04 in order to label residual cathepsin activity. At increasing concentrations of the diene-derivatized DCG-04 analogue **32** the intensity of labeling by the fluorescent probe was reduced, demonstrating that probe **32** is able to inhibit the activity of cysteine cathepsins in vitro.

The diene-functionalized cathepsin probe **32** was then used in a Diels–Alder ligation procedure. Cell extracts were treated with probe **32** (5 μM) for 1 hour at 37 °C and subjected to the Diels–Alder ligation protocol with increasing concentrations of maleimide tag **2** (Figure 7 B). As before, the intensities both of specific and of background fluorescence were dependent on the concentration of the dienophile. The labeling profile shows two bands after treatment with probe **32** (lanes 3–6) that are not visible in non-treated samples (lane 7). The labeling corresponds to labeling by N_3-BODIPY(TMR)-DCG-04 (lane 9) and was blocked by addition of an excess of the nonfluorescent cathepsin probe DCG-04 (lane 8). This confirms that the two bands indeed involve labeling of active cathepsins by means of the two-step Diels–Alder ligation procedure. From the observed molecular weights and a previously reported DCG-04 labeling profile in RAW cell extracts,[50] we assume that these two labeled proteases are cathepsin Z and cathepsin B.

Finally, the orthogonality of the two-step Diels–Alder procedure with respect to the Staudinger–Bertozzi ligation was es-

tablished by performing both procedures in one experiment for the independent labeling of different proteolytic activities. HEK cell lysates were first exposed to the azide-derivatized proteasome probe **3**, which is selective for the β1 subunit,[51] followed by the diene-containing probe **1 b** to label the β2 and β5 proteasome subunits. The cell extracts were then treated with the biotin-functionalized phosphine reagent **4** for Staudinger ligation,[52] prior to denaturation, cysteine masking, and Diels–Alder reaction with fluorescent maleimide **2**. After SDS-PAGE analysis, the labeled proteins were visualized by fluorescence scanning and streptavidin Western blotting (Figure 8).

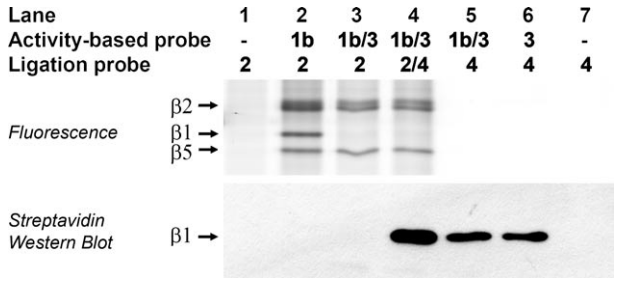


Figure 8. Diels–Alder and Staudinger ligations can be performed in the same sample. HEK cell lysates (50 μg total protein per reaction) were first exposed to the azide-derivatized β1-selective proteasome probe **3** (5 μM) for 1 h at 37 °C, then to the diene-functionalized probe **1 b** (5 μM) for 1 h at 37 °C, and then to the biotin-phosphine **4** (100 μM) for 1 h at 37 °C. After protein denaturation, masking of cysteine residues, and c/m precipitation, Diels–Alder ligation was performed with the BODIPY(TMR)-maleimide **2** (25 μM) overnight at 37 °C (pH 6.0). The labeled proteins were resolved by SDS-PAGE and detected by fluorescence readout followed by streptavidin Western blotting. Proteasome β-subunits are designated on the basis of known labeling profiles in HEK cell extracts.[53]

When the ligation was performed with maleimide **2** only, fluorescent labeling of the diene-modified active proteasome β-subunits was observed (upper panel, lane 2). As would be expected, preincubation with probe **3** completely blocked fluorescent labeling of the β1 subunit because this subunit was now targeted by azide-derivatized probe **3** (upper panel, lane 3). Ligation with Staudinger reagent **4** only gave selective labeling of the β1 subunit (lower panel, lanes 5 and 6).

Most importantly, the labeling profile obtained by the combination of the two ligation methods in one sample (lane 4) is very similar to that obtained by the separate methods (lanes 3 and 5). By this combined strategy, β1 activity was selectively labeled by Staudinger–Bertozzi ligation (lower panel) whereas at the same time the β2 and β5 subunits were fluorescently labeled by Diels–Alder ligation (upper panel). This experiment shows that the Diels–Alder ligation is fully compatible with Staudinger ligation conditions and that there is no cross-reactivity between the ligation reagents and the probes used for both methods.

Conclusions

In conclusion, we have developed a Diels–Alder-based ABPP strategy that has been successfully used to monitor the activity of endogenously expressed proteases in cellular extracts.[54,55] A drawback to the procedure described here is the need to mask cysteine residues prior to dienophile addition, which precludes in vivo application. A potential solution to this problem would be the use of a dienophile that is not a Michael acceptor and should thus be much more selective. Efforts to achieve this goal are currently being made in our lab. We feel that the Diels–Alder strategy presented here represents a useful alternative to click and Staudinger–Bertozzi ligation approaches. An attractive aspect of the Diels–Alder approach is that it is fully orthogonal with respect to the Staudinger ligation and can be performed in the same sample. This provides the potential to modify two different biomolecules—two enzymatic activities or a metabolite and an enzyme, for example—with different labels in a single experiment and thereby to study their roles or behavior simultaneously.

Experimental Section

General: All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (purum), ethyl acetate (EtOAc, puriss.), diethyl ether (Et_2O), and light petroleum ether (PetEt, puriss.) were obtained from Riedel–de Haën. Dichloroethane (DCE), dichloromethane (DCM), dimethyl formamide (DMF), and dioxane (Biosolve) were stored over molecular sieves (4 Å). Methanol (MeOH) and *N*-methylpyrrolidone (NMP) were obtained from Biosolve. Reactions were monitored by TLC analysis (DC-Alufolien, Merck, Kieselgel60, F254) with detection variously by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 g L^{-1}) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g L^{-1}) in sulfuric acid (10%) 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 (Screening Devices, 0.040–0.063 nm). LC/MS analysis was performed with a LCQ Advantage Max (Thermo Finnegan) instrument with a Gemini C18 column (Phenomene x). The applied buffers were H_2O (A), MeCN (B), and aq. TFA (1.0%, C). ^1H and ^{13}C APT-NMR spectra were recorded with Jeol JNM-FX-200 (200/50) or Bruker AV400 (400/100 MHz) instruments with a pulsed field gradient accessory. Chemical shifts (δ) are given in ppm relative to tetramethylsilane as internal standard. Coupling constants are given in Hz. All ^{13}C-APT spectra presented are proton-decoupled. In-gel fluorescence readout was measured with a Typhoon Variable Mode Imager (Amersham Biosciences) and with Cy3/TAMRA settings (excitation wavelength 532 nm, emission wavelength 580 nm). Western blotting was performed as indicated. Markers used were Dual Color protein standard (DC) and Biotinylated protein marker (BM).

Ethyl (*E*)-hepta-4,6-dienoate (6): Triethyl orthoacetate (2.8 mL, 15 mmol, 5.0 equiv) and propionic acid (one drop) were added to a solution of penta-1,4-dien-3-ol (**5**, 0.29 mL, 3.0 mmol) in toluene. The reaction mixture was stirred under reflux for 3 h, 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%). ^1H NMR (200 MHz, $CDCl_3$): $\delta = 6.44–5.99$ (m, 2H), 5.81–5.60 (m, 1H), 5.05 (ddd, $J = 13.29, 11.48, 1.62$ Hz, 2H), 4.14 (q, $J = 7.14, 7.14, 7.13$ Hz, 2H), 2.37 (s, 4H), 1.25 ppm (t, $J = 7.14, 7.14$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 172.12, 136.46, 132.18, 131.55, 115.05, 59.72, 33.31, 27.37, 13.75$ ppm.

2-(Anthracen-9-yl)acetonitrile (8): A solution of 9-(chloromethyl)-anthracene (**7**, 2.27 g, 10 mmol) in DMSO (15 mL) was heated to

60 °C, followed by the 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 h and was then allowed to cool to room temperature and stirred overnight. After addition of H_2O (40 mL), the mixture was filtered and washed with H_2O, and the residue was dried in vacuo to yield compound **8** as yellow crystals (2.04 g, 9.4 mmol, 94%). ^{1}H NMR (200 MHz, $CDCL_3$): δ = 8.53 (s, 1H), 8.13 (ddd, J_1 = 20.68 Hz, J_2 = 8.43 Hz, J_3 = 0.42 Hz, 4H), 7.59 (dddd, J_1 = 14.79 Hz, J_2 = 7.67 Hz, J_3 = 6.63 Hz, J_4 = 1.08 Hz, 4H), 4.61 ppm (s, 2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 131.21, 129.33, 128.66, 127.07, 125.13, 122.77, 117.65, 77.62, 77.00, 76.35, 15.96 ppm.

(*E*)-Hepta-4,6-dienoic acid (10b): Ethyl (*E*)-hepta-4,6-dienoate (**6**, 0.43 g, 2.8 mmol) was dissolved in a mixture of 1,4-dioxane/MeOH/1 M NaOH (1:1:1, *v*/*v*/*v*) and the mixture was stirred for 1 h. After addition of DCM, the mixture was washed with a saturated $NaHCO_3$ solution (1×). The aqueous layer was then acidified with HCl (1 M), followed by extraction with DCM (3×). The combined organic layers were dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by column chromatography (PetEt→25% EtOAc in PetEt), yielding hepta-4,6-dienoic acid (**10b**, 0.24 g, 1.9 mmol, 68%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 6.43–6.00 (m, 2H), 5.78–5.61 (m, 1H), 5.26–4.87 (m, 2H), 2.56–2.33 ppm (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 179.26, 136.67, 132.04, 115.74, 33.47, 27.26 ppm.

2-(Anthracen-9-yl)acetic acid (10c): 2-(Anthracen-9-yl)acetonitrile (**8**, 2.04 g, 9.4 mmol) was dissolved in 2-methoxyethanol (15 mL), and NaOH (0.94 g, 23.5 mmol, 2.5 equiv) was added. The reaction mixture was heated at reflux for 3 h before addition of H_2O (60 mL). The mixture was then washed with Et_2O (2×) and the aqueous layer was acidified to pH 2 with HCl (1 M), after which precipitation was allowed to take place overnight. The residue was filtered, washed with H_2O, dried in vacuo, and concentrated in the presence of toluene to give the acid **10c** (1.24 g, 5.2 mmol, 56%).

2-((2*E*,4*E*)-Hexa-2,4-dienyloxy)acetic acid (10d): (2*E*,4*E*)-Hexa-2,4-dien-1-ol (**9**, 3.0 g, 30 mmol) was added under argon to a suspension of NaH (60% in mineral oil, 2.5 g, 61 mmol, 2 equiv) in freshly distilled THF. The reaction mixture was stirred for 30 min, followed by addition of bromoacetic acid (4.2 g, 30 mmol, 1 equiv) and stirring overnight under argon. The reaction was quenched with aqueous KOH solution (3 M). The aqueous layer was then washed with Et_2O (2×), acidified with aqueous HCl solution (6 M), and extracted with $CHCl_3$ (3×). The combined organic layers were dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography (DCM + 1% AcOH→5% MeOH in DCM + 1% AcOH) yielded the acid **10d** (3.4 g, 22 mmol, 73%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 11.58 (s, 1H), 6.20 (dd, J = 15.20, 10.45 Hz, 1H), 6.04 (ddd, J = 14.73, 10.47, 1.34 Hz, 1H), 5.71 (qd, J = 13.57, 6.71, 6.70, 6.70 Hz, 1H), 5.62–5.53 (m, 1H), 4.08 (t, J = 3.20, 3.20 Hz, 4H), 1.73 ppm (d, J = 7.04 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 175.58, 134.55, 130.60, 130.27, 124.61, 71.51, 65.80, 17.75 ppm.

3-(2-Furyl)propanoyl-OSu (11a): A solution of 3-(2-furyl)propanoic acid (**10a**, 0.98 g, 7.0 mmol) in DCE/DMF was put under argon, and HOSu (3.2 g, 28 mmol, 4.0 equiv) and EDC·HCl (5.3 g, 28 mmol, 4.0 equiv) were added. The reaction mixture was stirred under argon overnight, after which EtOAc was added, and the mixture was washed with aqueous HCl solution (1 M, 2×). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo and the crude product was purified by column chromatography (PetEt→40% EtOAc in PetEt), yielding the OSu-ester **11a** (1.2 g, 4.9 mmol, 71%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.38–7.28 (m, 1H), 6.29 (dd, J = 3.18, 1.89 Hz, 1H), 6.11 (dd, J = 3.20, 0.80 Hz, 1H), 3.14–2.90 (m, 4H), 2.84 ppm (s, 4H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 169.04, 167.46, 152.32, 141.50, 109.99, 105.60, 29.09, 25.14, 22.51 ppm.

(*E*)-Hepta-4,6-dienoyl-OSu (11b): (*E*)-Hepta-4,6-dienoic acid (**10b**, 0.17 g, 1.3 mmol) was subjected to the same procedure as described above for **11a**, giving the OSu-ester **11b** (0.23 g, 1.0 mmol, 78%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.30 (td, J = 16.90, 10.22, 10.22 Hz, 1H), 6.14 (dd, J = 15.11, 10.51 Hz, 1H), 5.76–5.65 (m, 1H), 5.09 (dd, J = 51.12, 13.45 Hz, 2H), 2.80 (s, 4H), 2.71 (t, J = 7.39, 7.39 Hz, 2H), 2.54–2.47 ppm (m, 2H).

2-(Anthracen-9-yl)acetyl-OSu (11c): 2-(Anthracen-9-yl)acetic acid (**10c**, 0.86 g, 3.6 mmol) was subjected to the same procedure as described above for **11a**, giving the OSu-ester **11c** (0.98 g, 2.9 mmol, 81%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 8.48 (s, 1H), 8.22 (d, J = 9.12 Hz, 2H), 8.03 (d, J = 8.4 Hz, 2H), 7.64–7.45 (m, 4H), 4.92 (s, 2H), 2.76 ppm (s, 4H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 168.89, 166.58, 131.25, 130.43, 129.13, 128.16, 126.67, 125.01, 123.55, 122.85, 30.30, 25.27 ppm.

2-((2*E*,4*E*)-Hexa-2,4-dienyloxy)acetyl-OSu (11d): 2-((2*E*,4*E*)-Hexa-2,4-dienyloxy)acetic acid (**10d**, 3.42 g, 21.9 mmol) was subjected to the same procedure as described above for **11a**, giving the OSu-ester **11d** (4.41 g, 17.4 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.26 (dd, J = 15.22, 10.44 Hz, 1H), 6.07 (dd, J = 14.09, 11.48 Hz, 1H), 5.81–5.71 (m, 1H), 5.66–5.53 (m, 1H), 4.41 (s, 2H), 4.16 (d, J = 6.60 Hz, 2H), 2.85 (s, 4H), 1.77 ppm (d, J = 6.68 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 168.73, 165.91, 135.31, 131.28, 130.35, 124.34, 72.06, 64.36, 25.52, 18.08 ppm.

MBHA-HMPB-Thr(*t*Bu)-Ile-Ile-Boc (12): 4-Methylbenzhydrylamine (MBHA) resin (5.0 g, 6.0 mmol) was coupled to 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) linker (4.3 g, 18 mmol, 3 equiv) in the presence of BOP (8.0 g, 18 mmol, 3 equiv) and DiPEA (6.3 mL, 36 mmol, 6 equiv) in NMP. After overnight shaking, the resin was washed with NMP (3×) and DCM (3×). The coupling was monitored for completion by the Kaiser test. The resulting MBHA-HMPB resin (~6.0 mmol) was coevaporated with DCE (2×). The resin was then condensed with Fmoc-Thr(*t*Bu)-OH (7.2 g, 18 mmol, 3 equiv) in the presence of DIC (3.1 mL, 20 mmol, 3.3 equiv) and DMAP (0.11 g, 0.90 mmol, 15 mol%) in DCM for 2 h. After the resin had been filtered and washed with DCM, the condensation cycle was repeated. The resin was then washed with NMP (2×), DCM (2×), and ether (2×) and dried in vacuo overnight. The loading of the resin was determined to be 0.43 mmol g^{-1} by spectrophotometric analysis. The obtained resin was washed with DCM and subjected to two coupling cycles with Fmoc-Ile-OH and Boc-Ile-OH, respectively, as follows: after deprotection with piperidine in NMP (20%, 20 min), the resin was washed with NMP (2×) and DCM (2×) and coupled to Fmoc-Ile-OH (5.3 g, 15 mmol, 2.5 equiv) or Boc-Ile-OH (3.5 g, 15 mmol, 2.5 equiv) in the presence of BOP (6.6 g, 15 mmol, 2.5 equiv) and DiPEA (3.1 mL, 18 mmol, 3 equiv) in NMP. The reaction mixture was shaken overnight or for 5 h, respectively, followed by washing with NMP (3×) and DCM (3×). Couplings were monitored for completion by the Kaiser test. Washing with ether and drying in vacuo overnight yielded the fully protected resin tripeptide **12**.

Boc-Ile-Ile-Thr(*t*Bu)-OH (13): MBHA-HMPB-Thr(*t*Bu)-Ile-Ile-Boc resin (**12**, ~6.0 mmol) was subjected to mild acidic cleavage with TFA in DCM (1%, 10 min, 6×). The collected fractions were concentrated in the presence of toluene to yield the crude tripeptide **13**, which was used without further purification. LC/MS analysis: R_f 8.58 min (linear gradient 10→90% B in 15 min); *m/z*: 502.3 $[M+H]^+$, 524.5 $[M+Na]^+$, 1025.3 $[2M+Na]^+$, 1521.0 $[3M+H_2O]^+$.

Boc-Ile-Ile-Thr(*t*Bu)-methyl ester (14): TMS diazomethane (2 M in hexane, 24 mL, 48 mmol, 8 equiv) was added in four equal portions over 1.5 h to a solution of crude Boc-Ile-Ile-Thr(*t*Bu)-OH (**13**, ~3.0 g, ~6.0 mmol) in MeOH/toluene (1:1, *v/v*, 25 mL). The reaction mixture was then stirred for 2 h, before being concentrated in the presence of toluene. Purification by column chromatography (DCM→0.5% MeOH in DCM) yielded the partially purified product **14** (pure fraction 0.55 g, 1.1 mmol) as a colorless foam. The impure fraction (2.1 g, ~4.1 mmol) was dissolved in MeOH/toluene (1:1, *v/v*) and TMS diazomethane (2 M in diethyl ether, 4.1 mL, 8.2 mmol, 2 equiv) was added. After 30 s, the reaction mixture was concentrated in vacuo. Purification by column chromatography (DCM→0.5% MeOH in DCM) yielded a second batch of the title compound **14** (total yield 2.0 g, 3.8 mmol, 64% from MBHA-HMPB resin). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.51 (d, J = 8.12 Hz, 1H), 6.43 (d, J = 9.25 Hz, 2H), 4.48 (dd, J = 9.09, 1.61 Hz, 1H), 4.40 (dd, J = 8.56, 6.49 Hz, 1H), 4.24 (dq, J = 6.21, 6.16, 6.16, 1.59 Hz, 1H), 3.70 (s, 3H), 3.98–3.88 (m, 1H), 2.07–1.97 (m, 2H), 1.92–1.81 (m, 4H), 1.44 (s, 9H), 1.14 (d, J = 6.28 Hz, 3H), 1.11 (s, 9H), 0.99–0.86 ppm (m, 12H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 171.05, 170.86, 67.21, 57.83, 57.50, 57.46, 52.14, 37.86, 37.23, 37.22, 37.20, 37.03, 28.32, 28.29, 24.88, 24.79, 24.76, 20.92, 15.53, 15.08, 11.41, 11.31 ppm; LC/MS analysis: R_f 9.68 min (linear gradient 10→90% B in 15 min); *m/z*: 538.5 $[M+Na]^+$, 1053.5 $[2M+Na]^+$.

Boc-Ile-Ile-Thr(*t*Bu)-hydrazide (15): Hydrazine monohydrate (11.1 mL, 228 mmol, 60 equiv) was added to a solution of the fully protected tripeptide methyl ester **14** (2.0 g, 3.8 mmol) in MeOH and the reaction mixture was heated at reflux overnight, before being concentrated in the presence of toluene. The white precipitate was filtered and washed with MeOH to give the hydrazide **15** (0.88 g, 1.7 mmol, 45%). The filtrate was concentrated in the presence of toluene and recrystallized from toluene/MeOH to yield a second batch of the product **15** (0.61 g, 1.2 mmol, 31%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.50 (s, 1H), 7.79–7.71 (m, 1H), 7.54–7.45 (m, 2H), 6.03–5.95 (m, 2H), 4.47–4.35 (m, 1H), 4.28 (d, J = 7.27 Hz, 1H), 4.08 (d, J = 1.20 Hz, 1H), 3.94 (d, J = 6.40 Hz, 1H), 1.97–1.72 (m, 4H), 1.63–1.49 (m, 2H), 1.45 (s, 9H), 1.21 (s, 9H), 1.08 (d, J = 6.28 Hz, 3H), 0.92 ppm (dd, J = 14.72, 7.62 Hz, 12H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 172.90, 171.27, 169.84, 79.50, 74.62, 66.18, 58.82, 58.74, 57.90, 57.84, 57.74, 56.79, 36.38, 36.14, 27.64, 27.51, 24.32, 24.24, 18.04, 14.96, 14.85, 14.75, 10.36 ppm; LC/MS analysis: R_f 6.39 min (linear gradient 10→90% B in 15 min); *m/z*: 516.1 $[M+H]^+$, 460.1 $[M-t\text{Bu}+H]^+$.

Boc-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyloxirane (16): A solution of Boc-Ile-Ile-Thr(*t*Bu)-hydrazide (**15**, 0.52 g, 1.0 mmol) in DMF/EtOAc (1:1, *v/v*) was cooled under argon to a temperature of −30 °C [N_2 (l)+DCE]. After addition of HCl (4 M in dioxane, 0.70 mL, 2.8 mmol, 2.8 equiv) and *t*BuONO (0.13 mL, 1.1 mmol, 1.1 equiv), the reaction mixture was stirred under argon for 1 h. A solution of leucinyl-(*R*)-2-methyloxirane TFA salt (**17**,[45] 1.1 mmol, 1.1 equiv) and DiPEA (0.19 mL, 1.1 mmol, 1.1 equiv) in DMF was then added to the acyl azide reaction mixture at −30 °C. After addition of more DiPEA (0.66 mL, 3.8 mmol, 3.8 equiv), the reaction mixture was stirred overnight under argon (−30 °C→RT). The mixture was then extracted with EtOAc and washed with H_2O (3×), and the combined organic layers were dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography (PetEt→25% EtOAc in PetEt) afforded the fully protected epoxyketone **16** as white crystals (058 g, 0.89 mmol, 89%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.64 (d, J = 7.06 Hz, 1H), 6.85 (d, J = 5.84 Hz, 1H), 6.46 (d, J = 8.57 Hz, 1H), 5.07 (d, J = 8.31 Hz, 1H), 4.54–4.38 (m, 1H), 4.37–4.25 (m, 2H), 4.20–4.06 (m, 1H), 3.99–3.87 (m, 1H), 3.13 (dd, J = 93.10, 5.02 Hz, 2H), 1.97–1.53 (m, 5H), 1.52 (s, 3H), 1.44 (s, 9H), 1.28 (s, 9H), 1.06 (d, J = 6.44 Hz, 3H), 1.37–1.02 (m, 4H), 0.99–0.82 ppm (m, 18H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 208.01, 171.74, 171.13, 169.52, 155.91, 79.24, 75.27, 66.48, 59.11, 59.00, 57.29, 56.77, 52.35, 50.62, 39.64, 37.27, 37.06, 28.21, 27.96, 25.30, 24.90, 24.60, 23.26, 21.20, 16.62, 16.38, 15.35, 15.20, 11.23, 11.04 ppm; LC/MS analysis: R_f 11.44 min (linear gradient 10→90% B in 15 min); *m/z*: 599.13 $[M-t\text{Bu}+H]^+$, 655.3 $[M+H]^+$, 677.3 $[M+Na]^+$, 1331.4 $[2M+Na]^+$.

3-(2-Furyl)propanoyl-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyloxirane (1a): The tetrapeptide epoxyketone **16** (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 TFA salt of the deprotected compound was dissolved in DCE/DMF and neutralized with DiPEA (0.17 mL, 1.0 mmol, 4.0 equiv), followed by addition of a solution of the OSu-ester **11a** (0.18 g, 0.75 mmol, 3.0 equiv) in DCE/DMF. The reaction mixture was stirred under argon for 1.5 h. DCM was then added, and the mixture was washed with H_2O (1×). The aqueous layer was extracted with EtOAc (1×), 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 the title compound **1a** (0.13 g, 0.20 mmol, 82%) as a white solid. ^{1}H NMR (200 MHz, $CDCl_3$/MeOD): δ = 8.34 (d, J = 9.35 Hz, 1H), 7.87 (d, J = 7.38 Hz, 1H), 7.79 (d, J = 7.88 Hz, 1H), 7.26 (s, 1H), 7.15 (d, J = 8.80 Hz, 1H), 6.24 (dd, J = 3.12, 1.88 Hz, 1H), 6.00 (dd, J = 3.18, 0.71 Hz, 1H), 4.80–4.50 (m, 3H), 4.28–4.01 (m, 2H), 3.29 (d, J = 4.77 Hz, 1H), 3.03–2.90 (m, 2H), 2.88 (d, J = 4.86 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 = 6.20 Hz, 3H), 0.93–0.71 ppm (m, 18H); LC/MS analysis: R_f 8.50 min (linear gradient 10→90% B in 15 min); *m/z*: 621.3 $[M+H]^+$, 1241.3 $[2M+H]^+$; HRMS: calcd. for $[C_{32}H_{52}N_4O_8Na]^+$ 643.36774; found: 643.36751.

(*E*)-Hepta-4,6-dienoyl-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyloxirane (1b): The same procedure as described above (for **1a**) was used with the OSu-ester **11b** (0.13 g, 0.60 mmol, 3.0 equiv) to yield the title compound **1b** (78 mg, 0.13 mmol, 64%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 8.27 (d, J = 8.08 Hz, 1H), 7.85 (d, J = 7.86 Hz, 1H), 7.63 (d, J = 6.91 Hz, 1H), 7.01 (d, J = 8.92 Hz, 1H), 6.37–6.00 (m, 2H), 5.66 (td, J = 14.52, 6.19, 6.19 Hz, 1H), 5.03 (ddd, J = 13.25, 11.12, 1.58 Hz, 2H), 4.79–4.49 (m, 3H), 4.28–4.08 (m, 2H), 3.10 (dd, J = 80.88, 4.92 Hz, 2H), 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 = 6.45 Hz, 3H), 0.96–0.76 ppm (m, 18H); LC/MS analysis: R_f 8.94 min (linear gradient 10→90% B in 15 min); *m/z*: 607.3 $[M+H]^+$, 1213.3 $[2M+H]^+$, 1819.1 $[3M+H]^+$; HRMS: calcd. for $[C_{32}H_{54}N_4O_7Na]^+$ 629.38847; found: 629.38831.

2-(Anthracen-9-yl)acetyl-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyloxirane (1c): The same procedure as described above (for **1a**) with the OSu-ester **11c** (0.25 g, 0.75 mmol, 3.0 equiv) afforded the title compound **1c** (70 mg, 0.098 mmol, 39%). ^{1}H NMR (200 MHz, $CDCl_3$/MeOD): δ = 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), 3.09 (dd, J = 79.9, 4.94 Hz, 2H), 1.80–1.24 (m, 8H), 1.50 (s, 3H), 1.00–0.64 ppm (m, 22H); LC/MS analysis: R_f 10.17 min (linear gradient 10→90% B in 15 min); *m/z*: 717.3 $[M+H]^+$, 1455.4 $[2M+Na]^+$; HRMS: calcd. for $[C_{41}H_{56}N_4O_7Na]^+$ 739.40412; found: 739.40443.

2-(Hexa-2,4-dienyloxy)acetamido-Ile-Ile-Thr-leucinyl-2-methyloxirane (1d): The same procedure as described above (for **1a**) with the OSu-ester **11d** (0.21 g, 0.81 mmol, 3.2 equiv) afforded the title

compound **1 d** (0.16 g, 0.25 mmol, quant.) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.43 (d, J = 8.99 Hz, 1H), 7.94 (d, J = 7.41 Hz, 1H), 7.45 (d, J = 9.08 Hz, 1H), 7.30 (s, 1H), 6.19 (dd, J = 15.16, 10.43 Hz, 1H), 6.05 (dd, J = 14.02, 11.42 Hz, 1H), 5.79–5.66 (m, 1H), 5.63–5.54 (m, 1H), 4.86–4.69 (m, 1H), 4.55 (td, J = 9.77, 5.65, 5.65 Hz, 3H), 4.18–3.99 (m, 1H), 3.97 (s, 2H), 3.89 (d, J = 15.42 Hz, 2H), 3.12 (dd, J = 182.56, 4.90 Hz, 2H), 1.76 (d, J = 6.73 Hz, 2H), 1.71–1.58 (m, 2H), 1.52 (s, 3H), 1.43–1.17 (m, 2H), 1.11 (d, J = 6.29 Hz, 3H), 1.08–0.94 (m, 2H), 0.90 (d, J = 6.47 Hz, 6H), 0.87–0.75 ppm (m, 12H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 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 ppm; HRMS: calcd. for $[C_{33}H_{56}N_4O_8Na]^+$ 659.39904; found: 659.39896.

Methyl maleimido-*N*-carboxylate (19): A solution of maleimide (**18**, 0.99 g, 10 mmol) and *N*-methylmorpholine (1.1 mL, 10 mmol, 1.0 equiv) in EtOAc was cooled to 0 °C. Methyl chloroformate (0.80 mL, 10 mmol, 1.0 equiv) was added and the reaction mixture was stirred at 0 °C for 2 h. The mixture was then washed with saturated aqueous $NaHCO_3$ solution (3×) and the aqueous layer was extracted with EtOAc (1×). The combined organics were dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to yield the crude product **19** (0.80 g, 5.2 mmol, 52%), which was used without further purification. ^{1}H NMR (200 MHz, [D$_6$]acetone): δ = 7.05 (s, 2H), 3.89 ppm (s, 3H); ^{13}C NMR (50 MHz, [D$_6$]acetone): δ = 166.93, 148.80, 136.27, 54.12 ppm; LC/MS analysis: R_f 1.29 min (linear gradient 10→90% B in 15 min); *m/z*: 156.0 $[M+H]^+$.

1,2-Bis(2-azidoethoxy)ethane (21): Triethyleneglycol (**20**, 0.30 g, 2.0 mmol) was dissolved in DCM and put under argon, after which tosyl chloride (1.14 g, 6 mmol, 3 equiv), Et_3N (0.83 mL, 6.0 mmol, 3.0 equiv), and *N*,*N*-dimethyl-4-aminopyridine (12 mg, 0.1 mmol, 5 mol%) were added. After 16 h, the reaction mixture was washed with H_2O and the organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. NaN_3 (0.26 g, 4.0 mmol, 2.0 equiv) and tetrabutylammonium iodide (37 mg, 0.1 mmol, 5 mol%) were added to a solution of the resulting yellow oil in DMF. The reaction mixture was stirred for 16 h at 80 °C before being washed with saturated aqueous $NaHCO_3$. The organic layer was dried over $MgSO_4$, filtered, and concentrated to a yellow oil. Purification by column chromatography (toluene→15% EtOAc in toluene) afforded the bis-azide **21** as a colorless oil (0.31 g, 1.6 mmol, 78%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 3.68 (m, 8H), 3.39 ppm (t, J = 5.1 Hz, 4H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 70.3, 69.8, 50.3 ppm.

2-(2-(2-Azidoethoxy)ethoxy)ethanamine (22): An aqueous HCl solution (5%, 10 mL) was added to a cooled solution (0 °C) of 1,2-bis(2-azidoethoxy)ethane (**21**, 2.0 g, 10 mmol) in toluene (10 mL), after which triphenylphosphine (2.5 g, 9.5 mmol, 0.95 equiv) was added. The reaction mixture was allowed to warm to RT and stirred for 16 h, after which the aqueous layer was separated and concentrated in vacuo to yield the crude monoazide **22** (1.67 g, 7.9 mmol, 79%).

***tert*-Butyl 2-(2-(2-azidoethoxy)ethoxy)ethylcarbamate (23)**: Triethylamine (1.5 mL, 11 mmol, 2.0 equiv) and Boc_2O (1.3 g, 5.9 mmol, 1.1 equiv) were added to a solution of crude 2-[2-(2-azidoethoxy)ethoxy]ethanamine HCl salt (**22**, 1.5 g, 5.4 mmol) in DCM. 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 Boc-protected compound **23** (pure yield 1.3 g, 4.9 mmol, 90%) as a colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 5.07 (s, 1H), 3.73–3.59 (m, 6H), 3.58–3.50 (m, 2H), 3.45–3.24 (m, 6H), 1.45 ppm (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 154.91, 77.46, 69.36, 69.12, 68.96, 49.50, 39.28, 27.27 ppm; LC/MS analysis: R_f 7.00 min (linear gradient 10→90% B in 15 min); *m/z*: 274.8 $[M+H]^+$.

***tert*-Butyl 2-(2-(2-(2,5-dioxopyrrol-1-yl)ethoxy)ethoxy)ethylcarbamate (25)**: The Boc-protected azide **23** (1.0 g, 3.6 mmol) was dissolved in THF and triphenylphosphine (1.2 g, 4.4 mmol, 1.2 equiv) was added. The reaction mixture was stirred for 5 h, after which a few drops of H_2O were added. After the mixture had been stirred for an additional 2 h, toluene was added, and the mixture was extracted with HCl solution (1 M, 4×). The combined aqueous layers were made basic with saturated aqueous $NaHCO_3$ solution and KOH solution (3 M) and were extracted with DCM (3×) and EtOAc (3×). The combined organics were dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to give the crude partially protected amine **24** (0.65 g, 2.6 mmol, 72%); LC/MS analysis: R_f 3.86 min (linear gradient 10→90% B in 15 min); *m/z*: 249.0 $[M+H]^+$, 496.9 $[2M+H]^+$.

The crude compound **24** was then dissolved in saturated aqueous $NaHCO_3$ solution and cooled to 0 °C, and the activated maleimide **19** (0.52 g crude, ~3.4 mmol, 1.3 equiv) was added. The reaction mixture was stirred at 0 °C for 30 min and then at room temperature for 45 min. The mixture was extracted with chloroform (3×) and the combined organics were dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography (PetEt→70% EtOAc in PetEt) yielded the (Boc)-amine-PEG-maleimide **25** (0.36 g, 1.1 mmol, 31% over two steps). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 6.72 (s, 2H), 5.04 (s, 1H), 3.85–3.41 (m, 10H), 3.39–3.20 (m, 2H), 1.45 ppm (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 170.37, 155.66, 133.86, 78.70, 69.81, 69.51, 67.42, 40.00, 36.67, 28.06 ppm; LC/MS analysis: R_f 6.37 min (linear gradient 10→90% B in 15 min); *m/z*: 328.9 $[M+H]^+$, 351.1 $[M+Na]^+$.

(*N*)-Bodipy(TMR)-2-(2-(2-(2,5-dioxopyrrol-1-yl)ethoxy)ethoxy)-ethylamine (2): The Boc-protected compound **25** (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 TFA salt of the deprotected compound was dissolved in DCE and neutralized with DiPEA (0.20 mL, 1.2 mmol, 6.0 equiv), followed by addition of BODIPY(TMR)-OSu[46] (99 mg, 0.20 mmol, 1.0 equiv). The reaction mixture was stirred under argon overnight. DCE was added, and the mixture was washed with H_2O (1×). The aqueous layer was extracted with EtOAc (2×), 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 (toluene→30% acetone in toluene) afforded the title compound **2** (41 mg, 0.068 mmol, 34%) as a purple solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.89–7.83 (m, 2H), 7.07 (s, 1H), 6.99–6.95 (m, 2H), 6.94 (d, J = 4.07 Hz, 1H), 6.61 (s, 2H), 6.53 (d, J = 4.04 Hz, 1H), 6.35 (t, J = 4.93, 4.93 Hz, 1H), 3.85 (s, 3H), 3.68 (t, J = 5.49, 5.49 Hz, 2H), 3.58 (t, J = 5.39, 5.39 Hz, 2H), 3.52–3.41 (m, 6H), 3.41–3.35 (m, 2H), 2.77 (t, J = 7.44, 7.44 Hz, 2H), 2.53 (s, 3H), 2.36 (t, J = 7.45, 7.45 Hz, 2H), 2.21 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 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 ppm; LC/MS analysis: R_f 8.55 min (linear gradient 10→90% B in 15 min); *m/z*: 589.4 $[M-F]^+$, 1216.8 $[2M+H]^+$.

Diethyl (2*S*,3*S*)-oxirane-2,3-dicarboxylate (27): (−)-Diethyl D-tartrate (**26**, 29 g, 0.14 mol) was cooled to 0 °C, after which a solution of HBr in acetic acid (33%, 120 mL) was added dropwise over

45 min. After complete addition, the reaction mixture was stirred at 0 °C for 15 min and then at RT overnight. Next, the mixture was poured onto crushed ice/H_2O (300 mL) and extracted with Et_2O (3×). The combined organics were washed with H_2O (3×), dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. Remaining solvents were concentrated in the presence of toluene. The crude oil was dissolved in EtOH, and acetyl chloride (5.1 mL, 0.07 mol, 0.5 equiv) was added. The reaction mixture was stirred under reflux for 3.5 h before being concentrated at a temperature of 30 °C. The remaining yellowish oil was dissolved in Et_2O (175 mL), cooled to 0 °C, and put under argon. A solution of DBU (21 mL, 0.14 mol, 1.0 equiv) in Et_2O (90 mL) was added dropwise over 100 min. The reaction mixture was then stirred at 0 °C for 1 h, more DBU (2.1 mL, 14 mmol, 0.1 equiv) was added, and the reaction mixture was stirred for an additional 2 h, followed by addition of H_2O. The mixture was washed with $KHSO_4$ solution (1 M) and H_2O and the organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography (PEtEt→15% EtOAc in PEtEt) yielded the title compound **27** (15 g, 79 mmol, 56% over 3 steps). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.32–4.22 (m, 4H), 3.66 (s, 2H), 1.32 ppm (t, J = 7.15, 7.15 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.69, 62.14, 51.96, 13.96 ppm.

(2*S*,3*S*)-3-(Ethoxycarbonyl)oxirane-2-carboxylic acid (28): A solution of compound **27** (14 g, 76 mmol) in absolute EtOH (200 mL) was cooled to 0 °C and a solution of KOH (5.0 g, 76 mmol, 1.0 equiv) in absolute EtOH (100 mL) was added dropwise over 20 min. The reaction mixture was next stirred at 0 °C for 3 h and then at RT for 2 h, before being concentrated in vacuo. H_2O (200 mL) was added to the residue, and washing was performed with DCM (1× 30 mL). The aqueous layer was acidified with concentrated HCl (7.0 mL), NaCl (60 g) was added, and the mixture was extracted with EtOAc (4× 200 mL). The combined organics were dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to give the title compound **28** (11 g, 66 mmol, 86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.24 (br s, 1H), 4.33–4.20 (m, 2H), 3.74–3.61 (m, 2H), 1.32 ppm (t, J = 7.10, 7.10 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 168.54, 166.92, 61.95, 51.49, 51.42, 13.38 ppm.

MBHA-Rink amide-Lys(Boc)-Ahx-Tyr(*t*Bu)-Leu(Fmoc) (29): 4-Methylbenzhydrylamine-functionalized (MBHA-functionalized) Rink amide resin (3.2 g, 0.64 mmol g^{-1}, 2.1 mmol) was washed with DCM and deprotected in piperidine/NMP (1:4, *v*/*v*) for 20 min. After washing with NMP (2×) and DCM (2×), the resin was coupled to Fmoc-Lys(Boc)-OH (2.4 g, 5.2 mmol, 2.5 equiv) in the presence of BOP (2.3 g, 5.2 mmol, 2.5 equiv) and DiPEA (1.1 mL, 6.2 mmol, 3.0 equiv) in NMP; the reaction mixture was shaken overnight, followed by washing with NMP (2×) and DCM (2×). The remaining free amines were capped with acetic anhydride (0.98 mL, 10 mmol, 5.0 equiv) in the presence of DiPEA (3.6 mL, 21 mmol, 10 equiv) in DCM for 30 min. The resin was then washed with ether and dried in vacuo overnight, and the loading of the resin was determined by spectrophotometric analysis to be 0.30 mmol g^{-1} (3.7 g, 1.1 mmol, 53%). The obtained resin (3.0 g, 0.90 mmol) was subjected to three cycles of Fmoc solid-phase synthesis as follows: after deprotection in piperidine/NMP (1:4, *v*/*v*) for 30 min, the resin was washed with NMP (2×) and DCM (3×) and coupled to ε-Ahx-Fmoc (0.80 g, 2.3 mmol, 2.5 equiv) in the presence of BOP (1.0 g, 2.3 mmol, 2.5 equiv) and DiPEA (0.45 mL, 2.7 mmol, 3.0 equiv) in NMP for 3 days, followed by washing with NMP (3×) and DCM (3×). The second and third cycles were performed in the same way, with coupling to Fmoc-Tyr(*t*Bu)-OH (1.0 g, 2.3 mmol, 2.5 equiv) for 5 h and to Fmoc-Leu-OH (0.80 g, 2.3 mmol, 2.5 equiv) overnight, respectively. Couplings were monitored for completion by the Kaiser test. Washing with ether and drying in vacuo overnight yielded the resin-bound compound **29**.

(2*S*,3*S*)-3-(Ethoxycarbonyl)oxirane-2-carboxyl-Leu-Tyr-Ahx-Lys·TFA (30): The resin-bound compound **29** (~0.90 mmol) was deprotected in piperidine/NMP (1:4, *v*/*v*) for 30 min. The resin was washed with NMP (2×) and DCM (3×) before being subjected to a condensation cycle with the free acid **32** (0.36 g, 2.3 mmol, 2.5 equiv) in the presence of BOP (1.0 g, 2.3 mmol, 2.5 equiv) and DiPEA (0.45 mL, 2.7 mmol, 3.0 equiv) in NMP; the reaction mixture was shaken overnight, followed by washing with NMP (3×) and DCM (3×). The condensation cycle was repeated, after which the Kaiser test indicated complete coupling. Cleavage from the resin was then accomplished by treatment with TFA/TIS/H_2O (95:2.5:2.5, *v*/*v*/*v*) for 2 h. After filtration and concentration in vacuo in the presence of toluene, the residue was recrystallized first from acetone/MeOH/EtOAc and then from MeOH/Et_2O, yielding a 2:1 mixture of the fully deprotected ester **30** and the free acid **31** (total yield 0.73 g, 0.93 mmol, quant.) according to NMR analysis. ^{1}H NMR (400 MHz, MeOD): δ = 6.98 (d, J = 7.87 Hz, 2H), 6.66 (d, J = 7.76 Hz, 2H), 4.44 (t, J = 7.23, 7.23 Hz, 1H), 4.37 (dd, J = 7.59, 5.82 Hz, 1H), 4.30 (dd, J = 7.96, 5.15 Hz, 1H), 4.26–4.16 (m, 1H), 3.79–3.61 (m, 1H), 3.61–3.46 (m, 1H), 3.17–3.07 (m, 2H), 3.06–2.98 (m, 2H), 2.98–2.91 (m, 2H), 2.91–2.73 (m, 2H), 2.20 (t, J = 6.87, 6.87 Hz, 2H), 1.66–1.63 (m, 2H), 1.59–1.31 (m, 9H), 1.26 (t, J = 6.99, 6.99 Hz, 3H), 1.23–1.13 (m, 2H), 0.88 (d, J = 5.77 Hz, 3H), 0.84 ppm (d, J = 5.75 Hz, 3H); LC/MS analysis: R_f 4.97 min (linear gradient 10→90% B in 15 min); *m*/*z*: 677.4 $[M+H]^+$, 1353.2 $[2M+H]^+$.

(2*S*,3*S*)-3-(Ethoxycarbonyl)oxirane-2-carboxyl-Leu-Tyr-Ahx-((*N*)-(*E*)-hepta-4,6-dienoyl)Lys-H_2N (32): A solution of **30** (0.31 g, 0.39 mmol crude, non-hydrolyzed/hydrolyzed 2:1) in DCE/DMF under argon was made basic (pH 8.5) with DiPEA (0.13 mL, 0.78 mmol, 2.0 equiv), after which a solution of the OSu-ester **11b** (0.23 g, 1.0 mmol, 2.6 equiv) in DCE/DMF was added. After the reaction mixture had been stirred overnight under argon, it was concentrated in vacuo. The portion of the residue that was soluble in MeOH/acetone was purified by column chromatography ($CHCl_3$→10% MeOH in $CHCl_3$), yielding the diene-modified title compound **32** (0.16 g, 0.20 mmol, 76% from non-hydrolyzed starting compound). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 8.54 (d, J = 8.19 Hz, 1H), 8.11 (d, J = 8.24 Hz, 1H), 7.82–7.74 (m, 2H), 7.30 (s, 1H), 6.96 (d, J = 8.40 Hz, 2H), 6.61 (d, J = 8.32 Hz, 2H), 6.28 (td, J = 17.03, 10.26, 10.26 Hz, 1H), 6.04 (dd, J = 15.16, 10.53 Hz, 1H), 5.74–5.64 (m, 1H), 5.02 (dd, J = 50.10, 13.54 Hz, 2H), 4.37–4.28 (m, 2H), 4.23–4.08 (m, 3H), 3.73–3.69 (m, 1H), 3.62–3.57 (m, 1H), 3.07–2.88 (m, 4H), 2.82 (dd, J = 13.69, 5.57 Hz, 1H), 2.67 (dd, J = 13.64, 8.85 Hz, 1H), 2.26 (dd, J = 14.23, 7.06 Hz, 2H), 2.17–2.05 (m, 4H), 1.59 (td, J = 9.47, 6.89, 6.89 Hz, 1H), 1.54–1.26 (m, 12H), 1.23 (t, J = 7.10, 7.10 Hz, 3H), 1.20–1.12 (m, 2H), 0.83 ppm (dd, J = 14.79, 6.46 Hz, 6H).

In vitro competition assay versus MV151: EL-4 cells were cultured on Dulbecco's modified Eagle's medium supplemented with fetal calf serum (10%), penicillin (10 units mL^{-1}), and streptomycin (10 μg mL^{-1}) in a CO_2 (5%) humidified incubator at 37 °C. Cells were harvested, washed with PBS (2×), and lysed in digitonin lysis buffer [Tris (pH 7.5, 50 mM), sucrose (250 mM), $MgCl_2$ (5 mM), dithiothreitol (DTT) (1 mM), ATP (2 mM), digitonin (0.025%)] for 30 min on ice, followed by sonication. After centrifugation at 16100 *g* for 15 min at 4 °C, the supernatants containing the cytosolic fraction were collected and the protein concentration was determined by Bradford assay. The lysates (10 μg total protein per ex-

periment) were exposed to the indicated concentrations of **1a**, **1b**, **1c**, or **1d** (1 μL 10× solution in DMSO) for 2 h at 37 °C in a total reaction volume of 10 μL (buffer/DMSO 9:1, *v/v*), prior to incubation with MV151 (1 μM) for 1 h at 37 °C in a total reaction volume of 11 μL (buffer/DMSO 9:2, *v/v*). The reaction mixtures were heated to 55 °C for 20 min with 4× Laemmli's sample buffer (4 μL) containing 2-mercaptoethanol and resolved by SDS-PAGE (12.5%). In-gel visualization of the fluorescent labeling was performed directly in the wet gel slabs with use of Cy3/Tamra settings.

General procedure for Diels–Alder-based proteasome labeling in vitro: EL-4 cell lysates (50 μg total protein per experiment) were exposed to **1a**, **1b**, **1c**, or **1d** (1 μM, 1 μL, 10 μM DMSO) for 2 h at 37 °C in the presence or in the absence of epoxomicin (100 μM, 1 μL, 1 mM in DMSO) in a total reaction volume of 11 μL (buffer/DMSO 9:2, *v/v*). The proteins were then denatured for 15 min at room temperature in urea (8 M) and treated with DTT (5 mM) for 30 min at 55 °C, after which cysteine residues were capped with DTNB (50 mM) for 3.5 h at RT. Next, the mixtures were subjected to c/m precipitation and the proteins were taken up in Diels–Alder buffer [NaH_2PO_4 (5 mM), NaCl (20 mM), $MgCl_2$ (0.2 mM), pH 6.0] containing urea (2 M), followed by exposure to the indicated concentrations of the BODIPY(TMR)-maleimide **2** (1.7 μL 10× solution in DMSO) overnight at 37 °C in a total reaction volume of 17 μL (buffer/DMSO 15:2, *v/v*). Where indicated, reaction times were reduced to 1, 2 or 4 h. After c/m precipitation, the proteins were taken up in Laemmli's sample buffer (10 μL) containing 2-mercaptoethanol, heated to 55 °C for 15 min, and resolved by SDS-PAGE (12.5%). In-gel visualization of the fluorescent labeling was performed directly in the wet gel slabs with use of Cy3/Tamra settings.

Diels–Alder-based proteasome labeling in situ: EL-4 cells were cultured on Dulbecco's modified Eagle's medium supplemented with fetal calf serum (10%), penicillin (10 units mL^{-1}), and streptomycin (10 μg mL^{-1}) in a CO_2 (5%) humidified incubator at 37 °C. Some 1×10^6 cells per experiment were grown overnight in medium (5.5 mL), before being exposed to increasing concentrations of **1b** for 2 h at 37 °C in a total volume of 6 mL (medium containing 0.25% DMSO). As a control, cells were exposed to MV151 (1 μM). The cells were then harvested, washed with PBS (2×) and lysed in digitonin lysis buffer [Tris (pH 7.5, 50 mM), sucrose (250 mM), $MgCl_2$ (5 mM), DTT (1 mM), ATP (2 mM), digitonin (0.025%)] for 30 min on ice followed by sonication. After centrifugation at 16100 *g* for 15 min at 4 °C, the supernatants containing the cytosolic fraction were collected and the protein concentration was determined by Bradford assay. In case of a competition experiment, the lysates were exposed to MV151 and visualized as described above. For Diels–Alder ligation, the lysates from cells treated with compound **1b** (10 μM, 20 μg total protein per experiment) were denatured, capped, and subjected to c/m precipitation as described above, after which the proteins were taken up in Diels–Alder buffer [15 μL; NaH_2PO_4 (5 mM), NaCl (20 mM), $MgCl_2$ (0.2 mM), pH 6.0] containing urea (2 M) and exposed to the indicated concentrations of the BODIPY(TMR)-maleimide **2** (1.7 μL 10× solution in DMSO) overnight at 37 °C. After c/m precipitation, the proteins were taken up in Laemmli's sample buffer (10 μL) containing 2-mercaptoethanol, heated to 55 °C for 15 min, and resolved by SDS-PAGE (12.5%). In-gel visualization of the fluorescent labeling was performed directly in the wet gel slabs with use of Cy3/Tamra settings.

In vitro competition assay versus N_3-BODIPY(TMR)-DCG-04: RAW cell lysates were prepared by incubation of harvested RAW cells in MES lysis buffer [MES (pH 5.5, 50 mM), NaCl (50 mM), DTT (5 mM), digitonin (0.013%)] for 30 min on ice followed by sonication. After centrifugation at 16100 *g* for 15 min at 4 °C, the supernatants containing the cytosolic fraction were collected and the protein concentration was determined by Bradford assay. The lysates (50 μg total protein per experiment) were exposed to the indicated concentrations of compound **32** (1 μL 10× solution in DMSO) for 1 h at 37 °C in a total reaction volume of 10 μL (buffer/DMSO 9:1, *v/v*), prior to incubation with N_3-BODIPY(TMR)-DCG-04 (0.5 μM) for 1 h at 37 °C in a total reaction volume of 11 μL (buffer/DMSO 9:2, *v/v*). The reaction mixtures were boiled for 3 min with 4× Laemmli's sample buffer (4 μL) containing 2-mercaptoethanol and resolved by SDS-PAGE (12.5%). In-gel visualization of the fluorescent labeling was performed directly in the wet gel slabs with use of Cy3/Tamra settings.

General procedure for Diels–Alder-based cathepsin labeling in vitro: RAW cell lysates (50 μg total protein per experiment) were exposed to compound **32** (5 μM, 0.5 μL 100 μM DMSO) for 1 h at 37 °C in the presence or in the absence of DCG-04 (25 μM, 2.5 μL 100 μM in DMSO) in a total reaction volume of 12 μL (buffer/DMSO 9:3, *v/v*). The proteins were then subjected to denaturation, cysteine capping, c/m precipitation, exposure to the BODIPY(TMR)-maleimide **2** overnight at 37 °C, and again c/m precipitation (see proteasome labeling procedure). The labeled proteins were taken up in Laemmli's sample buffer (10 μL) containing 2-mercaptoethanol, heated to 55 °C for 15 min, and resolved by SDS-PAGE (12.5%). In-gel visualization of the fluorescent labeling was performed directly in the wet gel slabs with use of Cy3/Tamra settings.

Combined Diels–Alder and Staudinger ligation for labeling of proteasome β-subunits in vitro: EL-4 cell lysates (50 μg total protein per experiment) were exposed to compound **3** (5 μM, 1 μL 50 μM DMSO) for 1 h at 37 °C in a total reaction volume of 10 μL (buffer/DMSO 9:1, *v/v*), followed by exposure to compound **1b** (5 μM, 1 μL 50 μM DMSO) for 1 h at 37 °C. The biotin-phosphine **4** (100 μM) was then added (1.2 μL 1 mM in DMF) and the lysates were again incubated for 1 h at 37 °C. The proteins were then denatured for 15 min at room temperature in urea (8 M) and treated with DTT (5 mM) for 30 min at 55 °C, after which cysteine residues were capped with DTNB (50 mM) for 3.5 h at RT. The mixtures were next subjected to c/m precipitation and the proteins were taken up in Diels–Alder buffer [NaH_2PO_4 (5 mM), NaCl (20 mM), $MgCl_2$ (0.2 mM), $Cu(NO_3)_2$ (10 mM), pH 6.0] containing urea (2 M), followed by exposure to the indicated concentrations of the BODIPY(TMR)-maleimide **2** (1.7 μL 10× solution in DMSO) overnight at 37 °C in a total reaction volume of 17 μL (buffer/DMSO 15:2, *v/v*). Following c/m precipitation, the proteins were taken up in Laemmli's sample buffer (10 μL) containing 2-mercaptoethanol, heated to 55 °C for 15 min, and resolved by SDS-PAGE (12.5%). In-gel visualization of the fluorescent labeling was performed directly in the wet gel slabs with use of Cy3/Tamra settings, which was followed by Western blotting. Blots were blocked with BSA (1%) in TBS-Tween 20 (0.1% Tween 20) for 60 min at RT, hybridized for 40 min with streptavidin/HRP (1:10000) in blocking buffer, washed, and visualized with the aid of an ECL+ Western Blotting detection kit (Amersham Biosciences).

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