



Universiteit
Leiden
The Netherlands

Chemical tools to monitor and control human proteasome activities

Bruin, G. de

Citation

Bruin, G. de. (2016, June 1). *Chemical tools to monitor and control human proteasome activities*. Retrieved from <https://hdl.handle.net/1887/39834>

Version: Not Applicable (or Unknown)

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/39834>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/39834> holds various files of this Leiden University dissertation.

Author: Bruin, G. de

Title: Chemical tools to monitor and control human proteasome activities

Issue Date: 2016-06-01

Towards β 2 selective inhibitors with reduced basicity

Introduction

Proteasomes are large proteolytic machineries responsible for the degradation of the majority of proteins in eukaryotic cells. Inhibition of protein degradation through blockage of the proteolytic sites of the proteasome is cytotoxic for certain cancers. Bortezomib and carfilzomib are approved drugs for the treatment of multiple myeloma (MM) and mantle cell lymphoma, while various proteasome inhibitors are currently being evaluated in clinical trials for a variety of cancers. Constitutive proteasomes, which are expressed in every cell, have three different proteolytic activities, namely caspase-like (β 1c), trypsin-like (β 2c) and chymotrypsin-like (β 5c). Immune cells and cells exposed to inflammatory cytokines, express an additional proteasome-type, termed immunoproteasome, in which β 1i, β 2i and β 5i replace β 1c, β 2c and β 5c as catalytic activities. These subunits have slightly changed substrate specificities compared to their constitutive counterparts. The chymotryptic activities of the proteasome (β 5c and β 5i) have long been considered to be the only suitable subunits to target for drug development and bortezomib, carfilzomib and various clinical candidates were developed to target the β 5 subunits.¹ However, bortezomib efficiently inhibits β 1c and β 1i with similar potency as the β 5 active sites,² while carfilzomib inhibits both β 1 and β 2 activities at higher concentrations (see also chapter 3). It has been demonstrated that selective β 5 inhibition is not cytotoxic to most MM cell lines, but that partial co-inhibition of either β 1 or β 2 is necessary for cytotoxicity.³ In order to be able to investigate the effect of β 2 inhibition on MM cells, selective β 2 inhibitors have been developed. The first in-class β 2 selective inhibitors bears an arginine residue at P1 and/or P3 (NC-002 and NC-022, Figure 1). While NC-022 was more potent than NC-002 against purified proteasomes, it suffered from poor cell permeability caused by two positively charge Arg residues. Using NC-002 it was shown for the first time that selective β 2 inhibition sensitizes MM cells to bortezomib and carfilzomib.⁴ However, to overcome the lability of the arginine epoxyketone moiety (intramolecular attack of the guanidine group to the epoxyketone moiety), low yielding synthesis and poor cell

permeability in solid tumours, a second generation $\beta 2$ inhibitor was developed (LU-102, Figure 1).⁵ LU-102 bears a (4)-aminomethyl-phenylalanine (4-CH₂NH₂)Phe vinyl sulfone at P1, which can be straightforwardly synthesized on large scale and shows greater stability than the Arg epoxyketone of NC-002. Introduction of a P3 4-(CH₂NH₂)Phe in LU-112 further increased the potency and selectivity in lysate compared to LU-102, but dramatically impaired cell permeability (Figure 1).

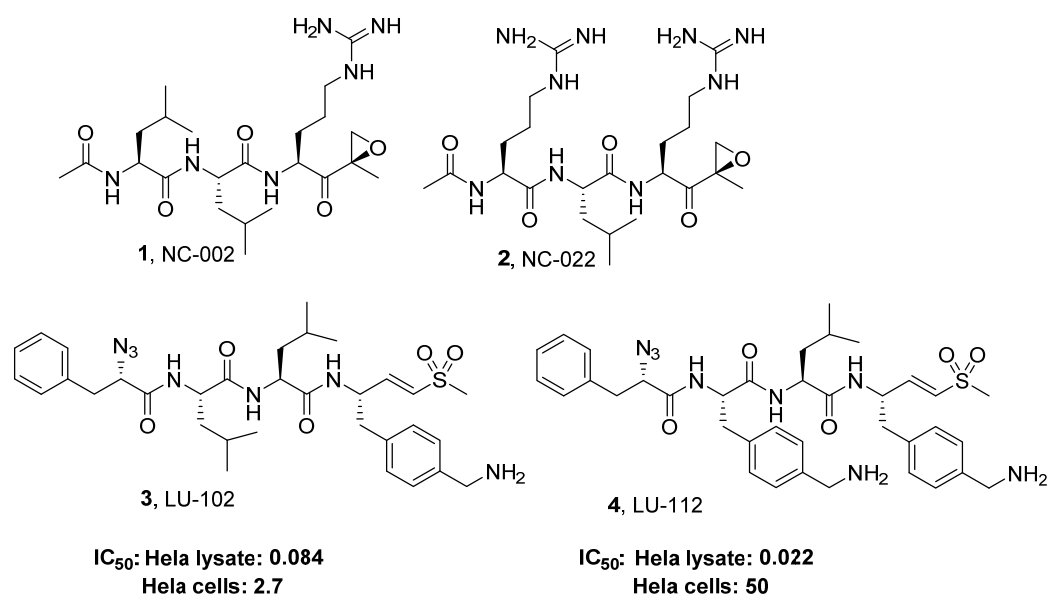
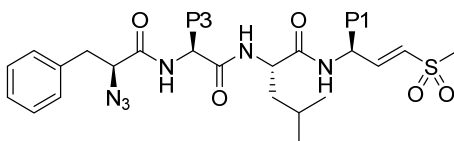


Figure 1. Structures of $\beta 2$ selective inhibitors. IC₅₀ values (μ M) have been determined in Hela cell lysates (1 h treatment) or intact Hela cells (4 h treatment).⁵

With LU-102 it has been shown that selective $\beta 2$ inhibition does not only sensitize MM cells to bortezomib and carfilzomib⁵, but also overcomes resistance to bortezomib and carfilzomib, a major problem which arises during treatment of patients with proteasome inhibitors.⁶ While LU-102 shows nanomolar potency in cell lysates, much higher IC₅₀ values are found in living cells.^{6, 7} Therefore, high concentrations of LU-102 are necessary to achieve efficient $\beta 2$ inhibition. In order to increase the cell permeability, one possibility is to lower the charge of the molecule at physiological pH. For this, basic amino acids with pK_a closer to physiological pH could be incorporated. In this chapter, amine-containing amino acids with various pK_a values are explored. In table 1 the structures of compounds and associated pK_a values described in this chapter are shown. The electron withdrawing properties of the double (Lys(4-ene)), triple (Lys(4-yn)) or amide bond (diaminopropionic acid-Gly, Dap(Gly)) in the Lys analogues cause significantly lower pK_a values of the ϵ -amine compared to Lys.⁸



Compound	P1	P3	Compound	P1	P3
3, (LU-102)			10		
5			11		
6			12		
7			13		
8			14		
9					
pK_a=	 9.3	 9.7	 8.9	 8.1	 6.5

Table 1. Structures of compounds synthesized in this study. pK_a values of conjugated acids are shown.⁸

Synthesis

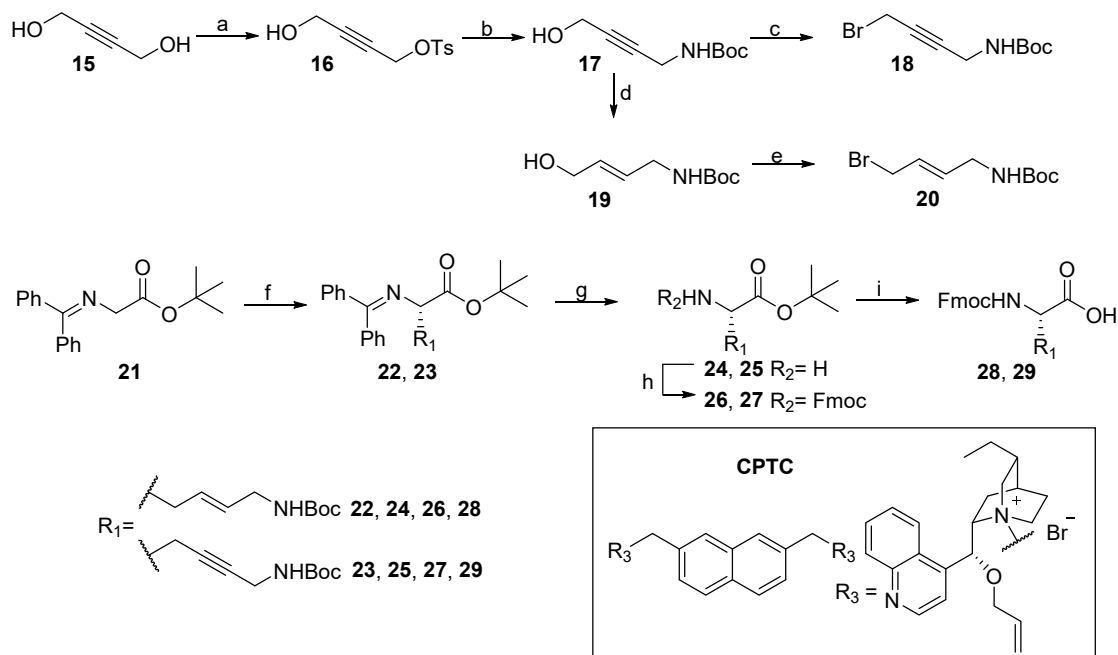
This section describes the synthesis of compounds **5-14**. Since no syntheses of asymmetric L-Lys(4-ene) or L-Lys(4-yn) exist, an enantioselective synthesis for both amino acids was developed, with as key step the catalytic enantioselective phase-transfer alkylation of a glycine derivative. Also the synthesis of the amide bond containing analogue of Lys is described (Dap(Gly)), which is made by peptide coupling between the β -amine of L-diaminopropionic acid (Dap) and Gly. The appropriate building blocks of the Lys analogues and

His could be converted to their corresponding vinyl sulfones and were incorporated at the P1, P3 or at P1 and P3 of tetrapeptide vinyl sulfones.

Synthesis of L-Lys(4-ene) or L-Lys(4-yn) containing inhibitors

The synthesis of α -amino acids by catalytic enantioselective phase transfer alkylation of a glycine derivative has been highly optimized by Park *et al.*⁹ They developed a dimeric cinchona-derived chiral phase transfer catalyst (CPTC, Scheme 1) and applied this in the synthesis of a wide range of α -amino acids, including allyl-Gly and propargyl-Gly. Based on these results, it was envisioned that this method could also be applied to the enantioselective synthesis of L-Lys(4-ene) and L-Lys(4-yn). In order to do so, first the appropriate bromides **18** and **20** were synthesized, which could be used in the chiral phase transfer alkylation of glycine derivative **21** (Scheme 1). Subsequent protection group manipulations provided the required building blocks **28** and **29**, which could be used for further synthesis of the desired inhibitors (Scheme 2).

The synthesis of bromides **18** and **20** commenced with the mono-tosylation of diol **15**. Next, the O-Ts moiety of compound **16** was substituted by ammonia¹⁰ followed by Boc protection of the amine providing compound **17**. In order to obtain the *E*-alkene, propargyl alcohol **17** could be selectively reduced by LiAlH₄ to give allyl alcohol **19**.^{11, 12}



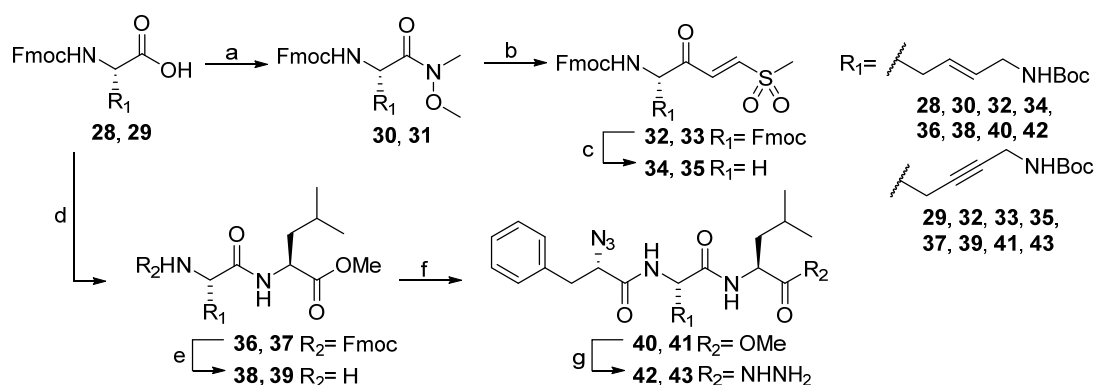
Scheme 1. Synthesis of lysine(4-ene) and lysine(4-yn) building blocks. Reagents and conditions: a. TsCl, pyridine, DCM, 63%; b. 1. 25% aq. NH₃; 2. Boc₂O, Et₃N, THF, DCM, 42%; c. PPh₃, CBr₄, DCM, 0°C 68%; d. LiAlH₄, THF, Δ , 38%; e. PPh₃, CBr₄, DCM, 0°C, 58%; f. **19** or **20**, CPTC, Tol/CHCl₃, 50% aq. KOH **22**: 84%, 79% ee; **23**: 84%, 80% ee; g. 15% aq. citric acid, THF, 0°C-rt; h. FmocOSu, DiPEA, DCM, **26**: 83%, **27**: 89% (over steps g/h); i. 1. TFA; 2. Boc₂O, DiPEA, MeCN, **28**: 82%, **29**: 63%

The low yielding reduction of alkyne **17** to alkene **19** is possibly caused by the harsh conditions (strong reducing agent and elevated temperature), which could result in partial Boc removal. Alcohol **17** and **19** were reacted in an Appel reaction providing bromides **18** and **20**. Although several steps towards the bromides were rather low-yielding, the reactions could be easily performed on large scale and sufficient quantities of both bromides were obtained.

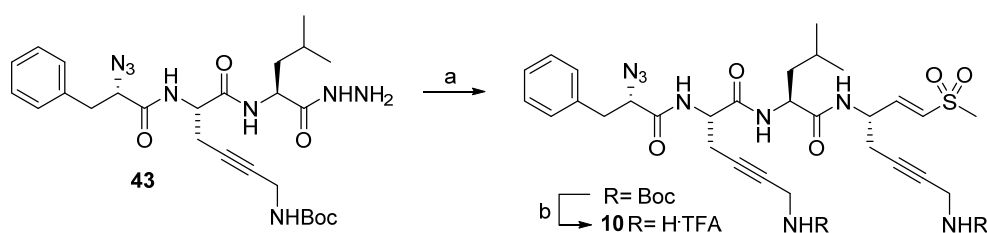
Both bromides were used in the chiral phase transfer alkylation of Gly-derivative **21**, providing compounds **22** and **23** in high yields and good enantiomeric excess (*ee*, 79% for **22** vs. 80% for **23**), as determined by chiral HPLC analysis.⁹ Mildly acidic hydrolysis of the imine provided amines **24** and **25**, which were Fmoc protected yielding compounds **26** and **27**. Subsequent Boc and t-Bu ester removal by treatment with TFA and Boc protection of the ϵ -amine, yielded building blocks **28** and **29** in good yields.

For the synthesis of the corresponding vinyl sulfones, **28** and **29** were converted to Weinreb amides **30** and **31** (Scheme 2). Using standard procedures for the synthesis of vinyl sulfones⁵, the Weinreb amides were reduced to the aldehydes directly followed by a Horner-Wadsworth-Emmons reaction to provide Fmoc protected vinyl sulfones **32** and **33**. The Fmoc group could be removed by treatment with diethylamine, providing free amines **34** and **35**.

For the incorporation of L-Lys(4-ene) or L-Lys(4-yn) at the P3 site, building blocks **28** and **29** were coupled to H-Leu-OMe by a standard peptide coupling yielding dipeptides **36** and **37** (Scheme 2). Subsequent Fmoc removal, peptide coupling to N₃Phe-OH and hydrazinolysis of the methylester provided hydrazides **42** and **43**. Standard azide couplings (see Scheme 3 for an example) between hydrazides **42**, **43**, or N₃Phe-Leu-Leu-NHNH₂⁵ and vinyl sulfones **34**, **35**, or H-Leu-VS followed by Boc removal provided the desired final compounds.



Scheme 2. Synthesis of Lys(4-ene) and Lys(4-yn) vinyl sulfones and peptide hydrazides. Reagents and conditions: a. HCTU, N,O-dimethyl hydroxylamine, **30**: 100%, **31**: 85%; b. 1. LiAlH₄, THF. 2. diethyl((methylsulfonyl)-methyl)-phosphonate, NaH, THF, **32**: 40%, **33**: 70%; c. Et₂NH, MeCN, **34**: 70%, **35**: 47%; d. H-Leu-OMe, HCTU, DiPEA, DCM, **36**: 96%, **37**: 76%; e. Piperidine, DMF, **38**: 94%, **39**: 100%; f. N₃Phe-OH, HCTU, DiPEA, DCM, **40**: 60%, **41**: 91%; g. hydrazine monohydrate, MeOH, 100%.

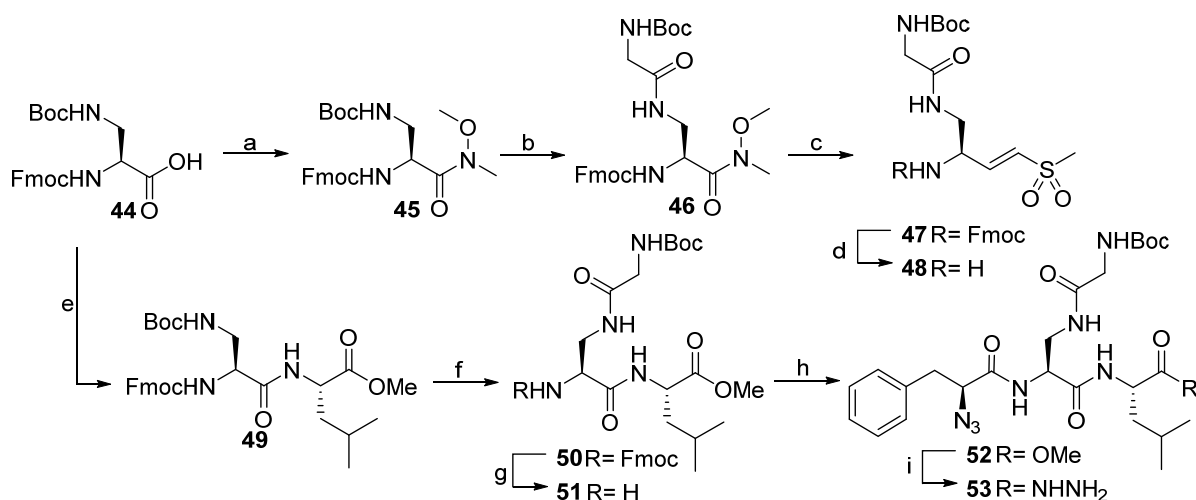


Scheme 3. Synthesis of compound 10 as example of an azide coupling followed by Boc removal. Reagents and conditions: a. 1. *t*BuONO, HCl, DMF. 2. H-Lys(4-yn)-VS, DiPEA; b. TFA, 79% over 2 steps

Synthesis of Dap(Gly) containing inhibitors

The synthesis of Dap(Gly) vinyl sulfone and peptide hydrazide for the synthesis of P3 Dap(Gly) compounds is shown in Scheme 4. The synthesis of the vinyl sulfone commenced with the conversion of commercially available Fmoc-Dap(Boc)-OH **44** to Weinreb amide **45**. Subsequent Boc removal and peptide coupling with Boc-Gly-OH provided compound **46**, followed by conversion of the Weinreb amide to the vinyl sulfone using similar procedures as described above, eventually yielding vinyl sulfone free amine **48**.

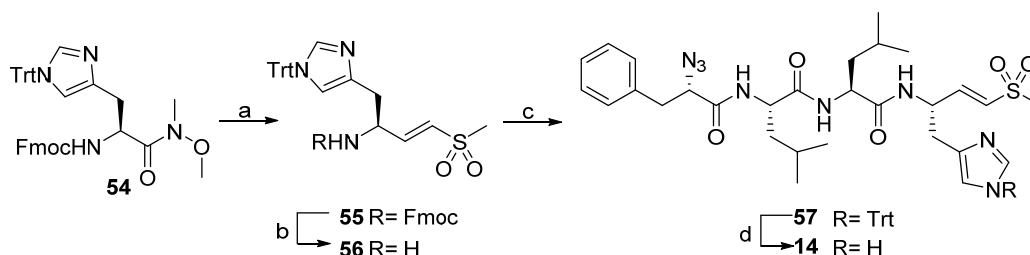
For the synthesis of the peptide hydrazide, Fmoc-Dap(Boc)-OH **44** was first coupled to H-Leu-OMe, followed by Boc removal and coupling of Boc-Gly-OH, providing dipeptide **50**. Fmoc removal, peptide coupling to N₃Phe-OH and hydrazinolysis of the methylester resulted in peptide hydrazide **53**. The desired inhibitors with Dap(Gly) at P1 and/or P3 were obtained by standard azide couplings between the appropriate hydrazides and vinyl sulfones, and Boc removal (in the same way as shown in Scheme 3).



Scheme 4. Synthesis of Dap(Gly) vinyl sulfone and peptide hydrazide. Reagents and conditions: a. HCTU, N,O-dimethyl hydroxylamine, 96%; b. HCTU, Boc-Gly-OH, DiPEA, DCM, 100%. c. 1. LiAlH₄, THF. 2. diethyl((methylsulfonyl)-methyl)-phosphonate, NaH, THF, 43%; d. Et₂NH, MeCN, 100%; e. HCTU, H-Leu-OMe, DiPEA, DCM, 94%; f. 1. TFA; 2. HCTU, Boc-Gly-OH, DiPEA, DCM, 100% g. Piperidine, DMF, 100%; h. N₃Phe, HCTU, DiPEA, DCM, 68%; i. hydrazine monohydrate, MeOH, 100%.

Synthesis of His containing inhibitor **14**

The synthesis of P1 His compound **14** is depicted in Scheme 5. The properly protected His Weinreb amide¹³ **54** was converted to the vinyl sulfone, using similar procedures as described above. Vinyl sulfone free amine **56** was reacted in an azide coupling reaction with N₃Phe-Leu-Leu-NHNH₂ to provide tetrapeptide vinyl sulfone **57**. Subsequent trityl removal by TFA with the help of TIPS as cation scavenger yielded compound **14**.



Scheme 5. Synthesis of compound **14.** Reagents and conditions: a. 1. LiAlH₄, THF. 2. diethyl((methylsulfonyl)-methyl)-phosphonate, NaH, THF, 65%; b. Et₂NH, MeCN, 64%; c. 1. N₃Phe-Leu-Leu-NHNH₂, tBuONO, HCl, DMF. 2. **56**, DiPEA, 53%; d. TFA, TIPS, DCM, 38%.

Biological evaluation

All newly synthesized compounds were evaluated for proteasome inhibition in Raji cell lysates (a human B-cell lymphoma cell line expressing constitutive and immunoproteasomes) and compared to LU-102 **3** by activity-based protein profiling (ABPP) (Figure 2). Cell lysates were incubated with inhibitors at four different concentrations (0.1, 1, 10 and 100 μM) for 1 h, followed by labelling of residual proteasome activity by the activity-based probe cocktail as described in chapter 3.⁷ For all compounds, a dramatic loss of potency against the $\beta 2$ subunits is found. Compounds **5** (P1: Lys(4-ene)) and compounds **8** (P1: Lys(4-yn)) both show some $\beta 2$ selectivity, although a more than 10 fold decrease in potency was observed. Incorporation of Lys(4-ene) or Lys(4-yn) at P3 or at P1 and P3 resulted in compounds with even lower potency (compounds **6**, **7**, **9**, **10**). The compounds with Dap(Gly) at P1 and/or P3 (**11**, **12**, **13**) were all very weak inhibitors, with almost no selectivity for $\beta 2$ over $\beta 5$. Finally, compounds **14** (P1: His) inhibited $\beta 2$ with similar potency as compounds **5** and **8** (complete inhibition at 10 μM), however, with poor selectivity over the $\beta 5$ subunits. Since all compounds showed much lower activity than LU-102 (**3**), it was anticipated that these compounds would also show poor activity in living cells. Indeed, compounds **8**, **9**, and **10** did not display any inhibitory activity in living Hela cells up to 100 μM (data not shown).

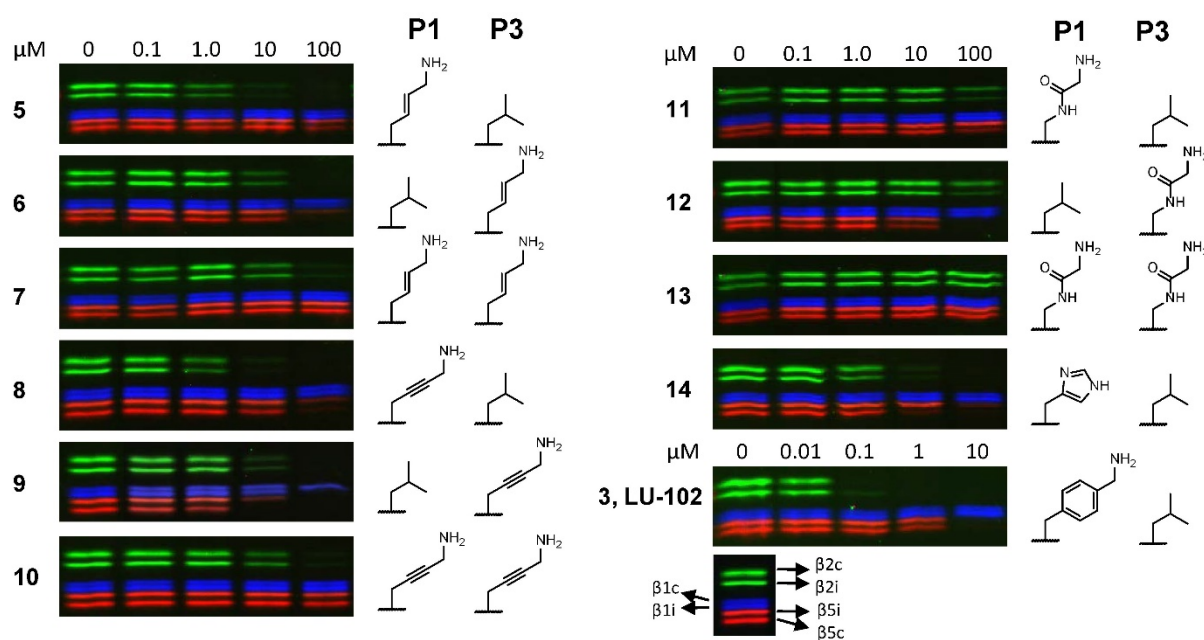


Figure 2. Inhibition profiles of compounds 5-14, compared to 3 (LU-102) in Raji lysates.

Discussion

All synthesized tetrapeptide vinyl sulfones showed reduced activity against $\beta 2$ compared to LU-102 (**3**). The low activity of these inhibitors could originate from several factors, such as lower basicity of the amine, lack of interactions due to loss of the aromatic ring or a lack of interactions between the side chain amine and the $\beta 2$ subunit due to a shorter side chain of the Lys analogues compared to the benzylamine side chain in LU-102.

The crystal structure of LU-102 in complex with yeast proteasome and superposition on mammalian constitutive proteasomes (murine and bovine) showed that the amino-group interacts with Asp53 in the S1 pocket of the $\beta 2$ subunit via hydrogen bonding.⁵ Due to absence of an acidic residue in the S1 pockets of $\beta 1$ and $\beta 5$, these interactions are the driving force for the $\beta 2$ selectivity of compounds equipped with P1 basic residues. So in order to maintain $\beta 2$ selectivity, compounds should have a strong interaction with Asp53. Compounds **5** (P1: Lys(4-ene)) and **8** (P1: Lys(4-yn)) show similar potency, indicating that the lower pK_a value of Lys(4-yn) compared to Lys(4-ene) does not result in additional loss of activity suggesting that both residues are able to interact with Asp53. In contrast, compound **6** shows a much lower activity, which might indicate that lower basicity of the amine (pK_a 8) is too low in order to establish a strong interaction with Asp53. However, the loss of activity of compound **6** could also be caused by unfavourable interactions between the side chain amide bond and the $\beta 2$ subunit or by a changed orientation of the side chain caused by the amide.

The S1 pocket of $\beta 2$ is spacious and therefore able to accommodate the P1 aromatic residue of LU-102. Absence of interactions between the much less sterically demanding Lys analogues

and the protein could be a reason for the lower potency of compounds **5** and **8**. Another important factor is the length of the side chains, which is one carbon atom shorter in case of the Lys analogues compared to the 4-aminomethyl-Phe residue of LU-102. This most likely results in a larger distance between Asp53 and the P1 amine of the inhibitors, causing a weaker interaction and thus lower potency of the compounds. In fact, in a previous study, Lys at P1 also showed a ten-fold lower potency compared to LU-102⁵, indicating that the low potency of compounds **5** and **8** is not a result of the lower basicity of the amine.

In case of His at P1 (compound **14**) the distance between Asp53 and the basic residue is even larger, most likely resulting in the absence of an interaction between the imidazole moiety and Asp53. However, compound **14** is still moderately active indicating that His at P1 might be stabilized by other interactions, similarly to the phenyl group of LU-102. Interestingly, compound **14** shows a 10-fold preference for β 5c over β 5i. This was unexpected, since β 5i prefers large (see chapter 5)¹⁴ and β 5c small (see chapter 7) residues at P1.¹⁵ This selectivity probably originates from a combination of Leu at P3 and His at P1. Leu at P3 is disfavoured by β 5i, see also chapter 4 in which all compounds with hydrophobic P1 residues and Leu at P3 showed selectivity for β 5c over β 5i.¹⁶ His at P1 might, due to its relatively small size, suffer less from unfavourable interactions with the relatively small S1 pocket of β 5c compared to the Lys analogues and 4-aminomethyl-Phe of LU-102.

The superposition of the crystal structure of LU-112 in complex with yeast proteasome on mammalian proteasome (murine and bovine) showed that the P3 amine group did not interact with an acidic residue of the proteasome. However, the P3 amine group is stabilized by several surrounding polar residues. These interactions can probably not be established with the shorter P3 residues of compound **6**, **7**, **9**, **10**, **12** and **13**. In addition, similarly to the S1 pocket, the S3 pocket is also spacious and the P3 phenyl moiety of LU-112 is stabilized by several van der Waals interactions.⁵ However, the P3 Leu moiety of LU-102 does not show any favourable interaction with the protein. Likely, compounds with Lys analogues at P3 do also not benefit from van der Waals interactions with the protein and do therefore not show increased potency.

Conclusion

In order to obtain β 2 selective inhibitors that are less charged at physiological pH, several Lys analogues with lower pK_a values were explored as basic residues in potential β 2 targeting inhibitors. A straightforward enantioselective synthesis of Lys(4-ene) and Lys(4-yn) was developed, which gives access to amino acid building blocks suitable for standard Fmoc chemistry. In addition, these amino acids could be converted to their corresponding vinyl sulfones and were incorporated as P1 and/or P3 residues in tetrapeptide vinyl sulfones.

Moreover, a Lys analogue equipped a peptide bond in the side chain (Dap(Gly)) was incorporated as P1 and/or P3 residues in potential proteasome inhibitors. Finally, His was explored as basic residue at P1. Evaluation by ABPP revealed that all compounds targeted $\beta 2$ with much lower potency than LU-102. The low activity of compounds with Lys(4-ene) and Lys(4-ene) at P1 and/or P3 is most likely not caused by the lower pK_a value of the amine group, but by the suboptimal distance between the side chain amine and Asp53 of $\beta 2$ and by the lack of van der Waals interactions.

Experimental

Synthetic procedures

General procedures

Acetonitrile (ACN), dichloromethane (DCM), N,N-dimethylformamide (DMF), methanol (MeOH), diisopropylethylamine (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, Iris Biotech) were used as received. Column chromatography was performed on Screening Devices b.v. Silica Gel, with a particle size of 40-63 μ m and pore diameter of 60 Å. 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 $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ (25 g/L) and $(\text{NH}_4)_4\text{Ce}(\text{SO}_4)_4\cdot 2\text{H}_2\text{O}$ (10 g/L) in 10% sulphuric acid, a solution of KMnO_4 (20 g/L) and K_2CO_3 (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 ^{13}C NMR spectra were recorded on a Bruker AV-300 (MHz), AV-400 (400 MHz) or AV-600 (600 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, CD_3OD or CDCl_3 as internal standard. High resolution mass spectra were recorded by direct injection (2 μ L of a 2 μ M 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). LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemini C_{18} 50 $\times$ 4.60 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI. The applied buffers were H_2O , ACN and 1.0% aq. TFA. Method: xx $\rightarrow$ xx% MeCN, 13.0 min (0 $\rightarrow$ 0.5 min: 10% MeCN; 0.5 $\rightarrow$ 8.5 min: gradient time; 8.5 $\rightarrow$ 10.5 min: 90% MeCN; 10.5 $\rightarrow$ 13.0 min: 10% MeCN). HPLC purification was performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5 μ m 250 $\times$ 10 mm column and a GX281 fraction collector. Enantiomeric excess (*ee*) was determined using chiral HPLC analysis (Daicell Chiralcel OD column (250 $\times$ 5.4 mm), hexane/isopropanol (99/1), flowrate: 1 mL/min, detection: UV254). All tested compounds are >95% pure on the basis of LC-MS and NMR.

General procedure for azide couplings.

Compounds **5-14** were prepared via azide coupling of peptide hydrazides and properly deprotected vinyl sulfone amines. The appropriate hydrazide was dissolved in DMF or 1:1 DMF:DCM (v/v) and cooled to -30°C. *t*BuONO (1.1 equiv.) and HCl (4M solution in 1,4-dioxane, 2.8 equiv.) were added, and the mixture was stirred for 3h at -30 °C after which TLC analysis (10% MeOH/DCM, v/v) showed complete consumption of the starting material. The vinyl sulfone as a free amine was added to the reaction mixture as a solution in DMF. DiPEA (5 equiv.) was added to the reaction mixture, and this mixture was allowed to warm to RT slowly overnight. The mixture was diluted with EtOAc and extracted with H_2O (3 $\times$). The organic layer was dried over MgSO_4 and purified by flash column chromatography (1-5% MeOH in DCM) and HPLC purification (if necessary).

General procedure for peptide couplings

Free acid (1.2 equiv.), HCTU (1.2 equiv.) and free amine (1 equiv.) are dissolved in DCM (0.1 M), followed by the addition of DiPEA (3.5 equiv or 4.5 equiv in case of 2-morpholinoacetic acid HCl). After stirring overnight (or alternatively 1-3 h, until completion), the reaction mixture is concentrated and re-dissolved in EtOAc, washed with 1 N HCl (2x), sat. NaHCO_3 (2x) and brine (in case of morpholino acetic acid coupling, no 1N HCl washings). The organic layer is dried over Na_2SO_4 , filtered and concentrated, followed by purification by column chromatography.

General procedure for Boc removal

Boc protected compounds are treated with TFA (0.1 M) for 30 minutes, followed by co-evaporation with toluene (2x).

General procedure for Fmoc removal

Fmoc protected compounds are treated dissolved in 20% piperidine in DMF and stirred until completion of the reactions (about 30 minutes), followed by concentration of the reaction mixture and purification by column chromatography.

N₃Phe-Leu-Leu-Lys(-4-ene)-VS (5)

This compound was obtained by the general protocol for azide coupling on a 60 μ mol scale. Purification by column chromatography (0 $\rightarrow$ 2% MeOH/DCM) provided the Boc protected compound, which was deprotected using the standard procedure for Boc removal, providing the title compound after purification by HPLC followed by lyophilization (6.1 mg, 8.5 μ mol, 14%). ¹H NMR (600 MHz, MeOD) δ 7.39 – 7.24 (m, 5H), 6.89 – 6.85 (m, 1H), 6.84 – 6.67 (m, 1H), 5.97 – 5.82 (m, 1H), 5.82 – 5.63 (m, 1H), 4.73 (dtd, J = 9.1, 5.2, 1.6 Hz, 1H), 4.42 – 4.30 (m, 2H), 4.30 – 4.20 (m, 1H), 3.60 – 3.55 (m, 2H), 3.27 (dd, J = 14.0, 4.9 Hz, 1H), 3.05 (dd, J = 14.1, 8.6 Hz, 1H), 3.03 (s, 3H), 2.58 (dddd, J = 10.7, 9.5, 5.4, 2.7 Hz, 1H), 2.51 – 2.37 (m, 1H), 1.79 – 1.53 (m, 6H), 1.08 – 0.89 (m, 12H). ¹³C NMR (151 MHz, MeOD) δ 174.89, 174.81, 174.48, 171.83, 146.86, 146.65, 137.83, 134.27, 134.14, 131.84, 131.67, 130.49, 130.44, 129.64, 129.60, 128.10, 128.07, 126.50, 126.33, 65.45, 53.97, 53.94, 53.45, 50.37, 42.75, 42.69, 42.18, 42.11, 41.55, 41.50, 38.64, 37.45, 25.98, 25.81, 23.45, 23.35, 22.00, 21.79. LC-MS (linear gradient 10 $\rightarrow$ 90% MeCN, 0.1% TFA, 13.0 min Rt (min): 6.15 (ESI-MS (m/z): 604.06 ($M+H^+$)). HRMS: calculated for C₂₉H₄₆N₇O₅S 604.32756 [$M+H^+$]; found 604.32751.

N₃Phe-Lys(-4-ene)-Leu-Leu-VS (6)

This compound was obtained by the general protocol for azide coupling on a 50 μ mol scale. Purification by column chromatography (0 $\rightarrow$ 2% MeOH/DCM) provided the Boc protected compound, which was deprotected using the standard procedure for Boc removal, providing the title compound after purification by HPLC followed by lyophilization (7.04 mg, 9.7 μ mol, 20%). Isolated with 15% *cis* isomer. Peaks reported correspond to *trans* isomer. ¹H NMR (600 MHz, MeOD) δ 7.35 – 7.19 (m, 5H), 6.80 (dd, J = 15.2, 5.3 Hz, 1H), 6.60 (dd, J = 15.2, 1.6 Hz, 1H), 5.83 – 5.72 (m, 1H), 5.72 – 5.54 (m, 1H), 4.61 (dtd, J = 10.3, 5.1, 1.6 Hz, 1H), 4.41 (t, J = 7.1 Hz, 1H), 4.34 (dd, J = 10.0, 5.2 Hz, 1H), 4.14 (dd, J = 8.6, 5.2 Hz, 1H), 3.49 (d, J = 6.5 Hz, 2H), 3.20 (dd, J = 13.9, 5.3 Hz, 1H), 3.02 – 2.95 (m, 1H), 2.98 (s, 3H), 2.52 (dd, J = 13.8, 7.4 Hz, 1H), 2.44 (q, J = 6.8 Hz, 1H), 1.77 – 1.41 (m, 6H), 1.04 – 0.86 (m, 12H). ¹³C NMR (151 MHz, MeOD) δ 174.51, 172.69, 171.62, 148.43, 137.79, 133.81, 130.85, 130.42, 129.63, 128.11, 126.17, 65.35, 54.12, 53.58, 53.54, 43.08, 42.75, 42.13, 41.80, 38.73, 36.06, 25.95, 25.82, 23.40, 23.38, 21.98, 21.91. LC-MS (linear gradient 10 $\rightarrow$ 90% MeCN, 0.1% TFA, 13.0 min Rt (min): 6.12 (ESI-MS (m/z): 604.13 ($M+H^+$)). HRMS: calculated for C₂₉H₄₆N₇O₅S 604.32756 [$M+H^+$]; found 604.32758.

N₃Phe-Lys-4-ene-Leu-Lys(-4-ene)-VS (7)

This compound was obtained by the general protocol for azide coupling on a 50 μ mol scale. Purification by column chromatography (0 $\rightarrow$ 2% MeOH/DCM) provided the Boc protected compound, which was deprotected using the standard procedure for Boc removal, providing the title compound after purification by HPLC followed by lyophilization (8.6 mg, 10.2 μ mol, 20%). ¹H NMR (600 MHz, MeOD) δ 7.39 – 7.33 (m, 2H), 7.33 – 7.24 (m, 3H), 6.87 (dd, J = 15.2, 5.1 Hz, 1H), 6.70 (dd, J = 15.2, 1.7 Hz, 1H), 5.97 – 5.79 (m, 2H), 5.77 – 5.64 (m, 2H), 4.71 (dtd, J = 9.0, 5.6, 1.7 Hz, 1H), 4.42 (dd, J = 8.0, 6.6 Hz, 1H), 4.37 – 4.30 (m, 1H), 4.23 – 4.15 (m, 1H), 3.56 (dd, J = 19.1, 6.6 Hz, 4H), 3.25 (dd, J = 14.0, 5.2 Hz, 1H), 3.04 (s, 3H), 3.07 – 3.01 (m, 1H), 2.63 – 2.54 (m, 2H), 2.54 – 2.42 (m, 2H), 1.81 – 1.67 (m, 2H), 1.58 (ddd, J = 13.8, 8.9, 4.6 Hz, 1H), 1.04 (d, J = 6.5 Hz, 3H), 0.99 (d, J = 6.5 Hz, 3H). ¹³C NMR (151 MHz, MeOD) δ 174.57, 173.21, 171.82, 146.79, 137.80, 134.17, 133.77, 131.73, 130.46, 130.42, 129.64, 128.12, 126.45, 126.27, 65.30, 54.53, 53.67, 50.53, 42.70, 42.18, 42.12, 41.59, 40.40, 38.73, 37.29, 35.81,

25.95, 23.46, 21.72. LC-MS (linear gradient 10→90% MeCN, 0.1% TFA, 13.0 min Rt (min): 4.77/4.84 different salt-forms (ESI-MS (m/z): 617.07 ($M+H^+$)). HRMS: calculated for $C_{29}H_{45}N_8O_5S$ 617.32281 [$M+H^+$]; found 617.32275.

N₃Phe-Leu-Leu-Lys(-4-yl)-VS (8)

This compound was obtained by the general protocol for azide coupling on a 100 μ mol scale. Purification by column chromatography (0→1.5% MeOH/DCM) provided the Boc protected compound, which was deprotected using the standard procedure for Boc removal, providing the title compound (20.4 mg, 28.5 μ mol, 92 %) as a white powder after lyophilisation. 1H NMR (400 MHz, MeOD) δ 7.35 – 7.20 (m, 5H), 6.87 (dd, J = 15.3, 5.0 Hz, 1H), 6.73 (dd, J = 15.2, 1.5 Hz, 1H), 4.81 – 4.71 (m, 1H), 4.35 (dd, J = 10.1, 4.5 Hz, 2H), 4.19 (tt, J = 8.6, 4.9 Hz, 1H), 3.22 (dd, J = 14.1, 4.8 Hz, 1H), 3.04 – 2.93 (m, 4H), 2.77 – 2.56 (m, 2H), 1.81 – 1.47 (m, 6H), 1.03 – 0.80 (m, 12H). ^{13}C NMR (101 MHz, MeOD) δ 174.81, 174.49, 171.86, 145.39, 137.79, 132.53, 130.43, 129.61, 128.07, 84.22, 75.48, 65.47, 53.80, 53.47, 49.96, 42.66, 41.53, 41.41, 38.62, 30.37, 25.95, 25.79, 24.39, 23.47, 23.35, 21.95, 21.73. LC-MS (linear gradient 10→90% MeCN, 0.1% TFA, 13.0 min Rt (min): 6.84 (ESI-MS (m/z): 602.7 ($M+H^+$)). HRMS: calculated for $C_{29}H_{44}N_7O_5S$, 602.31191 [$M+H^+$]; found 602.31195.

N₃Phe-Lys-4(-yl)-Leu-Leu-VS (9)

This compound was obtained by the general protocol for azide coupling on a 90 μ mol scale. Purification by column chromatography (0→1.5% MeOH/DCM) provided the Boc protected compound, which was deprotected using the standard procedure for Boc removal, providing title compound (34.2 mg, 48 μ mol, 53%) as a white powder after lyophilisation. Isolated with 10% *cis* isomer. Peaks reported correspond to *trans* isomer. 1H NMR (400 MHz, MeOD) δ 7.40 – 7.15 (m, 5H), 6.79 (dd, J = 15.2, 5.2 Hz, 1H), 6.60 (dd, J = 15.2, 1.4 Hz, 1H), 4.68 – 4.58 (m, 1H), 4.52 (t, J = 7.1 Hz, 1H), 4.38 (dd, J = 9.6, 5.3 Hz, 1H), 4.15 (dd, J = 8.6, 5.2 Hz, 1H), 3.74 (s, 2H), 3.20 (dd, J = 13.9, 5.3 Hz, 1H), 3.03 – 2.91 (m, 4H), 2.74 – 2.46 (m, 2H), 1.81 – 1.42 (m, 6H), 1.06 – 0.85 (m, 12H). ^{13}C NMR (101 MHz, MeOD) δ 174.39, 171.70, 148.37, 137.72, 130.84, 130.38, 129.61, 128.09, 83.97, 75.47, 65.28, 53.40, 43.06, 42.76, 41.82, 38.78, 30.49, 25.90, 23.35, 22.86, 21.95. LC-MS (linear gradient 10→90% MeCN, 0.1% TFA, 13.0 min Rt (min): 6.78 (ESI-MS (m/z): 602.7 ($M+H^+$)). HRMS: calculated for $C_{29}H_{44}N_7O_5S$, 602.31191 [$M+H^+$]; found 602.31171.

N₃Phe-Lys(-4-yl)-Leu-Lys(-4-yl)-VS (10)

This compound was obtained by the general protocol for azide coupling on a 90 μ mol scale. Purification by column chromatography (0→1.5% MeOH/DCM) provided the Boc protected compound, which was deprotected using the standard procedure for Boc removal, providing the title compound (29.2 mg, 34.7 μ mol, 79%) as a white powder after lyophilisation. Isolated with 10% *cis* isomer. Peaks reported correspond to *trans* isomer. 1H NMR (400 MHz, MeOD) δ 7.36 – 7.20 (m, 5H), 6.88 (dd, J = 15.2, 5.1 Hz, 1H), 6.74 (dd, J = 15.2, 1.5 Hz, 1H), 4.82 – 4.70 (m, 1H), 4.53 (t, J = 7.2 Hz, 1H), 4.37 (dd, J = 10.3, 4.9 Hz, 1H), 4.16 (dd, J = 8.6, 5.3 Hz, 1H), 3.81 – 3.69 (m, 4H), 3.21 (dd, J = 13.9, 5.2 Hz, 1H), 3.05 – 2.94 (m, 4H), 2.79 – 2.56 (m, 2H), 1.81 – 1.63 (m, 2H), 1.63 – 1.51 (m, 1H), 1.02 – 0.88 (m, 6H). ^{13}C NMR (101 MHz, MeOD) δ 174.59, 172.11, 145.33, 137.74, 132.57, 130.40, 129.63, 128.11, 84.21, 75.55, 65.28, 53.68, 53.61, 50.09, 49.64, 42.66, 41.64, 38.79, 30.35, 25.90, 24.30, 23.47, 22.64, 21.70. LC-MS (linear gradient 10→90% MeCN, 0.1% TFA, 13.0 min Rt (min): 5.51 (ESI-MS (m/z): 613.7 ($M+H^+$)). HRMS: calculated for $C_{29}H_{41}N_8O_5S$ 614.29934 [$M+H^+$]; found 614.29935.

N₃Phe-Leu-Leu-Dap(Gly)-VS (11)

This compound was obtained by the general protocol for azide coupling on a 50 μ mol scale. Purification by column chromatography (0→4% MeOH/DCM) provided the Boc-protected product, which was deprotected using the standard procedure for Boc removal. Purification by HPLC (30-50% MeCN, 0.1 % TFA, 10 min gradient) provided the title compound (3.16 mg, 8.6%) as a white powder after lyophilisation. 1H NMR (600 MHz, MeOD) δ 7.34 – 7.22 (m, 5H), 6.83 (dd, J = 15.3, 5.1 Hz, 1H), 6.72 (dd, J = 15.3, 1.6 Hz, 1H), 4.80 (dtd, J = 8.2, 5.3, 1.4 Hz, 1H), 4.36 (dd, J = 9.4, 5.3 Hz, 1H), 4.27 (dd, J = 10.2, 4.8 Hz, 1H), 4.21 (dd, J = 8.4, 5.0 Hz, 1H), 3.66 (d, J = 3.5 Hz,

2H), 3.56 (dd, $J = 13.7, 5.4$ Hz, 1H), 3.41 (dd, $J = 13.8, 8.6$ Hz, 1H), 3.23 (dd, $J = 14.0, 5.0$ Hz, 1H), 3.04 – 2.98 (m, 4H), 1.75 – 1.64 (m, 2H), 1.61 – 1.52 (m, 4H), 1.02 – 0.88 (m, 12H). ^{13}C NMR (151 MHz, MeOD) δ 174.96, 174.74, 171.89, 168.19, 144.45, 137.81, 132.78, 130.48, 129.64, 128.11, 65.58, 53.81, 53.66, 51.12, 42.88, 42.61, 41.67, 41.59, 41.32, 38.65, 25.98, 25.80, 23.47, 23.37, 21.97, 21.72. LC-MS (linear gradient 10 $\rightarrow$ 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 5.97 (ESI-MS (m/z): 621.33 ($M+H^+$)). HRMS: calculated for $\text{C}_{28}\text{H}_{45}\text{N}_8\text{O}_6\text{S}$ 621.31773 [$M+H$] $^+$; found 621.31744

N₃Phe-Dap(Gly)-Leu-Leu-VS (12)

This compound was obtained by the general protocol for azide coupling on a 50 μmol scale. Purification by column chromatography (0 $\rightarrow$ 4% MeOH/DCM) provided the Boc-protected product, which was deprotected using the standard procedure for Boc removal. Purification by HPLC (30-50% MeCN, 0.1 % TFA, 10 min gradient) provided the title compound (7.71 mg, 21%) as a white powder after lyophilisation. Isolated with 10% *cis* isomer. Peaks reported correspond to *trans* isomer. ^1H NMR (600 MHz, MeOD) δ 7.33 – 7.23 (m, 5H), 6.83 (dd, $J = 15.2, 5.4$ Hz, 1H), 6.64 (dd, $J = 15.2, 1.5$ Hz, 1H), 4.64 (ddt, $J = 10.3, 5.2, 2.6$ Hz, 1H), 4.53 (t, $J = 6.1$ Hz, 1H), 4.37 (t, $J = 7.6$ Hz, 1H), 4.19 – 4.14 (m, 1H), 3.67 – 3.64 (m, 2H), 3.63 – 3.57 (m, 1H), 3.53 (dd, $J = 13.9, 5.6$ Hz, 1H), 3.23 (dd, $J = 13.9, 5.2$ Hz, 1H), 3.00 (d, $J = 8.9$ Hz, 1H), 2.99 (s, 3H), 1.75 – 1.67 (m, 2H), 1.67 – 1.60 (m, 3H), 1.48 (ddd, $J = 13.9, 9.0, 5.2$ Hz, 1H), 1.00 (d, $J = 6.5$ Hz, 3H), 0.97 (d, $J = 6.6$ Hz, 3H), 0.96 – 0.92 (m, 6H). ^{13}C NMR (151 MHz, MeOD) δ 174.48, 171.94, 171.61, 168.25, 148.38, 137.82, 130.80, 130.41, 129.65, 129.63, 128.13, 65.48, 54.30, 54.19, 53.59, 53.20, 43.12, 42.72, 42.06, 41.75, 41.53, 38.96, 25.93, 25.88, 23.52, 23.34, 21.98, 21.74. LC-MS (linear gradient 10 $\rightarrow$ 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 5.86 (ESI-MS (m/z): 621.27 ($M+H^+$)). HRMS: calculated for $\text{C}_{28}\text{H}_{45}\text{N}_8\text{O}_6\text{S}$ 621.31773 [$M+H$] $^+$; found 621.31757

N₃Phe-Dap(Gly)-Leu-Dap(Gly)-VS (13)

This compound was obtained by the general protocol for azide coupling on a 50 μmol scale. Purification by column chromatography (0 $\rightarrow$ 4% MeOH/DCM) provided the Boc-protected product, which was deprotected using the standard procedure for Boc removal. Purification by HPLC (10-50% MeCN, 0.1 % TFA, 10 min gradient) provided the title compound (2.93 mg, 6.7%) as a white powder after lyophilisation. ^1H NMR (600 MHz, MeOD) δ 7.36 – 7.22 (m, 5H), 6.84 (dd, $J = 15.3, 5.0$ Hz, 1H), 6.72 (dd, $J = 15.3, 1.6$ Hz, 1H), 4.90 – 4.83 (m, 1H), 4.48 (t, $J = 6.8$ Hz, 1H), 4.30 (dd, $J = 10.6, 4.6$ Hz, 1H), 4.17 (dd, $J = 8.4, 5.5$ Hz, 1H), 3.79 – 3.67 (m, 5H), 3.58 (dd, $J = 13.7, 5.6$ Hz, 1H), 3.42 (dd, $J = 7.5, 4.8$ Hz, 1H), 3.39 (dd, $J = 7.5, 4.8$ Hz, 1H), 3.22 (dd, $J = 13.9, 5.5$ Hz, 1H), 3.04 – 2.97 (m, 4H), 1.76 – 1.63 (m, 2H), 1.59 (ddd, $J = 13.9, 9.4, 4.6$ Hz, 1H), 1.00 (d, $J = 6.5$ Hz, 3H), 0.95 (d, $J = 6.5$ Hz, 3H). ^{13}C NMR (151 MHz, MeOD) δ 174.65, 171.83, 168.44, 168.23, 144.51, 137.73, 132.70, 130.45, 129.64, 128.15, 68.14, 65.38, 54.38, 54.25, 53.72, 50.78, 42.84, 42.58, 41.77, 41.68, 41.54, 41.49, 38.89, 25.92, 23.51, 21.63. LC-MS (linear gradient 10 $\rightarrow$ 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 4.66 (ESI-MS (m/z): 651.27 ($M+H^+$)). HRMS: calculated for $\text{C}_{28}\text{H}_{45}\text{N}_8\text{O}_6\text{S}$ 651.30314 [$M+H$] $^+$; found 651.30286

N₃Phe-Leu-Leu-His-VS (14)

To a solution of N₃Phe-Leu-Leu-His(Trt)-VS **57** (45 mg, 53 μmol , 1 equiv.) in DCM (3 mL) were added TFA (30 μL) and triisopropylsilane (TIPS) (75 μL). After 30 min, TLC showed no formation of product, therefore another 30 μL of TFA was added. After 30 min, still no product formation was observed, therefore TFA (1 mL) and TIPS (75 μL) were added. After stirring for 1 h, TLC analysis revealed completion of the reaction and the reaction mixture was diluted with toluene and concentrated. Purification by column chromatography (1-8% MeOH/DCM) provided the product as a white powder after lyophilisation (28 mg, 38%). Isolated with 7% *cis* isomer. Peaks reported correspond to *trans* isomer. ^1H NMR (600 MHz, MeOD) δ 8.08 (s, 1H), 7.35 – 7.19 (m, 5H), 7.10 (s, 1H), 6.84 (dd, $J = 15.2, 5.1$ Hz, 1H), 6.63 (dd, $J = 15.2, 1.5$ Hz, 1H), 4.90 – 4.85 (m, 1H), 4.41 – 4.34 (m, 1H), 4.28 (dd, $J = 9.8, 5.1$ Hz, 1H), 4.18 (dd, $J = 8.5, 5.0$ Hz, 1H), 3.21 (dd, $J = 14.0, 4.9$ Hz, 1H), 3.09 – 3.03 (m, 1H), 3.00 (dd, $J = 14.3, 8.3$ Hz, 2H), 2.96 (s, 3H), 1.70 – 1.47 (m, 6H), 0.97 (d, $J = 6.4$ Hz, 3H), 0.95 – 0.89 (m, 9H). ^{13}C NMR (151 MHz, MeOD) δ 174.66, 174.39, 171.85, 146.26, 137.82, 135.94, 133.13, 132.14, 130.44, 129.60, 128.05, 118.46, 65.51, 53.73,

53.48, 50.89, 42.71, 41.56, 41.43, 38.62, 31.05, 25.91, 25.79, 23.47, 23.40, 21.90, 21.89. LC-MS (linear gradient 10→90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 6.22 (ESI-MS (m/z): 615.20 ($M+H^+$)). HRMS: calculated for $C_{29}H_{43}N_8O_5S$ 615.30716 [$M+H$] $^+$; found 615.30719.

4-hydroxybut-2-yn-1-yl 4-methylbenzenesulfonate (16)

2-butyne-1,4-diol **15** (68.87 g, 800 mmol, 1 equiv.) was dissolved in DCM (2000 mL) and pyridine (129 mL, 1.6 mol), followed by the portion wise addition of 4-toluenesulfonyl chloride (76.2 g, 400 mmol, 0.5 equiv.) over 15 min. Reaction completion was confirmed by TLC (50% EtOAc/pent) after 2 h. The reaction mixture was washed with 1M HCl (3x), brine (3x) and dried over Na_2SO_4 , filtered and concentrated. Purification by column chromatography yielded the title compound (60.7 g, 253 mmol, 63%). 1H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 8.2 Hz, 2H), 7.34 (d, J = 8.1 Hz, 2H), 4.69 (s, 2H), 4.12 (s, 2H), 3.12 (s, 1H), 2.41 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 145.24, 132.28, 129.72, 127.75, 87.73, 76.72, 57.95, 50.05, 21.31.

1-(tert-butoxycarbonylamino)-4-hydroxy-2-butyne (17)

Alcohol **16** (58.29 g, 243 mmol, 1 equiv.) was dissolved in ammonium hydroxide (450 mL, 25% NH_3 in H_2O), resulting in immediate formation of a white precipitate. TLC (50% EtOAc/pent) confirmed reaction completion after 1h. The ammonium hydroxide was removed *in vacuo* and the mixture co-evaporated with toluene (2x). The resulting solid was dissolved in THF (950 mL) and di-tert-butyl dicarbonate (63.53 g, 291 mmol, 1.2 equiv.) was added and the solution was cooled to 0°C. Triethylamine (40.6 mL, 291 mmol, 1.2 equiv.) was added slowly over 20 min and after stirring overnight, the reaction mixture was concentrated, redissolved in DCM (500 mL) and washed with water (3x). The aqueous layer was back extracted twice with DCM and the combined organic layers were washed with brine (1x), dried over Na_2SO_4 , filtered and concentrated. Purification by column chromatography (10→25 % EtOAc/n-Pentane) yielded the title compound (19.04 g, 102.8 mmol, 42%). 1H NMR (400 MHz, $CDCl_3$) δ 5.25 (s, 1H), 4.26 (s, 2H), 3.95 (s, 2H), 3.71 (s, 1H), 1.45 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.76, 81.53, 80.12, 50.58, 30.59, 28.35.

1-(tert-butoxycarbonylamino)-4-bromo-2-butyne (18)

To a solution of alcohol **17** (9.26 g, 50 mmol, 1 equiv.) in DCM (79 mL) at 0°C, triphenylphosphine (19.67 g, 75 mmol, 1.5 equiv.) was added, followed by portion wise addition of tetrabromomethane (3.90 g, 11.8 mmol, 1.5 equiv.). After stirring for 2 h, the reaction mixture was concentrated and purification by column chromatography (1→5% EtOAc/pent) yielded the title compound (8.50 g, 34.2 mmol, 68%). 1H NMR (400 MHz, $CDCl_3$) δ 5.00 (s, 1H), 3.99 (s, 2H), 3.92 (s, 2H), 1.46 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.31, 83.32, 80.02, 30.67, 28.35, 14.48.

tert-butyl (E)-(4-hydroxybut-2-en-1-yl)carbamate (19)

Alcohol **17** (7.9 g, 42.7 mmol) was dissolved in THF (30 mL) and added drop wise over 15 minutes to a solution of $LiAlH_4$ (1.95 g, 51.2 mmol, 1.2 equiv.) in THF (400 mL) at 0°C. After completion of addition, the solution was heated to reflux and stirred for 2 h, after which TLC analysis (50% EtOAc/pent) confirmed completion of the reaction. The reaction was quenched with 3M aq. KOH solution until no further gas evolution was observed, diluted with EtOAc (100 mL), washed with 1M HCl (3x), $NaHCO_3$ (3x), brine (1x) and dried over Na_2SO_4 , filtered and concentrated. Purification by column chromatography (10→30 % EtOAc/pent) yielded the title compound as pure *E*-isomer (3.00 g, 16.05 mmol, 38 %). 1H NMR (400 MHz, $CDCl_3$) δ 5.72 (dt, 2H), 4.89 (s, 1H), 4.09 (dd, J = 4.9, 1.3 Hz, 2H), 3.70 (d, J = 5.1 Hz, 2H), 2.93 (s, 1H), 1.42 (tt, J = 15.5, 5.2 Hz, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.03, 130.87, 128.04, 79.51, 62.65, 41.99, 28.44.

1-(tert-butoxycarbonylamino)-4-bromo-2-butene (20)

Alcohol **19** (3.00 g, 16.1 mmol, 1 equiv.) was dissolved in dry DCM (160 mL). Triphenylphosphine (6.31 g, 24.08 mmol, 1.5 equiv.) was added and the solution cooled to 0°C, followed by slow and portion wise addition of the tetrabromomethane (7.99 g, 24.1 mmol, 1.5 equiv.). After stirring for 1 h, the reaction mixture was concentrated

and purification by column chromatography (0→10% EtOAc/pent) yielded the title compound (2.32 g, 9.28 mmol, 58%). ¹H NMR (400 MHz, CDCl₃) δ 6.02 – 5.65 (m, 2H), 4.66 (s, 1H), 3.97 (d, *J* = 6.6 Hz, 2H), 3.79 (s, 2H), 1.47 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 132.22, 127.71, 41.72, 32.15, 28.51.

***tert*-butyl (S,E)-6-((*tert*-butoxycarbonyl)amino)-2-((diphenylmethylene)amino)hex-4-enoate (22)**

Bromide **20** (2.25 g, 8.99 mmol, 1 equiv.), N-(diphenylmethylene)glycine *tert*-butyl ester (**36**) (2.65 g, 8.99 mmol, 1 equiv.) and the CPTC (0.046 g, 0.045 mmol, 0.005 equiv.) were dissolved in toluene/chloroform (31.5 mL, 7/3 v/v) and cooled to 0°C. A 50% (w/w) aqueous KOH solution which had been cooled to 4°C was added drop wise (13.5 mL). The reaction was stirred over two nights at 4°C and the reaction progression was followed by TLC (15% EtOAc/pent). The solution was then diluted with EtOAc, washed with water (1x), brine (1x), dried over Na₂SO₄, filtered and concentrated. Purification by column chromatography (0→10% EtOAc/pent) yielded the title compound as a white solid (2.98 g, 6.44 mmol, 84%, *ee* 79.3%). ¹H NMR (400 MHz, CDCl₃) δ 7.67 – 7.29 (m, 8H), 7.16 (dd, *J* = 7.3, 2.4 Hz, 2H), 5.60 – 5.42 (m, 2H), 4.46 (s, 1H), 3.98 (t, *J* = 6.4 Hz, 1H), 3.65 (s, 2H), 2.60 (t, *J* = 5.4 Hz, 2H), 1.44 (s, 9H), 1.41 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 155.75, 128.56, 128.15, 128.00, 81.23, 66.03, 42.50, 36.59, 28.50, 28.20.

***tert*-butyl (S)-6-((*tert*-butoxycarbonyl)amino)-2-((diphenylmethylene)amino)hex-4-ynoate (23)**

Bromide **18** (1.90 g, 7.67 mmol, 1 equiv.), N-(diphenylmethylene)glycine *tert*-butyl ester **21** (2.27 g, 7.67 mmol, 1 equiv.) and the CPTC (0.039 g, 0.038 mmol, 0.005 equiv.) were dissolved in toluene/chloroform (27 mL, 7/3 v/v) and cooled to 0°C. A 50% (w/w) aqueous KOH solution which had been cooled to 4°C was added drop wise (11.5 mL). The reaction was stirred over two nights at 4°C and the reaction progression was followed by TLC (5% EtOAc/pent). The solution was then diluted with EtOAc, washed with water (1x), brine (1x), dried over Na₂SO₄, filtered and concentrated. Purification by column chromatography (1→10% EtOAc/pent) yielded the title compound as a white solid (2.98 g, 6.44 mmol, 84%, *ee* 80.2 %). ¹H NMR (400 MHz, CDCl₃) δ 7.75 – 7.19 (m, 10H), 4.74 (s, 1H), 4.16 (dt, *J* = 9.0, 4.5 Hz, 1H), 3.92 – 3.77 (m, 2H), 2.90 – 2.66 (m, 2H), 1.46 (s, 9H), 1.42 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 171.30, 169.67, 155.19, 139.58, 136.25, 130.34, 128.92, 128.66, 128.38, 128.18, 128.01, 81.47, 80.28, 79.56, 77.87, 64.99, 30.68, 28.32, 28.01, 23.53.

H-Lys(-4-ene)(Boc)-OtBu (24)

Compound **22** (2.74 g, 5.90 mmol, 1 equiv.) was dissolved in THF (33 mL) and cooled to 0°C. A citric acid solution (38 mL, 15 % w/w in water) was added and precipitation of a white solid was observed. The ice bath was removed, and the reaction mixture was allowed to warm to rt and was stirred for 2 hours during which time the solution turned clear again. The reaction was followed by TLC (10% EtOAc/pent) and quenched with a sat. K₂CO₃ solution until no further gas evolution was observed (approx. 20 mL). The reaction mixture was diluted with EtOAc, washed with water (2x), brine (1x). The organic layer was dried over Na₂SO₄, filtered, concentrated and purified by column chromatography (0→5 % MeOH/DCM) yielding the title compound (yield given over two steps, see synthesis of **26**). ¹H NMR (400 MHz, CDCl₃) δ 5.71 – 5.40 (m, 2H), 4.75 (s, 1H), 3.70 (t, *J* = 5.5 Hz, 2H), 3.40 (t, *J* = 6.0 Hz, 1H), 2.58 – 2.18 (m, 2H), 1.68 (s, 2H), 1.46 (s, 9H), 1.44 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 174.47, 155.76, 130.63, 127.14, 81.18, 79.27, 54.49, 42.34, 37.71, 28.44, 28.12.

H-Lys(-4-yn)(Boc)-OtBu (25)

Compound **23** (5.02 g, 10.9 mmol, 1 equiv.) was dissolved in THF (59 mL) and cooled to 0°C. A citric acid solution (69 mL, 15 % w/w in water) was added and precipitation of a white solid was observed. The ice bath was removed, and the reaction mixture was allowed to warm to rt and was stirred for 2 h during which time the solution turned clear again. The reaction was followed by TLC (5 % MeOH/DCM) and quenched with a sat. K₂CO₃ solution until no further gas evolution was observed (approx. 20 mL). The reaction mixture was diluted with EtOAc, washed with water (2x), brine (1x). The organic layer was dried over Na₂SO₄, filtered, concentrated and purified by column chromatography (0→5 % MeOH/DCM) yielding the title compound (2.94 g, 9.87 mmol, 91 %). ¹H NMR

(400 MHz, CDCl_3) δ 4.92 (s, 1H), 3.89 (d, J = 5.3 Hz, 2H), 3.48 (t, J = 5.5 Hz, 1H), 2.65 – 2.50 (m, 2H), 1.75 (s, 2H), 1.47 (s, 9H), 1.44 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 173.19, 155.32, 81.53, 79.75, 79.09, 78.75, 53.76, 30.67, 28.39, 28.04, 25.40.

Fmoc-Lys(-4-ene)(Boc)-OtBu (26)

H-Lys-4-ene(Boc)-OtBu **24** (5.90 mmol, 1 equiv.) was dissolved in dry DCM (60 mL) and FmocOSu (2.38 g, 7.08 mmol, 1.2 equiv.) was added followed by the drop wise addition of DiPEA (1.2 mL, 7.08 mmol, 1.2 equiv.). After stirring overnight, the reaction mixture was diluted with EtOAc, washed with 1M HCl (1x), sat. aq. NaHCO_3 (2x) and brine (1x). The organic layer was dried over Na_2SO_4 , filtered and concentrated. Purification by column chromatography (0 $\rightarrow$ 20% EtOAc/pent) yielded the title compound as a white powder (2.56 g, 4.91 mmol, 83% over two steps). ^1H NMR (400 MHz, CDCl_3) δ 7.76 (d, J = 7.5 Hz, 2H), 7.60 (d, J = 7.5 Hz, 2H), 7.40 (t, J = 7.5 Hz, 2H), 7.32 (t, J = 7.4 Hz, 2H), 5.63 – 5.44 (m, 2H), 5.39 (d, J = 8.1 Hz, 1H), 4.56 (s, 1H), 4.44 – 4.35 (m, 2H), 4.22 (t, J = 7.0 Hz, 1H), 3.69 (s, 2H), 2.64 – 2.40 (m, 2H), 1.47 (s, 9H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 170.83, 155.77, 143.92, 141.42, 131.23, 127.82, 127.18, 125.99, 125.22, 120.10, 82.48, 79.48, 67.06, 53.94, 47.30, 42.36, 35.59, 28.51, 28.18. $[\alpha]_D^{20}$ = 17.6 (C=1, CHCl_3). LC-MS (linear gradient 10 $\rightarrow$ 90% MeCN, 0.1% TFA, 13.0 min) Rt (min): 10.95 (ESI-MS (m/z): 523.80 ($\text{M}+\text{H}^+$)). HRMS: calcd. for $\text{C}_{30}\text{H}_{38}\text{N}_2\text{O}_6$, 523.27579 [$\text{M}+\text{H}^+$]; found 523.27997

Fmoc-Lys(-4-yn)(Boc)-OtBu (27)

H-Lys-4-yl(Boc)-OtBu **25** (2.89 g, 9.68 mmol, 1 equiv.) was dissolved in dry DCM (97 mL) and FmocOSu (3.92 g, 11.6 mmol, 1.2 equiv.) was added followed by the drop wise addition of DiPEA (2.0 mL, 11.6 mmol, 1.2 equiv.). After stirring overnight, the reaction mixture was diluted with EtOAc, washed with 1M HCl (1x), sat. aq. NaHCO_3 (2x) and brine (1x). The organic layer was dried over Na_2SO_4 , filtered and concentrated. Purification by column chromatography (0 $\rightarrow$ 20 % EtOAc, n-Pentane) yielded the title compound as a white foam (4.94 g, 9.48 mmol, 98 %). ^1H NMR (400 MHz, CDCl_3) δ 7.80 – 7.74 (m, 2H), 7.62 (d, J = 7.4 Hz, 2H), 7.40 (t, J = 7.5, 2H), 7.32 (tt, J = 7.4, 1.4 Hz, 2H), 5.67 (d, J = 8.0 Hz, 1H), 4.68 (s, 1H), 4.44 – 4.34 (m, 3H), 4.24 (t, J = 7.2 Hz, 1H), 3.88 (s, 2H), 2.73 (dt, J = 4.9, 2.3 Hz, 2H), 1.49 (s, 9H), 1.44 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 169.57, 155.74, 143.97, 141.39, 127.83, 127.19, 125.27, 120.10, 82.84, 79.51, 77.84, 77.36, 67.25, 53.00, 47.24, 30.76, 28.46, 28.08, 23.41. $[\alpha]_D^{20}$ = 23.0 (C=1, CHCl_3). LC-MS (linear gradient 10 $\rightarrow$ 90% MeCN, 0.1% TFA, 13.0 min): Rt (min): 9.22 (ESI-MS (m/z): 520.87 ($\text{M}+\text{H}^+$)). HRMS: calcd. for $\text{C}_{30}\text{H}_{37}\text{N}_2\text{O}_6$ 521.26461 [$\text{M}+\text{H}^+$]; found 521.26459.

Fmoc-Lys(-4-ene)(Boc)-OH (28)

Fmoc-L-Lys-4-ene(Boc)-OtBu **26** (2.48 g, 4.76 mmol, 1 equiv.) was dissolved in 100% TFA (47.6 mL) and stirred for 2 hours, after which TLC analysis (10% EtOAc/pent) showed completion of the reaction, in combination with TLC-MS and HPLC-MS to ensure completion of ester hydrolysis and not only removal of the Boc group. The reaction mixture was concentrated and co-evaporated with toluene (3x). The residue was redissolved in MeCN (48 mL) and Boc_2O (1.25 g, 5.71 mmol, 1.2 equiv.) and DiPEA (1.15 mL, 6.91 mmol, 1.45 equiv.) were added. A white precipitate formed immediately and gas evolution was observed. After stirring overnight, the reaction mixture was concentrated and the residue was dissolved in EtOAc, washed with 0.1 M HCl (2x), water (2x) and brine (2x), dried over Na_2SO_4 , filtered and concentrated. Purification by column chromatography (0 $\rightarrow$ 2% MeOH/DCM) yielded the title compound (1.82 g, 3.90 mmol, 82%). ^1H NMR (400 MHz, CDCl_3) δ 7.74 (t, J = 7.9 Hz, 2H), 7.56 (d, J = 7.9 Hz, 2H), 7.37 (t, J = 7.7 Hz, 2H), 7.31 (d, J = 8.2 Hz, 2H), 6.94 (s, 1H), 6.74 (d, J = 8.6 Hz, 1H), 5.55 (dt, J = 15.8, 9.2 Hz, 2H), 4.60 (s, 1H), 4.43 (q, J = 12.0, 11.4 Hz, 2H), 4.23 (t, 1H), 4.13 (t, J = 6.6 Hz, 1H), 3.63 (d, J = 48.0 Hz, 2H), 2.79 – 2.46 (m, 2H), 1.52 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 185.56, 155.73, 144.25, 143.86, 141.33, 129.97, 127.65, 119.96, 81.68, 66.59, 53.08, 47.34, 42.76, 34.77, 29.82, 28.49.

Fmoc-Lys(-4-yn)(Boc)-OH (29)

Fmoc-Lys-4-yl(Boc)-OtBu **27** (4.93 g, 9.48 mmol, 1 equiv.) was dissolved in 100% TFA (95 mL) and stirred for 2 h, after which TLC analysis (10 % EtOAc/pent) showed completion of the reaction, in combination with TLC-MS and

HPLC-MS to ensure completion of ester hydrolysis and not only removal of the Boc group. The reaction mixture was concentrated and co-evaporated with toluene (3x). The residue was redissolved in MeCN (95 mL) and Boc_2O (2.48 g, 11.38 mmol, 1.2 equiv.) and DiPEA (2 mL, 11.38 mmol, 1.2 equiv.) were added. A white precipitate formed immediately and gas evolution was observed, and the pH was adjusted until basic by the addition of 1 mL DiPEA. After stirring overnight, the reaction mixture was concentrated and the residue was dissolved in EtOAc, washed with 0.1 M HCl (2x), water (2x) and brine (2x), dried over Na_2SO_4 , filtered and concentrated. Purification by column chromatography (0→1% MeOH/DCM) yielded the title compound (2.80 g, 6.02 mmol, 63%). ^1H NMR (400 MHz, CDCl_3) δ 7.73 (d, J = 7.5 Hz, 2H), 7.57 (dd, J = 17.7, 7.4 Hz, 2H), 7.48 (d, J = 4.1 Hz, 1H), 7.36 (d, J = 7.5 Hz, 2H), 7.28 (d, J = 7.2 Hz, 2H), 4.72 – 4.60 (m, 1H), 4.43 – 4.33 (m, 1H), 4.30 – 4.13 (m, 2H), 3.93 – 3.72 (m, 2H), 2.89 (d, J = 4.2 Hz, 2H), 1.51 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 174.93, 157.99, 155.85, 144.33, 143.80, 141.44, 141.35, 127.69, 127.07, 125.22, 119.98, 82.27, 79.67, 77.59, 66.95, 52.55, 47.29, 32.36, 28.46, 23.03.

Fmoc-Lys(-4-ene)(Boc)-N(OMe)Me (30)

The title compound was prepared by the general procedure for peptide coupling on a 0.5 mmol scale. Purification by column chromatography (10→40% EtOAc/pent) yielded the title compound in a quantitative yield. ^1H NMR (400 MHz, CDCl_3) δ 7.76 (d, J = 7.5 Hz, 2H), 7.60 (t, J = 7.0 Hz, 2H), 7.40 (t, J = 7.6, 2H), 7.31 (tdd, J = 7.4, 3.0, 1.2 Hz, 2H), 5.64 – 5.51 (m, 3H), 4.80 (q, J = 7.0 Hz, 1H), 4.69 – 4.50 (m, 1H), 4.47 – 4.27 (m, 2H), 4.22 (t, J = 7.2 Hz, 1H), 3.77 (s, 3H), 3.71 – 3.63 (m, 2H), 3.22 (s, 3H), 2.57 – 2.44 (m, 1H), 2.44 – 2.32 (m, 1H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 173.58, 171.90, 156.04, 143.98, 143.87, 141.38, 130.85, 127.81, 127.17, 126.55, 125.26, 120.08, 67.14, 61.80, 50.79, 47.20, 35.50, 32.21, 28.49.

Fmoc-Lys(-4-yl)(Boc)-N(OMe)Me (31)

The title compound was prepared by the general procedure for peptide coupling on a 3.0 mmol scale. Purification by column chromatography (10→40 % EtOAc/pent) yielded the title compound as a white foam (1.29 g, 2.54 mmol, 85 %). ^1H NMR (400 MHz, CDCl_3) δ 7.73 (d, J = 7.5 Hz, 2H), 7.60 (t, J = 7.3 Hz, 2H), 7.37 (td, J = 7.6, 1.4 Hz, 2H), 7.32 – 7.25 (m, 2H), 6.13 (d, J = 8.8 Hz, 1H), 5.04 (bs, 1H), 4.90 (bs, 1H), 4.35 (dd, J = 7.4, 1.8 Hz, 2H), 4.21 (t, J = 7.1 Hz, 1H), 3.86 (bs, 2H), 3.72 (s, 3H), 3.21 (s, 3H), 2.79 – 2.55 (m, 2H), 1.40 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 170.49, 155.80, 155.25, 143.60, 141.07, 127.57, 126.93, 125.07, 119.83, 79.51, 79.14, 77.84, 77.36, 67.02, 61.52, 49.71, 46.91, 32.01, 30.60, 28.20, 22.74. LC-MS (linear gradient 10→90% MeCN, 0.1% TFA, 13.0 min): Rt (min): 8.20 (ESI-MS (m/z): 507.8 ($\text{M}+\text{H}^+$)). HRMS: calcd. for $\text{C}_{28}\text{H}_{33}\text{N}_3\text{O}_6$ 508.24421 [$\text{M}+\text{H}^+$]; found 508.24405. $[\alpha]_D^{20}$ = 4.4 ($\text{C}=1$, CHCl_3).

Fmoc-Lys(-4-ene)(Boc)-VS (32)

Weinreb amide **30** (255 mg, 0.5 mmol, 1 equiv.) was dissolved in dry Et_2O (5 mL) and cooled to -30°C . LiAlH_4 (2M in THF, 0.25 mL, 0.5 mmol, 1 equiv.) was added drop wise. After 1 h, TLC analysis indicated completion of the reaction. The reaction mixture was quenched with 1M HCl (approx. 2 mL), diluted with EtOAc and wash 1M HCl (2x) and brine (2x). The organic layer was dried over Na_2SO_4 , filtered and concentrated and used directly in the next step. Diethyl((methylsulfonyl)methyl)-phosphonate (173 mg, 0.75 mmol, 1.5 equiv.) was dissolved in THF (20 mL) and cooled to 0°C followed by the addition of NaH (60 % w/w in mineral oil, 24 mg, 0.6 mmol, 1.2 equiv.). After stirring for 30 min, the freshly obtained aldehyde in THF (5 mL) was added drop wise to the reaction mixture. After 2.5 h, the reaction was diluted with EtOAc, washed with 1M HCl (1x) and brine (1x). The organic layer was dried over Na_2SO_4 , filtered and concentrated. NMR analysis of the crude product indicated significant amounts of aldehyde remaining. Therefore reaction was repeated with 0.7 equiv diethyl((methylsulfonyl)-methyl)-phosphonate and 0.5 equiv NaH. Purification by column chromatography (10→40 % EtOAc, n-Pentane) yielded the title compound (105 mg, 0.2 mmol, 40 %). ^1H NMR (400 MHz, CDCl_3) δ 7.76 (d, J = 7.4 Hz, 2H), 7.58 (dd, J = 7.3, 3.2 Hz, 2H), 7.40 (t, J = 7.3 Hz, 2H), 7.36 – 7.28 (m, 2H), 6.83 (dd, J = 15.2, 4.6 Hz, 1H), 6.44 (d, J = 15.1 Hz, 1H), 5.65 – 5.40 (m, 2H), 5.31 – 5.10 (m, 1H), 4.80 – 4.59 (m, 1H), 4.54 – 4.30 (m, 3H), 4.20 (q, J = 6.6, 5.9 Hz, 1H), 3.68 (q, J = 7.1, 6.0 Hz, 2H), 2.91 (s, 3H), 2.46 – 2.22 (m, 2H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 155.66,

146.86, 143.76, 143.60, 141.35, 132.12, 130.06, 127.84, 127.72, 127.12, 125.86, 124.95, 120.07, 66.81, 51.00, 47.20, 42.86, 42.22, 36.69, 28.41.

Fmoc-Lys(-4-yn)(Boc)-VS (33)

Weinreb amide **31** (1.29 g, 2.54 mmol, 1 equiv.) was dissolved in dry Et₂O (26 mL) and cooled to -30°C. LiAlH₄ (2M in THF, 1.3 mL, 2.54 mmol, 1 equiv.) was added drop wise. After < 10 minutes, TLC analysis indicated completion of the reaction. The reaction mixture was quenched with 1M HCl (approx. 10 mL), diluted with EtOAc and washed with 1M HCl (2x) and brine (2x). The organic layer was dried over Na₂SO₄, filtered and concentrated and used directly in the next step. Diethyl((methylsulfonyl)methyl)- phosphonate (0.88 g, 3.81 mmol, 1.5 equiv.) was dissolved in THF (20 mL) and cooled to 0°C followed by the addition of NaH (60 % w/w in mineral oil, 0.12 g, 3.05 mmol, 1.2 equiv.). After stirring for 30 min, the freshly obtained aldehyde in THF (10 mL) was added drop wise to the reaction mixture. After 1 h, TLC (2.5 % MeOH, DCM) indicated completion of the reaction. The reaction was diluted with EtOAc, washed with 1M HCl (1x) and brine (1x). The organic layer was dried over Na₂SO₄, filtered and concentrated. Purification by column chromatography (10→40 % EtOAc, n-Pentane) yielded the title compound (0.94 g, 1.79 mmol, 70 %). ¹H NMR (400 MHz, CDCl₃) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.60 (t, *J* = 7.3 Hz, 2H), 7.40 (td, *J* = 7.5, 1.2 Hz, 2H), 7.36 – 7.29 (m, 2H), 6.88 (dd, *J* = 15.1, 4.8 Hz, 1H), 6.55 (dt, *J* = 15.1, 1.6 Hz, 1H), 5.58 (d, *J* = 8.6 Hz, 1H), 4.93 (s, 1H), 4.60 (s, 1H), 4.44 (d, *J* = 7.0 Hz, 2H), 4.21 (t, *J* = 6.7 Hz, 1H), 3.87 (s, 2H), 2.94 (s, 3H), 2.75 – 2.41 (m, 2H), 1.44 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 155.56, 145.51, 143.69, 141.38, 130.96, 127.90, 127.20, 125.09, 120.13, 80.70, 77.28, 67.13, 50.02, 47.20, 42.87, 28.42, 24.54.

H-Lys(-4-ene)(Boc)-VS (34)

To a solution of vinyl sulfone **32** (86 mg, 0.16 mmol, 1 equiv.) in MeCN (2 mL) was added diethylamine (0.85 mL). After 1h, TLC analysis (2.5 % MeOH, DCM) showed completion of the reaction and the mixture was diluted with toluene, evaporated to dryness and co-evaporated with toluene (2x). Purification by column chromatography (0→5% MeOH, DCM) yielded the title compound as a yellow oil (37 mg, 0.12 mmol, 75%). ¹H NMR (400 MHz, CDCl₃) δ 6.94 (dd, *J* = 15.0, 4.8 Hz, 1H), 6.60 (dd, *J* = 15.0, 1.3 Hz, 1H), 5.67 – 5.47 (m, 2H), 4.63 (s, 1H), 3.76 – 3.61 (m, 3H), 2.95 (s, 3H), 2.43 – 2.31 (m, 1H), 2.25 – 2.10 (m, 1H), 1.57 (s, 2H), 1.45 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 155.83, 150.89, 131.82, 128.91, 126.93, 51.66, 42.97, 42.36, 39.72, 28.50.

H-Lys(-4-yn)(Boc)-VS (35)

To a solution of vinyl sulfone **33** (0.94 g, 1.79 mmol, 1 equiv.) in MeCN (18 mL) dropwise added diethylamine (8 mL). After 1h, TLC analysis (2.5 % MeOH, DCM) showed completion of the reaction and the mixture was diluted with toluene (30 mL), evaporated to dryness and co-evaporated with toluene (2x). Purification by column chromatography (0→5% MeOH, DCM) yielded the title compound as a yellow oil (0.26 g, 0.84 mmol, 47%). ¹H NMR (400 MHz, CDCl₃) δ 6.94 (dd, *J* = 15.3, 4.8 Hz, 1H), 6.68 (d, *J* = 15.1 Hz, 1H), 5.11 (bs, 1H), 3.89 (s, 2H), 3.76 (d, *J* = 5.9 Hz, 1H), 2.98 (s, 3H), 2.46 (qd, *J* = 16.8, 5.9 Hz, 2H), 1.76 (s, 2H), 1.45 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 155.38, 149.25, 129.66, 79.95, 79.81, 78.24, 50.81, 42.77, 30.52, 28.33, 27.24.

Fmoc-Lys(-4-ene)(Boc)-Leu-OMe (36)

The title compound was prepared by the general procedure for peptide coupling on a 0.5 mmol scale. Column chromatography (0→30% EtOAc/pentane) provided the product (285 mg, 0.48 mmol, 96%). Product isolated with 10% of minor diastereomer. Peaks reported for major diastereomer. ¹H NMR (400 MHz, CDCl₃) δ 7.74 (d, *J* = 7.5 Hz, 2H), 7.57 (d, *J* = 6.3 Hz, 2H), 7.41 – 7.35 (m, 2H), 7.32 – 7.26 (m, 2H), 6.86 (d, *J* = 8.1 Hz, 1H), 5.81 (d, *J* = 8.2 Hz, 1H), 5.65 – 5.49 (m, 2H), 5.00 (t, *J* = 5.8 Hz, 1H), 4.65 – 4.53 (m, 1H), 4.46 – 4.24 (m, 3H), 4.19 (t, *J* = 7.1 Hz, 1H), 3.71 (s, 3H), 3.69 – 3.56 (m, 2H), 2.57 – 2.40 (m, 2H), 1.71 – 1.50 (m, 3H), 1.42 (s, 9H), 0.88 (d, *J* = 4.2 Hz, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 174.32, 173.57, 131.05, 130.99, 127.71, 54.24, 52.29, 50.36, 50.32, 42.34, 41.37, 37.92, 28.42, 24.94, 22.90, 21.84.

Fmoc-Lys(-4-yn)(Boc)-Leu-OMe (37)

The title compound was prepared by the general procedure for peptide coupling on a 0.6 mmol scale. Column chromatography (10→30% EtOAc/pentane) provided the product (220 mg, 0.38 mmol, 76%). ¹H NMR (400 MHz, CDCl₃) δ 7.73 (d, *J* = 7.5 Hz, 2H), 7.57 (dd, *J* = 7.7, 2.9 Hz, 2H), 7.37 (t, *J* = 7.5 Hz, 2H), 7.32 – 7.24 (m, 2H), 6.03 (d, *J* = 7.9 Hz, 1H), 5.28 (s, 1H), 4.73 – 4.58 (m, 1H), 4.51 – 4.27 (m, 3H), 4.19 (t, *J* = 7.1 Hz, 1H), 3.99 – 3.77 (m, 2H), 3.71 (d, *J* = 6.8 Hz, 3H), 2.89 – 2.40 (m, 2H), 1.73 – 1.53 (m, 3H), 1.42 (s, 9H), 0.90 (t, *J* = 5.2 Hz, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 173.24, 169.88, 155.84, 155.49, 143.61, 141.20, 127.71, 127.04, 125.06, 119.96, 79.83, 78.32, 67.27, 53.53, 52.35, 50.92, 46.96, 41.20, 30.71, 28.32, 24.74, 23.28, 22.73, 21.80.

H-Lys(-4-ene)(Boc)-Leu-OMe (38)

Fmoc-L-Lys-4-ene(Boc)-Leu-OMe **36** (273 mg, 0.46 mmol) was deprotected using the standard procedure for Fmoc removal, providing the title compound (161 mg, 0.43 mmol, 94%) after purification by column chromatography (0→3% MeOH, DCM). Product was isolated with 10% of minor diastereomer. Peaks reported for major diastereomer. ¹H NMR (400 MHz, CDCl₃) δ 7.64 (d, *J* = 8.6 Hz, 1H), 5.58 – 5.49 (m, 2H), 4.80 (s, 1H), 4.57 (td, *J* = 8.8, 4.7 Hz, 1H), 3.71 (s, 3H), 3.69 – 3.61 (m, 2H), 3.40 (dd, *J* = 8.3, 4.3 Hz, 1H), 2.53 (dt, *J* = 13.6, 5.0 Hz, 1H), 2.24 (dt, *J* = 13.5, 7.9 Hz, 1H), 1.74 (s, 2H), 1.68 – 1.52 (m, 3H), 1.41 (s, 9H), 0.92 (dd, *J* = 6.1, 3.4 Hz, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 174.32, 173.57, 131.05, 130.99, 127.71, 54.24, 52.29, 50.36, 50.32, 42.34, 41.37, 37.92, 28.42, 24.94, 22.90, 21.84.

H-Lys(-4-yn)(Boc)-Leu-OMe (39)

Fmoc L-Lys-4-yl(Boc)-Leu-OMe **37** (220 mg, 0.38 mmol) was deprotected using the standard procedure for Fmoc removal, providing the title compound (189 mg, 100%) after purification by column chromatography (0→3 % MeOH, DCM). ¹H NMR (400 MHz, CDCl₃) δ 7.74 (d, *J* = 8.7 Hz, 1H), 4.95 (s, 1H), 4.70 – 4.51 (m, 1H), 3.88 (s, 2H), 3.74 (s, 3H), 3.51 (t, *J* = 5.9 Hz, 1H), 2.77 – 2.52 (m, 2H), 1.89 (s, 2H), 1.72 – 1.55 (m, 3H), 1.44 (s, 9H), 1.00 – 0.89 (m, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 173.44, 173.02, 155.37, 79.32, 79.17, 53.66, 52.32, 50.48, 41.43, 30.72, 28.39, 25.38, 24.94, 22.94, 21.84.

N₃Phe-Lys(-4-ene)(Boc)-Leu-OMe (40)

The title compound was prepared by the general procedure for peptide coupling on a 0.38 mmol scale. Column chromatography (0→50% EtOAc/pentane) provided the product (124 mg, 0.23 mmol, 60%). Product was isolated with 10% of minor diastereomer. Peaks reported for major diastereomer. ¹H NMR (400 MHz, CDCl₃) δ 7.39 – 7.24 (m, 5H), 6.99 (d, *J* = 7.3 Hz, 1H), 6.54 (d, *J* = 7.5 Hz, 1H), 5.53 – 5.44 (m, 2H), 4.88 (s, 1H), 4.64 – 4.53 (m, 1H), 4.38 (q, *J* = 7.3 Hz, 1H), 4.24 (dd, *J* = 7.8, 4.2 Hz, 1H), 3.77 (s, 3H), 3.73 – 3.59 (m, 2H), 3.32 (dd, *J* = 14.1, 4.2 Hz, 1H), 3.08 (dd, *J* = 14.0, 7.8 Hz, 1H), 2.46 – 2.37 (m, 1H), 2.37 – 2.26 (m, 1H), 1.64 (dtd, *J* = 16.3, 11.9, 10.4, 6.8 Hz, 3H), 1.46 (s, 9H), 0.95 (t, *J* = 5.3 Hz, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 173.38, 170.12, 168.30, 155.91, 135.90, 131.79, 129.69, 129.48, 128.78, 127.43, 125.83, 65.29, 52.77, 52.55, 51.04, 42.44, 41.17, 38.48, 35.37, 28.52, 24.95, 22.90, 21.91.

N₃Phe-Lys(-4-yn)(Boc)-Leu-OMe (41)

The title compound was prepared by the general procedure for peptide coupling on a 0.23 mmol scale. Column chromatography (0→40% EtOAc/pentane) provided the product (111 mg, 0.21 mmol, 91 %). ¹H NMR (400 MHz, CDCl₃) δ 7.44 – 7.15 (m, 5H), 7.00 (d, *J* = 7.8 Hz, 1H), 5.22 (s, 1H), 4.68 – 4.57 (m, 1H), 4.57 – 4.46 (m, 1H), 4.22 (dd, *J* = 8.0, 4.3 Hz, 1H), 3.99 – 3.78 (m, 2H), 3.75 (s, 3H), 3.30 (dd, *J* = 14.0, 4.3 Hz, 1H), 3.06 (dd, *J* = 14.1, 8.0 Hz, 1H), 2.67 – 2.55 (m, 1H), 2.37 (dd, *J* = 16.7, 8.7 Hz, 1H), 1.75 – 1.56 (m, 3H), 1.43 (s, 9H), 1.00 – 0.89 (m, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 173.29, 169.27, 168.52, 135.82, 129.54, 129.38, 128.80, 128.70, 127.35, 80.21, 78.14, 65.10, 52.49, 51.76, 51.15, 41.27, 38.44, 30.79, 29.73, 28.41, 24.88, 22.86, 22.80, 21.97.

N₃Phe-Lys(-4-ene)(Boc)-Leu-NHNH₂ (42)

Tripeptide **40** (124 mg, 0.23 mmol, 1 equiv.) was dissolved in MeOH (5 mL) and hydrazine monohydrate (0.34 mL, 6.9 mmol, 30 equiv.) was added drop wise. After 3 h, TLC analysis showed completion of the reaction and the reaction mixture was evaporated to dryness and co-evaporated with toluene (3x) yielding the title compound in a quantitative yield. ¹H NMR (400 MHz, MeOD) δ 7.38 – 7.19 (m, 5H), 5.57 – 5.34 (m, 2H), 4.43 – 4.32 (m, 2H), 4.15 (dd, J = 8.6, 5.0 Hz, 1H), 3.60 (qd, J = 16.1, 15.4, 4.4 Hz, 2H), 3.19 (dd, J = 14.0, 5.1 Hz, 1H), 2.97 (dd, J = 13.9, 8.6 Hz, 1H), 2.50 – 2.26 (m, 2H), 1.71 – 1.50 (m, 3H), 1.43 (s, 9H), 0.94 (dd, J = 16.7, 6.4 Hz, 6H). ¹³C NMR (101 MHz, MeOD) δ 173.55, 172.71, 171.39, 158.15, 137.81, 132.12, 130.42, 129.64, 129.58, 128.04, 126.99, 125.99, 120.12, 111.88, 65.35, 54.21, 51.59, 43.01, 41.96, 38.81, 36.08, 28.76, 25.74, 23.33, 22.16.

N₃Phe-Lys(-4-yn)(Boc)-Leu-NHNH₂ (43)

Tripeptide **41** (73 mg, 0.14 mmol, 1 equiv.) was dissolved in MeOH (2 mL) and hydrazine monohydrate (0.2 mL, 4.1 mmol, 30 equiv.) was added drop wise and the solution was then refluxed at 80°C for 1h. The reaction mixture was evaporated to dryness and co-evaporated with toluene (3x) yielding the title compound in a quantitative yield. NMR-analysis could not be performed due to poor solubility in chloroform, methanol and mixture thereof.

Fmoc-Dap(Boc)-N(OMe)Me (45)

The title compound was prepared by the general procedure for peptide coupling on a 0.47 mmol scale, with 2 equiv of *N,O*-dimethylhydroxylamine. Column chromatography (20-40% EtOAc/pentane) provided the product (211 mg, 96%). ¹H NMR (300 MHz, CDCl₃) δ 7.76 (d, J = 7.4 Hz, 2H), 7.67 – 7.55 (m, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.6 Hz, 2H), 5.93 (d, J = 6.1 Hz, 1H), 4.96 – 4.83 (m, 1H), 4.83 – 4.69 (m, 1H), 4.39 (d, J = 7.0 Hz, 2H), 4.21 (t, J = 6.9 Hz, 1H), 3.78 (s, 3H), 3.57 (s, 1H), 3.44 (d, J = 13.2 Hz, 1H), 3.22 (s, 3H), 1.43 (s, 9H). ¹³C NMR (75 MHz, CDCl₃) δ 156.16, 143.85, 141.43, 127.82, 127.17, 125.26, 120.09, 120.06, 79.81, 67.17, 61.76, 52.04, 49.86, 47.27, 42.09, 28.43.

Fmoc-Dap(Gly-Boc)-N(OMe)Me (46)

Weinreb amide **44** (211 mg, 0.45 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with Boc-Gly-OH using the standard procedure for peptide couplings. Column chromatography (60→100% EtOAc/pentane) provided the product (267 mg, 100%). ¹H NMR (400 MHz, CDCl₃) δ 7.75 (d, J = 7.5 Hz, 2H), 7.65 – 7.56 (m, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.35 – 7.27 (m, 2H), 6.72 (s, 1H), 6.12 (d, J = 6.9 Hz, 1H), 5.20 (s, 1H), 4.85 (s, 1H), 4.37 (d, J = 7.0 Hz, 2H), 4.20 (t, J = 7.0 Hz, 1H), 3.77 (s, 3H), 3.84 – 3.51 (m, 4H), 3.20 (s, 3H), 1.42 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 170.30, 156.42, 143.93, 143.80, 141.40, 141.36, 127.82, 127.17, 125.28, 120.08, 120.06, 80.35, 67.27, 61.82, 51.12, 47.19, 44.40, 41.38, 32.44, 28.38.

Fmoc-Dap(Gly-Boc)-VS (47)

To a solution of Fmoc-Dap(Gly-Boc)-N(OMe)Me **46** (157 mg, 0.3 mmol) at -20°C in THF (4 mL) was added LiAlH₄ (2M in THF, 150 μ L, 1 equiv.) dropwise in 10 min. TLC analysis (3% MeOH in DCM) revealed completion of the reaction and the reaction was quenched by the addition of 1M HCl. EtOAc was added and the layers were separated. The organic layer was washed with brine, dried over Na₂SO₄, filtered and concentrated, providing the crude aldehyde which was directly used in the next step. Diethyl((methylsulfonyl)methyl) phosphonate (1.5 equiv, 0.45 mmol, 104 mg) was dissolved in THF (4 mL) and cooled to 0 °C under an argon atmosphere. NaH (1.3 equiv, 0.39 mmol, 15.6 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 (5 mL)) was slowly added and the mixture was stirred for 1 h while slowly warming it to RT. After this time TLC analysis indicated complete conversion of the aldehyde. EtOAc was added and the mixture was extracted with 1 M aq. HCl (2x) and brine, dried over Na₂SO₄ and concentrated. Column chromatography (20→100% EA:pent) yielded the title compound (105 mg, 43% (contains 0.4 equiv of Diethyl((methylsulfonyl)methyl) phosphonate based on NMR)). ¹H NMR (400 MHz, MeOD) δ 7.79 (d, J = 7.5 Hz, 2H), 7.65 (d, J = 7.2 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.33 (t, J = 7.3 Hz, 2H), 6.82 (dd, J = 15.2, 5.1 Hz, 1H), 6.59 (d,

$J = 15.3$ Hz, 1H), 4.55 – 4.39 (m, 3H), 4.30 – 4.18 (m, 1H), 3.69 (d, $J = 4.9$ Hz, 2H), 3.43 (ddd, $J = 42.5, 13.5, 7.0$ Hz, 2H), 2.99 (s, 3H), 1.45 (s, 9H).

H-Dap(Gly-Boc)-VS (48)

Fmoc-Dap(Gly-Boc)-VS **47** (105 mg, 0.13 mmol) was dissolved in MeCN (2 mL) and cooled to 0°C. Diethylamine (2 mL) was added and after stirring for 1 h, the reaction mixture was concentrated and purified by column chromatography (0→40% MeOH/DCM), providing the title compound (0.13 mmol, 100%). ^1H NMR (400 MHz, MeOD) δ 6.87 (dd, $J = 15.2, 5.4$ Hz, 1H), 6.67 (d, $J = 15.2$ Hz, 1H), 3.85 – 3.69 (m, 3H), 3.50 – 3.23 (m, 2H), 3.00 (s, 3H), 1.45 (s, 9H). ^{13}C NMR (101 MHz, MeOD) δ 173.21, 147.58, 132.23, 80.78, 53.08, 44.80, 44.62, 42.72, 28.69.

Fmoc-Dap(Boc)-Leu-OMe (49)

The title compound was prepared by the general procedure for peptide coupling on a 0.5 mmol scale. Column chromatography (10→50% EtOAc/pentane) provided the product (259 mg, 94%). ^1H NMR (400 MHz, CDCl_3) δ 7.72 (d, $J = 7.5$ Hz, 2H), 7.56 (d, $J = 7.2$ Hz, 2H), 7.35 (t, $J = 7.4$ Hz, 2H), 7.30 – 7.21 (m, 2H), 7.15 (s, 1H), 6.38 (s, 1H), 5.38 (s, 1H), 4.54 (s, 1H), 4.32 (d, $J = 7.0$ Hz, 3H), 4.18 (t, $J = 7.2$ Hz, 1H), 3.67 (s, 3H), 3.49 (s, 2H), 1.58 (dq, $J = 16.8, 8.7, 6.7$ Hz, 3H), 1.40 (s, 9H), 0.87 (d, $J = 5.5$ Hz, 6H). ^{13}C NMR (101 MHz, CDCl_3) δ 173.13, 170.32, 156.72, 143.79, 141.30, 127.80, 127.15, 125.24, 120.03, 80.21, 67.53, 56.15, 52.45, 51.08, 47.05, 42.91, 40.85, 28.35, 24.87, 22.87, 21.77.

Fmoc-Dap(Gly-Boc)-Leu-OMe (50)

Fmoc-Dap(Boc)-Leu-OMe **49** (211 mg, 0.45 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with Boc-Gly-OH using the standard procedure for peptide couplings. Column chromatography (20→100% EtOAc/pentane) provided the product in a quantitative yield. ^1H NMR (400 MHz, CDCl_3) δ 7.73 (d, $J = 7.5$ Hz, 2H), 7.56 (d, $J = 7.4$ Hz, 3H), 7.47 – 7.40 (m, 1H), 7.37 (t, $J = 7.4$ Hz, 2H), 7.28 (d, $J = 7.5$ Hz, 2H), 6.08 (d, $J = 6.7$ Hz, 1H), 5.73 (t, $J = 5.7$ Hz, 1H), 4.66 – 4.48 (m, 1H), 4.44 – 4.26 (m, 3H), 4.17 (t, $J = 7.0$ Hz, 1H), 3.93 – 3.84 (m, 1H), 3.82 (d, $J = 5.8$ Hz, 1H), 3.75 – 3.62 (m, 4H), 3.31 – 3.19 (m, 1H), 1.75 – 1.58 (m, 3H), 1.40 (s, 9H), 0.88 (d, $J = 5.7$ Hz, 6H). ^{13}C NMR (101 MHz, CDCl_3) δ 174.02, 170.94, 170.50, 155.85, 143.52, 143.47, 140.98, 127.48, 126.85, 124.93, 119.71, 79.90, 67.15, 53.83, 52.36, 51.03, 46.73, 44.26, 41.55, 39.67, 28.06, 24.60, 22.60, 21.15.

H-Dap(Gly-Boc)-Leu-OMe (51)

Fmoc-Dap(Gly-Boc)-Leu-OMe **50** is deprotected using the standard procedure for Fmoc removal, providing the title compound (189 mg, 100%) after purification by column chromatography (50% EtOAc/pent followed by 0→10 % MeOH, EtOAc). Complex NMR due to presence of rotamers. Peaks of major rotamer are reported. ^1H NMR (400 MHz, CDCl_3 , MeOD) δ 4.51 (t, $J = 7.3$ Hz, 1H), 3.79 – 3.72 (m, 5H), 3.58 (t, $J = 6.3$ Hz, 1H), 3.55 – 3.46 (m, 1H), 3.46 – 3.33 (m, 1H), 1.70–1.51 (m, 3H), 1.45 (s, 9H), 1.01 – 0.91 (m, 6H). ^{13}C NMR (101 MHz, MeOD) δ 174.17, 173.40, 171.84, 157.02, 80.38, 54.35, 52.61, 51.24, 44.15, 43.60, 43.50, 40.26, 28.38, 25.06, 22.94, 21.45.

N₃Phe-Dap(Gly-Boc)-Leu-OMe (52)

The title compound was prepared by the general procedure for peptide coupling on a 0.5 mmol scale. Column chromatography (20→80% EtOAc/pentane) provided the product (192 mg, 68%). ^1H NMR (400 MHz, CDCl_3) δ 7.71 (d, $J = 7.8$ Hz, 1H), 7.40 (d, $J = 6.9$ Hz, 2H), 7.31 – 7.20 (m, 5H), 5.83 (t, $J = 5.7$ Hz, 1H), 4.65 – 4.46 (m, 2H), 4.17 (dd, $J = 8.4, 4.0$ Hz, 1H), 3.84 (dd, $J = 16.6, 5.9$ Hz, 1H), 3.72 (s, 3H), 3.77 – 3.66 (m, 2H), 3.27 (dd, $J = 14.1, 4.0$ Hz, 1H), 3.20 – 3.07 (m, 1H), 2.99 (dd, $J = 14.0, 8.6$ Hz, 1H), 1.64 (d, $J = 6.6$ Hz, 3H), 1.40 (s, 9H), 0.97 – 0.82 (m, 6H). ^{13}C NMR (101 MHz, CDCl_3) δ 174.20, 171.13, 170.18, 169.10, 156.43, 136.07, 129.49, 128.73, 127.31, 80.08, 65.14, 52.64, 52.57, 51.39, 44.44, 41.30, 39.98, 38.50, 28.37, 24.92, 22.89, 21.54.

N₃Phe-Dap(Gly-Boc)-Leu-NHNH₂ (53)

N₃Phe-Dap(Gly-Boc)-Leu-OMe **52** (0.34 mmol) was dissolved in MeOH (3 mL), followed by the addition of NH₂⁻ NH₂·H₂O (497 μ L, 10 mmol, 30 equiv.). The reaction mixture was stirred at rt for 4h, concentrated and co-evaporated with toluene (2x) thereby providing the product in a quantitative yield. ¹H NMR (400 MHz, MeOD) δ 7.27 (qd, J = 8.6, 7.7, 4.0 Hz, 5H), 4.51 (t, J = 6.3 Hz, 1H), 4.41 (dd, J = 9.5, 5.5 Hz, 1H), 4.18 (dd, J = 9.0, 4.8 Hz, 1H), 3.83 – 3.65 (m, 2H), 3.52 (tt, J = 13.8, 6.8 Hz, 2H), 3.22 (dd, J = 14.0, 4.7 Hz, 1H), 2.96 (dd, J = 13.9, 9.0 Hz, 1H), 1.75 – 1.51 (m, 3H), 1.44 (s, 9H), 0.95 (d, J = 6.4 Hz, 3H), 0.91 (d, J = 6.4 Hz, 3H). ¹³C NMR (101 MHz, MeOD) δ 173.70, 173.21, 171.64, 171.51, 158.32, 137.81, 130.35, 129.55, 128.01, 80.74, 65.50, 54.37, 51.97, 44.64, 41.81, 41.63, 38.98, 28.70, 25.73, 23.41, 22.02.

Fmoc-His(Trt)-VS (55)

To a solution of Fmoc-His(Trt)-N(OMe)Me **54** (1.99 g, 3 mmol) at 0°C in THF (30 mL) was added LiAlH₄ (2M in THF, 1.8 mL, 3.6 mmol, 1.2 equiv.) dropwise in 10 min. TLC analysis (3% MeOH in DCM) revealed completion of the reaction after 2 h and the reaction was quenched by the addition of 1M HCl. EtOAc was added and the layers were separated. The organic layer was washed with brine, dried over Na₂SO₄, filtered and concentrated, providing the crude aldehyde which was directly used in the next step. Diethyl((methylsulfonyl)methyl) phosphonate (267 mg, 1.26 mmol, 1.5 equiv.) was dissolved in THF (8.5 mL) and cooled to 0 °C under an argon atmosphere. NaH (44 mg, 60% w/w in mineral oil, 1.1 mmol, 1.3 equiv.) was slowly added and the mixture was stirred at 0°C for 45 min. Next, the freshly obtained aldehyde (510 mg, 0.85 mmol, 1 equiv.) in THF (3 mL) was slowly added and the mixture was stirred for 3 h while slowly warming to RT. After this time TLC analysis indicated complete conversion of the aldehyde. EtOAc was added and the mixture was extracted with 1 M aq. HCl (2x) and brine, dried over Na₂SO₄ and concentrated. Column chromatography (twice, 10→80% EA/pent) yielded the title compound (378 mg, 65% (contains 0.2 equiv of Diethyl((methylsulfonyl)methyl) phosphonate based on NMR)). ¹H NMR (400 MHz, CDCl₃) δ 7.78 (d, J = 7.5 Hz, 2H), 7.64 (dt, J = 11.8, 5.8 Hz, 2H), 7.50 – 7.26 (m, 13H), 7.19 – 7.11 (m, 7H), 7.06 (d, J = 7.8 Hz, 1H), 6.85 (dd, J = 15.0, 4.6 Hz, 1H), 6.66 (s, 1H), 6.47 (d, J = 15.0 Hz, 1H), 4.78 (d, J = 5.3 Hz, 1H), 4.47 – 4.33 (m, 2H), 4.33 – 4.20 (m, 1H), 3.04 (dd, J = 14.7, 4.7 Hz, 1H), 2.90 (s, 3H), 2.85 (dd, J = 14.7, 5.2 Hz, 1H).

H-His(Trt)-VS (56)

Fmoc-His(Trt)-VS (266 mg, 0.39 mmol) was dissolved in 1:1 MeCN/diethylamine (5 mL) and cooled to 0°C. After stirring for 1 h, the reaction mixture was concentrated and purified by column chromatography (100% EtOAc, followed by 0→10% MeOH/DCM), providing the title compound (115 mg, 64%). ¹H NMR (400 MHz, CDCl₃) δ 7.42 (d, J = 1.2 Hz, 1H), 7.36 (dd, J = 5.1, 1.7 Hz, 10H), 7.19 – 7.10 (m, 5H), 6.98 (dd, J = 15.0, 4.8 Hz, 1H), 6.65 (s, 1H), 6.62 (dd, J = 15.1, 1.6 Hz, 1H), 4.02 (ddt, J = 6.9, 5.3, 2.7 Hz, 1H), 2.91 (s, 3H), 2.85 (dd, J = 14.3, 5.3 Hz, 1H), 2.67 (dd, J = 14.3, 7.8 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 150.89, 142.36, 139.04, 136.97, 129.77, 128.90, 128.21, 119.80, 75.40, 51.98, 42.93, 35.61.

N₃Phe-Leu-Leu-His(Trt)-VS (57)

The title compound was obtained by the general protocol for azide coupling on a 100 μ mol scale. Purification by column chromatography (0→2% MeOH/DCM) provided the title compound (45 mg, 53%). ¹H NMR (400 MHz, CDCl₃) δ 8.28 (d, J = 8.1 Hz, 1H), 7.37 (dd, J = 5.2, 1.8 Hz, 9H), 7.34 – 7.27 (m, 4H), 7.27 – 7.22 (m, 2H), 7.16 – 7.05 (m, 6H), 6.86 (d, J = 7.7 Hz, 1H), 6.79 (dd, J = 15.1, 4.6 Hz, 1H), 6.68 – 6.62 (m, 2H), 6.45 (dd, J = 15.1, 1.5 Hz, 1H), 5.01 – 4.91 (m, 1H), 4.56 – 4.46 (m, 1H), 4.43 – 4.34 (m, 1H), 4.31 (dd, J = 7.5, 4.1 Hz, 1H), 3.30 (dd, J = 14.1, 4.1 Hz, 1H), 3.08 (dd, J = 14.1, 7.6 Hz, 1H), 2.98 – 2.91 (m, 1H), 2.90 (s, 3H), 2.80 (dd, J = 14.7, 5.7 Hz, 1H), 1.76 (ddd, J = 13.6, 8.9, 4.7 Hz, 1H), 1.67 – 1.55 (m, 2H), 1.55 – 1.42 (m, 2H), 1.41 – 1.21 (m, 1H), 0.93 (d, J = 6.5 Hz, 6H), 0.79 (d, J = 6.6 Hz, 3H), 0.76 (d, J = 6.5 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 171.67, 171.64, 169.26, 146.56, 142.19, 138.71, 136.30, 135.81, 130.27, 129.82, 129.73, 129.61, 128.74, 128.29, 128.25, 128.14, 127.36, 120.18, 75.49, 65.43, 52.26, 52.01, 49.86, 42.92, 41.25, 40.36, 38.29, 31.22, 25.04, 24.53, 23.22, 23.12, 21.82, 21.78.

Biochemical experiments

General

Lysates of cells were prepared by treating cell pellets with 4 volumes of lysis buffer containing 50 mM Tris pH 7.5, 2 mM DTT, 5 mM MgCl₂, 10% glycerol, 2 mM ATP, and 0.05% digitonin for 60 min. Protein concentration was determined using Qubit® protein assay kit (ThermoFisher). All cell lysate labelling experiments were performed in assay buffer containing 50 mM Tris pH 7.5, 2 mM DTT, 5 mM MgCl₂, 10% glycerol, 2 mM ATP. Cell lysate labelling and competition experiments were performed at 37°C. The probe cocktail consists of: 100 nM Cy5-NC-001, 30 nM BODIPY(FL)-LU-112, 100 nM BODIPY(TMR)-NC-005-VS, used as premixed 10x concentrated cocktail in DMSO which is incubated with cell lysate for 60 min. Prior to fractionation on 12.5% SDS-PAGE (TRIS/glycine), samples were boiled for 3 min in a reducing gel loading buffer. The 7.5x10 cm (L x W) gels were run for 15 min at 80V followed by 120 min at 130V. In-gel detection of (residual) proteasome activity was performed in the wet gel slabs directly on a ChemiDoc™ MP System using Cy2 setting to detect BODIPY(FL), Cy3 settings to detect BODIPY(TMR) and Cy5 settings to detect Cy5.

Competition experiments in cell lysate

Cell lysates (diluted to 10-15 µg total protein in 9 µL buffer) were exposed to the inhibitor (10x stock in DMSO) at indicated concentrations for 1 h at 37 °C, followed by addition of probe cocktail (10x stock, 1.1 µL) and SDS-PAGE as described above.

References

1. Adams, J. The development of proteasome inhibitors as anticancer drugs. *Cancer Cell* **5**, 417-421 (2004).
2. Altun, M. et al. Effects of PS-341 on the activity and composition of proteasomes in multiple myeloma cells. *Cancer Res.* **65**, 7896-901 (2005).
3. Britton, M. et al. Selective inhibitor of proteasome's caspase-like sites sensitizes cells to specific inhibition of chymotrypsin-like sites. *Chem. Biol.* **16**, 1278-1289 (2009).
4. Mirabella, Anne C. et al. Specific cell-permeable inhibitor of proteasome trypsin-like sites selectively sensitizes myeloma cells to bortezomib and carfilzomib. *Chem. Biol.* **18**, 608-618 (2011).
5. Geurink, P.P. et al. Incorporation of non-natural amino acids improves cell permeability and potency of specific inhibitors of proteasome trypsin-like sites. *J. Med. Chem.* **56**, 1262-1275 (2013).
6. Kraus, M. et al. The novel β 2-selective proteasome inhibitor LU-102 synergizes with bortezomib and carfilzomib to overcome proteasome inhibitor resistance of myeloma cells. *Haematologica* (2015).
7. de Bruin, G. et al. A set of activity-based probes to visualize human (immuno)proteasome activities. *Angew. Chem. Int. Ed.*, **13**, 4199-4203 (2016).
8. pKa values obtained from a predictor: <https://epoch.uky.edu/ace/public/pKa.jsp>.
9. Park, H.-g. et al. Highly enantioselective and practical cinchona-derived phase-transfer catalysts for the synthesis of α -amino acids. *Angew. Chem. Int. Ed.* **41**, 3036-3038 (2002).
10. Thimon, C., Panza, L. & Morin, C. Synthesis of a Glycosylated ortho-Carboranyl Amino Acid. *Synlett* **2003**, 1399-1402 (2003).
11. Porter, N.A., Ziegler, C.B., Khouri, F.F. & Roberts, D.H. General synthesis of polyunsaturated fatty acid hydroperoxides involving a novel vinylcyclopropyl bromide ring opening. *J. Org. Chem.* **50**, 2252-2258 (1985).
12. Wender, P.A., Holt, D.A. & Sieburth, S.M. in *Organic Syntheses* (John Wiley & Sons, Inc., 2003).
13. Valverde, I.E. et al. 1,2,3-triazoles as amide bond mimics: triazole scan yields protease-resistant peptidomimetics for tumor targeting. *Angew. Chem. Int. Ed.* **52**, 8957-8960 (2013).
14. de Bruin, G. et al. Structure-based design of β 1i or β 5i specific inhibitors of human immunoproteasomes. *J. Med. Chem.* **57**, 6197-6209 (2014).
15. Huber, Eva M. et al. Immuno- and constitutive proteasome crystal structures reveal differences in substrate and inhibitor specificity. *Cell* **148**, 727-738 (2012).
16. Huber, E.M. et al. Systematic analyses of substrate preferences of 20S proteasomes using peptidic epoxyketone inhibitors. *J. Am. Chem. Soc.* **137**, 7835-7842 (2015).

