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Asymmetric synthesis of lysine analogues with reduced basicity and their incorporation in proteasome inhibitors

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Abstract: Most known β2 selective proteasome inhibitor suffer from relatively poor cell permeability as the result of a net positive charge caused by the basic moiety at P1. Here we describe the synthesis of oligopeptide vinyl sulfones that contain different amino acids bearing amino groups with reduced basicity at P1 and/or P3. For this, we developed the first enantioselective synthesis of lysine(4-ene) and lysine(4-yn). These amino acids, as well as histidine and diaminopropionic acid-glycine, were incorporated at the P1 and/or P3 position of oligopeptide vinyl sulfones. All inhibitors inhibit β2, however, with loss of potency compared to our most potent and selective β2 inhibitor, LU-102. These results notwithstanding, our study described here provides important insights for the future design of β2 selective proteasome inhibitors.

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.[1,2] Constitutive proteasomes, which are expressed in every cell type, have three different proteolytic activities, namely caspase-like (β1c), trypsin-like (β2c) and chymotrypsin-like (β5c). Immune cells and cells exposed to inflammatory cytokines, express an additional type of proteasome, termed immunoproteasome, in which β1i, β2i and β5i replace β1c, β2c and β5c as catalytic activities.[3] These subunits have slightly changed substrate specificities compared to their constitutive counterparts. The chymotryptic activities of the proteasome (β5c and β5i) 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 β5 subunits.[4] However, bortezomib efficiently inhibits β1c and β1i with similar potency as the β5 active sites,[5] while carfilzomib inhibits both β1 and β2 activities at higher concentrations.[6,7] Selective β5 inhibition is not cytotoxic to most MM cell lines, and partial co-inhibition of either β1 or β2 is necessary for cytotoxicity.[8] In order to be able to investigate the effect of β2 inhibition on MM cells, selective β2 inhibitors have been developed. The first in-class β2 selective inhibitors bear an arginine residue at P1 and/or P3 (NC-002 and NC-022, Figure 1). While NC-022 is the most potent proteasome inhibitor of the two, its Arg residues render the molecule impermeable to cells. Using NC-002 we showed that selective β2 inhibition sensitizes MM cells to bortezomib and carfilzomib.[9] With the aim to overcome the lability of the arginine epoxyketone (intramolecular attack of the guanidine group to the epoxyketone moiety), low yielding synthesis and poor cell permeability in solid tumours, a second generation β2 inhibitor was developed (LU-102, Figure 1).[10] LU-102 bears a (4)-aminomethyl-phenylalanine ((4-CH_2NH_2)Phe) vinyl sulfone at P1, which can be synthesized on large scale. Introduction of a P3 4-(CH_2NH_2)Phe in LU-112 further increased the potency and selectivity in lysate compared to LU-102, but this increased efficacy is at the cost of cell permeability (Figure 1).

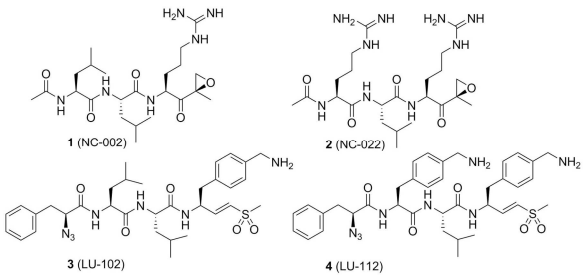

Figure 1. Structure of β2-selective inhibitors previously reported. IC_{50} on Hela cell lysates: **3**: 0.084 μM; **4**: 0.022 μM, IC_{50} on Hela cells: **3**: 2.7 μM; **4**: 50 μM. IC_{50} values (μM) have been determined in Hela cell lysates (1 h treatment) and intact Hela cells (4 h treatment).

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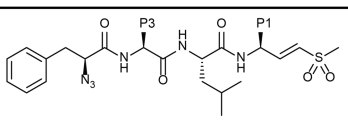
With LU-102 we found that selective β2 inhibition does not only sensitize MM cells to bortezomib and carfilzomib[10], but also overcomes resistance to bortezomib and carfilzomib, a major problem which arises during treatment of patients with proteasome inhibitors.[6] Whereas LU-102 possesses nanomolar potency in cell lysates, much higher IC_{50} values are found in living cells.[6,7] Therefore, high concentrations of LU-102 are necessary to achieve efficient β2 inhibition. In order to increase cell permeability, one option would be to lower the charge of the molecule at physiological pH. For this, basic amino acids with pK_a closer to physiological pH would be required. Optional amino acids with lowered pK_a values are histidine and the lysine analogues Lys(4-ene), Lys(4-yn) and diamino-propionic acid-Gly (Dap(Gly)) (Table 1), which, as a result of the electron withdrawing properties of the alkene, alkyne or amide moiety, exhibit significant lower pK_a values of the protonated ε-amine compared to lysine.[11] Here we describe the synthesis of these lysine analogues and their incorporation in tetrapeptide vinylsulfones. We developed an enantioselective synthesis for both amino acids, which includes as key step the catalytic enantioselective phase-transfer alkylation of a glycine derivative. We also describe the synthesis of Dap(Gly), an amide bond containing analogue of lysine, which was prepared by a peptide coupling between the β-amine of L-diaminopropionic acid (Dap) and glycine. The appropriate histidine and lysine analogous building blocks were used for installation as P3 residues, as well as converted into their corresponding vinyl sulfones at the P1 position All synthesized inhibitors (Table 1) were tested for proteasome inhibition by competitive activity-based protein profiling (cABPP).

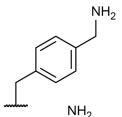
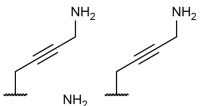
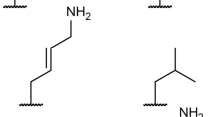
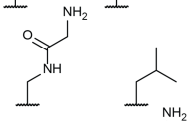
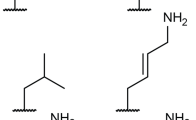
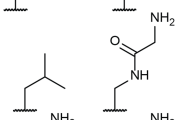
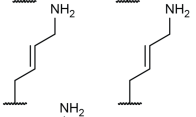
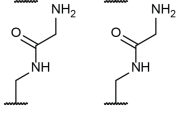
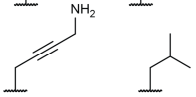
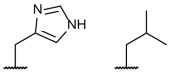
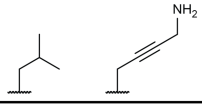
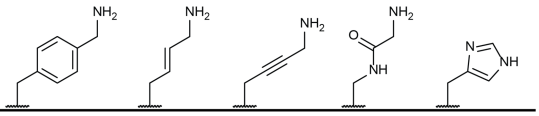
Results and Discussion

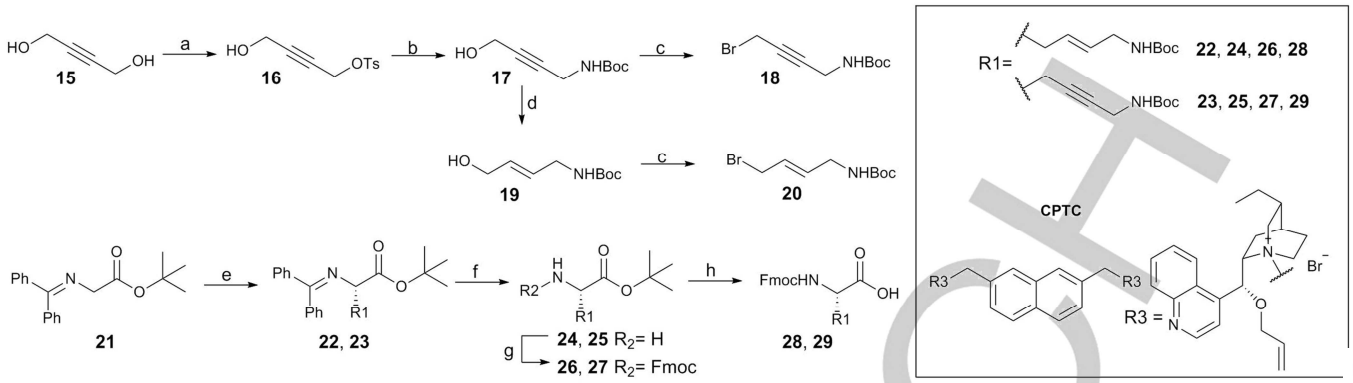
Synthesis of L-Lys(4-ene) and L-Lys(4-yn) containing inhibitors

The synthesis of α-amino acids by catalytic enantioselective phase transfer alkylation of a glycine derivative has been highly optimized by Park and coworkers.[12] In their procedure, a dimeric cinchona-derived chiral phase transfer catalyst (CPTC, Scheme 1) is applied in the synthesis of a wide range of α-amino acids, including allyl-glycine and propargyl-glycine. We envisioned that this method could be applied as well to the enantioselective synthesis of L-Lys(4-ene) and L-Lys(4-yn). For this purpose we prepared bromides **18** and **20**, and used these 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 the 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 ammonia[13] 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 $LiAlH_4$ to give allyl alcohol **19**.[14,15] 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. Alcohols **17** and **19** were converted via an Appel reaction into bromides **18** and **20**. Although several steps towards the bromides were rather low-yielding, the reactions could be easily performed on large scale and we obtained sufficient quantities of both **18** and **20**. Both bromides were used in the chiral phase transfer alkylation of glycine-derivative **21**,[12] providing compounds **22** and **23** in high yields and good enantiomeric excess (*ee,* 79% for **22** and 80% for **23**), as determined by chiral HPLC analysis. Mildly acidic hydrolysis of the imine moieties 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, provided 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 sulfones[10], 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**.

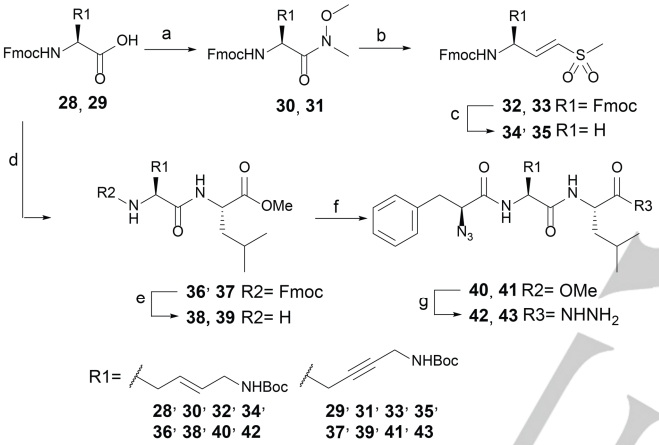
Table 1. Structures of compounds synthesized in this study. pKa values of conjugated acids are shown.



Cmp	P1	P3	Cmp	P1	P3
3 LU-102			10		
5			11		
6			12		
7			13		
8			14		
9					
					
pKa =	9.3	9.7	8.9	8.1	6.5



Scheme 1. Synthesis of Lys(4-ene) and Lys(4-yn) building blocks. Reagents and conditions: a. TsCl, pyridine, DCM, 63%; b. 1. 25% aq. NH_3; 2. Boc_2O, Et_3N, THF, DCM, 42%; c. PPh_3, CBr_4, DCM, 0℃, **18**: 68%, **20**: 58%; d. $LiAlH_4$, THF, Δ, 38%; e. **18** or **20**, CPTC, Tol/$CHCl_3$, 50% aq. KOH **22**: 84%, 79% ee; **23**: 84%, 80% ee; f. 15% aq. citric acid, THF, 0℃-rt; g. Fmoc OSu, DiPEA, DCM, **26**: 83%, **27**: 89% (over steps f/g); h. 1. TFA; 2. Boc_2O, DiPEA, MeCN, **28**: 82%, **29**: 63%

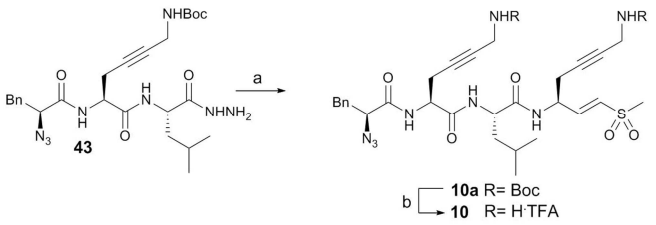


Scheme 2. Synthesis of Lys(4-ene) and Lys(4-yn) vinyl sulfones and peptide hydrazides. Reagents and conditions: a. HCTU, *N,O*-dimethyl hydroxylamine, **30**: 100%, **31**: 85%; b. 1. $LiAlH_4$, THF. 2. diethyl((methylsulfonyl)-methyl)-phosphonate, NaH, THF, **32**: 40%, **33**, 70%; c. Et_2NH, MeCN, **34**: 70%, **35**: 47%; d. H-Leu-OMe, HCTU, DiPEA, DCM, **36**: 96%, **37**: 76%; e. piperidine, DMF, **38**: 94%, **39**: 100%; f. N_3Phe-OH, HCTU, DiPEA, DCM, **40**: 60%, **41**: 91%; g. hydrazine monohydrate, MeOH, 100%.

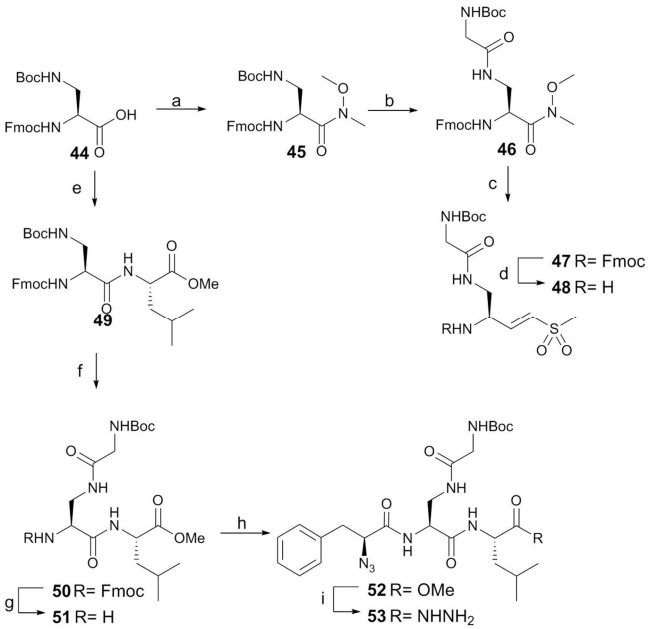
The Fmoc groups in **32** and **33** were removed by treatment with diethylamine, providing free amines **34** and **35**. For the incorporation of L-Lys(4-ene) or L-Lys(4-yn) at the P3 site, building blocks **28** and **29** were condensed with H-Leu-OMe, yielding dipeptides **36** and **37** (Scheme 2). Subsequent Fmoc removal, peptide coupling to N_3Phe-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 N_3Phe-Leu-Leu-$NHNH_2$[10] and vinyl sulfones **34**, **35**, or H-Leu-VS followed by Boc removal provided the desired final compounds.

Synthesis of Dap(Gly) containing inhibitors

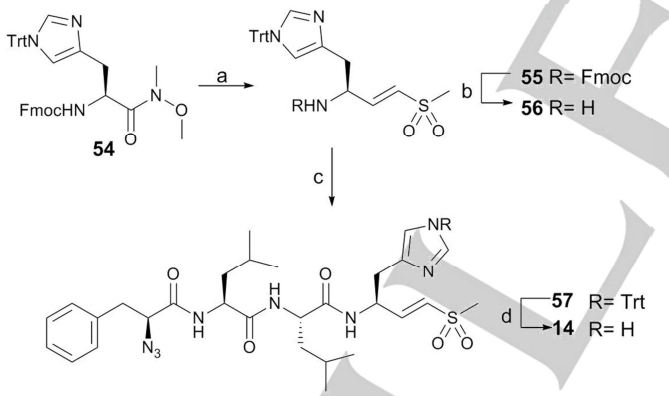
The synthesis of Dap(Gly) vinyl sulfone and peptide hydrazide for the synthesis of P3 Dap(Gly) compounds is shown in Scheme 4. The synthesis of the vinyl sulfone commenced with the conversion of commercially available Fmoc-Dap(Boc)-OH **44** to Weinreb amide **45**. Subsequent Boc removal and condensation with Boc-Gly-OH provided compound **46**. Conversion of the Weinreb amide to the vinyl sulfone using similar procedures as described above yielded **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 N_3Phe-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 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.



Scheme 4. Synthesis of Dap(Gly) vinyl sulfone and peptide hydrazide. Reagents and conditions: a. HCTU, *N,O*-dimethyl hydroxylamine, 96%; b. 1. TFA 2. HCTU, Boc-Gly-OH, DiPEA, DCM, 100%. c. 1. $LiAlH_4$, THF. 2. diethyl((methylsulfonyl)-methyl)-phosphonate, NaH, THF, 43%; d. Et_2NH, 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. N_3Phe, HCTU, DiPEA, DCM, 68%; i. hydrazine monohydrate, MeOH, 100%.



Scheme 5. Synthesis of compound 14. Reagents and conditions: a. 1. $LiAlH_4$, THF. 2. diethyl((methylsulfonyl)-methyl)-phosphonate, NaH, THF, 65%; b. Et_2NH, MeCN, 64%; c. 1. N_3Phe-Leu-Leu-$NHNH_2$, *t*BuONO, HCl, DMF. 2. **56**, DiPEA, 53%; d. TFA, TIPS, DCM, 38%.

Synthesis of His containing inhibitor

The synthesis of P1 His compound **14** is depicted in Scheme 5. The properly protected His Weinreb amide[16] **54** was converted to vinyl sulfone **56**, using procedures as described above. Free amine **56** was reacted in an azide coupling reaction with N_3Phe-Leu-Leu-$NHNH_2$ to provide tetrapeptide vinyl sulfone **57**. Subsequent trityl removal by TFA with the help of TIPS as cation scavenger yielded compound **14**.

Biological evaluation

All peptide vinyl sulfones 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** in a competitive activity-based protein profiling (cABPP) assay (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 our recently described activity-based proteasome probe cocktail.[7] For all compounds, a dramatic loss of potency against the β2 subunits was found. Compounds **5** (P1: Lys(4-ene)) and **8** (P1: Lys(4-yn)) both show some β2 selectivity, although a more than 10-fold decrease in potency compared to LU-102 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 β2 over β5. Finally, compounds **14** (P1: His) inhibited β2 with similar potency as compounds **5** and **8** (complete inhibition at 10 µM), however, with poor selectivity over the β5 subunits. Since all compounds showed much lower activity than LU-102 (**3**), we 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).

All synthesized tetrapeptide vinyl sulfones showed reduced activity against β2 compared to LU-102 (**3**). The low activity of these inhibitors could originate from 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 β2 subunit due to a shorter side chain of the lysine analogues compared to the benzyl amine 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 β2c subunit via hydrogen bonding (Glu53 in case of β2i).[10] Due to absence of an acidic residue in the S1 pockets of β1 and β5, these interactions are the driving force for the β2 selectivity of compounds equipped with P1 basic residues. Thus, in order to maintain β2 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

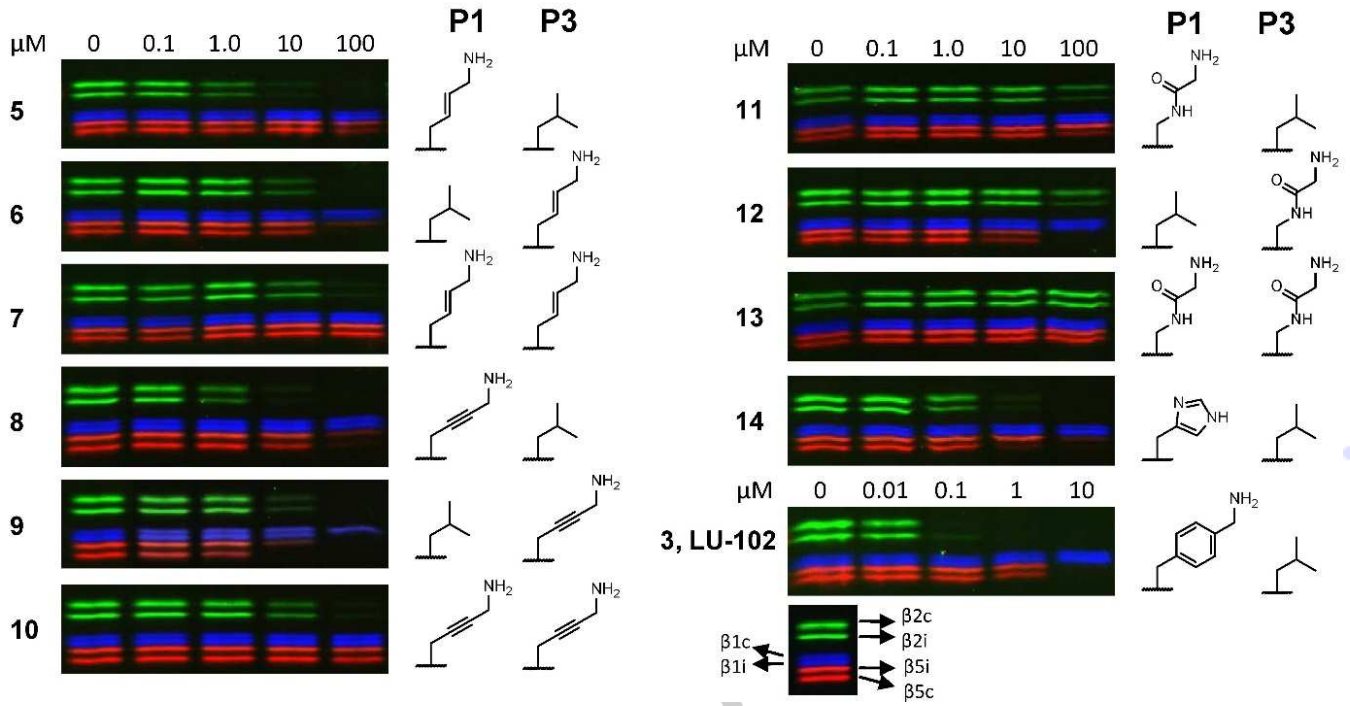


Figure 2. Inhibition profiles of compounds 5-14, compared to 3 (LU-102) in Raji lysates. Lysates were incubated with compounds at indicated concentration for 1 hour, followed by labelling of residual proteasome activity with the ABP cocktail.

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 β2 subunit or by a changed orientation of the side chain caused by the amide.

The S1 pocket of β2 is spacious and therefore able to accommodate the P1 aromatic residue of LU-102. Absence of interactions between the much less sterically demanding lysine 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 lysine 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, lysine at P1 also showed a ten-fold lower potency compared to LU-102[10], 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 β5c over β5i. This was unexpected, since β5i prefers large[17] and β5c small residues at P1.[18] This selectivity probably originates from a combination of Leu at P3 (disfavoured by β5i)[19] and histidine at P1. Histidine at P1 might, due to its relatively small size, suffer less from unfavourable interactions with the relatively small S1 pocket of β5c compared to the lysine 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.[10] However, the P3 Leu moiety of LU-102 does not show any favourable interaction with the protein. Likely, compounds with lysine analogues at P3 do also not benefit from van der Waals interactions with the protein and do therefore not show increased potency.

Conclusions

In order to obtain β2 selective inhibitors that are less charged at physiological pH, we explored several lysine

analogues with lower pK_a values as basic residues in potential β2 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 lysine analogue equipped a peptide bond in the side chain (Dap(Gly)) was incorporated as P1 and/or P3 residues in potential proteasome inhibitors. Finally, histidine was explored as basic residue at P1. Evaluation by cABPP revealed that all compounds targeted β2 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 β2 and by the lack of van der Waals interactions.

Experimental Section

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 µm 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 $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (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 µL of a 2 µM solution in water/acetonitrile 50/50 (v/v) and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60,000 at m/z 400 (mass range m/z = 150-2,000) and dioctylphthalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemini C_{18} 50 × 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→xx% MeCN, 13.0 min (0→0.5 min: 10% MeCN; 0.5→8.5 min: gradient time; 8.5→10.5 min: 90% MeCN; 10.5→13.0 min: 10% MeCN). HPLC purification was performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5µm 250×10 mm column and a GX281 fraction collector. Enantiomeric excess (*ee*) was determined using chiral HPLC analysis (Daicell Chiralcel OD column (250 x 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×). 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.

N_3Phe-Leu-Leu-Lys(-4-ene)-VS (5). This compound was obtained by the general protocol for azide coupling on a 60 µmol scale. Purification by column chromatography (0→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 µmol, 14%). ^1H NMR (600 MHz, CD_3OD) 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). ^{13}C NMR (151 MHz, CD_3OD) 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→90% MeCN, 0.1% TFA, 13.0 min Rt (min): 6.15 (ESI-MS (m/z): 604.06 ($M+H^+$)). HRMS: calculated for $C_{29}H_{46}N_7O_5S$ 604.32756 [$M+H^+$]; found 604.32751.

N_3Phe-Lys(-4-ene)-Leu-Leu-VS (6). This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (0→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 µmol, 20%). Isolated with 15% *cis* isomer. Peaks reported correspond to *trans* isomer. ^{1}H NMR (600 MHz, CD_3OD) 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). ^{13}C NMR (151 MHz, CD_3OD) 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→90% MeCN, 0.1% TFA, 13.0 min Rt (min): 6.12 (ESI-MS (m/z): 604.13 ($M+H^+$)). HRMS: calculated for $C_{29}H_{46}N_7O_5S$ 604.32756 [$M+H^+$]; found 604.32758.

N_3Phe-Lys-4-ene-Leu-Lys(-4-ene)-VS (7). This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (0→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 µmol, 20%). ^{1}H NMR (600 MHz, CD_3OD) 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). ^{13}C NMR (151 MHz, CD_3OD) 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.

N_3Phe-Leu-Leu-Lys(-4-yl)-VS (8). This compound was obtained by the general protocol for azide coupling on a 100 µmol 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 µmol, 92 %) as a white powder after lyophilisation. ^{1}H NMR (400 MHz, CD_3OD) 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, CD_3OD) 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, 12.5 min Rt (min): 6.84 (ESI-MS (m/z): 602.70 ($M+H^+$)). HRMS: calculated for $C_{29}H_{44}N_7O_5S$, 602.31191 [$M+H^+$]; found 602.31195.

N_3Phe-Lys-4(-yl)-Leu-Leu-VS (9). This compound was obtained by the general protocol for azide coupling on a 90 µmol 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 µmol, 53%) as a white powder after lyophilisation. Isolated with 10% *cis* isomer. Peaks reported correspond to *trans* isomer. ^{1}H NMR (400 MHz, CD_3OD) 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, CD_3OD) 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.

N_3Phe-Lys(-4-yl)-Leu-Lys(-4-yl)-VS (10). This compound was obtained by the general protocol for azide coupling on a 90 µmol 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 µmol, 79%) as a white powder after lyophilisation. Isolated with 10% *cis* isomer. Peaks reported correspond to *trans* isomer. ^{1}H NMR (400 MHz, CD_3OD) 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, CD_3OD) δ 174.59, 172.11, 171.84, 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.

N_3Phe-Leu-Leu-Dap(Gly)-VS (11). This compound was obtained by the general protocol for azide coupling on a 50 µmol 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. ^{1}H NMR (600 MHz, CD_3OD) 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, CD_3OD) 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→90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 5.97 (ESI-MS (m/z): 621.33 ($M+H^+$)). HRMS: calculated for $C_{28}H_{45}N_8O_6S$ 621.31773 [$M+H]^+$; found 621.31744

N_3Phe-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→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. ^{1}H NMR (600 MHz, CD_3OD) 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, CD_3OD) 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→90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 5.86 (ESI-MS (m/z): 621.27 ($M+H^+$)). HRMS: calculated for $C_{28}H_{45}N_8O_6S$ 621.31773 [$M+H]^+$; found 621.31757

N_3Phe-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→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. ^{1}H NMR (600 MHz, CD_3OD) 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, CD_3OD) 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→90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 4.66 (ESI-MS (m/z): 651.27 ($M+H^+$)). HRMS: calculated for $C_{28}H_{45}N_8O_6S$ 651.30314 [$M+H]^+$; found 651.30286

N_3Phe-Leu-Leu-His-VS (14). To a solution of N_3Phe-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. ^{1}H NMR (600 MHz, CD_3OD) 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, CD_3OD) 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%). ^{1}H 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 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^{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%). ^{1}H 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^{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%).^{1}H 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℃. 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 %). ^{1}H 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^{o}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%). ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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^{o}C. A 50% (w/w) aqueous KOH solution which had been cooled to 4℃ was added dro p wise (13.5 mL). The reaction was stirred over two nights at 4℃ and the reaction progression was followed by TL C (15% EtOAc/pent). The solution was then diluted with EtOAc, washed with water (1x), brine (1x), dried over Na_2SO_4, 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%). ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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℃. A 50% (w/w) aqueous KOH sol ution which had been cooled to 4℃ was added drop wise (1 1.5 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 Na_2SO_4, 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 %). ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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℃. 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. K_2CO_3 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 Na_2SO_4, filtered, concentrated and purified by column chromatography (0→5 % MeOH/DCM) yielding the title compound (yield given over two steps, see synthesis of **26**). ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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℃. 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. K_2CO_3 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 Na_2SO_4, filtered, concentrated and purified by column chromatography (0→5 % MeOH/DCM) yielding the title compound (2.94 g, 9.87 mmol, 91 %). ^{1}H 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→20% EtOAc/pent) yielded the title compound as a white powder (2.56 g, 4.91 mmol, 83% over two steps). ^{1}H 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→90% MeCN, 0.1% TFA, 13.0 min) Rt (min): 10.95 (ESI-MS (m/z): 523.80 ($M+H^+$)). HRMS: calcd. for $C_{30}H_{38}N_2O_6$, 523.27579 [$M+H^+$]; 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→20 % EtOAc, n-Pentane) yielded the title compound as a white foam (4.94 g, 9.48 mmol, 98 %).^{1}H 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→90% MeCN, 0.1% TFA, 13.0 min): Rt (min): 9.22 (ESI-MS (m/z): 520.87 ($M+H^+$)). HRMS: calcd. for $C_{30}H_{37}N_2O_6$ 521.26461 [$M+H^+$]; 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→2% MeOH/DCM) yielded the title compound (1.82 g, 3.90 mmol, 82%). ^{1}H 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%). ^{1}H 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. ^{1}H 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 %). ^{1}H 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 ($M+H^+$)). HRMS: calcd. for $C_{28}H_{33}N_3O_6$ 508.24421 [$M+H^+$]; found 508.24405. $[\alpha]_D^{20}$ = 4.4 (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℃. $LiAlH_4$ (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^{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 %). ^{1}H 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 Et_2O (26 mL) and cooled to -30℃. $LiAlH_4$ (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 Na_2SO_4, 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^{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 Na_2SO_4, filtered and concentrated . Purification by column chromatography (10→40 % EtOAc, n-Pentane) yielded the title compound (0.94 g, 1.79 mmol, 70 %). ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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%). ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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%). ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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 diasteromer. ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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%). ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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 diasteromer. ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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). ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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.

N_3Phe-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 diasteromer. ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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.

N_3Phe-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 %). ^{1}H NMR (400 MHz,

$CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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.

N_3Phe-Lys(-4-ene)(Boc)-Leu-$NHNH_2$ (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. ^{1}H NMR (400 MHz, CD_3OD) 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). ^{13}C NMR (101 MHz, CD_3OD) 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.

N_3Phe-Lys(-4-yn)(Boc)-Leu-$NHNH_2$ (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℃ 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%). ^{1}H NMR (300 MHz, $CDCl_3$) 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). ^{13}C NMR (75 MHz, $CDCl_3$) 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%).^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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℃ in THF (4 mL) was added $LiAlH_4$ (2M in THF, 150 µL, 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 Na_2SO_4, 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 ℃ 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℃ 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 (2×) and brine, dried over Na_2SO_4 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)). ^{1}H NMR (400 MHz, CD_3OD) 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℃. 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%). ^{1}H NMR (400 MHz, CD_3OD) 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, CD_3OD) 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%). ^{1}H 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. ^{1}H 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. ^{1}H NMR (400 MHz, $CDCl_3$, CD_3OD) 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, CD_3OD) 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.

N_3Phe-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%). ^{1}H 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.

N_3Phe-Dap(Gly-Boc)-Leu-$NHNH_2$ (53). N_3Phe-Dap(Gly-Boc)-Leu-OMe **52** (0.34 mmol) was dissolved in MeOH (3 mL), followed by the addition of $NH_2NH_2{\cdot}H_2O$ (497 µL, 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. ^{1}H NMR (400 MHz, CD_3OD) 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). ^{13}C NMR (101 MHz, CD_3OD) 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℃ in THF (30 mL) was added $LiAlH_4$ (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 Na_2SO_4, 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 ℃ 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℃ 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 (2×) and brine, dried over Na_2SO_4 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)). ^{1}H NMR (400 MHz, $CDCl_3$) 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℃. 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%). ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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.

N_3Phe-Leu-Leu-His(Trt)-VS (57). The title compound was obtained by the general protocol for azide coupling on a 100 µmol scale. Purification by column chromatography (0→2% MeOH/DCM) provided the title compound (45 mg, 53%). ^{1}H NMR (400 MHz, $CDCl_3$) 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). ^{13}C NMR (101 MHz, $CDCl_3$) 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 $MgCl_2$, 10% glycerol, 2 mM ATP, and 0.05% digitonin for 60 min on ice. 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 $MgCl_2$, 10% glycerol, 2 mM ATP. Cell lysate labelling and competition experiments were performed at 37℃. 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 ℃, followed by addition of probe cocktail (10x stock, 1.1 µL) and SDS-PAGE as described above.

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Keywords: asymmetric synthesis • amino acids • inhibitors • proteasome • activity-based protein profiling

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