Synthetic tools to illuminate matrix metalloproteinase and proteasome activities
Geurink, P.P.

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Selective Inhibitors of Proteasome’s Trypsin-like Sites
Synthesis and biological evaluation

5.1 Introduction

The mammalian 20S proteasome catalytic core contains two sets of three catalytically active β subunits, which display a different substrate specificity, namely β1 (caspase-like) cleaves after acidic residues, β2 (trypsin-like) cleaves after basic residues and β5 (chymotrypsin-like) cleaves after bulky, hydrophobic residues. In specific cell types involved in the immune surveillance system four additional active subunits can be expressed. In the so-called immunoproteasome the β1i, β2i and β5i replace their corresponding constitutive counterparts and, in addition to that, β5 is replaced by β5t in cortical thymic epithelial cells. To study the role of each individual catalytic subunit in the generation of oligopeptides, the development of cell permeable inhibitors that target one specific subunit has become an important field of research. Peptide-based inhibitors targeting the β5 subunit can be created by introduction of hydrophobic amino acids, as was, for example, shown in Chapter 4. In addition, Van Swieten et al. reported on the development of a cell permeable β1 selective inhibitor containing hydrophobic amino acids as well. In contrast, the search for highly selective inhibitors for the β2 and/or β2i active sites with good cell permeability remains a challenging task. One reason for this might be that introduction of basic amino acids is often required to target the trypsin-like site more selectively. These basic amino acids (Arg, Lys) are positively charged at neutral pH, making it very difficult to cross the cell membrane. A second problem is the synthesis of inhibitors bearing an electrophilic trap (for instance the epoxy ketone) in combination with basic amino acids (especially at the P1 position, next to the warhead), for they are susceptible to cyclisation via nucleophilic attack of the free amine/guanidine on the electrophilic trap. Some modified peptides that target
the β2 subunit selectively have been described in the literature (see Figure 1A). In a P2-P4 side chain positional scanning study Bogyo et al.⁹ found the β2 selective inhibitor Ac-YRLN-VS 1 and showed that the P3 substituent (Arg) is of considerable importance in selectivity enhancement. In addition, the group of Tomatis¹⁰,¹¹ reported on the vinyl ethyl ester tripeptide HMB-VSL-VE 2, which was able to selectively target the β2 active site, both in purified proteasome and in living cells.

This chapter describes the development of inhibitors targeting the trypsin-like subunits (β2 and β2i) by modification of the P1 site, which plays a key role in subunit binding, with basic residues. The initial set of inhibitors synthesized and studied is shown in Figure 1C. The general structure is based on the tripeptide vinyl sulfone Z-L₃VS 3, which targets all proteasome active sites (Figure 1B).¹² The P1 leucine side chain was replaced by either a panel of phenylalanine derivatives containing an amine with varying basicity (benzyl amine 4a, aniline 5a, pyridine 6) or a lysine (7) side chain. In addition to the vinyl sulfone electrophilic trap, the epoxyketone featured by natural proteasome inhibitor epoxomicin was incorporated as well (4b, 5b), since it displays a specific reactivity towards proteasome active sites (see also Chapter 4).³,¹³ The N-terminal benzoxycarbonyl group was replaced by the structurally related azidophenylalanine, which opens the possibility for additional modifications,¹⁴-¹⁶ yet it does not significantly influence the inhibitory properties compared to the benzoxycarbonyl group.¹⁷

![Figure 1](image-url)

**Figure 1.** (A) Examples of two published β2 selective proteasome inhibitors. (B) Modifications of broad-spectrum proteasome inhibitor ZL₃VS at the basis of the here presented inhibitors. (C) Initial panel of inhibitors prepared and studied in this chapter.
5.2 Results and Discussion

Retro-synthetically, the modified oligopeptides can be prepared from tripeptide hydrazide \( N_3 \text{Phe-Leu-Leu-NHNH}_2 \) and the properly protected warhead amines in an (epimerization free) azide coupling. The synthesis of P1-benzyl amine containing vinyl sulfone and epoxyketone warheads leading to inhibitors 4a and 4b is shown in Scheme 1. The synthetic scheme commenced with the introduction of the aminomethylene substituent on L-phenylalanine 8, by performing an electrophilic aromatic substitution with \( N \)-(hydroxymethyl)trichloroacetamide under acidic conditions. In this reaction both the ortho and the para substituted isomers were formed, which could be separated by column chromatography. The desired para substituted isomer was obtained in 35% yield. After Cbz-protection of the \( \alpha \)-amine compound 9 was obtained. Basic removal of the trichloroacetamide group followed by Boc protection of the formed amine gave 10, which was coupled to \( N,O \)-dimethylhydroxylamine to give Weinreb-amide 11. Upon a reaction with 2-lithiumpropene the \( \alpha',\beta' \)-unsaturated ketone 12 was obtained. Stereoselective reduction to the allylic alcohol 13 and subsequent asymmetric epoxidation and Dess-Martin oxidation resulted in epoxyketone 14. This compound was \( \alpha \)-amine deprotected by hydrogenation, which finalized the synthesis of compound 15. The vinyl sulfone analogue was created by \( \alpha \)-amine deprotection of compound 11, followed by tritylation (16). Reduction of the Weinreb-amide, followed by a Horner-Wadsworth-Emmons reaction and de-tritylation finally resulted in compound 18.


Reagents and conditions: (a) i) \( N \)-(hydroxymethyl)trichloroacetamide, \( \text{H}_2\text{SO}_4, \text{H}_2\text{O} \); ii) benzyl chloroformate, \( \text{Na}_2\text{CO}_3, \text{H}_2\text{O, 1:4-dioxane, 35%} \); (b) i) 20% \( \text{NaOH, EtOH/H}_2\text{O 1:1} \); ii) \( \text{Boc}_2\text{O, Na}_2\text{CO}_3, \text{THF, H}_2\text{O, 75%} \); (c) \( \text{NH} \cdot \text{Me} \cdot \text{OMe} \cdot \text{HCl, HCTU, DiPEA, DCM, 98%} \); (d) 2-bromopropene, \( \text{tBuLi, THF, -78 °C, 94%} \); (e) \( \text{NaBH}_4, \text{CeCl}_3 \cdot 7\text{H}_2\text{O, MeOH, 0 °C, 92%} \); (f) i) \( \text{tBuOOH, VOAcacl}_2, \text{DCM, 0 °C} \); ii) Dess-Martin periodinane, DCM, 56%; (g) \( \text{H}_2, \text{Pd black, TFA, MeOH} \); (h) i) \( \text{H}_2, \text{Pd/C, AcOH, EtOH} \); ii) \( \text{TrCl, Et}_3\text{N, DMAP, DCM, 38%} \); (i) i) \( \text{LiAlH}_4, \text{Et}_2\text{O, 0 °C} \); ii) diethyl (methylsulfonyl)methylphosphonate, \( \text{NaH, THF, 0 °C, 85%} \); (j) 1% TFA/DCM.
Aniline containing warheads 24 and 29 (Scheme 2) were made from Weinreb-amide 21 by following a similar reaction sequence as described for 18 and 15 respectively (see Scheme 1). Compound 21 was made from Fmoc protected para-nitrophenylalanine 19. Reduction of the nitro group followed by Boc-protection of the formed amine and formation of the Weinreb-amide gave fully protected 20, which was converted into free α-amine 21 by removal of the Fmoc group.

Scheme 2. Synthesis of warheads 24 and 29.

Reagents and conditions: (a) i) NH₄HCO₂H, Pd/C, MeOH; ii) Boc₂O, NaHCO₃, H₂O, 1,4-dioxane; iii) NH₂OMe·HCl, HCTU, DiPEA, DCM, 99%; (b) DBU, THF, 85%; (c) TrCl, Et₃N, DMAP, DCM, 96%; (d) i) LiAlH₄, Et₂O, 0 °C; ii) diethyl ((methylsulfonyl)methyl)phosphonate, NaH, THF, 0 °C, 85%; (e) 1% TFA/DCM; (f) benzyl chloroformate, DiPEA, THF, 85%; (g) 2-bromopropene, tBuLi, THF, −78 °C, 94%; (h) NaBH₄, CeCl₃·7H₂O, MeOH, 0 °C, 92%; (i) i) tBuOOH, VO(Acac)₂, DCM, 0 °C; ii) Dess-Martin periodinane, DCM, 56%; (j) H₂, Pd black, TFA, MeOH.

The synthesis of lysine (33) and pyridine (37) functionalized vinyl sulfones is depicted in Scheme 3. These compounds were produced in a procedure similar to that for 18, from ε-amine-Boc-protected Weinreb-amide 30 and commercially available Boc-β-(4-pyridyl)-L-alanine 34 respectively.

Scheme 4 shows the azide coupling of amine warheads 18 and 24 with tripeptide hydrazide 38, giving, after TFA mediated deprotection and RP-HPLC purification, inhibitors 4a and 5a. The other inhibitors were made in a similar reaction from the appropriate amines in varying yields of 7-48% after RP-HPLC. LC-MS and NMR analysis showed for neither compound any sign of epimerization of the final products.

The inhibition potential of the inhibitors for each of the catalytically active subunits was assessed in competition assays employing extracts of human embryonic kidney cells (HEK-293T) and mouse lymphoma cells (EL-4) in combination with the fluorescent broad spectrum proteasome probe MV151 (see also Chapter 4). The gel images are shown in Figure 2. Competitive inhibition of a proteasome active site is reflected by the disappearance of the corresponding band.
Selective inhibitors of proteasome’s trypsin-like sites


Reagents and conditions: (a) TrCl, DiPEA, DCM, 68%; (b) i) LiAlH₄, Et₂O, 0 °C; ii) diethyl (methylsulfonyl)methylphosphonate, NaH, THF, 0 °C, 64%; (c) 1% TFA/DCM; (d) NH(Me)OMe·HCl, HCTU, DiPEA, DCM, quant.; (e) TFA, DCM.

Scheme 4. Azide coupling towards the target inhibitors.

Reagents and conditions: (a) i) tBuONO, HCl, DMF, DCM, −30 °C; ii) compound 18 or 24, DiPEA; iii) TFA, DCM, then RP-HPLC, yields: 7-48%.

It is apparent from these results (Figure 2) that the selectivity for β2 decreases with decreasing basicity (compare compounds 4a and 7 with 5a and 6). When the substituent becomes less basic the inhibitor targets both β5 and β2. This phenomenon can be explained by the fact that the nature of the substituent becomes more hydrophobic and is therefore more favoured by β5. In general, the β1 subunit is not affected by any compound and is even upregulated at higher concentrations (a related effect was also seen in Chapter 4 in case β5-specific inhibitors were employed). There appears to be little difference between the experiments in HEK-293T and EL-4 lysate with respect to the potency towards β2, however the selectivity for β2 over β5 is difficult to determine since the β5(i) and β1(ii) bands are overlapping. Apparently, the inhibitors do not distinguish between the constitutive subunits and their immuno counterparts.

Interestingly, the vinyl sulfones seem to display better characteristics, in terms of selectivity and potency, compared to the epoxyketones (compare compounds 4a and 5a with 4b and 5b). This observation is remarkable since epoxyketones are generally more active than their vinyl sulfone counterparts (in a head-to-head comparison). Benzyl amine derivative 4a and lysine derivative 7 are the most β2 selective inhibitors in
this series, however in terms of potency, compound 4a is about a 10 fold more potent than 7. Capable of (almost) complete inhibition of β2(i) at a concentration of 0.5 μM, while leaving the other subunits untouched, compound 4a is the most valuable compound derived from this series.

Three inhibitors were selected from this panel and tested for their capability to cross the cell membrane. Primary amine containing compounds 4a and 7 were selected because of their enhanced preference for β2 and compound 5a was tested for its ability to target both β2 and β5. Living HEK-293T cells were incubated with each of the three inhibitors at 0.5, 5 and 50 μM final concentrations for 4 hours, after which all residual proteasome activity was labelled with cell permeable probe MV151. The cells were lysed, all proteins denatured and resolved by SDS-PAGE. As a control the broad-spectrum proteasome inhibitor AdaAhx3L7VS,25 which is known to be able to cross the cell membrane, was used. From the results shown in Figure 3 it follows that the primary amine in compound 4a does not result in impermeability towards the cell membrane and is still able of inhibiting (almost) all β2 activity at 5 μM. Aniline containing compound 5a was also able to cross the cell membrane, after which it targets both β2 and β5. Lysine derived inhibitor 7 appears to be unable of crossing the cell membrane as evidenced from Figure 3.
Selective inhibitors of proteasome’s trypsin-like sites

Figure 3. Competition assay in live HEK-293T cells. The cells were treated with compounds 4a, 5a and 7 at the indicated final concentrations for 4 hours, followed by incubation with MV151 (5 μM final concentration) for 2 hours. After cell lysis and denaturation the samples were resolved by SDS-PAGE and analyzed by fluorescence scanning. Controls used: 0 = no inhibitor, D = DMSO, Ada = AdaAh3xL3VS (20 μM).

For direct labelling of β2 a new fluorescent probe was made by reacting compound 4a with a green fluorescent Bodipy-alkyne in a ‘click’ reaction (see Figure 4A). This reaction however was not as straightforward as could be expected from earlier results (see Chapter 4). Upon reaction of both compounds with CuSO4 and sodium ascorbate in an aqueous medium compound 4a was completely consumed, however the formed product had a mass of 1 Da less compared to the expected product mass and it was dramatically more hydrophobic compared to the starting material, as evidenced from LC-MS measurements. It was reasoned that the free benzylic amine was oxidized and hydrolyzed into its corresponding benzaldehyde (Figure 4A). This reaction has been previously observed by Srogl and Voltrova, who describe a copper/ascorbic acid dyad catalytic system for the selective aerobic oxidation of amines (both benzylic and aliphatic). Indeed, upon addition of ammonium acetate and NaCNBH3 a reductive amination took place, resulting in the desired product 39.

The ability of compound 39 to label proteasome actives both in HEK-293T cell lysate and living cells was assessed in a competition assay as described above. A dual-wavelength fluorescence read-out was performed allowing visualisation of one of the two fluorescent dyes at a time. The results are shown in Figure 4B. From this it becomes clear that the introduction of the bulky, hydrophobic bodipy moiety has resulted in the loss of the inhibitor’s selectivity for β2 over β5. Both subunits are inhibited equally well, leaving only β1 untouched. Probably the large hydrophobic moiety is too close to the active site and introduction of a spacer between tag and warhead may reinstall β2 selectivity. Interestingly, introduction of the bodipy has had a detrimental effect on cell permeability. At a concentration of 5 μM both subunits (β2 and β5) seem not to be competed away at all, although a faint band for each subunit is visible in the lower gel. Even at a concentration of 50 μM not all proteasome activity is silenced. This

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observations must come from the cell penetrating properties of the probe, since all β2 and β5 proteasomal activity is inhibited at a 5 μM concentration in cell lysate.

Figure 4. (A) Synthesis of fluorescent probe 39 in a ‘click’ reaction with inhibitor 4a and Bodipy-alkyne. As a side reaction the benzylamine was converted to the corresponding benzaldehyde. Reagents and conditions: (a) Bodipy-alkyne, CuSO₄, sodium ascorbate, H₂O/tBuOH/toluene 1:1:1, 80 °C; (b) NH₄OAc, NaCNBH₄, MeOH, RP-HPLC, 29%. (B) Competition assay in HEK-293T cell lysate (left) and living cells (right) with compound 39 at the indicated final concentrations. Residual proteasome activity was labelled with MV151. Fluorescence read-out at λₑₓ 532 nm, λₑᵐ 560 nm (MV151, upper panels) and λₑₓ 488 nm, λₑᵐ 520 nm (compound 39, lower panels). Controls used: Ada = AdaAhx₃L₃VS (20 μM final concentration), D = DMSO, 0 = no inhibitor.

As discussed in the introduction of this Chapter vinyl ethyl ester tripeptide HMB-VSL-VE 2 was identified as a potent, cell permeable β2 selective inhibitor. Other inhibitors containing the vinyl ethyl ester warhead have been made, which are known to target other subunits as well. It is therefore likely that the majority of the β2 selectivity comes from the unique HMB-Val-Ser peptide sequence. For this reason, a combination of the HMB-Val-Ser peptide sequence and the P1-functionalized warheads discussed so far may result in inhibitors with an even enhanced preference for the β2(i) subunit. To this end compounds 40 and 41 (see Figure 5A) were synthesized via the method outlined above from HMB-Val-Ser(tBu)-NHNH₂. First, both compounds were tested for their inhibitory activity in HEK-293T cell lysate in a competition assay as discussed earlier. The results are depicted in Figure 5A. When comparing compound 40 and 4a it becomes clear that substitution of the N₃PheLeu₂ for the HMB-Val-Ser motif the general potency is decreased by a factor two. In addition, the selectivity for β2 over β5 is substantially increased. Only a part of the β5 activity is inhibited at 50 μM by 40, whereas compounds 4a completely blocks β5 at this concentration. This difference is even more pronounced for the inhibition in living cells by 40 (Figure 5B). The β2 band has almost disappeared at a concentration of 5 μM and β5 is not affected at all at concentrations up to 50 μM. The most striking result from this assay is the apparent
Selective inhibitors of proteasome’s trypsin-like sites

The selectivity of compound 40 for β2 over β2i in EL-4 lysate (Figure 5A). At a concentration of 0.5 μM β2 is almost completely blocked, whereas the compound starts to inhibit β2i only at 5 μM. The attachment of the HMB-Val-Ser peptide sequence to the aniline derived vinyl sulfone (41) only resulted in a drop of potency of the inhibitor compared to 5a. The characteristics in terms of selectivity remain unchanged (it still targets both β2 and β5). These observations invite the conclusion that the HMB-Val-Ser sequence on its own is not enough to active β2 selectivity, but that by selection of a suitable P1 substituent this objective might be reached after all.

Figure 5. Competition assay of (A) compounds 40 and 41 in HEK-293T and EL-4 cell lysate and (B) compound 40 in HEK-293T living cells. Residual proteasome activity was labelled with MV151 as described above. Controls used: Ada = AdaAhx3L3VS (20 μM final concentration), D = DMSO, 0 = no inhibitor.

5.3 Conclusion

In summary, the effect of introduction of different amines of varying basicity, at the P1 position in oligopeptide proteasome inhibitors with respect to the selectivity for proteasome’s trypsin-like sites was studied. As expected, it was found that the β2 selectivity increases with increasing basicity of the side chain. All compounds were ineffective towards β1, but upon decreasing basic character of the substituent β5 was targeted. The most β2 selective compounds identified were lysine derived 7 and 4-aminomethylene phenylalanine derived 4a, of which the latter one proved to be most potent. It was shown that 4a was capable of inhibiting β2 selectively both in cell lysate and in living cells. This demonstrates that the nature of the side chain amine is such, that it is basic enough to direct the inhibitor towards β2, yet it allows the inhibitor to cross the cell membrane. Introduction of a hydrophobic fluorescent tag into 4a, to label and visualize the β2 subunit selectively, resulted in a decreased selectivity for β2 over β5. A good alternative would be the use of two-step labelling, in which a biological sample is first treated with inhibitor 4a, after which the construct is captured at the azide moiety via, for instance the Staudinger-Bertozzi ligation or ‘click’ chemistry. Preliminary results however, showed that the azide in 4a is relatively inreactive towards...
a biotin-phosphane reagent. Therefore, improvements have to made, for instance by introduction of a more accessible azide or a spacer between warhead and modification site/tag.

In addition, the 4-aminomethylene phenylalanine vinyl sulfone warhead was coupled to the HMB-Val-Ser peptide, of which a preference for β2 has been reported. This resulted in inhibitor 40, which is completely ineffective towards β5 up to 50 μM in vivo and has a comparable potency towards β2. Interestingly, compound 40 was found to be able to distinguish between the constitutive active subunit β2 and its immunoproteasome counterpart β2i, showing a ten fold higher preference for β2 in EL-4 lysate.

**Experimental section**

**General Procedures:**

Tetrahydrofuran was distilled over LiAlH₄ prior to use. Acetonitrile (ACN), dichloromethane (DCM), N,N-dimethylformamide (DMF), methanol (MeOH), disopropylethylamine (DiPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Acros, Merck, Aldrich, Sigma) were used as received. O-(1H-6-Chlorobenzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) was purchased at Iris Biotech (Marktrewitz, Germany). Traces of water were removed from reagents used in reactions that require anhydrous conditions by co-evaporation with toluene. Solvents that were used in reactions were stored over 4 Å molecular sieves, except methanol and acetonitrile which were stored over 3 Å molecular sieves. Column chromatography was performed on Screening Devices b.v. Silica Gel, with a particle size of 40-63 μm and pore diameter of 60 Å. The eluents toluene, ethyl acetate and petroleum ether (40-60 °C boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F254). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₂·2H₂O (10 g/L) in 10% sulfuric acid, a solution of KMnO₄ (20 g/L) and K₂CO₃ (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. ¹H- and ¹³C-NMR spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, CD₃OD or CDCl₃ as internal standard. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile 50/50 (v/v) and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60,000 at m/z 400 (mass range m/z = 150-2,000) and dioctylphthalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). Optical rotations [α]D²³ were recorded on a Propol automatic polarimeter. LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.60 mm column (detection at 200-600 nm), coupled to a Finnigan LCO Advantage Max mass spectrometer with ESI. The applied buffers were H₂O, ACN and 1.0% aq. TFA. HPLC purifications were performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5 μm 250 × 10 mm column and a GX281 fraction collector. The applied buffers were: 0.1% aq. TFA and ACN.

**General procedure I: azide coupling of N₃Phe-Leu-Leu-NHNH₂ or HMB-Val-Ser(tBu)-NHNH₂ to an amine-warhead followed by acidic deprotection**

N₃Phe-Leu-Leu-NHNH₂ 38 or HMB-Val-Ser(tBu)-NHNH₂ (1 eq.) was dissolved in a 9:1 mixture of DCM/DMF (10 mL/mmol) and cooled to −35 °C. To this were added tert-butylisocyanate (1.1 eq.) and
Selective inhibitors of proteasome's trypsin-like sites

HCl (2.8 eq. as a 4 M solution in 1,4-dioxane) and the mixture was stirred for 3 h at –35 °C. Next, a mixture of the deprotected amine (1.1 eq.) and DiPEA (5 eq.) in DMF (1 mL) were added. The reaction was slowly warmed to room temperature and stirred for another 12 h before being diluted with DCM and extracted with 1M aq. HCl (2×), saturated aq. Na₂CO₃ (2×) and brine. After drying (MgSO₄) and concentrating the obtained crude product was dissolved in DCM (2.5 mL/mmol). TFA (2.5 mL/mmol) was added and the mixture was stirred for 30 min, after which it was concentrated under reduced pressure in the presence of toluene (3×). The obtained crude product was purified by RP-HPLC.

N₂-Phe-Leu-Leu-NHNH₂ (38)
This compound was synthesized via general Boc-based peptide coupling procedures using HCTU from H-Leu-OMe, Boc-Leu-H and N₂-Phe-H. The last step involved the introduction of the hydrazide by stirring of a mixture containing tripeptide N₂-Phe-Leu-Leu-OMe (1.51 g, 3.49 mmol) and hydrazine monohydrate (30 eq., 105 mmol, 5.1 mL) in MeOH (30 mL) for 15 h at RT. The title compound was obtained after coevaporation of the mixture with toluene (3×) as a colourless solid (yield: 1.51 g, 3.49 mmol, quant.). LC-MS: Rt (min): 6.87 (ESI-MS (m/z): 432.13 (M + H+)).

(S)-2-(((benzyl oxy)carbonyl)amino)-3-(4-((2,2,2-trichloroacetamido)methyl)phenyl)propanoic acid (9)
L-Phenylalanine (8, 8.26 g, 50.0 mmol) was added in portions to concentrated H₂SO₄ (35 mL) maintaining the temperature at 25 °C. N-(hydroxymethyl)trichloroacetamide (1.05 eq., 52.5 mmol, 10.1 g) was added in portions while maintaining the temperature at 20–25 °C. The cooling bath was removed and the light-brown cloudy solution was stirred at room temperature for 1 h. The reaction mixture was added to ice (500 mL) and the pH was adjusted to pH 5.5 with 8 M aq. NaOH solution while maintaining the quench temperature at 15-20 °C. The white solid was filtered off and washed with ice-cold H₂O. The residue was dissolved in a 1:1 mixture of H₂O/dioxane (100 mL) and the pH was adjusted to pH 9 by addition of Na₂CO₃. Next, benzyl chloroformate (7.32 mL, 50.0 mmol) was added and the mixture was stirred for 4 h. Concentrated aq. HCl was added until pH 1 and the mixture was extracted twice with EtOAc. The combined organic layers were extracted with brine, dried (MgSO₄) and concentrated under reduced pressure. The resulting crude product was purified by column chromatography (25% → 60% EtOAc/PE) and the title compound was obtained as a colourless solid (yield: 8.29 g, 17.5 mmol, 35%). ¹H NMR (400 MHz, CDCl₃): δ = 10.16 (s, 1H), 7.31-7.21 (m, 6H), 7.13 (d, / = 7.88 Hz, 2H), 7.09 (d, / = 7.97 Hz, 2H), 5.58 (d, / = 8.21 Hz, 1H), 5.05-4.97 (m, 2H), 4.60 (dd, / = 13.68, 6.42 Hz, 1H), 4.40 (d, / = 5.54 Hz, 2H), 3.14 (dd, / = 13.57, 4.65 Hz, 1H), 3.01 (dd, / = 13.81, 6.53 Hz, 1H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 174.70, 162.06, 155.85, 135.68, 135.31, 135.15, 129.57, 128.31, 128.28, 127.76, 127.57, 92.30, 66.91, 54.41, 44.54, 36.99 ppm.

(S)-2-(((benzyl oxy)carbonyl)amino)-3-(4-((tert-butoxycarbonyl)amino)methyl)phenyl)propanoic acid (10)
Compound 9 (2.82 g, 5.94 mmol) was treated with 20% w/w NaOH in H₂O/EtOH (1:1) for 1 h after which TLC analysis indicated complete conversion of starting material. Next, 3 M aq. HCl was added until pH 7 and the mixture was concentrated under reduced pressure. The resulting crude compound was dissolved in THF (40 mL) and cooled to 0 °C. Boc₂O (1.5 eq., 8.91 mmol, 2.0 g) was added and the solution was basified by addition of Na₂CO₃ until pH 9. The mixture was stirred at RT for 3 h, after which it was acidified with 10% w/v aq. HCl until pH 2 and extracted with EtOAc (3×). The combined organic layers were extracted with brine, dried over MgSO₄ and concentrated under reduced pressure. The resulting crude mixture was purified by column chromatography (20% → 100% EtOAc/PE) and the title compound...
was obtained as a colourless solid (yield: 1.90 g, 4.45 mmol, 75%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 9.32$ (s, 1H), 7.36-7.28 (m, 5H), 7.16-7.02 (m, 4H), 5.33 (d, $J = 7.64$ Hz, 1H), 5.09 (q, $J = 12.32$, 12.32, 12.29 Hz, 2H), 4.95 (s, 1H), 4.65 (d, $J = 6.41$ Hz, 1H), 4.26-4.19 (m, 2H), 3.20-3.04 (m, 2H), 1.45 (s, 9H) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta = 174.79$, 156.16, 155.77, 137.47, 136.15, 134.80, 129.61, 128.46, 128.14, 128.03, 79.85, 66.99, 54.52, 44.31, 37.30, 28.36 ppm.

(S)-2-(((benzyloxy)carbonyl)amino)-3-(4-(((tert-butoxycarbonyl)amino)methyl)phenyl)-N-methoxy-N-methylpropionamide (11)

Carboxylic acid 10 (4.45 g, 10.4 mmol) was dissolved in DCM (75 mL). To this were added NH(Me)OMe·HCl (1.5 eq., 15.6 mmol, 1.55 g), HCTU (1.5 eq., 15.6 mmol, 6.45 g) and DiPEA (4.5 eq., 46.7 mmol, 7.72 mL) and the mixture was stirred for 2 h until TLC analysis indicated a completed reaction. The solvent was evaporated under reduced pressure and the residue was dissolved in EtOAc. This was extracted with 1 M aq. HCl (2×), saturated aq. Na$_2$CO$_3$ (2×) and brine, dried over MgSO$_4$ and concentrated under reduced pressure. The product was purified by column chromatography (10% → 75% EtOAc/PE) and obtained as colourless oil (yield: 4.81 g, 10.2 mmol, 98%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 7.29-7.22$ (m, 5H), 7.14 (d, $J = 8.12$ Hz, 2H), 7.09 (d, $J = 8.17$ Hz, 2H), 6.02 (d, $J = 8.49$ Hz, 1H), 5.35 (s, 1H), 5.00 (dd, $J = 28.51$, 12.34 Hz, 2H), 4.96-4.94 (m, 1H), 4.21 (d, $J = 5.20$ Hz, 2H), 3.62 (s, 3H), 3.10 (s, 3H), 3.02 (dd, $J = 13.63$, 5.63 Hz, 1H), 2.85 (dd, $J = 13.27$, 7.70 Hz, 1H), 1.43 (s, 9H) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta = 171.54$, 155.50, 137.23, 136.02, 135.04, 129.04, 127.92, 127.48, 127.42, 126.98, 78.64, 66.11, 61.01, 51.78, 43.76, 37.46, 31.52, 27.96 ppm. $\alpha$D$^2$ = +10.1 (c = 1, CHCl$_3$). HRMS: calcd. for C$_{25}$H$_{33}$N$_3$O$_6$ 472.24421 [M+ H]$^+$; found 472.24402.

2-Bromopropene (3.5 eq., 14.0 mmol, 1.25 mL) was dissolved in THF (50 mL) and cooled to –78 °C. tBuLi (6.5 eq., 26.0 mmol, 16.3 mL; 1.6 M in hexane) was added slowly and the mixture was stirred for 1 h at –78 °C after which Weinreb amide 11 (1 eq., 4.0 mmol, 1.89 g) was added in THF (5 mL). The mixture was allowed to warm to –20 °C in 6 h after which TLC analysis indicated complete consumption of the Weinreb amide. A saturated aqueous NH$_4$Cl solution and EtOAc were added and the layers were separated. The organic layer was extracted with brine, dried over MgSO$_4$ and concentrated under reduced pressure. The product was purified by column chromatography (20% → 50% EtOAc/PE) and obtained as a colourless oil (yield: 1.71 g, 3.77 mmol, 94%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 7.33-7.24$ (m, 5H), 7.11 (d, $J = 8.12$ Hz, 2H), 6.97 (d, $J = 8.00$ Hz, 2H), 6.03 (s, 1H), 5.85 (s, 1H), 5.77 (d, $J = 8.18$ Hz, 1H), 5.30 (dd, $J = 14.10$, 6.11 Hz, 1H), 5.12-5.08 (m, 1H), 5.04 (dd, $J = 26.54$, 12.35 Hz, 2H), 4.21 (d, $J = 5.41$ Hz, 2H), 3.09 (dd, $J = 13.79$, 5.88 Hz, 1H), 2.89 (dd, $J = 13.76$, 5.97 Hz, 1H), 1.84 (s, 3H), 1.44 (s, 9H) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta = 199.28$, 155.66, 155.34, 141.99, 137.40, 136.13, 134.60, 129.27, 128.17, 127.80, 127.71, 126.50, 79.01, 66.45, 55.13, 43.96, 38.76, 28.14, 17.44 ppm. HRMS: calcd. for C$_{26}$H$_{32}$N$_2$O$_5$ 453.23840 [M+ H]$^+$; found 453.23818.

Ketone 12 (2.81 g, 4.30 mmol) was dissolved in MeOH (25 mL) and cooled to 0 °C. To this were added CeCl$_3$·7H$_2$O (1.5 eq., 6.45 mmol, 2.43 g) and NaBH$_4$ (1.4 eq., 6.0 mmol, 227 mg) portionwise and the mixture was stirred for 5 min, after which TLC analysis indicated a complete conversion. Glacial acetic acid (10 mL) was added and the mixture was concentrated under reduced pressure. The resulting residue was dissolved in EtOAc and extracted with half saturated aq. NaHCO$_3$ (2×) and brine, dried over MgSO$_4$ and concentrated in vacuo. The title compound was obtained as a colourless oil (yield: 1.79 g, 3.94 mmol, 92%). $^1$H
Selective inhibitors of proteasome’s trypsin-like sites

NMR (400 MHz, CDCl3): $\delta = 7.31-7.01$ (m, 9H), 5.30 (d, $J = 9.18$ Hz, 1H), 5.06 (s, 1H), 5.00 (d, $J = 5.19$ Hz, 1H), 4.96-4.91 (m, 3H), 4.21 (d, $J = 4.41$ Hz, 1H), 4.16-4.11 (m, 1H), 4.07-3.98 (m, 1H), 2.85 (d, $J = 12.55$ Hz, 1H), 2.65 (dd, $J = 13.60$, 10.41 Hz, 1H), 1.77 (s, 3H), 1.44 (s, 9H) ppm. 13C NMR (100 MHz, CDCl3): $\delta = 155.96, 155.85, 144.44, 137.34, 136.44, 129.34, 128.19, 127.77, 127.66, 127.25, 112.17, 79.25, 76.65, 66.27, 54.06, 44.15, 33.69, 28.22, 18.73 ppm.

$\alpha$ = –18.7 (c = 1, CHCl3). HRMS: calcd. for C26H34N2O5 455.25405 [M+ H]+; found 455.25392.

benzyl ((S)-3-(4-((tert-butyloxycarbonylamino)methyl)phenyl)-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)carbamate (14)

Allylic alcohol 13 (1.79 g, 3.94 mmol) was dissolved in DCM (25 mL) and cooled to 0 °C after which vanadyl acetylacetonate (0.1 eq., 0.4 mmol, 107 mg) and tBuOOH (3 eq., 12.0 mmol, 2.18 mL; 5.5 M in decane) were added and the mixture was stirred at 0 °C until TLC analysis indicated complete consumption of starting material after 2 h. The mixture was concentrated under reduced pressure, redissolved in EtOAc and extracted with half sat. aq. NaHCO3, H2O and brine, dried over MgSO4 and concentrated under reduced pressure. The resulting product was quickly purified by column chromatography (20% → 60% EtOAc/PE) and immediately subjected to the next step because of the possible instability of the intermediate. The compound was dissolved in DCM (25 mL) and Dess-Martin periodinane (3 eq., 11.0 mmol, 4.50 g) was added. The mixture was stirred at RT for 12 h after which TLC analysis indicated complete conversion. Next, a 1:4 (v/v) mixture (150 mL) of NaHCO3 sat. aq./Na2S2O3 (1 M aq.) and the resulting emulsion was stirred vigorously for 30 min. after which the layers were separated and the aqueous layer extracted with DCM. The combined organic layers were extracted with sat. aq. NaHCO3, H2O and brine, dried over MgSO4 and concentrated under reduced pressure. The title compound was obtained after column chromatography (20% → 30% EtOAc/PE) as a colourless oil (yield: 1.03 g, 2.20 mmol, 56%). 1H NMR (400 MHz, CDCl3): $\delta = 7.33-7.22$ (m, 5H), 7.16 (d, $J = 7.94$ Hz, 2H), 7.08 (d, $J = 7.95$ Hz, 2H), 5.51 (d, $J = 8.19$ Hz, 1H), 5.06-5.01 (m, 1H), 4.97 (d, $J = 4.39$ Hz, 2H), 4.60 (d, $J = 12.65$, 7.86 Hz, 1H), 4.24 (d, $J = 4.36$ Hz, 2H), 3.26 (d, $J = 4.62$ Hz, 1H), 3.08 (d, $J = 13.98$, 4.48 Hz, 1H), 2.87 (d, $J = 4.53$ Hz, 1H), 2.70 (d, $J = 13.88$, 8.12 Hz, 1H), 1.49 (s, 3H), 1.44 (s, 9H) ppm. 13C NMR (100 MHz, CDCl3): $\delta = 207.77, 155.74, 155.66, 137.61, 135.97, 134.62, 129.32, 128.26, 127.91, 127.76, 79.15, 66.62, 58.99, 54.07, 52.12, 44.07, 36.61, 28.21, 16.34 ppm. $\alpha$ = +82.2 (c = 1, CHCl3).

$\textit{tert}$-butyl 4-((S)-2-amino-3-((R)-2-methyloxiran-2-yl)-3-oxopropyl)benzylcarbamate TFA salt (15)

Cbz protected amine 14 (107 mg, 0.23 mmol) was dissolved in MeOH (5 mL) and to this was added TFA (1.2 eq., 0.27 mmol, 21 μL). Argon was bubbled through the solution for 15 min., after which Pd black (10 mg) was added and the flask was charged with hydrogen gas. After 10 min, TLC analysis indicated complete conversion of starting material and all solids were removed by filtration over Celite. Toluene (10 mL) was added and the mixture was concentrated under reduced pressure followed by coevaporation with toluene (2×) in order to remove excess TFA. The purity of the deprotected amine (as TFA salt) was confirmed by LC-MS analysis and the compound was subjected to the next step without further purification.

(S)-$\textit{tert}$-butyl 4-(3-(methoxy(methyl)amino)-3-oxo-2-(tritylamino)propyl)benzylcarbamate (16)

Compound 11 (1.43 g, 3.04 mmol) was dissolved in a 50:1 mixture EtOH/AcOH (25 mL) and argon was bubbled through this solution for 15 min. Next, Pd/C (10% w/w, 0.1 g) was added and hydrogen was bubbled through the mixture until TLC indicated complete consumption of starting material after 4 h. Argon was bubbled through for another 15 min. after which the mixture was filtered over Celite and the filtrate concentrated under reduced pressure. The deprotected amine (as AcOH salt) was obtained in a crude yield of
1.21 g (max. 3.04 mmol) and was subsequently dissolved in DCM (20 mL). To this were added Et$_3$N (2 eq., 6.08 mmol, 0.85 mL), DMAP (0.1 g) and trityl chloride (1.5 eq., 4.56 mmol, 1.30 g). The mixture was stirred for 6 h after which it was concentrated under reduced pressure, redissolved in ETOAc and extracted with 10 mM aq. HCl and brine, dried over MgSO$_4$ and concentrated under reduced pressure. The resulting mixture was purified by column chromatography (10% → 50% ETOAc/PE) and the title compound was obtained as colourless foam (yield: 0.68 g, 1.17 mmol, 38%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 7.47 (s, 1H), 7.34 (d, J = 7.33 Hz, 6H), 7.26-7.20 (m, 4H), 7.18-7.05 (m, 9H), 5.10 (s, 1H), 4.28 (s, 2H), 4.00 (t, J = 5.60 Hz, 1H), 3.18 (s, 3H), 2.92 (dd, J = 13.24, 5.63 Hz, 1H), 2.77 (dd, J = 13.24, 5.63 Hz, 1H), 2.69 (s, 3H), 1.44 (s, 9H) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 174.80, 155.74, 145.92, 137.18, 137.13, 130.25, 128.70, 127.33, 127.15, 125.86, 79.07, 70.59, 54.09, 44.19, 41.86, 31.96, 28.20 ppm. $\lambda$ = +58.6 (c = 1, CHCl$_3$).

(S,E)-tert-butyl 4-(4-(methylsulfonyl)-2-(tritylamino)but-3-en-1-yl)benzylcarbamate (17)

Weinreb amide 16 (0.65 g, 1.12 mmol) was dissolved in Et$_2$O (15 mL), put under an argon atmosphere and cooled to 0 °C. LiAlH$_4$ (2 eq., 2.25 mmol, 0.56 mL of a 4 M solution in Et$_2$O) was added slowly and the mixture was stirred at 0 °C for 1 h after which TLC analysis indicated complete conversion of the starting compound. 0.1 M aq. HCl (15 mL) was slowly added and the layers were separated. The organic layer was extracted with 0.1 M aq. HCl and brine, dried over MgSO$_4$ and concentrated under reduced pressure. Diethyl ((methylsulfonyl)methyl)phosphonate (1.5 eq., 1.68 mmol, 0.39 g) was dissolved in THF (20 mL) and cooled to 0 °C under an argon atmosphere. NaH (1.5 eq., 1.68 mmol, 67.2 mg, 60% w/w in mineral oil) was slowly added and the mixture was stirred at 0 ºC for 30 min. Next, the freshly obtained aldehyde (in THF (2 mL)) was slowly added and the mixture was stirred for 2 h while slowly warming it to RT. After this time TLC analysis indicated complete conversion of the aldehyde. EtOAc (20 mL) was added and the mixture was extracted with 10 mM aq. HCl (2×) and brine, dried over MgSO$_4$ and concentrated under reduced pressure. The title compound was obtained after column chromatography (20% → 50% EtOAc/PE) as a colourless foam (yield: 0.57 g, 0.95 mmol, 85%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 7.46 (d, J = 7.6 Hz, 6H), 7.28 (t, J = 7.20, 6.80 Hz, 6H), 7.20 (t, J = 7.20, 7.20 Hz, 3H), 7.13 (d, J = 7.60 Hz, 2H), 6.87 (d, J = 8.00 Hz, 2H), 6.57 (dd, J = 14.80, 7.00 Hz, 1H), 5.96 (d, J = 14.80 Hz, 1H), 4.80 (s, 1H), 4.24 (d, J = 5.60 Hz, 2H), 3.49 (q, J = 6.00 Hz, 1H), 2.61 (s, 3H), 2.54 (dd, J = 13.20, 5.20 Hz, 1H), 2.33 (dd, J = 13.20, 8.20 Hz, 1H), 1.44 (s, 9H) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 155.59, 150.21, 145.74, 137.42, 135.28, 129.53, 128.35, 128.02, 127.70, 127.14, 126.44, 78.91, 71.05, 55.33, 43.79, 42.43, 41.86, 28.09 ppm. $\lambda$$_D$ = +58.6 (c = 1, CHCl$_3$). HRMS: calcd. for C$_{36}$H$_{40}$N$_2$O$_4$S $\lambda$$_D$ = 619.26010 [M+ Na]$: found 619.26001.

(S,E)-tert-butyl 4-(4-(methylsulfonyl)-2-aminobut-3-en-1-yl)benzylcarbamate (18)

Trityl protected amine 17 (0.54 g, 0.90 mmol) was treated with 1% v/v TFA/DCM (15 mL) at RT. To this yellow solution was added H$_2$O (1 mL) which resulted in a colourless suspension. After stirring the mixture for 30 min., 10 mM aq. HCl (20 mL) was added and DCM was removed under reduced pressure. The aqueous layer was extracted with Et$_2$O (3×) and basified with NaHCO$_3$ until pH 9, after which it was extracted with DCM (3×). The latter combined organic layers were dried over MgSO$_4$ and concentrated under reduced pressure. The resulting deprotected amine proved to be pure on LC-MS analysis and was subjected to the next step without further purification.
**Selective inhibitors of proteasome's trypsin-like sites**

**N3Phe-Leu-Leu-Phe(4-CH$_2$NH$_2$)VS TFA salt (4a)**

This compound was synthesized according to General procedure I on a 100 µmol scale by addition of amine. The title compound was obtained after RP-HPLC purification (gradient: 20% → 60% MeOH/0.1% aq. TFA) as a colourless solid (yield: 15.4 mg, 20.1 µmol, 20%). $^1$H NMR (400 MHz, CD$_3$OD): $\delta = 7.39-7.21$ (m, 9H), 6.78 (dd, $J = 15.20$, 5.34 Hz, 1H), 6.55 (dd, $J = 15.21$, 1.52 Hz, 1H), 4.82-4.77 (m, 1H), 4.36-4.27 (m, 2H), 4.17 (dd, $J = 8.61$, 4.80 Hz, 1H), 4.07 (s, 2H), 3.19 (dd, $J = 14.05$, 4.75 Hz, 1H), 3.02-2.95 (m, 3H), 2.92 (s, 3H), 1.63-1.43 (m, 6H), 0.93 (t, $J = 5.65$, 5.65 Hz, 6H), 0.88 (d, $J = 6.24$ Hz, 6H) ppm. $^{13}$C NMR (100 MHz, CD$_3$OD): $\delta = 174.45$, 174.27, 171.95, 146.65, 139.63, 137.85, 133.01, 131.90, 131.30, 130.47, 130.26, 129.67, 128.13, 65.56, 53.74, 53.49, 52.46, 44.11, 42.83, 41.61, 40.29, 38.71, 25.95, 25.86, 23.47, 23.46, 21.96, 21.94 ppm. LC-MS: $R_t$ (min): 6.99 (ESI-MS (m/z): 654.20 (M + H$^+$)). HRMS: calcd. for C$_{33}$H$_{47}$N$_7$O$_5$S 654.34321 [M+ H$^+$]; found 654.34322.

**N3Phe-Leu-Leu-Phe(4-CH$_2$NH$_2$)EK TFA salt (4b)**

This compound was synthesized according to General procedure I on a 100 µmol scale by addition of amine. The title compound was obtained after RP-HPLC purification (gradient: 20% → 60% MeOH/0.1% aq. TFA) as a colourless solid (yield: 17.6 mg, 23.5 µmol, 24%). $^1$H NMR (400 MHz, CD$_3$OD): $\delta = 7.36-7.20$ (m, 9H), 4.68 (dd, $J = 9.34$, 4.20 Hz, 1H), 4.38-4.28 (m, 2H), 4.12 (dd, $J = 8.58$, 4.79 Hz, 1H), 4.05 (s, 2H), 3.21 (d, $J = 4.97$ Hz, 1H), 3.15 (dd, $J = 14.18$, 4.69 Hz, 1H), 3.08 (dd, $J = 13.84$, 4.06 Hz, 1H), 2.95-2.87 (m, 2H), 2.72 (dd, $J = 13.90$, 9.34 Hz, 1H), 1.52-1.43 (m, 6H), 1.41 (s, 3H), 0.94-0.83 (m, 12H) ppm. $^{13}$C NMR (100 MHz, CD$_3$OD): $\delta = 208.54$, 174.48, 174.13, 171.69, 139.71, 137.87, 132.95, 131.15, 130.47, 130.14, 129.65, 128.10, 65.59, 60.25, 54.51, 53.40, 53.15, 52.79, 44.14, 42.13, 41.79, 38.73, 37.11, 25.83, 23.49, 22.03, 21.94, 16.81 ppm. LC-MS: $R_t$ (min): 7.36 (ESI-MS (m/z): 634.20 (M + H$^+$)). HRMS: calcd. for C$_{34}$H$_{47}$N$_7$O$_5$ 634.37114 [M+ H$^+$]; found 634.37090.

**S-(9H-fluoren-9-yl)methyl (3-(4-(tert-butyloxycarbonylamino)phenyl)-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)carbamate (20)**

To a suspension of Fmoc-Phe(4-NO$_2$)-OH (19, 1.27 g, 2.93 mmol) in MeOH (60 mL) was added ammonium formate (10 eq., 30.0 mmol, 1.95 g) which resulted in a clear solution. Pd/C (10% w/w, 0.5 g) was added and the mixture was stirred at RT for 14 h after which TLC analysis indicated complete consumption of starting material. All solids were removed by filtration over Celite and the filtrate was concentrated under reduced pressure. In order to remove excess ammonium formate the resulting product was coevaporated with a 3:1 (v/v) mixture of MeOH/H$_2$O (5×). Next, the residue was dissolved in H$_2$O (40 mL) containing NaHCO$_3$ (11.7 mmol, 0.99 g) and cooled to 0 °C. To this was added Boc$_2$O (4.40 mmol, 0.99 g) in 1,4-dioxane (20 mL) and the mixture was allowed to stir at RT for 14 h. The mixture was acidified with 10% w/v aq. HCl until pH 1 and extracted three times with EtOAc. The combined organic layers were dried over MgSO$_4$ and concentrated under reduced pressure. Finally the Weinreb amide was created in a peptide couplings procedure similar to that for 11. The title compound was obtained after column chromatography (10% → 50% EtOAC/PE) as a colourless oil (yield: 1.59 g, 2.91 mmol, 99%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 7.70-7.66$ (m, 2H), 7.53 (dd, $J = 12.68$, 7.49 Hz, 2H), 7.33 (dd, $J = 13.74$, 6.75 Hz, 4H), 7.27-7.21 (m, 2H), 7.11 (d, $J = 8.33$ Hz, 2H), 6.17 (d, $J = 8.85$ Hz, 1H), 5.06-4.98 (m, 1H), 4.32 (dd, $J = 10.25$, 7.60 Hz, 1H), 4.25-4.19 (m, 1H), 4.14 (t, $J = 7.25$, 7.25 Hz, 1H), 3.60 (s, 3H), 3.13 (s, 3H), 3.05 (dd, $J = 13.68$, 5.45 Hz, 1H), 2.90 (dd, $J = 13.46$, 7.49 Hz, 1H), 1.47 (s, 9H) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta = 171.76$, 155.65, 152.62, 143.49, 143.42, 140.78, 137.22, 130.36, 129.47, 127.25, 126.67, 124.85, 124.81, 119.51, 118.14, 79.76, 66.61, 61.13, 51.93, 46.66, 37.40, 31.64, 27.96 ppm.
(S)-tert-butyl (4-(2-amino-3-(methoxy(methyl)amino)-3-oxopropyl)phenyl)carbamate (21)

To a solution of compound 20 (1.50 g, 2.75 mmol) in THF (20 mL) was added DBU (1.5 mmol, 229 µL). After 10 min. TLC analysis indicated complete consumption of starting material. 1 M aq. HCl (30 mL) and EtOAc (25 mL) were added and the layers were separated. The organic layer was extracted with 1 M aq. HCl and the combined aqueous layers were basified with Na₂CO₃ until pH 10. This layer was extracted with EtOAc (3×) and the latter combined organic layers were dried over MgSO₄ and concentrated under reduced pressure. The title compound was obtained as a colourless oil (yield: 0.75 g, 2.34 mmol, 85%).

1H NMR (400 MHz, CDCl₃) δ = 7.62 (s, 1H), 7.33 (d, J = 8.14 Hz, 2H), 7.09 (d, J = 8.43 Hz, 2H), 4.02-3.95 (m, 1H), 3.57 (s, 3H), 3.16 (s, 3H), 2.98 (dd, J = 13.30, 5.56 Hz, 1H), 2.65 (dd, J = 13.25, 7.71 Hz, 1H), 1.93 (s, 2H), 1.49 (s, 9H) ppm.

13C NMR (100 MHz, CDCl₃) δ = 175.13, 152.66, 137.04, 131.64, 129.29, 118.28, 79.54, 60.96, 52.38, 40.41, 31.82, 27.94 ppm. [α]D²³ = +19.6 (c = 1, CHCl₃).

HRMS: calcd. for C₁₆H₂₅N₃O₄ 324.19178 [M+ H]+; found 324.19193.

(S,E)-tert-butyl (4-(4-(methylsulfonyl)-2-(tritylamino)but-3-en-1-yl)phenyl)carbamate (23)

To a solution of Weinreb amide 22 (0.61 g, 1.11 mmol) in a synthetic procedure similar to that for compound 17 and obtained after column chromatography (10% → 40% EtOAc/PE) as a colourless foam (yield: 0.24 g, 0.41 mmol, 37%).

1H NMR (400 MHz, CDCl₃): δ = 7.45 (d, J = 7.49 Hz, 6H), 7.28-7.15 (m, 12H), 6.81 (d, J = 8.38 Hz, 2H), 6.58 (s, 1H), 5.92 (d, J = 15.11 Hz, 1H), 3.45 (dd, J = 12.80, 6.98 Hz, 1H), 2.58 (s, 3H), 2.28 (dd, J = 13.36, 5.25 Hz, 1H), 2.14 (s, 3H), 1.49 (s, 9H) ppm.

[α]D²³ = −14.8 (c = 1, CHCl₃).
(S)-benzyl (3-(4-(tert-butyloxycarbonylamino)phenyl)-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)carbamate (25)

To a solution of amine 21 (0.79 g, 2.43 mmol) in THF (20 mL) was added DIPEA (1.2 eq., 2.91 mmol, 482 µL), and benzylchloroformate (1.1 eq., 2.67 mmol, 392 µL) and the mixture was stirred for 4 h in which a colourless solid precipitated. EtOAc was added and the mixture was extracted with 1 M aq. HCl (2×), sat. aq. NaHCO3 and brine, dried over MgSO4 and concentrated under reduced pressure. The title compound was obtained after column chromatography (10% → 75% EtOAc/PE) as a colourless foam (yield: 0.95 g, 2.06 mmol, 85%).  

1H NMR (400 MHz, CDCl3): δ = 7.33-7.23 (m, 7H), 7.14 (s, 1H), 7.05 (d, J = 8.21 Hz, 2H), 5.80 (d, J = 8.71 Hz, 1H), 5.04 (dd, J = 26.77, 12.39 Hz, 2H), 4.98-4.94 (m, 1H), 3.62 (s, 3H), 3.13 (s, 3H), 3.01 (dd, J = 13.65, 5.91 Hz, 1H), 2.85 (dd, J = 13.58, 7.35 Hz, 1H), 1.49 (s, 9H) ppm.  

13C NMR (100 MHz, CDCl3): δ = 171.72, 155.64, 152.64, 137.21, 136.07, 130.35, 129.52, 128.13, 127.69, 127.64, 118.18, 79.88, 66.41, 61.21, 51.95, 37.53, 31.705 ppm.


(S)-benzyl (1-(4-(tert-butyloxycarbonylamino)phenyl)-4-methyl-3-oxopent-4-en-2-yl)carbamate (26)

This compound was prepared from Weinreb amide 25 (0.90 g, 1.96 mmol) in a synthetic procedure similar to that for compound 12 and obtained after column chromatography (10% → 25% EtOAc/PE) as a colourless oil (yield: 0.70 g, 1.58 mmol, 81%).  

1H NMR (400 MHz, CDCl3): δ = 7.37-7.20 (m, 7H), 6.92 (d, J = 8.47 Hz, 2H), 6.81 (s, 1H), 6.00 (s, 1H), 5.86 (d, J = 1.20 Hz, 1H), 5.66 (d, J = 8.15 Hz, 1H), 5.30 (dd, J = 14.10, 6.04 Hz, 1H), 5.07 (q, J = 12.28, 12.28, 12.25 Hz, 2H), 3.07 (dd, J = 13.85, 6.11 Hz, 1H), 2.89 (dd, J = 13.86, 5.86 Hz, 1H), 1.84 (s, 3H), 1.49 (s, 9H) ppm.  

13C NMR (100 MHz, CDCl3): δ = 199.48, 155.44, 152.61, 142.11, 137.24, 136.17, 129.91, 129.69, 128.31, 127.92, 127.84, 126.73, 118.22, 80.17, 66.63, 55.33, 38.83, 28.16, 17.54 ppm. = +77.1 (c = 1, CHCl3). HRMS: calcd. for C25H30N2O5 439.22275 [M+ H]+; found 439.22276.

benzyl ((2S,3R)-1-(4-(tert-butyloxycarbonylamino)phenyl)-3-hydroxy-4-methylpent-4-en-2-yl)carbamate (27)

This compound was prepared from ketone 26 (0.70 g, 1.85 mmol) in a synthetic procedure similar to that for compound 13 and obtained after column chromatography (10% → 25% EtOAc/PE) as a colourless oil (yield: 0.71 g, 1.85 mmol, quant.).  

1H NMR (400 MHz, CDCl3): δ = 7.32-7.18 (m, 7H), 7.04 (d, J = 8.15 Hz, 2H), 5.17 (d, J = 9.01 Hz, 1H), 5.05-4.91 (m, 4H), 4.14-4.10 (m, 1H), 4.05-3.97 (m, 1H), 3.01 (s, 1H), 2.86-2.78 (m, 1H), 2.64 (dd, J = 14.18, 9.64 Hz, 1H), 1.76 (s, 3H), 1.50 (s, 9H) ppm.  

13C NMR (100 MHz, CDCl3): δ = 156.03, 152.82, 144.45, 136.48, 136.33, 132.60, 129.65, 128.24, 127.79, 127.70, 118.49, 112.48, 80.17, 76.61, 66.37, 54.06, 33.46, 28.20, 18.71 ppm. = –13.8 (c = 1, CHCl3). HRMS: calcd. for C25H32N2O5 441.23840 [M+ H]+; found 441.23843.

(S)-benzyl (1-(4-(tert-butyloxycarbonylamino)phenyl)-4-methyl-3-oxopent-4-en-2-yl)carbamate (28)

This compound was prepared from allylic alcohol 27 (0.70 g, 1.58 mmol) in a synthetic procedure similar to that for compound 14 and obtained after column chromatography (10% → 30% EtOAc/PE) as a colourless oil (yield: 0.35 g, 0.76 mmol, 48%).  

1H NMR (400 MHz, CDCl3): δ = 7.28-7.17 (m, 7H), 7.10 (s, 1H), 6.96 (d, J = 8.42 Hz, 2H), 6.50 (s, 1H), 5.27 (d, J = 8.21 Hz, 1H), 5.00-4.87 (m, 2H), 4.51 (dt, J = 7.98, 7.96, 4.96 Hz, 1H), 3.19 (d, J = 4.85 Hz, 1H), 2.98 (dd, J = 14.06, 4.82 Hz, 1H), 2.82 (d, J = 4.81 Hz, 1H) 2.62 (dd, J = 14.04, 7.84 Hz, 1H), 1.47 (s, 3H), 1.43 (s, 9H) ppm.  

13C NMR (100 MHz, CDCl3): δ = .207.87, 155.70, 155.61, 137.34, 136.02, 129.93, 129.78, 128.40, 128.04, 127.91, 118.53, 112.31, 82.57, 66.80, 59.12, 54.15, 52.24, 36.62, 28.25, 16.47 ppm. = +91.3 (c = 1, CHCl3).
**tert-butyl (4-((S)-2-amino-3-((R)-2-methyloxiran-2-yl)-3-oxopropyl)phenyl)carbamate TFA salt (29)**

This compound was prepared from Cbz protected amine 28 in a synthetic procedure similar to that for compound 15. The purity was checked by LC-MS analysis and the amine (as TFA salt) was subjected to the next step without further purification.

![Chemical Structure](image)

**N\(_2\)Phe-Leu-Leu-Phe(4-NH\(_2\))VS (5a)**

This compound was synthesized according to General procedure I on a 100 µmol scale by addition of amine 24. The title compound was obtained after RP-HPLC purification (gradient: 10% → 90% ACN/0.1% aq. TFA) as a colourless solid (yield: 8.2 mg, 10.8 µmol, 11%). \(^1\)H NMR (400 MHz, CD\(_3\)OD): 6 = 8.25 (d, J = 8.29 Hz, 1H), 7.93 (d, J = 7.42 Hz, 1H), 7.32 (d, J = 8.47 Hz, 2H), 7.26-7.19 (m, 8H), 6.77 (dd, J = 15.20, 5.35 Hz, 1H), 6.53 (dd, J = 15.21, 1.52 Hz, 1H), 4.81-4.73 (m, 1H), 4.33-4.27 (m, 1H), 4.27-4.22 (m, 1H), 4.14 (dd, J = 8.58, 4.82 Hz, 1H), 3.16 (dd, J = 14.07, 4.83 Hz, 1H), 2.99-2.90 (m, 3H), 2.89 (s, 3H), 1.59-1.38 (m, 6H), 0.89 (dd, J = 6.13, 3.43 Hz, 6H), 0.85 (d, J = 6.11 Hz, 6H) ppm. 13C NMR (100 MHz, CD\(_3\)OD): 6 = 174.55, 174.47, 171.98, 146.53, 138.54, 137.85, 137.85, 132.22, 132.01, 130.48, 129.68, 128.14, 123.43, 65.57, 53.73, 53.70, 52.53, 42.79, 41.83, 41.60, 41.63, 39.97, 38.72, 38.72, 25.94, 25.86, 23.48, 23.46, 21.94 ppm. LC-MS: Rt (min): 6.95 (ESI-MS (m/z): 640.0 (M + H\(^+\)). HRMS: calcd. for C\(_{32}\)H\(_{45}\)N\(_7\)O\(_5\)S [M+ H\(^+\)] 640.32756; found 640.32756.

**N\(_2\)Phe-Leu-Leu-Phe(4-NH\(_2\))EK (5b)**

This compound was synthesized according to General procedure I on a 100 µmol scale by addition of amine 29. The title compound was obtained after RP-HPLC purification (gradient: 30% → 50% ACN/0.1% aq. TFA) as a colourless solid (yield: 4.1 mg, 6.60 µmol, 6.6%). \(^1\)H NMR (400 MHz, CD\(_3\)OD): 6 = 7.36 (d, J = 8.47 Hz, 2H), 7.28-7.16 (m, 9H), 4.66 (dd, J = 9.33, 4.29 Hz, 1H), 4.35-4.27 (m, 2H), 4.10 (dd, J = 8.46, 4.68 Hz, 1H), 3.19 (d, J = 4.88 Hz, 1H), 3.15-3.10 (m, 2H), 2.98-2.88 (m, 2H), 2.73 (dd, J = 8.77, 5.04 Hz, 1H), 1.56-1.42 (m, 8H), 1.38 (s, 3H), 0.90-0.82 (m, 12H) ppm. LC-MS: Rt (min): 7.37 (ESI-MS (m/z): 620.20 (M + H\(^+\)). HRMS: calcd. for C\(_{33}\)H\(_{45}\)N\(_7\)O\(_5\) [M+ H\(^+\)] 620.35549; found 620.35532.

**H-Lys(Boc)-N(Me)OMe (30)** (2.72 g, 6.44 mmol) was dissolved in DCM (40 mL) and to this were added DiPEA (2 eq., 12.9 mmol, 2.13 mL) and tritylchloride (1.1 eq., 7.08 mmol, 1.97 g). The reaction mixture was stirred for 15 h, after which TLC analysis indicated complete consumption of starting material, extracted with H\(_2\)O (4×) and dried over MgSO\(_4\). The title compound was obtained after purification by column chromatography (DCM → 3% MeOH/DCM) as a colourless foam (yield: 2.30 g, 4.32 mmol, 68%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): 6 = 7.49 (d, J = 7.47 Hz, 6H), 7.23 (t, J = 7.60, 7.60 Hz, 6H), 7.14 (t, J = 7.25 Hz, 3H), 4.63 (s, 1H), 3.81 (s, 1H), 3.31 (s, 3H), 3.15-3.03 (m, 2H), 2.70 (s, 3H), 1.81-1.68 (m, 1H), 1.61-1.29 (m, 14H) ppm. 13C NMR (100 MHz, CDCl\(_3\)): 6 = 175.06, 155.58, 145.92, 128.52, 127.17, 125.77, 78.26, 70.68, 59.98, 51.52, 40.00, 34.85, 31.77, 29.92, 28.04, 21.55 ppm.

**((S)-5-(trityl-amino)-5-(methoxy-methyl-carbamoyl)-pentyl)-carbamic acid tert-butyl carbamate (31)**

H-Lys(Boc)-N(Me)OMe (30) (2.72 g, 6.44 mmol) was dissolved in DCM (40 mL) and to this were added DiPEA (2 eq., 12.9 mmol, 2.13 mL) and tritylchloride (1.1 eq., 7.08 mmol, 1.97 g). The reaction mixture was stirred for 15 h, after which TLC analysis indicated complete consumption of starting material, extracted with H\(_2\)O (4×) and dried over MgSO\(_4\). The title compound was obtained after purification by column chromatography (DCM → 3% MeOH/DCM) as a colourless foam (yield: 2.30 g, 4.32 mmol, 68%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): 6 = 7.49 (d, J = 7.47 Hz, 6H), 7.23 (t, J = 7.60, 7.60 Hz, 6H), 7.14 (t, J = 7.25 Hz, 3H), 4.63 (s, 1H), 3.81 (s, 1H), 3.31 (s, 3H), 3.15-3.03 (m, 2H), 2.70 (s, 3H), 1.81-1.68 (m, 1H), 1.61-1.29 (m, 14H) ppm. 13C NMR (100 MHz, CDCl\(_3\)): 6 = 175.06, 155.58, 145.92, 128.52, 127.17, 125.77, 78.26, 70.68, 59.98, 51.52, 40.00, 34.85, 31.77, 29.92, 28.04, 21.55 ppm.

**((S,E)-tert-butyl (5-(trityl-amino)-7-(methylsulfonyl)hept-6-en-1-yl)-carbamate (32)**

This compound was prepared from Weinreb amide 31 (0.81 g, 1.52 mmol) in a synthetic procedure similar to that for compound 17 and obtained after column chromatography (0% → 30% EtOAc/PE) as a colourless foam (yield: 0.53 g, 0.97 mmol, 64%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): 6 = 7.51-7.46 (m, 6H), 7.29-7.23 (m, 6H), 7.20-7.14 (m,
Selective inhibitors of proteasome's trypsin-like sites

(3R,5S)-tert-butyl (3-amino-7-(methylsulfonyl)hept-6-en-1-yl)carbamate (33)

This compound was prepared from trityl protected amine 32 in a synthetic procedure similar to that for compound 18. The purity was checked by LC-MS analysis and the amine was subjected to the next step without further purification.

\[
^13\text{C NMR (100 MHz, CDCl}_3\text{}): \delta = 155.64, 150.93, 145.89, 128.38, 128.01, 127.69, 126.42, 78.62, 71.06, 53.72, 42.56, 39.77, 35.29, 29.54, 28.14, 22.08 \text{ ppm.}
\]

\[
\text{N}_3\text{Phe-Leu-Leu-LysVS TFA salt (7)}
\]

This compound was synthesized according to General procedure I on a 360 \(\mu\)mol scale by addition of amine 33. The title compound was obtained after RP-HPLC purification (gradient: 20% \(\rightarrow\) 65% ACN/0.1% aq. TFA) as a colourless solid (yield: 96.5 mg, 134 \(\mu\)mol, 37%). \(^1\text{H NMR (400 MHz, CD}_3\text{OD)}:\delta = 7.16-7.04 (m, 5H), 6.65 (dd, J = 15.20, 5.10 Hz, 1H), 6.46 (dd, J = 15.21, 1.40 Hz, 1H), 4.44 (td, J = 9.60, 4.87, 4.87 Hz, 2H), 4.04 (dd, J = 8.56, 4.80 Hz, 1H), 0.72 (d, J = 5.77 Hz, 3H) ppm. \(^{13}\text{C NMR (100 MHz, CD}_3\text{OD)}:\delta = 174.72, 174.64, 171.90, 147.77, 137.79, 131.19, 130.46, 129.64, 128.10, 128.01, 127.69, 126.42, 78.62, 71.06, 53.72, 42.56, 39.77, 35.29, 29.54, 28.14, 22.08 \text{ ppm.}
\]

\[\text{HRMS: calcd. for } C_{29}H_{47}N_7O_5S [M+ H]^+ 606.343; \text{ found } 606.34299.\]

\[
\text{(S,E)-tert-butyl (5-amino-7-(methylsulfonyl)hept-6-en-1-yl)carbamate (33)}
\]

\[
\text{To a mixture of Boc-\(\beta\)-(4-pyridyl)-L-alanine (34, 300 mg, 1.08 mmol) and NH(Me)OMe \cdot HCl (1.2 eq., 1.30 mmol, 129 mg) in DCM (10 mL) was added HCTU (1.2 eq., 1.30 mmol, 536 mg) and DiPEA (3.5 eq., 3.78 mmol, 625 \mu\text{L}) and the mixture was stirred for 1 h after which TLC analysis indicated complete conversion of starting material. The solvent was evaporated under reduced pressure en the residue was taken up in EtOAc, extracted with sat. aq. NaHCO}_3 (2×) and brine, dried over MgSO}_4 and concentrated in vacuo. The title compound was obtained after purification by column chromatography (EtOAc \(\rightarrow\) 2% MeOH/EtOAc) as a pale yellow solid (yield: 334 mg, 1.08 mmol, quant.). \(^1\text{H NMR (400 MHz, CD}_3\text{OD)}:\delta = 8.40 (d, J = 4.59 Hz, 2H), 7.29 (d, J = 4.88 Hz, 2H), 6.97 (d, J = 7.26 Hz, 1H), 4.77-4.68 (m, 1H), 3.76 (s, 3H), 3.15 (s, 3H), 3.02 (dd, J = 13.59, 4.85 Hz, 1H), 2.83-2.77 (m, 1H), 1.32 (s, 9H) ppm. \(^{13}\text{C NMR (100 MHz, CD}_3\text{OD)}:\delta = 173.49, 157.58, 149.88, 149.50, 126.48, 80.55, 62.52, 52.71, 38.03, 32.49, 28.66 ppm. }^{[\alpha]}_D^{23} = -2.9 (c = 1, MeOH). \text{HRMS: calcd. for } C_{15}H_{43}N_3O_4 [M+ H]^+ 310.17613; \text{ found 310.17629.}
\]

\[
\text{(S)-tert-butyl (1-(methoxy(methyl)amino)-1-oxo-3-(pyridin-4-yl)propan-2-yl)carbamate (35)}
\]

\[
\text{To a mixture of Boc-\(\beta\)-(4-pyridyl)-L-alanine (34, 300 mg, 1.08 mmol) and NH(Me)OMe \cdot HCl (1.2 eq., 1.30 mmol, 129 mg) in DCM (10 mL) was added HCTU (1.2 eq., 1.30 mmol, 536 mg) and DiPEA (3.5 eq., 3.78 mmol, 625 \mu\text{L}) and the mixture was stirred for 1 h after which TLC analysis indicated complete conversion of starting material. The solvent was evaporated under reduced pressure en the residue was taken up in EtOAc, extracted with sat. aq. NaHCO}_3 (2×) and brine, dried over MgSO}_4 and concentrated in vacuo. The title compound was obtained after purification by column chromatography (EtOAc \(\rightarrow\) 2% MeOH/EtOAc) as a pale yellow solid (yield: 334 mg, 1.08 mmol, quant.). \(^1\text{H NMR (400 MHz, CD}_3\text{OD)}:\delta = 8.40 (d, J = 4.59 Hz, 2H), 7.29 (d, J = 4.88 Hz, 2H), 6.97 (d, J = 7.26 Hz, 1H), 4.77-4.68 (m, 1H), 3.76 (s, 3H), 3.15 (s, 3H), 3.02 (dd, J = 13.59, 4.85 Hz, 1H), 2.83-2.77 (m, 1H), 1.32 (s, 9H) ppm. \(^{13}\text{C NMR (100 MHz, CD}_3\text{OD)}:\delta = 173.49, 157.58, 149.88, 149.50, 126.48, 80.55, 62.52, 52.71, 38.03, 32.49, 28.66 ppm. }^{[\alpha]}_D^{23} = -2.9 (c = 1, MeOH). \text{HRMS: calcd. for } C_{15}H_{43}N_3O_4 [M+ H]^+ 310.17613; \text{ found 310.17615.}
\]

\[
\text{(S,E)-tert-butyl (4-(methylsulfonyl)-1-(pyridin-4-yl)but-3-en-2-yl)carbamate (36)}
\]

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\text{Weinreb amide 35 (0.33 g, 1.08 mmol) was dissolved in THF (15 mL), put under an argon atmosphere and cooled to 0°C. LiAlH}_4 (1.5 eq., 1.68 mmol, 1.68 \text{mL of a 1 M solution in THF}) was added slowly and the mixture was stirred at 0°C for 1 h after which TLC analysis indicated complete conversion of the starting compound. 0.1 M aq. HCl (1 mL) was slowly added and the mixture was stirred vigorously for 5 min. The organic layer was extracted with sat. aq. NaHCO}_3 (2×) and brine, dried over MgSO}_4 and concentrated under reduced pressure. Diethyl ((methylsulfonyl)methyl)phosphonate (1.5 eq., 1.68 mmol, 0.39 g) was dissolved in THF (20 mL) and cooled to 0°C under an argon atmosphere. NaH (1.3 eq., 1.46 mmol, 58.0 mg, 60% w/w in
mineral oil) was slowly added and the mixture was stirred at 0 °C for 30 min. Next, the freshly obtained aldehyde (in THF (2 mL)) was slowly added and the mixture was stirred for 2 h while slowly warming it to RT. After this time TLC analysis indicated complete conversion of the aldehyde. The reaction was quenched by addition of 1 M aq. HCl (1 mL) after which the mixture was diluted with EtOAc and extracted with sat. aq. NaHCO₃ (2×) and brine, dried over MgSO₄ and concentrated under reduced pressure. The title compound was obtained after column chromatography (40% → 80% Acetone/PE) as a colourless solid (yield: 117 mg, 0.36 mmol, 32%).

1H NMR (400 MHz, CDCl₃): δ = 8.50 (d, J = 5.28 Hz, 2H), 7.15 (d, J = 5.90 Hz, 2H), 6.92 (dd, J = 15.10, 4.76 Hz, 1H), 6.53 (d, J = 15.10 Hz, 1H), 5.56 (d, J = 8.99 Hz, 1H), 4.80-4.72 (m, 1H), 3.02-2.84 (m, 2H), 2.93 (s, 3H), 1.39 (s, 9H) ppm. 13C NMR (100 MHz, CDCl₃): δ = 154.87, 149.59, 146.40, 145.41, 130.04, 124.51, 80.10, 51.10, 42.58, 39.48, 28.06 ppm. [α]D²³ = −6.0 (c = 1, MeOH). HRMS: calcd. for C₁₅H₂₂N₂O₄S [M+ H]+ 327.13730; found 327.13741.

N₃Phe-Leu-Leu-Phe(4-N)VS (6) This compound was synthesized according to General procedure I on a 300 μmol scale by addition of amine 36 (after Boc deprotection with TFA/DCM 1:1 v/v, 37). The title compound was obtained after RP-HPLC purification (gradient: 25% → 45% ACN/0.1% aq. TFA) as a colourless syrup (yield: 89.0 mg, 142 μmol, 48%). 1H NMR (400 MHz, CD₂OD): δ = 8.62 (d, J = 6.12 Hz, 2H), 7.85 (d, J = 6.16 Hz, 2H), 7.21-7.06 (m, 5H), 6.81 (dd, J = 15.13, 5.03 Hz, 1H), 6.62 (dd, J = 15.23, 1.46 Hz, 1H), 5.00-4.93 (m, 1H), 4.20-4.14 (m, 1H), 4.10-4.04 (m, 2H), 3.31 (dd, J = 13.99, 4.46 Hz, 1H), 3.11-3.03 (m, 2H), 2.88 (s, 3H), 2.86-2.82 (m, 1H), 1.51-1.08 (m, 6H), 0.80 (dd, J = 7.81, 6.26 Hz, 6H), 0.75 (d, J = 6.00 Hz, 6H) ppm. 13C NMR (100 MHz, CD₃OD): δ = 174.63, 174.48, 171.74, 160.53, 145.75, 142.38, 137.86, 132.60, 130.47, 129.63, 129.45, 128.07, 65.49, 53.64, 53.55, 50.74, 42.76, 41.57, 41.45, 40.32, 38.64, 25.92, 25.80, 23.49, 23.37, 21.86, 21.80 ppm. LC-MS: Rᵣ (min): 6.86 (ESI-MS (m/z): 626.2 (M + H⁺)). HRMS: calcd. for C₃₁H₄₃N₇O₅S [M+ H]+ 626.31191; found 626.31172.

Bodipy-triazole-Phe-Leu-Leu-Phe(4-CH₂NH₂)VS TFA salt (39) Compound 4a (5.68 mg, 8.69 μmol) and Bodipy-alkyne (1.5 eq., 13.0 μmol, 4.28 mg) were dissolved in a 1:1:1 mixture of H₂O/tBuOH/Tol (1.5 mL) and to this were added CuSO₄ (0.1 eq., 0.87 μmol, 0.87 μL of a 1M solution in H₂O) and sodium ascorbate (0.15 eq., 1.3 μmol, 1.3 μL of a 1M solution in H₂O) and the reaction was stirred at 80 °C for 4 h. LC-MS analysis revealed complete consumption of the azide and formation of a single product (Rᵣ (min.): 10.41 (ESI-MS (m/z): 981.20 (M + H⁺)), which was assigned to be the corresponding benzaldehyde. The mixture was concentrated under reduced pressure and dissolved in MeOH (1.5 mL). To this were added NH₄OAc (10 eq., 70 μmol, 5.4 mg) and NaCNBH₄ (2 eq., 15 μmol, 1.0 mg) and the reaction was stirred for 15 h, after which LC-MS analysis indicated a complete disappearance of the aldehyde peak. The reaction was quenched by addition of aqueous HCl (100 μL, 1M) and the mixture was concentrated under reduced pressure. The title compound was obtained after RP-HPLC purification (gradient: 30% → 70% ACN/0.1% aq. TFA) as a red/brown solid (yield: 2.1 mg, 2.14 μmol, 29%). 1H NMR (400 MHz, CD₂OD): δ = 7.77 (s, 1H), 7.31 (d, J = 7.91 Hz, 2H), 7.25 (d, J = 8.08 Hz, 2H), 7.03-6.97 (m, 5H), 6.75 (dd, J = 15.17, 5.40 Hz, 1H), 6.50 (dd, J = 15.26, 1.28 Hz, 1H), 6.08 (s, 2H), 5.52 (dd, J = 10.52, 5.15 Hz, 1H), 4.85-4.81 (m, 1H), 4.29-4.22 (m, 2H), 3.95 (s, 2H), 3.37-3.34 (m, 2H), 2.97-2.91 (m, 4H), 2.87 (s, 3H), 2.72-2.67 (m, 2H), 2.40 (s, 6H), 2.33 (s, 6H), 1.88-1.79 (m, 2H), 1.64-1.41 (m, 8H), 0.93-0.75 (m, 12H) ppm. LC-MS: Rᵣ (min): 8.42 (ESI-MS (m/z): 982.40 (M + H⁺)). HRMS: calcd. for C₅₂H₇₀BF₂N₉O₅S [M+ H]+ 982.53545; found 982.53653.
Selective inhibitors of proteasome’s trypsin-like sites

(Val-Ser-Phe(4-CH₂NH₂)-methyl vinyl sulfone)-3-hydroxy-2-methylbenzamide (40)

This compound was synthesized according to General procedure I on a 245 µmol scale by addition of amine 18.

The title compound was obtained after RP-HPLC purification (gradient: 10% → 25% ACN/0.1% aq. TFA) as a colourless solid (yield: 57.3 mg, 83.2 µmol, 34%). ¹H NMR (400 MHz, CD₂OD): δ = 7.22 (d, J = 8.48 Hz, 2H), 7.19 (d, J = 8.45 Hz, 2H), 6.95 (t, J = 7.80 Hz, 1H), 6.77-6.69 (m, 3H), 6.65 (dd, J = 15.17, 1.46 Hz, 1H), 4.81-4.76 (m, 1H), 4.29 (t, J = 5.53 Hz, 1H), 4.17 (d, J = 7.16 Hz, 1H), 3.90 (d, J = 5.45 Hz, 2H), 3.69 (dd, J = 10.78, 5.01 Hz, 1H), 3.60 (dd, J = 10.76, 6.19 Hz, 1H), 2.92 (dd, J = 13.83, 6.47 Hz, 1H), 2.84-2.80 (m, 1H), 2.82 (s, 3H), 2.10-2.06 (m, 1H), 0.89 (dd, J = 6.67, 4.95 Hz, 6H) ppm. ¹³C NMR (100 MHz, CD₃OD): δ = 174.00, 173.61, 171.83, 157.08, 146.95, 139.62, 139.11, 132.93, 131.78, 131.18, 127.60, 123.26, 119.16, 117.19, 61.62, 56.49, 52.60, 44.04, 42.84, 40.16, 31.40, 21.00, 19.87, 19.04, 13.04 ppm. LC-MS: Rt (min): 4.19 (ESI-MS (m/z): 575.20 (M + H⁺)). HRMS: calcd. for C₂₈H₃₈N₄O₇S [M+ H]+ 575.25340; found 575.25336.

(Val-Ser-Phe(4-NH₂)-methyl vinyl sulfone)-3-hydroxy-2-methylbenzamide (41)

This compound was synthesized according to General procedure I on a 100 µmol scale by addition of amine 24. The title compound was obtained after RP-HPLC purification (gradient: 10% → 25% ACN/0.1% aq. TFA) as a colourless solid (yield: 14.2 mg, 21.0 µmol, 21%). ¹H NMR (400 MHz, CD₂OD): δ = 7.33 (d, J = 8.45 Hz, 2H), 7.19 (d, J = 8.46 Hz, 2H), 7.00 (t, J = 7.81 Hz, 1H), 6.84-6.70 (m, 4H), 4.33 (dd, J = 6.11, 5.13 Hz, 1H), 4.22 (d, J = 7.14 Hz, 1H), 3.72 (dd, J = 10.74, 5.01 Hz, 1H), 3.64 (dd, J = 10.75, 6.27 Hz, 1H), 3.03 (dd, J = 13.89, 5.94 Hz, 1H), 2.90-2.85 (m, 1H), 2.88 (s, 3H), 2.11 (s, 3H), 2.10-2.06 (m, 1H), 0.94 (t, J = 6.28 Hz, 6H) ppm. ¹³C NMR (100 MHz, CD₃OD): δ = 174.12, 173.55, 171.89, 157.13, 146.75, 139.97, 139.12, 132.23, 131.98, 130.63, 127.60, 124.19, 123.27, 119.14, 117.18, 61.62, 61.36, 56.46, 52.54, 42.80, 39.85, 31.40, 19.87, 19.04, 13.04 ppm. LC-MS: Rt (min): 3.99 (ESI-MS (m/z): 561.07 (M + H⁺)). HRMS: calcd. for C₂₉H₃₆N₄O₇S [M+ H]+ 561.23775; found 561.23775.

Biological evaluation

Competition assays in cell-lysate

Whole cell lysates of HEK-293T or EL-4 were made by sonication in 3 volumes of lysis buffer containing 50 mM Tris pH 7.5, 1 mM DTT, 5 mM MgCl₂, 250 mM sucrose, 2 mM ATP, 0.025% digitonin. Protein concentration was determined by the Bradford assay. Cell lysates (13.5 µg total protein for HEK lysates and 9 µg total protein for EL-4 lysates) were exposed to the inhibitors for 1 h at 37 °C prior to incubation with MV151 (0.5 µM) for 1 h at 37 °C. Reaction mixtures were boiled with Laemmli’s buffer containing β-mercaptoethanol for 5 min. before being resolved by 12.5% SDS-PAGE. In-gel detection of residual proteasome activity was performed in the wet gel slabs directly on the Typhoon Variable Mode Imager (Amersham Biosciences) using the Cy3/Tamra settings (λₜₜₙ 532nm, λₑₐₜ 560 nm) to detect MV151 and Cy2/Fam settings (λₑₜₙ 488 nm, λₑₐₜ 520 nm) to detect compound 39.

Competition assays in living cells

Human embryonic kidney cells (some 1 × 10⁶) were cultured in 6-well plates in DMEM containing 10% fetal calf serum, 10 units/mL penicillin and 10 µg/mL streptomycin in a 7% CO₂ humidified incubator at 37 °C overnight. Part of the medium was taken and to this was added the appropriate inhibitor in DMSO (1 µL of a 1,000× stock solution), after which the medium was added to the cells. The cells were incubated with the inhibitors for 4 h at 37 °C and this was followed by
addition of MV151 (1 μL of a 5 mM stock solution in DMSO) and incubation for 2 h at 37 °C. Next, the medium was removed and the cells were washed with PBS and harvested. After flash freezing in liquid N₂, the cells were resuspended in 4 volumes of homogenation buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl₂, 1 mM DTT, 2 mM ATP, 0.025% digitonin) containing 10 μM AdaKBio, sonicated (12 W, 1 min.) and centrifuged at 16,000 rcf at 0 °C for 20 min. The supernatant was collected and the protein concentration was determined by the Bradford assay. All samples were normalized to the same protein concentration with lysis buffer. After boiling the samples with Laemmli’s buffer containing β-mercaptoethanol for 5 min. and resolving by 12.5% SDS-PAGE the residual proteasome activity was detected as described above.

References

(17) Unpublished results.
Selective inhibitors of proteasome’s trypsin-like sites


(21) A modified procedure of the synthesis for the corresponding leucine epoxyketone described in patent WO/2007/149512, Proteolix Inc., 2007 was applied.


