Discovery and development of inhibitors selective for human constitutive proteasome and immunoproteasome active sites

Xin, B.; Xin B.

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Incorporation of the constrained peptidomimetic, 5-methylpyridin-2-one into peptide vinyl sulfones and peptide epoxyl ketones is detrimental for proteasome inhibition

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4.1 Introduction

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. The constitutive proteasome core 20S particle contains three distinct catalytic activities, β1 (cleaving after acidic amino acids), β2 (cleaving after basic amino acids) and β5 (cleaving after hydrophobic amino acids).[1,2] In higher vertebrates, immunoproteasomes are expressed in specific tissue. Within immunoproteasomes 20S core particles, β1, β2 and β5 are replaced by β1i, β2i and β5i, respectively.[3] Cortical thymic epithelial cells uniquely express β5t subunits, which replace β5i in immunoproteasomes to form thymoproteasomes.[4]

Proteasome inhibitors are important tools in fundamental research on the activity and physiological roles of proteasomes. They are found in nature and on the basis of these natural products, many synthetic analogues have been developed.[5] Proteasomes are validated drug targets in oncology and the peptide boronic acid, bortezomib[6] and the peptide epoxyketone, carfilzomib,[7] are used in the clinic for the treatment of multiple myeloma and mantle cell lymphoma. Often, proteasome inhibitors are composed of short (2-4 residue) oligopeptides, the C-terminus of which is modified to contain an electrophilic trap and the N-terminus of which is capped. Next to peptide boronic acids and peptide epoxyketones – the compound classes to which bortezomib and carfilzomib belong – other electrophilic traps including Michael acceptors (predominantly peptide vinyl sulfones) and aldehydes are often encountered. Arguably, the small size, together with the caps at both termini, protects proteasome inhibitors to a certain extent against proteolysis. In general, however, peptide-based drug candidates suffer from being labile to proteolysis in physiological systems.[8] With the aim to counter this caveat, researchers often turn towards peptidomimetics, functional groups featuring structural elements that mimic those present in natural peptides while conferring protease stability. Several research groups have recently reported on the use of peptidomimetics in proteasome inhibitor design. Baudy-Floch *et al.* reported on the use of retro hydrazine azapeptoids as peptidomimetics in proteasome inhibitors (Figure 1A, **1**).[9] Despite of low inhibitory activities compared to bortezomib, these inhibitors showed some selectivity and specificity towards β5c. Ettari *et al.* incorporated a pyridin-2-one moiety in a peptide vinyl sulfone scaffold (Figure 1A, **2**).[10] In this way, they identified a number of peptide vinyl sulfones capable of inhibiting the constitutive proteasome β5 subunit.

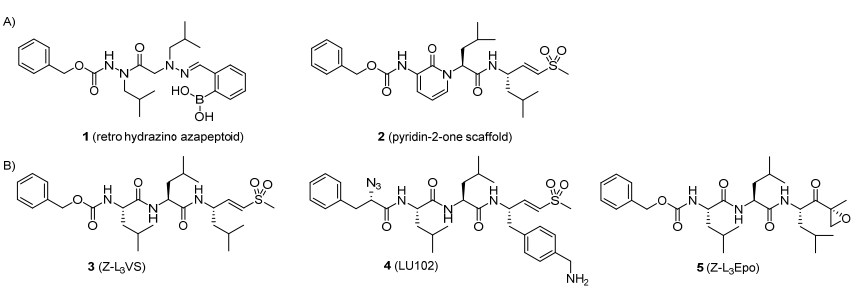


Figure 1: A) previous studies of peptidometics of proteasome inhibitors B) Structures of the lead compounds Z-L_3VS, Z-L_3Epo and LU102.

In the past decade, research has focused on the development of inhibitors specific for one or a number of the catalytic subunits of either constitutive proteasomes or immunoproteasomes.[11] Peptide vinyl sulfones (exemplified by Z-L_3VS) and peptide epoxyketones (exemplified by Z-L_3Epo) were previously taken as starting points. Variation of amino acid residues at positions P1 (the C-terminal residue carrying the electrophilic trap) through to P4 (the N-terminus – at which site a fluorophore or a bioorthogonal group can be installed as a reporter entity) were

carried out. In this way, inhibitors specific for each of the catalytic activities of human constitutive proteasomes and immunoproteasomes were identified, as well as inhibitors specific for a subset of these six catalytic activities.[12] An example of the latter class comprises the peptide vinyl sulfone, LU-102 bearing a basic (p-aminomethyl)-phenylalanine at P1, which proved specific for β2/β2i over the four other catalytic activities.

With the aim to study the merits of peptidomimetics in search for new proteasome inhibitors, it was decided to focus on the broad-spectrum inhibitors, Z-L_3VS and Z-L_3Epo as well as the in-class subunit-specific inhibitor, LU102 as lead structures. Sugar amino acid-based dipeptide isosteres were previously investigated as replacement of an internal (P2-P3) dipeptide in tetrapeptide epoxyketones.[13] In these studies, it was found that the resulting compounds exhibited little to no proteasome inhibitory activity. This observation may be explained by the general finding that the activity of peptide-based proteasome inhibitors relies on their ability to form an antiparallel beta-sheet structures with the amino acid residues in the proteasome active sites.[14,15] Based on this reasoning, attention was turned to the 5-methylpyridin-2(1*H*)-one moiety, which when introduced into an oligopeptide loses a single N-H functionality (thus one H-bond donor) but otherwise leaves the H-bond donor-acceptor pattern of an oligopeptide intact. The pyridine-2(1H)-one unit has been adopted in many biologically active synthetic inhibitors for various enzymes.[16] This chapter reports on the results on the synthesis and evaluation as proteasome inhibitors of peptide vinyl sulfones and peptide epoxyketones bearing a 5-methylpyridin-2(1*H*)-one moiety as replacement of a leucine residue in the lead peptide-based proteasome inhibitors, Z-L_3VS, Z-L_3Epo and LU-102.

4.2 Results and Discussion

The set of modified peptides studied here is depicted in Figure 2 (compounds **6**-**18**). In their design 5 N-terminal caps were included, one (in compounds **1**-**3**) bearing an azide for potential two-step labeling or bioconjugation and four others (in **4**-**13**) to modulate solubility according to literature precedents. The Z-protecting group is widely used in proteasome inhibitors as N-terminal cap. The 2,5-dichlorobenzoyl moiety was adopted in MLN2238, a second-generation peptide boronate proteasome inhibitor and also in MLN9798, which is a boronic ester prodrug with oral bioavailability.[17] It was also reported that the pyrazine-2-carboxyl cap in bortezomib forms a specific interaction with the amino acid residues in proteasome active sites.[14] The 5-methylisoxazole-3-carboxyl cap features in the proteasome inhibitor, CPSI, which showed selectivity for β5 and β5i.[18] In total a set of 3 (scaffolds) times 5 (N-caps) equals 15 compounds were targeted initially, of which a total of 13 compounds could actually be realized.

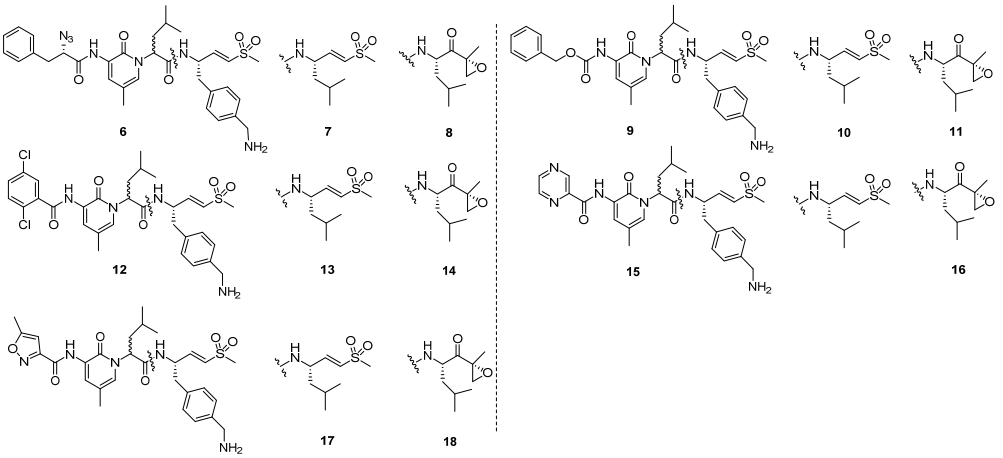
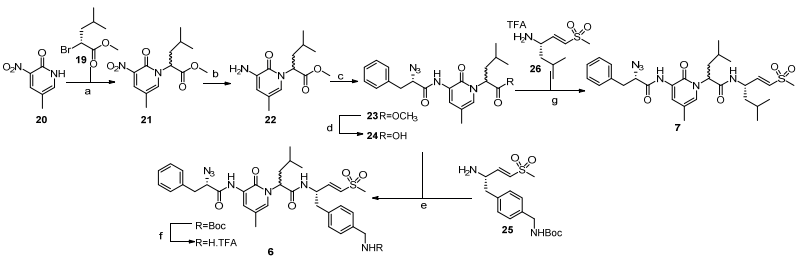


Figure 2: Structures of compounds **6**-**18**.

As representative examples of the synthesis strategy followed, the synthesis of compounds **6** and **7** is depicted in Scheme 1. (*R*)-Methyl-2-bromo-4-methylpentanoate **19** was synthesized according to the literature procedure.[19] Alkylation of 5-methyl-3-nitropyridin-2(1*H*)-one **20** with alkylating agent **19** was accomplished using sodium hydride as the base and DMF as the solvent. According to chiral HPLC analysis, compound **21** was obtained as a 1:1 mixture of enantiomers and the synthesis was continued with this mixture. Later biological results showed that the stereocenter on the position did not contribute too much to the proteasome inhibitory activity. Therefore, it was decided to continue the synthesis with the racemic product. After reduction of **21** (H_2, Pd/C), the resulting crude **22** was condensed with (*S*)-2-azido-3-phenylpropanoic acid with phosphoryl chloride in pyridine at 0 °C to give **23** (yield 61%). Treatment with LiOH in THF and water yielded **24**. Compound **6** was obtained after condensing compound **24** with *p*-(*N*-Boc)-benzylamino-phenylalanine vinyl sulfone **25**[11a] in DCM with HCTU and DiPEA, followed by removal of the Boc group using TFA. Compound **7** was obtained by condensing compound **24** with (*S*,*E*)-5-methyl-1-(methylsulfonyl)hex-1-en-3-amine **26**[20] in DCM with HCTU and DiPEA. The two diasteoreoisomers **7a** and **7b** could be separated by reverse-phase HPLC, yielding two diastereomerically pure compounds for assessment as proteasome inhibitors, the absolute configuration of which however could not be determined. The two diasteoreoisomers of compound **6** could not be separated and they were tested as a mixture. As shown in Figure 2, various different N-cap on P4 position were also introduced to see whether these would affect the inhibitory activities. At the same time, two different amino acids on P1 were incorporated while keeping the same electrophile (vinyl sulfone). Furthermore, the electrophile was also changed from vinyl sulfone to epoxyketone to investigate the effects on proteasome inhibitory activities. During the synthesis, of two compounds (not represented in Figure 2) initially targeted, sufficient quantity could not be obtained to collect decent NMR data and it was decided to leave these two compounds out.

Scheme 1. Synthesis of compounds **6** and **7**.



Reagents and conditions: (a) NaH/DMF, 0 °C; (b) H_2,Pd/C(10%), EtOH; (c) (*S*)-2-azido-3-phenylpropanoic acid, $POCl_3$/pyridine, 0 °C; (d) LiOH/THF/H_2O; (e) HCTU/DiPEA/DCM; (f) TFA; (g) HCTU/DiPEA/DCM.

The inhibitory properties of compounds **6**-**18** as proteasome inhibitors were determined in a competitive activity-based protein profiling assay in HEK cell lysate using the broad-spectrum activity-based proteasome probe, BODIPY-epoxomicin **27** (Figure 3) as the read-out.[21] As can be seen (Table 1 and Figure 4), incorporation of 5-methylpyridin-2-one at P3 led to a dramatic drop in proteasome inhibitory activity (compare **6**-**18** with Z-L_3VS, Z-L_3Epo and LU102).

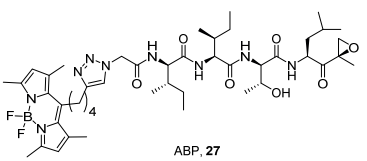


Figure 3. Structure of activity-based probe **27**.

Compound **6** inhibits β2 with an apparent IC_{50} value of 319 μM and did not inhibit β1 and β5 up to 1mM final concentration. Compound **7a** proved the best inhibitor of the series, and inhibits β1, β2 and β5 with IC_{50} values of 60.2 μM, 55.6 μM and 228 μM, respectively. Its' diastereoisomer **7b** revealed a similar broad-spectrum inhibitory activity but is less potent, with apparent IC_{50} values for β1, β2 and β5 of 297 μM, 131 μM and 724 μM, respectively. Compounds **9** and **10** only inhibited β1 and β2 at high concentration. Compound **11a** and diastereoisomeric **11b** showed different inhibitory preferences. Compound **11a** only inhibited β5 (apparent IC_{50} 83.4 μM) while **11b** only inhibited β1 (apparent IC_{50} 259 μM). Compound **13** inhibits β2 with an apparent IC_{50} value of 321 μM and compound **17a** blocks β1 with an apparent IC_{50} value of 359 μM. None of the other compounds show significant proteasome inhibitions at concentrations up to 1000 μM.

Table 1: The apparent IC_{50} (μM) value of compound **6**-**18**.[a]IC_{50} values were determined using a competitive ABPP assay in HEK cell lysate

Compds:	beta1	beta2	beta5	Compds:	beta1	beta2	beta5
Z-L3VS	32	3.6	<1	**11b**	259	>1000	>1000
Z-L3Epo	>1000	3.55	<1	**12**	>1000	>1000	>1000
LU102	>1000	<1	1.15	**13**	>1000	321	>1000
6	>1000	319	>1000	**14**	>1000	>1000	>1000
7a	60.2	55.6	228	**15**	>1000	>1000	>1000
7b	297	131	724	**16a**	>1000	>1000	>1000
8a	>1000	>1000	>1000	**16b**	>1000	>1000	>1000
8b	>1000	>1000	>1000	**17a**	359	>1000	>1000
9	997	947	>1000	**17b**	>1000	>1000	>1000
10	580	630	>1000	**18a**	>1000	>1000	>1000
11a	>1000	>1000	83.4	**18b**	>1000	>1000	>1000

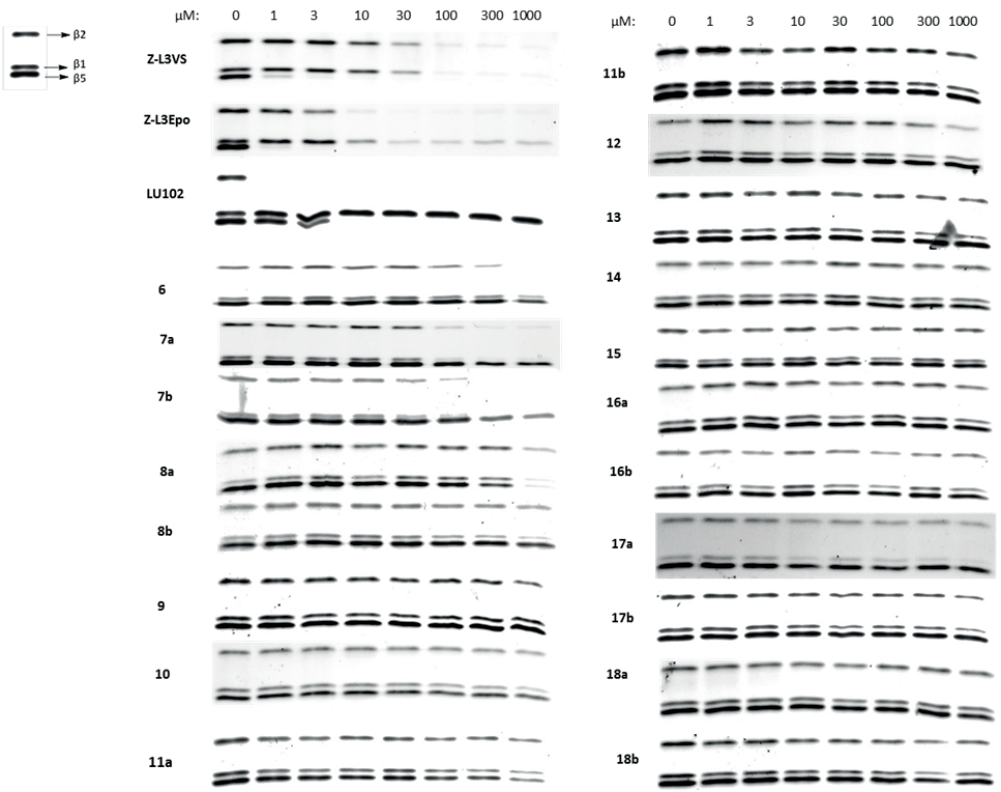


Figure 4. The inhibition profile of compound Z-L3VS, Z-L3Epo, LU102 and **6**-**18**.

Based on the above results, it was decided to investigate whether incorporation of the 5-methylpyridin-2-one moiety at P2 would have a positive effect on proteasome inhibitory activity. Furthermore, it was reasoned that the decrease in inhibitory activity of compounds **6**-**18** when compared to their parent compounds was due to the loss of the amide hydrogen at P2, in other words that the absence of a potential hydrogen bonding interaction has detrimental effect on inhibitory potency. To test both issues, a further three compounds were designed, the structures of which are depicted in Figure **5**.

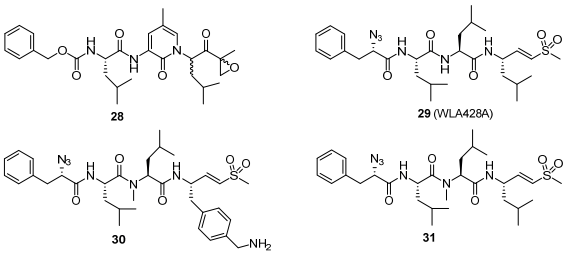
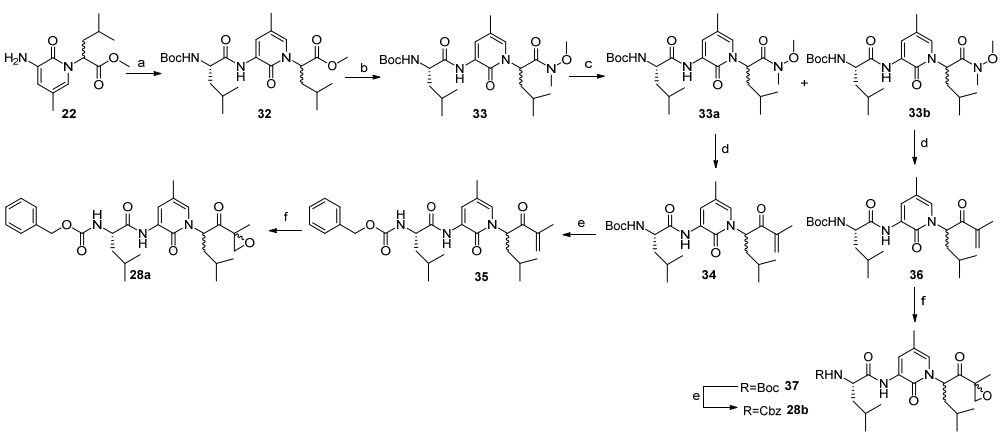


Figure 5: Structures of compounds **28**-**31.**

The synthesis of compound **28** commenced with condensing Boc-*L*-Leu-OH with compound **22** to give **32** (Scheme 2). Weinreb amide **33** was prepared via hydrolysis of **32** with LiOH in THF and H_2O and then coupling with *N*,*O*-dimethylhydroxylamine hydrochloride with HCTU. The diastereoisomers of compound **33** were separated by preparative HPLC to give compound **33a** and **33b**. The absolute stereochemistry of the individual compounds could not be determined and therefore it was decided to continue the synthesis with both diastereoisomers separately. The enones (**34** and **36**) were prepared via the addition of 2-vinyllithium, which was prepared through the transformation of 2-bromopropene with *tert*-butyllithium, to the Weinreb amide **33a** and **33b**. Compound **34** was treated with trifluoroacetic acid and compound **35** was obtained after coupling with CbzCl. Epoxidation of **35** with H_2O_2 in MeOH yielded compound **28a** as a mixture of two diastereoisomers. Compound **36** was first reacted with H_2O_2, which also gave compound **36** as diastereoisomers. Again, the synthesis was continued with the mixture. Treatment of TFA and subsequent coupling with CbzCl gave compound **28b** as a mixture of two diastereoisomers.

Scheme 2. Synthesis of compounds **28a** and **28b**.

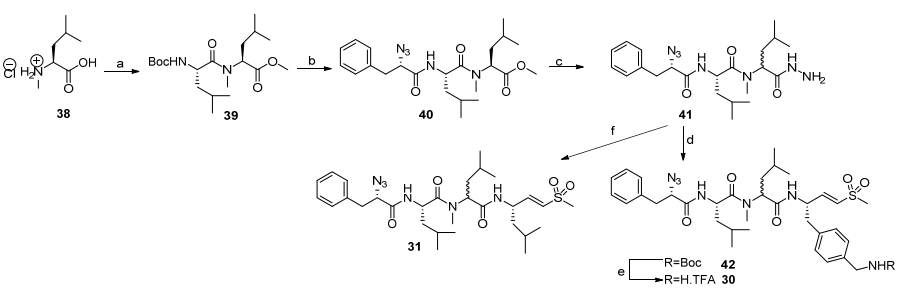


Reagents and conditions: (a) Boc-Leu-OH, $POCl_3$/pyridine, 0 °C; (b) i) LiOH/THF/H_2O; ii) *N*,*O*-dimethylhydroxylamine hydrochloride, HCTU/DiPEA/DCM; (c) reverse HPLC separation (60% MeCN/H_2O, with 0.1% TFA); (d) 2-bromopropene, *t*BuLi/Et_2O, -78 °C; (e) i) TFA; ii) CbzCl, DiPEA/DCM; (f) H_2O_2, DiPEA/MeOH.

The synthesis of compounds **30** and **31** commenced with treatment of *N*-methyl-L-leucine hydrochloride **38** with thionyl chloride in methanol, followed by coupling with Boc-*L*-leucine, yielding compound **39** (Scheme 3). Removal of the Boc group and coupling with (*S*)-2-azido-3-phenylpropanoic acid gave **40**. After treatment of **40** with hydrazine, hydrazoic acid **41** was obtained in quantitative yield. Condensation of **41** and **25** and subsequent removal of the Boc group in TFA gave **30**. Compound **31** was prepared by the condensation of **41** and compound **26**. NMR data analysis revealed compound **30** and **31** to exist as a mixture of two compounds. Since the NMR data revealed that compound **40** existed as a single diastereomer, racemization likely happened upon treatment of compound **40** with hydrazine. It is well known that nitrogen methylated amino acids are prone to epimerization under basic condition. As before, the stereoisomers were tested as mixtures.

The inhibition properties of compounds **28**-**31** were determined in the same way as described above. The results (Table 2 and Figure 6) reveal that incorporation of 5-methylpyridin-2-one moiety on P2 also led to a dramatic drop in proteasome inhibitory activity (**28a** and **28b** compared with Z-L_3Epo). Compound **28a** selectively inhibits β1 and β2 (β1, IC_{50} 241; β1, IC_{50} 119) while its diastereoisomer **28b** showed no inhibition at all.

Scheme 3. Synthesis of compounds **30** and **31**.



Reagents and conditions: (a) i) $SOCl_2$, MeOH; ii) Boc-Leu-OH, HCTU/DiPEA/DCM; (b) i) TFA; ii) (*S*)-2-azido-3-phenylpropanoic acid, HCTU/DiPEA/DCM; c) $N_2H_4.H_2O$, MeOH; d) i) *t*BuONO, HCl, DMF/DCM, -30 °C; ii) (*S*,*E*)-tert-butyl 4-(2-amino-4-(methylsulfonyl)-but-3-enyl)benzylcarbamate **25**, DiPEA/DMF; (e) TFA; (f) i) *t*BuONO, HCl, DMF/DCM, -30 °C; ii) (*S*,*E*)-5-methyl-1-(methylsulfonyl)hex-1-en-3-amine, DiPEA/DMF.

Compared with **11ab**, **28a** showed better inhibitory activity, which indicates that the S2 pocket has better tolerance for the 5-methylpyridin-2-one moiety than the S3 pocket. When comparing **30** with LU102, the inhibitory activities of β5 subunit show a considerable drop (**30**, IC_{50} 54.9 μM; LU102, IC_{50} <1 μM). Comparing **31** with **29** reveals that the inhibitory activities of β2 and β5 dramatically drop (**31**, IC_{50} 836 and 878 μM; **29**, IC_{50} <1 μM). The inhibitory activities

of β1 in contrast are in the same range (**31**, IC_{50} 54.9 μM; **29**, IC_{50} 26.8 μM). These results indicate that the hydrogen on P2 position of **29** is also important for the inhibitory activity.

Table 2: The apparent IC_{50} (μM) values of compounds **28**-**30**. [a]IC_{50} values were determined using a competitive ABPP assay in Hek cell lysate.

Compounds:	beta1	beta2	beta5	Compounds:	beta1	beta2	beta5
29	26.8	<1	<1	**30**	>1000	1.1	54.9
28a	241	119	>1000	**31**	54.9	836	878
28b	>1000	>1000	>1000				

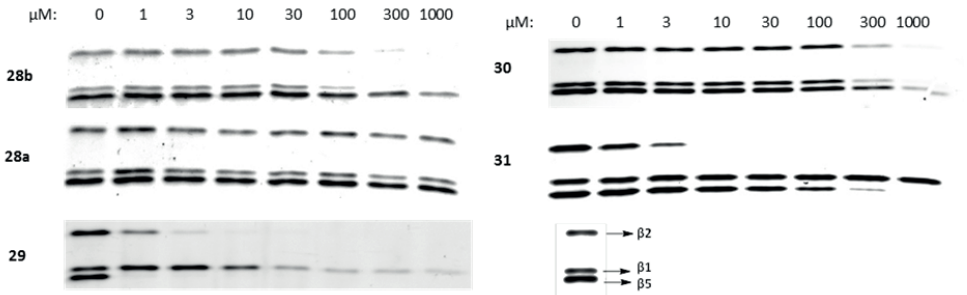


Figure 6. The inhibition profile of compounds **28**-**31**.

4.3 Conclusions

In conclusion, this chapter described the design and synthesis of novel proteasome inhibitors with the incorporation of 5-methylpyridin-2-one moiety as conformationally constrained peptidomimetics. The results show that the S2 pocket has better tolerance for the 5-methylpyridin-2-one moiety than the S3 pocket. The hydrogen in the amide bond of P2 position is critical for the inhibition of proteasome activity, which is probably because of the important hydrogen bond interaction between the inhibitor and the proteasome. Thus, though peptidomimetics may find use in proteasome inhibitor design different from the strategy described in this chapter, comparing the here presented results with the results which were obtained previously in modulating alpha-amino acid side chains to arrive at potent and selective proteasome inhibitors may actually indicate that the design of new proteasome inhibitors based on alpha amino acids is the best way forward.

6.4 Experimental section

Synthesis

General procedures

All reagents were of commercial grade and used as received unless indicated otherwise. Methylene chloride (DCM), dimethylformamide (DMF) and pyridine 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 (ThermoFinnigan) equipped with a Gemini C18 column (Phenomenex). HRMS was recorded on a LTQ Orbitrap (ThermoFinnigan). 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.(S)-2-amino-4-methyl-1-((R)-2-methyloxiran-2-yl)pentan-1-one TFA salt (H-Leu-EK) was prepared according to literature method.[22]

General procedure A

Acid (1.1 eq.) and amine were dissolved in pyridine, which was dried over 4 Å molecular sieves before use. The solution was cooled to -15 °C and phosphorous oxychloride (1.1 eq.) was added dropwise under vigorous stirring. Crushed ice and water were added after 1h, followed by addition of EtOAc. The organic layer was washed by 0.5 M HCl, H_2O, sat. aq.$NaHCO_3$, brine, dried over $MgSO_4$ and purified by silica gel flash column chromatography.

General procedure B

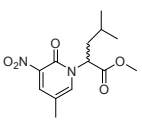
The ester was dissolved in MeOH/H_2O (4:1) and LiOH (2.0 eq.) was added at 0 °C. The solution was stirred at r.t. for 6h and TLC-MS analysis showed the complete conversion of the starting material. MeOH was removed i*n vacuo* and the pH was adjusted to 4 with 0.5 M HCl. The mixture was extracted with EtOAc (3×). The combined organic layer was washed by brine, dried over MgSO4 and concentrated *in vacuo*. The crude product was used without further purification.

General procedure C

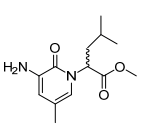
Amine and acid (1.2 eq.) were dissolved in DCM, followed by addition of HCTU (1.2 eq.) and DiPEA(3.5 eq.). TLC analysis indicated completion after 6 h. The reaction mixture was dilutedwith EtOAc, washed withH_2O and brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by HPLC yielded the title compound.

General procedure D

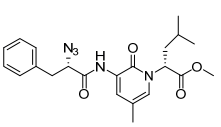
The appropriate Boc-protected C-terminally modified leucine derivative was dissolved in TFA and stirred for 20 min. Co-evaporation with toluene (3x) afforded the TFA-salt, which was used without further purification.



Methyl(*RS*)-4-methyl-2-(5-methyl-3-nitro-2-oxopyridin-1(2*H*)-yl)pentane-ate(21). 2-Hydroxy-5-methyl-3-nitropyridine (1.4 g, 8.9 mmol) was dissolved in dried DMF (40 mL) at 0 °C. NaH (60% disp., 390 mg, 9.8 mmol, 1.1 eq.) was added and the mixture was stirred for 20 min. A solution of (*R*)-methyl-2-bromo-4-methylpentanoate **19** (2.5g, 12.0 mmol, 1.35 eq.) in DMF was added to the mixture. The mixture was slowly warmed upto r.t. in 1h and stirred overnight. The solvent was removed *in vacuo* and the residue dissolved in EtOAc (100 mL). The mixture was washed with H_2O, brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by silica gel column chromatography (5% EtOAc-pentane→20% EtOAc-pentane) yielded the title compound (840 mg, 3.0mmol, 33%). The enantiomeric excess (ee) of the product was determined by HPLC (Chiralpak HD (250×4.6mm), *n*-hexane/*i*-PrOH 9:1, 1.0 mL/min, λ 254 nm: τ_{max}15.781 min, τ_{min}17.806 min, 0% ee). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (d, J = 2.0 Hz, 1H), 7.54 (s, 1H), 5.81 (dd, *J*= 10.2 Hz, 5.4 Hz,1H), 3.77 (s, 3H), 2.25 (s, 3H), 2.09-2.02 (m, 1H), 1.92-1.84 (m, 1H), 1.53-1.41 (m, 1H), 0.98-0.95 (m, 6H). ^{13}C NMR (100MHz, $CDCl_3$): δ 170.55, 153.88, 140.76, 140.52, 137.91, 113.07, 56.29, 53.09, 40.23, 24.83, 22.85, 21.54, 17.27. LC-MS (linear gradient 10→90% MeCN/H_2O, 0.1% TFA, 13.5 min):R_t (min): 6.53 (ESI-MS (m/z):283.00, ($M+H^+$)). HRMS calculated for $C_{13}H_{18}N_2O_5$ 283.12885 $[M+H]^+$; found 283.12881.

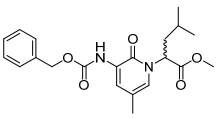


Methyl (*RS*)-2-(3-amino-5-methyl-2-oxopyridin-1(2*H*)-yl)-4-methyl-pentanoate (**22**). Compound **21** (840 mg, 3.0mmol) was dissolved in EtOH (50 mL). Catalyst Pd/C 10% (32 mg, 0.3 mmol, 0.1 eq.) was added and the reaction was stirred under an H_2 atmosphere for 3h. The mixture was filtrated over celite and concentrated *in vacuo* to yield the title compound as green oil (731 mg, 2.9mmol, 97%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.50 (s, 1H), 6.40 (s, 1H), 5.67 (dd, *J*= 10.0 Hz, 5.6 Hz, 1H), 4.22 (s, 2H), 3.72 (s, 3H), 2.03 (s, 3H), 2.00-1.90 (m, 2H), 1.48-1.43 (m, 1H), 0.96-0.92 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.43, 156.99, 136.81, 119.65, 116.06, 114.97, 55.94, 52.60, 39.54, 24.63, 23.06, 21.59, 18.10. HRMS calculated for $C_{13}H_{20}N_2O_3$253.15467 $[M+H]^+$; found 253.15462.

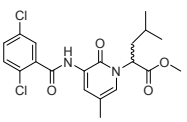


(*RS*)-Methyl 2-(3-((*S*)-2-azido-3-phenylpropanamido)-5-methyl-2-oxopyridin-1(2*H*)-yl)-4-methylpentanoate(23). This compound was synthesized according to the general procedure **A** described above on a 0.38 mmol scale. Purification by silica gel column chromatography (5% EtOAc-pentane→25% EtOAc-pentane) yielded the title compound (102 mg, 0.24 mmol, 63%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.15 (d, *J* = 3.8 Hz), 8.30 (s, 1H), 7.35-7.25 (m, 5H), 6.84 (s, 1H), 5.65-5.61 (m, 1H), 4.26 (dd, *J*= 9.2 Hz, 4.1 Hz, 1H), 3.74 (s, 3H), 3.42 (dd, *J* = 14.0 Hz,

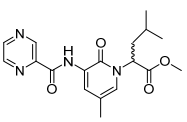
4.1 Hz, 1H), 3.04 (dd, *J*= 14.0 Hz, 9.2 Hz, 1H), 2.15 (s, 3H), 2.04-1.87 (m, 2H), 1.47-1.38 (m, 2H), 0.93 (dd, *J*= 15.2 Hz, 9.0 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.91, 167.70, 156.52, 136.17, 129.46, 128.87, 127.59, 127.38, 125.41, 115.93, 66.06, 56.46, 52.85, 39.45, 39.01, 24.66, 23.00, 21.54, 18.23. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.67 (ESI-MS (m/z): 426.07, ($M+H^+$)). HRMS calculated for $C_{22}H_{27}N_5O_4$ 426.21358 $[M+H]^+$;found 426.21296.



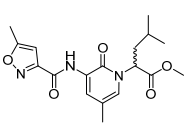
(*RS*)-Methyl-2-(3-(benzyloxycarbonyl)-5-methyl-2-oxopyridin-1(2*H*)-yl)-4-methylpenta-n oate (**23a**). Compound **22** (100mg, 0.4 mmol) was dissolved in DCM (5 mL), followed by the addition of DiPEA (140 µL, 0.8 mmol, 2 eq.). After the addition of CbzCl (115 µL, 0.8 mmol, 2 eq.), the reaction mixture was stirred at r.t. overnight. The mixture was concentrated *in vacuo* and purification by silica gel column chromatography (5% EtOAc-pentane→10% EtOAc-pentane) yieldedthe title compound (100 mg, 0.26 mmol, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (s, 1H), 7.89 (s, 1H),7.45-7.27 (m, 5H), 6.74 (s, 1H), 5.67-5.63 (m, 1H), 5.19 (d, *J* = 2.5 Hz, 2H), 3.72 (s, 3H), 2.12 (s, 3H), 2.00-1.88 (m, 2H), 1.26 (s, 1H), 0.95-0.88 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.95, 156.29, 153.36, 136.03, 128.59, 128.28, 128.06, 123.68, 122.61, 115.92, 66.99, 56.27, 52.72, 39.51, 24.60, 23.02, 21.44, 18.24. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.74 (ESI-MS (m/z):387.07, ($M+H^+$)).



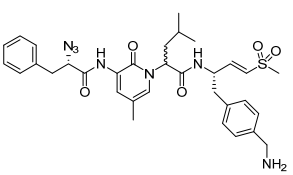
(*RS*)-Methyl-2-(3-(2,5-dichlorobenzamido)-5-methyl-2-oxopyridin-1(2*H*)-yl)-4-methylpentan o-ate (**23b**).This compound was synthesized according to the general procedure **A** described above on a 0.38 mmol scale. Purification by silica gel column chromatography (5% EtOAc-pentane→10% EtOAc-pentane) yielded the title compound (58. mg, 0.14 mmol, 37%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.13 (s, 1H), 8.44 (d, *J* = 2.1 Hz, 1H), 7.71 (dd, *J* = 2.1, 0.9 Hz, 1H), 7.44-7.34 (m, 2H), 6.87 (dd, *J* = 2.3, 1.2 Hz, 1H), 5.68 (dd, *J* = 10.3, 5.4 Hz, 1H), 3.75 (s, 3H), 2.19 (s, 3H), 2.09-1.84 (m, 2H), 1.50-1.42 (m, 1H), 1.03-0.86 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.92, 163.62, 156.62, 135.96, 133.30, 131.82, 130.04, 129.48, 128.18, 125.68, 125.38, 116.16, 56.37, 52.89, 24.69, 23.05, 21.52, 18.32. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 9.01 (ESI-MS (m/z):425.07, ($M+H^+$)). HRMS calculated for $C_{20}H_{22}Cl_2N_2O_4$ 425.10294 $[M+H]^+$; found 425.10279.



(*RS*)-Methyl 4-methyl-2-(5-methyl-2-oxo-3-(pyrazine-3-carboxamido)pyridin-1(2*H*)-yl)-pentanoate (**23c**). This compound was synthesized according to the general procedure **A** described above on a 0.38 mmol scale. Purification by silica gel column chromatography (5% EtOAc-pentane→10% EtOAc-pentane) yielded the title compound (58 mg, 0.19 mmol, 50%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.72 (s, 1H), 9.45 (d, *J* = 1.4 Hz, 1H), 8.80 (d, *J* = 2.4 Hz, 1H), 8.63 (dd, *J* = 2.5, 1.5 Hz, 1H), 8.48 (d, *J* = 2.2 Hz, 1H), 6.89 (dd, *J* = 2.3, 1.2 Hz, 1H), 5.70 (dd, *J* = 10.2, 5.6 Hz, 1H), 3.76 (s, 3H), 2.20 (s, 3H), 2.07-1.93 (m, 2H), 1.52-1.43 (m, 1H), 0.99-0.86 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.92, 161.61, 156.73, 147.59, 144.38, 144.28, 142.94, 127.91, 125.57, 115.96, 56.51, 52.83, 39.46, 24.67, 23.00, 21.54, 18.26. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 7.50 (ESI-MS (m/z):359.13, ($M+H^+$)). HRMS calculated for $C_{18}H_{22}N_4O_4$ 359.17138 $[M+H]^+$; found 359.17141.

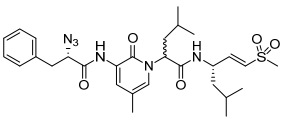


(*RS*)-Methyl 4-methyl-2-(5-methyl-3-(5-methylisoxazole-3-carboxamido)-2- oxopyridin -1(2*H*)-yl)pentanoate (**23d**). This compound was synthesized according to the general procedure **A** described above on a 0.38 mmol scale. Purification by silica gel column chromatography (5% EtOAc-pentane→30% EtOAc-pentane) gave the title compound (66 mg, 0.18 mmol, 47%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.63 (s, 1H), 8.35 (d, *J* = 2.1 Hz, 1H), 6.86 (dd, *J* = 2.3, 1.2 Hz, 1H), 6.48 (d, *J* = 1.1 Hz, 1H), 5.68 (dd, *J* = 10.3, 5.5 Hz, 1H), 3.75 (s, 3H), 2.50 (s, 3H), 2.17 (s, 3H), 2.06-1.90 (m, 2H), 1.51-1.39 (m, 1H), 0.97-0.93 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.56, 170.94, 158.69, 157.72, 156.43, 127.78, 125.50, 115.74, 101.21, 56.36, 52.81, 24.64, 23.01, 21.49, 18.24, 12.44. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.16 (ESI-MS (m/z):362.13, ($M+H^+$)).

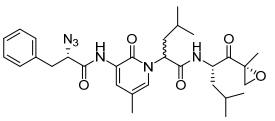


(*RS*)-N-((*S*,*E*)-1-(4-(aminomethyl)phenyl)-4-(methylsulfonyl)but-3-en-2-yl)-2-(3-((*S*)-2-azido-3-phenylpropanamido)-5-methyl-2-oxopyridin-1(2*H*)-yl)-4-methylpe ntanamideTFA salt (**6**). Compound **23** was hydrolyzed to acid (**24**) according to general procedure B on a 0.06 mmol scale. The title compound was synthesized according to the general procedure **C** using **24** and warhead (**25**) as starting materials, followed by the the removal of the Boc protecting group according to the general procedure **D**. Purification by HPLC (40%-60% MeCN-H_2O) yielded the title compound (3.8 mg, 0.005 mmol, 8%). ^{1}H NMR (400 MHz, MeOD): δ 8.28-8.20 (m, 1H), 7.30-6.96 (m, 10H), 6.76-6.69 (m, 1H), 6.58 (t, *J* = 10.0 Hz, 1H), 5.46-5.31 (m, 1H), 4.37-4.34 (m, 1H), 4.12 (s, 1H), 4.01 (s, 1H), 3.27-2.82 (m, 6H), 2.17-2.13 (m, 3H), 1.78-1.52 (m, 2H), 1.46-1.21 (m, 2H), 0.82-0.73 (m, 6H). ^{13}C NMR (100 MHz, MeOD): δ 171.09, 170.19, 157.73, 146.60,139.52, 137.67, 133.75, 131.86-129.54, 128.32, 128.21, 127.77, 116.96, 80.13, 66.36, 58.31, 52.54, 44.77, 42.73, 40.40, 39.97, 39.24, 28.79, 25.83, 25.64, 23.19, 22.17, 21.97, 18.04. LC-MS (linear gradient

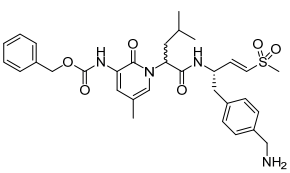
10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 6.45 (ESI-MS (m/z):648.20, ($M+H^+$)). HRMS calculated for$C_{33}H_{41}N_7O_5S$ 648.29626 $[M+H]^+$; found 648.29595.



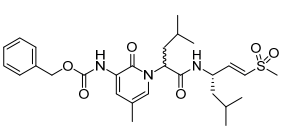
(*RS*)-2-(3-((*S*)-2-Azido-3-phenylpropanamido)-5-methyl-2-oxopyridin-1(2*H*)-yl)-4-methyl-*N*-((*S*,*E*)-5-methyl-1-(methylsulfonyl)hex-1-en-3-yl)pentanamide (**7**). This compound was synthesized according to the general procedure **C** on a 0.066 mmol scale using **24** and **26** as starting materials. Using HPLC purification (60% MeCN-H_2O), the two diastereoisomers were separated.**7a**: (5 mg, 9 µmol, 13%). ^{1}H NMR (400 MHz, MeOD): δ 8.15 (d, *J* = 2.1 Hz, 1H), 7.19-7.10 (m, 6H), 6.83-.78 (m, 1H), 6.71-6.67 (m, 1H), 5.66-5.62 (m, 1H), 4.68-4.65(m, 1H), 4.50-4.47(m, 1H), 3.17-3.07 (m, 2H), 3.00 (s, 3H), 2.18 (s, 3H), 1.99-1.88 (m, 2H), 1.58-1.34 (m, 4H), 0.85-0.69 (m, 12H). ^{13}C NMR (100 MHz, MeOD) δ 171.35, 169.91, 157.85, 148.15, 137.64, 130.96, 130.51, 129.66, 128.42, 128.18, 127.89, 127.48, 117.20, 66.30, 58.01, 49.52, 43.08, 42.72, 40.47, 39.25, 25.93, 23.31, 21.87, 17.95. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.70 (ESI-MS (m/z):584.93.($M+H^+$)). HRMS calculated for $C_{29}H_{40}N_6O_5S$ 585.28537$[M+H]^+$; found 585.28514. **7b**: (5.3 mg, 9.1 µmol, 14%). ^{1}H NMR (400 MHz, MeOD): δ 8.14 (s, 1H), 7.19-7.12 (m, 6H), 6.81-6.76 (m, 1H), 6.71-6.67 (m, 1H), 5.64-5.52 (m, 1H), 4.70-4.67 (m, 1H), 4.50-4.47 (m, 1H), 3.12-3.06 (m, 2H), 2.90 (s, 3H), 2.19 (s, 3H), 2.06-1.86 (m, 2H), 1.75-1.45 (m, 4H), 1.02-0.94 (m, 12H). ^{13}C NMR (100 MHz, MeOD): δ 171.68, 169.89, 158.02, 148.37, 137.69, 130.82, 130.54, 130.51, 129.69, 128.21, 128.05, 127.66, 117.10 66.39, 58.49, 49.57, 43.04, 42.69. 40.45, 39.29, 26.19, 25.95, 23.40, 23.11, 21.96, 21.66, 17.99. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.84 (ESI-MS (m/z):584.93, ($M+H^+$)). HRMS calculated for $C_{29}H_{40}N_6O_5S$ 585.28537 $[M+H]^+$; found 585.28503.



(*RS*)-2-(3-((*S*)-2-Azido-3-phenylpropanamido)-5-methyl-2-oxopyridin-1(2*H*)-yl)-4-methyl-*N*-((*S*)-4-methyl-1-((*R*)-2-methyloxiran-2-yl)-1-oxopentan-2-yl)pentanamide (**8**). This compound was synthesized according to the general procedure **C** described above on a 0.045 mmol scale using **24** and warhead (H-Leu-EK) as starting materials. After HPLC purification (75%-85% MeCN-H_2O), the two diastereoisomers were separated.**8a**:(3.4 mg, 6 µmol, 13%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.12 (s, 1H), 8.29 (d, *J* = 2.2 Hz, 1H), 7.40-7.24 (m, 5H), 6.95 (s, 1H), 6.55 (d, *J* = 7.6 Hz, 1H), 5.58 (t, *J* = 7.8 Hz, 1H), 4.45 (ddd, *J* = 10.5, 7.6, 3.0 Hz, 1H), 4.28 (dd, *J* = 9.0, 4.2 Hz, 1H), 3.43 (dd, *J* = 14.1, 4.2 Hz, 1H), 3.29 (d, *J* = 5.0 Hz, 1H), 3.06 (dd, *J* = 14.0, 9.0 Hz, 1H), 2.90 (d, *J* = 5.0 Hz, 1H), 2.13 (s, 3H), 1.52 (s, 3H), 0.95-0.89 (m, 6H), 0.82-0.78 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.30, 167.71, 136.16, 129.54, 128.96, 127.51, 127.44, 125.59, 124.61, 116.58, 100.12, 66.09, 59.31, 52.58, 50.90, 39.69, 39.07, 38.69, 25.43, 24.71, 23.23, 22.20, 21.31, 18.27, 16.92. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 9.48 (ESI-MS (m/z): 564.80, ($M+H^+$)). HRMS calculated for $C_{30}H_{40}N_6O_5$ 565.31329 $[M+H]^+$; found 565.31296. **8b**: (5.5 mg, 0.01 mmol, 22%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.15 (s, 1H), 7.36-7.28 (m, 5H), 6.90 (s, 1H), 6.68 (d, *J* = 8.1 Hz, 1H), 5.56 (t, *J* = 7.8 Hz, 1H), 4.53-4.47 (m, 1H), 4.29 (dd, *J* = 9.1, 4.0 Hz, 1H), 3.45 (dd, *J* = 14.2, 4.1 Hz, 1H), 3.22 (d, *J* = 5.0 Hz, 1H), 3.05 (dd, *J* = 14.0, 9.1 Hz, 1H), 2.85 (d, *J* = 5.0 Hz, 1H), 2.13 (s, 3H), 1.45 (s, 3H), 1.03-0.83 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.59, 167.69, 156.79, 136.28, 129.57, 128.96, 127.48, 125.74, 124.67, 116.65, 77.48, 77.16, 76.84, 66.15, 59.27, 52.64, 51.11, 39.77, 39.09, 38.81, 25.48, 24.91, 23.48, 22.79, 21.31, 18.32, 16.95. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 9.61 (ESI-MS (m/z):564.80, ($M+H^+$)). HRMS calculated for $C_{30}H_{40}N_6O_5$ 565.31329 $[M+H]^+$; found 519.31302.

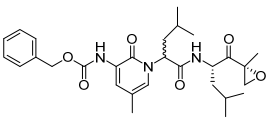


Benzyl 1-((*RS*)-1-((*S*,*E*)-1-(4-(aminomethyl)phenyl)-4-(methylsulfonyl)but-3-en-2-ylamino)-4-methyl-1-oxopentan-2-yl)-5-methyl-2-oxo-1,2-dihydropyridin-3-yl carbamateTFA salt (**9**). This compound was synthesized using the same procedures as described above for the preparation of compound **6** on a 0.035 mmol scale. Purification by HPLC (40%-60% MeCN-H_2O) yielded the title compound (9.9 mg, 0.016 mmol, 46%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24-8.19 (m, 2H), 7.97 (d, *J* = 8.8 Hz, 1H), 7.81 (d, *J* = 16.0 Hz, 1H), 7.53 (d, *J* = 8.3 Hz, 1H), 7.45-7.29 (m, 5H), 7.14 (d, *J* = 7.8 Hz, 1H), 7.08 (d, *J* = 7.8 Hz, 1H), 7.02- 6.97 (m, 1H), 6.94 (d, *J* = 7.9 Hz, 1H), 6.89-6.78 (m, 1H), 6.48-6.31 (m, 1H), 5.59-5.44 (m, 1H), 5.31-5.13 (m, 2H), 5.06-4.67 (m, 1H), 3.94-3.81 (m, 2H), 3.49 (s, 2H), 2.91 (s, 1.5H), 2.79 (s, 1.5H), 2.19-1.97 (m, 3H), 1.87-1.81 (m, 2H), 1.37-1.29 (m, 1H), 0.97-0.76 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.53, 169.48, 161.63, 161.26, 156.38, 156.31, 146.12, 146.05, 137.38, 136.64, 135.97, 131.38, 131.28, 130.43, 130.23, 130.18, 130.12, 129.56, 129.10, 128.82, 128.78, 128.65, 128.58, 128.46, 128.39, 128.25, 128.11, 124.19, 117.16, 76.84, 67.50, 67.38, 51.35, 50.18, 50.16, 43.53, 42.78, 42.77, 42.57, 38.95, 24.72, 24.67, 23.05, 22.81, 21.83, 21.57, 18.28. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 6.38 (ESI-MS (m/z): 609.00,($M+H^+$)). HRMS calculated for $C_{32}H_{40}N_4O_6S$ 609.27413 $[M+H]^+$; found 609.27386.

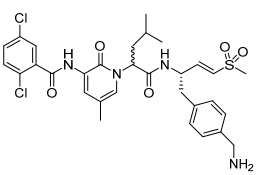


Benzyl 5-methyl-1-((*RS*)-4-methyl-1-((*S*,*E*)-5-methyl-1-(methylsulfonyl)hex-1-en-3-ylamino)-1-oxopentan-2-yl)-2-oxo-1,2-dihydropyridin-3-ylcarbamate (**10**). This compound was synthesized using the same procedures as described above for the preparation of compound **7** on a 0.038 mmol scale. Purification by HPLC (55%-65%

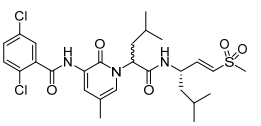
MeCN-H_2O) yielded the title compound (15 mg, 0.028mmol, 74%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05-7.95 (m, 1H), 7.84-7.80 (m, 1H), 7.46-7.32 (m, 5H), 7.01-6.85 (m, 1H), 6.94-6.87 (m, 1H), 6.85-6.66 (m, 2H), 6.49-6.45 (m, 0.5H), 6.14-6.06 (m, 0.5H), 5.54-5.48 (m, 1H), 5.21 (s, 2H), 4.66-4.56 (m, 1H), 2.94 (s, 1.5H), 2.73 (s, 1.5H), 2.14-2.10 (m, 3H), 2.08-1.77 (m, 2H), 1.70-1.22 (m, 4H), 1.00-0.66 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.31, 168.99, 168.84, 156.38, 156.32, 156.25, 153.28, 147.19, 147.07, 135.80, 135.77, 129.52, 128.66, 128.46, 128.42, 128.38, 128.23, 123.67, 123.43, 117.47, 67.29, 67.26, 67.01, 48.26, 48.12, 42.85, 42.73, 42.62, 42.47, 42.42, 42.24, 39.52, 38.68, 38.22, 37.92, 24.90, 24.78, 24.75, 22.83, 22.73, 22.65, 22.55, 22.10, 21.97, 21.92, 21.79, 21.60, 18.20, 18.16. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.70 (ESI-MS (m/z):546.13, ($M+H^+$)). HRMS calculated for $C_{28}H_{39}N_3O_6S$ 546.26323 $[M+H]^+$; found 546.26310.



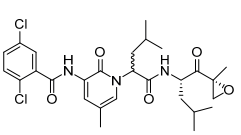
Benzyl 5-methyl-1-((*R* or *S*)-4-methyl-1-((*S*)-4-methyl-1-((*R*)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxopentan-2-yl)-2-oxo-1,2-dihydropyridin-3-ylcarbamate (**11**). This compound was synthesized using the same procedures as described above for the preparation of compound **8** on a 0.035 mmol scale. After HPLC purification (75%-85% MeCN-H_2O), the two diastereoisomers were separated.**11a**: (3 mg,5.7 µmol, 16%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (s, 1H), 7.85 (s, 1H), 7.47-7.30 (m, 5H), 6.85 (s, 1H), 6.60 (d, *J* = 7.5 Hz, 1H), 5.57 (t, *J* = 7.9 Hz, 1H), 5.21 (s, 2H), 4.47-4.41 (m, 1H), 3.30 (d, *J* = 5.0 Hz, 1H), 2.89 (d, *J* = 5.0 Hz, 1H), 2.10 (s, 3H), 1.98-1.74 (m, 2H), 1.47-1.17 (m, 4H), 0.94-0.77 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.06, 169.44, 156.49, 153.47, 128.76, 128.51, 128.44, 128.29, 122.92, 122.83, 116.65, 67.25, 59.31, 52.59, 50.85, 39.71, 38.50, 25.41, 24.71, 23.23, 22.96, 22.13, 21.29, 18.32, 16.92. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 9.71(ESI-MS (m/z):526.00, ($M+H^+$)). HRMS calculated for $C_{29}H_{39}N_3O_6$ 526.29116 $[M+H]^+$; found 526.29101. **11b**: (2.8 mg,5.3 µmol, 15%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (s, 1H), 7.88 (s, 1H), 7.49-7.32 (m, 5H), 6.87-6.71 (m, 2H), 5.54 (t, *J* = 7.8 Hz, 1H), 5.21 (s, 2H), 4.52-4.46 (m, 1H), 3.21 (d, *J* = 5.0 Hz, 1H), 2.84 (d, *J* = 5.0 Hz, 1H), 2.11 (s, 3H), 2.00-1.92 (m, 1H), 1.81-1.74 (m, 1H), 1.57-1.46 (m, 2H), 1.44 (s, 4H), 1.28-1.21 (m, 1H), 0.98-0.88 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.71, 169.73, 156.64, 153.46, 136.08, 128.75, 128.48, 128.43, 128.31, 123.09, 122.90, 116.75, 67.21, 59.26, 52.63, 51.03, 39.84, 38.56, 25.45, 24.88, 23.50, 22.89, 22.18, 21.31, 18.37, 16.95. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 9.77 (ESI-MS (m/z):526.07, ($M+H^+$)). HRMS calculated for $C_{29}H_{39}N_3O_6$ 526.29116 $[M+H]^+$; found 526.29088.



N-(1-((*RS*)-1-((*S*,*E*)-1-(4-(aminomethyl)phenyl)-4-(methylsulfonyl)but-3-en-2-ylamino)-4-methyl-1-oxopentan-2-yl)-5-methyl-2-oxo-1,2-dihydropyridin-3-yl)-2,5-dichlorobenzamide TFA salt (**12**). This compound was synthesized using the same procedures as described above for the preparation of compound **6** on a 0.046 mmol scale. Purification by HPLC(40%-60% MeCN-H_2O) yielded the title compound (11.4 mg, 0.018 mmol, 39%). ^{1}H NMR (400 MHz, $CDCl_3$)δ 9.15-9.12 (m, 1H), 8.48-8.46 (m, 1H), 8.38-8.18 (s, 3H), 7.86-7.66 (m, 1H), 7.55 (d, *J* = 8.2 Hz, 1H), 7.47-7.35 (m, 3H), 7.18 (d, *J* = 7.7 Hz, 1H), 7.10 (t, *J* = 9.1 Hz, 2H), 6.97 (d, *J* = 7.6 Hz, 1H), 6.91-6.81 (m, 1H), 6.48-6.34 (m, 1H), 5.64-5.49 (m, 1H), 5.05-4.75 (m, 1H), 4.09-3.69 (m, 2H), 3.05-2.75 (m, 5H), 2.21-2.16 (m, 3H), 1.94-1.85 (m, 2H), 1.38 (s, 1H), 1.01-0.71 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.48, 169.39, 163.63, 156.65, 146.19, 146.10, 137.38, 136.57, 135.76, 135.57, 133.73, 133.57, 132.27, 132.08, 132.02, 131.90, 131.39, 131.33, 130.60, 130.50, 130.41, 130.26, 130.15, 129.59, 129.17, 129.13, 129.05, 127.85, 127.73, 126.89, 125.93, 117.13, 117.07, 56.15, 51.49, 50.12, 43.61, 42.79, 42.67, 39.52, 24.70, 23.09, 22.86, 21.84, 21.56, 18.31. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 6.48 (ESI-MS (m/z): 646.87, ($M+H^+$)). HRMS calculated for $C_{31}H_{36}Cl_2N_4O_5S$ 647.18562 $[M+H]^+$; found 647.18565.

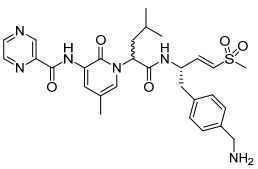


2,5-Dichloro-N-(5-methyl-1-((*RS*)-4-methyl-1-((*S*,*E*)-5-methyl-1-(methylsulfonyl)hex-1-en-3-ylamino)-1-oxopentan-2-yl)-2-oxo-1,2-dihydropyridin-3-yl)benzamide (**13**). This compound was synthesized using the same procedures as described above for the preparation of compound **7** on a 0.038 mmol scale. Purification by HPLC(50%-70% MeCNH_2O) yielded the title compound (11 mg, 0.019 mmol, 50%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.17-9.15 (m, 1H), 8.52-8.48 (m, 1H), 7.85-7.67 (m, 1H), 7.43-7.41 (m, 2H), 7.06-6.98 (m, 1H), 6.86-6.62 (m, 2H), 6.49-6.46 (m, 0.5H), 6.19-6.15(m, 0.5H) 5.56-547 (m, 1H), 4.70-4.53 (m, 1H), 2.94 (s, 1.5H), 2.78 (s, 1.5H), 2.19 (d, *J* = 3.1 Hz, 3H), 2.10-1.80 (m, 2H), 1.74-1.19 (m, 4H), 1.03-0.67 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.95, 168.81, 163.65, 163.60, 156.86, 147.28, 147.17, 135.69, 133.60, 132.10, 131.91, 130.47, 129.71, 129.35, 129.29, 128.15, 126.60, 126.36, 124.78, 124.48, 117.65, 48.43, 48.32, 42.99, 42.88, 42.73, 38.53, 38.02, 25.04, 24.97, 24.94, 24.91, 22.93, 22.86, 22.76, 22.74, 22.34, 22.18, 21.92, 21.68, 18.30. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.89 (ESI-MS (m/z): 583.80($M+H^+$)). HRMS calculated for $C_{27}H_{35}Cl_2N_3O_5S$ 584.17472 $[M+H]^+$; found 584.17490.

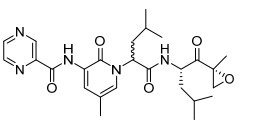


2,5-Dichloro-*N*-(5-methyl-1-((*RS*)-4-methyl-1-((*S*)-4-methyl-1-((*R*)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxopentan-2-yl)-2-oxo-1,2-dihydropyridin-3-yl)benzamide

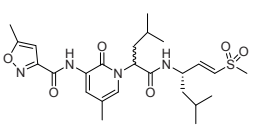
(**14**). This compound was synthesized using the same procedures as described above for the preparation of compound **8** on a 0.038 mmol scale. Purification by HPLC (75%-85% MeCN-H_2O) yielded the title compound (12.3 mg, 0.022 mmol, 58%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.15 (s, 1H), 8.46-8.44 (m, 1H), 7.73 (s, 1H), 7.42-7.37 (m, 2H), 7.00-6.94 (m, 1H), 6.80-6.65 (m, 1H), 5.66-5.49 (m, 1H), 4.54-4.43 (m, 1H), 3.31-3.21 (m, 1H), 2.90-2.85 (m, 1H), 2.17 (s, 3H), 2.05-1.90 (m, 1H), 1.85-1.70 (m, 2H), 1.56-1.40 (m, 5H), 1.31-1.18 (m, 1H), 1.00-0.74 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.05, 207.71, 169.52, 169.27, 163.61, 156.87, 156.75, 136.09, 135.95, 133.50, 133.44, 131.98, 131.90, 131.87, 130.33, 130.29, 129.50, 129.39, 128.07, 126.08, 125.90, 124.74, 124.61, 116.84, 116.77, 59.31, 59.24, 56.13, 55.86, 52.62, 51.06, 50.91, 39.90, 39.66, 38.86, 38.68, 25.46, 25.42, 24.95, 24.76, 23.49, 23.28, 22.90, 22.81, 22.28, 22.21, 21.31, 21.28, 18.36, 18.32. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 9.86 (ESI-MS (m/z): 564.00, ($M+H^+$)). HRMS calculated for $C_{28}H_{35}Cl_2N_3O_5$ 564.20265 $[M+H]^+$; found 564.20260.



N-(1-((*RS*)-1-((*S,E*)-1-(4-(aminomethyl)phenyl)-4-(methylsulfonyl)but-3-en-2-ylamino)-4-methyl-1-oxopentan-2-yl)-5-methyl-2-oxo-1,2-dihydropyridin-3-yl)pyrazine-2-carboxamide TFA salt (**15**). This compound was synthesized using the same procedures as described above for the preparation of compound **6** on a 0.072 mmol scale. Purification by HPLC (50%-55% MeCN-H_2O) yielded the title compound (15.2 mg, 0.026 mmol, 36%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.54 (d, *J* = 7.3 Hz, 1H), 9.42 (s, 1H), 8.86-8.77 (m, 1H), 8.65 (dt, *J* = 38.1, 1.8 Hz, 1H), 8.55-8.30 (m, 4H), 7.59 (dd, *J* = 55.9, 8.4 Hz, 1H), 7.25-6.78 (m, 5H), 6.53-6.37 (m, 1H), 5.85-5.46 (m, 1H), 4.91 (d, *J* = 97.4 Hz, 1H), 4.11-3.79 (m, 3H), 2.94 (s, 2H), 2.91-2.78 (m, 3H), 2.33-2.10 (m, 3H), 1.02-0.76 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.60, 169.52, 161.60, 161.53, 156.79, 156.70, 147.96, 146.15, 144.39, 144.23, 143.31, 137.50, 136.60, 131.46, 131.43, 130.30, 129.59, 129.08, 127.46, 127.30, 126.60, 126.04, 117.15, 42.77, 42.62, 39.52, 39.27, 24.68, 23.08, 22.82, 21.83, 21.50, 18.32. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 5.55 (ESI-MS (m/z): 581.00, ($M+H^+$)). HRMS calculated for $C_{29}H_{36}N_6O_5S$ 581.25407 $[M+H]^+$; found 581.25371.

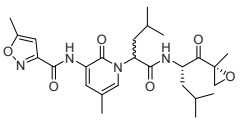


***N*-(5-methyl-1-((*RS*)-4-methyl-1-((*S*)-4-methyl-1-((*R*)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxopentan-2-yl)-2-oxo-1,2-dihydropyridin-3-yl)pyrazine-2-carboxamide** (**16**). This compound was synthesized using the same procedures as described above for the preparation of compound **8** on a 0.058 mmol scale. After HPLC purification (60% MeCN-H_2O), the two diastereoisomers were separated.**16a:** (6.1 mg, 0.012 mmol, 21%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.69 (s, 1H), 9.46 (d, *J* = 1.5 Hz, 1H), 8.80 (d, *J* = 2.5 Hz, 1H), 8.65 (dd, *J* = 2.5, 1.5 Hz, 1H), 8.47 (d, *J* = 2.2 Hz, 1H), 7.01-6.99 (m, 1H), 6.64 (d, *J* = 7.6 Hz, 1H), 5.68 (dd, *J* = 8.6, 7.0 Hz, 1H), 4.50-4.44 (m, 1H), 3.30 (d, *J* = 5.0 Hz, 1H), 2.90 (d, *J* = 5.0 Hz, 1H), 2.17 (s, 3H), 2.10-1.94 (m, 1H), 1.86-1.78 (m, 1H), 1.51 (s, 4H), 1.49-1.39 (m, 2H), 1.31-1.18 (m, 1H), 0.97-0.93 (m, 6H), 0.80-0.77 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.04, 169.40, 161.72, 156.92, 147.75, 144.49, 144.47, 143.06, 127.81, 125.70, 124.72, 116.67, 59.31, 55.77, 52.59, 50.90, 39.72, 38.75, 25.44, 24.76, 23.20, 22.90, 22.22, 21.31, 18.31, 16.92. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.60 (ESI-MS (m/z): 497.80, ($M+H^+$)). HRMS calculated for $C_{26}H_{35}N_5O_5$ 498.27110 $[M+H]^+$; found 498.27092. **16b:** (8.7 mg, 0.017 mmol, 29%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.70 (s, 1H), 9.46 (d, *J* = 1.4 Hz, 1H), 8.80 (d, *J* = 2.4 Hz, 1H), 8.65-8.54 (m, 1H), 8.49 (d, *J* = 2.2 Hz, 1H), 6.95 (s, 1H), 6.78 (d, *J* = 8.2 Hz, 1H), 5.64-5.62 (m, 1H), 4.55-4.49 (m, 1H), 3.23 (d, *J* = 5.0 Hz, 1H), 2.85 (d, *J* = 5.0 Hz, 1H), 2.18 (s, 3H), 2.06-1.97 (m, 1H), 1.82-1.72 (m, 2H), 1.58-1.46 (m, 2H), 1.44 (s, 3H), 1.36-1.22 (m, 1H), 0.97-0.94 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.69, 169.67, 161.69, 157.05, 147.68, 144.57, 144.48, 143.03, 127.80, 125.92, 124.80, 116.75, 59.26, 55.87, 52.63, 51.10, 39.77, 38.79, 25.47, 24.93, 23.48, 22.78, 22.29, 21.31, 18.35, 16.94. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.73 (ESI-MS (m/z): 498.00,($M+H^+$)). HRMS calculated for $C_{26}H_{35}N_5O_5$ 498.27110 $[M+H]^+$; found 498.27087.

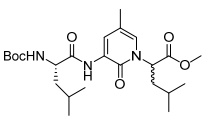


5-Methyl-N-(5-methyl-1-((*RS*)-4-methyl-1-((*S,E*)-5-methyl-1-(methylsulfonyl)hex-1-en-3-ylamino)-1-oxopentan-2-yl)-2-oxo-1,2-dihydropyridin-3-yl)isoxazole-3-carboxamide (**17**). This compound was synthesized using the same procedures as described above for the preparation of compound **7** on a 0.057 mmol scale. After HPLC purification (55% MeCN-H_2O), the two diastereoisomers were separated. **17a**: (4.6 mg, 0.009 mmol, 16%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.59 (s, 1H), 8.40 (d, *J* = 2.2 Hz, 1H), 6.99 (s, 1H), 6.89-6.72 (m, 2H), 6.59-6.37 (m, 2H), 5.53 (t, *J* = 7.6 Hz, 1H), 4.51-4.58 (m, 1H), 2.95 (s, 3H), 2.52 (s, 3H), 2.17 (s, 4H), 1.93-1.86 (m, 1H), 1.52-1.26 (m, 4H), 0.98-0.93 (m, 6H), 0.78-0.73 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.94, 169.04, 158.73, 157.81, 156.78, 147.30, 129.68, 127.81, 126.10, 117.28, 101.39, 48.42, 43.01, 42.76, 37.91, 24.92, 22.95, 22.67, 22.17, 21.76, 18.27, 12.59. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.29 (ESI-MS (m/z): 520.87, ($M+H^+$)). HRMS calculated for $C_{25}H_{36}N_4O_6S$ 521.24283 $[M+H]^+$; found 521.24258. **17b**: (5.2 mg, 0.01 mmol, 18%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.59 (s, 1H), 8.41 (d, *J* = 2.2 Hz, 1H), 7.02 (s, 1H), 6.85-6.57 (m, 2H), 6.50 (d, *J* = 1.0 Hz, 1H), 6.20 (dd, *J* = 15.2, 1.7 Hz, 1H), 5.50 (t, *J* = 7.8 Hz, 1H), 4.93-4.44 (m, 1H), 2.80 (s, 3H), 2.51 (s, 3H), 2.18 (s, 3H), 2.10-2.03 (m, 1H), 1.88-1.81 (m, 1H), 1.69-1.63 (m, 1H), 1.59-1.34 (m, 3H), 1.06-0.87 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.90, 168.85, 158.75, 157.76, 156.73, 147.02, 129.31, 127.73, 126.32, 124.71, 117.38, 101.44, 48.28,

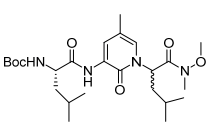
42.93, 42.67, 38.24, 25.06, 22.88, 22.75, 22.34, 21.94, 18.32, 12.58. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 5.43 (ESI-MS (m/z):520.87,($M+H^+$)). HRMS calculated for $C_{25}H_{36}N_4O_6S$ 521.24283 $[M+H]^+$; found 521.24257.



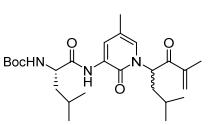
5-Methyl-N-(5-methyl-1-((*RS*)-4-methyl-1-((*S*)-4-methyl-1-((*R*)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxopentan-2-yl)-2-oxo-1,2-dihydropyridin-3-yl)isoxazole-3-carboxamide (**18**). This compound was synthesized using the same procedures as described above for the preparation of compound **8** on a 0.063 mmol scale. After HPLC purification (68 % MeCN-H_2O), the two diastereoisomers were separated. **18a**: (6.9 mg, 0.014 mmol, 22%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.61 (s, 1H), 8.34 (d, *J* = 2.2 Hz, 1H), 6.97 (dd, *J* = 2.2, 1.2 Hz, 1H), 6.65 (d, *J* = 7.5 Hz, 1H), 6.49 (d, *J* = 1.0 Hz, 1H), 5.62 (dd, *J* = 8.6, 7.1 Hz, 1H), 4.48-4.43 (m, 1H), 3.32 (d,*J*=5.0 Hz 1H), 2.89 (d, *J* = 5.0 Hz, 1H), 2.51 (s, 3H), 2.14 (s, 3H), 1.98-1.91 (m, 1H), 1.83-1.76 (m, 1H), 1.53 (s, 3H), 1.49-1.37 (m, 3H), 1.30-1.20 (m, 1H), 0.96-0.91 (m, 6H), 0.83-0.78 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.99, 171.76, 169.38, 158.83, 157.83, 156.64, 127.70, 125.73, 124.62, 116.44, 101.38, 59.34, 55.78, 52.59, 50.94, 39.65, 38.68, 25.43, 24.74, 23.23, 22.91, 22.21, 21.29, 18.30, 16.94, 12.57. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 9.13 (ESI-MS (m/z): 550.87, ($M+H^+$)). HRMS calculated for $C_{26}H_{36}N_4O_6$ 501.27076 $[M+H]^+$; found 501.27060. **18b**: (6.0 mg, 0.012 mmol, 19%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.64 (s, 1H), 8.37 (d, *J* = 2.2 Hz, 1H), 6.92 (dd, *J* = 2.2, 1.2 Hz, 1H), 6.81 (d, *J* = 8.0 Hz, 1H), 6.49 (d, *J* = 1.0 Hz, 1H), 5.60 (t, *J* = 7.8 Hz, 1H), 4.52-4.47 (m, 1H), 3.23 (d, *J* = 5.1 Hz, 1H), 2.84 (d, *J* = 5.0 Hz, 1H), 2.51 (s, 3H), 2.15 (s, 3H), 2.02-1.95 (m, 1H), 1.81-1.74 (m, 1H), 1.59-1.47 (m, 2H), 1.44 (s, 4H), 1.36-1.19 (m, 1H), 0.99-0.90 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.62, 171.70, 169.71, 158.89, 157.79, 156.81, 127.70, 125.95, 124.68, 116.54, 101.39, 59.29, 55.83, 52.64, 51.14, 39.65, 38.66, 25.46, 24.90, 23.48, 22.83, 22.24, 21.29, 18.34, 16.96, 12.57. LC-MS (linear gradient 10 →90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 9.24 (ESI-MS (m/z):500.87,($M+H^+$)). HRMS calculated for $C_{26}H_{36}N_4O_6$ 501.27076 $[M+H]^+$; found 501.27060.



(*RS*)-Methyl 2-(3-((*S*)-2-(tert-butoxycarbonyl)-4-methylpentanamido)-5-methyl-2-oxopyridin-1(2*H*)-yl)-4-methylpentanoate (**32**). This compound was synthesized according to the general procedure **A** described above on a 4.5 mmol scale. Purification by silica gel column chromatography (5% EtOAc-pentane→25% EtOAc-pentane) gave the title compound (940 mg, 2.0mmol, 44%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28 (d, *J* = 2.2 Hz, 1H), 6.79 (dd, *J* = 2.4, 1.4 Hz, 1H), 5.63 (td, *J* = 10.6, 5.4 Hz, 1H), 5.00 (t, *J* = 7.7 Hz, 1H), 4.37-4.26 (m, 1H), 3.73 (d, *J* = 1.9 Hz, 3H), 2.12 (s, 3H), 2.09-1.79 (m, 4H), 1.79-1.64 (m, 3H), 1.59 (q, *J* = 4.6 Hz, 1H), 1.52 (d, *J* = 2.3 Hz, 1H), 1.44 (s, 12H), 1.36-1.19 (m, 7H), 1.03-0.85 (m, 17H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.00, 171.03, 156.58, 128.14, 125.15, 116.10, 56.45, 52.87, 41.95, 41.01, 39.50, 31.56, 30.42, 29.82, 28.38, 24.68, 23.19, 23.09, 21.52, 18.31. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min):8.85 (ESI-MS (m/z): 465.80, (M^+)). HRMS calculated for $C_{24}H_{39}N_3O_6$ 466.29116 $[M+H]^+$; found 466.29094.

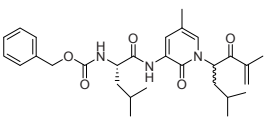


Tert-butyl (*S*)-1-(1-((*R* or *S*)-1-(methoxy(methyl)amino)-4-methyl- 1-oxopentan-2-yl)-5-methyl-2-oxo-1,2-dihydropyridin-3-ylamino)-4-methyl-1-oxopentan-2-ylcarbamate (**33**). Compound **32** was hydrolyzed to acid according to general procedure **B** on a 2.0mmol scale and the crude product was used directly in next step. The crude product was coupled with N,O-dimethylhydroxylamine hydrochloride according to general procedure **C**. Using HPLC purification (60% MeCN-H_2O), the two diastereoisomers were separated. **33a**: (124 mg, 0.25 mmol, 13%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81 (s, 1H), 8.27 (d, *J* = 2.2 Hz, 1H), 7.08 (s, 1H), 6.27 (s, 1H), 4.95 (d, *J* = 8.0 Hz, 1H), 4.28 (s, 1H), 3.77 (s, 3H), 3.20 (s, 3H), 2.13 (s, 3H), 1.91-1.51 (m, 5H), 1.44 (s, 10H), 1.02-0.90 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.75, 156.39, 155.44, 127.50, 125.15, 115.5, 99.86, 61.80, 54.25, 51.51, 41.83, 40.10, 32.27, 28.27, 23.05, 22.97, 21.80, 18.21. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.63 (ESI-MS (m/z): 495.93, ($M+H^+$)). HRMS calculated for $C_{25}H_{42}N_4O_6$ 495.31771 $[M+H]^+$; found 495.31747. **33b**: (181 mg, 0.37 mmol, 18%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (s, 1H), 8.27 (d, *J* = 2.2 Hz, 1H), 7.13-7.01 (m, 1H), 6.27 (s, 1H), 4.96 (d, *J* = 7.9 Hz, 1H), 4.28 (s, 1H), 3.78 (s, 3H), 3.20 (s, 3H), 2.12 (s, 3H), 1.92-1.50 (m, 5H), 1.44 (s, 10H), 0.98-0.88 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.90, 171.30, 156.51, 127.67, 125.24, 115.66, 100.12,61.99, 54.26, 51.59, 41.95, 40.24, 32.41, 24.99, 24.73, 23.22, 21.88, 18.36. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.78 (ESI-MS (m/z): 495.00, ($M+H^+$)). HRMS calculated for $C_{25}H_{42}N_4O_6$ 495.31771 $[M+H]^+$; found 495.31751.

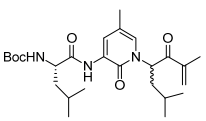


Tert-butyl (*S*)-1-(1-((*R* or *S*)-2,6-dimethyl-3-oxohept-1-en-4-yl)-5-methyl-2-oxo-1,2-dihydropyridin-3-ylamino)-4-methyl-1-oxopentan-2-ylcarbamate (**34**). A solution of 2-bromopropene (75 µL, 0.75 mmol, 3 eq.) in dry Et_2O (10 mL) was cooled down to −78 °C under argon atmosphere and stirred for 15 min before adding tBuLi (0.7 mL 1.6 M in pentane, 1.2 mmol, 4.5 eq.).The reaction mixture was stirred for 15 min. Weinreb amide **33a** (124 mg, 0.25 mmol) was coevaporated with toluene and dissolved in 5 mL Et_2O. This solution was added dropwise to the reaction mixture during 10 min at −78 °C. The resulting reaction mixture was quenched after 2 h with sat. aq. NH_4Cl (5 mL). The water layer was extracted with EtOAc (3 times) and the combined organics were

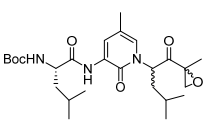
washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by flash column chromatography (2% EtOAc/pentane→20% EtOAc/pentane) yielded the product as clear oil (82 mg, 0.17 mmol, 68%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.88 (s, 1H), 8.27 (d, *J* = 2.1 Hz, 1H), 6.80 (s, 1H), 6.47 (dd, *J* = 9.4, 5.8 Hz, 1H), 6.36 (s, 1H), 5.90 (d, *J* = 1.6 Hz, 1H), 5.14 (dd, *J* = 20.0, 7.7 Hz, 1H), 4.27 (d, *J* = 9.8 Hz, 1H), 2.10 (s, 3H), 1.86 (s, 3H) 1.84-1.49 (m, 5H), 1.44 (s, 9H), 1.31-1.23 (m, 1H), 1.01-0.89 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.34, 171.89, 155.93, 142.44, 127.81, 124.93, 124.17, 116.13, 54.25, 41.66, 40.23, 28.33, 24.70, 23.13, 23.07, 22.05, 21.80, 18.23, 17.92. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 9.44 (ESI-MS (m/z):476.07, $(M+H^+)$). HRMS calculated for $C_{26}H_{41}N_3O_5$ 476.31190$[M+H]^+$; found 476.31164.



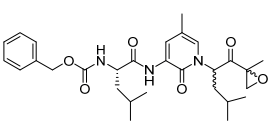
Benzyl (*S*)-1-(1-((*S* or *R*)-2,6-dimethyl-3-oxohept-1-en-4-yl)-5-methyl-2-oxo-1,2-dihydropyridin-3-ylamino)-4-methyl-1-oxopentan-2-ylcarbamate (**35**). Compound **34** was treated with TFA according to the general procedure **D** on a 0.093 mmol scale. Deprotected **34** was dissolved in DCM (2 mL), followed by the addition of DiPEA (0.42 mmol, 4.5 eq.) and CbzCl (0.12 mmol, 1.3 eq.). The reaction mixture was stirred at r.t. overnight. The crude product was concentrated *in vacuo* and purification by silica gel flash column chromatography (2% EtOAc/pentane→20% EtOAc/pentane) yielded the title compound (33 mg, 0.064 mmol, 69%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.78 (s, 1H), 8.25 (d, *J* = 2.2 Hz, 1H), 7.41-7.29 (m, 5H), 6.89-6.75 (m, 1H), 6.48 (dd, *J* = 9.4, 5.8 Hz, 1H), 6.35 (s, 1H), 5.92 (t, *J* = 1.6 Hz, 1H), 5.40 (d, *J* = 8.4 Hz, 1H), 5.22-4.99 (m, 2H), 4.38 (dt, *J* = 13.0, 6.0 Hz, 1H), 2.10 (s, 3H), 1.88 (s, 3H), 1.79-1.56 (m, 4H), 1.46-1.29 (m, 2H), 1.00-0.86 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.36, 171.46, 156.01, 142.53, 128.60, 128.17, 127.88, 125.23, 124.55, 116.11, 67.22, 54.41, 41.89, 40.33, 29.80, 24.77, 23.09, 22.05, 17.96. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 9.44 (ESI-MS (m/z):510.13,$(M+H^+)$). HRMS calculated for $C_{29}H_{39}N_3O_5$ 510.29625 $[M+H]^+$; found 510.29586.



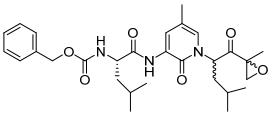
Tert-butyl (*S*)-1-(1-((*S* or *R*)-2,6-dimethyl-3-oxohept-1-en-4-yl)-5-methyl-2-oxo-1,2-dihydropyridin-3-ylamino)-4-methyl-1-oxopentan-2-ylcarbamate (**36**). This compound was prepared in the same way was described for the preparation of compound on 0.37 mmol scale. Purification by silica gel flash column chromatography (2% EtOAc/pentane→20% EtOAc/pentane) yielded the title compound (114 mg, 0.24 mmol, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.89 (s, 1H), 8.27 (d, *J* = 2.2 Hz, 1H), 6.86-6.67 (m, 1H), 6.46 (dd, *J* = 9.3, 5.9 Hz, 1H), 6.36 (d, *J* = 1.0 Hz, 1H), 5.91 (q, *J* = 1.5 Hz, 1H), 5.24-5.01 (m, 1H), 4.29 (s, 1H), 2.10 (s, 3H), 1.87 (s, 3H), 1.83-1.51 (m, 5H), 1.44 (s, 9H), 1.29 (s, 1H), 1.02-0.89 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.34, 171.90, 155.93, 142.49, 128.09, 127.78, 124.95, 124.18, 116.13, 54.26, 41.64, 40.18, 28.32, 24.92, 24.72, 23.12, 23.03, 22.06, 21.80, 18.21, 17.90. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 9.38 (ESI-MS (m/z):476.13, $(M+H^+)$). HRMS calculated for $C_{26}H_{41}N_3O_5$ 476.31190 $[M+H]^+$; found 476.31122.



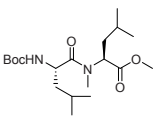
Tert-butyl (*S*)-4-methyl-1-(5-methyl-1-((*R* or *S*)-4-methyl-1-((*RS*)-2-methyloxiran-2-yl)-1-oxopentan-2-yl)-2-oxo-1,2-dihydropyridin-3-ylamino)-1-oxopentan-2-ylcarbamate (**37**). Compound **36** (67 mg, 0.14 mmol) was dissolved in MeOH (5 mL) and DiPEA(0.1 mL, 0.56 mmol, 4 eq.) was added. H_2O_2 (57 µL, 0.56 mmol, 4 eq.) was added after the reaction mixture was cooled to 0°C. The reaction was stirred at 0 °C for 24 h and H_2O_2 (57 µL, 0.56 mmol, 4.0 eq.) was added again. The reaction was stirred at 0 °C for another 24h after which TLC analysis showed the complete conversion of the starting material. The crude product was concentrated *in vacuo* and purification by flash column chromatography (2% EtOAc/pentane→20% EtOAc/pentane) yielded the title compound (38 mg, 0.08 mmol, 54%).^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.77 (s, 1H), 8.28 (s, 1H), 6.99-6.85 (m, 1H), 5.88-5.83 (m, 1H), 5.00 (s, 1H), 4.26 (s, 1H), 3.59 (d, *J* = 5.0 Hz, 1H), 2.93 (d, *J* = 4.9 Hz, 1H), 2.13 (s, 3H), 1.89-1.66 (m, 4H), 1.53 (s, 3H), 1.44 (s, 9H), 1.31-1.22 (m, 2H), 1.00-0.89 (m, 12H).^{13}C-NMR (100 MHz, $CDCl_3$) δ 206.67, 171.84, 156.61, 127.73, 125.14, 115.43, 59.32, 53.83, 52.54, 41.75, 38.41, 29.81, 28.38, 24.83, 23.30, 23.16, 21.88, 21.04, 18.41, 16.71.HRMS calculated for$C_{26}H_{41}N_3O_6$ 492.30681$[M+H]^+$; found 492.30655.



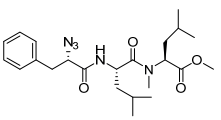
(*S*)-Benzyl 4-methyl-1-(5-methyl-1-((*S* or *R*)4-methyl-1-((*RS*)2-methyloxiran-2-yl)-1-oxopentan-2-yl)-2-oxo-1,2-dihydropyridin-3-ylamino)-1-oxopentan-2-ylcarbamate (**28a**). The title compound was prepared in the same way was described for the preparation of compound**37** on a 0.064 mmol scale. Purification by HPLC(65%-75% MeCN-H_2O) yielded the title compound (8.1 mg, 0.015 mmol, 23%). ^{1}H NMR (400 MHz, $CDCl_3$) δ8.70 (s, 1H), 8.27 (s, 1H), 7.36-7.34 (m, 5H), 6.95 (s, 1H), 5.87-5.93 (m, 1H), 5.23-5.09 (m,3H), 4.36 (s, 1H), 3.68-3.50 (m, 1H), 3.32-3.20 (m, 1H), 2.94 (d, *J* = 4.9 Hz, 1H), 1.87-1.57 (m, 4H), 1.54 (s, 3H), 1.49-1.33 (m, 1H), 0.97-0.91 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.65, 171.40, 156.62, 128.66, 128.23, 127.62, 125.45, 115.57, 67.32, 59.39, 54.64, 54.12, 52.70, 52.62, 41.91, 38.41, 24.86, 23.35, 21.04, 18.44, 16.76. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 9.14 (ESI-MS (m/z):526.20, $(M+H^+)$). HRMS calculated for $C_{29}H_{39}N_3O_6$ 526.29116 $[M+H]^+$; found 526.29087.



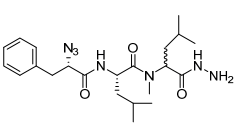
(*S*)-Benzyl 4-methyl-1-(5-methyl-1-((*R* or *S*)4-methyl-1-((*RS*)2-methyloxiran-2-yl)-1-oxopentan-2-yl)-2-oxo-1,2-dihydropyridin-3-ylamino)-1-oxopentan-2-ylcarbamate (**28b**). The title compound was prepared in the same way was described for the preparation of compound **35**on 0.076 mmol scale. Purification by HPLC (72%-82% MeCN-H_2O) yielded the title compound (6.8 mg, 0.013 mmol, 17%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.93-8.62 (m, 1H), 8.28 (s, 1H), 7.46-7.28 (m, 5H), 6.95 (s, 0.5H), 6.73-6.68 (m, 0.5H), 6.00-5.66 (m, 1H), 5.27-5.04 (m, 3H), 4.36 (s, 1H), 3.64-3.37 (m, 1H), 3.30 (s, 1H), 3.20 (s, 0.5H), 2.94 (d, *J*=6.0 Hz, 0.5H), 2.19-2.01 (m, 3H), 1.79-1.66 (m, 3H), 1.66-1.48 (m, 3H), 1.06-0.88 (m, 12H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 206.63, 171.40, 156.63, 156.61, 156.22, 136.25, 129.06, 128.87, 128.67, 128.51, 128.32, 128.23, 127.63, 125.56, 125.54, 125.49, 125.42, 116.19, 115.62, 75.26, 74.15, 70.62, 67.32, 59.37, 59.20, 54.73, 54.15, 54.07, 52.70, 52.63, 52.62, 44.04, 41.88, 38.44, 24.94, 24.89, 24.82, 24.77, 23.34, 23.23, 23.16, 23.14, 21.91, 21.15, 18.44, 18.26, 18.24, 16.75, 13.78, 13.41. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min):9.29 (ESI-MS (m/z):526.13, ($M+H^+$)). HRMS calculated for $C_{29}H_{39}N_3O_6$ 526.29116[M+H]$^+$; found 526.29087.



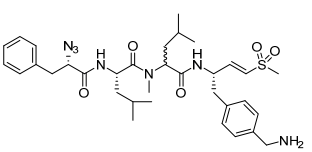
Methyl *N*-((tert-butoxycarbonyl)-*L*-leucyl)-*N*-methyl-*L*-leucinate (**39**). *N*-Methyl-L-Leu-OH hydrochloride (182 mg, 1 mmol) was dissolved in MeOH (10 mL) and cooled to 0 ºC. $SOCl_2$ (0.15 mL, 2eq.) was slowly added and then stirred at r.t. over night. The mixture was coevaporated with toluene (3x) and used directly in the next step. The crude product obtained above was then coupled with Boc-L-Leu-OH according to the general procedure **C** described above on a 1.0 mmol scale. Purification by silica gel column chromatography (5% EtOAc/pentane→25% EtOAc/pentane) yielded the title compound(81 mg, 0.22 mmol, 22%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.42-5.30 (m, 1H), 5.20 (d, *J* = 9.2 Hz, 1H), 4.76-4.56 (m, 1H), 3.70 (s, 3H), 3.00 (s, 3H), 1.84-1.65 (m, 3H), 1.57-1.36 (m, 12H), 1.05-0.84 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.12, 172.31, 155.84, 79.55, 54.43, 52.20, 49.06, 42.01, 37.08, 30.94, 28.35, 24.80, 24.68, 23.48, 23.35, 21.91, 21.38. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min):8.51 (ESI-MS (m/z):372.80, ($M+H^+$)). HRMS calculated for $C_{19}H_{36}N_2O_5$ 373.26970 [M+H]$^+$; found 373.26968.



Methyl *N*-(((*S*)-2-azido-3-phenylpropanoyl)-*L*-leucyl)-*N*-methyl-*L*-leucinate (**40**). Compound **39** (81.3 mg, 0.22 mmol) was deprotected according to the general procedure **D** and followed by the general procedure **C**. Purification by silica gel column chromatography (5% EtOAc/pentane→30% EtOAc/pentane) yielded the title compound (88.1 mg, 0.20 mmol, 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36-7.16 (m, 5H), 6.99 (d, *J* = 8.7 Hz, 1H), 5.30-5.26 (m, 1H), 5.03-4.89 (m, 1H), 4.21-4.18 (m, 1H), 3.69 (s, 3H), 3.32-3.28 (m, 1H), 3.12-3.02 (m, 1H), 3.00 (s, 3H), 1.81-1.61 (m, 2H), 1.52-1.43 (m, 4H), 1.04-0.81 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.98, 172.12, 168.24, 136.09, 129.59, 128.65, 127.23, 65.34, 54.66, 52.27, 47.86, 41.56, 38.36, 37.04, 31.03, 24.94, 24.55, 23.45, 23.26, 21.72, 21.46. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min):8.80 (ESI-MS (m/z):446.27, ($M+H^+$)).HRMS calculated for $C_{23}H_{35}N_5O_4$ 446.27618 [M+H]$^+$; found 446.27736.

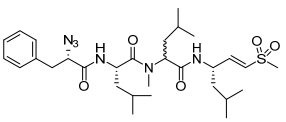


(*S*)-2-((*S*)-2-azido-3-phenylpropanamido)-*N*-((*RS*)-1-hydrazinyl-4-methyl-1-oxopentan-2-yl)-*N*,4-dimethylpentanamide (**41**). Compound **40** (81.3 mg, 0.18 mmol) was dissolved in MeOH (5 mL) and followed by the addition of hydrazine monohydrate (0.26 mL, 5.4 mmol, 30 eq.). The reaction solution was stirred at r.t overnight and refluxed for 2h. The title compound was obtained after coevaporation of the mixture with toluene (3x) with quantitative yield. The product was used directly in next step.



(*S*)-*N*-((*RS*)-1-(((*S*,*E*)-1-(4-(aminomethyl)phenyl)-4-(methylsulfonyl) but-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)-2-((*S*)-2-azido-3-phenylpropanamido) -*N*,4-dimethylpentanamide TFA salt (**30**). The hydrazide **41** (22 mg, 0.05 mmol,) was dissolved in 1:1 DMF:DCM (v/v) and cooled to -30ºC. *t*BuONO (7.3 µl, 55 µmol, 1.1 eq.) and HCl (35 µl,4M solution in 1,4-dioxane, 0.14 mmol, 2.8 eq.) were added, and the mixture was stirred for 3 h at -30ºC after which TLC analysis (10% MeOH/DCM, v/v) showed complete consumption of the starting material. Compound **25** was added to the reaction mixture as a solution in DMF with DiPEA (44 µl, 0.25 mmol, 5.0 eq.) and this mixture was allowed to warm up to room temperature slowly overnight. The mixture was diluted with EtOAc and extracted with H_2O (3x) and brine. The organic layer was dried over $MgSO_4$ and concentrated in *vacuo*, followed by the general procedure **D**. Purification by HPLC (45%-55% MeCN-H_2O) yielded the title compound (3.3 mg, 4.2 µmol, 8%). ^{1}H NMR (400 MHz, MeOD) δ 7.46-7.19 (m, 9H), 6.84-6.78 (dd, *J* = 15.2, 5.4 Hz, 1H), 6.58 (dd, *J* = 15.2, 1.6 Hz, 1H), 5.00-4.95 (m, 1H), 4.83-4.71 (m, 1H), 4.16-4.00 (m, 3H), 3.25-3.03 (m, 2H), 3.03-2.90 (m, 5H), 2.80 (s, 2H), 2.31 (s, 1H), 1.88-1.18 (m, 6H), 1.02-0.84 (m, 12H). ^{13}C NMR (100 MHz, MeOD) δ 174.51, 172.75, 171.32, 147.09, 146.59, 139.77, 137.72, 133.03, 131.86, 131.73, 131.25, 131.21, 130.43, 130.37, 130.27, 129.69, 129.61, 128.13, 128.09, 65.23, 64.45, 60.00, 57.08, 52.74, 52.38, 44.04, 42.81, 42.71, 41.31, 40.21, 39.96, 38.84, 38.56, 38.28, 32.02, 29.73, 26.11, 26.00, 25.76, 23.83,

23.64, 23.53, 23.25, 22.03, 21.95, 21.86. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min):6.41(ESI-MS (m/z):668.07, ($M+H^+$)). HRMS calculated for $C_{34}H_{49}N_7O_5S$ 668.35886 $[M+H]^+$; found 668.35885.



(*S*)-2-((*S*)-2-azido-3-phenylpropanamido)-*N*,4-dimethyl-*N*-((*RS*)-4-methyl-1-(((*S*,*E*)-5-methyl-1-(methylsulfonyl)hex-1-en-3-yl)amino)-1-oxopentan-2-yl)pentanamide (**31**). Compound was prepared by the same procedure as described for the preparation of compound **30**. Purification by HPLC (55%-65% MeCN-H_2O) yielded the title compound (3.3 mg, 5.5 µmol, 11%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.39-7.17 (m, 5H), 6.96-6.75 (m, 2H), 6.60-6.41 (m, 1H), 6.28 (d, *J* = 8.4 Hz, 1H), 4.98-4.80 (m, 1H), 4.80-4.57 (m, 2H), 4.30-4.19 (m, 1H), 3.34-3.29(m, 1H), 3.10-3.05 (m, 1H), 3.00 (s, 2H), 2.97-2.94 (m, 3H), 2.77 (s, 1H), 1.80-1.18 (m, 9H), 1.06-0.76 (m, 18H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 173.43, 172.29, 169.99, 169.90, 168.73, 168.37, 147.71, 147.57, 135.92, 135.58, 129.73, 129.64, 129.60, 129.54, 129.48, 129.42, 128.89, 128.76, 127.54, 127.41, 65.40, 65.01, 58.84, 48.45, 47.89, 47.83, 47.74, 47.48, 42.98, 42.95, 42.75, 41.62, 40.33, 38.43, 38.36, 37.93, 36.00, 29.42, 25.10, 24.84, 24.83, 24.76, 24.71, 24.53, 23.59, 23.48, 23.27, 23.07, 23.01, 22.32, 21.83, 21.66, 21.65, 21.49. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 12.5 min): R_t (min): 8.68 (ESI-MS (m/z):604.87, ($M+H^+$)). HRMS calculated for $C_{30}H_{48}N_6O_5S$ 605.34797 $[M+H]^+$; found 605.34821.

Biological analysis

Competition assay in cell lysate

Lysates of HEK-293T cells were prepared by sonication in 3 volumes of lysis buffer containing 50 mMTris 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 BODIPY-FL-epoxomicin**27** (1µM each) for an additional 1 h at 37 °C, followed by 5 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 (λex 488 nm, λem 520 nm). Intensities of bands were measured by fluorescent densitometry and normalized to the intensity of bands in mock-treated extracts. Average values of two independent experiments were plotted against inhibitor concentrations. Apparent IC_{50} values were calculated using GraphPad Prism software.

4.5 References

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