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Exploring dual electrophiles in peptide-based proteasome inhibitors: carbonyls and epoxides†

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Peptide epoxyketones are potent and selective proteasome inhibitors. Selectivity is governed by the epoxyketone dual electrophilic warhead, which reacts with the N-terminal threonine 1,2-amino alcohol uniquely present in proteasome active sites. We studied a series of C-terminally modified oligopeptides featuring adjacent electrophiles based on the epoxyketone warhead. We found that the carbonyl moiety in the natural warhead is essential, but that the adjacent epoxide can be replaced by a carbonyl, though with considerable loss of activity.

Introduction

The ubiquitin-proteasome system (UPS) is the major cytosolic and nuclear protein degradation pathway in many organisms.[1] The 26S proteasome, composed of a catalytic 20S core particle and a 19S regulator particle, is responsible for the turnover of proteins tagged for degradation through poly-ubiquitin chains. In higher vertebrates, the constitutive proteasome core 20S particle contains three distinct catalytic activities, namely β1 (cleaving after acidic amino acids), β2 (cleaving after basic amino acids) and β5 (cleaving after hydrophobic amino acids).[2] In immunoproteasomes, these catalytic subunits are replaced by β1i, β2i and β5i, respectively.[3] Cortical thymic epithelial cells uniquely express β5t subunits, which may replace β5i to form thymoproteasomes.[4] To date numerous proteasome inhibitors have been reported. These include both natural products and synthetic compounds.[5] Of note, proteasomes are validated drug targets in oncology and the peptide boronic acid, bortezomib[6] as well as the peptide epoxyketone, carfilzomib,[7] are used in the clinic for the treatment of multiple myeloma and mantle cell lymphoma.

Carfilzomib is a member of the family of peptide epoxyketone proteasome inhibitors. The first compound identified in this class is the natural product; epoxomicin (Fig. 1, **1**).[8] Epoxomicin features an epoxyketone electrophilic trap, also referred to as the warhead, which renders this compound highly selective towards the proteasome catalytic activities are characterized by an N-terminal threonine in which the hydroxyl acts as the nucleophile with the amine as catalytic base. Such catalytic sites are rare in nature and peptide epoxyketones appear ideally suited to react with the 1,2-amino-alcohol moiety in these N-terminal threonine residues.[9] In the inhibition mechanism, the carbonyl of the epoxyketone warhead is first attacked by the N-terminal threonine γ-hydroxyl (Fig. 2) after which the α-amine attacks the epoxide ring, resulting in morpholine ring formation.

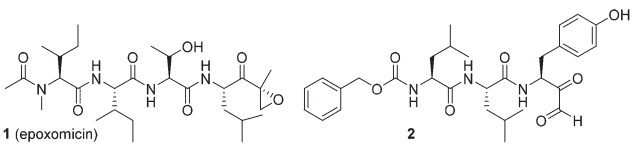

Fig. 1 Structures of epoxomicin (**1**) and peptide α-keto-aldehyde (**2**) that are at the basis of the here presented studies.

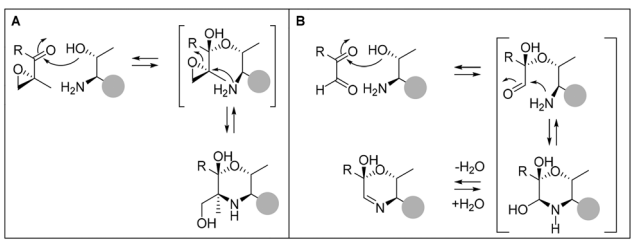

Fig. 2 Mechanism of peptide epoxyketone (A) and peptide α-keto-aldehyde (B) mediated proteasome inactivation.

A large number of epoxyketone warhead containing inhibitors of proteasome have been reported[10] including the aforementioned clinical drug, carfilzomib. A related class of proteasome inhibitors more recently investigated in detail and that also capitalize on the reactive 1,2-amino alcohol present

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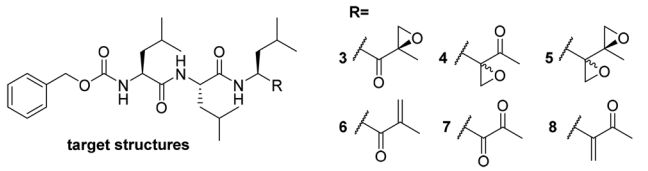


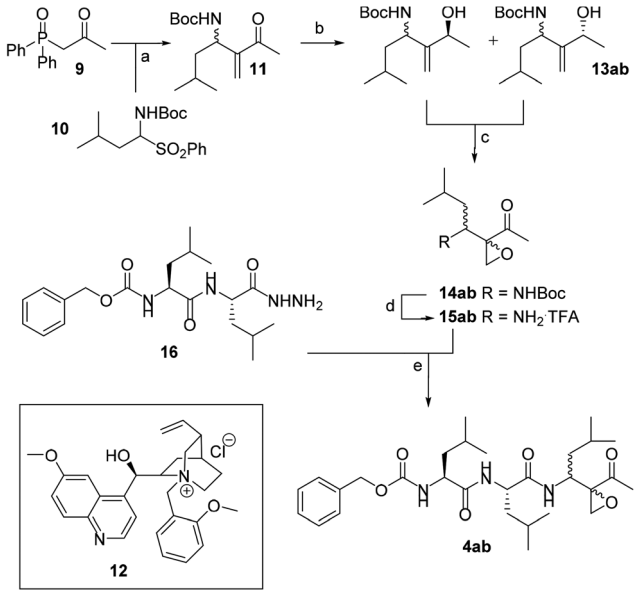
Fig. 3 Target structures **3–8** that are at the basis of the here-presented studies.

in proteasome active sites comprises the peptide α-keto-aldehydes (Fig. 1, **2**).[11] In a subsequent study, Groll and co-workers reported a series of *N*-benzyloxycarbonyl-trileucinyl-1,2-dicarbonyl derivatives based on this motif and identified the α-ketoamide electrophile as the most effective inhibitor of this series.[12] In α-keto-aldehyde **2**, the carbonyl occupying the position normally occupied by the amide carbonyl of the scissile peptide bond is attacked first by the N-terminal threonine γ-hydroxyl to reversibly form the hemi-ketal. This then further condenses to form through a cyclic carbinolamine intermediate the corresponding 5,6-dihydro-2*H*-1,4-oxazine. In contrast to epoxyketone-mediated inhibition, this process is reversible, but the analogy is remarkable: two adjacent electrophilic carbons are brought near to the proteasome N-terminal threonine 1,2-amino alcohol to form after double nucleophilic attack a six-membered adduct. This analogy invites the question whether hybrid structures varying in the number and position of carbonyl moieties (featuring an sp^2 hybridized electrophilic carbon) and epoxides (with an sp^3 electrophilic carbon) would be effective proteasome inhibitors. We decided to put this question to the test by the design, synthesis and evaluation as proteasome inhibitors of compounds **3–8** (Fig. 3).

N-Benzyloxycarbonyl-trileucinyl epoxyketone (ZL3-epoxyketone) **3** is a known[10a] and easily accessible broad-spectrum proteasome inhibitor and a close analogue of the natural product, epoxomicin. The compound features an α-keto-epoxide as the electrophilic trap with the two electrophilic carbons hybridized as sp^2–sp^3. In compound **4** the direction of the epoxyketone is inverted and the two electrophilic carbons are now sp^3–sp^2-hybridized. Compound **5** features two adjacent epoxides (sp^3–sp^3) and compound **7**, as close analogue to lead structure **2**, features two adjacent carbonyls (sp^2–sp^2). Compounds **6** and **8** finally feature an enone system, thus Michael acceptors with the two electrophilic carbons now separated by one carbon. We thought to include these latter compounds because their ease of synthesis and because conjugate addition has been shown to be another valid strategy in the design of peptide-based proteasome inhibitors.[13]

Results and discussion

The synthesis of inverted epoxyketone **4** commenced with an enantioselective Mannich reaction (Scheme 1). Phosphonate **9** and sulfonate **10** were prepared following literature procedures,[14] and used in a one-pot procedure to form compound

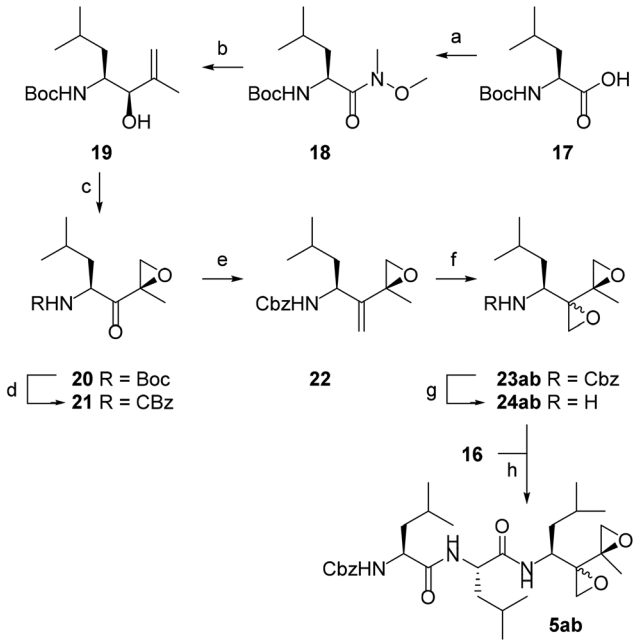


Scheme 1 Synthesis of **4ab**. Reagents and conditions: (a) (i) **12** (cat.), toluene–DCM, aq. 50% K_3PO_4, –20 °C, 60 h; (ii) aq. 37% HCHO, aq. 50% K_3PO_4, RT, 60 h; (b) $CeCl_3{\cdot}7H_2O$, $NaBH_4$, MeOH; (c) (i) *t*BuOOH, VO(acac)$_2$, DCM; (ii) Dess–Martin periodinane, DCM; (d) 50% TFA–DCM; (e) (i) *t*BuONO, HCl, DMF–DCM, –30 °C; (ii) **15a** or **15b**, DiPEA–DMF.

11 *via* an enantioselective Mannich reaction catalyzed by quinine-based catalyst **12**, followed by Horner olefination. According to chiral HPLC analysis, compound **11** was obtained as a 1 : 3 mixture of enantiomers and we continued the synthesis with this mixture. After reduction of **11** with $CeCl_3{\cdot}7H_2O$ and $NaBH_4$, compounds **13ab** were obtained in equal amounts and were separated by silica gel column chromatography. It should be noted that both compounds exist as enantiomeric mixture, in a 1 : 3 ratio. We could not determine the absolute stereochemistry of the individual compounds and therefore decided to continue the synthesis with both.

After diastereoselective epoxidation (*t*BuOOH and VO(acac)$_2$) and Dess–Martin oxidation of the hydroxyl group, diastereomeric compounds **14a** and **14b** were obtained. Treatment with TFA yielded the leucine keto-epoxides **15a** and **15b**. Compound **16** was prepared according to literature methods.[15] Finally, compounds **4a** and **4b** were prepared by standard azide peptide coupling of hydrazide **16** with either of the Leu-keto-epoxides **15a** and **15b**. During reverse HPLC purification of **4a** and **4b**, there was a small peak next to the main peak, which had the same mass as the product according to the LC/MS analysis. We argued that this most probably is the enantiomeric impurity which was introduced in compound **11**. This impurity was removed HPLC purification, and NMR-analysis showed clearly that only one diastereomer was isolated for both compounds **4a** and **4b**. Therefore, we assume that the sole difference between **4a** and **4b** lies in the orientation of the epoxide ring. Again, we could not establish the absolute stereochemistry.

The synthesis of the di-epoxides commenced with Wittig olefination of the Cbz-Leu-epoxyketone **21**, prepared following

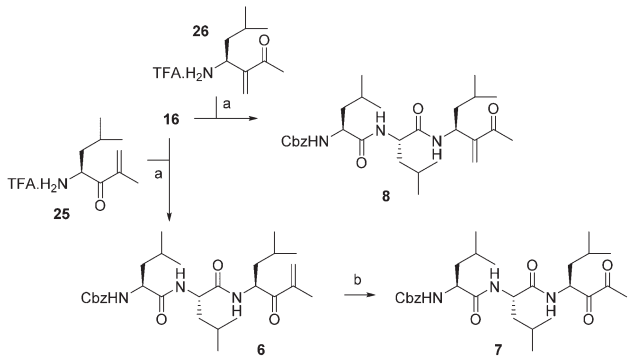


Scheme 2 Synthesis of **5ab**. Reagents and conditions: (a) *N*,*O*-dimethylhydroxylamine hydrochloride, HCTU, DiPEA, DCM; (b) (i) 2-bromopropene, *t*BuLi, Et_2O; (ii) $NaBH_4$, $CeCl_3{\cdot}7H_2O$, MeOH; (c) (i) $VO(acac)_2$, *t*BuOOH, DCM; (ii) Dess Martin periodinane, DCM; (d) (i) 50% TFA–DCM; (ii) CbzCl, DiPEA, DCM; (e) $[Ph_3PCH_3]^+Br^-$, KHMDS, THF, RT to −78 °C; (f) *m*-CPBA, DCM; (g) Pd/C, 1,4-cyclohexadiene, THF–MeOH; (h) (i) *t*BuONO, HCl, DMF–DCM, −30 °C; (ii) **24a** or **24b**, DiPEA–DMF.

the route of synthesis as published in the patent literature.[16] Briefly, Boc-leucine **17** was transformed into Weinreb amide **18** to which was added 2-vinyllithium, prepared for this purpose through transmetallation of 2-bromopropene with *tert*-butyllithium. The intermediate enone was stereoselectively reduced under Lüche conditions to provide after separation of the formed diastereomers allylic alcohol **19** in good yield and enantiomeric purity. Sharpless allylic epoxidation followed by Dess Martin oxidation gave leucine epoxyketone **20**. Removal of the Boc group and installment of the *N*-Cbz protective group gave key intermediate **21** in good overall yield (Scheme 2). Wittig olefination of Cbz-Leu-epoxyketone **21** gave alkene **22**, and ensuing epoxidation with *m*-CPBA yielded epoxides **23a** and **24b** in equal amounts. The absolute stereochemistry of these two compounds, which were obtained as enantiomerically pure di-epoxides, again could not be determined.

After Cbz-removal (to give **24a/b**) and condensation of these warheads with dipeptide **16**, **5a** and **5b** were obtained. (*S*)-4-Amino-2,6-dimethylhept-1-en-3-one **25**, which we obtained in route to leucine epoxyketone **21**, was condensation with **16** to obtain **6** (Scheme 3). Ozonolysis of **6** gave diketone **7**. Compound **16** was converted in a similar sequence of events to give **8** as an inseparable mixture of diastereomers.

The inhibition properties of all compounds were determined in a competitive activity-based protein profiling assay using broad-spectrum proteasome activity-based probe BODIPY-epoxomicin **27**[17] as the read-out. The results (Fig. 4) reveal that interchanging the position of the epoxide and the



Scheme 3 Synthesis of **6** and **7**. Reagents and conditions: (a) (i) *t*BuONO, HCl, DMF–DCM, −30 °C; (ii) **25** or **26**, DiPEA–DMF. (b) O_3, PPh_3, DCM, −78 °C to RT.

ketone part led to a dramatic drop in proteasome inhibitory potency (**4a** and **4b** compared with **3**).

Compound **4b** proved almost completely inactive whereas diastereoisomer **4a** inhibited β1 and β2 at high concentration (>300 μM). In case of di-epoxides **5a** and **5b**, inhibition of β1 and β2 subunits was observed at concentration above 100 μM, while β5 is not even completely blocked at 1 mM. Dicarbonyl derivative **7** displayed some β5-selectivity in a manner similar to ZL3-epoxyketone **3**. Enones **6** and **8** showed similar inhibitory potential. At high concentration (100 μM), β1 was completely inhibited whereas β2 and β5 were only partially inhibited. Although being slightly less potent than **3**, diketo compound **7** showed complete β5 inhibition already at 10 μM, whereas β1 and β2 were inhibited at much higher concentrations.

Our results underscore the fact that the epoxyketone geometry as present in the natural product epoxomicin and synthetic compounds featuring the same adjacent sp^3–sp^2 electrophilic carbons is the most effective design where it comes to proteasome inhibition. The sp^2-carbonyl in peptide epoxyketones situated at the position resembling the amide carbonyl in a scissile amide bond of a proteasome substrate appears highly important for effective inhibition. Interestingly, peptide vinyl sulfones – another major class of proteasome inhibitors – position an sp^2-hybridized electrophilic carbon for nucleophilic 1,4-addition at the same location whereas arguably the covalent but reversible inhibition effected by peptide boronic acids[18] proceeds starting from an sp^2-hybridized boron as well. Apparently, a situation in which the reactive carbon/carbon-replacing atom in a substrate/inhibitor is offered to the active site in an sp^2 geometry to yield after nucleophilic addition of the threonine–OH an sp^3-adduct is ideal. The secondary electrophilic trap – the tertiary epoxide carbon in epoxomicin – is amenable for modification to provide sp^2 analogues, as is evidenced by peptide diketone **7** in a result that complements the literature findings[11,12] on related compounds. It is however also obvious that inhibitory potency is partially compromised in such sp^2–sp^2 hybridized compounds.

Our set of compounds completes a series of peptide epoxyketone analogues as potential proteasome inhibitors that has

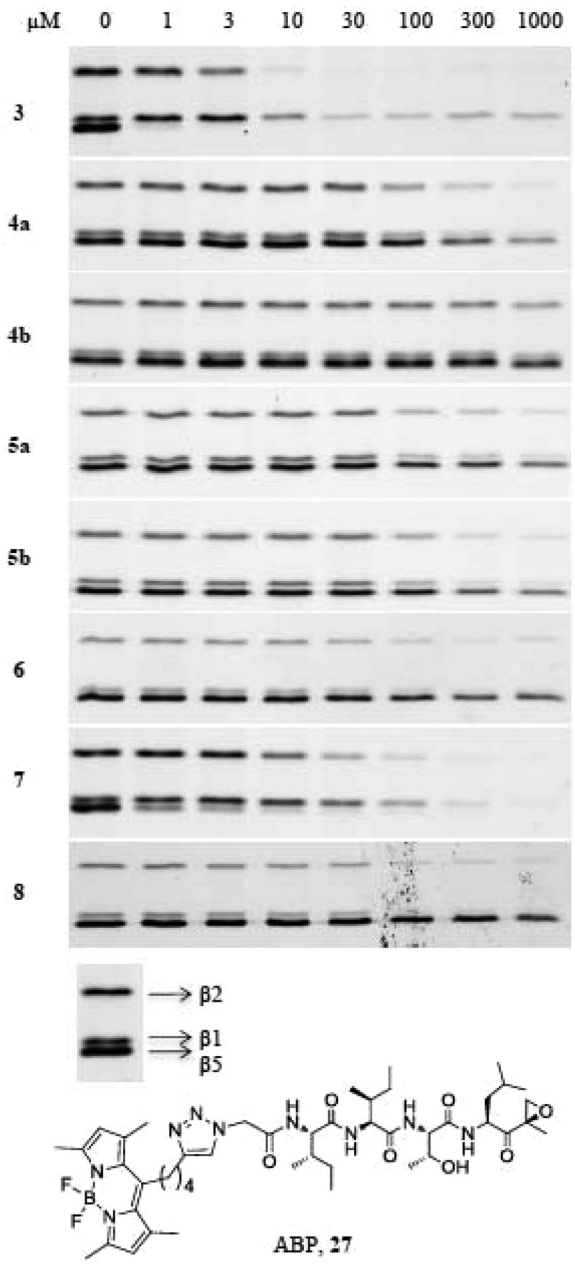


Fig. 4 Inhibition profile of compounds **3–8** and the structure of ABP (**27**).

been growing in the literature in recent years. We could not determine the absolute stereochemistry of the various epoxides we studied. However, in all cases we obtained the respective diastereoisomeric pairs as enantiomerically pure compounds, neither of which proved potent proteasome inhibitors. This result, coupled with our previous[19] finding that a diastereomeric epoxyketone with respect to the chirality of the epoxide carbon in epoxomicin derivatives also results in a drastic drop in activity, leads to the conclusion that the geometry and absolute stereochemistry of the epoxomicin warhead is indeed ideally suited for optimal proteasome inactivation.

Experimental

General

All reagents were of commercial grade and used as received unless indicated otherwise. Methylene chloride (DCM), dimethylformamide (DMF) and tetrahydrofuran (THF) were stored over 4 Å molecular sieves. Reactions were conducted under an argon atmosphere. Reactions were monitored by TLC analysis by using DC-fertigfolien (Schleicher & Schuell, F1500, LS254) with detection by UV absorption (254 nm), spraying with an aqueous solution of $KMnO_4$ (7%) and KOH (2%). Column chromatography was performed on silica gel from Screening devices (0.040–0.063 mm). ^{1}H-NMR and ^{13}C-APT-NMR spectra were recorded on Bruker AV-400 (400 MHz) or Bruker AV-600 (600 MHz) machines. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as an internal standard. Coupling constants are given in Hz. Peak assignments are based on 2D ^{1}H-COSY and ^{13}C-HSQC NMR experiments. All presented ^{13}C-APT spectra are proton decoupled. LC/MS analysis was performed on a LCQ Advantage Max (Thermo Finnigan) equipped with a Gemini C18 column (Phenomenex). HRMS was recorded on a LTQ Orbitrap (Thermo Finnigan). For reverse phase HPLC purification of the final compounds, an automated Gilson HPLC system equipped with a C18 semiprep column (Gemini C18, 250 × 10 mm, 5μ particle size, Phenomenex) was used.

General procedure for Boc deprotection

The appropriate Boc-protected C-terminally modified leucine derivative was dissolved in TFA–DCM (1 : 1, v/v) and stirred for 20 min. Co-evaporation with toluene (3×) afforded the TFA-salt, which was used without further purification.

General procedure for azide peptide coupling

The hydrazide (**16**) was dissolved in 1 : 1 DMF–DCM (v/v) and cooled to −30 °C. *t*BuONO (1.1 eq.) and HCl (4 M solution in 1,4-dioxane, 2.8 eq.) were added, and the mixture was stirred for 3 h at −30 °C after which TLC analysis (10% MeOH–DCM, v/v) showed complete consumption of the starting material. The warhead-TFA salt was added to the reaction mixture as a solution in DMF with 5.0 eq. of DiPEA and this mixture was allowed to warm up to room temperature slowly overnight. The mixture was diluted with EA and extracted with H_2O (3×) and brine. The organic layer was dried over $MgSO_4$ and purified by reverse phase HPLC.

***tert*-Butyl (*S*)-(6-methyl-3-methylene-2-oxoheptan-4-yl)carbamate (11).** Diphenylphosphorylketone **9** (798 mg, 1.54 mmol, 1.1 eq.), compound **10** (917 mg, 2.80 mmol), and catalyst **12** (134 mg, 0.28 mmol, 0.1 eq.) were added to the flask and the solids were suspended in a toluene–DCM (7 : 3) mixture (14.0 mL), and the mixture was cooled to −20 °C. Aqueous K_3PO_4 (50% w/w, 3.8 mL, 14.0 mmol, 5.0 eq.) was then added in one portion. After the resulting biphasic mixture had been vigorously stirred at the same temperature for 60 h, aq. formaldehyde (37% w/w, 1.0 mL, 14.0 mmol, 5.0 eq.) was added, followed by another portion of aq. K_3PO_4 (50% w/w, 15.4 mL,

56.0 mmol, 20.0 eq.). The mixture was stirred at room temperature for 60 h. The organic layer was then charged directly on a silica gel chromatographic column, the aqueous phase was extracted with toluene (2 × 7.0 mL), and the extracts were charged as well. Purification by column chromatography (1% EtOAc–pentane→5% EtOAc–pentane) gave **11** (469.7 mg, 1.8 mmol, 66% yield). The enantiomeric excess (ee) of the product was determined by HPLC (Chiralcel OD (250 × 4.6 mm), *n*-hexane–i-PrOH 99 : 1, 1.0 mL min^{-1}, λ = 254 nm: τ_{max} = 5.612 min, τ_{min} = 6.526 min, 50.8% ee). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.04 (s, 1H), 5.94 (s, 1H), 5.18 (s, 1H), 4.44 (s, 1H) 2.33 (s, 3H), 1.54–1.44 (m, 3H), 1.42 (s, 9H), 2.04 (s, 3H), 0.93–0.89 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.91, 155.32, 148.78, 126.62, 79.23, 52.68, 43.96, 28.50, 25.32, 23.57, 22.65, 22.29. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 7.68 (ESI-MS (*m*/*z*): 255.80 (M^+)). HRMS calculated for $C_{14}H_{26}NO_3$ 256.19072 $[M + H]^+$; found 256.19085. $[\alpha]_D^{21}$ +9.8 (*C* = 1, $CHCl_3$).

***tert*-Butyl ((2*R*/*S*,4*R*)-2-hydroxy-6-methyl-3-methyleneheptan-4-yl)carbamate (13a and 13b).** A solution of compound **11** (400 mg, 1.57 mmol) in methanol was put under argon atmosphere and $CeCl_3{\cdot}7H_2O$ (882 mg, 2.36 mmol, 1.5 eq.) was added. The solution was cooled to 0 °C, before $NaBH_4$ (71.2 mg, 1.88 mmol, 1.2 eq.) was added in 3 portions over 10 min. After stirring for 30 min., the reaction was quenched with glacial acetic acid and toluene was added after an additional 20 min at 0 °C. The solvents were removed *in vacuo* and the oily residue was dissolved in an EtOAc–H_2O mixture. The organic layer was washed with H_2O and brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Purification by column chromatography (2% EtOAc–pentane→10% EtOAc–pentane) gave **13a** (153.4 mg, 0.6 mmol, 38% yield) and **13b** (105.0 mg, 0.4 mmol, 26% yield). **13a**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.11 (s, 1H), 5.02 (s, 1H), 4.75 (s, 1H), 4.34 (q, *J* = 6.8 Hz, 1H), 4.21(s, 1H), 1.69–1.66 (m, 1H), 1.41 (s, 11H), 1.35 (d, *J* = 8 Hz, 3H), 0.95 (d, *J* = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.04, 155.07, 110.34, 79.88, 69.45, 48.96, 44.71, 28.52, 25.25, 23.35, 22.39, 22.00. **13b**: ^{1}H NMR (100 MHz, $CDCl_3$): δ 5.18 (s, 1H), 5.05 (s, 1H), 4.69 (s, 1H), 4.31 (q, *J* = 6.4 Hz, 1H), 4.20 (q, *J* = 7.6 Hz, 1H), 1.70–1.65 (m, 1H), 1.45 (s, 11H), 1.38 (d, *J* = 6.4 Hz, 3H), 0.99 (d, *J* = 6.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.85, 155.54, 109.21, 79.82, 68.79, 50.48, 44.76, 28.54, 25.15, 23.22, 22.84, 22.24. HRMS calculated for $C_{14}H_{28}NO_3$ 258.20637 $[M + H]^+$; found 258.20654.

***tert*-Butyl ((*S*)-1-((*R*/*S*)-2-acetyloxiran-2-yl)-3-methylbutyl)-carbamate (14a and 14b).** Compound **13a** (100 mg, 0.39 mmol) was dissolved in anhydrous DCM and cooled to 0 °C. Next, $VO(acac)_2$ (11 mg, 0.04 mmol, 0.1 eq.) and *t*BuOOH (0.21 ml 5.5 M in decane, 1.17 mmol, 3.0 eq.) were added and the reaction mixture was stirred for 2 h, while allowing the temperature to rise slowly to room temperature. The resulting purple solution was concentrated *in vacuo* and the residue was dissolved in EtOAc and washed with half saturated $NaHCO_3$ solution. The aqueous layer was extracted with EtOAc and the combined organic layer was washed with H_2O and brine. The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude product was used in the next step without any purification. Dess–Martin periodinane (496 mg, 1.17 mmol, 3.0 eq.) was suspended in anhydrous DCM, put under argon atmosphere and cooled to 0 °C. The crude product was co-evaporated with toluene, dissolved in anhydrous DCM and added to the first solution. The reaction mixture was stirred overnight and allowed to warm up to room temperature. Sat. aq. $NaHCO_3$ was added and the layers were separated. The aqueous layer was extracted with EtOAc and the combined organic layers were washed with H_2O, brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by column chromatography (1% EtOAc–pentane→10% EtOAc–pentane) to yield the title compound (**14a**, 84.0 mg, 0.31 mmol, 79%). **14b** was prepared in the same method and on the same scale, yielding the title compound (**14b**, 42.1 mg, 0.15 mmol, 40%). **14a**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.48–4.43 (m, 2H), 2.92–2.88 (m, 2H), 2.05 (s, 3H), 1.68–1.63 (m, 1H), 1.43 (s, 10H), 1.29–1.21 (m, 1H), 0.96–0.90 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.60, 155.48, 79.57, 64.92, 49.30, 47.00, 40.92, 28.33, 24.90, 24.59, 23.55, 21.25. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 7.65 (ESI-MS (*m*/*z*): 271.80 (M^+)). **14b**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.33 (d, *J* = 10 Hz, 1H), 3.50–3.44 (m, 1H), 3.15 (d, *J* = 4.8 Hz, 1H), 2.97 (d, *J* = 5.2 Hz, 1H), 2.01 (s, 3H), 1.67–1.51 (m, 2H), 1.44 (s, 10H), 1.29–1.21 (m, 1H), 0.93–0.89 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 208.64, 155.48, 79.41, 52.59, 51.26, 40.73, 28.43, 24.92, 24.48, 23.17, 22.04. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 7.87 (ESI-MS (*m*/*z*): 271.80 (M^+)). HRMS calculated for $C_{14}H_{26}NO_4$ 272.18563 $[M + H]^+$; found 272.18592.

Benzyl ((*S*)-1-(((*S*)-1-(((*S*)-1-((*R*/*S*)-2-acetyloxiran-2-yl)-3-methylbutyl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (4a and 4b). Compound **15a** was prepared by the general procedure for Boc deprotection (**14a**, 14.9 mg. 55 μmol, 1.1 eq.), followed by the general procedure for azide coupling using **16** (19.6 mg, 50 μmol, 1 eq.). Purification by preparative HPLC (60%→90% MeCN–H_2O) yielded the title compound (**4a**, 4.9 mg, 9.2 μmol, 18%). Compound **4b** was prepared in the same method and same scale, yielding the title compound (**4b**, 6.9 mg, 13.0 μmol, 26%). **4a**: ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.26–7.15 (m, 5H), 5.03 (s, 2H), 4.75 (t, *J* = 2.4 Hz, *J* = 3.2 Hz, 1H), 4.20 (m, 1H), 4.03 (t, *J* = 7.2 Hz, *J* = 7.2 Hz, 1H), 2.80 (d, *J* = 4.4 Hz, 1H), 2.74 (d, *J* = 3.2 Hz, 1H), 1.91 (s, 3H), 1.61–1.38 (m, 8H), 1.08–1.01 (m, 1H), 0.85–0.78 (m, 18H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 208.80, 175.62, 174.88, 158.58, 129.48, 129.01, 128.85, 67.76, 65.82, 55.09, 53.31, 49.64, 45.94, 41.94, 41.52, 40.21, 26.04, 25.95, 25.87, 24.52, 23.94, 23.39, 23.36, 21.94, 21.24. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.39 (ESI-MS (*m*/*z*): 532.20, ($M + H^+$)). **4b**: ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.26–7.16 (m, 5H), 5.00 (s, 2H), 4.26 (t, *J* = 5.2 Hz, *J* = 4.8 Hz, 1H), 4.04 (m, 1H), 3.95 (m, 1H), 2.90 (m, 2H), 1.88 (s, 3H), 1.61–1.27 (m, 9H), 0.86–0.74 (m. 18H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 208.95, 175.57, 174.32, 158.58, 138.21,

129.47, 129.02, 128.89, 67.89, 63.26, 55.14, 53.29, 50.59, 49.57, 42.05, 41.50, 40.64, 25.92, 25.90, 24.45, 23.74, 23.41, 21.96, 21.86, 21.78. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.39 (ESI-MS (*m*/*z*): 532.20, ($M + H^+$)). HRMS calculated for $C_{29}H_{46}N_3O_6$ 532.33811 $[M + H]^+$; found 532.33796.

***tert*-Butyl (*S*)-(1-(methoxy(methyl)amino)-4-methyl-1-oxopenta-*n*-2-yl)carbamate (18).** Boc-Leu-OH (4.63 g, 20 mmol) was dissolved in DCM (200 mL), followed by addition of HBTU (9.10 g, 24 mmol, 1.2 eq.). After stirring for 5 min DiPEA (10.5 mL, 60 mmol, 3.0 eq.) and *N*,*O*-dimethylhydroxylamine·HCl (2.35 g, 24 mmol, 1.2 eq.) was added. TLC analysis indicated completion after 2 h. The reaction mixture was diluted with EtOAc, washed with 1 M aqueous HCl solution, sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by column chromatography (10% EtOAc–pentane→40% EtOAc–pentane) yielded the title compound as a clear oil (4.6 g, 17 mmol, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.07 (d, J = 9.0 Hz, 1H), 4.73 (s, 1H), 3.79 (s, 3H), 3.20 (s, 3H), 1.78–1.66 (m, 1H), 1.44 (s, 11H), 0.97 (d, J = 6.5 Hz, 3H), 0.93 (d, J = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.63, 79.43, 61.57, 48.97, 42.11, 32.08, 28.35, 24.73, 23.34, 21.58. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 15 min): R_t (min): 7.81 (ESI-MS (*m*/*z*): 274.93, ($M + H^+$)).

***tert*-Butyl ((3*R*,4*S*)-3-hydroxy-2,6-dimethylhept-1-en-4-yl)-carbamate (19).** A solution of 2-bromopropene (3.3 mL, 33.1 mmol, 3 eq.) in dry Et_2O (40 mL) was cooled down to −78 °C under argon atmosphere and stirred for 15 min before adding *t*BuLi (31.0 mL 1.6 M in pentane, 49.7 mmol, 4.5 eq.). The reaction mixture was stirred for 15 min. Weinreb amide **18** (3.0 g, 11.1 mmol) was coevaporated with toluene and dissolved in 10 mL Et_2O. This solution was added dropwise to the reaction mixture during 30 min. The resulting reaction mixture was allowed to warm up to rt and quenched after 2 h with sat. aq. NH_4Cl (40 ml). The water layer was extracted with EtOAc (3×) and the combined organics were washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by flash column chromatography (1% EtOAc–pentane→10% EtOAc–pentane) yielded the product as clear oil (1.60 g, 6.3 mmol, 57%). The product obtained from last step (1.60 g, 6.3 mmol) was dissolved in methanol (60 mL), followed by addition of $CeCl_3·7H_2O$ (3.52 g, 9.5 mmol, 1.5 eq.). After the solution turned clear, it was cooled down to 0 °C and $NaBH_4$ (0.34 g, 8.8 mmol, 1.4 eq.) was added portion-wise. After 5 min TLC analysis indicated complete conversion and the reaction mixture was quenched with glacial AcOH (6 mL). The mixture was concentrated, coevaporated with toluene, dissolved in EtOAc, washed with H_2O and brine, dried over $MgSO_4$ and concentrated *in vacuo.* Purification by column chromatography (10% EtOAc–pentane→50% EtOAc–pentane) yielded the title compound (1.39 g, 5.4 mmol, 86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.03 (s, 1H), 4.93 (s, 1H), 4.14 (s, 1H), 3.83 (s, 1H), 1.75 (s, 3H), 1.63 (d, J = 5.9 Hz, 1H), 1.44 (s, 9H), 1.31–1.15 (m, 2H), 0.97–0.82 (m, 6H). ^{13}C NMR (100 MHz, CDCl3): δ 156.22, 144.96, 111.95, 111.34, 77.87, 51.07, 37.20, 28.48, 24.69, 23.87, 21.60, 19.52.

***tert*-Butyl ((*S*)-4-methyl-1-((*R*)-2-methyloxiran-2-yl)-1-oxopentan-2-yl)carbamate (20).** Compound **19** (0.786 g, 3.1 mmol) was coevaporated with toluene, dissolved in DCM (30 mL) and cooled down to 0 °C. After addition of $VO(acac)_2$ (0.041 g, 0.15 mmol, 0.05 eq.) the solution turned light blue-green and subsequent addition of *t*BuOOH (1.7 mL 5.5 M solution, 9.4 mmol, 3.0 eq.) resulted in a dark brown-purple reaction mixture. After stirring for 1 h the reaction mixture was removed from the ice bath. After 15 min, the reaction mixture was concentrated *in vacuo*, dissolved in EtOAc and washed with a 1 : 1 mixture of sat. aq. $NaHCO_3$ and H_2O. The aqueous layer was extracted with EtOAc (3×) and the combined organics were washed with H_2O and brine, dried over $MgSO_4$ and concentrated *in vacuo* to give the crude intermediate as a yellow oil. Dess–Martin periodinane (1.974 g, 4.7 mmol, 1.5 eq.) was dissolved in DCM (30 mL) and cooled down to 0 °C. The intermediate was coevaporated with toluene, dissolved in DCM (30 mL) and added to the Dess–Martin periodinane solution. After 1.5 h the reaction mixture was removed from the ice bath. After another 1.5 h TLC analysis indicated full conversion of the intermediate and the reaction was quenched with sat. aq. $NaHCO_3$ (30 mL). The aqueous layer was extracted with DCM (2 × 15 mL). The combined organics were washed with H_2O (3×) and brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by column chromatography (1% EtOAc–pentane→5% EtOAc–pentane) yielded the title compound (391 mg, 1.4 mmol, 46%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.89 (d, J = 8.4 Hz, 1H), 4.38–4.27 (m, 1H), 3.29 (d, J = 4.9 Hz, 1H), 2.89 (d, J = 5.0 Hz, 1H), 1.78–1.66 (m, 1H), 1.52 (s, 3H), 1.48 (dd, J = 9.8, 3.2 Hz, 1H), 1.41 (s, 9H), 1.22–1.12 (m, 1H), 0.95 (dd, J = 12.8, 6.6 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 209.54, 155.63, 79.61, 58.98, 52.29, 51.37, 40.28, 28.31, 25.06, 23.41, 21.26, 16.75.

Benzyl ((*S*)-4-methyl-1-((*R*)-2-methyloxiran-2-yl)-1-oxopentan-2-yl)carbamate (21). *tert*-Butyl ((*S*)-4-methyl-1-((*R*)-2-methyloxiran-2-yl)-1-oxopentan-2-yl)-carbamate (**20**) (312 mg, 1.15 mmol) was dissolved in 30% TFA in DCM and the reaction was stirred at room temperature for 30 min. All solvent was removed *in vacuo* and co-evaporated with toluene for three times. The deprotected compound was dissolved in 15 mL DCM and DiPEA (0.60 mL, 3.45 mmol, 3 eq.) was added. Then, CbzCl (0.25 mL, 1.73 mmol, 1.5 eq.) was added and the reaction was stirred at room temperature overnight. All solvent was removed *in vacuo* and the residue was dissolved in EtOAc. The organic layer was washed by H_2O, brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Purification by column chromatography (1% EtOAc–pentane→5% EtOAc–pentane) yielded the title compound (290.0 mg, 0.95 mmol, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37–7.23 (m, 5H), 6.90 (d, J = 8.4 Hz, 1H), 5.42 (d, J = 8.4 Hz, 1H) 5.07–4.99 (m, 2H), 4.42–4.36 (m, 1H), 3.27 (d, J = 5.2 Hz, 1H), 2.96 (d, J = 4.8 Hz, 1H), 1.74–1.65 (m, 1H), 1.53–1.34 (m, 4H), 1.28–1.20 (m, 1H), 0.97–0.88 (m. 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 209.27, 156.22, 136.21, 128.70, 127.94, 127.41, 126.88, 66.84, 59.01, 52.20, 51.82, 40.21, 25.01, 23.37, 21.19, 16.67. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 7.88 (ESI-MS (*m*/*z*): 305.93,

(M + H^+)). HRMS calculated for $C_{17}H_{24}NO_4$ 306.16998 $[M + H]^+$; found 306.17003.

Benzyl ((*S*)-5-methyl-2-((*S*)-2-methyloxiran-2-yl)hex-1-en-3-yl)-carbamate (22). Methyltriphenylphosphonium bromide (543 mg, 1.52 mmol, 1.6 eq.) was suspended in 10 mL anhydrous THF and then potassium bis(trimethylsilyl)amide (2.7 mL, 0.5 M in toluene, 1.2 mmol, 1.4 eq.) was added drop by drop. The reaction was stirred at room temperature for 1 h and then cooled to −78 °C. Compound **18** (290.0 mg, 0.95 mmol) was dissolved in 5 mL anhydrous THF and added to the reaction mixture. The reaction was stirred at −78 °C for 1 h and then stirred overnight while allowing to warm up to room temperature. The reaction was quenched by saturated aqueous NH_4Cl (30 mL) and then extracted with EtOAc (3 × 30 mL). The combined organic layer was washed by brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Purification by column chromatography (1% EtOAc–pentane→10% EtOAc–pentane) yielded the title compound (240 mg, 0.79 mmol, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35–7.26 (m, 5H), 5.20–5.03 (m, 5H), 4.16–4.10 (m, 1H), 2.85 (d, J = 4.8 Hz, 1H), 2.73 (d, J = 5.2 Hz, 1H), 1.70–1.60 (m, 1H), 1.49–1.25 (m, 5H), 0.96–0.89 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.68, 151.38, 136.53, 129.40, 128.47, 128.40, 128.05, 110.73, 66.83, 56.85, 54.25, 49.78, 44.98, 25.11, 23.28, 21.56. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 6.81 (ESI-MS (m/z): 304.07, (M + H^+)). HRMS calculated for $C_{18}H_{26}NO_3$ 304.19072 $[M + H]^+$; found 304.19086.

Benzyl ((*S*)-3-methyl-1-((2*R*/*S*, 2′*R*)-2′-methyl-[2,2′-bioxiran]-2-yl)butyl)-carbamate (23a and 23b). Compound **22** (240 mg, 0.79 mmol) was dissolved in 15 mL anhydrous DCM and *m*-CPBA (585.0 mg. 2.37 mmol, 3 eq.) was added. The reaction was stirred at room temperature overnight. DCM was removed *in vacuo* and the residue was dissolved EtOAc and washed by saturated NaS_2O_3, saturated $NaHCO_3$, brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Purification by column chromatography (1% EtOAc–pentane→10% EtOAc–pentane) yielded the title compounds (**23a**, 99 mg, 0.31 mmol, 39% and **23b**, 65.0 mg, 0.20 mmol, 26%). **23a**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41–7.29 (m, 5H), 5.17–5.08 (m, 2H), 4.72–4.69 (m, 1H), 4.34–4.27 (m, 1H), 2.74 (d, J = 5.2 Hz 1H), 2.68 (d, J = 7.6 Hz, 1H), 2.65–2.63 (m, 2H), 1.72–1.66 (m, 1H), 1.47(s, 3H), 1.44–1.35 (m, 2H), 0.99–0.85 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.37, 128.74, 128.62, 128.42, 128.27, 66.90, 60.85, 54.49, 51.89, 48.42, 47.56, 40.13, 23.79, 22.52, 21.20, 18.73. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 7.56 (ESI-MS (m/z): 319.93, (M + H^+)). **23b**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41–7.27 (m, 5H), 5.12–5.08 (m, 2H), 4.89 (d, J = 9.2 Hz, 1H), 4.13–4.07 (m, 1H), 2.80–2.73 (m, 3H), 2.53 (d, J = 5.2 Hz, 1H), 1.68–1.61 (m, 1H), 1.47(s, 3H), 1.47–1.33 (m, 2H), 0.99–0.88 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.26, 128.70, 128.63, 128.56, 128.28, 66.84, 61.48, 57.03, 51.17, 49.76, 49.70, 40.60, 23.91, 22.77, 21.65, 19.04. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 7.49 (ESI-MS (m/z): 319.93, (M + H^+)). HRMS calculated for $C_{18}H_{26}NO_4$ 320.18563 $[M + H]^+$; found 320.18573.

Benzyl ((*S*)-4-methyl-1-(((*S*)-4-methyl-1-(((*S*)-3-methyl-1-((2*S* or *R*, 2′*R*)-2′-methyl-[2,2′-bioxiran]-2-yl)butyl)amino)-1-oxopentan-2-yl)amino)-1-oxopentan-2-yl)carbamate (5a and 5b). Compound **23a** (39 mg, 0.12 mmol) was dissolved in 3 mL anhydrous THF and 3 mL anhydrous MeOH. 1,4-Cyclohexadiene (0.4 mL, 5.52 mmol, 46 eq.) and 10% Pd–C (68 mg) were added. After stirring at room temperature for 2 h, the reaction mixture was filtered through celite and solvents were removed *in vacuo*. The crude products **24a** was directly used in next step. **24b** was prepared in the same method and the same scale. Compound **5a** and **5b** were prepared by the general procedure for azide peptide coupling used **16** (43.1 mg, 0.11 mol, 1 eq.), **24a** and **24b**. Purification by preparative HPLC (50%→100% MeCN–H_2O) yielded the title compound (**5a**, 8.1 mg, 14.9 μmol, 14% and **5b**, 9.4 mg, 17.2 μmol, 16%). **5a**: ^{1}H NMR (400 MHz, MeOD): δ 7.26–7.17 (m, 5H), 4.98 (s, 2H), 4.39–4.35 (m, 1H), 4.28–4.25 (m, 1H), 4.06–4.02 (m, 1H), 2.67 (d, J = 5.2 Hz, 1H), 2.54–2.52 (m, 2H), 2.45 (d, J = 5.2 Hz, 1H), 1.61–1.37 (m, 9H), 1.29 (s, 3H), 0.88–0.79 (m, 18H). ^{13}C NMR (100 MHz, MeOD): δ 175.37, 174.62, 158.52, 138.17, 129.47, 129.02, 128.82, 67.68, 61.59, 55.74, 54.87, 53.23, 52.59, 46.71, 41.94, 41.80, 40.10, 25.91, 25.85, 25.64, 24.18, 23.41, 23.37, 21.97, 21.86, 21.38, 18.93. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.22 (ESI-MS (m/z): 546.27, (M + H^+)). **5b**: ^{1}H NMR (600 MHz, MeOD): δ 7.25–7.16 (m, 5H), 5.00–4.94 (m, 2H), 4.29–4.25 (m, 2H), 4.06–4.01 (m, 1H), 2.59–2.49 (m, 4H), 1.58–1.20 (m, 12H), 0.83–0.73 (m, 18H). ^{13}C NMR (150 MHz, MeOD): δ 175.17, 174.55, 158.53, 138.14, 129.48, 129.02, 128.82, 67.69, 63.11, 56.90, 54.97, 53.39, 52.61, 47.91, 47.68, 41.93, 41.76, 40.49, 25.84, 25.74, 25.67, 24.10, 23.39, 23.35, 22.18, 22.02, 21.53, 18.75. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.22 (ESI-MS (m/z): 546.27, (M + H^+)). HRMS calculated for $C_{30}H_{48}N_3O_6$ 546.35376 $[M + H]^+$; found 546.35375.

Benzyl ((*S*)-1-(((*S*)-1-(((*S*)-2,6-dimethyl-3-oxohept-1-en-4-yl) amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (6). Compound **25** was prepared according to the literature procedures, followed by the general procedure for azide peptide coupling using **16** (121.7 mg, 0.31 mol, 1 eq.) and compound **25** (92.0 mg. 0.34 mmol, 1.1 eq.). Purification by column chromatography (10% EtOAc–pentane→30% EtOAc–pentane) yielded the title compound (130.0 mg, 0.25 mmol, 81%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35–7.29 (m, 5H), 6.90 (d, J = 8.4 Hz, 1H), 6.65 (d, J = 7.6 Hz, 1H), 6.11 (s, 1H), 5.90 (s, 1H), 5.51 (d, J = 8.4 Hz, 1H) 5.40–5.35 (m, 1H), 5.13–5.06 (m, 2H), 4.55 (m, 1H), 4.27 (m, 1H), 1.89 (s, 3H), 1.68–1.34 (m, 9H), 0.96–0.86 (m. 18H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 200.71, 172.19, 171.41, 156.34, 142.26, 136.34, 128.64, 128.28, 128.12, 126.65, 67.12, 53.52, 51.82, 51.26, 42.73, 41.59, 41.29, 25.05, 24.79, 23.47, 22.96, 22.80, 22.37, 22.24, 21.86, 17.92. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.69 (ESI-MS (m/z): 516.20, (M + H^+)). HRMS calculated for $C_{29}H_{46}N_3O_5$ 516.34320 $[M + H]^+$; found 516.34302.

Benzyl ((*S*)-4-methyl-1-(((*S*)-4-methyl-1-(((*S*)-6-methyl-2,3-dioxoheptan-4-yl)amino)-1-oxopentan-2-yl)amino)-1-oxopentan-

2-yl)-carbamate (7). Ozone was bubbled into a stirred solution of **6** (130 mg, 0.25 mmol) in DCM at −78 °C for 15 min until the blue colour of the saturated ozone solution persisted. The solution was then purged with oxygen until the solution became colourless. Triphenylphosphine (131.1 mg, 0.50 mmol, 2 eq.) was added after purging with argon for 5 min and the reaction was stirred overnight while warming up to room temperature. Purification by preparative HPLC (50%→100% MeCN–H_2O) yielded the title compound (18.5 mg, 35.8 μmol, 14%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38–7.31 (m, 5H), 6.93 (d, J = 5.6 Hz, 1H), 6.54 (d, J = 8.0 Hz, 1H), 5.32 (d, J = 7.2 Hz, 1H), 5.14–5.11 (m, 2H), 4.93–4.90 (m, 1H), 4.47–4.42 (m, 1H), 4.18 (m, 1H), 2.35 (s, 3H), 1.68–1.43 (m, 9H), 0.93–0.87 (m. 18H) ^{13}C NMR (100 MHz, $CDCl_3$): δ 196.86, 172.50, 171.91, 156.53, 136.08, 128.74, 128.48, 128.23, 67.42, 53.82, 52.37, 51.43, 41.21, 40.33, 39.67, 25.13, 24.84, 24.20, 23.26, 23.04, 22.91, 22.12, 22.05, 21.61. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.52 (ESI-MS (m/z): 518.13, ($M + H^+$)). HRMS calculated for $C_{28}H_{44}N_3O_6$ 518.32246 $[M + H]^+$; found 518.32245.

Benzyl ((*S*)-4-methyl-1-(((*S*)-4-methyl-1-(((*S*)-6-methyl-3-methylene-2-oxoheptan-4-yl)amino)-1-oxopentan-2-yl)amino)-1-oxopentan-2-yl)carbamate (8). Compound **26** was prepared by the general procedure for Boc deprotection (compound **11**, 52 mg. 0.19 mmol, 1.1 eq.), followed by the general procedure for azide peptide coupling used **16** (67.8 mg, 0.17 mol, 1 eq.). Purification by column chromatography (10% EtOAc–pentane→30% EtOAc–pentane) yielded the title compound (68.2 mg, 0.13 mmol, 78%).), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.33–7.27 (m, 5H), 6.72 (d, J = 8.4 Hz, 1H), 6.42 (d, J = 7.6 Hz, 1H), 6.00 (s, 1H), 5.92 (s, 1H), 5.13–5.05 (m, 2H), 4.79–4.73 (m, 1H), 4.22–4.39 (m, 1H), 4.25–4.24 (m, 1H), 2.30 (s, 3H), 1.68–1.29 (m, 9H), 0.92–0.85 (m. 18H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.71, 172.34, 170.85, 156.35, 148.23, 136.26, 128.63, 128.28, 128.10, 126.87, 67.13, 53.63, 52.01, 50.34, 43.57, 41.56, 41.27, 25.35, 24.90, 23.10, 22.90, 22.81, 22.27, 22.06, 22.00. LC-MS (linear gradient 10→90% MeCN–H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.43 (ESI-MS (m/z): 516.20, ($M + H^+$)). HRMS calculated for $C_{29}H_{46}N_3O_5$ 516.34320 $[M + H]^+$; found 516.34316.

Competition assay in cell lysate

Lysates of HEK-293 T cells were prepared by sonication in 3 volumes of lysis buffer containing 50 mM Tris pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 250 mM sucrose, 2 mM ATP, and 0.025% digitonin. Protein concentration was determined by the Bradford assay. Cell lysates (15 μg total protein) were incubated with the inhibitors for 1 h at 37 °C prior to incubation with green-BODIPY-epoxomicin **27** (0.5 μM each) for an additional 1 h at 37 °C, followed by 3 min boiling with a reducing gel-loading buffer and fractionation on 12.5% SDS-PAGE. In-gel detection of residual proteasome activity was performed in the wet gel slabs directly on a BioRad Imager using the Cy2/Fam settings (λ_{ex} 488 nm, λ_{em} 520 nm).

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